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3Log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

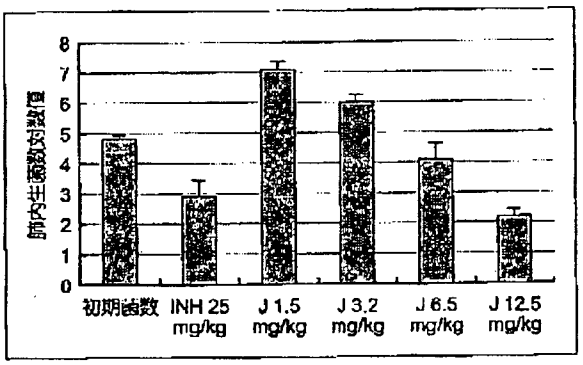

図4　急性感染症モデルにおけるTMC-207の治療効果

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）

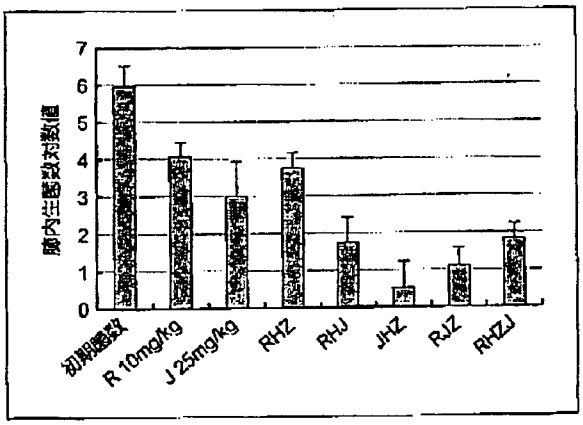

図5　慢性結核症モデルでのTMC-207と既存薬の併用による治療効果

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3Log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.

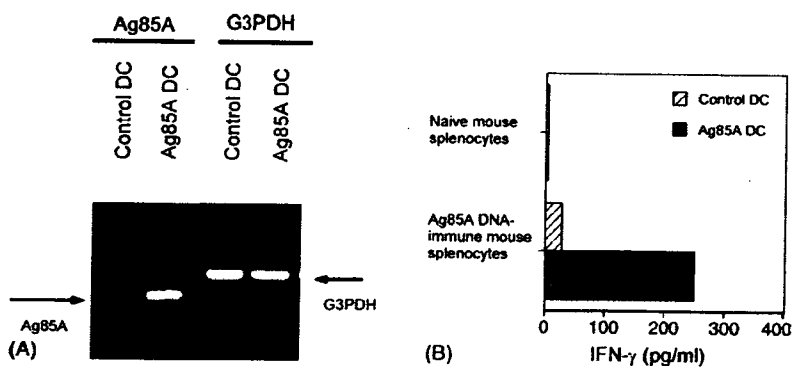

Fig. 1. Ag85A gene expression in Ag85A-transduced DCs and antigen presentation capacity of the cells. (A) Ag85A gene expression by DCs transduced with pMX-Ag85A. DCs were transduced with Ag85A-expressing retrovirus and harvested to prepare total RNA. Ag85A gene expression was evaluated by RT–PCR with 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.

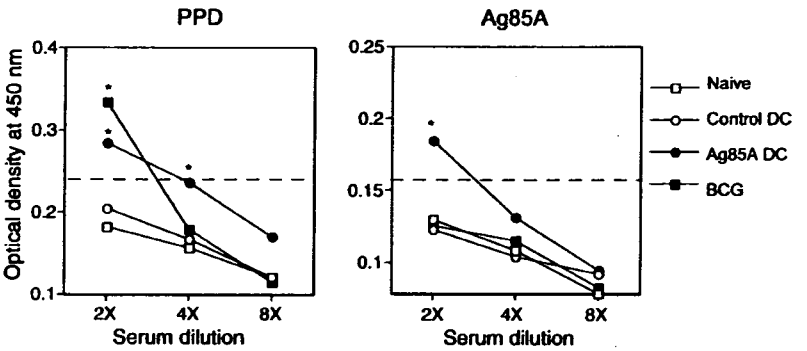

Fig. 2. Detection of PPD- and Ag85A-reactive Abs in the sera of Ag85A gene-transduced DC-immune mice. The sera of control untransduced DC-, Ag85A gene-transduced DC-, or BCG-immune mice or naïve mice were examined for binding to PPD (left panel) or purified Ag85A protein (right panel) by ELISA. The mean 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

