Vol. 22, No. 11, 2006 化学療法の領域

特集 多剤耐性結核の現状と今後

7．新たな抗結核薬開発の必要性と世界の現状

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世界の総人口の約3分の1に相当する約20億人の人が結核菌に感染している。また近年，ヒト免疫不全ウイルス（以下HIV）/後天性免疫不全症候群（以下AIDS）の世界的な蔓延に伴い，結核患者数も増加に転じ，過去のどの時代よりも多い人が結核に感染している状態となっている。また，多剤耐性結核菌の出現が治療を難治化し，交通網の発達により，世界中の人々が多剤耐性結核菌を含む結核菌の感染の危機に直面している。このような現状を解決するために，新しい結核治療薬の開発が切望されている。現在40年ぶりの新規抗結核薬となるOPC-67683およびTMC-207が，臨床試験段階に入っている。

Key Words：結核症／新規抗結核薬／OPC-67683／TMC-207／MFLX

I 世界の結核の現状

世界で知られる種々な感染症の中で，結核は死亡者数第1位の単独の感染症として今も尚猛威を振るっている[1]。1993年にWHOは，結核緊急事態宣言を発表した。世界の人口の32%（約20億人）が結核菌に感染しており，そのうち毎年800万人が伝染性の活動性結核を発病し，200万人が死亡していると報告されている[2]。結核発病の年間増加率は，世界で3%であるが，東欧では7%程度と高く，アフリカ諸国では10%以上に達するとされている[3]。

日本においては，かつて「国民病」といわれた結核は，ここ30年間に激減したが，1985年以降感染の減少率は鈍化し，1997年には新規登録患者数，罹患率等が増加に転じ，1999年に厚生大臣による「結核緊急事態宣言」が出されるに至っている。現在は再び減少に転じているが，現在も年間3万人以上の新規発病患者が報告されている。

現在の結核の問題は，大きく多剤耐性菌の問題，ヒト免疫不全ウイルス（HIV）/後天性免疫不全症候群（AIDS）の世界的な蔓延に伴う結核患者の増加の問題が挙げられる。

多剤耐性結核（MDR-TB）は，主には不完全な治療により発生し，その発生は結核治療において致命的な状況をもたらしている。一旦発生した多剤耐性菌は，一昔前とは異なり，交通手段の発達，それによる人々の移動の増加に伴い，町から町へ，国から国へ，更には大陸から大陸へ，すばやく広がる。72カ国で調査した結果[4]，多剤耐性結核は以前考えられていた以上に蔓延し，悪化の一途を辿っている。専門家らは，世界で年に18.5～41.5万の新規の多剤耐性結核が発現するであろうと推定している[5]。日本では，治療中の活動性感染者の中で約2,500人が多剤耐性結核に感染しており，適切な治療法がないのが現状である。

今日，結核は過去15年以上前から蔓延しているHIV/AIDSとの致死的な相乗作用によって複雑化されている。結核とHIVとの二重感染は，潜在性結核感染を活動性結核に転換する最も有力な危険因子となる。一方で，結核菌感染はHIVの病態経過を加速させAIDSを発病させる。現在，世界

Current global situation of TB and need for new drugs

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で1,200万人の患者が二重感染しており，結核はHIV陽性患者の間で主な死亡原因となっている。潜在性結核感染の患者がHIVに二重感染すると，活動性結核に進展する危険性が30～50倍増加する[6]。

結核は，かつて撲滅されるだろうと考えられていた。しかし，近年巻き返しが起こり，歴史上のどの時点よりも多くの人々が感染している。もし，この傾向が続くとすると20年後には，10億人もが新規に感染し，3,600万人は死亡するであろうと考えられている[6]。

II　今日の化学療法の現状

今日の結核の化学療法は，リファンピシンとピラジナミドの登場した1970年代まで遡る。British Medical Research Councilによって確立された現在の標準治療は，初め2カ月間のリファンピシン，イソニアジド，エタンブトール，ピラジナミドの併用による初期治療と，それに続く4カ月間のリファンピシンとイソニアジドによる維持治療からなっている。WHOはDOTS（directly observed treatment, short course：直接監視下短期化学療法）療法の中で，この治療法を推奨している。しかし，6カ月の治療期間は相当長い期間であり，服薬し続けることは容易なことではない。特に発展途上国においては，コンプライアンスが悪く，不完全な治療により薬剤耐性の問題が深刻化している。多剤耐性結核に対する治療には十分な効力がなく，且つ高価で重篤な副作用のある二次選択薬を使った組み合わせで治療しなければならないのが現状である。

過去40年間，政府機関や企業が新しい抗結核薬の開発にあまり興味を示さなかった結果として，新しい作用機序を持った新しい抗結核薬は1966年のリファンピシン以来上市されていない。したがって，今日WHOを初め世界結核薬開発同盟（Global Alliance for TB Drug Development）は，次の4つを可能にする新しい化学療法剤の登場を求めている。（1）総治療期間の短縮，（2）多剤耐性結核に対する有効性の改善，（3）潜在性結核感染を標的とする，（4）投与方法等を改良しコンプライアンスを向上させる。

III　現在開発途上にある抗結核薬

前臨床段階の化合物や初期の化合物を含めると数多くの化合物が報告されているが，今回は具体的に臨床試験に移行している化合物について紹介してみたい。

1．モキシフロキサシン（MFLX）

モキシフロキサシン（MFLX）（図1）は，グラム陽性菌およびグラム陰性菌を主体とする感染症の治療薬として開発され，幅広い抗菌スペクトラムを有する薬剤である。従来からニューキノロンには抗結核活性があることはわかっていたが，*in vivo*での効力は低く，臨床的有効性についても賛否両論であった。

1990年代後半にJ Grosset ら[7]およびWR Bishai ら[8]のグループにより，MFLXは従来のキノロンに比較し，*in vitro*および*in vivo*共に優れた抗結核活性があることが示された。その後，同研究グループより実験的マウス結核症モデルを用い

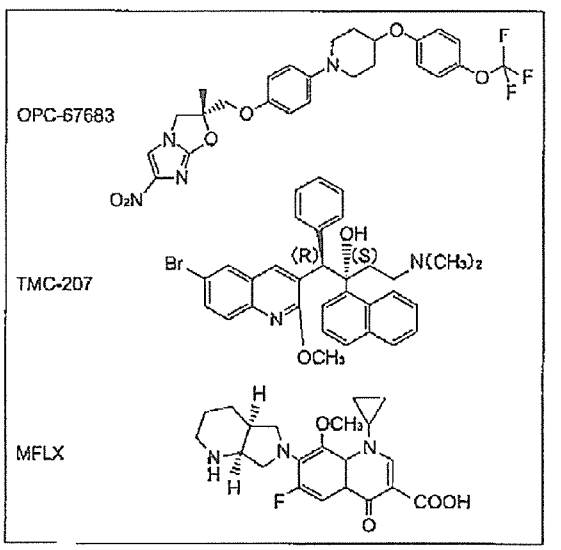

図1　現在開発中の主な抗結核薬

MFLXはグラム陽性菌およびグラム陰性菌を主体とする感染症の治療薬として開発され，幅広い抗菌スペクトラムを有する薬剤である。OPC-67683は大塚製薬株式会社微生物研究所にて新規に見出された，ニトロイミダゾオキサゾール誘導体である。TMC-207はヤンセンファーマ株式会社により見出された，ジアリルキノリン誘導体である。

たMFLXと既存薬剤との組み合わせに関する研究が行われ，イソニアジドにMFLXを置き換えることで，1カ月から2カ月の短期化を達成できることを示した（表1）[9]。また，結核治療において再発という問題を考えることが重要であるが，同グループにより，再発率についてもMFLXをイソニアジドに置き換えることで，既存薬剤より短期治療であっても既存薬と治療機関と同等以上の再発率であることが示された（表2）[10]。

これらの結果を基に，米国CDC（Centers for Disease Control and Prevention）を中心として立ち上げられたコンソーシアム，Tuberculosis Trials Consortium（TBTC）がMFLXを含むレジメについて臨床試験を行った。マウスを用いた実験結果からはMFLXをイソニアジドに置き換えたレジメが最も優れた成績を示していたが，イソ

表1　肺内生菌数の平均対数値

	治療期間（月）					
治療レジメ	0	2	3	4	5	6
A. Infected, untreated	7.80±0.21	7.63±0.41	7.24±0.41	8.06±0.81	7.68±0.51	7.34±0.50
B.2RHZ/4RH		3.36±0.32	1.89±0.40	0.39±0.32	0	0
C.2RHMZ/4RHM		2.74±0.48	1.26±0.33	−0.29±0.58	0	0
D.2RHM/4RH		3.70±0.25	2.11±0.26	1.32±0.37	−0.10±0.57	−0.65±0.32
E.2RMZ/4RM		0.90±0.58	−0.47±0.43	0	0	0
F.2MHZ/4MH		4.21±0.25	3.67±0.12	3.39±0.18	2.63±0.35	1.98±0.22

（文献9より抜粋）

結核菌H37Rv株をマウスにエアロゾル感染させ，19日目から治療を開始し，経時的な肺内生菌数の計測を行っている。治療は，非治療群（A群），標準療法（B群）に対し，モキシフロキサシンを標準療法に追加した群（C群），標準療法使用薬剤の何れか1剤と置き換えた群（D，E，F群）を設定し，6カ月までの治療効果を比較検討している。

