Table 4 Production of IFN-γ by stimulated T cells and IL-12 p70 by *Mycobacterium leprae*-infected macrophage-derived dendritic cell (DC)-like cells (MACDC)*

		CD4		CD8		
Stimulator	M. leprae (MOI) T/DC	20	40‡	10‡	20	IL-12† (pg/ml)
MØ	0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	4.6 ± 0.9
	5	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	3.0 ± 1.0
	20	0.1 ± 0.0	0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.0
MACDC	0	6.2 ± 0.8§	3.1 ± 0.7§¶	5.4 ± 1.0§	2.9 ± 0.8§**	11.4 ± 9.1¶**
	5	73.0 ± 2.3§	22.6 ± 0.8¶	34.3 ± 2.1§	11.1 ± 1.0§	43.6 ± 7.4**
	20	173.5 ± 6.9§	82.3 ± 2.1§	84.9 ± 3.1§	31.1 ± 1.9**	56.1 ± 8.8¶
	80	19.2 ± 1.4	9.5 ± 1.0	15.6 ± 0.9	7.7 ± 0.9	Not detected

*The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with an indicated dose of autologous macrophages or MACDC. MACDC were differentiated from macrophages, either uninfected or infected for 5 days with an indicated dose of *M. leprae*, by using IFN-γ, rGM-CSF and rIL-4. The concentration of IFN-γ produced by stimulated T cells was measured by ELISA.
†Macrophages and MACDC (2×10^5/well) were stimulated in the presence of CD40L for 24 h and the concentration of IL-12 p70 was measured. Representative of three separate experiments (IFN-γ and IL-12 production) is shown. Assays were done in triplicate, and results are expressed as mean ± SD.
‡IFN-γ (pg/ml).
§$P < 0.0005$.
¶$P < 0.0001$.
**$P < 0.001$.

produce IL-12 p70 by stimulation with CD40L. The infection of MACDC with *M. leprae* further upregulated the cytokine production. However, macrophages did not produce the cytokine by any stimuli such as CD40L or *M. leprae* infection.

Susceptibility of MACDC to CTL-killing activity

We examined the expression of *M. leprae*-derived antigens on the MACDC by using leprosy patient sera and pAbs to subcellular components of the bacteria, as it has been reported that leprosy sera detected *M. leprae*-derived antigens on the bacteria-infected DC [8]. *M. leprae*-infected MACDC expressed molecules which reacted with sera obtained from leprosy patients (Fig. 3). Furthermore, these mycobacteria-infected MACDC were also positively stained with pAb to cell membrane fraction of *M. leprae* but did not react to pAbs against cell wall or cytosol fractions. These results may suggest that MACDC expressed cell membrane components on the surface. In order to clarify the significance of expression of membrane components, we assessed whether *M. leprae*-infected MACDC could be more efficiently killed by *M. leprae* cell membrane-specific CTL than the bacteria-infected macrophages (Fig. 4). In a previous report, we showed that $CD8^+$ T cells, stimulated *in vitro* with *M. leprae*-derived cell membrane fraction-pulsed DC, produced intracellular perforin [21]. In this experiment, we used these perforin-producing $CD8^+$ T cells as an effector population. While $CD8^+$ T cells stimulated with DC unpulsed with any specific antigens did not kill either macrophages or MACDC regardless of the bacterial infection (data not shown), $CD8^+$ T cells stimulated with cell membrane-pulsed DC did kill *M. leprae*-infected, but not uninfected, target cells. More than 50% of *M. leprae*-infected MACDC were killed

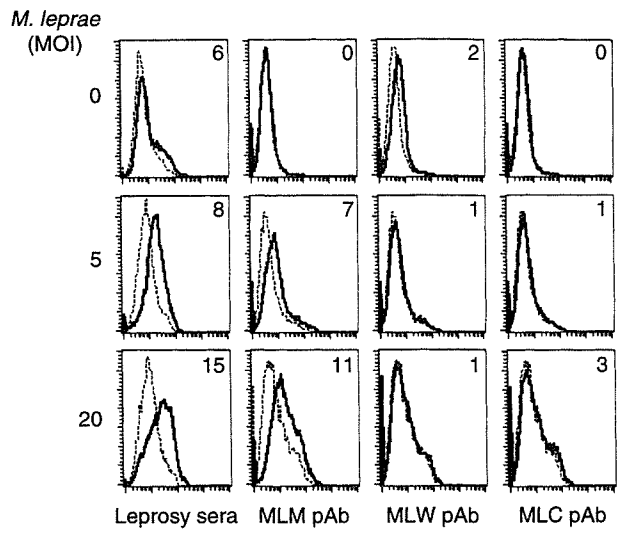

Figure 3 Expression of *Mycobacterium leprae*-derived molecules on macrophage-derived dendritic cell-like cells (MACDC). MACDC were differentiated from macrophages either uninfected or infected with an indicated dose of *M. leprae* for 5 days and were stained with lepromatous leprosy patients' sera and polyclonal antibody (pAb) to *M. leprae* subcellular fractions. The number represents the difference in mean fluorescence intensity between dotted and solid lines. A representative of three independent experiments is shown. MLC, *M. leprae* cytosol fraction; MLM, *M. leprae* cell membrane fraction; MLW, *M. leprae* cell-wall fraction. Dotted line, normal rabbit immunoglobulin G; solid line, pAb.

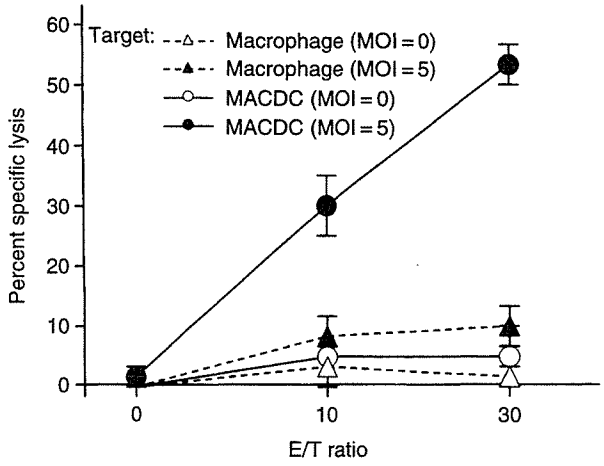

Figure 4 Susceptibility of *Mycobacterium leprae*-infected macrophage-derived dendritic cell-like cells (MACDC) to *M. leprae*-derived membrane antigen-specific CTL. *M. leprae*-derived membrane antigen-specific $CD8^+$ CTL were differentiated from $CD8^+$ T cells by stimulation with the antigen-pulsed autologous mature DC as described previously [21] and were used as effector cells. Macrophages and MACDC either uninfected or infected with *M. leprae* (MOI 5) for 5 days were cocultured with effector cells for 5 h at the indicated E/T ratio. lactate dehydrogenase released by cells were measured. The mean ± SD of triplicate assay and a representative of three independent experiments is shown.

at the E/T ratio of 30, and these MACDC were more efficiently killed than the bacteria-infected macrophages.

Discussion

We tried to induce phenotypic change in mycobacteria-infected macrophages and examined its effect on the host defence activity such as antigen-presenting capacity and the sensitivity to $CD8^+$ CTL. It is known that *M. leprae*-infected macrophages produce IL-10 and stimulate T cell less efficiently [22], and in our hands too, the infected macrophages did not stimulate T cells vigorously, even after being treated with exogenous IFN-γ (Table 4). We have previously demonstrated that monocyte-derived DC also exhibited similar *in vitro* susceptibility to *M. leprae* infection, but in contrast to macrophages, the DC stimulated autologous T cells [8]. However, when the DC were infected with a low number of bacteria, they did not vigorously stimulate T cells, and additional stimulation by bacterial subcellular components was necessary for induction of significant T-cell activation [21]. The exact reason for the less efficient T-cell-activating ability of *M. leprae* is not fully uncovered but might be due to scarcity of antigens on the surface of *M. leprae* as a consequence of large number of pseudogenes in their genome [23]. On the other hand, from the aspect of host defence, these observations indicate the necessity to recruit professional APC, which can initiate T-cell responses by responding to a small number of the bacteria.

In animal models, *M. tuberculosis* induces disease similar to human tuberculosis [24–27], in which the bacteria infect both macrophages and DC [1, 14, 25, 28]. Although these APC are found in the lesion microenvironment, macrophages and DC seem to respond differently following infection. Macrophages produce IL-10 upon an infection with the mycobacterium, and the secreted IL-10 lead naïve T cells to unresponsiveness against the bacterial antigens, although they promote the formation of tuberculous granuloma by residing in close opposition with activated T cells [29]. However, they produce no detectable level of IL-12. In contrast to macrophages, DC initiate both type-1 $CD4^+$ and $CD8^+$ T-cell activation and further act as a primary producer of IL-12 p70 following mycobacterial infection. The DC-mediated IL-12 triggers rapid differentiation of both T-cell subsets into type-1 T cells which cognately interact with DC. These T cells produce IFN-γ, which in turn, contributes to induce mycobacteriocidal action to APC such as macrophages [14, 29, 30]. Considering these facts, we tried to induce phenotypic changes in mycobacteria-infected macrophages, so that T cells could be stimulated. On treatment with rIFN-γ, rGM-CSF and rIL-4, *M. leprae*-infected macrophages were phenotypically transformed to DC-like cells (MACDC). These cells expressed CD1a and CD83 antigens but lacked the expression of CD14 and produced IL-12 and induced responses, such as proliferation and IFN-γ production, of both $CD4^+$ and $CD8^+$ T-cell subsets. These results are partly supported by previous report that Th1-polarizing potential was observed when macrophages, not infected with any bacteria, were treated with GM-CSF and IL-4 [31]. Although, monocyte-derived DC required higher dose of bacterial infection for T-cell activation, MACDC showed a distinctive feature, in that they initiated T-cell proliferation more efficiently than the DC with rather small number of *M. leprae* (Table 1). Therefore, it may be reasoned out that MACDC and DC contribute distinctively to the host defence; the former might be more important in the microenvironment where a small number of *M. leprae* exist. This hypothesis might be associated with the previous finding that $CD1a^+$ and $CD83^+$ cells were enrolled in tuberculoid leprosy lesion [22], although it was not clarified whether they originated from macrophages or monocytes.

IFN-γ contributed to the efficient development of MACDC by upregulating the expression of CD1a and CD86 molecules on macrophages (Fig. 2). In murine system, IFN-γ is known to be associated with production of the reactive nitrogen intermediates which can directly kill intracellular mycobacteria [32]. Although there is no definite evidence suggesting the association of such intermediate products with mycobacterial killing in human, IFN-γ can activate macrophages and kill the intracellular

bacteria. In this study, we showed that IFN-γ did not directly endow macrophages with T-cell-stimulating activity but contribute to efficient differentiation of macrophages to MACDC by upregulating the expression of CD86 and CD1a molecules (Fig. 2). Furthermore, the IFN-γ obviously upregulated T-cell-activating ability of MACDC (Table 3).