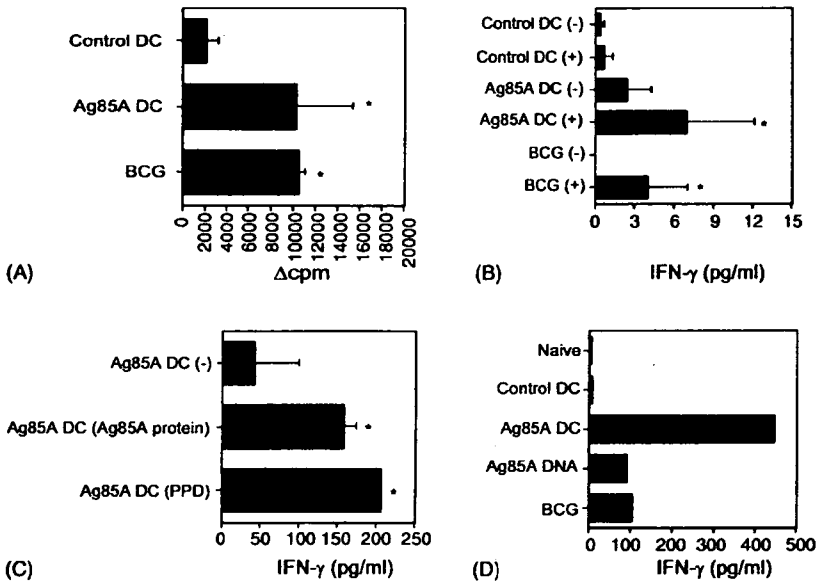

Fig. 3. (A) PPD-specific splenocyte proliferation of mice immunized with DCs transduced with Ag85A gene. BALB/c mice were immunized with Ag85A gene-transduced DCs two times at a 1-week interval. The data of mice immunized with BCG twice were also shown as controls. Spleen cells of the immunized mice 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.

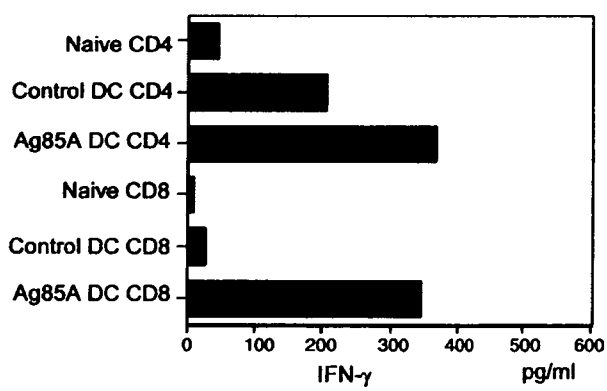

Fig. 4. Ag85A gene-transduced DC immunization elicited the antigen-specific CD4$^+$ and CD8$^+$ T cells. CD4$^+$ and CD8$^+$ T cell-enriched spleen cells of control untransduced DC-, or Ag85A gene-transduced DC-immune mice were cultured with in vitro-prepared Ag85A gene-transduced DCs for 4 days and 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-

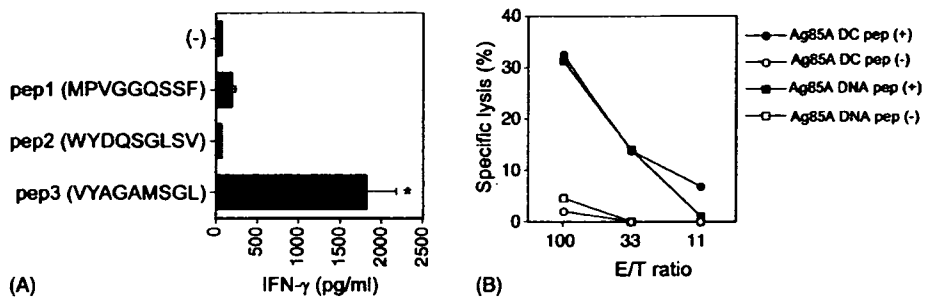

Fig. 5. Cytotoxic activity of Ag85A-transduced DC-immune splenocytes to VYAGAMSGL peptide-pulsed RAW264.7 cells. (A) IFN-γ production by spleen cells of BALB/c mice immunized with Ag85A plasmid DNA in the presence of candidate CTL epitope peptides. The spleen cells produced the significant level of IFN-γ 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.