H＝イソニアジド：25mg/kg，M＝モキシフロサシン：100mg/kg，R－リファンピシン：10mg/kg，
Z＝ピラジナミド：150mg/kg

表2　治療終了後の再発率

	治療期間（月）						
	3		4		5		6
治療レジメ	数	CFU分布*	数	CFU分布*	数	CFU分布*	数
2RHZ/4RH	11/12	2.7～3.7	5/12	2.1～3.4	1/16	0.6	0/12
1RMZ/4RM	4/12	1.7～3.0	0/12	0	0/12	0	Not done
2RMZ/3RM	2/12	0.3～2.9	0/12	0	0/13	0	Not done
5RMZ	4/12	3.4～3.7	0/12	0	0/12	0	Not done

（文献10より抜粋）

結核菌H37Rv株をマウスにエアロゾル感染させ，19日目から治療を開始し，3カ月，4カ月，5カ月および6カ月の期間治療を行い，治療終了後3カ月後のマウス肺内生菌の有無と菌数を測定することで，再発率を算出している。

H＝イソニアジド：25mg/kg，M＝モキシフロサシン：100mg/kg，R－リファンピシン：10mg/kg，
Z＝ピラジナミド：150mg/kg
＊再発したマウスの肺内菌数の対数値

ニアジドは第1選択薬としてFirst-lineで使用される薬剤であり，本薬剤を治療レジメから外すことに倫理的抵抗があったためか，MFLXを含む新しいレジメ試験はエタンブトールとの置き換えにより，2カ月間の投与でエタンブトール群との比較試験が行われた。その結果，4週目および6週目の喀痰中の結核菌陰性率は，MFLXを含むレジメにおいて有意に高かったが，期待に反して2カ月目の陰性化率においては，両レジメとも同等の結果であった[11]。現在，実験的マウス結核症モデルにおいて最も強い効力が確認されたMFLX，リファンピシンおよびピラジナミドの3剤により臨床試験が行われており，その結果が待たれるところである。

また，MFLXの後に開発されたガチフロキサシンについても，現在WHOの主導のもと臨床試験が行われているが，詳細なデータは発表されていない。

2．OPC-67683

OPC-67683（図1）は大塚製薬株式会社微生物研究所にて新規に見出された，ニトロイミダゾオキサゾール誘導体である。*in vitro*の活性は現在開発中の化合物を含め全ての抗結核薬の中で最も強い活性を示している。本薬剤の作用機作の1つとして，サブクラスミコール酸であるメトキシおよびケトミコール酸の生合成を阻害することが判明しており，既存の抗結核薬とは異なった新しい作用メカニズムにより効力を発揮する。これら本薬剤の特徴を簡単に示す[12]。

1）結核菌に対する*in vitro*の効力

臨床分離株（67株）に対するMIC$_{90}$値は0.024 μg/mL（範囲：0.003～0.024 μg/mL）を示し，既存薬とは交叉耐性を示さないことから，多剤耐性結核菌にも有効と考えられる（表3）。また，既存の抗結核薬と拮抗作用がないことが確認されている。これは，単剤での治療が難しい結核治療を考えると，既存薬との組み合わせで，新しいレジメが構築できる可能性を示すものである。また，本化合物はグラム陽性菌，グラム陰生菌，主な腸内細菌などに対しては全く活性を示さず，結核菌を主体とする抗酸菌特異的な活性を示す。

2）細胞内結核菌に対する効力

結核菌は細胞内に寄生し増殖することが知られており，また既存薬剤の細胞内結核菌に対する効力が十分ではないことがいわれている。したがって細胞内結核菌に効力を示すことは，結核治療薬の1つの大きなプロファイルとして要求されるところである。OPC-67683の細胞内結核菌に対する活性をヒト単球由来のTHP-1細胞をマクロファージに分化誘導し，結核菌標準株である

表3　OPC-67683の既存薬剤感受性株および耐性株に対する最小発育阻止濃度

臨床分離株（試験菌株数）		最小発育阻止濃度（μg/mL）	
		MIC$_{90}$*	95%信頼区間
RFP感受性株	(31)	0.01248	0.01097～0.01535
RFP耐性株	(36)	0.01221	0.01050～0.01583
INH感受性株	(31)	0.01194	0.01054～0.01452
INH耐性株	(36)	0.01279	0.01094～0.01679
EB感受性株	(56)	0.01213	0.01081～0.01440
EB耐性株	(11)	0.01341	0.01073～0.02450
SM感受性株	(49)	0.01203	0.01077～0.01416
SM耐性株	(18)	0.01340	0.01068～0.02298

（文献12より抜粋）

臨床分離の67菌株に対する最小発育阻止濃度の範囲は，0.006～0.024 μg/mLであった。

＊ MIC$_{90}$値はプロビット法により算出した。

RFP：リファピシン，INH：イソニアジド，EB：エタンブトール，SM：ストレプトマイシン

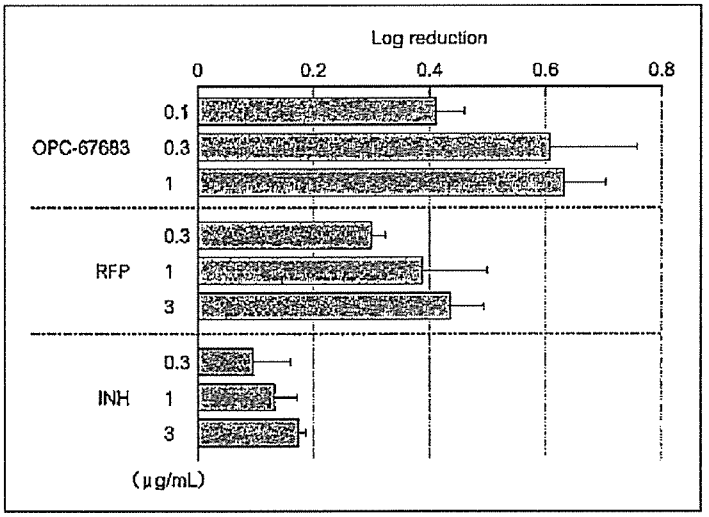

図2　短時間接触における細胞内結核菌に対する殺菌力

ヒト単球由来THP-1細胞をマクロファージに分化誘導し，結核菌H37Rv株を感染させた後，OPC-67683，RFPまたはINHを4時間接触させ，68時間後の細胞内生菌数を求め，コントロールからの対数減少度で効力を表した。

(文献12より抜粋)

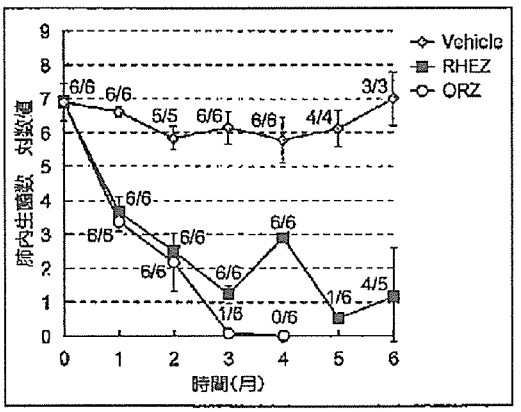

図3　OPC-67683を含む新しいレジメと既存薬標準療法との治療効果の比較試験成績

結核菌Kurono株を気管内接種し，慢性結核症モデルを作製し，2RHEZ/4RHと2ORZ/2ORのスケジュールで連日強制経口投与を行い，肺内生菌数をモニタリングすることで，OPC-67683を含む新しいレジメの効力を，標準療法と比較した。

O:OPC-67683;2.5mg/kg，R:リファンピシン;5mg/kg，H:イソニアジド;10mg/kg，E:エタンブトール;100mg/kg，Z：ピラジナミド；100mg/kg

(文献12より抜粋)

H37Rv株を感染させ，効力を測定した結果，短時間の作用（4時間，0.1 μg/mL）においても強い効力を示すことがわかっている（図2）。

3）実験モデルでの薬物動態

マウスに0.156～40 mg/kgまで経口投与し，経時的に血中および肺内濃度を測定した結果，用量相関的に吸収が確認され，0.625 mg/kg投与時に約100 ng/mLのC_{max}（最高血中濃度）が得られた。また，肺内へは血中の約3倍移行することが確認され，ほとんどの結核症の発症の場が肺であることを考えると望ましいプロファイルであると考えられる。

4）実験的マウス結核症モデル

結核菌Kurono株を感染させ，結核症が慢性化した4週目より治療を開始し，単剤での治療効果を確認したところ，0.313 mg/kg以上の投与量において有意に治療開始時の菌量を減少させている。同実験において，既存薬の同様の効果を発揮する用量は，OPC-67683より8倍～500倍高い用量を必要とする。

また，同モデルにおいて現在の4剤併用療法（リファンピシン，イソニアジド，エタンブトール，およびピラジナミドの4剤併用の2カ月強化療法と，リファンピシンとイソニアジドの2剤併用の4カ月の維持療法）を対照に，より強い効力を発揮する併用レジメを検討した結果，OPC-67683，リファンピシン，ピラジナミドの3剤2カ月強化療法にOPC-67683とリファンピシンの2カ月維持療法において，既存の標準療法を少なくとも2カ月以上短期化できることが確認され

ている（図3）。

5）薬物相互作用

前述したようにHIV/AIDSとの混合感染症が大きな問題となっており，その治療において，リファンピシンが持つCYP3A4の酵素誘導と，それにより代謝を受けるプロテアーゼ阻害剤の併用が困難なことが大きな問題となっている[13]。したがって，併用薬が多い治療を強いられるこの分野においては，何よりも薬物相互作用がない薬剤が求められる。OPC-67683のヒトを含む各種動物のCYPへの影響を確認したところ，代謝や活性阻害に影響をしないことがわかっている。

