The role of antigenic peptide in CD4\(^{+}\) T helper phenotype development in a T cell receptor transgenic model

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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-\(\gamma\)/IL-12 signals in Th1 induction, we generated TCR transgenic mice (P25 TCR-Tg) expressing TCR \(\alpha\)- and \(\beta\)-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-\(\gamma\) 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-\(\gamma\) 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-\(\gamma\) and lymphotoxin (TNF-\(\beta\)) 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-\(\gamma\) 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...
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-γ/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 accepted 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 an 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 (TCRβ11) in an I-Ab-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 TCRβ11+ T cells (22). Using Peptide-25-reactive Vβ11+ 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-Aβ binding and requires processing by APC to trigger Ag85B-specific TCRβ11+ T cells (22). Active immunization of C57BL/6 mice with Peptide-25 can induce the differentiation of CD4+ TCRβ11+ 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-Aβ. 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-Aβ 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 as a recipient cells for TCR (25). PLAT-E is a packaging cell line that produces retroviruses (26). Chinese hamster ovary cells expressing I-Aβ (I-Aβ-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–allophycocyanin (11B11), anti-Vβ11–PE (RR3-15), anti-CD4–FITC or -PE (OK1.25), 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′-ATCCATAAGCTTTGGTACAC for TCR α-chain and 5′-GCAATTCCACCCACTGCTCA for TCR β-chain). The first PCR amplification was carried out using gene-specific primer 2 (5′-GCAATTCAGAGGCTGCAATT -CATGTCCA for TCR α-chain and 5′-GGCCATCCACCCACTGCTCA for TCR β-chain). The second PCR amplification was carried out with nested gene-specific primer 1 (5′-GCCGTCACTCTTGATGGCTAAC for TCR β-chain). The second PCR amplification was carried out with nested gene-specific primer 2 (5′-GCCGTCACTCTTGATGGCTAAC for TCR β-chain). The PCR products were subcloned in Bluescript SK+ and sequenced 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

These 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 (30) of the expression vector pHSE3 (provided by H. Pircher), a poly(A) signal from the human immunoglobulin heavy chain enhancer (30). The full-length BP1 TCR α and β cDNAs were subcloned into the SalI and BamHI sites of the expression vector pHSE3 plasmid under control of the H-2Kβ 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 co-injected into fertilized eggs of C57BL/6 mouse. We finally obtained a TCR-Tg line of mice expressing TCR-Vα11, Jα15 and Cα1, and TCR-β with the use of Vβ11, Jβ2.3 and Cβ2 (Accession No.: AB183190).

Preparation of naive CD4+ T cells and APC

Splenocytes 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-Aβ (M5/114,15,2) (Miltenyi Biotec, Bergisch Gladbach, Germany). MEL-14high T cells were purified from splenic CD4+ T cells by positive selection using MACS after treatment with anti-CD62L (MEL-14). MEL-14 high 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-AβCHO 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 x 10^6/culture) were stimulated with various concentrations of peptide in the presence of irradiated spleen cells (5 x 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.

Ex vivo differentiation of CD4+ T cells

For the first culture, purified splenic naive CD4+ T cells (5 x 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 x 10^5/culture) in a 48-well plate. In some experiments, we used Peptide-25 loaded I-Aβ-CHO (2.5 x 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 Ficoll–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–FITC and anti-IL-4–allophycocyanin. Isootype-matched control antibodies were used as well. The cells stained were gated on live Vβ11- or CD4-positive cells and analyzed on a FACS Calibur 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-Aβ-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-Aβ+ mouse origin (23) with the use of 5'-RACE. BP1-TCR α-chain was found to be composed of Vβ5 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-chains of peptides, we used Peptide-25 loaded I-Aβ-CHO (2.5 x 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 Ficoll–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.

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These results indicate that recombinant TCR-α to Peptide-25 in the presence of splenic APC (data not shown). TG40-BP1/CD4 in the presence of splenic APC among the various peptides only Peptide-25 could induce IL-2 production by TG40-BP1/CD4 (TCR+ CD4+) among the various strains of mice. Although we do not show data here, 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 with alanine, G248A. The APL stimulation at 10 \(\mu\)g/ml of TG40-BP1/CD4 induced marginal IL-2 production, and the stimulation 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 *Immunological 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.

**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 transgenics 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 transgenics 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-V\(^{β}\)11, followed by FACS analysis. We obtained founder mice expressing V\(^{α}\)5-\(^{β}\)11, V\(^{α}\)5-\(^{β}\)11 and V\(^{α}\)5-\(^{β}\)11 T cells. In the present study, we have mainly analyzed TCR transgenic (P25 TCR-Tg) mice expressing both TCR-V\(^{α}\)5 and V\(^{β}\)11.

**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 V\(^{β}\)11\(^+\) in WT mice (22). Over 98% of splenic CD4\(^+\) T cells from the RAG-2 deficient P25 TCR-Tg mice expressed TCR-β11-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-β11 (A.K. and K.T., unpublished observation). We did not observe any significant Kn\(^{7}\) 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 vitro.

**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 intracellular 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-Ab-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 TCRV\(^b\)11+ 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 V\(^b\)11+ 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 \(\alpha\)-chain in five different Peptide-25-reactive V\(^b\)11+ Th1 clones. All Peptide-25-reactive V\(^b\)11+ Th1 clones expressed V\(\alpha\)5, while each clone showed slightly different amino acid sequences in CDR3 regions of both V\(\alpha\)5 and V\(^b\)11 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 V\(\alpha\)5 and V\(^b\)11 chain. TG40 transfectants (TG40-BP1) expressing \(\alpha\) and \(\beta\) chains from the BP1 clone constructed functional TCRs that recognize Peptide-25 in the context of I-A\(^\alpha\) on APC resulting in IL-2 production even in the absence of CD4 expression (Fig. 1A). Enhanced 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](https://academic.oup.com/intimm/article-abstract/16/12/1691/657375/1696)
Naive CD4+ T cells from P25 TCR-Tg mice were stimulated for 6 days from WT mice, and mRNA expression of neither T-bet nor IFN-γ in splenic T cells from P25 TCR-Tg mice were similar to these. This may have lower avidity for the TCR compared with Peptide-25. No IL-4-producing cells were observed, suggesting that CD4+ T cells in P25 TCR-Tg mice could differentiate into 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.

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 B7h, we are in favor of the hypothesis 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/A-Ab and APL/A-Ab to TCR.

Expression of cell surface activation markers on splenic T cells from P25 TCR-Tg mice were stimulated for 6 days in vitro with Peptide-25-loaded I-Ab-CHO. Naive CD4+ T cells from P25 TCR-Tg mice were stimulated for 6 days as shown in T cells stimulated with Peptide-25 and APL fully preserves the I-Ab-binding amino acids of Peptide-25, the APL/I-Ab 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.
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-Ab and TCR may directly complex and TCR may primarily influence the determination of TCR-Tg naive CD4+ T cells in response to Peptide-25-loaded antigen presenting cells (I-Ab-CHO). 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-Ab-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 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-Ab-CHO.

In conclusion, we have presented data showing that naive CD4+ T cells from P25 TCR-Tg mice stimulated with Peptide-25/I-Ab which 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.

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Abbreviations
APL altered peptide ligand of Peptide-25
I-Aβ-CHO Chinese hamster ovary cells expressing I-Aβ

References


**Th1 development by antigenic peptide**

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