なワクチン作成の試みでした。BCGの正式名称は、開発者のCalmetteとGuérin博士の名を冠した、bacille de Calmette et Guérinです。BCGは、両博士がパスツール研究所で、ウシ型結核菌を13年間230代にわたって培養してできた弱毒菌です。BCGは生ワクチンで、上述の結核菌病原因子ESAT6やCFP10などをのぞいて結核菌とほぼ同一の抗原を産生しています。ですから、一部データを誇張した報告はありますが、一般的に不活化ワクチンで生ワクチンであるBCGの効果を上回ることは難しいようです。

ワクチン効果をあらわす獲得防御免疫の主役は、新潟大学で河村・光山等が指摘したように、マクロファージを活性化するIFN-gamma産生T細胞[9]で、感染時に適時、分化・活性化されることが肝要と推察されます。最近は、好中球の集積を促すIL-17を産生する細胞も肉芽腫形成を担うことから[10]、ワクチンによる獲得防御免疫の誘導に必要とされています。このような防御的T細胞を活性化・記憶細胞化できる抗原を利用することが結核ワクチンの開発に必要なステップでしょう。

ワクチン候補分子を眺めると、前述のESAT6やCFP10、細胞壁主要成分であるミコール酸を転移して結核菌の細胞壁を構築する酵素Antigen 85（ちなみ私の恩師である山田毅先生等がalpha抗原としてクローニングされました[11]）、細胞接着／侵入や休眠に関わるHBHAやMDP1[12]など、結核菌の生存や病原性に必須の分子であることが多いようです。抗原性があることに加え、結核菌が必ず産生しなければならない分子、すなわち結核菌の生存に必須な分子をワクチン抗原として利用するこが肝要かもしれません。そう考えると細菌学的な解析もワクチン開発に役立つことが期待されます。

死に至る感染症をワクチンで予防できることは、たいへん望ましく魅力的ですから、私も日本ビーシージー製造株式会社等と共同で結核のワクチン開発を進めています。BCGは小児に対する効果はあるのだから、問題となっている成人期に不活化ワクチンで追加免疫を行うというのが今は現実的だと考えています。BCGの再接種はコッホ反応をおこし副反応が強すぎるので、適切な成分ワクチンを作成しブーストすることが望ましいと思われます。特に成人型肺結核のおもな原因である内因性再燃を押さえるには、増殖期に結核菌が産生する蛋白質のみならず、休止期に産生するMDP1[12]のような抗原も利用し、潜在期に菌を充分に攻撃して弱らせておくことが重要と考え開発を進めています（図２）。

化学療法薬の開発状況

現在の結核治療は、世界保健機関の推奨する主力２薬剤を含む複数薬の直接監視下短期化学療法（DOTS）を基本として遂行されていますが、それでも最低６ヶ月の投薬期間が必要です。結核治療としてはかなり短縮されましたが、一般の感染症に比べると異例の投薬期間です。長期にわたる治療は、患者の精神的苦痛を伴うことから中断も少なくありません。

薬剤耐性結核は“Man made disease”とよばれるように、中途半端な治療が薬剤耐性菌出現の元凶です。この根本的な要因に結核菌の休眠（dormancy）現象があります。結果として多剤耐性結核菌（MDR-TB）や超多剤耐性結核菌（XDR-TB）が出現すると、治療は困難に直面し

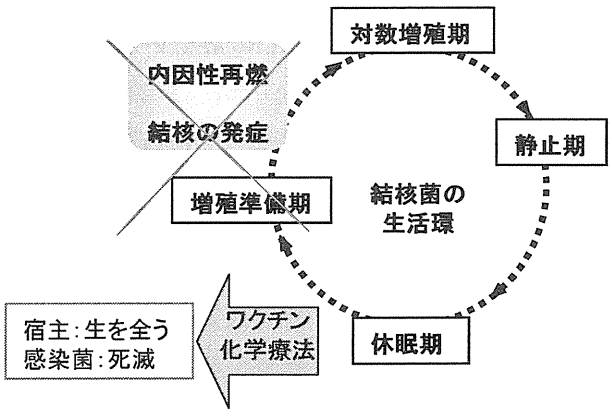

図２　結核菌の生活環と制圧のストラテジー

一般細菌が静止期移行すみやかに死滅するのに対して、結核菌は休眠期に移行して分裂せず長期間生きながらえることができる。休眠菌は一定の割合で、再び増殖を開始し内因性再燃によって結核を発症させる。発症を抑止できれば、宿主の死とともに感染結核菌も死滅する。結核菌の住み処はヒトに限定されているため、内因性再燃を制御することが根本的な結核対策につながる。発症を抑止するワクチンや休眠菌を殺傷できる薬剤の開発が対策の鍵となるだろう。