3．TMC-207

TMC-207（図1）は，ヤンセンファーマ株式会社により見出されたジアリルキノリン誘導体である。グループ会社であるジョンソン・エンド・ジョンソン株式会社から1994年の第44回ICAACにて発表された。当初のコードはR207910であったが，後にグループ会社のTibotec Pharmaceuticals Limitedが開発を行うことになり，コード名がTMC-207に変更されている。本化合物も既存の抗結核薬にはない新しい作用メカニズムであるATP合成酵素の阻害活性により抗結核活性を発揮することが示されている[14]。

1）結核菌に対する *in vitro* の効力

臨床分離株（6株）に対するMIC値は0.030～0.120 μg/mLであり，試験菌株数は少ないが，リファンピシン耐性株，イソニアジド耐性株，エタンブトール耐性株，ピラジナミド耐性株，およびフルオロニューキノロン耐性株に対しても同様に活性を示す。ヘリコバクター，ノカルディア，大腸菌，肺炎球菌，黄色ブドウ球菌などにはほとんど活性を示さず，結核菌を含む一部抗酸菌に特異的に活性を示す。結核菌に対する作用は，殺菌作用が発現までに時間を要する結果が示されているが，10 MIC濃度以上12日間作用において，初期菌数から3 Logの減少させることで殺菌作用を有することが確認されている。

2）実験モデル動物での薬物動態

マウスに単回経口投与し，血中および主要臓器

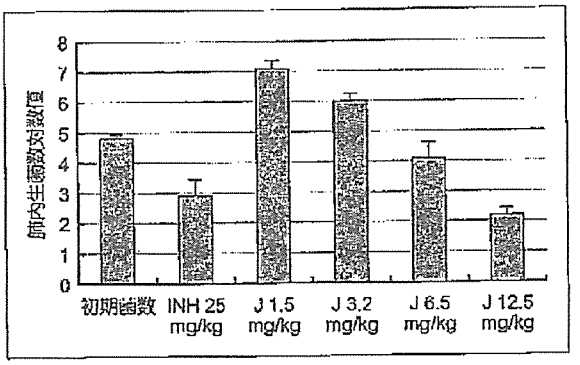

図4　急性感染症モデルにおけるTMC-207の治療効果

結核菌H37Rv株をマウスに感染させた後，週5回，4週間の治療を行った結果の肺内菌数の対数値を示している。

J：TMC-207

（文献14より改変）

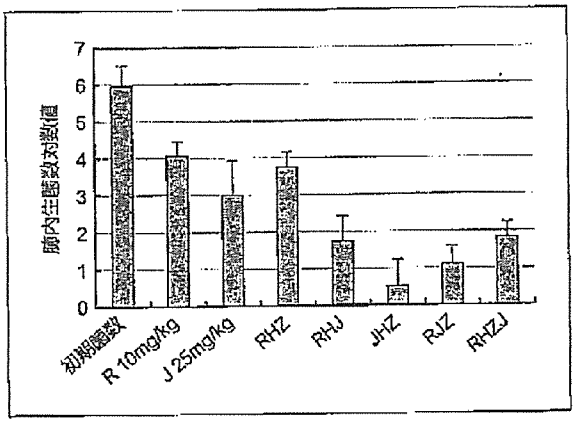

図5　慢性結核症モデルでのTMC-207と既存薬の併用による治療効果

結核菌H37Rv株を感染させ，12日間放置し，結核症を発症させ，その後週5回，4週間治療を行った結果の肺内生菌数を表している。

J：TMC-207，R：リファンピシン，H：イソニアジド，Z：ピラジナミド　（文献14より改変）

内濃度を測定した結果，6.25 mg/kg 投与時に C_{max} 値で 0.40 μg/mL，25 mg/kg 投与時に C_{max} 値で 1.1 μg/mL の血中濃度を示し，肺内では血中濃度の 13 倍から 16 倍の C_{max} 値を示している。

3）実験的マウス結核症モデル

マウスに結核菌を感染させた日より治療を開始し，週5回，4週間投与を行った結果，最小効果投与量は 6.5 mg/kg で，12.5 mg/kg 投与時には 3 Log の減少を示している（図4）。また，感染後 12 日目より，週5回，4週間投与を行った結果，TMC-207 の 25 mg/kg 単独投与を行った群はリファンピシン，イソニアジド，およびピラジナミドの3剤併用量を行った群よりも強い効果を示しており，強いポテンシャルを有した化合物であることがわかる（図5）。しかしながら，著者らも記しているように結核菌の耐性化などの問題を考えると，決して単剤では使用されるべきものではなく，既存薬との併用を考える必要がある。図5に示すように，各既存薬剤の1つと TMC-207 を置き換えた場合，リファンピシンと置き換えた組み合わせ（JHZ）が最も強い効果を示しており，既存薬より強い治療効果が期待される。しかしながら，JHZ にリファンピシンを加えると効力が減弱することから，リンファンピシンと拮抗している可能性が示唆され，懸念されるところである。

4．臨床第 I 相試験

ヒトでの第 I 相試験は，健常人男性を用いた二重盲検試験として実施されている。TMC-207 の投与は，40%の HP-β-CD（hydroxypropyl-β-cyclodextrin）に 10 または 40 mg/mL の溶液で行われている。投与量としては，10，30，100，300，450，および 700 mg の単回投与試験，50，150，450 mg の 14 日間連投試験が行われている。これらの結果，十分な安全性が確認され，用量相関的な吸収とマウスでの薬効濃度の8倍までの吸収がヒトで確認されている。

Ⅳ 終わりに

現在上記に示した3種類の化合物が抗結核薬として臨床試験段階にある。モキシフロキサシンおよびガチフロキサシンは臨床第Ⅱ相試験および Ⅲ 相試験段階にあるが，既にキロノン耐性結核菌は多数報告されており，幅広い抗菌スペクトラムを有することが，長期服薬を強いられる結核治療において，常在細菌への影響，一般細菌のキノロン耐性化の誘導など懸念される。これらの点は今後モニタリングしていく必要があると考えられる。しかしながら，マウスを用いた動物実験の結果が示すように，モキシフロキサシンを含む新しいレジメにより，結核の治療がヒトにおいても短期化されれば，その恩恵は大きいものと考えられる。

OPC-67683 および TMC-207 は共に臨床第Ⅱ相試験段階にあり，従来の抗菌薬とは全く異なる新しい化学構造および作用機作を有しており，40 年来新薬が開発されていない結核の化学療法に大きな革命をもたらしてくれることを期待してやまない。

その他[15]，世界結核薬開発同盟が手がけている PA-824，Lupin 社のピロール誘導体（LL-3858），Sequella 社の SQ-109 なども第 I 相試験または移行段階にある。結核の治療は単剤で達成できるものではなく，新しい薬理作用を有した画期的な治療薬の開発がまだまだ必要である。特に，難治化している原因と考えられている Dormant 株に対し，効力を有するような化合物の登場が待たれるところである。

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Immunization with dendritic cells retrovirally transduced with mycobacterial antigen 85A gene elicits the specific cellular immunity including cytotoxic T-lymphocyte activity specific to an epitope on antigen 85A

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Abstract

In the present study, we evaluated antigen 85A (Ag85A) gene-transduced dendritic cells (DCs) vaccine against *Mycobacterium tuberculosis*. Murine bone marrow-derived DCs were retrovirally transduced with mycobacterial Ag85A gene and injected to BALB/c mice intravenously. The DC vaccine was capable of inducing purified protein derivative (PPD)- and the antigen-specific spleen cell proliferation and IFN-γ production from both $CD4^+$ and $CD8^+$ T cells in spleens of the immune mice. In addition, the DC vaccination induced cytotoxic T-lymphocytes (CTL) and IFN-γ-producing cells specific for a 9-mer CTL epitope on Ag85A molecule. This eliciting cellular immunity led to protection against wasting disease due to *M. tuberculosis* infection and induction of moderate bacterial clearance.

Keywords: Antigen 85A; Dendritic cells; *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) remains one of most serious public health problems being prevailed worldwide along with AIDS and malaria, resulting in 8 million new cases and 2 million deaths each year [1]. The appearance of multidrug-resistant *Mycobacterium tuberculosis* strains has worsened the problem. The only TB vaccine currently available is the attenuated *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG), which has been reported to have a variable protective efficacy especially in adult TB [2]. Therefore, there remains an urgent need for more effective vaccines for TB [3].

Protection against intracellular bacteria such as *M. tuberculosis* critically depends on induction of cellular immune responses. Administration of soluble proteins would be insufficient to stimulate these responses. The reason why BCG vaccine has been utilized for decades is that the vaccine is able to induce specific cellular immunity although the efficacy is controversial as mentioned before. Immunization with dendritic cells (DCs) is one of promising strategies for eliciting effective cellular immunity against intracellular pathogens as DCs are the most potent antigen-presenting cells (APCs). DCs capture the pathogens or apoptotic cells. Then they migrate to regional lymphoid organs, where they present antigens to naïve T cells [4,5]. DCs possess the distinct ability to prime naïve helper T-lymphocytes (Th) and cytotoxic T-lymphocytes (CTL). Thus there has been much interest in the

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use in immune modulation of infectious diseases and cancer. Vaccination with DCs pulsed with tumor-associated antigens has been shown to generate specific anti-tumor immunity in vivo in murine tumor models [6–9]. DC vaccination has been also examined in the field of infectious diseases [10–13]. We also showed that vaccination with DCs retrovirally transduced with a gene for a dominant CTL epitope derived from *Listeria monocytogenes* elicited significant protective immunity against lethal listerial challenge infection [14].