Another peculiar feature of *M. leprae*-infected MACDC is that they showed an enhanced susceptibility to killing activity of *M. leprae* cell membrane antigen-specific $CD8^+$ CTL, although the identification of immunodominant antigenic determinants remains unknown. When compared to *M. leprae*-infected macrophages, the *M. leprae*-infected MACDC were more efficiently killed by CTL. It is interesting to note that *M. leprae*-infected MACDC expressed antigens reactive to pAb to cell membrane components, but not antigens recognized by pAb to cell wall or cytosol components. In addition, our previous data showed that cell membrane was the most efficient antigen among *M. leprae* subcellular components for the activation of $CD8^+$ CTL [21]. When we compared *M. leprae*-infected MACDC and the macrophages, there were no difference in the expression of MHC class I and class II antigens, but only the former expressed *M. leprae*-derived antigens on the surface. Therefore, the expression of cell membrane antigens on MACDC might be closely associated with the enhanced susceptibility to killing activity.

Taken together, an induction of phenotypic change on *M. leprae*-infected macrophages resulted in enhanced type-1 T-cell-stimulating ability and an upregulated susceptibility to CTL activity. These observations may be further useful for developing immunotherapeutic tools against intracellular pathogens which threaten humans worldwide.

Acknowledgments

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Role of the polypeptide region of a 33 kDa mycobacterial lipoprotein for efficient IL-12 production

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Abstract

Mycobacterium leprae lipoprotein, LpK, induced IL-12 production from human monocytes. To determine the components essential for cytokine production and the relative role of lipidation in the activation process, we produced lipidated and non-lipidated truncated forms of LpK. While 0.5 nM of lipidated LpK-a having N-terminal 60 amino acids of LpK produced more than 700 pg/ml IL-12 p40, the non-lipidated LpK-b having the same amino acids as that of LpK-a required more than 20 nM of the protein to produce an equivalent dose of cytokine. Truncated protein having the C-terminal 192 amino acids of LpK did not induce any cytokine production. Fifty nanomolar of the synthetic lipopeptide of LpK produced only about 200 pg/ml IL-12. Among the truncated LpK, only LpK-a and lipopeptide stimulated NF-kB-dependent reporter activity in TLR-2 transfectant. However, when monocytes were stimulated with lipopeptide in the presence of non-lipidated protein, they produced IL-12 synergistically. Therefore, both peptide regions of LpK and lipid residues are necessary for efficient IL-12 production.

Keywords: Lipoprotein; IL-12; Mycobacteria; TLR-2

1. Introduction

Bacterial lipoproteins, containing *N*-acyldiglyceride-cysteine residues at their amino termini, have been well studied in gram-positive and gram-negative bacteria [1,2]. Acylation of the amino group of cysteine in the consensus lipid-binding sequence takes place by attachment of the diacylglycerol moiety in a thioether linkage and subsequent cleavage of the proprotein by a specific signal peptidase. One of the functional characteristics of such acylated proteins is the production of interleukin-12 (IL-12)[1] from host antigen (Ag)-presenting cells (APCs). Lipoproteins stimulated APCs and these APCs in turn activated both type 1 $CD4^+$ and $CD8^+$ T cells, to produce interferon-γ (IFN-γ), which endows bacteriocidal activities to APCs mainly macrophages. Therefore, lipoproteins play a central role as an inducer of host defense activities to control the growth of intracellular parasitic bacteria such as mycobacteria. Such lipoproteins were isolated from *Mycobacterium tuberculosis*, of which the 19- and 38-kDa proteins have been reported to be capable of activating both innate and adaptive immunity [3–5]. There are only a few reports of lipoprotein from other mycobacterial species, but recently we have identified a novel 33 kDa lipoprotein, LpK, from *Mycobacterium leprae* [6].

Mycobacterium leprae induce a chronic infectious disease, termed leprosy which has been characterized by

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[1] *Abbreviations used:* IL-12, interleukin-12; M., *Mycobacterium*; Ag, antigen; APC, antigen-presenting cell; IFN, interferon; PVDF, polyvinyl difluoride; PBMC, peripheral blood mononuclear cell; LPS, lipopolysaccharide; PG, peptidoglycan; TLR, toll-like receptor; DCs, dendritic cells.

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progressive peripheral nerve injury and skin lesions [7]. One representative spectrum of the disease is a paucibacillary form of leprosy, in which the disease lesion is localized. The localization of the lesion is a consequence of the suppression of bacterial spread and, in this process, IL-12 producing APCs seem to play a central role in activating innate and type 1 cellular immunity [8–11]. Since the newly identified lipoprotein LpK was found to be capable of inducing IL-12 production in human peripheral monocytes, it can be predicted that LpK is one of the antigens in *M. leprae* with the potential to contribute to the host defense against leprosy.

Although it may be assumed that the immuno-dominant region of the lipoprotein is the lipid region, the immuno-dominant region of LpK in terms of IL-12 production has not been studied, and it remains to be clarified whether the acylated lipopeptide region alone could represent the immuno-stimulatory domain of the lipoprotein.

In this study, we expressed various forms of truncated LpK, assessed its IL-12 producing activity and attempted to clarify the role of peptide lipidation in the context of cytokine production.

2. Materials and methods

2.1. Bacterial strains, plasmids, and lipopeptides

Escherichia coli DH5α strain (Toyobo, Tokyo, Japan) was used for all cloning and recombinant expression experiments. The plasmids used for the expression in *E. coli* were pGEM-T Easy Vector (Promega, Madison, WI), and pGFPuv (Clontech, Palo Alto, CA). Clones were selected on Luria–Bertani medium agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar) or broth supplemented with ampicillin at 100 μg/ml. All other chemicals were purchased from Wako Chemicals (Richmond,VA), Sigma–Aldrich (St. Louis, MO) or Amersham–Pharmacia (Piscataway, NJ). The LpK lipopeptide containing the N-terminal 12 amino acids of LpK was synthesized by Bachem AG (Germany). The structure of the lipopeptide is as follows: Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Leu-Pro-Asp-Trp-Leu-Ser-Gly-Phe-Leu-Thr-Gly-Gly-OH. The corresponding unlipidated LpK peptide containing only the N-terminal 12 amino acids was also synthesized.

2.2. Cloning and sequencing of the truncated forms of the lpk gene

To clone the *lpk* gene, the DNA of interest was amplified by PCR by taking the genomic DNA from *M. leprae* (Thai-53 strain) as a template for PCR, and the expressed LpK lipoprotein was purified as previously described [6]. The primers used for the amplification of the gene coding protein constructs in Fig. 1 were as follows: For LpK-a, the sense primer 5′ACATGCA TGCCCTGGTGTTGGTCCTGTGG3′ (a-s) and the antisense primer 5′CGGAATTCTTAGTGATGGTGA TGGTGATGGCCTGCCCGCTGCCG3′ (a-as) were used. For LpK-b amplification, primers 5′ACATGCA TGCCCTGTTGCCTGATTGGTTGT3′ (b-s) and the antisense primer a-as were utilized. Similarly, for LpK-c, the sense primer used was a-s and antisense 5′GGAA TTCTTAGTGATGGTGATGGTGATGGCTAAGCT TAGTGATCC3′ (c-as), for LpK-d, primers used were b-s and c-as. LpK-e utilized the sense primer 5′ACAT

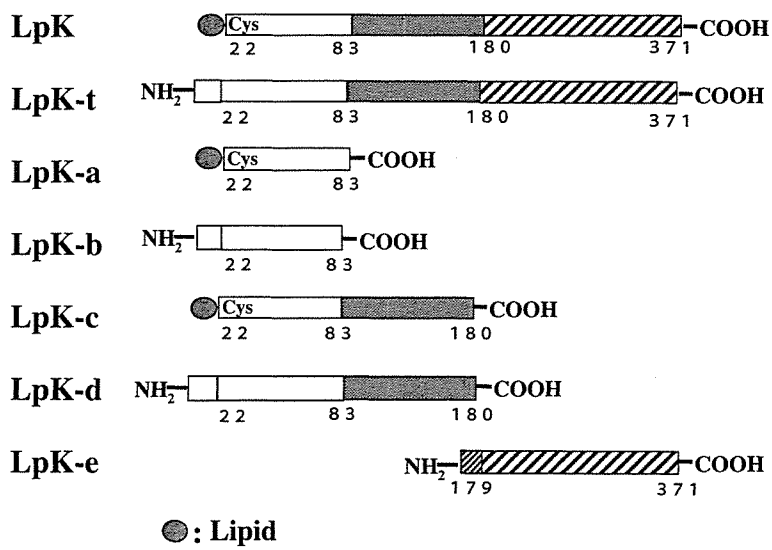

Fig. 1. Schematic representation of the constructs of LpK and truncated LpK. The lipidated constructs are LpK, LpK-a, and LpK-c. Non-lipidated constructs include LpK-t, -b, -d, and -e. The thatched region indicates the C-terminal half of the LpK protein. Numbering shows the position of the amino acid of LpK in the prolipoprotein form.

GCATGCCCTTAGCGAGCGTACTGA3′ and the previously described antisense primer for LpK amplification [6]. For LpK-t, the sense primer a-s and the same antisense primer for LpK amplification was used. All antisense primers contained the histidine tag coding sequence at the C-terminus of the protein for easy protein detection. The gene was first cloned into pGEM-T Easy Vector (Promega), and further inserted into the expression vector. All other genetic manipulations were done according to established cloning techniques [12]. All lipidated and non-lipidated *lpk* genes were expressed in *E. coli*. Restriction enzymes were purchased from New England Biolabs (Beverly, MA), Takara Shuzo (Shiga, Japan) or Toyobo (Osaka, Japan) and used according to the manufacturer's specifications. For DNA sequencing, plasmid DNA samples were purified using a Qiagen MiniPrep Kit (Qiagen, Valencia, CA). DNA sequence analysis was performed on an ABI Prism Genetic Analyser (PE Biosystems, Foster City, CA) using the dideoxy dye termination PCR method.

2.3. Detection of the expressed proteins and protein purification

Escherichia coli transformants were lysed in 6 M urea, 0.5% CHAPS, and 1 mM DTT containing 50 mM Tris–Cl and run on a 12% SDS–polyacrylamide gel [13]. The resolved proteins were transferred to a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA). The proteins were then detected using penta-His mAb (Qiagen), and color developed with 5-bromo,4-chloro, 3-indoylphosphate/nitroblue tetrazolium (BCIP/NBT). The overexpressed protein was also gel filtrated through a HiLoad 26/60 Superdex 75 prep grade column (Amersham–Pharmacia), using buffer containing 6 M urea, 50 mM Tris–Cl (pH 8.0), and 0.1% CHAPS at a flow rate of 2 ml/min. After collecting around 30 fractions, SDS–polyacrylamide gel electrophoresis was performed; the proteins were stained either by Silver Stain 'Daiichi' (Dai-ichi Pure Chemicals, Tokyo, Japan) or Coomassie blue brilliant stain. Western blotting was performed using a penta-His mAb. The fraction containing the desired protein was used for further evaluation. By SDS–PAGE of the protein and further staining with a silver stain, no apparent contamination of *E. coli*-derivatives was observed. The concentrations of LpK and its mutant proteins were determined using a Bio-Rad Protein Assay kit according to the manufacturer's instructions.

