some patients show high positivity against only the mycoloyl glycolipids (TDM-T and TMM-T), whereas others were positive against only the phospholipid ($Ac\text{-}PIM_2$). Some were positive to TMM-T, but not to TDM-T, indicating that not only the carbohydrate moiety, but also the mycolyl residues or acyl numbers of the trehalose esters was exclusively recognized, as we have already reported for recognition of the mycolic acid structure (24). Recently, lipid-antigen presenting molecules group 1-CD1 (such as CD1a, CD1b and CD1c) and group 2-CD1 (CD1d and CD1e) in human dendritic cells or macrophages were investigated (25–27), and T-cell responses in humans infected with *M. tuberculosis* to CD-1 restricted lipid antigens were reported (28). However, lipid antigen presentation to B-cells in humoral immune responses has not yet been reported. Mycobacterial lipid antigen epitopes may be presented by novel mechanisms different from the classical MHC class I and class II restricted protein antigens.

To improve the ELISA sensitivity, we also evaluated a combination of mycobacterial glycolipids. Combinations of three types of TDM (TDM from *M. tuberculosis* $H_{37}Rv$, *M. bovis* BCG Connaught and *M. avium* complex) and three types of TMM (TMM from *M. tuberculosis* $H_{37}Rv$, *M. bovis* BCG Connaught and *M. avium* complex) as ELISA antigens gave 90% (36/40) positive results in sera from 40 active TB patients (data not shown). Furthermore, an additional combination of diacyl phosphatidylinositol dimannosides and phenol glycolipids (PGL-O and -K) from *M. tuberculosis* Aoyama B surprisingly gave a 97.5% (39/40) positive result (data not shown). This suggests that mycobacterial lipid antigens can be recognized distinctively and individually in sera from TB patients and multiplication of lipid antigens in an ELISA could possibly give better discriminating power serologically.

In a separate paper (29) based on the quantitative analysis of anti-mycobacterial lipid antibodies, we reported that IgG antibody titers in TB patient sera varied greatly according to the stage of the disease. This differs from the TST result, which shows a long-lasting positive once changed to positive from negative.

Patients with a positive smear showed higher positive rates and higher average IgG antibody titers, indicating that IgG antibody responses reflect well the bacterial burden. IgG antibody responses were also related to the severity and size of pathological lesions identified by chest X-ray. In general, patients with larger and more severe pathological lesions showed higher positive rates against all three antigens, while patients with smaller or less severe lesions showed lower rates.

In our study, we tested antibody titers of suspected TB patients, rather than patients in hospital, and therefore subjects were in the early stages of the active disease with minimal clinical symptoms and preliminary chest X-ray results. However, our multiple-antigen ELISA still showed higher positive rates than other serodiagnostic kits, and immune-suppressed patients such as TST-negative and diabetes mellitus-TB patients showed antibody positivity, irrespective of age. Taken together, a multiple-antigen ELISA based on humoral immune responses may be the most useful tool for the rapid diagnosis of active disease due to mycobacterial infection, even for TST-negative cases and subjects in an immunosuppressed state. To establish the most reliable serodiagnosis, tests with higher sensitivity and specificity based on the best combination of antigens need to be identified.

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# 肺結核診断におけるプロテオミクス

*Proteomic and diagnostic analysis of tuberculous antigens*

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Key words
結核感染と発病の診断，ツベルクリン皮内反応，細胞性免疫，体液性免疫，結核菌抗原のプロテオーム解析，ESAT-6ファミリー分泌抗原

## Summary

結核は現在でも人類に蔓延する伝染病の中で最も重要なものの１つであり，AIDSや免疫不全患者では予後が悪く早期の診断・治療・予防が必要である。ツベルクリン反応はきわめて感度の高い皮膚反応で，細胞性免疫の指標として長年用いられてきたが，一方で抗酸菌共通抗原を多数含むため交叉反応性が強く，結核かまたは他の抗酸菌感染症かの鑑別が困難なだけでなく，BCG接種による陽性転化か否かの区別がつきがたい。よって感染防御免疫確立の指標とはならない。結核菌分泌タンパク質抗原のプロテオーム解析が発展し，結核菌主要タンパク質抗原の性質が解明されつつあり，その中に結核ワクチン抗原として有用なもの(HSP-65やAg85Bなど)や結核特異抗原として診断に有用な低分子分泌タンパク質抗原(ESAT-6やCFP-10など)が見出され，これらの刺激によるヒト末梢血リンパ球IFN-γ産生能を測定する特異的な結核感染(細胞性)免疫診断法が開発され，ツベルクリン反応に代わるものとして注目されている。

## はじめに

結核は，ハンセン病と並び人類始まって以来の長い関わりをもつ慢性伝染病で，時代とともに蔓延衰退を繰り返してきた。化学療法発見以前には人類死亡原因の中で最高位を占め，診断・治療・予防のあらゆる面で難病の第１とされた。20世紀後半，衛生環境の改善と抗結核薬の開発により結核罹患率は先進工業国で順調に減少を示しているが，1980年代にAIDS合併症として再び増加傾向を示した。今日結核は，大都市や高齢者，ホームレスなど社会的弱者，医療関係者などデンジャーグループ特定集団に偏在する傾向があり，世界でなお200万人を超える結核死が毎年報告されている。

## Ⅰ　ツベルクリン反応と結核の診断法

結核の診断は，基本的には菌の検出(染色や培養)により行われるが，検出感度や迅速性から考えるとこれらの方法のみでは不十分であり，遺伝子診断や臨床症状により総合的に診断される。免疫学的には結核に感染すると細

胞性免疫が成立するが，それを検出するのにツベルクリン反応(ツ反)が用いられる。Robert Kochは旧ツベルクリン(old tuberculin；OT，結核菌培養濾液を滅菌濃縮したもの)を用いて治療に応用しようとしたが，これは失敗に終わった。その後1907年，von PirquetがOTが結核の診断に役立つことを見出し，その後Mantauxが現在の皮内反応(Mantaux reaction)を始めた。1920年代Seibertらがツ反惹起活性成分がタンパク質抗原であることを示し，糖・核酸成分を除去した精製ツベルクリン(purified protein derivative；PPD)として結核の診断やBCGで誘導される細胞性免疫の診断に用いられるようになった。PPDは結核菌が産生するストレスタンパク質[熱ショックタンパク質(heat shock protein；HSP)やDnaK，GroEL，GroESなど]や分泌タンパク質(α抗原)など，Tリンパ球に対する強い抗原性を示すタンパク質抗原を多数含んでいる。

ヒトではこの抗原液PPDを皮内に接種し，約48時間後にみられる局所の発赤や硬結の程度から結核感染の診断を行うものできわめて高い感度(97%以上)の診断法であるが，BCG接種によっても陽性転化するため，その判別が困難である。またツ反陽性化しても感染防御免疫が成立したことの証拠にはならない。PPDで惹起されるツ反は典型的な遅延型過敏症(delayed-type hypersensitivity；DTH)で，血管拡張，浮腫，単核細胞浸潤がみられ，結核病巣の原型と考えられ，Th1型の$CD4^+$T細胞の分裂増殖と活性化，機能分化が誘導される。感作T細胞の多くのクローンはPPDに含有される数多くのタンパク質抗原に対してMHC (major histocompatibility complex)クラスII拘束性であり，特異的に反応して種々のT細胞サイトカインが産生される。一方，ツ反陽性が結核に対する感染防御免疫確立の証拠となるか否かはきわめて重要な問題である。R. Kochの時代からツ反型のDTH皮内反応は，結核菌に対する免疫応答としてTh1型の反応と認識されていることに変わりはないが，動物実験の結果をみるに限り，DTHと結核感染防御免疫とは必ずしも一致しない。DTHの発現なしに防御免疫の確立はありえないが，DTHの発現があれば防御免疫が十分とはいえない。現在わが国の制度下で実施されるBCG被接種者では最終的に90%近くの中・高校生がツ反陽性となっているにもかかわらず，15～20歳台の若年者集団に感染がみられることを考え併せると，BCG接種によってたとえツ反全てを陽性転化させても結核感染を100%防御することは困難である。わが国のように，BCG接種を制度的に採用している国々での大きな課題は，これまでのツ反が真に結核防御免疫の指標となるのかどうか，ということである。最近，結核感染防御免疫機構が次第に解明されて，結核防御免疫の発見に最も重要なTh1免疫誘導性サイトカインであるIFN-γをPPDに特異的に反応して産生させ，T細胞の分化誘導の程度を定量的に調べる方法が報告された[1)2)]。IFN-γ産生性T細胞の主体は$Th1型CD4^+$T細胞であるが，最近では$CD8^+$細胞にもこの機能をもつものが知られており，IFN-γはマクロファージを活性化し，結核菌が示すファゴソームリソゾーム融合(phagosome-lysosome fusion；P-L fusion)阻害効果の抑制と，iNOS誘導により合成されるNOラジカルがマクロファージ内で結核菌増殖を抑制することから，IFN-γ産生能を診断の指標とすることは意義がある。最近，PPD特異的IFN-γ定量キットが開発され皮内反応に代わるものとして試みられているが[3)]，ここでもPPDの高い交叉反応性が問題となった。