てしまいます。新規の抗結核薬は1967年のリファンピシン以来、市場投入されていませんが、多剤耐性結核菌にも効果をあらわす複数の新規抗結核薬が臨床試験中です。その中には、国産で大塚製薬の松本真　等が開発したOPC-67683(delamanid)も含まれています[13]。

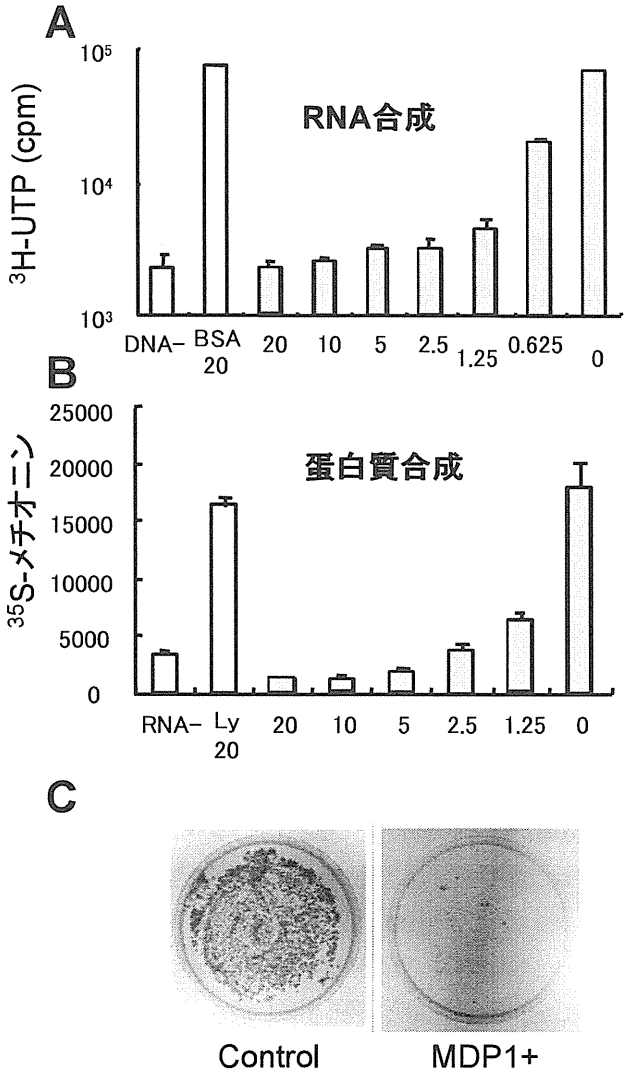

図3　結核菌の蛋白質MDP1は転写／翻訳を阻害し、菌の増殖を抑制する

A、MDP1によるRNA合成阻害。縦軸は、RNAの合成量を^{3}H-dUTPの取り込み量で表している。MDP1を20、10、5、2.5、1.25および0μMそれぞれ加えた。RNA合成酵素はT7ファージ由来のものを用いた。B、MDP1による蛋白質合成阻害。縦軸は、蛋白質合成量を^{35}S-メチオニンの取り込み量で表している。大腸菌リボゾームとファージRNAを鋳型に蛋白質合成を行った。C、BCG由来のMDP1を発現した速育型抗酸菌*Mycobacterium smegmatis*（MDP1+）を寒天培地にて増殖させたもの。コントロール（左）に比べ、増殖が顕著に抑制される。

結核治療が長期の投薬期間を必要とするのは、結核菌が潜在期のみならず活動期においても一定の割合で休眠状態に移行するからです。休眠菌に対して現行の結核薬は効果が低く、増殖期に入ってからようやく菌を殺せるため長い治療期間が必要となります。さらに人類の1/3に潜伏感染する菌は殆どが休眠状態であるため、結核予備軍である潜在性結核の処置を効率よく行うことが難しいのが現状です。

もしも休眠菌を殺傷することができれば、結核治療を短縮、薬剤耐性菌の出現抑制、さらには潜伏感染菌の殺傷による結核の撲滅も不可能ではありません。このような理由から昨今では休眠菌対策が重要課題と位置づけられています[14]。私自身も遅発育性抗酸菌に必須の蛋白質で、休眠期に特に発現し、結核菌の増殖を停止させる分子MDP1を同定しています（図3）[15)-17)]。休眠菌に必須の代謝を標的にした新しいタイプの創製を目指したいと考えています。