2.4. Measurement of IL-12 production by human PBMC

Human PBMCs from healthy individuals were isolated on Ficoll–Paque Plus (Amersham–Pharmacia, Upsala, Sweden) and cultured for 1 h in 10 cm dishes. The non-adherent cells were removed by washing several times with RPMI 1640 (Sigma) containing 2% FCS. By flow cytometric analyses, among the plastic adherent cells, 95–98% of the cells were CD14 positive. T cells and B cells constituted less than 1% and $CD1a^+$ dendritic cells constituted less than 0.1% of the adherent cell population. These adherent cells were then detached and cultured in triplicate in 96-well plates (10^5 cells/well) with purified lipoproteins at various concentrations. Twenty to twenty-four hours later, the culture supernatants were collected and assayed for human IL-12 p40 production using an OptEIA Set (Pharmingen, SanDiego, CA). The amount of lipopolysaccharide (LPS) in the purified lipoprotein was measured quantitatively with a Limulus Amoebocyte Lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be <10 pg/μg protein, an amount that did not stimulate IL-12. Also, the contribution of $CD1a^+$ dendritic cells within the plastic adherent cells in the IL-12 production was examined. No significant difference in the cytokine production was observed by depleting the $CD1a^+$ cells using immunomagnetic beads (Dynabeads 450, Dynal, Oslo, Norway).

2.5. Cell transfection and luciferase assay

Human embryonic kidney cells (HEK293) were obtained from the American Type Cell Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% FCS, 50 mg/ml penicillin/streptomycin, and non-essential amino acids (Invitrogen, Carlsbad, CA), at 37 °C in a humidified incubator of 5% CO_2. The cDNA of human Toll-like receptor 2 (TLR) was PCR-amplified using a human spleen cDNA library (BD Biosciences, San Jose, CA) and inserted into pCIneo (Promega, Madison, WI). HEK293 cells (2×10^4) were transiently transfected with a mixture of plasmids: 200 ng pCIneo hTLR2, 25 ng p5× NF-kB-luc (Stratagene, La Jolla, CA), and 10 ng pRL-TK-*Renilla* luciferase plasmid (Promega) using the FuGENE 6 reagent (Roche molecular Biochemicals, Indianapolis, IN), as previously described [14]. Thirty-six hours after transfection, cells were treated with or without various amounts of LpK and its truncated forms, or peptidoglycan (PG) as positive control (for TLR2-dependent luciferase activity) for further 6 h. The cells were lysed in 70 μl of 1× passive lysis buffer (Promega) and luciferase activity in 10 μl of the cell lysate was measured using Promega Dual-Luciferase Reporter Assay System according to the protocol provided by the manufacturer. Data were expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency.

2.6. Statistical evaluation

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

3. Results

3.1. The role of LpK lipidation in IL-12 production

To verify the role of lipid modification of LpK in terms of IL-12 production from human monocytes, various forms of truncated LpK were produced in *E. coli*. LpK-a is a lipidated protein, while other truncated proteins (LpK-t, LpK-b, LpK-d, and LpK-e) are non-lipidated (Fig. 1). The cytokine producing ability of mature lipidated LpK was first compared with non-lipidated LpK-t having the whole conserved amino acid residues of LpK. The expression vector for LpK-t was constructed by eliminating the nucleic acids coding for the N-terminal hydrophobic region up to the cysteine residue which is acylated in LpK. While 2.5 nM LpK produced more than 1000 pg/ml IL-12 p40 in human monocytes, 2.5 nM LpK-t produced IL-12 more than 9-fold less efficiently, and it required more than 10 nM to produce an equivalent dose of the cytokine (Fig. 2). This result indicated that the presence of the N-terminal lipid entity significantly enhanced the IL-12 producing activity of LpK protein from monocytes. To confirm this point, we examined the IL-12 inducing ability of another truncated protein, LpK-a. The expression plasmid encoding *lpk-a* was constructed by taking the N-terminal 82 amino acid coding nucleic acids of *lpk* including the signal peptide region. LpK-b having no lipid residue, but only the amino acid residues of LpK-a, was also produced. The uptake of radio-labeled glycerol was used to confirm the presence of lipid modification. *E. coli* expressing LpK-a was co-cultured for 5 h in the presence of [^{14}C]glycerol and the cells were lysed. When the protein was run on an SDS–polyacrylamide gel, autoradiography showed a 12 kDa radio-labeled band, which corresponded to the predicted molecular mass (not shown). The expression of the protein at the same position was confirmed by Western blotting. Such a radio-labeled band was not observed in the case of LpK-b, which indicated that LpK-a was lipidated.

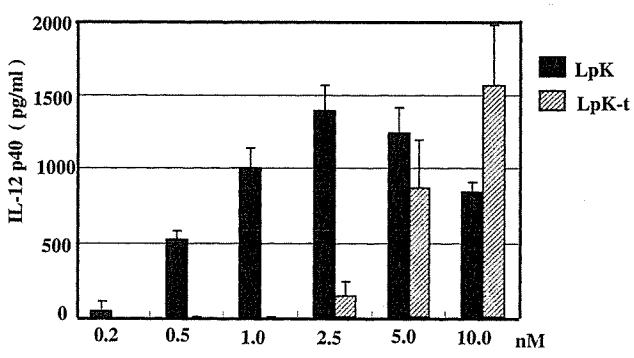

Fig. 2. LpK induces significant IL-12 production from human blood monocytes when compared to truncated LpK-t. Monocytes were isolated from healthy human blood cells as described in Section 2.4. IL-12 p40 production was measured by ELISA. The results shown are obtained from one experiment, but were consistent with three different experiments. Mean ± standard deviation of a triplicate assay is shown. By Student's *t* test, the *p* values obtained were: $p < 0.005$ for values between LpK and LpK-t at 2.5 nM and $p < 0.05$ at 5.0 nM.

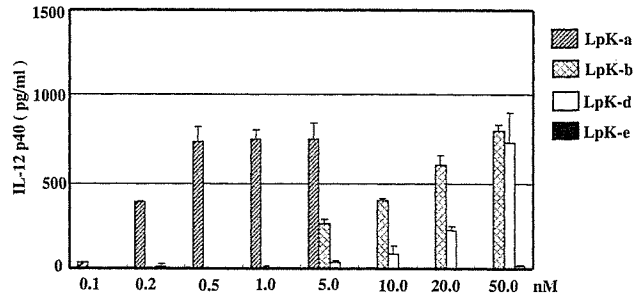

Fig. 3. Among the truncated proteins, LpK-a was the most efficient in inducing IL-12 from monocytes. Various truncated forms of LpK were expressed in *E. coli* and purified, then evaluated for their IL-12 inducing ability. Among LpK-a, -b, -d, -e, and lipidated LpK-a was the most efficient in inducing IL-12, but LpK-e did not induce cytokine production. The results were obtained from one experiment, but were consistent with three different experiments. Mean ± standard deviation of a triplicate assay is shown. By Student's *t* test, the following *p* values were obtained: $p < 0.01$ for values between LpK-a and LpK-b, LpK-a and LpK-e; $p < 0.005$ for values between LpK-a and LpK-d, LpK-b, and LpK-e; $p < 0.001$ for values between LpK-b and LpK-d; and $p < 0.05$ for values between LpK-d and LpK-e at 5.0 nM.

Next, we determined the ability of LpK-a to produce IL-12 in monocytes, and compared it with that of non-lipidated LpK-b. LpK-a was significantly more efficient at cytokine production than LpK-b (Fig. 3). While 0.5 nM LpK-a produced more than 700 pg/ml IL-12, non-lipidated LpK-b required more than 20 nM of protein to produce an equivalent dose of IL-12. One possible reason for the less efficient cytokine production by LpK-b is that it lacks some immuno-stimulatory domain, which may be the acylated structure itself or its contribution to the conformation of the protein. To further analyze the effect of other truncated LpK, we produced non-lipidated LpK protein, LpK-d, and LpK-e (Fig. 1). LpK-c, a lipidated protein having the N-terminal half of the LpK protein could not be expressed in *E. coli* for unknown reasons. LpK-d covers the N-terminal 158 amino acid residues of the LpK protein and LpK-e has amino acid corresponding to the C-terminal half of the LpK protein with a single overlapping amino acid with LpK-d. However, LpK-d induced IL-12 less efficiently compared to that produced by LpK-a. The IL-12 producing activity of LpK-d was comparable to that of LpK-b, but LpK-e had no such IL-12 stimulating ability (Fig. 3). These results may indicate that acylated N-terminal 60 amino acids of LpK is responsible for the strong immuno-stimulatory activity of LpK.

3.2. Immuno-stimulatory activity of synthetic lipopeptide

As shown in Fig. 3, the ability of LpK to induce IL-12 production in monocytes resides in the N-terminal

region of the protein, including the acylated portion. Therefore, we synthesized lipopeptide with N-terminal 12 amino acid residues of LpK, having the N-terminal cysteine residue conjugated to palmitoylated triacylated glycerol, which is presumed to be having the same lipid composition as that of LpK purified from *E. coli*. Fig. 4 represents the IL-12 production from monocytes by synthetic LpK lipopeptide and non-lipidated peptide having the same N-terminal 12 amino acids of the lipopeptide. It was surprising to note that, 50 nM synthetic lipopeptide produced less than 200 pg/ml IL-12. Further increasing the concentration of lipopeptide, did not result in further elevation of the level of cytokine production (not shown). The synthetic non-lipidated peptide having the same amino acids as that of the lipopeptide almost totally lacked the ability to induce IL-12 production in monocytes (Fig. 4). These results suggested that lipidation of the N-terminal peptide was necessary, but was not as efficient as LpK or LpK-a, for the production of IL-12.

3.3. Association of LpK protein and TLR-2

We examined whether NF-kB-driven luciferase activity was upregulated in TLR2 transfected HEK 293 cells by LpK and its truncated protein. PG, a well-defined TLR-2-associated Ag, was used as a positive control. Significantly higher luciferase activity was observed when the TLR-2 transfected HEK293 cells were stimulated with PG or lipidated lipoprotein including LpK and LpK-a, but not in mock transfected HEK293 cells (Fig. 5). On the contrary, no significant or no antigen-dose-dependent luciferase activity was induced by any of the non-lipidated proteins such as LpK-b, LpK-d, and LpK-e. But, significant activity was observed in cells when stimulated with synthetic lipopeptide LpK, despite lacking the ability to induce IL-12 efficiently. These results suggested that TLR-2 stimulation of LpK protein is essential, but not adequate for efficient production of the cytokine.

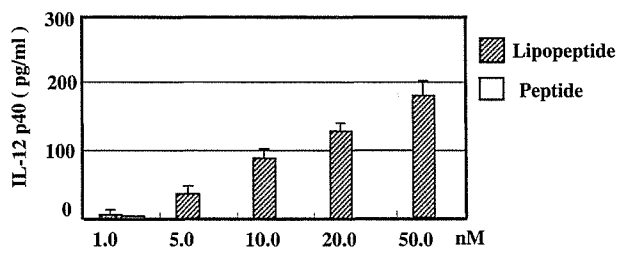

Fig. 4. The synthetic lipopeptide derived from the sequence of N-terminal LpK showed ability to induce IL-12, but to a limited extent. The same peptide sequence as that of the lipopeptide sequence without the acyl attachment did not induce any cytokine production. A representative of three independent experiments is shown. Each experiment was performed in triplicate and the mean ± standard deviation is shown. By Student's t test, the p values obtained were: $p < 0.05$ for values between lipopeptide and peptide at 5.0 nM.