## II 抗酸菌タンパク質抗原の多様性と結核診断

PPDがきわめて多種類のタンパク質抗原を含むことからもわかるように，結核菌はきわめて多数のタンパク質抗原を合成する。しかも菌体内のみならず菌体外に分泌されるタンパク質抗原は，結核感染初期に宿主に免疫反応を惹起させる。結核菌および類縁抗酸菌が産生する代表的なタンパク質抗原を表1に示し，その特徴を述べる。

### 1．65kDa HSP

結核菌をはじめ他の*Mycobacterium*属でも産生されるストレスタンパク質(HSP)で，大腸菌GroELとの相同性が高い。HSPは自然界に広く分布し，分類的に近縁の種の中ではかなりの構造類似性が保たれている。高温などストレス環境で産生量が増え，菌体内では複合体として存在する。分子内にヘリックス構造と疎水性領域を有し，T細胞に対して強い抗原性を示す。抗原エピトープは複数個存在し，結核菌特異的なものとmycobacteria共通のものがあるばかりでなく，哺乳類HSP抗

表1．結核菌(および類縁抗酸菌)の主なタンパク質抗原

| 菌種 | サブユニットサイズ(kDa) | 名称(別名) | 機能 | 免疫学的性質 |
|---|---|---|---|---|
| *M. tuberculosis* | 71 | CIE Ag 63(DnaK) | 熱ショックタンパク質；大腸菌DnaKおよびヒト*hsp*-70と50%以上相似 | マウスおよびヒト抗体と反応；結核患者T細胞と増殖反応，自己反応性抗原 |
| *M. bovis* BCG | 70 | (DnaK) | | |
| *M. leprae* | 70 | (DnaK) | | マウスおよびヒト抗体と反応；結核患者のCTLと増殖反応，γδT細胞により認識される |
| *M. tuberculosis* | 65 | CIE Ag 82(GroEL) | 熱ショックタンパク質；大腸菌GroELおよびヒト*hsp*-60と50%以上相似 | |
| *M. bovis* BCG | 65 | 64-kDa antigen(GroEL) MbaA, CIE Ag 82 | | *M. tuberculosis* complex特異的に患者血清抗体と反応；結核患者およびBCG接種者のT細胞と増殖反応 |
| *M. tuberculosis* | 38 | CIE Ag 78, Pab(PhoS) US Japan Ag 5 | リン酸輸送における"結合タンパク質"；シグナルペプチドリポタンパク質コンセンサスあり；糖タンパク質 | モノクローナル抗体により認識される |
| *M. tuberculosis* | 23 | CIE Ag 62(SodA) | スーパーオキシドジスムターゼ；大腸菌SodAおよびヒトミトコンドリアMnSodと50%以上相似 | |
| *M. leprae* | 28 | (SodA) | | |
| *M. tuberculosis* | 12 | (GroES) | 熱ショックタンパク質 GroELと共同作用 | モノクローナル抗体により認識される。T細胞に対して強い増殖反応誘導 |
| *M. bovis* BCG | 12 | MPB 57, BCG-α (GroES) MCP-1 | | |
| *M. leprae* | 14 | (GroES) | | 結節らいおよびレプロミンテストで反応陽性 |
| *M. tuberculosis* | 30/31 | CIE Ag 85 antigen, P32, MPT 44 | 3～4種の分泌関連タンパク質をコードするmultigeneファミリーを形成；成熟タンパク質ではシグナルペプチドは分解されている。最近Ag85A, B, Cの各タンパク質はミコリルトランスフェラーゼ活性をもつことがわかった | MAbsにより認識される。ハンセン病と結核患者抗体と交叉性，リウマチ性関節疾患患者の関節液とT細胞により認識される。フィブロネクチン結合活性を有し，現在最もワクチン抗原として有望なタンパク質とされる |
| *M. bovis* BCG | 30/31 | CIE AG 85 A, MPB 44 | | |
| *M. bovis* BCG | 30/31 | CIE Ag 85B, MPB 59, α antigen, US Japan Ag 6 | | |
| *M. kansasii* | 30/31 | α Antigen, antigen α 2 | 鉄調節タンパク質：強いシグナル配列 | らい腫型ハンセン病患者血清と反応 |
| *M. leprae* | 30/31 | | | |
| *M. leprae* | 28 | | | |
| *M. bovis* BCG | 23 | MPB 64 | *M. tuberculosis* complexに特有の主要分泌タンパク質 | |
| *M. tuberculosis* | 19 | Lipoprotein | 強力なシグナルペプチドおよびリピド部分をもつ。リポタンパク質 | マウスおよびヒト抗体と反応；患者T細胞と増殖反応 |
| *M. bovis* | 19 | | | |
| *M. leprae* | 18 | L5 | 低分子熱ショックタンパク質群の1種 | (同上) |
| *M. bovis* BCG | 18 | MPB 70 | *M. bovis*の主要分泌タンパク質 | *M. bovis*感染で特異抗体と反応 |
| | | MPB 80 | シグナルペプチド；20～25kDaの変異タンパク質あり：糖タンパク質 | |
| *M. tuberculosis* | | ESAT-6 family antigens | STCF(培養初期分泌タンパク質) | 結核菌群(ヒト型菌および*M. bovis*)に含まれる最も特異的な抗原で感染防御抗原としてAg 85や19kDa抗原と合わせて最も可能性が高い |
| *M. bovis* | 4～6 | | | |
| *M. kansasii* | | ESAT-6(1.7kbp DNA) | early secretory antigenic target-6の略。代表的な低分子T細胞抗原 | |
| | 10.4 | TB10.4 | ESAT-6とともにSTCFに含まれるAla-richタンパク質でBCG，MACにも存在 | |
| | 10 | CFP-10 | ESAT-6 family中，最も強力な細胞性免疫抗原としてIFN-γ誘導活性あり | 結核患者，BCG陽転者と健常者の鑑別が可能 |
| *M. tuberculosis* | 7.3 | TB7.3 | Biotin結合部位あり，pI3.8. ESAT-6 familyには属さず | IFN-γ誘導能はやや低い。結核とBCG陽性の鑑別は困難 |
| *M. bovis* BCG | | | | |
| *M. avium* | | | | |

(文献6，その他より改変)

表2．*Mycobacterium* species内での低分子抗原（*tb7.3*, *tb10.4*および*esat-6*）の分布

| Species | Source | Distribution of the following gene | | | |
|---|---|---|---|---|---|
| | | *tb7.3* | *tb10.4* | *cfp-10* | *esat-6* |
| *M. tuberculosis* H37Rv | ATCC 27294 | + | + | + | + |
| *M. bovis* MNC27 | SSI | + | + | + | + |
| BCG Danish 1331 | SSI | + | + | − | − |
| BCG Tokyo | WHO | + | + | − | − |
| *M. avium* | ATCC 15769 | + | + | − | − |
| *M. intracellulare* | ATCC 15985 | + | + | − | − |
| *M. kansasii* | ATCC 12478 | − | + | + | + |
| *M. marinum* | ATCC 927 | + | + | + | + |
| *M. scrofulaceum* | ATCC 19275 | − | − | − | − |
| *M. fortuitum* | ATCC 6841 | − | − | − | − |
| *M. xenopi* | Isolated from a Danish patient | + | − | − | − |
| *M. szulgai* | Isolated from a Danish patient | + | − | + | + |

（文献9より引用）

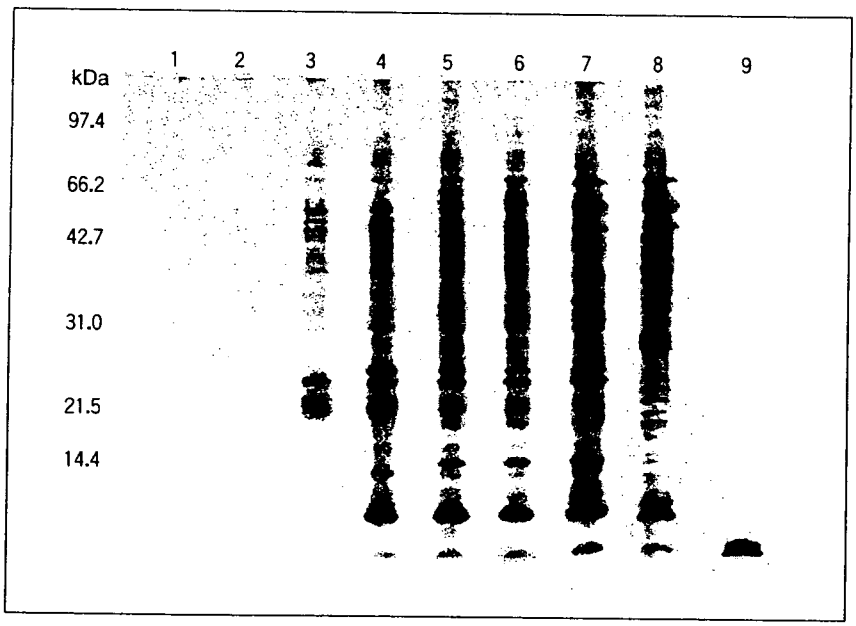


図1．結核菌培養濾液タンパク質のSDS-PAGE分析パターン

*M. tuberculosis*は，0～42日までの種々の期間培養し，上清を濃縮後SDS-PAGEで分離した。染色は銀染色でタンパク質各バンドを検出した。

レーン1（day 0），レーン2（day 2），レーン3（day 3），レーン4（day 5），レーン5（day 7），レーン6（day 14），レーン7（day 42），レーン8（STCF），レーン9（ESAT-6）