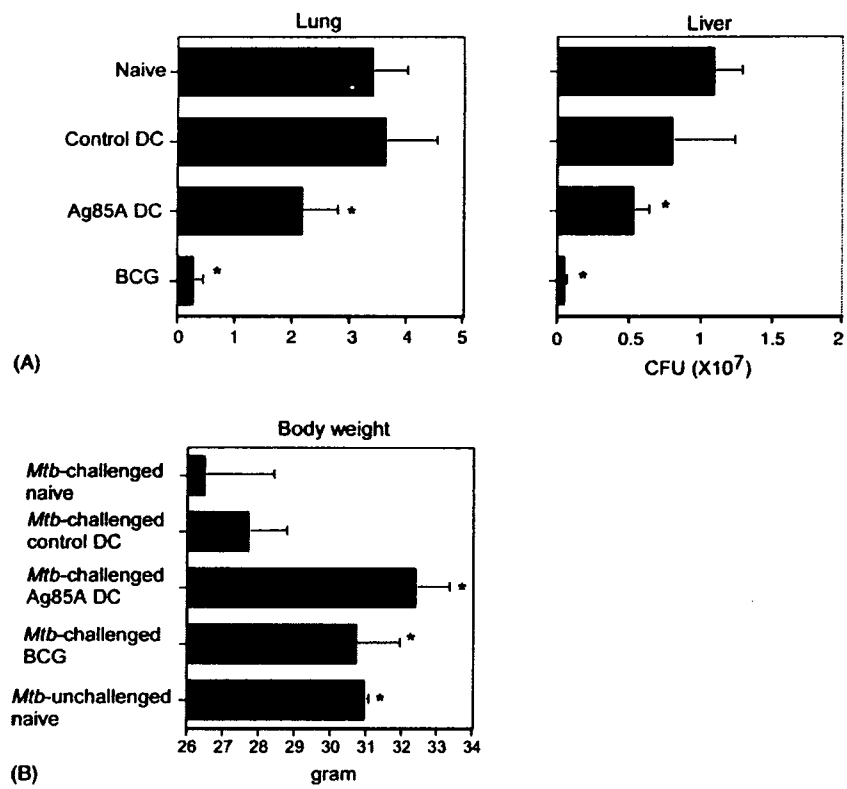

Fig. 6. (A) In vivo protective activity of mice immunized with DCs transduced with Ag85A gene against virulent *M. tuberculosis* challenge. BALB/c mice were immunized with Ag85A gene-transduced DCs twice at a 1-week interval. These mice were challenged i.v. with 5×10^5 CFU of live *M. tuberculosis* H37Rv. 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,

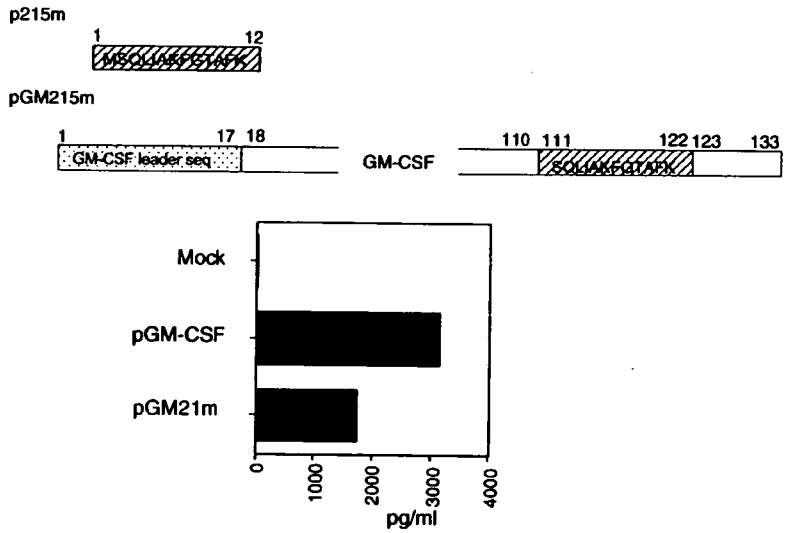

Fig. 1. (A) The schema of gene products deduced from the cDNA constructs prepared in this study (p215m and pGM215m). The hatched boxes indicate LLO 215–226 peptide and a dotted box indicate murine GM-CSF leader sequence. Amino acid numbers of each domain were shown above each schema. (B) Expression of 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, MSQLIAK-FGTAFK 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