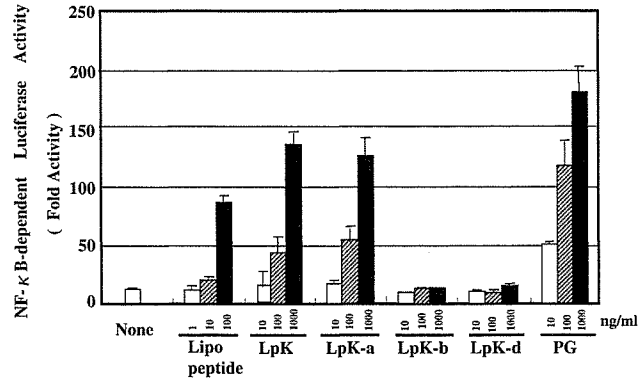

Fig. 5. Association of TLR2 and truncated LpK proteins. NF-kB-dependent reporter gene activity of the TLR2 transfectant was measured after stimulation with LpK or its truncated forms as described in Section 2.5. Peptidoglycan (PG) was used as a positive control for TLR2 dependent luciferase activity. Data are expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency in the Dual luciferase reporter assay. The result shown is representative of three different experiments. Assays were done in triplicate and the mean ± standard deviation is shown.

3.4. The role of the non-lipidated protein component of LpK in IL-12 production

To confirm that the stimulation of monocytes with non-lipidated components of LpK protein is required for efficient IL-12 production, we stimulated monocytes with lipopeptide in the presence of various concentrations of non-lipidated proteins (Fig. 6). When monocytes were co-stimulated with lipopeptide and LpK-b or LpK-d, they produced IL-12 in a manner dependent on the concentration of truncated non-lipidated LpK proteins, although induction of IL-12 by LpK-b was a little lower in this set of experiments due to donor variations. However, the combination of lipopeptide and LpK-e or synthetic peptide did not induce any cytokine production. These results suggested that lipopeptide by itself is ineffective in producing IL-12, but is markedly synergistic with certain immuno-dominant regions of LpK, which apparently seem to correspond to the N-terminal 60 amino acid residues.

4. Discussion

The clinical manifestations of leprosy appear based on the immunological spectrum according to the level of cell-mediated immunity to *M. leprae*. Lepromatous leprosy patients manifest disseminated infection, their T cells respond weakly to the bacilli and their lesions express type 2 cytokines. In contrast, tuberculoid patients mount a strong Th1 response to *M. leprae*. When a Th1 cell-mediated immune response is generated, clinically apparent leprosy infection is localized, leading to the formation of a granuloma. For efficient induction of Th1 response, IL-12 is envisaged to play an

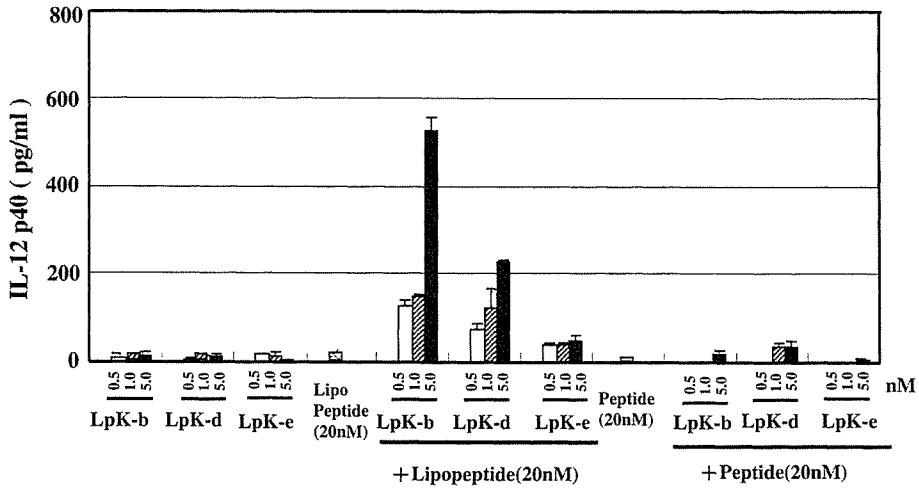

Fig. 6. The presence of lipopeptide enhances the IL-12-inducing ability of non-lipidated truncated proteins of LpK, namely Lpk-b and LpK-d, but not that of LpK-e. Values expressed are the mean values ± SD of triplicate samples and are representative of three independent experiments.

important role. Presently, a number of studies are being conducted to precisely evaluate the relationship between IL-12 and mycobacterial diseases [15–20]. Early leprosy progression is controlled by type 1 cytokines, including IL-12, as observed in single-skin lesion tuberculoid leprosy, and resistance to experimental *M. leprae* infection has been correlated with the early production of IL-12 at the site of infection [21,22]. Also, active tuberculosis is associated with reduced type 1 responses to *M. tuberculosis*, the response of which is efficiently induced by IL-12. Furthermore, human patients with inherited IL-12 or IL-12-receptor deficiency showed higher susceptibility to even primary mycobacterial infection, including environmental and less pathogenic mycobacteria such as *Mycobacterium bovis* bacillus Calmette Guérin [23]. These data indicate that IL-12 is closely associated with the activation of protective immunity against mycobacterial infection.

IL-12 is chiefly produced from APCs such as monocyte/macrophages and dendritic cells (DCs), which are the most susceptible cells in vivo to *M. tuberculosis* and *M. leprae* infection. The activation of APCs takes place by the engagement of antigenic molecules with the ligands ubiquitously expressed on the cell surface, and is closely associated with innate immunity. Therefore, molecules capable of inducing IL-12 production from monocytes largely contribute to evoke anti-mycobacterial host defense activities. Recently, we isolated an *M. leprae* lipoprotein, LpK, and indicated that LpK stimulated monocytes to produce IL-12 [6]. Although the receptor molecules for LpK are unknown, TLR-2 is reported to be involved in interactions with mycobacterial lipoprotein such as 19-kDa *M. tuberculosis*-derived lipoprotein, and PG [3,24,25]. In the present study, we produced various form of truncated LpK and analyzed the role of TLR-2 and lipidated or non-lipidated proteins in the production of IL-12 from monocytes, since monocytes are the APCs that first encounter *M. leprae* in vivo.

NF-kB-driven luciferase activity in TLR-2-transfected HEK 293 cells was upregulated when stimulated with LpK, as well as the lipid modified truncated LpK-a and lipopeptide, while the activity was not seen when stimulated with the non-lipidated LpK proteins. However, it was observed that non-lipidated LpK-t and LpK-b, in addition to lipidated forms of LpK, efficiently induced IL-12 production in monocytes. Although there are reports suggesting that heat shock protein 70, which is a non-lipidated protein, utilizes TLR-2 to transduce its proinflammatory signal [26], the lack of activation by non-lipidated LpK protein, especially LpK-t, indicates the absence of TLR-2 involvement in the non-lipidated LpK region for efficient IL-12 production. However, the significant production of IL-12 in monocytes was not induced by sole ligation of TLR-2 with a lipid component, as indicated by the lack of efficient IL-12 production by a synthetic LpK lipopeptide. This observation might be on line with the findings that glycolipids do not induce cytokine production from monocytes. But inflammatory cytokine TNF-α, which is also induced via ligation to TLR2 [27], has been efficiently induced by the lipopeptide (data not shown). Therefore, it can be emphasized that additional stimulation by the polypeptide region of LpK through some unidentified receptors is required for the efficient production of IL-12 from monocytes. When higher concentrations of non-lipidated LpK proteins such as LpK-t and LpK-b, were pulsed to monocytes, they produced comparable level of IL-12 to that produced by 1–2 nM of lipidated LpK. This implies that the protein component by itself can activate monocytes. Recently, a number of receptor molecules on macrophages and DCs, such as mannose receptors, complement receptors, DC-SIGN, and CD14 molecules, have been extensively analyzed, and

have been shown to be capable of binding mycobacterial components, while the receptors associated with IL-12 production from monocytes have not been fully elucidated. However, the results suggest that the non-lipidated protein region of LpK is involved in IL-12 production in monocytes and we are now examining the host surface molecules involved in polypeptide binding for efficient IL-12 production.

From the aspect of IL-12 production, the N-terminal portion of *M. leprae*-LpK is important since LpK-a carrying both a lipidated and hydrophobic protein region stimulated IL-12 as efficiently as parent LpK. Therefore, LpK-a, for which lipidation is confirmed by integration of radio-labeled glycerol into the protein, can be a substitute for LpK. In addition to innate immunity, adaptive immunity such as IFN-γ production from type 1 $CD4^+$ T cells also plays an important role in the protective immunity against mycobacterial infection [28–30]. Although IL-12 does not directly act as an initiator of adaptive immunity, it can work as a bridge between innate and adaptive immunity [9,31]. We are now evaluating the antigenicity of LpK and LpK-a in DC-mediated, MHC or CD1 molecule-restricted cellular immunity in vitro and also underway to determine the efficacy of LpK in vaccine development by conducting animal experiments.

In conclusion, *M. leprae* lipoprotein produced IL-12 in monocytes by stimulating them through two or more signaling pathways, and both components, the triacylated lipid region and the polypeptide region were required for efficient IL-12 production. Therefore, the potential contribution of LpK to protective immunity against *M. leprae* infection is highly anticipated.

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The role of antigenic peptide in CD4$^+$ T helper phenotype development in a T cell receptor transgenic model

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Keywords: altered peptide ligand, IFN-γ, Th1, Th2, Th1-inducing peptide, transgenic mouse

Abstract

CD4$^+$ Th1 cells play a critical role in the induction of cell-mediated immune responses that are important for the eradication of intracellular pathogens. Peptide-25 is the major Th1 epitope for Ag85B of *Mycobacterium tuberculosis* and is immunogenic in I-A^b mice. To elucidate the role of the TCR and IFN-γ/IL-12 signals in Th1 induction, we generated TCR transgenic mice (P25 TCR-Tg) expressing TCR α- and β-chains of Peptide-25-reactive cloned T cells and analyzed Th1 development of CD4$^+$ T cells from P25 TCR-Tg. Naive CD4$^+$ T cells from P25 TCR-Tg differentiate into both Th1 and Th2 cells upon stimulation with anti-CD3. Naive CD4$^+$ T cells from P25 TCR-Tg preferentially develop Th1 cells upon Peptide-25 stimulation in the presence of I-A^b splenic antigen-presenting cells under neutral conditions. In contrast, a mutant of Peptide-25 can induce solely Th2 differentiation. Peptide-25-induced Th1 differentiation is observed even in the presence of anti-IFN-γ and anti-IL-12. Furthermore, naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg also differentiate into Th1 cells upon Peptide-25 stimulation. Moreover, Peptide-25-loaded I-A^b-transfected Chinese hamster ovary cells induce Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg in the absence of IFN-γ or IL-12. These results imply that interaction between Peptide-25/I-A^b and TCR may primarily influence determination of the fate of naive CD4$^+$ T cells in their differentiation towards the Th1 subset.