Promising candidate antigens for TB vaccines include antigen (Ag) 85 family molecules such as Ag85A or Ag85B, heat shock proteins such as Hsp60 and ESAT-6 (reviewed in [15]). We used Ag85A as a vaccine target in this study. Ag85A molecule is a mycobacterial major secreted protein which belongs to the Ag85 family consisting of three structurally related components, Ag85A (p32A; 32 kDa), Ag85B (p30, MPT59, α antigen; 30 kDa), and Ag85C (reviewed in [16]). The Ag85 family molecules are cross-reactive antigens and are highly conserved among *Mycobacterium* spp. The genes encode proteins with fibronectin-binding capacities [17] and mycolyltransferase activities, which are involved in the final stage of mycobacterial cell wall assembly [18]. Ag85A protein was reported to stimulate B- and T-cell responses in TB patients and immunization with Ag85A protein induced the protective immunity against *M. tuberculosis* in guinea pigs [19]. In addition, reports of naked DNA vaccines against TB employing Ag85A gene have accumulated [20–24]. In addition, we reported recently that vaccination with attenuated *Listeria* carrying Ag85A expression plasmid elicited significant protective immunity against *M. tuberculosis* challenge [25]. More recently, vaccination with Ag85A-expressing vaccinia virus was shown to be effective in boosting antimycobacterial immunity in human trial [26]. According to these reports, Ag85A molecule seems to be one of the most promising candidates for future subunit TB vaccines.

In the present study, we developed a retrovirally transduced DC vaccine expressing Ag85A, and assessed its ability to generate the antigen-specific cellular immunity and to induce protective immunity against murine *M. tuberculosis* infection.

2. Materials and methods

2.1. Recombinant retroviral vector

BCG Ag85A gene was amplified from a plasmid, pMB49 [27] by PCR with following primers: 5′-ATAAGAATGCGGCCGCACCATGCAGCTTGTTGACAGG-3′ (forward primer) and 5′-ATAGTTTAGCGGCCGCTGTTCGGAGCTAGGCGC-3′ (reverse primer) (underlined letters indicate NotI sites). These PCR fragments were digested with NotI and inserted into a NotI site of pMX [28]. The nucleotide sequence designed in the plasmid was confirmed by DNA sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Large-scale purification of the plasmid was conducted using the Qiagen Plasmid Mega Kit System (Qiagen, Chatsworth, CA) and endotoxin was removed by Triton X-114 phase separation. Retroviral supernatant was generated by transfection of pMX-Ag85A proviral construct into Phoenix ecotropic packaging cell line [purchased from American Type Culture Collection (Manassas, VA) and used with the permission of Dr. GP Nolan (Stanford University School of Medicine, Stanford, CA)].

2.2. Reverse transcription (RT)–PCR analysis for Ag85A gene detection

Bone marrow-derived DCs transduced with pMX-Ag85A were harvested and total RNA was prepared from the cells by Isogen RNA extraction solution (Nippon Gene, Tokyo, Japan). The single-stranded cDNA was synthesized with Molony murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) and then used for PCR analysis. The images were recorded using AE-6900M densitograph (ATTO, Tokyo, Japan). Primers used for Ag85A gene detection are: 5′-AGGCCAACAGGCACGTCAA-3′ (forward primer) and 5′-ACATGTCGGAGGCCTTGTA-3′ (reverse primer). As a control, the same RT–PCR was performed with primers for glycelaldehyde-3-phosphate dehydrogenase (G3PDH).

2.3. Mice

BALB/c mice were purchased from SLC Japan (Hamamatsu, Japan). These mice were maintained in a specific-pathogen-free condition at the Experimental Animal Institute, Hamamatsu University School of Medicine. All mice used in this study were between 8 and 14 weeks of age. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University, School of Medicine.

2.4. Peptides and protein

Lyophilized peptides were purchased from Invitrogen Corporation (Carlsbad, CA). The three Ag85A CTL-epitope candidate peptides are synthesized based on Denis et al. [29]. They are, pep1 (MPVGGQSSF; corresponding to amino acid residues (aa) 70–78 of Ag85A which is predicted to bind H2-L^d according to SYFPEITHI computer algorithm [http://www.syfpeithi.de]), pep2 (WYDQSGLSV; aa 60–68 of Ag85A predicted to bind H2-K^d), and pep3 (VYAGAMSGL; aa 144–152 of Ag85A predicted to bind H2-K^d). The purity of peptides was confirmed by mass spectrometry. All peptides were dissolved in 5% dimethyl sulfoxide in distilled water to a concentration of 1 mM and were stored at −80 °C until used. Purified recombinant (r) Ag85A protein was kindly provided by Dr. John T. Belisle

(Colorado State University, Fort Collins, CO) through the NIH, NIAID Contract NO1 AI-75320 entitled "Tuberculosis Research Materials and Vaccine Testing".

2.5. Culture of bone marrow-derived DCs and transduction with retrovirus

Bone marrow-derived DCs were cultured using a method described by Inaba et al. [30] with some modifications as in our previous work [14]. To determine the phenotype of cultured DCs, we stained them with PE-, or FITC-conjugated monoclonal antibodies (mAbs) against cell surface molecules [CD40, CD80, CD86, H2-A^d (all from BD Biosciences, San Diego, CA)] and analyzed using EPICS Profile-II (Beckman Coulter, Fullerton, CA). Transduction of retroviruses was also carried out as in our previous work [14]. Briefly, 1×10^6 bone marrow-derived DCs were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (RPMI/10FCS) for 48 h and resuspended in 1 ml of the retroviral supernatant supplemented with 8 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO), 1000 units/ml of murine rGM-CSF, and 1000 units/ml of murine rIL-4. These cells were centrifuged at $2500 \times g$ at 32 °C for 2 h. After centrifugation, cells were cultured in RPMI/10FCS in 5% CO_2 atmosphere. The transduction process was repeated on days 3 and 4.

2.6. Immunization

After washing twice in phosphate-buffered saline (PBS), 1×10^5 transduced DCs in 0.2 ml of PBS were injected intravenously into mouse twice at a 2-week interval. As a control, mice were also immunized with 2×10^6 CFU of BCG (substrain Tokyo; Japan BCG Inc., Tokyo, Japan) subcutaneously twice at a 2-week interval. In some experiments, 2 μg of Ag85A expression plasmid (pCI-Ag85A) was immunized with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA) four times at 1-week intervals as in our previous work on MPT51 molecule [31].

2.7. Detection of PPD-, or Ag85A-specific antibodies (Abs) by ELISA

The 96-well ELISA plates (EIA/RIA plate A/2; Costar, Cambridge, MA) were coated with 25 μg/ml of purified protein derivative (PPD; Japan BCG Inc., Osaka, Japan) or 5 μg/ml of purified Ag85A protein at 4 °C overnight, washed with PBS containing 0.05% Tween 20 (PBS/Tween), and blocked with 30% Block Ace (Dainippon Seiyaku, Tokyo, Japan) solution in PBS at 37 °C for 2 h. After washing, the sera diluted with RPMI1640 medium were added to the plates and incubated at 4 °C overnight. After washing, horseradish peroxydase (HRP)-conjugated goat anti-mouse IgG antibodies (Abs) were added to the plates at room temperature for 2 h. After washing, the bound HRP-conjugated Abs were detected by HRP substrate reagent (Techne, Minneapolis, MN). The absorbance at 450 nm was determined with EZS-ABS microplate reader (Iwaki, Tokyo, Japan).

2.8. Lymphocyte proliferation assay

Spleen cells were prepared from the immune mice and treated with Tris-buffered 0.83 M NH_4Cl buffer for 1 min at room temperature to remove red blood cells. Then the spleen cells (5×10^5 per well) were incubated for 48 h at 37 °C in 96-well round-bottom tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) in the presence or absence of 5 μg/ml of PPD (Japan BCG Inc.). The de novo DNA synthesis was assessed by adding 0.5 μCi/well of [methyl-^{3}H] thymidine (6.7 Ci/mmol; ICN Biochemicals, Irvine, CA) for the last 12 h of culture. The cultured cells were harvested onto glass fiber filters, and the radioactivity was counted by a liquid scintillation counter (LSC-5100; Aloka, Tokyo, Japan). The [methyl-^{3}H] thymidine incorporation was calculated in counts per minute (cpm).

2.9. Quantification of IFN-γ by sandwich ELISA

Pools of spleen cell suspensions (2×10^6 ml^{-1}) from groups of mice immunized with DCs were cultured in RPMI/10FCS in 24-well plates in the presence of PPD (Japan BCG Inc.) (10 μg/ml), Ag85A protein (5 μg/ml), or peptides (5 μM) at 37 °C in 5% CO_2 atmosphere. The culture supernatants were harvested after 5 days, aliquoted, and stored at −20 °C until assayed for IFN-γ. Concentration of IFN-γ in the culture supernatants was determined by sandwich ELISA as described in our previous work [31]. Briefly, the 96-well ELISA plates (EIA/RIA plate A/2; Costar) were coated with 2 μg/ml of capture Ab (anti-murine IFN-γ mAb, R4-6A2; BD Biosciences) at 4 °C overnight and washed with PBS/Tween and blocked with PBS/Tween containing Block Ace (Dainippon Seiyaku) at 37 °C for 2 h. After washing, the culture supernatants to be tested and serially diluted IFN-γ standard solutions were added to the plates and incubated at 4 °C overnight. After washing, 0.5 μg/ml of detection Ab (biotinylated anti-murine IFN-γ mAb, XMG1.2; BD Biosciences) was added to the plates. The plates were incubated at room temperature for 2 h and washed. The plates were then added with 0.1 μg/ml of HRP-conjugated streptavidin (Vector laboratories Inc., Burlingame, CA) and incubated at room temperature for 30 min. After washing, bound HRP-conjugated streptavidin was detected by HRP substrate reagent (Techne). The absorbance at 450 nm was determined with EZS-ABS microplate reader (Iwaki).