from the immunized mice were treated with Tris-buffered ammonium chloride solution to remove red blood cells as described in our previous report [7].

2.8. Bacterial infection

A seed of *L. monocytogenes* EGD strain was cultured overnight in trypticase soy broth (Beckton Dickinson and Company, Cockeysville, MD) at 37 °C in a bacterial shaker and suitably diluted with PBS. The exact infection dose was assessed retrospectively by plating. Mice were immunized four times with DNA vaccine plasmids as described above, or immunized by a single intraperitoneal injection with a sublethal dose (1×10^4 CFU) of *L. monocytogenes*. One month later, the mice were challenged intraperitoneally with 1×10^5 CFU of *L. monocytogenes*. Bacterial numbers of the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates (Beckton Dickinson and Company).

2.9. Statistics

Data from multiple experiments were expressed as the mean ± S.D. Statistical analyses were performed by using StatView-J 5.0 statistics program (SAS Institute, Inc., Cary, NC). Data were analysed with one factor-analysis of variance followed by the Fisher's protected least significant difference (PLSD) test.

3. Results

3.1. Construction of plasmids for DNA immunization

In order to evaluate vaccination with plasmid DNA encoding murine GM-CSF inserted with a dominant Th epitope, we constructed pGM215m plasmid (Fig. 1A). As a control, we prepared another plasmid, p215m, a minigene plasmid for expression of LLO 215–226 peptide alone (Fig. 1A). In order to confirm expression of the GM-CSF-LLO 215–226 protein (GM215) by transfection of pGM215m, we transiently transfected 293T cells with pGM215m or pGM-CSF control plasmid and prepared the culture supernatants. As shown in Fig. 1B, we were able to detect expression of GM215 protein in the culture supernatant of pGM215m-transfected 293T cells by using GM-CSF-specific ELISA.

3.2. Proliferative responses of spleen cells of mice immunized with pGM215m plasmid and the epitope-specific IFN-γ production by the spleen cells

In order to examine the effect of immunization with pGM215m, we performed lymphocyte proliferation assay after immunization of C3H/He mice with the plasmid by using gene gun bombardment. We chose the immunization method as it is an appropriate vaccination route to evaluate the effect of GM-CSF expression plasmid on Langerhans cells and also it is a reliable and reproducible method from our previous experience [8]. As shown in Fig. 2, immunization with pGM215m plasmid induced LLO 215–226-specific proliferative responses of spleen cells from the immunized mice. Immunization with p215m plasmid showed less proliferative responses to LLO 215–226 peptide stimulation, which were not significantly different from those observed in naïve mice (data not shown).