Introduction

Naive CD4$^+$ Th cells recognize an antigenic peptide through their TCR in the context of MHC class II molecules on antigen-presenting cells (APC) and undergo differentiation to effector cells that can produce cytokines and chemokines. During this process, naive CD4$^+$ T cells can differentiate to at least two functionally distinct subsets of cells, represented by Th1 and Th2 (1). Th1 cells produce IFN-γ and lymphotoxin (TNF-β) in addition to IL-2 and are responsible for directing cell-mediated immune responses leading to the eradication of intracellular pathogens such as *Mycobacterium*, viruses and parasites (1–4). Th1 cells also regulate IgG2a and IgG3 antibody production via IFN-γ production, which is involved in the opsonization and phagocytosis of particulate microbes. Th2 cells secrete IL-4, IL-5 and IL-13 as effector cytokines and are responsible for humoral immune responses for the eradication of helminths. Th2 cells also cause inflammatory damage during allergic diseases, such as asthma and atopic dermatitis. The process by which an uncommitted Th cell develops into a mature Th1 or Th2 subset is a matter of fact for regulating the immune response to various antigens.

Considerable progress has been made in identifying the factors that govern the progression of cell differentiation during the generation of Th subsets (2–4). Using T cells stimulated with polyclonal activators or T cells from mice expressing transgenic antigen receptors of known specificities, it has become clear that Th1 and Th2 subsets develop

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from the same T cell precursor (5–7), which is a naive $CD4^+$ T cell. There is a body of evidence to indicate that the cytokines IL-12 and IL-4 are key determinants of the Th1 and Th2 response, respectively (4). For example, IL-12 directs Th1 development from antigen-stimulated naive $CD4^+$ T cells and activates STAT4 in Th1 cells (8,9). In terminally differentiated Th1 cells, successive IFN-γ production can occur through TCR ligation or IL-12 and IL-18 stimulation. Using mice deficient in either cytokines or STAT, it has been shown that activation of the IFN-γR/STAT1 is also important for the differentiation of $CD4^+$ T cells into Th1 cells (10,11). The IL-4R/STAT6 signaling pathway plays a central role in the differentiation of naive $CD4^+$ T cells into Th2 cells (12–14). The balance of IFN-γ and IL-4 levels present during T cell activation is considered to be the major influence on Th1 versus Th2 differentiation. Although the strength of the interaction mediated through TCR and MHC/peptide complex is suspected to affect the lineage commitment of Th cells to Th1 cells and clonal expansion (15–17), it remains unclear whether Th1 cells can develop from naive $CD4^+$ T cells upon antigenic peptide stimulation in the presence of APC under neutral conditions.

Ag85B (also known as α antigen or MPT59) is the most potent antigen species yet purified for both humans and mice (18). Ag85B can elicit strong Th1 response *in vitro* from PPD^+ asymptomatic individuals (19–21). We have shown that *in vitro* stimulation of lymph node cells from *Mycobacterium tuberculosis*-primed C57BL/6 mice with Ag85B induces the production of IFN-γ and IL-2 and expansion of $CD4^+$ T cells expressing Vβ11 of TCR (TCRVβ11) in an $I\text{-}A^b$-restricted manner (22,23). We identified the 15-mer peptide (Peptide-25), covering amino acids residues 240–254 (FQDAYNAAGGHNAVF) of Ag85B, as the major epitope for Ag85B-specific $TCRV\beta11^+$ T cells (22). Using Peptide-25-reactive $V\beta11^+$ T cell clones (BP1, BP4, BM5, BM7 and BM12) and substituted Peptide-25 mutants, we determined which amino acid residues within Peptide-25 were critical for TCR recognition (23,24). Peptide-25 contains the motif that is conserved for $I\text{-}A^b$ binding and requires processing by APC to trigger Ag85B-specific $TCRV\beta11^+$ T cells (22). Active immunization of C57BL/6 mice with Peptide-25 can induce the differentiation of $CD4^+$ TCR $V\beta11^+$ Th1 that produce IFN-γ and TNF-α and protect against subsequent infection with live *M. tuberculosis* H37Rv (23).

Here we generate transgenic mice (P25 TCR-Tg) expressing functional TCR that interacts with Peptide-25 in conjunction with $I\text{-}A^b$. We report that naive $CD4^+$ T cells in the spleen of P25 TCR-Tg mice respond specifically to Peptide-25 in the presence of APC from $I\text{-}A^b$ mice and differentiate to Th1 cells in the absence of IFN-γ or IL-12 under neutral conditions.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). STAT1 deficient mice were kindly provided by Dr R. D. Schreiber, Center for Immunology, Washington University School of Medicine. These mice were maintained under specific pathogen-free conditions in our animal facility according to our Institute's guidelines, and used at 8–15 weeks of age.

Cell lines

Five different Peptide-25-reactive $CD4^+$ Th1 clones (BP1, BP4, BM5, BM7 and BM12) were established *in vitro* by culturing lymph node cells from C57BL/6 mice immunized with heat-killed *M. tuberculosis* H37Rv as described (23). TG40 is a variant T cell hybridoma cell line lacking the expression of surface TCR-α and -β chains that has been used a recipient cells for TCR (25). PLAT-E is a packaging cell line that produces retroviruses (26). Chinese hamster ovary cells expressing $I\text{-}A^b$ ($I\text{-}A^b$-CHO) (27) were kindly provided by Dr Y. Fukui (Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan).

Reagents and antibodies

All peptides including Peptide-25 and its substituted mutants were synthesized by Funakoshi Co. Ltd (Tokyo, Japan). Anti-IFN-γ–FITC (XMG1.2), anti-IL-4–allophycocyaninn (11B11), anti-Vβ11–PE (RR3-15), anti-CD4–FITC or -PE (GK1.5), anti-CD8–PE (53.6.72), anti-CD25–FITC (7D4), anti-CD28–FITC (37.51), anti-CD69–FITC (H1.2F3), anti-CD44–FITC (IM7), anti-CD45RB–PE (16A) and anti-LFA1–FITC (2D7) were purchased from BD Biosciences PharMingen (San Diego, CA). Purified anti-CD3 ε (2C11), anti-IFN-γ (R4-6A2) and anti-IL-12 (C17.8) were purchased from BD Biosciences PharMingen.

Subcloning of TCR

Total cellular RNA was isolated from BP1 by using acid guanidinium–phenol–chloroform method. cDNA was synthesized with random hexamer primers and superscript II cDNA kit (GIBCO BRL, Grand Island, NY). 5′-Rapid amplification of cDNA end (5′-RACE) was performed using the 5′-RACE System Ver.2.0 (Life Technologies, Rockville, MD) according to the manufacturer's instructions. The first strand of cDNA was synthesized with gene-specific primer 1 (5′-ATCCATAGCTTTCATGTCCA for TCR α-chain and 5′-GCCATTCACCCACCAGCTCA for TCR β-chain). The first PCR amplification was carried out by using gene-specific primer 2 (5′-GCGAATTCTGAGACCGAGGATCTTTTAACTGGTAC for TCR α-chain and 5′-GCGTCGACTCTGCTTTTGATGGCTCAAAC for TCR β-chain). The second PCR amplification was carried out with nested gene-specific primer (5′-GCGTCGACACAGCAGGTTCTGGGTTCTGGAT for TCR α-chain and 5′-GCGTCGACAAGGAGACCTTGGGTGGAGTCAC for TCR β-chain). The PCR fragment was subcloned in Bluescript SK^+ and sequences by automatic DNA sequencer (ABI PRISM 3700 DNA analyzer, Applied Biosystems, Foster City, CA).

Retrovirus-mediated gene transfer

Full length cDNAs genes encoding the TCR α- and β-chains of BP1 were inserted into a retroviral vector pMX-IRES-GFP vector, pMX-BP1-α and pMX-BP1-β, respectively, and were transfected into a retroviral packaging cell line, PLAT-E with LipofectAMINE Plus Reagent (GIBCO BRL) (28) The cultured supernatant of PLAT-E after 24 h culture was collected, and added to TG40 cells together with DOTAP Liposomal Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) (29). Transfection was monitored by the cell surface expression of TCR by FACS analysis. TG40 cells were transfected with each of plasmids or in their combinations

and selected TG40 cells expressing TCR-αβ (TG40-BP1). TG40-BP1 cell line for expression of CD4 (TG40-BP1/CD4) was established by electroporation of the expressible constructs of full length CD4 cDNA into TG40-BP1 cells by Gene Pulser (Bio-Rad laboratories, Hercules, CA).

Establishment of transgenic mice

The transgenic TCR-α and -β genes were isolated from BP-1 as described in the previous session. The DNA sequences of the PCR products revealed that BP1-TCR-α was composed of Vα5, Jα15 and Cα1, and the TCR-β chain of Vβ11, Jβ2.3 and Cβ2. The pHSE3′ plasmid contains the H-2K^b promoter (provided by H. Pircher), a poly(A) signal from β-globin and the immunoglobulin heavy chain enhancer (30). The full-length BP1 TCR α and β cDNAs were subcloned into the *Sal*I and *Bam*HI sites of the expression vector pHSE3′ plasmid under control of the H-2K^b promoter. The constructs were excised from these plasmids by *Xho*I cleavage for TCR-α chain and *Apa*LI cleavage for TCR-β, and purified by using QIAEX II gel extraction system (Qiagen Inc., Valencia, CA). The purified expression constructs for TCR α and β cDNAs were co-injected into fertilized eggs of C57BL/6 mice. We finally obtained a TCR-Tg line of mice expressing TCR-Vα5-Vβ11 (P25 TCR-Tg). P25 TCR-Tg mice were bred to STAT1 deficient mice (STAT1 deficient P25 TCR-Tg) on a C57BL/6 background in our animal faculty under specific pathogen-free conditions.

Preparation of naive CD4$^+$ T cells and APC

Splenic T cells from either P25 TCR-Tg or littermate C57BL/6 mice were enriched by passing splenocytes through a nylon wool column. To further purify primary CD4$^+$ T cells, the splenic T cells were incubated with a mixture of Microbead-bound monoclonal antibodies that were specific for CD8 (53-6.72), CD49b (DX5), B220 (RA3-6B2) and I-A^b (M5/114.15.2) (Miltenyi Biotec, Bergisch Gladbach, Germany). MEL-14^{high} T cells were purified from splenic CD4$^+$ T cells by positive sorting using MACS after treatment with anti-CD62L (MEL-14)-Microbeads (Miltenyi Biotec) and were used as naive CD4$^+$ T cells. The purity of CD4$^+$ naive T cells was >98%. Splenocytes from wild-type (WT) C57BL/6 mice were incubated with a mixture of anti-Thy1 (30-H12)-Microbeads and anti-CD49b-Microbeads (Miltenyi Biotec) to deplete T cells and NK cells. Cells were then recovered by passage through a MACS column according to the manufacturer's instructions. Recovered cells were irradiated with a total of 3500 Rad, and used as APC. I-A^b-CHO was incubated with 10 μg/ml Peptide-25 for 12 h and extensively washed and incubated with 50 μg/ml mitomycin C for 15 min in 37°C and used as APC in some experiments.