おわりに

結核菌は人類の誕生以来、人に非常に巧妙に寄生してきた病原体で、アフリカの東岸で近縁菌より進化し、人類の出アフリカにともなって世界に拡がったと推定されています。結核菌の病原性を探ると、その巧みな“生き様”に感嘆さえしますが、アキレス腱をみつけてなんとか菌も驚くような？戦略を立ててみたいとおもいます。一方、結核菌は単細胞ながら休眠現象によって人なみの長寿を実現しています。そのような長生きのしくみを解明し人の長寿にも応用できないかと考えます。新しい研究室でこのような研究をはじめようとしています。ご興味のある方は、気軽に声をかけていただければ幸甚です。これからどうかよろしくお願いいたします。

【文献】

1）Hershkovitz I, Donoghue HD, Minnikin DE, et al: Detection and molecular characterization of 9,000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean. PLoS One 2008; 3: e3426.

2) Gutierrez MC, Brisse S, Brosch R, et al: Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. PLoS Pathog 2005; 1; e5.
3) Yamamura Y: The pathogenesis of tuberculous cavities. Bibl Tuberc 1958; 13; 13-37.
4) Bekierkunst A: Acute granulomatous response produced in mice by trehalose-6,6-dimycolate. J Bacteriol 1968; 96; 958-961.
5) Yamagami H, Matsumoto T, Fujiwara N, et al: Trehalose 6,6'-dimycolate (cord factor) of *Mycobacterium tuberculosis* induces foreign-body- and hypersensitivity-type granulomas in mice. Infect Immun 2001; 69; 810-815.
6) Ishikawa E, Ishikawa T, Morita YS, et al: Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. J Exp Med 2009; 206: 2879-2888.
7) van der Wel N, Hava D, Houben D, et al: *M. tuberculosis* and *M. leprae* translocate from the phagolysosome to the cytosol in myeloid cells. Cell 2007; 129: 1287-1298.
8) MacGregor GA: BCG: bad news from India. Lancet 1980; 1: 318.
9) Kawamura I, Tsukada H, Yoshikawa H, et al: IFN-gamma-producing ability as a possible marker for the protective T cells against *Mycobacterium bovis* BCG in mice. J Immunol 1992; 148: 2887-2893.
10) Okamoto Yoshida Y, Umemura M, Yahagi A, et al: Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. J Immunol 2010; 184: 4414-4422.
11) Matsuo K, Yamaguchi R, Yamazaki A, et al: Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular alpha antigen. J Bacteriol 1988; 170: 3847-3854.
12) Matsumoto S, Matsumoto M, Umemori K, et al: DNA augments antigenicity of mycobacterial DNA-binding protein 1 and confers protection against *Mycobacterium tuberculosis* infection in mice. J Immunol. 175 2005;441-449.
13) Gler MT, Skripconoka V, Sanchez-Garavito E, et al: Delamanid for multidrug-resistant pulmonary tuberculosis. N Engl J Med 2012; 366: 2151-2160.
14) Ginsberg AM and Spigelman M: Challenges in tuberculosis drug research and development. Nat Med 2007; 13: 290-294.
15) Matsumoto S, Furugen M, Yukitake H, et al: The gene encoding mycobacterial DNA-binding protein I (MDP1) transformed rapidly growing bacteria to slowly growing bacteria. FEMS microbiology letters 2000; 182: 297-301.
16) Katsube T, Matsumoto S, Takatsuka M, et al: Control of cell wall assembly by a histone-like protein in Mycobacteria. J Bacteriol 2007; 189: 8241-8249.
17) Niki M, Niki M, Tateishi Y, et al: A novel mechanism of growth phase-dependent tolerance to isoniazid in mycobacteria. J Biol Chem 2012; 287: 27743-27752.