（文献5より引用）

原とも共通するエピトープを有することから，アジュバント関節炎やヒトの自己免疫疾患とも関連するのではないかと指摘されている。抗原決定基に非特異的な面が多いことから，ワクチン抗原としてはともかく，診断抗原としては適当でない。

### 2．38kDaヒト型結核菌特異抗原

結核菌の種特異抗原の1つで，診断用抗原としては重要なものの1つである。DanielとAnderson[4]はantigen 5と命名された38kDaタンパク質抗原を単離し，これが糖鎖を含む糖タンパク質抗原であることを明らかにした。このタンパク質は*M. tuberculosis*と*M. bovis*に特異的に産生され，感作モルモットと結核患者のTおよびBリンパ球に増殖反応を引き起こすが，ヒトの皮膚反応ではモルモットと比べて種特異性が低い。それでもこの抗原は，結核感染に対する体液性免疫反応においては分泌抗原としてELISA法では最も反応性が高いとされている。

### 3．Antigen 85 complex（α抗原）

結核菌タンパク質抗原中，最も特徴的な機能をもつ成分の1つで3種の類似タンパク質（85A，85Bおよび85C）からなる。おのおの別個の遺伝子によ

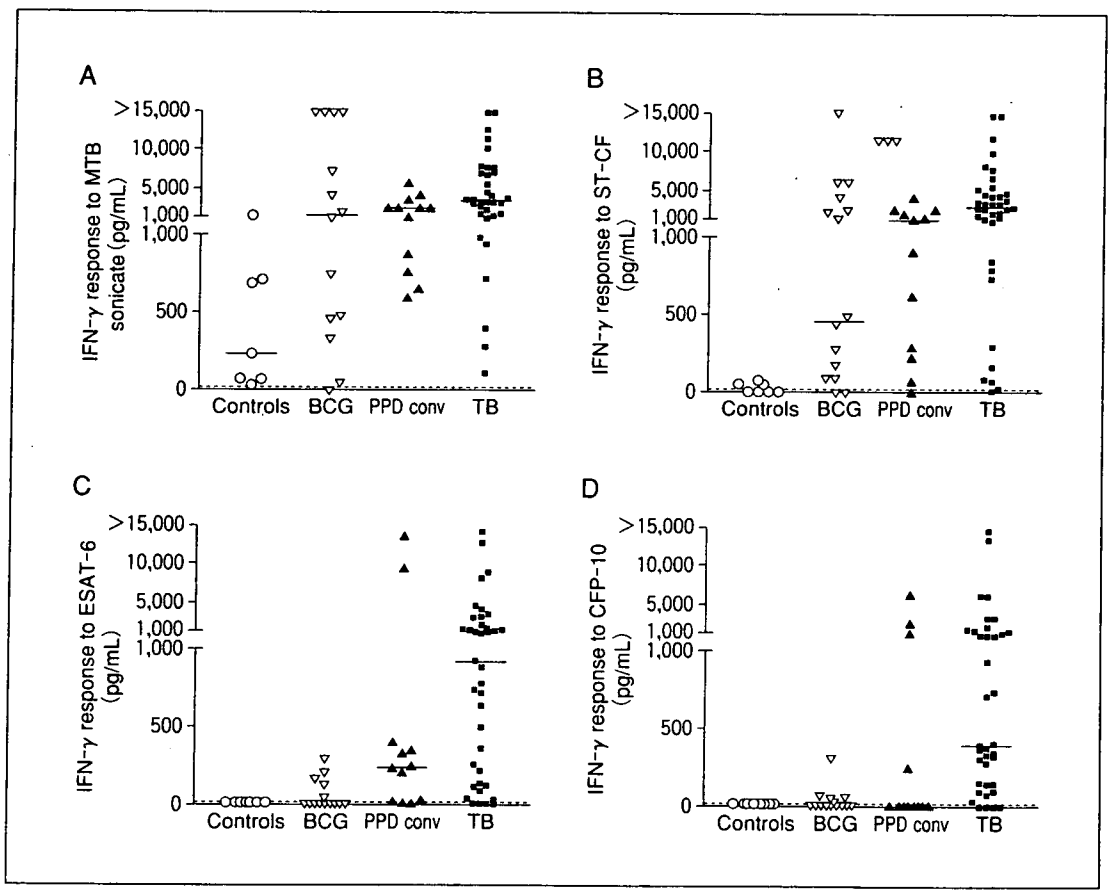


図2．結核患者，PPD陽転者，BCG接種者の結核菌各種抗原に対する細胞性免疫反応性の比較
結核患者（37名），PPD陽転者（12名），BCG接種者（14名，うち10名PPD陽性）および健常者（8名，PPD陰性，BCG非接種）の末梢血PBMCを各抗原で刺激し，上清中のIFN-γを測定してTリンパ球の活性とした。
MTB sonicateに対しては，結核患者（TB），PPD陽転者（PPD conv.），BCG接種者（BCG）のいずれもが同程度反応，ST-CFではBCG接種者の反応性が低下する。ESAT-6ではBCGとPPD conv.の反応性が低下する。CFP-10では，BCG，PPD conv.の反応性が著しく低下し，活動性結核患者のみ陽性となる。
A：MTB超音波処理上清抗原，B：MTB-ST-CF抗原，C：ESAT-6抗原，D：CEF-10抗原，IFN-γ産生
（文献7より引用）

りコードされているがエピトープは共通しており，それぞれのタンパク質の性質も共通点が多い。このうち85B抗原はα抗原(antigen 6, MPB59/MPT59など)と呼ばれ，分子量30kDaで発育菌から分泌され，その産生量も多い。30kDaタンパク質遺伝子は*M. bovis*と*M. kansasii*からクローニングされたが，この抗原は全ての*Mycobacterium*属菌種に存在する他ミコール酸を合成する近縁菌にも広く分布する。種特異的および属共通のエピトープを有し，mycoloyl transferase活性があり細胞壁合成に必須である。30kDaタンパク質は感作動物にはPPD以上に高い特異性で皮膚反応を引き起こし，またIgG抗体も活動性結核の血清診断に有用とされるが交叉反応性は高い。Th1型免疫誘導抗原として結核感染健常者のT細胞と強く反応するが，活動性結核患者のリンパ球との反応は弱く，リンパ球増殖反応の弱い宿主ほど結核が発病しやすく進行しやすい。*in vitro* IFN-γとTNF-α産生を誘導し，これはフィブロネクチン結合を介して起こるとされる。結核ワクチン抗原としては最も強力なものと考えられる。

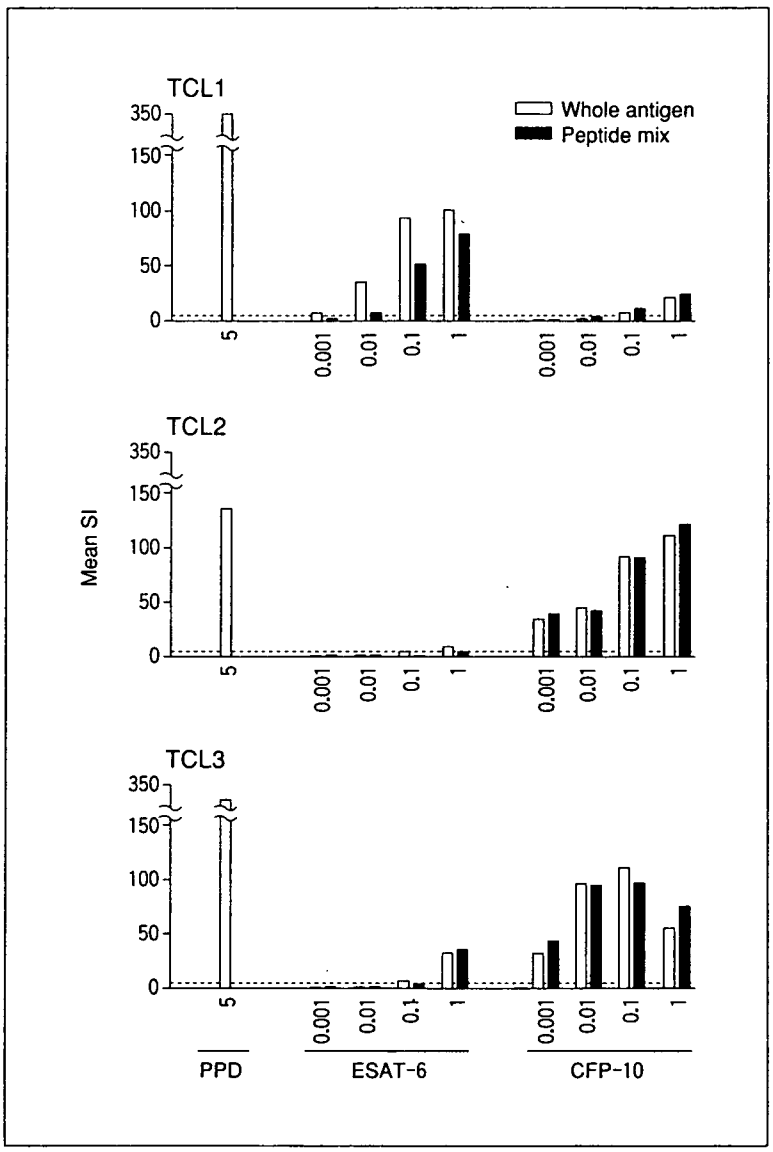


図3．異なった患者末梢血単球からのT-cell line(TCL-1，TCL-2およびTCL-3)のPPD，rESAT-6およびrCFP-10に対する反応性
結核患者由来の結核菌特異的Tリンパ球のPPD，rESAT-6およびrCFP-10各抗原に対する増殖活性は，患者によって異なり，ESAT-6抗原(whole antigenおよびpeptide mixture)に高い反応性を示すもの，CFP-10(whole antigenおよびpeptide mixture)に高い反応性を示すもの，両者に高い反応性を示すものなどさまざまであるが，PPDには普遍的に反応するものが多い。この理由は宿主側の抗原認識様式が遺伝的に支配されており(HLA型など)，抗原の種類によって提示されやすさに個人的な差があるためと考えられる。このことから，混合抗原は単一抗原より高い感度を示すこととなり，したがって多重抗原診断法が有利な理由となる。
(文献8より引用)