2.10. Preparation of $CD4^+$ and $CD8^+$ T cell subsets from immune splenocytes with Ag85A gene-transduced DCs

Spleen cells were prepared from the immune mice and treated with Tris-buffered 0.83 M NH_4Cl buffer for 1 min

at room temperature to remove red blood cells. Then they were washed twice with RPMI 1640 medium. $CD4^+$ and $CD8^+$ T cell subsets were prepared from spleen cells of immune mice using murine $CD4^+$ or $CD8^+$ T cell isolation kit according the manufacturer's instruction (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The $CD4^+$ or $CD8^+$ T cells (1×10^6 cells) and Ag85A gene-transduced DCs (1×10^5 cells) were cultured in 96-well round-bottom tissue plates (Greiner Bio-One GmbH) for 4 days. The culture supernatants were harvested and stored at −20 °C until assayed.

2.11. CTL assay

Eight weeks after the last immunization, immune spleen cells were cocultured in 12-well plates at density of 2×10^7 cells/well for 5 days with 2×10^7 cells/well syngeneic splenocytes that had been pretreated with 100 μg/ml of mitomycin C and pulsed with 1 μM of Ag85A pep3 peptide (VYAGAMSGL) for 1 h at 37 °C. Each well received also 10 units/ml of human rIL-2 (Hoffmann-La Roche, Nutley, NJ). Cell-mediated cytotoxicity was measured using a conventional ^{51}Cr release assay. The target cells used in this study were RAW264.7 (mouse macrophage cell line; $H2^d$) pulsed with 1 μM of the peptide for 1.5 h at 37 °C. Target cells at a concentration of 1×10^4 cells/well were incubated for 5 h in triplicate at 37 °C with serial dilutions of effector cells, and the specific lysis was determined as calculated by the formula: percent specific lysis = [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100.

2.12. Bacterial infection and evaluation of protective ability of Ag85A gene-transduced DC vaccine

Immunized BALB/c mice were infected with 5×10^5 CFU of *M. tuberculosis* H37Rv i.v. 2 months after the last immunization. Mice were sacrificed 4 weeks later and the bacterial numbers in the spleens, livers, and lungs were counted in CFU on Middlebrook 7H11 plates (BD Biosciences).

2.13. Statistical analysis

Data from multiple experiments were expressed as mean ± standard deviations (S.D.). Statistical analyses were performed with the StatView-J5.0 statistics program (SAS Institute Inc., Cary, NC). Data were analyzed by Fisher's protected least significant difference (PLSD).

3. Results

3.1. Retroviral transduction of bone marrow-derived DCs and expression of Ag85A gene in the cells

DCs were generated from murine bone marrow by culturing with rGM-CSF and rIL-4, as previously described [30]. DCs transduced with Ag85A-encoding retrovirus (Ag85A gene-transduced DCs) and control untransduced DCs expressed similar amounts of CD40, CD80, CD86, and MHC class II molecules (data not shown), indicating that retroviral transduction to DCs did not affect the phenotype of the DCs.

In order to confirm the expression of Ag85A gene in transduced DCs, RT–PCR was performed. As shown in Fig. 1A, an Ag85A gene-specific band was detected in the retrovirus-transduced DCs, but not in control untransduced DCs, indicating Ag85A gene expression in the transduced DCs.

We next examined the antigen presentation capacity of Ag85A gene-transduced DCs. When Ag85A gene-transduced DCs or untransduced DCs were incubated with spleen cells derived from Ag85A DNA vaccine-immune mice, Ag85A-transduced DCs, but not untransduced DCs rendered the spleen cells to produce IFN-γ (Fig. 1B), indicating that Ag85A gene-transduced DCs were capable of presenting the antigen (Ag85A) to T cells.

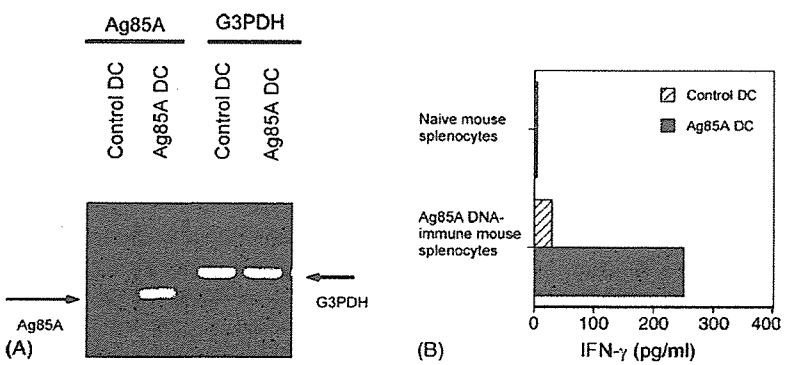

Fig. 1. Ag85A gene expression in Ag85A-transduced DCs and antigen presentation capacity of the cells. (A) Ag85A gene expression by DCs transduced with pMX-Ag85A. DCs were transduced with Ag85A-expressing retrovirus and harvested to prepare total RNA. Ag85A gene expression was evaluated by RT–PCR with Ag85A-specific primers. (B) Antigen presentation capacity of DCs transduced with Ag85A-expressing retrovirus. DCs transduced with or without Ag85A-expressing retrovirus were incubated with spleen cells of Ag85A DNA-immune mice or naïve mice for 2 days and the culture supernatant was examined for IFN-γ amounts with ELISA. Average values from two independent experiments are shown.

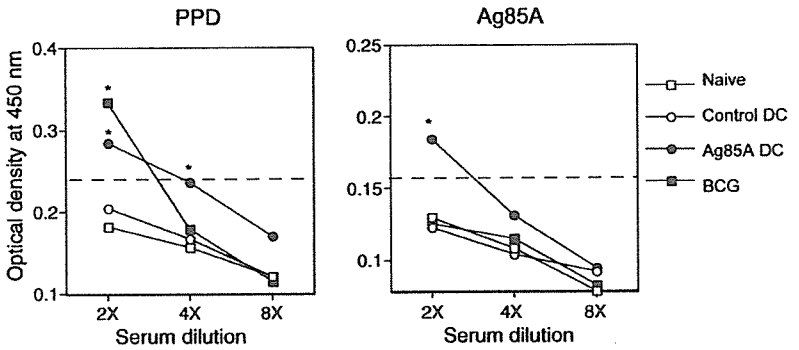

Fig. 2. Detection of PPD- and Ag85A-reactive Abs in the sera of Ag85A gene-transduced DC-immune mice. The sera of control untransduced DC-, Ag85A gene-transduced DC-, or BCG-immune mice or naïve mice were examined for binding to PPD (left panel) or purified Ag85A protein (right panel) by ELISA. The mean optical density at 450 nm of six mice in each group for PPD-reactive Abs and those of three mice in each group for Ag85A protein-reactive Abs are shown. Horizontal broken lines in figures indicate the two-fold greater values than the average values of 8× diluted sera of naïve mice. Asterisks indicate statistically significant ($p < 0.01$ for PPD, $p < 0.03$ for Ag85A) compared with the average value of 8× diluted sera of control untransduced DC-immune mice.

3.2. Ag85A gene-transduced DC vaccination was able to generate PPD- and Ag85A -reactive Abs in vivo

After injection of Ag85A gene-transduced DCs into BALB/c mice, we first examined the production of PPD-reactive Abs in the vaccinated mice. Ag85A molecule is one of the most abundant secreted proteins in *M. tuberculosis* and PPD contains the molecule. PPD-reactive Abs will be therefore produced if Ag85A molecule is successfully expressed in the vaccinated mice. Sera were prepared from the immunized mice 1 month after the last immunization and examined for antibodies for PPD. Sera from Ag85A gene-transduced DC-immune mice showed higher binding units to PPD than sera from control untransduced DC-immune mice and naïve mice (Fig. 2, left panel). Sera from BCG-vaccinated mice also showed PPD-binding activity. Furthermore, the sera were also examined for Abs specific for Ag85A protein (Fig. 2, right panel). Sera from Ag85A gene-transduced DC-immune mice showed binding activity to Ag85A protein. In this time, sera from BCG-immune mice did not show Ag85A protein-binding activity. These results suggest that Ag85A gene-transduced DC-vaccinated mice produced Ag85A-reactive Abs in the sera.

3.3. Ag85A gene-transduced DC vaccination induced PPD-specific spleen cell proliferation, and PPD- and Ag85A-specific IFN-γ production from the spleen cells

We then examined the proliferative response of spleen cells derived from Ag85A gene-transduced DC-immune mice in response to in vitro PPD stimulation. As shown in Fig. 3A, a significant proliferative response was observed in Ag85A gene-transduced DC-immune mice. The level of the response was comparable to that of BCG-immune mice. Only faint proliferative response was detected in control untransduced DC-immune mice.