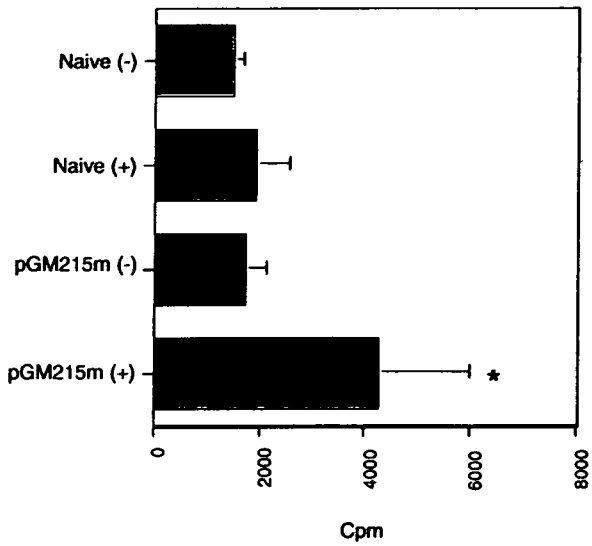

Fig. 2. Specific proliferative responses of spleen cells from pGM215m-immunized mice. C3H/He mice were immunized with pGM215m plasmid by using gene gun four times at 1-week intervals. Spleen cells from the immunized mice were harvested three weeks after the last immunization and cultured in vitro (5×10^5 cells/well) in the presence or absence of 1 μM of LLO 215–226 peptide for 2 days and pulsed with 0.5 μCi of [methyl-^{3}H] thymidine for last 12 h. Results of naïve C3H/He mice are also shown as a control. The values indicate cpm per well. The mean ± S.D. of cpm of four mice per group are shown. Asterisks indicate statistical significance ($p \leq 0.001$) compared with the value of spleen cells of naïve mice without LLO 215–226 stimulation.

Furthermore, we analyzed IFN-γ amounts in the supernatants of spleen cell culture after 5-day in vitro stimulation with LLO 215–226 peptide. Again, immunization with pGM215m induced higher amounts of IFN-γ than those of mice immunized with p215m after the in vitro stimulation (Table 1). We did not detect IL-4 pro-

Table 1
IFN-γ production by splenocytes from C3H/He mice immunized with pGM215m plasmid

Immunization	Stimulation[a]	IFN-γ (pg/ml)[b]
Naïve	–	85.1
	LLO 215	74.1
pGM215m	–	95.9
	LLO 215	1318.7

[a] Spleen cells of immunized mice (2×10^6 cells/well) were cultured in the absence (–) or presence of 1 μM of LLO 215–226 peptide (LLO 215).

[b] After 4 days, cytokine concentrations in culture supernatants were quantified with sandwich ELISA. The mean of duplicate wells of representative data was shown.

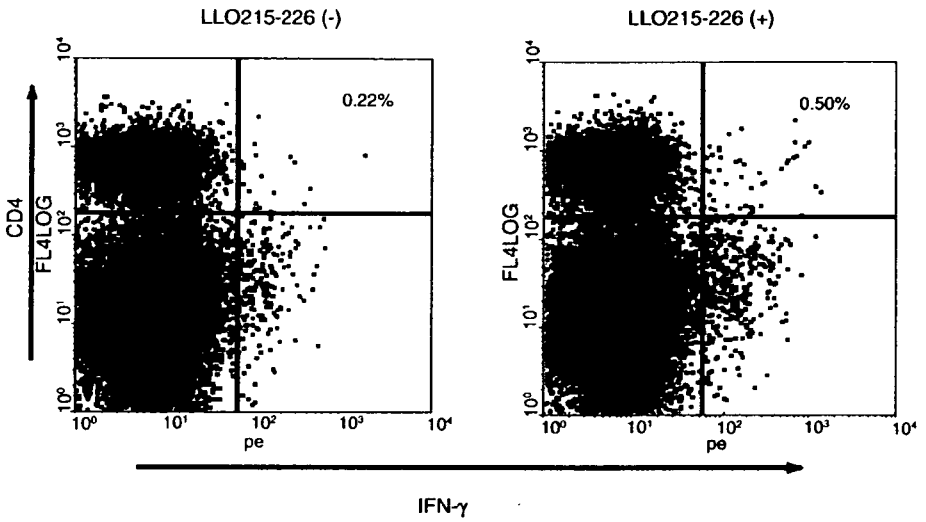

Fig. 3. Induction of LLO 215-specific CD4+ T cells after pGM215m immunization. Intracellular IFN-γ staining on CD4+ T-cell subset was performed using spleen cells after pGM215m immunization in the presence or absence of LLO 215–226 peptide. The percentages of IFN-γ-positive cells in CD4+ T cells are shown.

duction from spleen cells of the immunized mice with ELISA (the detection limit of IL-4 in our ELISA system was approximately 100 pg/ml; data not shown). In addition, we performed intracellular IFN-γ staining with spleen cells of mice immunized with pGM215m. After in vitro stimulation with LLO 215–226 peptide, CD4+ IFN-γ-producing cells was induced in the immune spleen cells (Fig. 3).