**4．低分子分泌タンパク質抗原(表2)**

結核菌(振盪)培養初期に分泌される低分子タンパク質(short term culture filtrate；STCF)は，最近結核の特異的診断用抗原として注目される強力なT細胞エピトープをもつ抗原である(図1)[5)6)]。この代表的なものがESAT-6(early secretory antigenic target-6)で，モノクローナル抗体による分析結果から，これらは分子量が不均一で3～31kDaに及ぶ単純タンパ

表3．結核専門病院を受診した初診患者(210名)の，6種の脂質抗原に対する血清IgG抗体陽性率と喀痰中結核菌排菌量の影響

| Gaffky numbers | Antigens | | | | | | total* |
|---|---|---|---|---|---|---|---|
| | TDM-T | TDM-I | TMM-T | TMM-I | PL-1 | PL-2 | |
| G－0 (n＝25) | 17(68.0%) | 17(68.0%) | 12(48.0%) | 12(48.0%) | 18(72.0%) | 21(84.0%) | 24(96.0%) |
| G(＋1～＋3) (n＝29) | 18(62.0%) | 14(48.3%) | 11(37.9%) | 11(37.9%) | 18(62.0%) | 21(72.4%) | 26(89.7%) |
| G(＋4～＋8) (n＝88) | 62(70.5%) | 47(53.4%) | 67(76.1%) | 48(54.5%) | 63(71.6%) | 68(77.3%) | 81(92.0%) |
| G(＋9 or＋10) (n＝43) | 36(83.7%) | 32(74.4%) | 32(74.4%) | 30(69.8%) | 31(72.1%) | 35(81.4%) | 42(97.7%) |
| Gaffky unknown (n＝25) | 22(88.0%) | 17(68.0%) | 21(84.0%) | 12(48.0%) | 19(76.0%) | 17(68.0%) | 23(92.0%) |
| total (n＝210，100%) | 155(73.8%) | 127(60.5%) | 143(68.1%) | 113(53.8%) | 149(70.9%) | 162(77.1%) | 196(93.3%) |

＊ 6種の抗原(TDM-T，TDM-I，TMM-T，TMM-I，PL-1，PL-2)のうちのいずれか1種以上に陽性となったものを結核(発病)陽性と算定した場合，93.3%の高い陽性率(感度)が得られる。(文献11より引用)

ク質でタンパク質自体の不均一性または重合度差によって区別されるとされる。類似のタンパク質にCFP-10，TB10.4がある。ESAT-6は*M. tuberculosis*および*M. bovis*に存在するRD-1遺伝子にコードされ，BCG株にはRD-1欠損があるため産生されない。*M. kansasii*, *M. marinum*および*M. smegmatis*には遺伝子が存在し，TB10.4，CFP-10とともにタンパク質の発現もみられる。これまで用いられてきたツ反は，結核感染による陽性反応かBCG接種による陽転かの鑑別は不可能であるが，このような結核菌特異抗原を用いた細胞性免疫反応を指標とすることにより，結核患者(発病者)，結核感染者(未発病者)およびBCG陽転者の正確な診断が可能となるものと思われる(**図2**)[7)-9)]。QuantiFERON® TB-2Gなどの測定用キットが最近開発された。ただ，さらに残った問題は，このような精製タンパク質抗原を用いた場合，これらの単独抗原に対する宿主(ヒト)免疫応答がHLA型などにより異なることで(**図3**)[9)]，診断感度を高めるためには複数抗原を組み合わせた多重抗原診断法が必要になるものと考えられる。

## おわりに

最近，タンパク質抗原と比べて結核菌表層脂質抗原に対する抗体反応が活動性結核の診断に有用であることが報告されつつあり(**表3**)[11)]，発病や化学療法による治療経過によく一致することとMHC拘束性をもたないことから，反応は普遍性が高く，結核の診断にはプロテオミクスに基づく抗原解析と併せてリピドミクス解析による診断法が発展することも期待される。

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Research paper

# Expression mapping using a retroviral vector for $CD8^+$ T cell epitopes: Definition of a *Mycobacterium tuberculosis* peptide presented by H2-$D^d$

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**Abstract**

Identification of $CD8^+$ T cell epitopes is important because detection of specific $CD8^+$ T cells after infection or immunization requires prior knowledge of epitope specificity. Furthermore, identification of $CD8^+$ T cell epitopes permits the development of specific preventive and therapeutic approaches to both infections and tumors. Thus far, $CD8^+$ T cell epitopes have been identified either using an overlapping peptide library covering an entire protein, or using algorithms designed to identify likely peptides that bind to major histocompatibility complex (MHC) class I molecules. The synthesis of overlapping peptides can be prohibitively expensive, and the algorithm programs used to predict $CD8^+$ T cell epitopes are not always accurate. Here we describe a retroviral expression system that specifically allows longer polypeptides and shorter peptides to be expressed in the cytoplasm, and thereby to be processed onto class I MHC molecules. T cells from mice that were immunized with a DNA vaccine encoding MPT-51 were probed against MHC-compatible cell lines retrovirally transduced with overlapping gene fragments encoding 120–140 amino acids of the MPT-51 molecule. After further testing of shorter peptide sequences, we identified a $CD8^+$ T cell epitope using cell lines expressing a relatively small number of algorithm-predicted candidate epitopes. We found that one of the requirements for cell surface display of the 20-mer peptide was the need for cotranslational ubiquitination. The restriction molecule was identified as $D^d$ following transduction with MHC class I genes followed by transduction with the oligonucleotide encoding the epitope. The retroviral expression system described here is cost-effective, particularly if the target molecule is large, and could be adapted to identifying T cell epitopes recognized in infectious disease and against tumor cell antigens.


*Keywords:* Epitope mapping; $CD8^+$ T cells; Retroviral expression system; Algorithm

*Abbreviations:* aa, amino acid; CTL, cytotoxic T lymphocytes; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot assay; 10FCS, 10% heat-inactivated fetal calf serum; HRP, horseradish peroxidase; MHC, major histocompatibility complex; mAb, monoclonal antibody; PBS, phosphate-buffered saline; RT, reverse transcription; TAP, transporter associated with antigen processing.

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## 1. Introduction

$CD8^+$ cytotoxic T lymphocytes (CTL) play a pivotal role in protective immunity against infection with intracellar pathogens, such as certain bacteria and viruses (Uchijima et al., 1998; Flynn and Chan, 2001; Gulzar and Copeland, 2004). During an infection, many proteins derived from the pathogen are secreted or produced in the host cells. These proteins are processed by the MHC class I pathway. The endogenous MHC class I pathway targets cytosolic proteins, either self-derived or pathogen-derived, that are processed to peptides by the proteasome. The resulting peptides are actively transported into the endoplasmic reticulum by transporter associated with antigen processing (TAP) molecules, and are finally presented to CTL in the context of MHC class I molecules (Ortmann et al., 1994; Heemels and Ploegh, 1995). Following CTL recognition of pathogen-derived MHC/peptides complexes on the cell surface, the infected cells are killed and infection eliminated by the secretion of effector molecules, such as perforin, granzyme, and interferon-γ (Barry and Bleackley, 2002).

Although intracellular pathogens possess a large number of peptides which have the potential to bind to MHC class I molecules, the $CD8^+$ T cell response is more limited, with only a minority of the potential binding motif-containing peptides from a given foreign protein being actually immunogenic (Sercarz et al., 1993; Barber and Parham, 1994). Various factors have been implicated in this limitation (epitope selection): (1) antigen processing by the proteasome (Eggers et al., 1995; Niedermann et al., 1996); (2) TAP-dependent peptide transport (Lauvau et al., 1999); (3) affinity of the peptide for the MHC (Chen et al., 1994; Sette et al., 1994); (4) the transport of MHC/peptide complexes to the cell surface (Levitsky et al., 1997); and (5) the response of the T-cell repertoire (Connolly, 1994; Cao et al., 1996).