In addition, we examined IFN-γ production from spleen cells of Ag85A gene-transduced DC-immune mice. Correlating with the proliferative response, spleen cells from mice immunized with Ag85A gene-transduced DCs produced high amounts of IFN-γ after in vitro stimulation with PPD. The IFN-γ amounts produced by the spleen cells of Ag85A gene-transduced DC-immune mice were higher than those by the spleen cells of BCG-vaccinated mice (Fig. 3B), suggesting that immunization with Ag85A gene-transduced DC efficiently generates PPD-specific IFN-γ-producing cells in vivo. Further, we also examined IFN-γ production from spleen cells of Ag85A gene-transduced DC-immune mice in response to purified Ag85A protein. As shown in Fig. 3C and D, the spleen cells of Ag85A gene-transduced DC-immune mice were capable of producing IFN-γ in response to purified Ag85A protein.

3.4. Ag85A gene-transduced DC immunization can generate the antigen-specific $CD4^+$ and $CD8^+$ T cells

In previous section, we examined immune responses of splenocytes derived from Ag85A DC-immune mice in response to PPD or purified Ag85A protein. $CD4^+$ T cells are speculated to respond to these exogenous antigens which should be presented on MHC class II molecules on APCs. We next examined whether Ag85A gene-transduced DC immunization is capable of inducing the antigen-specific $CD4^+$ or $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells in the spleens of Ag85A gene-transduced DC-immune mice were prepared with magnetic beads. They were cultured with Ag85A gene-transduced DCs and examined IFN-γ amounts in the culture supernatants. As shown Fig. 4, $CD4^+$ T cell- or $CD8^+$ T cell-enriched splenocytes of Ag85A gene-transduced DC-immune mice produced IFN-γ in the presence of Ag85A gene-transduced DCs. In this experiment, $CD4^+$ T cell-enriched splenocytes of control DC-immune mice also produced IFN-γ in the presence of Ag85A gene-transduced DCs, although the amounts were lower than those by $CD4^+$ T cell-enriched splenocytes of Ag85A gene-transduced DC-immune mice (Fig. 4). It may be caused by bovine serum

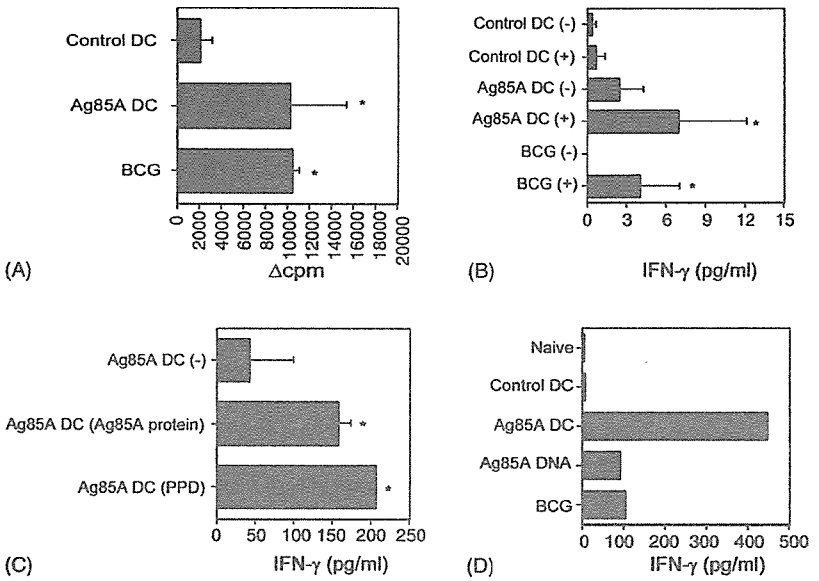

Fig. 3. (A) PPD-specific splenocyte proliferation of mice immunized with DCs transduced with Ag85A gene. BALB/c mice were immunized with Ag85A gene-transduced DCs two times at a 1-week interval. The data of mice immunized with BCG twice were also shown as controls. Spleen cells of the immunized mice were harvested 1 month after the last immunization and cultured in vitro in the presence or absence of 5 μg/ml of PPD for 48 h and pulsed with 0.5 μCi [methyl-^{3}H] thymidine for last 12 h. The values represent Δcpm (the value after in vitro stimulation in the presence of PPD subtracted by the value in the absence of PPD). The mean ± S.D. of quintuplicate determinations of a representative experiment from three independent experiments, are shown. Asterisks indicate statistically significant ($p < 0.001$) compared with the value of control untransduced DC immune mice (Control DC). (B) PPD-specific IFN-γ production from spleen cells of Ag85A gene-transduced DC-immune mice. BALB/c mice were immunized with Ag85A gene-transduced DCs two times at a 1-week interval. The data of mice immunized with BCG twice were also shown as controls. Spleen cells of the immunized mice were harvested 1 month after the last immunization and cultured in vitro in the presence or absence of 10 μg/ml of PPD for 72 h. The supernatants were harvested and assayed for IFN-γ with ELISA. The mean ± S.D. of five independent experiments are shown. Asterisks indicate statistically significant ($p < 0.001$) compared with the value of control untransduced DC-immune mice in the absence of PPD [Control DC(−)]. (C, D) Ag85A-specific IFN-γ production from spleen cells of mice immunized with Ag85A gene-transduced DCs. (C) BALB/c mice were immunized with Ag85A gene-transduced DCs twice at a 1-week interval. Spleen cells of the immune mice were harvested 1 month after the last immunization and cultured in vitro in the presence of 5 μg/ml of Ag85A protein or 10 μg/ml of PPD for 72 h. The supernatants were harvested and assayed for IFN-γ with ELISA. The mean ± S.D. of three independent experiments are shown. Asterisks indicate statistically significant ($p < 0.04$) compared with the value without Ag85A protein or PPD [Ag85A DC (−)]. (D) BALB/c mice were immunized with control untransduced DCs (Control DC), Ag85A gene-transduced DCs (Ag85A DC), Ag85A expression plasmid DNA (Ag85A DNA), or BCG. Spleen cells of the immunized mice were harvested 1 month after the last immunization and cultured in vitro in the presence of 5 μg/ml of Ag85A protein for 72 h. The supernatants were harvested and assayed for IFN-γ with ELISA. Average values from two independent experiments are shown.

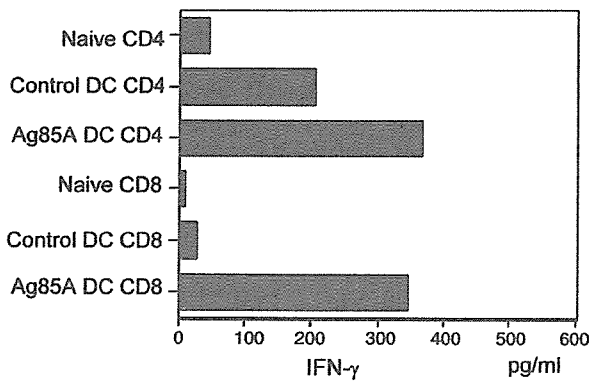

Fig. 4. Ag85A gene-transduced DC immunization elicited the antigen-specific CD4$^+$ and CD8$^+$ T cells. CD4$^+$ and CD8$^+$ T cell-enriched spleen cells of control untransduced DC-, or Ag85A gene-transduced DC-immune mice were cultured with in vitro-prepared Ag85A gene-transduced DCs for 4 days and the supernatants were examined for IFN-γ with ELISA. Naïve BALB/c mice were also examined as controls.

proteins contained in culture medium for DCs. Immunization with DCs taken up the proteins may induce CD4$^+$ T cells specific to these proteins, which would lead to the relatively high background value.

3.5. *Ag85A gene-transduced DC immunization can generate the antigen-specific CTL*

Denis et al. [29] reported several candidate CTL epitopes on Ag85A in BALB/c mice. In order to identify minimal CTL epitopes on Ag85A in BALB/c mice, we examined IFN-γ production by spleen cells derived from Ag85A DNA vaccine-immune BALB/c mice in response to several candidate CTL epitope peptides. We chose these peptides because results in Denis et al. [29] indicate that 20-mer peptides containing these 9-mer peptides showed stimulatory effects on splenocytes from Ag85A DNA-immune BALB/c mice, and also these peptides showed high scores to bind H2-K^d or H2-L^d molecules in a computer algorithm for epitope prediction (SYFPEITHI; http://www.syfpeithi.de). We demon-

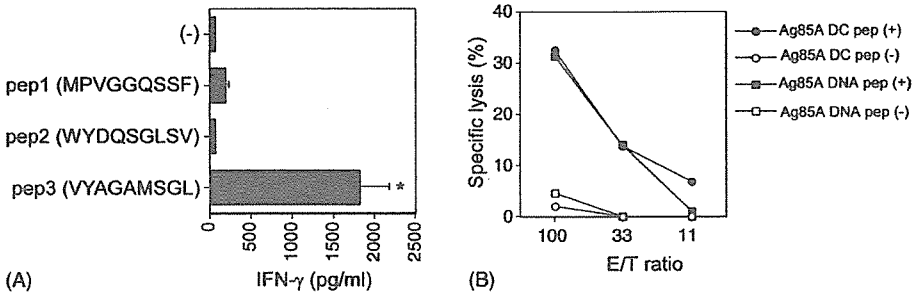

Fig. 5. Cytotoxic activity of Ag85A-transduced DC-immune splenocytes to VYAGAMSGL peptide-pulsed RAW264.7 cells. (A) IFN-γ production by spleen cells of BALB/c mice immunized with Ag85A plasmid DNA in the presence of candidate CTL epitope peptides. The spleen cells produced the significant level of IFN-γ only in the presence of VYAGAMSGL peptide. The mean ± S.D. of three independent experiments are shown. Asterisks indicate statistically significant ($p < 0.001$) compared with the value without any peptides [(−)]. (B) Spleen cells of Ag85A DC- or Ag85A plasmid DNA-immune mice (effectors) were incubated with the peptide-pulsed RAW264.7 cells (target cells) with the effector/target ratios (E/T ratio) indicated on the *x*-axis.

strated that only one peptide (VYAGAMSGL) among peptides examined was able to induce IFN-γ production by the spleen cells (Fig. 5A).