Cell culture

Stably transfected TG40-BP1 or TG40-BP1/CD4 cells (1 × 10^4/culture) were stimulated with various concentrations of peptide in the presence of irradiated spleen cells (5 × 10^5/culture) from various strains of mouse in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark). The cultured supernatants were collected and subjected to ELISA.

To examine Th differentiation *in vitro*, two-step cultures were employed. For the first culture, purified splenic naive CD4$^+$ T cells (5 × 10^5/culture) were activated for 6 days with 10 μg/ml of anti-CD3 or 10 μg/ml Peptide-25 or its substituted mutant in the presence of T- and NK cell-depleted C57BL/6 splenic APC (2.5 × 10^6/culture) in a 48-well plate. In some experiments, we used Peptide-25 loaded I-A^b-CHO (2.5 × 10^5/culture) as APC. For the second culture, the cells collected from the first culture were extensively washed and dead cells were removed by centrifugation through Ficol–Hypaque gradients. The viable primed CD4$^+$ T cells were re-stimulated with 10 μg/ml of anti-CD3 or 10 μg/ml of Peptide-25 in the presence of splenic APC or 1 μg/well of immobilized anti-CD3.

Intracellular cytokine staining and FACS analysis

We identified cytokine-producing cells by cytoplasmic staining with anti-cytokine antibody as previously described (24). First, 2 μM of Monensin (BD Biosciences PharMingen) was added to the secondary culture for the last 4 h of each stimulation. The cells were harvested at 24 h of the secondary culture and stained with 7-amino-actinomycin D and with anti-Vβ11–PE or anti-CD4–PE. The cells were fixed with 4% formaldehyde after washing with 0.05% azide–1% FCS–PBS, permeabilized with 0.1% saponin, and stained with both anti-IFN-γ–FITC and anti-IL-4–allophycocyanin. Isotype-matched control antibodies were also used. The cells stained were gated on live Vβ11- or CD4-positive cells and analyzed on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA).

ELISA

Amounts of IL-2, IL-4 and IFN-γ in the culture supernatant were measured by ELISA. All monoclonal antibodies specific for mouse IL-2, IL-4 and IFN-γ used for capture and detection of cytokines were purchased from BD Biosciences PharMingen. ELISA was performed following the instruction of BD Biosciences PharMingen.

ELISPOT assay

Cytokine producing cells were identified by ELISPOT assay, using the IFN-γ and IL-4 ELISPOT assay kits (R&D Systems, Minneapolis, MN). After naive CD4$^+$ T cells from P25 TCR-Tg mice were cultured with Peptide-25-loaded I-A^b-CHO for 20 h in a 96-well plate coated with capture antibodies, ELISPOT assay was performed following the manufacturer's instructions. Spots were analyzed by KS ELISPOT compact (Carl Zeiss, Oberkochen, Germany).

Results

Analysis of Peptide-25 recognition by reconstituted TCR-αβ pairs

To investigate the functional TCR able to bind a Peptide-25/MHC complex at the clonal level, we first determined the usage of TCR-α and -β chains of Peptide-25-reactive Vβ11$^+$ Th1 clone (BP1) that was of C57BL/6 (I-A^b) mouse origin (23) with the use of 5′-RACE. BP1-TCR α-chain was found to be composed of Vα5 and Jα15 and Cα (Accession No.: AB183189). BP1-TCR β-chain was also identified to be Vβ11, Jβ2.3 and Cβ2 (Accession No.: AB183190).

In order to analyze Peptide-25-recognition by TCR dimers composed of the TCR α- and β-chains of BP1, TCR α- and

β-chain were subcloned into a retrovirus vector and then transfected by retrovirus-mediated gene transfer into a TCR-αβ- and CD4-deficient recipient T cell hybridoma cell line, TG40 (28), and the reconstruction and functional specificity of the TCR was assessed by measuring IL-2 production (Fig. 1). TG40-BP1 produced substantial amounts of IL-2 in response to Peptide-25 plus APC in a dose dependent manner. Enforced expression of CD4 molecules on TG40-BP1 (TG40-BP1/CD4) augmented IL-2 production even upon a lower dose of Peptide-25 stimulation (0.3 μg/ml). TG40-BP1/CD4 produced much more IL-2 than TG40-BP1 when stimulated with higher concentrations of Peptide-25 (10 μg/ml) (Fig. 1A). TG40 transfectants of TCR-α alone or TCR-β alone did not respond to Peptide-25 in the presence of splenic APC (data not shown). These results indicate that recombinant TCR α- and β-chains can reconstruct functional TCR and recognize Peptide-25/I-A^b complex to become IL-2-producing cells.

The specificity of BP1 TCR for Peptide-25 and splenic APC from C57BL/6 mice was examined by culturing TG40-BP1/CD4 with various I-A^b-binding peptides in the presence of APC from different strains of mice. Although we do not show data here, among the various peptides only Peptide-25 could induce IL-2 production by TG40-BP1/CD4 in the presence of splenic APC from C57BL/6 (I-A^b) mice. The 11-mer from Peptide-25 was stimulatory while the 8-mer from Peptide-25 was ineffective. We then stimulated TG40-BP1/CD4 cells with a mutant of Peptide-25 as an altered peptide ligand (APL). The APL preserves those amino acid residues within Peptide-25 essential for I-A^b binding, while one of TCR-binding amino acid residues, glutamic acid at position 248 of Peptide-25, was substituted to alanine, G248A. The APL stimulation at 10 μg/ml of TG40-BP1/CD4 induced marginal IL-2 production, and the stimulatory activity was much lower than with Peptide-25 (Fig. 1B).

We then determined the amino acid sequences for the TCR-α and -β chains of four other Peptide-25-reactive Th1 clones (BP4, BM5, BM7 and BM12). All these Th1 clones responded to Peptide-25 for proliferation and IFN-γ production (23). Analysis of the TCR-α and -β chain amino acid sequences for each clone revealed no obvious differences from BP1 except in the sequence and in the length of CDR3 regions of TCR α- and β-chain (Supplementary table 1, available at *International Immunology* Online). Taking all these results together, the TCR-α5 and -β11 can reconstitute a functional TCR complex that is able to recognize and respond to Peptide-25 when presented in the context of I-A^b. As BP1 is the best Peptide-25-reactive Th1 clone with respect to IFN-γ production in response to Peptide-25, we chose BP1 TCR cDNAs for generating P25 TCR-Tg mice.

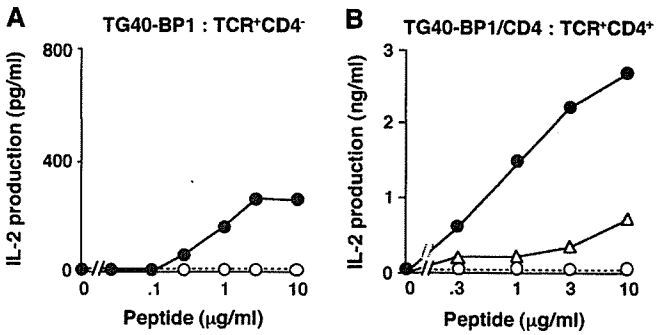

Fig. 1. IL-2 production of TG40 transfectants upon stimulation with Peptide-25. TG40 transfectants retrovirally introduced TCR-αβ of BP1, TG40-BP1 (TCR^+ $CD4^-$) and TG40-BP1 transfectants of CD4, TG40-BP1/CD4 (TCR^+ $CD4^+$) (1 × 10^4 cells/culture) were stimulated with various concentrations of Peptide-25 in the presence (closed circles) or absence (open circles) of irradiated C57BL/6 spleen cells (5 × 10^5 cells/culture) as APC in 96-well microplates. We also stimulated TG40-BP1/CD4 with APL (triangles) in the presence of C57BL/6 spleen cells (5 × 10^5 cells/culture) as APC. After incubation for 24 h, IL-2 in the cultured supernatants were titrated by ELISA.

Generation of Peptide-25-reactive TCR-Tg mice

We then analyzed the clonal basis of preferential Th1 development by single TCR-Tg mice line expressing TCR-α5 and -β11. We constructed transgenes for TCR α5- and β11-chains under the control of the H-2K^b promoter, the poly(A) signal from human β-globin gene and the immunoglobulin heavy chain enhancer. The transgenes were excised from the vector sequences and co-microinjected into fertilized eggs from C57BL/6 mice. Transgenic mice were screened by Southern blot analysis of tail DNA and by staining peripheral blood T cells with anti-Vβ11, followed by FACS analysis. We obtained founder mice expressing $Vα5^+$-$Vβ11^-$, $Vα5^-$-$Vβ11^+$ and $Vα5^+$-$Vβ11^+$ T cells. In the present study, we have mainly analyzed TCR transgenic (P25 TCR-Tg) mice expressing both TCR-Vα5 and -Vβ11.

FACS analysis revealed that >85% of splenic $CD4^+$ T cells from the P25 TCR-Tg mice expressed TCR β11-chain, while 5–7% of splenic $CD4^+$ T cells were $Vβ11^+$ in WT mice (22). Over 98% of splenic $CD4^+$ T cells from the RAG-2 deficient P25 TCR-Tg mice expressed TCR Vβ11-chain. Similar results were obtained by staining splenic $CD4^+$ T cells from P25 TCR-Tg mice with anti-idiotypic antibody (KN7) for the recombinant TCR αβ (A.K. and K.T., unpublished observation). We did not observe any significant $KN7^+$ lymph node cells from transgenic mice expressing TCR α-chain alone or β-chain alone. We compared the expression patterns of LFA-1, CD25, CD28, CD44, CD45RB and CD69 on splenic $CD4^+$ T cells from P25 TCR-Tg mice with those from WT mice. There were no significant differences in the expression pattern or mean fluorescence intensity of these cell surface molecules between the two groups. RT–PCR analysis revealed that T-bet and IFN-γ mRNA expressions were not detected in freshly prepared splenic $CD4^+$ cells of P25 TCR-Tg mice. Taking these results together, $CD4^+$ T cells from P25 TCR-Tg mice are not pre-activated *in vivo*.

Naive $CD4^+$ T cells from P25 TCR-Tg mice are able to differentiate into both Th1 and Th2

Naive $CD4^+$ T cells from P25 TCR-Tg and WT mice were purified from the spleen and stimulated *in vitro* with anti-CD3 in the presence of exhaustively T- and NK cell-depleted irradiated C57BL/6 splenocytes as APC. After 6 days in culture, the proliferated cells were harvested and re-stimulated for another day with anti-CD3 in the presence of APC. After culturing, IFN-γ- and IL-4-producing cells were analyzed by intracellular staining. The cultured supernatants were subjected to ELISA assay for cytokine titration. The results revealed that *in vitro* stimulation of naive $CD4^+$ T cells from

P25 TCR-Tg mice with anti-CD3 induced the propagation of both IFN-γ- and IL-4-producing cells to a similar extent as from WT mice (Fig. 2A). The IFN-γ and IL-4 production were confirmed by ELISA (Fig. 2B). It is also evident from Fig. 2 that P25 TCR-Tg T cells has a higher proportion of IFN-γ-producing cells and IFN-γ production upon anti-CD3 stimulation compared with T cells from WT mice. These results indicate that naive CD4$^+$ T cells from P25 TCR-Tg mice can differentiate into both Th1 and Th2 upon TCR cross-linking.