To better understand $CD8^+$ T cell responses during infection, it is important to identify epitopes that are recognized by $CD8^+$ T cells. Identification of the epitopes, which typically consist of 8–10-mer peptides, is essential to detect specific $CD8^+$ T cells by the tetramer assay (Altman et al., 1996), intracellular cytokine staining, or the enzyme-linked immunosorbent spot assay (ELISPOT). In addition, identification of these epitopes permits the development of specific preventive and therapeutic approaches in both infectious disease and cancer (Nagata et al., 2004; Chen et al., 2004; Faure et al., 2004; Romero et al., 2004).

Overlapping peptide libraries have been widely employed for the identification of T cell epitopes. The libraries, consisting of 15–20-mer peptides overlapping by at least nine residues, span the whole protein of interest (Van der Zee et al., 1989). Once a T cell epitope has been mapped in a 15–20-mer peptide, the minimal T cell epitope can be delineated by preparing a set of peptides, which have been truncated from the N- or C-terminal end of the known active peptide. This is a very powerful method for mapping T cell epitope(s), but it requires numerous rounds of peptide synthesis, which can be prohibitively expensive for a large protein.

Recently, a number of programs that utilize algorithms that have been useful in predicting T cell epitopes for peptide mapping studies have been developed (Parker et al., 1994; Rammensee et al., 1999; Reche et al., 2002). These programs list and rank the possible candidates of T cell epitopes within a given protein sequence. However, it has been shown that these program methods are not infallible, and in some cases are no better than the use of the simple anchor residue motifs (Andersen et al., 2000). In general the immunogenicity of individual peptides predicted by such programs must be confirmed empirically, using any number of T cell assays, although some programs have incorporated various factors involved in the epitope processing (Peters et al., 2003). Currently, the combination of an overlapping peptide library with an epitope prediction program seems to be the most comprehensive and effective method for identification of $CD8^+$ T cell epitopes within a protein (Suzuki et al., 2004).

In this report, we describe a novel cost-effective retroviral expression method for the identification of $CD8^+$ T cell epitopes within antigens of interest. As a model antigen, we used MPT51, a major secreted protein of *Mycobacterium tuberculosis* (Wilson et al., 2004), since we recently demonstrated that MPT51 can induce T-cell-mediated immune responses and protective immunity upon challenge with *M. tuberculosis* (Miki et al., 2004). The retroviral expression system makes it possible to express large DNA

fragments in cells so that the location of $CD8^+$ T cell epitopes can be rapidly identified without preparing numerous synthesized peptides. Minimal $CD8^+$ T cell epitopes can be identified from the candidate DNA fragments using a computer-assisted algorithm. This method is particularly useful for mapping epitopes from large target molecules where the cost of synthesizing peptides is particularly high.

## 2. Materials and methods

### 2.1. Mice

BALB/c mice (Japan SLC, Hamamatsu, Japan) were kept under specific pathogen-free conditions and fed autoclaved food and water ad libitum at the Institute for Experimental Animals, Hamamatsu University School of Medicine. Two-month-old female mice were used in all experiments. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

### 2.2. Cell lines

P815 mastocytoma cell line ($H2^d$) and BW5147 T lymphoma cell line ($H2^k$) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (RPMI/10FCS) in a humidified atmosphere of 5% $CO_2$.

### 2.3. Construction of a plasmid DNA vaccine, pCI-MPT51

The DNA encoding the mature MPT51 molecule (*D26486*) was amplified from a plasmid, pMB49 (Ohara et al., 1995), by PCR with following primer pairs: forward primer with ATG starting codon, 5′-CCTCTAGAATGGCCCATACGAGAACCTGA-3′; reverse primer with stop codon, 5′-CAGGCTCTAGACATCGGCACCTGGCTTAGC-3′ (underlined nucleotides indicate *Xba*I site). The PCR fragment was digested with *Xba*I and inserted into the *Xba*I site located downstream to the CMV immediate-early enhancer/promoter region of an expression plasmid pCI (Promega, Madison, WI). The nucleotide sequence was confirmed by automated DNA sequencing (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA) using a dye primer cycle sequencing kit (Applied Biosystems).

### 2.4. Construction of retroviral expression library

Three overlapping DNA fragments encoding fragments of the 266-amino acid (aa) mature MPT51 molecule of *M. tuberculosis* were generated from pCI-MPT51 by PCR amplification using the following primer pairs: for MPT51-F1 encoding aa 1–140, 5′-CCTCTAGAATGGCCCATACGAGAACCTGA-3′ (forward primer) and 5′-GTTCTAGATTAGTACAAAAAGCCCGACATC-3′(reverse primer); for MPT51-F2 encoding aa 78–198, 5′-ACTCTAGAATGGATGGCAGCAAGCAGTG-3′ and 5′-CCTCTAGATTACGGGTCCACACCCACACC-3′; and for MPT51-F3 encoding aa 135–266, 5′-CTTCTAGAATGTCGGGCTTTTTGTA-3′ and 5′-CAGGCTCTAGACATCGGCACCTGGCTTAG-3′ (the forward primers have the ATG starting codon, while the reverse primers contain the TAA stop codon; underlined nucleotides indicate the *Xba*I site). The PCR fragments were digested with *Xba*I and inserted into the *Xba*I site of pBluescript II SK(−) (Stratagene, La Jolla, CA), the orientation of DNA fragments was confirmed by DNA sequencing, and then *Xho*I–*Not*I fragments were cloned into the retroviral expression vector, pDON-AI (Takara Bio, Tokyo, Japan), the multiple cloning site of which had been modified by us. Furthermore, DNA fragments encoding aa 1–100 of the mature MPT51 were amplified by PCR as 40 aa peptide stretches overlapping by 20 residues. The primer pairs used were as follows: for MPT51 1–40, 5′-CCTCTAGAATGGCCCATACGAGAACCTGA-3′ and 5′-CCTCTAGATTAATCCGGGCCGGCGTTGAAG-3′; for MPT51 21–60, 5′-CCTCTAGAATGTTCCTAGCCGGTGGGCCGC-3′ and 5′-CCTCTAGATTACGAAATCCC CTTGCCCGCC-3′; for MPT51 41–80, 5′-CC TCTAGAATGGTCAGTAACTGG GTCA CCG-3′ and 5′-CCTCTAGATTAGCTGC CATCCTGCTCCCAG-3′; for MPT51 61–100, 5′-CCTCTAGAATGGTGGTGGCA CCGGCCGGTG-3′ and 5′-CCTCTAGATTAGCCCCGGTTAGCGGC-CAGC-3′. The resultant PCR products were inserted into pDON-AI after cloning into pBluescript II SK(−) as described above.

Forward and reverse primers for MPT51 1–20, MPT51 21–40, and MPT51 41–60 were synthesized, annealed, and then inserted into the *Xba*I site of pDON-AI. The nucleotide sequences of the oligonucleotides were as follows:

MPT51 1–20 (forward) 5′-CTAGAATGGCCCCRACGAGAACCTGATGGTGCCCAGCCCCAGCATGGGCAGGGACATCCCCGTGGCCTAAT-3′;
MTP51 1–20 (reverse) 5′-CTAGATTAGGCCACGGGGATGTCCCTGCCCATGCTGGGGCTGGGCACCATCAGGTTCTCGTAGGGGGCCATT-3′;
MTP51 21–40 (forward) 5′-CTAGAATGTTCCTGGCCGGCGGCCCCCACGCCGTGTACTTGCTGGACGCCTTCAACGCCGGCCCGACTAAT-3′;
MPT51 21–40 (reverse) 5′-CTAGATTAGTCGGGGCCGGCGTTGAAGGCGTCCAGCAGGTACACGGCGTGGGGGCCGCCGGCCAGGAACATT-3′;
MPT51 41–60 (forward) 5′-CTAGAATGGTGAGCAAGTGGGTGACCGCCGGCAACGCCATGAACACCCTGGCCGGCAAGGGCATCAGCTAAT-3′;
MPT51 41–60 (reverse) 5′-CTAGATTAGCTGATGCCCTTGCCGGCCAGGGTGTTCATGGCGTTGCCGGCGGTCACCCAGTTGCTCACCATT-3′.

In some experiments, the ubiquitin gene was fused to the 5′ side of the above DNA fragments encoding the 20-aa length peptides. The ubiquitin gene was amplified from mouse cDNA with a forward primer (5′-TCACTAGTATGCAGATCTTCGTG AAGAC-3′; underline indicates the *Spe*I site) with the ATG starting codon and a reverse primer (5′-AATCTAGA CACCACACCTCTCAGGCGA AG-3′; underline indicates the *Xba*I site). Using the reverse primer, a glutamine residue at aa number 76 ($G_{76}$) normally present in the ubiquitin protein was changed to a valine ($V_{76}$) to avoid cleavage with deubiquitinase. After digesting the PCR product with *Spe*I and *Xba*I, the ubiquitin gene was inserted into pBluescript II SK(−) and then the DNA fragments encoding the MPT51 20-mers were inserted in the *Xba*I site to obtain ubiquitin/MPT51 20-mer fusion products. The fused product was cut out with *Xho*I and *Not*I, and inserted in the *Xho*I–*Not*I site of a retroviral expression vector, pDON-AI. H2-$K^d$, H2-$D^d$, and H2-$L^d$ cDNA fused with the EGFP gene were also cloned into a retroviral vector.

The nucleotide sequences of the recombinant plasmids were confirmed by DNA sequencing using an ABI PRISM 310 genetic analyzer. Large-scale purification of expression vectors was achieved using a Qiagen Plasmid Mega kit system (Qiagen, Chatsworth, CA) and endotoxin was removed by Triton X-114 phase separation.