We next determined whether the peptide-specific CTL were generated following Ag85A gene-transduced DC vaccination. After in vitro restimulation of immune spleen cells with the peptide, spleen cells obtained from Ag85A gene-transduced DC-immune mice showed cytolytic activity to the peptide-pulsed RAW264.7 cells. The CTL activity was comparable to that by spleen cells from Ag85A DNA vaccine-immune mice (Fig. 5B). This result indicates that Ag85A gene-transduced DC immunization is capable of eliciting CTL specific for at least one CTL-epitope in Ag85A protein.

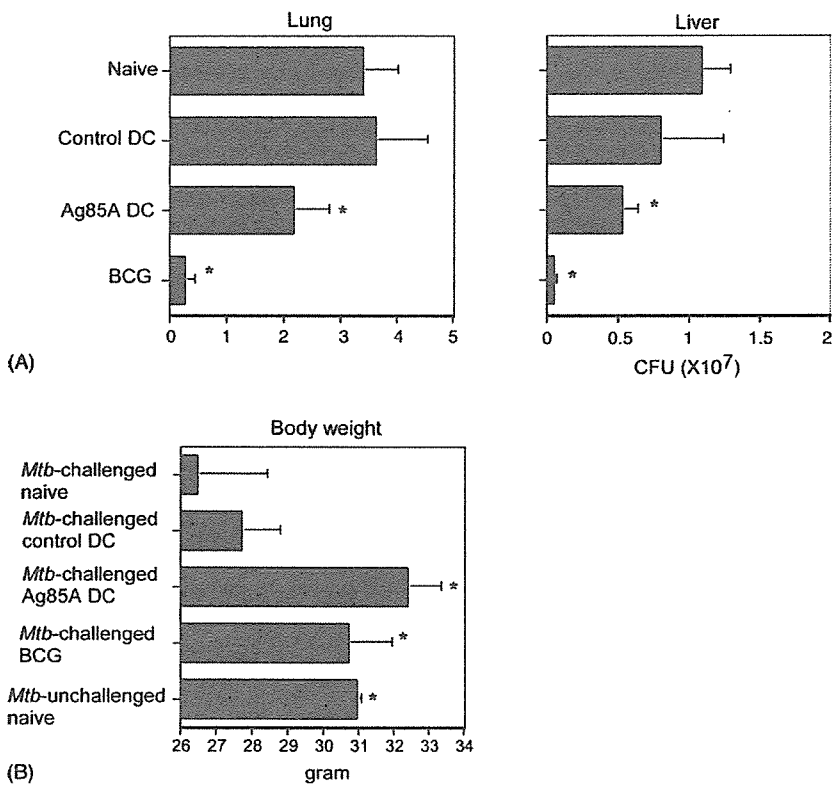

Fig. 6. (A) In vivo protective activity of mice immunized with DCs transduced with Ag85A gene against virulent *M. tuberculosis* challenge. BALB/c mice were immunized with Ag85A gene-transduced DCs twice at a 1-week interval. These mice were challenged i.v. with 5×10^5 CFU of live *M. tuberculosis* H37Rv. Numbers of the CFU in the lungs and the livers were determined 4 weeks later. The mean ± S.D. of five mice in each group are shown. Asterisks indicate statistically significant ($p < 0.05$) compared with the value of naïve mice. (B) Body weights of mice immunized with DCs transduced with Ag85A gene after virulent *M. tuberculosis* H37Rv challenge. Body weights of control untransduced DC-, Ag85A gene-transduced DC-, or BCG-immune mice or naïve mice were measured 4 weeks after i.v. challenge of *M. tuberculosis* H37Rv. Body weights of unchallenged naïve mice were also shown as controls. The mean ± S.D. of five mice in each group are shown. Asterisks indicate statistically significant ($p < 0.001$) compared with the value with *M. tuberculosis*-challenged naïve mice.

3.6. Ag85A gene-transduced DC immunization can provide moderate protective immunity against a subsequent challenge with viable M. tuberculosis

We finally evaluated the effects of Ag85A gene-transduced DC immunization on protective immunity against *M. tuberculosis* infection. Four weeks after i.v. injection with *M. tubeculosis* H37Rv, spleens, livers, and lungs were prepared from the immunized mice and the CFU of *M. tuberculosis* H37Rv in these tissues were evaluated. As shown in Fig. 6A, the CFU in lungs and livers of Ag85A gene-transduced DC-vaccinated mice, but not control untransduced DC-immune mice, were significantly lower than those of naïve mice ($p < 0.05$), although the difference was less than one log 10 order. But the CFU in spleens were not significantly different between Ag85A gene-transduced DC-vaccinated mice and naïve mice (data not shown). In addition, we evaluated body weights of the same mice as used in this challenge study. It is especially noteworthy that body weights of Ag85A gene-transduced DC-immune mice were as high as those of unchallenged naïve mice whereas naïve and control untransduced DC-immune mice showed significant loss of body weights (Fig. 6B).

4. Discussion

DCs have been shown to be the most powerful APCs that initiate the primary immune response. DC vaccines have been examined for the efficacy as vaccines against infectious diseases as well as cancer [6–13]. There are several strategies for using DCs as vaccines against intracellular bacteria, including ex vivo pulses with bacteria or bacterial antigens, or transfer of genes encoding antigens to DCs. Among them, retroviral transduction is advantageous for long-term antigen presentation in vivo, because the transgene integrates into the chromosome leading to gene expression throughout the life of the cell and its progeny [9]. In our previous work [14], we showed that immunization with DCs retrovirally transduced with a minimal CTL epitope derived from *Listeria monocytogenes* successfully induced the specific CTL and protective immunity against lethal listerial challenge. Here, we examined immunization with DCs retrovirally transduced with *M. tuberculosis*-derived Ag85A gene. The results shown here indicate that the DC immunization successfully induced the specific cellular immunity, including immune responses of $CD4^+$ T cells and $CD8^+$ CTL, as well as specific antibody responses. The de novo synthesized Ag85A proteins in DCs would be processed in MHC class I pathway to induce specific $CD8^+$ T cells. The Ag85A proteins are then secreted from DCs and would induce specific Abs. Specific $CD4^+$ T-cell responses to the proteins may be evoked through uptake of the secreted proteins by APCs or direct antigen presentation by Ag85A gene-transduced DCs. The conclusive description waits further analysis of the antigen presentation mechanisms in this system.

In this work, we identified a minimal CTL epitope on Ag85A molecule in BALB/c mice. We showed here that immunization with DCs retrovirally transduced with Ag85A gene could efficiently induce the CTL activity specific to a peptide in Ag85A molecule, VYAGAMSGL. Denis et al. [29] showed that vaccination of BALB/c mice with Ag85A plasmid DNA induced the CTL activity against target cells pulsed with at least three 20-mer peptides in Ag85A. We, however, observed CTL activity only to VYAGAMSGL-pulsed target cells. Generally, the number of the dominant CTL epitope in one protein is small (one or two). In our previous work for identifying T-cell epitopes on MPT51 molecule derived from *M. tuberculosis*, we only identified one dominant CTL epitope on the protein in BALB/c mice [30]. We therefore speculated that the peptide (VYAGAMSGL) is the dominant CTL epitope on Ag85A molecule in BALB/c mice. The peptide was highly predicted to bind to H2-K^d molecule in an MHC-binding peptide prediction algorithm [the binding score in SYFPEITHI (http://www.syfpeithi.de) is 25 and that in RANKPEP (http://www.mifoundation.org/Tools/) is 102.0].

Ag85A gene-transduced DC immunization was able to induce PPD- and Ag85A-specific immune responses. The immunization, however, led to the moderate level of protection against virulent *M. tuberculosis* challenge. Body weights of *M. tuberculosis*-challenged mice appeared to indicate that Ag85A gene-transduced DC immunization was very effective (Fig. 6B), but the immunization was not so strikingly effective in terms of clearance of *M. tuberculosis* from tissues (Fig. 6A). It seems to be a good possibility that the DC immunization was able to induce granuloma formation which restricts *M. tuberculosis* growth and at the same time permits persistence of *M. tuberculosis*. In addition, several other factors would be also speculated. First, the amount of DCs immunized to the mice may be critical. Indeed, when we immunized mice with 5×10^5 DCs instead of 1×10^5 DCs, we observed much more bacterial burden in tissues in the immune mice after *M. tuberculosis* challenge (data not shown). Too much immunization of DCs augmented T-cell response against pathogens including the IFN-γ production by T cells, but that may not be favorable for the protective capacity of the DC immunization. González-Juarrero et al. [32] reported that intranasal immunization with lung-derived DCs pulsed with Ag85A protein elicited IFN-γ production by $CD4^+$ T cells but showed exacerbation in terms of the protective capacity against *M. tuberculosis* infection. The exacerbation was attributed to florid pulmonary inflammatory responses by the DC immunization. Further assessment of optimal DC dosage to be immunized and careful examination of tissue pathology would be necessary. Second, condition of DCs to be vaccinated may be also important. In this work and our previous work [14], we used DCs incubated with medium supplemented with GM-CSF and IL-4, but we did not treat the DCs with maturation-inducing reagents, such as lipopolysaccharide or CpG oligodeoxynucleotides. We chose this condition because we think that DCs maturate after the injection into mice. Indeed, in our previous work [14],

immunization with DCs which were not treated with such reagents successfully induced protective immunity against *L. monocytogenes*. The culture condition of DCs which is most optimal for immunization should be clarified in further studies. In addition, the expression level of Ag85A in the transduced DCs may not have been strong enough to induce more protective immunity, although Ag85A gene expression was observed in RT–PCR analysis.