Induction of naive CD4$^+$ T cells from P25 TCR-Tg mice to Th1 differentiation upon Peptide-25 stimulation

To examine the differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon *in vitro* Peptide-25 stimulation, naive CD4$^+$ splenic T cells were purified from P25 TCR-Tg mice and stimulated *in vitro* for 6 days with Peptide-25 in the presence of T and NK cell-depleted irradiated C57BL/6 splenocytes as APC. The activated cells produced IL-2 and proliferated upon Peptide-25 stimulation in a dose dependent manner in the presence of APC, but they did not produce IL-2 in the absence of Peptide-25 or in the presence of APC from strains of mice other than C57BL/6 mice (data not shown).

In another set of cultures, we stimulated naive CD4$^+$ T cells from P25 TCR-Tg mice *in vitro* with Peptide-25. After 6 days in culture, the proliferated cells were re-stimulated for another day with immobilized anti-CD3. After culturing, IFN-γ- and IL-4-producing cells were analyzed by cytoplasmic staining, followed by FACS analysis. The cultured supernatants were subjected to ELISA for titration of cytokine levels. As a control, we also cultured the cells with APL or medium alone. Naive CD4$^+$ T cells stimulated with Peptide-25 in the presence of splenic APC became solely IFN-γ-producing cells under neutral conditions (Fig. 3A). IFN-γ production was detected on the first day of culture and increased for the rest of the culture period at day 5 (data not shown). IL-4 secretion was not detected even after 5 days of culture. Importantly, stimulation of the cells with APL, in place of Peptide-25, solely induced IL-4-producing cells (Fig. 3B). When we cultured naive CD4$^+$ T cells and splenic APC in the absence of Peptide-25 or APL in the primary culture, cells did not proliferate well (data not shown). These results indicate that naive CD4$^+$ T cells from P25 TCR-Tg mice can be activated leading to proliferation and differentiate solely into Th1 cells upon stimulation with Peptide-25 under neutral conditions.

Roles of IFN-γ/STAT1 and IL-12 signaling in the Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice

It is well known that in addition to the TCR signals IFN-γ and IL-12 play an important role in the Th1 development. To examine whether IFN-γ and IL-12 are required for Th1 development, we

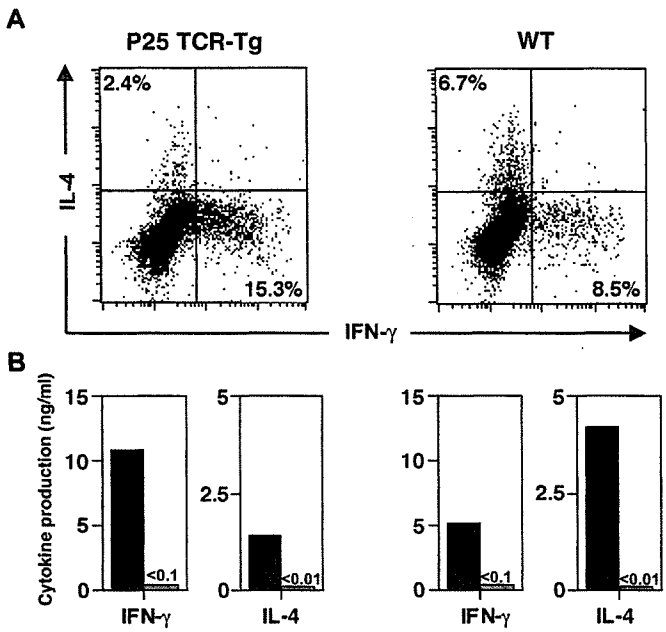

Fig. 2. Induction of Th1 and Th2 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with anti-CD3. Naive CD4$^+$ T cells from P25 TCR-Tg and WT mice were purified and cultured with 10 μg of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for 6 days. (A) After the culture, the cells were washed extensively and re-stimulated with 10 μg/ml of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for another day. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively. (B) After the culture, the cells were washed extensively and re-stimulated with (black bar) or without (hatched bar) 10 μg/ml of anti-CD3 in the presence of T- and NK-cell depleted C57BL/6 splenic APC for another day. IFN-γ and IL-4 in the cultured supernatants were titrated by ELISA.

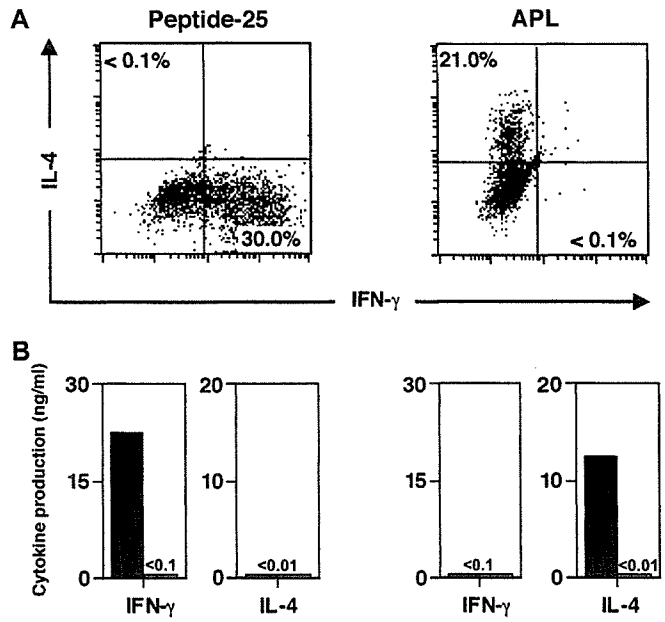

Fig. 3. Induction of Th1 and Th2 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25 and APL, respectively. Naive CD4$^+$ T cells from P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 or APL for 6 days. (A) On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for another day. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively. (B) On day 6, the cells were washed and re-stimulated with (black bar) or without (hatched bar) 1 μg/well of immobilized anti-CD3 for another day. IFN-γ and IL-4 in the cultured supernatants were titrated by ELISA.

cultured naive CD4$^+$ T cells from P25 TCR-Tg mice with Peptide-25 and splenic APC in the presence of anti-IFN-γ, anti-IL-12 or anti-IFN-γ and anti-IL-12 for 6 days. Results revealed that IFN-γ-producing cells were predominantly observed even when cultured in the presence of anti-IFN-γ and anti-IL-12 (Fig. 4). It was also evident that addition of anti-IL-12 partially reduced the proportion of IFN-γ-producing cells without enhancing IL-4-producing cells, while the addition of anti-IFN-γ treatment slightly increased the frequencies of both IFN-γ- and IL-4-producing cells. These results imply that IFN-γ and IL-12 are not essential for Th1 development of CD4$^+$ T cells from P25 TCR-Tg mice in response to Peptide-25. To evaluate further the role of IFN-γ in the Th1 development, we examined the differentiation fate of naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg mice upon Peptide-25 stimulation. This result revealed that Peptide-25-stimulated naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg mice became solely IFN-γ-producing cells after 6 days of culture under neutral conditions (Fig. 5).

Induction of IFN-γ-producing cells upon stimulation of naive CD4$^+$ T cells from P25 TCR-Tg mice with Peptide-25-loaded I-A^b-CHO

To elucidate the mechanism that ensures Th1 differentiation upon TCR stimulation with peptide/MHC, naive CD4$^+$ T cells were stimulated *in vitro* with Peptide-25-loaded I-A^b-CHO for 20 h and assayed for IFN-γ and IL-4 production by ELISPOT assay. IFN-γ-producing cells were induced upon treatment with Peptide-25-loaded I-A^b-CHO stimulation in a dose-dependent manner; however, IL-4-producing spots were not detected. Neither IFN-γ nor IL-4 spots were detected when naive CD4$^+$ T cells from P25 TCR-Tg mice were cultured *in vitro* without Peptide-25-loaded I-A^b-CHO for 20 h. These results indicate that activated CD4$^+$ T cells stimulated with Peptide-25/I-A^b produced IFN-γ in primary culture within 24 h.

To evaluate the role of IFN-γ and IL-12 in Th1 development, naive CD4$^+$ T cells from P25 TCR-Tg mice were stimulated for 6 days *in vitro* with Peptide-25-loaded I-A^b-CHO in the presence of anti-IFN-γ and anti-IL-12. At 24 h after the re-stimulation with immobilized anti-CD3, the frequency of IFN-γ producing cells was 14.5% for the live CD4$^+$ T cells (13% for the live TCRVβ11$^+$ T cells) (Fig. 6), indicating that naive CD4$^+$ T cells can differentiate into Th1 by TCR activation with Peptide-25/I-A^b stimulation even in the absence of IFN-γ and IL-12. In separate experiments, we confirmed IFN-γ-producing cells when CD4$^+$ naive T cells from RAG-2$^{-/-}$ P25 TCR-Tg mice were cultured with Peptide-25-loaded I-A^b-CHO even in the presence of anti-IFN-γ or anti-IL-12. Therefore, direct interaction between Peptide-25/I-A^b and TCR may determine the fate of naive CD4$^+$ T cells for differentiating into Th1 subsets.

Discussion

Peptide-25 is the major antigenic epitope for Ag85B of *M. tuberculosis*, is immunogenic in C57BL/6 (I-A^b) mice, and preferentially induces Vβ11$^+$ Th1 cells. It remains unclear why Peptide-25 can preferentially induce Th1 immune responses in C57BL/6 mice. We approached this question by analyzing naive CD4$^+$ T cells from transgenic mice, whose T cells express functional TCR capable of recognizing Peptide-25 in the context with I-A^b molecules. In the present study we generated TCR-Tg mice for the Th1-inducing peptide, Peptide-25, to elucidate the role of TCR signals in the decision of CD4$^+$ T cells to development into either a Th1 or Th2 cell. Our data support the notion that TCR signals may play a role in the determination of Th1 development under neutral conditions in the absence of IFN-γ or IL-12.