### *2.5. Retroviral infection and selection of G418-resistant transformants*

Retroviral supernatant was generated by transfecting recombinant pDON-AI proviral constructs into the Phoenix ecotropic packaging cell line (purchased from the American Type Culture Collection, Manassas, VA, and used with the permission of Dr. G.P. Nolan, Stanford University School of Medicine, CA) according to Dr. Nolan's protocol (Grignani et al., 1998). A confluent 100-mm plate of retrovirus-producing cells was maintained in DMEM/10FCS. After 2 days, the culture supernatant contain-

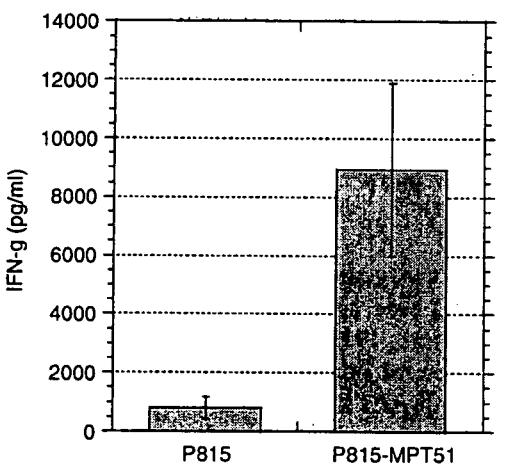


Fig. 1. P815 cells retrovirally transduced with gene encoding mature MPT51 stimulate splenocytes from BALB/c mice following gene gun immunization with pCI-MPT51. The immune splenocytes were stimulated with either wild-type P815 cells or MPT51-expressing P815 cells for 24 h and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

ing the retrovirus was used to infect P815 cells in the presence of 8 μg/ml polybrene (Sigma-Aldrich Japan, Tokyo, Japan). After 16 h, the P815 cells were washed in phosphate-buffered saline (PBS) and cultured in the presence of 400 μg/ml G418. After 2–3 weeks, surviving stable transformants were obtained and used for the study.

### 2.6. Semi-quantitative reverse transcription (RT) PCR

RT-PCR was performed as described previously (Yoshida et al., 1995). Total RNA was prepared from the G418-resistant transformants using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Single-stranded cDNA was synthesized with SuperScript II reverse transcriptase (Gibco BRL) and then used for PCRs. The PCR products were separated by 8% polyacrylamide gel electrophoresis and visualized by staining with ethidium bromide (Sigma-Aldrich Japan). The images were recorded using a densitograph AE-6900M (ATTO, Tokyo, Japan). The sequences of the primers used in this study were as follows:

Primer pairs for MPT51-F1, MPT51-F2, and MPT51-F3 were described above.

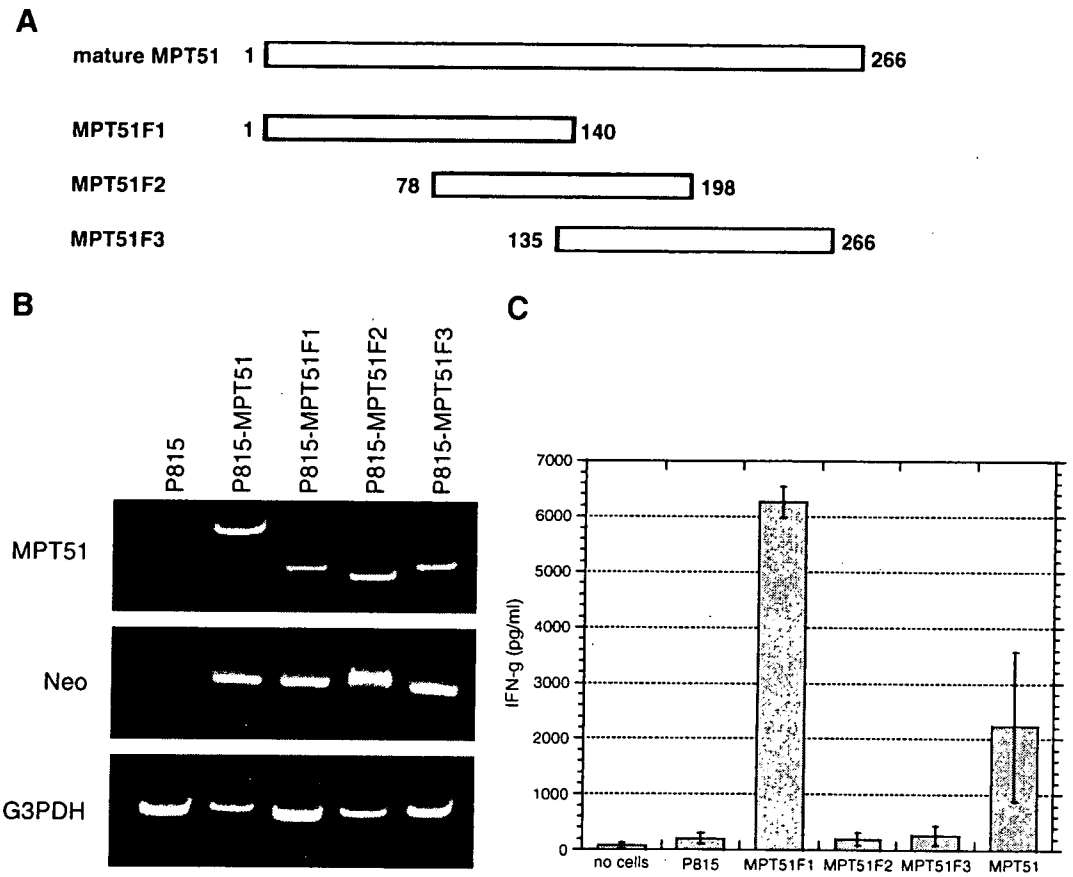


Fig. 2. Identification of fragments of a portion that contains a T cell epitope(s). (A) Regions of MPT51 protein, F1, F2, and F3 that were cloned into the retroviral expression vector. The numbers next to the indicated fragments correspond to the amino acid numbers of mature MPT51 protein. (B) P815 cells were infected with retroviruses encoding the indicated MPT51 fragments and G418-resistant stable transformants were obtained. Then, mRNA expression of the MPT51 gene, and MPT51F1, MPT51F2, and MPT51F3 gene fragments in the transformants were assessed by RT-PCR analysis. *Neo* and G3PDH mRNA expression were also assessed by RT-PCR. (C) The immune splenocyes were stimulated with either wild-type P815 cells or MPT51 fragment-expressing P815 cells for 24 h, and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

Neo forward 5′-CGACCTGTCCGGTGCCCT-GAATGAA-3′;
Neo reverse 5′-GTCCCTTCCCGCTTCAGTGA-CAACG-3′;
G3PDH forward 5′-ACCACAGTCCATGCCAT-CAC-3′;
G3PDH reverse 5′- TCCACCACCCTGTT-GCTGTA-3′.

### 2.7. Immunization of mice

Mice were immunized by employing a gene gun system with a plasmid DNA vaccine encoding the mature MPT51 molecule. For DNA immunization with the Helios gene gun system (Bio-Rad Laboratories, Hercules, CA), DNA-coated gold particles were prepared according to the manufacturer's instruction manual. The gold particles were coated with plasmid DNA in a ratio of 0.5 mg gold particles/1 μg DNA. To immunize the mice, the shaved abdominal skin area was wiped with 70% ethanol. The spacer of the gene gun was held directly against the abdominal skin, and the device was discharged at a helium discharge pressure of 400 psi. Each mouse received two immunizations containing 1 mg of plasmid DNA-coated gold particles in total.

### 2.8. Preparation of splenocyte culture supernatants for evaluation of IFN-γ production

Spleen cell suspensions ($1\times10^6$ cells/well) from mice immunized with a DNA vaccine encoding the MPT51 protein were stimulated by P815 stable

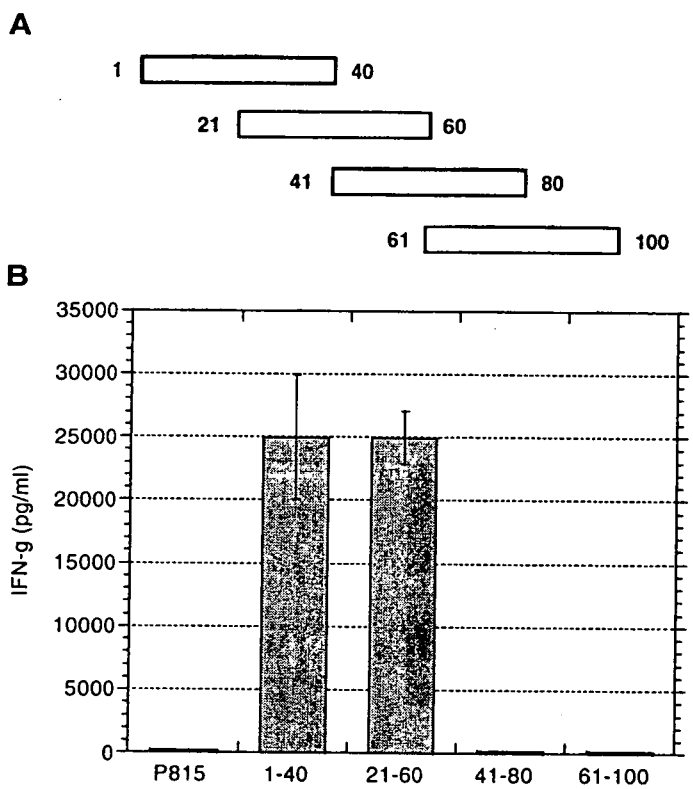


Fig. 3. MPT51 1–60 contains a T cell epitope(s). (A) Four 40-mer fragments of MPT51 overlapping 20 aa were cloned into the retroviral expression vector. (B) The immune splenocytes were stimulated with the P815 cells expressing the indicated 40-mer and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