We also evaluated the prime-boost regimen, namely, the regimen in which, mice were primed with Ag85A gene-transduced DC vaccine and boosted with BCG injection. Our data showed that the protocol was not effective compared with two BCG injection protocol in terms of *M. tuberculosis* clearance from tissues after the intravenous challenge (data not shown). Several investigators evaluated the regimen in which DNA immunization was used for priming and BCG vaccination for boosting. Ag85B DNA vaccination followed with BCG vaccination has been shown to be more effective than BCG immunization alone in protecting against *M. tuberculosis* infection [33,34]. However, Skinner et al. [35] reported that priming with Ag85A/ESAT-6 fusion DNA vaccination and boosting with BCG vaccination augmented antigen-specific IFN-γ-producing T cell number, but did not increase the protective efficacy of BCG against *M. tuberculosis*. Skinner et al. [35] pointed out several possible reasons including the difference of BCG strains used. A variety of factors must be considered for the successful prime-boost regimen.

Taken together, we showed here that immunization of DCs retrovirally transduced with Ag85A gene was able to elicit specific cellular immune responses containing $CD4^+$ and $CD8^+$ T-cell responses as well as specific Ab production. During this study, we identified a minimal CTL epitope on Ag85A molecule in BALB/c mice. But the responses lead to only a moderate level of protective immunity. Further study is clearly necessary to improve the effectiveness of DC vaccines against TB.

Acknowledgments

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Immunization with a gene encoding granulocyte-macrophage colony-stimulating factor inserted with a single helper T-cell epitope of an intracellular bacterium induces a specific T-cell subset and protective immunity

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Abstract

We evaluated here the effect of immunization with a gene encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) inserted with a helper T cell (Th) epitope, listeriolysin O (LLO) 215–226 derived from *Listeria monocytogenes* on induction of a specific Th by gene gun bombardment. Immunization of C3H/He mice with pGM215m plasmid encoding murine GM-CSF inserted with LLO 215–226 Th epitope gave the epitope-specific proliferative responses of $CD4^+$ T lymphocytes. In addition, specific interferon-γ production from the splenocytes was observed. Concomitantly, pGM215m-immunized mice showed significant protective immunity against lethal listerial challenge. These results suggest that immunization of a gene for GM-CSF inserted with a Th epitope is useful for eliciting a specific Th subset in vivo.

Keywords: DNA immunization; GM-CSF; Th epitope

1. Introduction

Helper T cells (Th) play pivotal roles in many aspects of infection immunity, especially for modulating immune responses by producing special sets of cytokines. For protection against intracellular bacteria, activation of macrophages is indispensable and type 1-helper T cells (Th1) are important for the activation. The DNA vaccination method which induces only a particular Th population without production of antibodies may be advantageous as antibodies could, in some cases, give undesirable consequences [1]. Here, we evaluated the effect of immunization with a gene encoding murine granulocyte-macrophage colony-stimulating factor (GM-CSF) inserted with a single H2-E^k-restricted Th epitope [residues 215–226 of listeriolysin O (LLO)] derived from *Listeria monocytogenes* [2] by gene gun bombardment.

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2. Materials and methods

2.1. Animals

C3H/He mice (between 6 and 18 weeks of age; Japan SLC, Hamamatsu, Japan) were maintained at the Institute for Experimental Animals, Hamamatsu University School of Medicine. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

2.2. Plasmid construction

The eukaryotic expression vector, pCI (Promega, Madison, WI) was used as a backbone plasmid for construction of plasmids for DNA immunization. The oligonucleotides used for p215m plasmid were, 5′-CCCGGG ATG AGC CAG CTG ATC GCC AAG TTT GGC ACC GCC TTT AAG TAG CCCGGG-3′ and the opposite-strand oligonucleotide,

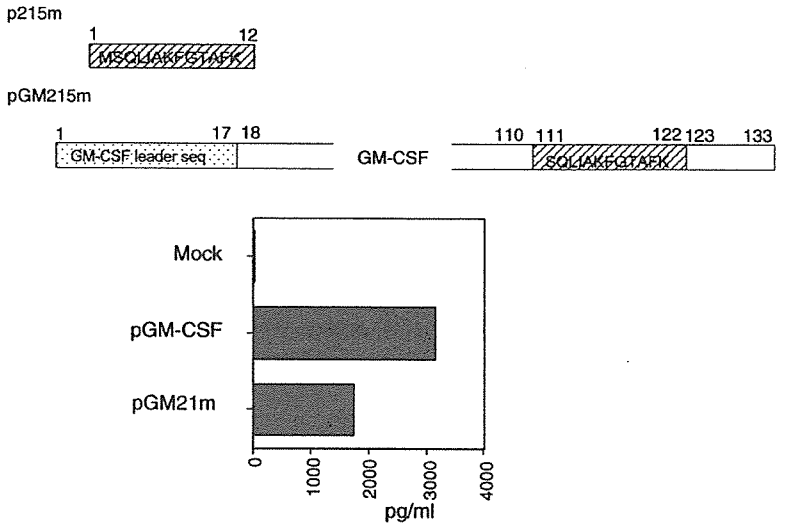

Fig. 1. (A) The schema of gene products deduced from the cDNA constructs prepared in this study (p215m and pGM215m). The hatched boxes indicate LLO 215–226 peptide and a dotted box indicate murine GM-CSF leader sequence. Amino acid numbers of each domain were shown above each schema. (B) Expression of GM-CSF inserted with LLO 215–226 peptide (GM215) in transfected cells. Supernatants of pGM-CSF- or pGM215m-transfected 293T cells were examined with ELISA specific to murine GM-CSF.

5′-CCCGGG CTA CTT AAA GGC GGT GCC AAA CTT GGC GAT CAG CTG GCT CAT CCCGGG-3′, which encode amino acid residues 215–226 of LLO, MSQLIAKFGTAFK and a termination codon. These oligonucleotides were annealed and inserted into the SmaI site of pCI (Fig. 1A). The codon usage of the oligonucleotide for LLO 215–226 peptide was optimized to that of *Mus musculus* [3]. pGM-CSF was constructed by inserting murine GM-CSF gene into the EcoRI/NotI sites of pCI. For pGM215m plasmid, a double-stranded oligonucleotide encoding LLO 215–226 was inserted in the unique EcoRV site of murine GM-CSF gene in pGM-CSF (Fig. 1A). The region is located in the region which should not affect the function of GM-CSF [4,5]. The nucleotide sequences of the resultant plasmids were confirmed by dideoxy sequencing by using ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Foster City, CA).

2.3. Mice immunization

For DNA immunization with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA), preparation of the cartridge of DNA-coated gold particle cartridge was followed to the manufacturer's instruction manual. Finally, 0.5 mg of gold particles was coated with 1 μg of plasmid DNA and the injection was carried out with 0.5 mg gold per shot twice. Mice were injected with 2 μg of plasmid DNA four times at 1-week intervals.

2.4. Enzyme-linked immunosorbent assay (ELISA) for GM-CSF

293T cells (human embryonal kidney cells) (approximately 5×10^6) were transfected with 2 μg of pGM-CSF or pGM215m using SuperFect Transfection Reagent (QIAGEN GmbH, Hilden, Germany). The supernatants were prepared 48 h after transfection and were assayed for GM-CSF using AN'ALYZA mouse GM-CSF Immunoassay Kit (G-T, Mineapolis, MN) according to the instruction manual.

2.5. Lymphocyte proliferation assay

Spleen cells (5×10^5 cells per well) from the immunized mice were incubated in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified 5% CO_2 atmosphere for 48 h at 37 °C in 96-well round-bottom tissue culture plates in the presence or absence of 1 μM of LLO 215–226 peptide. After 48 h in culture, de novo DNA synthesis was assessed by adding 0.5 μCi/well of [methyl-^{3}H] thymidine (10 Ci/mmol; ICN Biochemicals, Irvine, CA) for the last 12 h of culture. Triplicate cultures were harvested onto glass fiber filters, and the [methyl-^{3}H] thymidine incorporation was determined by counting the radioactivity (cpm) using a liquid scintillation counter.

2.6. ELISA for IFN-γ

Spleen cells were harvested from the immunized mice. Recovered cells were plated in 24-well plates at 2×10^6 cells/well in the presence or absence of 1 μM of LLO 215–226 peptide for 5 days. Concentration of IFN-γ in the culture supernatants was determined by a sandwich ELISA as described in our previous report [6].

2.7. Intracellular IFN-γ staining

The number of LLO 215–226-specific $CD4^+$ T-cell subset was examined by intracellular IFN-γ staining. Spleen cells