We determined usage of TCR α-chain in five different Peptide-25-reactive Vβ11$^+$ Th1 clones. All Peptide-25-reactive Vβ11$^+$ Th1 clones expressed Vα5, while each clone showed slightly different amino acid sequences in CDR3 regions of both Vα5 and Vβ11 chains (Supplementary table 1). Although each Th1 clone responds to Peptide-25 to a similar extent with regard to proliferation and IFN-γ production, it responds differently to a mutant of Peptide-25 where an amino acid required for TCR-binding had been substituted to alanine (data not shown). However, this may be due to the heterogeneity of the CDR3 regions of both Vα5 and Vβ11 chain. TG40 transfectants (TG40-BP1) expressing α and β chains from the BP1 clone constructed functional TCRs that recognize Peptide-25 in the context of I-A^b on APC resulting in IL-2 production even in the absence of CD4 expression (Fig. 1A). Enforced expression of CD4 in TG40-BP1 enhanced IL-2 production along with a low dose of Peptide-25 stimulation (Fig. 1B), suggesting that the avidity of the TCR and Peptide-25/I-A^b complex is potent enough to trigger TG40-BP1

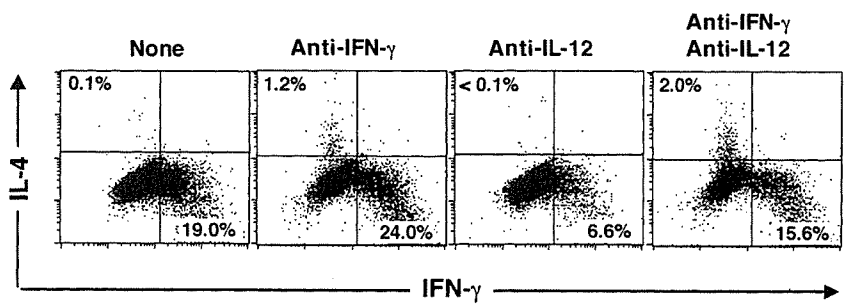

Fig. 4. Effect of anti-IFN-γ and anti-IL-12 on Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25. Naive CD4$^+$ T cells in the spleen of P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 for 6 days. Anti-IFN-γ (10 μg/ml), anti-IL-12 (10 μg/ml) or anti-IFN-γ (10 μg/ml) plus anti-IL-12 (10 μg/ml) were added at the onset of culture. On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for another day in the absence of antibodies. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

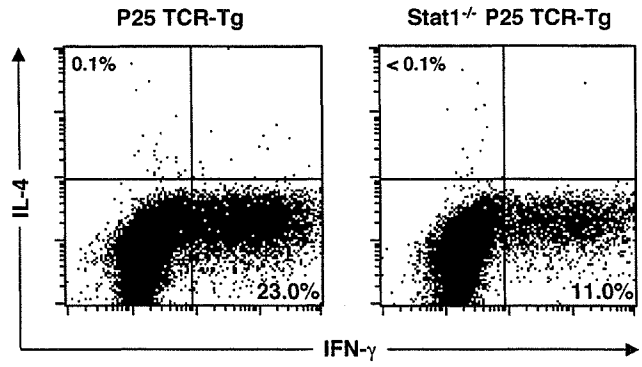

Fig. 5. Induction of Th1 differentiation of naive CD4$^+$ T cells from STAT1 deficient P25 TCR-Tg mice upon stimulation with Peptide-25. Naive CD4$^+$ T cells in the spleen from STAT1 deficient P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 for 6 days. On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for 24 h. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

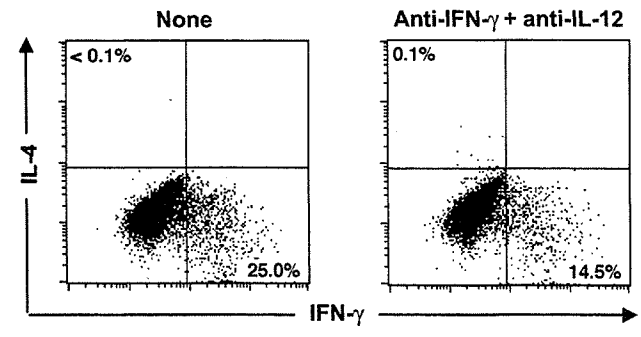

Fig. 6. Induction of Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25-loaded I-A^b-CHO. Naive CD4$^+$ T cells from P25 TCR-Tg mice were stimulated for 6 days *in vitro* with Peptide-25-loaded I-A^b-CHO in the presence or absence of anti-IFN-γ and anti-IL-12. Six days after the culture, the proliferated cells were harvested and re-stimulated with 1 μg/well of immobilized anti-CD3 for 24 h and subjected to cytoplasmic staining for IFN-γ and IL-4. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

transfectants. CD4 expression may facilitate the interaction between TG40-BP1 and APC, resulting in augmented IL-2 production. Intriguingly, the APL could stimulate TG40-BP1/CD4 IL-2 production to a much lesser extent even at higher peptide concentrations (Fig. 1B). As APL fully preserves the I-A^b-binding amino acids of Peptide-25, the APL/I-A^b complex may have lower avidity for the TCR compared with Peptide-25.

Expression profiles of cell surface activation markers on splenic T cells from P25 TCR-Tg mice were similar to these from WT mice, and mRNA expression of neither T-bet nor IFN-γ was observed, suggesting that CD4$^+$ T cells in P25 TCR-Tg mice are not pre-activated. Naive CD4$^+$ T cells from P25 TCR-Tg mice could differentiate into IFN-γ- and IL-4-producing cells upon anti-CD3 stimulation (Fig. 2), indicating that they keep their potential to differentiate into either Th1- or Th2-lineage cells upon TCR ligation. Interestingly, naive CD4$^+$ T cells differentiated solely to IFN-γ-producing cells, but not to IL-4-producing cells upon Peptide-25 stimulation (Fig. 3). This preferential Th1 differentiation induced by Peptide-25 stimulation was also dependent on APC from C57BL/6 mice. As we described, stimulation of naive CD4$^+$ T cells from P25 TCR-Tg mice with Peptide-25 at 10 μg/ml (6.0 μM) preferentially induces Th1 development. In contrast, when we stimulated the T cells with Peptide-25 at 0.1 μg/ml (0.06 μM), we observed a Th2-dominant response (data not shown). These observations are consistent with the published data (31) addressing that IFN-γ production is preferentially induced at 1.6–6.2 μM of OVA peptide in the OVA TCR-Tg mouse model. These results further support the notion that the Peptide-25 has an intrinsically highly potential to induce Th1. Intriguingly, stimulation with APL in place of Peptide-25 induced solely IL-4-producing cells (Fig. 3). When we analyzed APC cell surface marker expression after stimulation with either Peptide-25 or APL, we did not observe an activation-dependent alteration of cell surface marker expression such as CD80, CD86, or CD40 (data not shown). The differences between Peptide-25 and APL regarding Th1 and Th2 differentiation may be due to differences in avidity between Peptide-25/I-A^b and APL/I-A^b to TCR.

Differentiation of naive CD4$^+$ Th precursors to Th1 and Th2 is affected by the manner and environment that they encounter (2,32,33). The strength of interaction between the TCR and MHC/peptide complex affects the lineage commitment of Th cells (15,17,31,34). It is well known that Th1 cell development involves IFN-γ signaling through STAT1 and IL-12 signaling through STAT4 activation (35,36). Peptide-25-induced Th1 differentiation of naive CD4$^+$ T cells from P25 TCR-Tg mice was observed even in the presence of anti-IFN-γ and anti-IL-12 (Fig. 4). We obtained similar results using T cells of STAT1 deficient P25 TCR-Tg mice (Fig. 5). This indicates that both IFN-γ/STAT1 and IL-12 signals are not essential for preferential induction of P25 TCR-Tg naive CD4$^+$ T cells to Th1.

The activation and differentiation of naive CD4$^+$ T cells appears to require at least three separate signals. The first signal is delivered through the TCR/CD3 complex after its interaction with MHC/peptide complex on APC. The second signal is provided by a number of co-stimulatory or accessory molecules on the APC that interact with their ligands on T cells such as CD28/CD80/86, CTLA-4/CD80/86, LFA-1/ICAM-1, OX40/OX40L or ICOS/B7h (37–43). The dose or antigen concentration is also important in determining the Th1-dominated immune response. Third, cytokines such as IFN-γ, IL-12 or IL-18 play a role in the expansion of the committed Th1 cells (10,11,44–46). Stimulation of naive CD4$^+$ T cells from P25 TCR-Tg mice with Peptide-25-loaded I-A^b-CHO in primary culture lead to lower proliferation and cell recovery after culturing compared to stimulation with Peptide-25-loaded splenic APC (data not shown). Interestingly, anti-CD3 stimulation of the T cells, recovered from culture with Peptide-25-loaded I-A^b-CHO, could induce Th1 development preferentially as shown in T cells stimulated with Peptide-25 and splenic APC in primary culture (Fig. 6). As Chinese hamster ovary cells do not express detectable levels of CD80, CD86, ICAM-1, OX40L or B7h, we are in favor of the hypothesis that preferential induction of Th1 development in P25 TCR-Tg naive CD4$^+$ T cells may be independent of these well-known co-stimulating signals from APC.

A complex network of gene transcription events is likely to be involved in establishing an environment that promotes Th1 development. T-bet, a recently discovered member of T-box transcription factor is expressed selectively in thymocytes and Th1 cells, and controls the expression of the hallmark Th1 cytokine, IFN-γ (47). T-bet expression correlates with IFN-γ expression in Th1 and NK cells. Ectopic expression of T-bet both transactivates the IFN-γ gene and induces endogenous IFN-γ production (47). T-bet appears to initiate Th1 lineage development from naive Th cells both by activating Th1 genetic programs and by repressing the opposing Th2 programs (47). It has been reported that T-bet is regulated by IFN-γ signaling through STAT1 activation in the context of TCR ligation (10,11) and induces chromatin remodeling of the *ifn-γ* locus (48). As naive $CD4^+$ T cells are capable of differentiating into IFN-γ producing cells even in the presence of anti-IFN-γ, the interaction between Peptide-25/I-A^b and TCR may directly induce T-bet that leads to Th1 differentiation. We are currently investigating T-bet expression during Th1 differentiation in P25 TCR-Tg naive $CD4^+$ T cells in response to Peptide-25-loaded I-A^b-CHO.

There are several possibilities to account for the immunogenicity and adjuvant activity of Peptide-25 for Th1 development. First, Peptide-25 may activate DCs directly or indirectly through Th cells to enhance expression of co-stimulatory molecules leading to activate Th1 precursors by enhancing well-known transcription factors such as T-bet or unidentified 'master cytokine' for Th1 development. Second, the avidity of Peptide-25 to its specific TCR would be potent enough leading to Th1 development preferentially. Third, Peptide-25 might enhance activation or selection of unidentified T cell subpopulations that suppress GATA-3 leading to Th2 development.

In conclusion, we have presented data showing that naive $CD4^+$ T cells from P25 TCR-Tg mice stimulated with Peptide-25/I-A^b that polarize to Th1 differentiation preferentially in the absence of IFN-γ or IL-12. We propose the hypothesis that direct interaction of the specific antigenic peptide/MHC class II complex and TCR may primarily influence the determination of naive $CD4^+$ T cell fate in development towards the Th1 subset. Therefore, P25 TCR-Tg mice may provide us with new insights and help us understand how Th cell fate is determined.

Supplementary data

Supplementary data are available at *International Immunology* Online.

Acknowledgements

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Abbreviations

APL	altered peptide ligand of Peptide-25
I-A^b-CHO	Chinese hamster ovary cells expressing I-A^b
P25 TCR-Tg	TCR-Tg line of mice expressing TCR-Vα5-Vβ11
TCRVβ11	Vβ11 of TCR
TG40-BP1	TG40 cells expressing TCR-αβ
TG40-BP1/CD4	TG40-BP1 cell line for expression of CD4

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