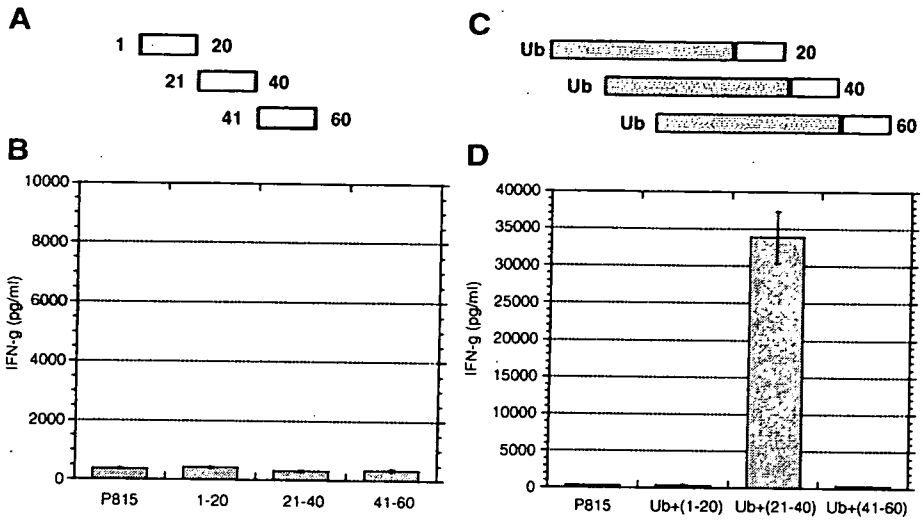


Fig. 4. Epitope expression on the 20-mer of MPT51 requires ubiquitination. (A) Three 20-mer fragments of MPT51 were inserted into the retroviral vector. (B) The immune splenocytes were stimulated with the P815 cells expressing the indicated 20-mer, and IFN-γ concentrations were measured by ELISA. (C) Construction of cotranslational ubiquitination of the 20-mer fragments. (D) The immune splenocytes were stimulated with the P815 cells expressing the ubiquitinated 20-mer and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

transformants ($2.5\times10^4$ per well) in RPMI/10FCS in 96-well plates at 37 °C in 5% $CO_2$. The IFN-γ concentration was measured by a sandwich enzyme-linked immunosorbent assay (ELISA).

### 2.9. Quantification of IFN-γ with cytokine ELISA

IFN-γ production was measured by ELISA. The 96-well ELISA plates (EIA/RIA Plate A/2; Costar, Cambridge, MA) were coated with 2 μg/ml capture antibody (anti-murine IFN-γ monoclonal antibody (mAb) R4-6A2; BD PharMingen, San Jose, CA) at 4 °C overnight, washed with PBS containing 0.05% Tween-20, and blocked with Block Ace (Dainippon Seiyaku, Tokyo, Japan) at 37 °C for 2 h. After washing, the culture supernatant to be tested and IFN-γ standards were added to the plates and incubated at 4 °C overnight. After further washing, 0.5 μg/ml biotin-labeled anti-murine IFN-γ detection mAb (XMG1.2; BD PharMingen) was added to the plates, which were then incubated at room temperature for 1 h. After washing the plates, 0.1 μg/ml horseradish peroxidase (HRP)-conjugated streptavidin (Vector Laboratories, Inc., Burlingame, CA) was added, followed by a 30-min incubation at room temperature. After washing, bound HRP-conjugated streptavidin was detected using the substrate 3-, 3′-, 5-, 5′-tetramethylbenzene dihydrochloride (Sigma-Aldrich Japan). After 5 min, the enzyme reaction was stopped by adding 2 M $H_2SO_4$, followed by measuring the absorbance at 450 nm using an EZS-ABS Microplate Reader (Iwaki, Tokyo, Japan).

### 2.10. Analysis of $CD8^+$ T cells using H2-$D^d$–peptide tetramer complexes

An H2-$D^d$–peptide tetramer complex was kindly supplied by the NIH Tetramer Facility. Spleen cells

Table 1
Candidates of T cell epitope in MPT51 21–40

| | Prediction score (9-mer) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | SYPEITHI | | BIMAS | | | RANKPEP | | |
| | $K^d$ | $L^d$ | $K^d$ | $D^d$ | $L^d$ | $K^d$ | $D^d$ | $L^d$ |
| 21–29 | | | | 0.4 | | 25.0 | | |
| 23–31 | 16.0 | 15.0 | 57.6 | 20.0 | 5.0 | 10.0 | 103.0 | 53.0 |
| 24–32 | 13.0 | 12.0 | 48.0 | 400.0 | 7.5 | | 76.0 | 40.0 |
| 25–33 | | 13.0 | | | | | 35.0 | 48.0 |
| 27–35 | | | | 0.4 | 10.0 | | | |
| 29–37 | 20.0 | | 120.0 | | | 34.0 | | |

from immunized mice were treated with ACK lysis buffer for 5 min at room temperature to remove RBC and washed twice with RPMI-1640 medium and resuspended in the RPMI/10FCS. $1\times10^6$ cells were stained with phycoerythrin-conjugated H2-$D^d$–peptide tetramer complexes, fluorescein isothiocyanate-conjugated anti-CD8 (53-6.7; BD PharMingen), and CyChrome-conjugated anti-CD4 (RM4-5; BD PharMingen) mAbs for 0.5 h at 4°C. After washing, the cells were resuspended in PBS containing 0.1% sodium azide and 1% bovine serum albumin, and then analyzed on an EPICS digital flow cytometer (EPICS XL; Beckman Coulter, Miami, FL).

## 3. Results

### *3.1. P815 cells retrovirally transduced with MPT51 can stimulate MPT51 immune splenocytes from BALB/c mice*

We examined whether MPT51-transduced P815 cells were capable of stimulating splenocytes from BALB/c mice immunized with pCI-MPT51. The immune splenocytes were stimulated with MPT51 gene-transduced or nontransduced P815 for 24 h, and the IFN-γ concentrations in the culture supernatants were determined by ELISA. As shown in Fig. 1, robust IFN-γ production was observed after stimula-

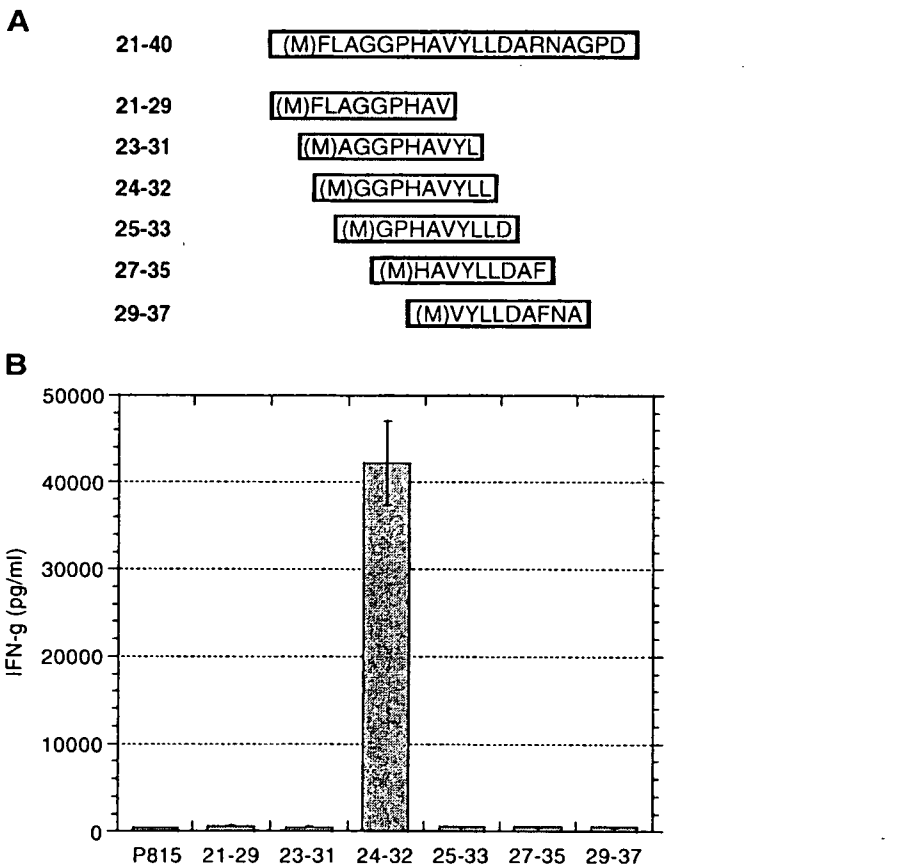


Fig. 5. Identification of a T cell epitope on MPT51. (A) Six 9-mer aa sequences predicted by algorithms. Double-stranded synthetic oligonucleotides encoding these sequences were inserted into the retroviral vector. (B) The immune splenocytes were stimulated with the P815 cells expressing the indicated putative epitopes and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

tion with P815 cells that had been transduced with MPT51. However, no significant IFN-γ production was observed in response to wild-type P815 cells. This shows that the stable transformant is able to express and process MPT51 peptides on MHC, which are then recognized by MPT51 immune splenocytes.