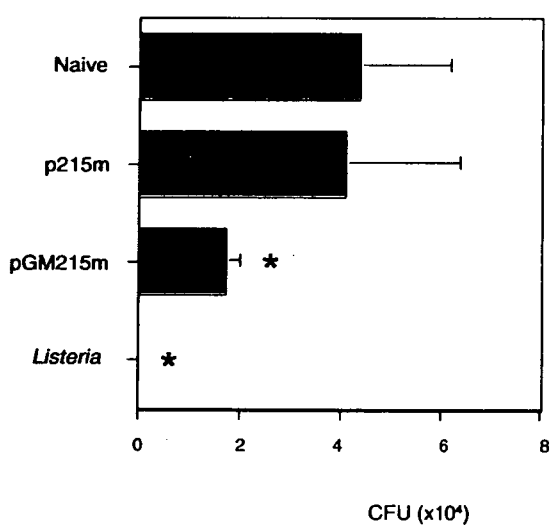

Fig. 4. Protective immunity induced by immunization with pGM215m. Mice were immunized with pGM215m four times at one-week intervals. One month after the last immunization, the immunized mice were challenged intraperitoneally with 1×10^5 CFU of *L. monocytogenes*. Bacterial numbers in the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates. Results of naïve mice and mice immunized with a sublethal dose of *L. monocytogenes* are also shown as controls. Results are expressed as the means ± S.D. for three mice for each group. Asterisks indicate statistical significance ($p < 0.05$) compared with the value of naïve mice.

3.3. Induction of protective immunity against listerial infection after immunization with pGM215m plasmid

In order to examine whether the immunity evoked by immunization with pGM215m plasmid is associated with an increased resistance to infection of virulent *L. monocytogenes*, the in vivo protection experiment was carried out. Seventy-two hours after listerial challenge, mice immunized with pGM215m plasmid were sacrificed and CFU from the spleens were counted. As shown in Fig. 4, immunization with p215m did not show significant protective effects. On the contrary, mice immunized with a sublethal dose of *L. monocytogenes* were able to eliminate challenged *L. monocytogenes* from the spleens. Immunization with pGM215m conferred moderate, but significant protective immunity against lethal listerial challenge.

4. Discussion

DNA vaccination may work through direct transfection of antigen presenting cells (APCs), or by secretion of the encoded protein by muscle or skin cells for the uptake by APCs. Therefore, two different strategies have been considered to induce a particular epitope-specific Th by DNA vaccination. One is an intracellular targeting of antigens [9–11]. Another strategy is taking an advantage of secreted proteins. In general, secreted proteins are phagocytosed by APCs and presented on MHC class II molecules. In this study, we used a plasmid expressing murine GM-CSF, a cytokine which is indispensable for development of APCs such as dendritic

cells and macrophages. GM-CSF is one of most studied cytokines for vaccine adjuvants [12,13]. GM-CSF expression plasmid injected into mouse muscle has been reported to lead to a local infiltration of potential APCs [14]. We therefore reasoned that immunization of a gene for GM-CSF-Th epitope fusion molecule may work well for induction of the epitope-specific Th subset.

Several reports showed that immunization with a DNA vaccine co-expressing both antigen and GM-CSF [15] or a bicistronic plasmid DNA for antigen and GM-CSF [16,17] is superior to co-immunization of DNA vaccines for antigen alone and for GM-CSF in terms of strength of the specific immunity induced by the vaccines. Linking antigen and GM-CSF expression closely in vivo may provide a microenvironment suitable for the uptake and presentation of antigen by dendritic cells or macrophages. Immunization of pGM215m plasmid fits this condition.

It has been reported that $CD4^+$ T cells, especially LLO 215–226-specific T cells, are involved in protective immunity against listerial challenge. Verma et al. [18] demonstrated that induction of $CD4^+$ T cell population responsive to LLO 215–226 in vivo elicits partial protective immunity by using *Salmonella* carrier system. They showed one-log order reduction in numbers of the bacterium in spleens and livers of the immunized mice. In another approach, we showed that significant induction of protective immunity to *L. monocytogenes* by immunization with a plasmid DNA expressing LLO 215–226 Th epitope that replaces the class II-associated invariant chain (Ii) peptide (CLIP) of the Ii [10] or immunization with a plasmid encoding LLO 215–216 Th epitope fused with the endosomal/lysosomal targeting signal of lysosome-associated membrane protein (LAMP)-1 [11]. pGM215m immunization shown here was more effective than LLO215–LAMP fusion DNA immunization [11] in terms of induction of the protective immunity (data not shown).