### 3.2. IFN-γ production in response to P815 cells expressing three overlapping MPT51 fragments

To map the approximate location of MPT51 peptides presented by MHC class I, three retrovirally transduced P815 transformants were produced (P815F1, P815F2, and P815F3) that expressed overlapping stretches of MPT51. These include F1 (aa 1–140), F2 (aa 78–198), and F3 (aa 136–266) (Fig. 2A). We confirmed the expression of each transgene by RT-PCR analysis. As shown in Fig. 2B, P815F1, P815F2, and P815F3 cells expressed almost the same amount of mRNA derived from each MPT51 gene fragment. Since these stable transformants were G418-resistant, they also expressed the *neo* gene. Employing these transformants as stimulators, we observed vigorous IFN-γ production by MPT51-immune spleen cells after stimulation with P815F1, suggesting that the 1–140 aa region probably contained T cell epitope(s) (Fig. 2C), while P815F2 and P815F3 did not induce IFN-γ production, suggesting that no class I-restricted MPT51 peptides were present in either of these regions.

### 3.3. IFN-γ production in response to P815 cells expressing shorter MPT51 peptides

In order to further map the T cell epitope within the first 140 amino acid section, we constructed plasmids encoding 40-mer stretches of MPT51 that were overlapping by 20 aa (Fig. 3A). As shown in Fig. 3B, P815 cells transduced with retrovirally encoded MPT51 aa 1–40 and MPT51 aa 21–60 were able to stimulate MPT51-immunized splenocytes to produce substantial amounts of IFN-γ. In contrast, P815 transformants that expressed the peptides MPT51 41–80 and 61–100 failed to stimulate the immune splenocytes. These results defined the T cell epitope(s) residing within aa 1–60, probably within aa 21–40.

### 3.4. Responses of immune splenocytes to P815 cells expressing 20-mer peptides

To further define the MPT51 epitope, we constructed three additional P815 cell lines that expressed the MPT51 peptides 1–20, 21–40, and 41–60 (Fig. 4A). However, MPT51-immune splenocytes failed to produce IFN-γ in response to P815 cells transduced with these products (Fig. 4B). Therefore, we fused the gene encoding murine ubiquitin to these products (Fig. 4C) to improve entry of the 20-mer peptides into the proteasome degradation pathway and thus enhance presentation via MHC class I. As shown in Fig. 4D, the P815 cell line expressing the fused construct containing ubiquitin and MPT51 aa 21–40 stimulated IFN-γ production by immune splenocytes, while the other fused products containing aa 1–20 and 41–60 failed to stimulate the immune splenocytes. These data confirmed that MPT51 21–40 probably contained the $CD8^+$ T cell recognized epitope.

### 3.5. Identification of $CD8^+$ T cell epitope on MPT51 21–40

Using three computer-based programs that are used to predict peptide binding to MHC molecules,

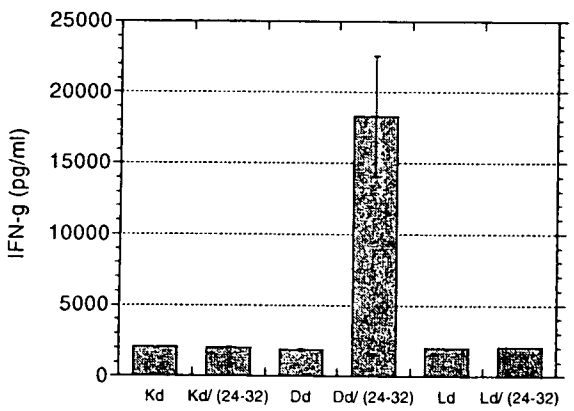


Fig. 6. MHC class I restriction molecule for the epitope, MPT51 24–32. BW5147 cells were cotransfected with MPT51 24–32 and the indicated cDNA encoding MHC class I molecules. The immune splenocytes were stimulated with the P815 cells expressing the indicated MHC class I molecules, and the epitope and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

SYFPEITHI Epitope Prediction (http://www.syfpeithi.de/), BIMAS HLA Peptide Binding Prediction (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform), and RANKPEP (http://www.mifoundation.org/Tools), we predicted several candidate T cell epitopes within the 20-mer peptide. Table 1 illustrates the results of screening the interaction of different peptides with $H2^d$ class I ligands using these programs. Since rank orders of score were not always the same depending on the algorithm used, we selected six candidate T cell epitopes that had the top three scores according to each algorithm, and then constructed oligonucleotides for each into a retroviral package for expression in the P815 cell line. As shown in Fig. 5B, IFN-γ production was observed solely in response to P815 cells expressing MPT51 24–32, indicating that the 24–32 aa contains the T cell epitope.

### 3.6. Identification of the MHC class I restriction molecule for MPT51 24–32

To determine the MHC class I restriction molecule for MPT51 24–32, we prepared three BW5147 ($H2^k$) lymphoma cell lines retrovirally transduced with one of the genes encoding H2-$K^d$, H2-$D^d$, or H2-$L^d$, followed by transfection of each line with the construct encoding MPT51 24–32. IFN-γ production by immune splenocytes was only observed after stimulating with the H2-$D^d$ cell line (Fig. 6), indicating that MPT51 24–32 is presented by H2-$D^d$ molecules.

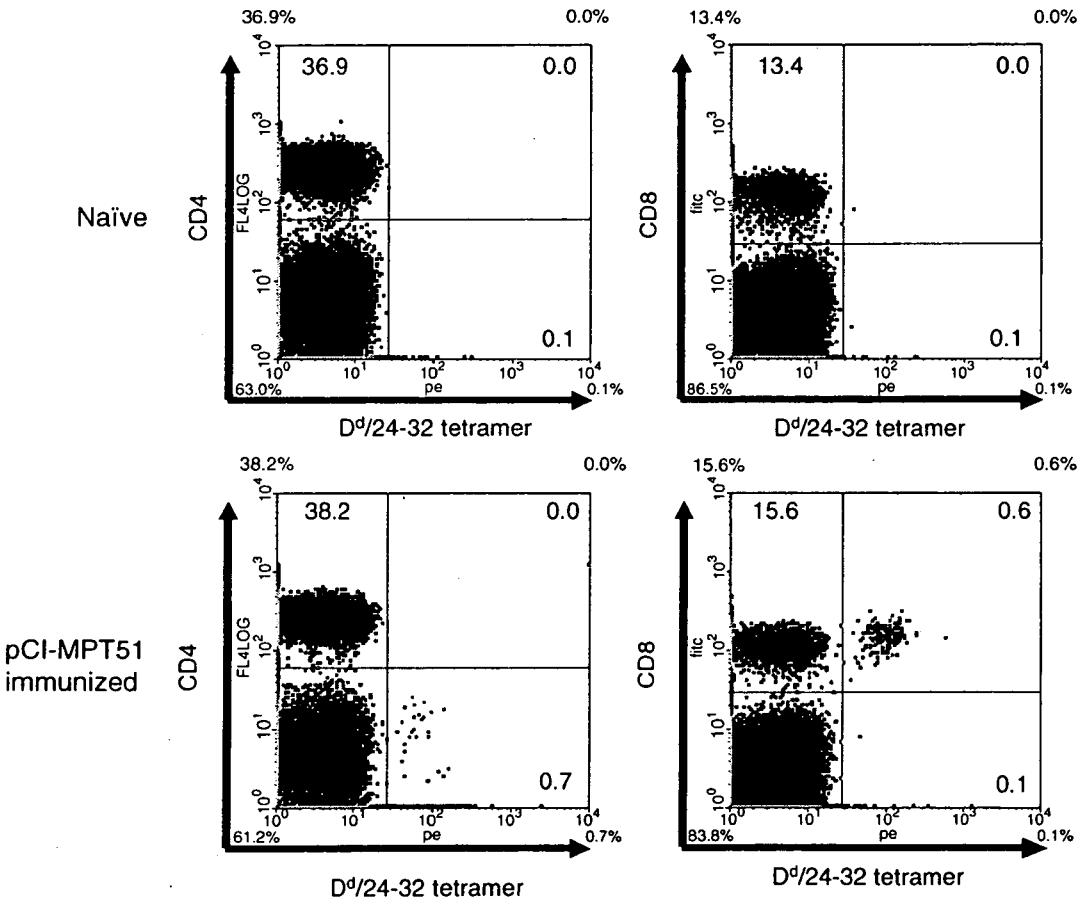


Fig. 7. Flow cytometric analysis for H2-$D^d$/MPT51 24–32 tetramer staining. Naïve (upper row) and immune (lower row) splenocytes were stained with PE-conjugated $D^d$–peptide tetramer complex and FITC-conjugated anti-CD4 (left column) or anti-CD8 (right column).