The immunization strategy shown here, i.e., immunization with GM-CSF gene inserted with a double-stranded oligonucleotide encoding a Th epitope, would be applicable to DNA vaccination for induction of CTL or antibodies as a molecular adjuvant for supplying Th. Investigators add a universal Th epitope such as 13 aminio-acid Pan HLA-DR Epitope (PADRE) [19] in multi-CTL epitope plasmid DNA construction for efficient CTL induction [20]. GM-CSF-Th epitope DNA vaccination would be the alternative strategy for induction of Th.

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Molecular Analysis of RANKL-Independent Cell Fusion of Osteoclast-Like Cells Induced by TNF-α, Lipopolysaccharide, or Peptidoglycan

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Abstract Focusing on the final step of osteoclastogenesis, we studied cell fusion from tartrate-resistant acid phosphatase (TRAP)-positive mononuclear cells into multinuclear cells. TRAP-positive mononuclear cells before generation of multinuclear cells by cell fusion were differentiated from RAW264.7 cells by treatment with receptor activator of nuclear factor kappa B ligand (RANKL), and then the cells were treated with lipopolysaccharide (LPS), followed by culturing for further 12 h. LPS-induced cell fusion even in the absence of RANKL. Similarly, tumor necrosis factor (TNF)-α and peptidoglycan (PGN) induced cell fusion, but M-CSF did not. The cell fusion induced by RANKL, TNF-α, and LPS was specifically blocked by osteoprotegerin (OPG), anti-TNF-α antibody, and polymyxin B, respectively. LPS- and PGN-induced cell fusion was partly inhibited by anti-TNF-α antibody but not by OPG. When TRAP-positive mononuclear cells fused to yield multinuclear cells, phosphorylation of Akt, Src, extracellular signal-regulated kinase (ERK), p38MAPK (p38), and c-Jun NH2-terminal kinase (JNK) was observed. The specific chemical inhibitors LY294002 (PI3K), PP2 (Src), U0126 (MAPK-ERK kinase (MEK)/ERK), and SP600125 (JNK) effectively suppressed cell fusion, although SB203580 (p38) did not. mRNA of nuclear factor of activated T-cells c1 (NFATc1) and dendritic cell-specific transmembrane protein (DC-STAMP) during the cell fusion was quantified, however, there was no obvious difference among the TRAP-positive mononuclear cells treated with or without M-CSF, RANKL, TNF-α, LPS, or PGN. Collectively, RANKL, TNF-α, LPS, and PGN induced cell fusion of osteoclasts through their own receptors. Subsequent activation of signaling pathways involving PI3K, Src, ERK, and JNK molecules was required for the cell fusion. Although DC-STAMP is considered to be a requisite for cell fusion of osteoclasts, cell fusion-inducing factors other than DC-STAMP might be necessary for the cell fusion. J. Cell. Biochem. 9999: 1–13, 2006.

Key words: TNF-α; lipopolysaccharide; peptidoglycan; cell fusion; osteoclasts

Abbreviations used: Akt, PKB, protein kinase B; c-Fos, cellular homolog of v-fos; c-Src, cellular homolog of v-src; DC-STAMP, dendritic cell-specific transmembrane protein; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; HRP; horse radish peroxidase; IL-1β, interluekine-1β; IL-6, interluekine-6; JNK, c-Jun NH2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK-ERK kinase; MyD88, myeloid differentiation factor 88; NFATc1, nuclear factor of activated T-cells c1; NF-κB, nuclear factor kappa B; OPG, osteoprotegrin ligand; P-, phosphorylated; PBS, phosphate-buffered saline; PGN, peptidoglycan; p38, p38MAPK; p65, 65 kD subunit of NF-κB (RelA); RANKL, receptor activator of nuclear factor kappa B ligand; SDS, sodium dodecyl sulfate; TBST, 150 mM NaCl and 0.1% Tween-20 in 25 mM Tris/HCl, pH 7.6; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; TRAP, tartrate-resistant acid phosphatase;

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