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Original Article

Multicolor Flow Cytometric Analyses of CD4$^+$ T Cell Responses to *Mycobacterium tuberculosis*-Related Latent Antigens

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SUMMARY: Although IFN-γ release assays (IGRAs) provide increased specificity over tuberculin skin tests, the early and sensitive detection of reactivation of latently infected *Mycobacterium tuberculosis* is required to control tuberculosis (TB). Recently, a multicolor flow cytometry has been developed to study CD4$^+$ T cell cytokine responses (IFN-γ/IL-2/TNF-α) to purified protein derivatives (PPD) and *M. tuberculosis*-specific antigens (ESAT-6/CFP-10) and provided useful information regarding anti-TB immunity. However, the diagnostic relevancy remains uncertain. Here, we analyzed three additional CD4$^+$ T cell cytokine responses (IL-10/IL-13/IL-17) to latent mycobacterial antigens (α-crystallin, methylated heparin-binding hemagglutinin [HBHA], and mycobacterial DNA-binding protein 1 [MDP-1]) as well as PPD and ESAT-6/CFP-10 in 12 IGRA$^+$ TB cases and 8 healthy controls. No significant difference in IFN-γ response was observed between TB cases and controls, which was likely due to the high variation among the individuals. However, we found a significant increase over healthy controls in (i) the IL-2 response to HBHA in recovery stage TB cases, (ii) the number of *M. tuberculosis*-specific polyfunctional CD4$^+$ T cells in on-treatment and recovery stage cases, and (iii) the IL-17 response to HBHA and MDP-1 in on-treatment and recovery stage cases. These results suggest that a combination of these T cell cytokine parameters could aid in accurate diagnosis of latent TB infection.

INTRODUCTION

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* infection. *M. tuberculosis* can establish a long-term persistent infection without causing any symptoms; this condition is referred to as latent TB infection (LTBI). It is estimated that one to two billion people worldwide are living with LTBI (1). A minority of people with LTBI develop clinical disease (active TB) when the host TB defense mechanism is altered by poor health conditions such as malnutrition, aging, and immunodeficiency caused by human immunodeficiency virus (HIV) infection (2). The lifetime risk of clinical disease development in LTBI individuals is estimated to be 5–10% (3,4); this group of individuals may benefit from prophylactic treatment. However, because the vast majority of people with LTBI will not develop active TB, it is not practical to treat all LTBI individuals. Therefore, development of a screening method to identify individuals who may benefit from preventative treatment is required.

Clinical IFN-γ release assays (IGRAs) that detect the IFN-γ response to *M. tuberculosis*-specific antigens such as ESAT-6 and CFP-10 have been developed and are widely utilized for the diagnosis of TB because they are more specific than conventional tuberculin skin tests (5). The sensitivity of IGRAs for the detection of active TB is approximately 85%, and the specificities for the detection of active TB and LTBI are greater than 85% and 98–100%, respectively (6); however, the predictive value of IGRAs for the development of active TB from LTBI is only 2.7% (7).

Although ESAT-6 and CFP-10 are the predominant secretory proteins released during the active stage of *M. tuberculosis*, several mycobacterial antigens are known to be associated with the latent stage of *M. tuberculosis* infection. The α-crystallin (Acr) protein is a member of the small heat shock protein family that has chaperone activity in vitro (8). Acr is required for the growth of *M.*

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tuberculosis in cultured macrophages, and its expression increases under hypoxic conditions (9). Heparin-binding hemagglutinin (HBHA) is a protein that functions as an adhesin for epithelial cells (10). Because the transition from the alveolar space to deeper organ sites is a crucial step in the pathogenesis of LTBI, HBHA may serve as a target antigen for the diagnosis of LTBI, as reported previously (11). Mycobacterial DNA-binding protein 1 (MDP-1) belongs to a group of orthologous DNA-binding proteins that constitute 8–10% of total protein in mycobacteria such as *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and *M. tuberculosis* (12,13). MDP-1 is upregulated in the stationary phase of *M. tuberculocis* infection and induces protective immunity against *M. tuberculosis* infection in mice (12,14). Therefore, in combination with *M. tuberculosis*-specific ESAT-6 and CFP-10, the latency-associated antigens may aid in monitoring the immune response to *M. tuberculosis* in LTBI.

$CD4^+$ T cells produce IFN-γ and play a critical role in controlling persistent *M. tuberculosis* infection. HIV-infected patients with a decreased baseline $CD4^+$ T cell count have a higher incidence of TB (15), and macaques coinfected with *M. tuberculosis* and simian immunodeficiency virus that display a decreased $CD4^+$ T cell count show earlier reactivation of latent *M. tuberculosis* infection (16). There are many subsets of $CD4^+$ T cells, such as T-helper 1 (Th1), Th2, Th17, and regulatory T cells (Tregs), and they produce a unique set of cytokines (17). All $CD4^+$ T subsets cooperate or interfere with each other to control infection, and the dominant subset may differ between active and latent *M. tuberculosis* infection cases. Multi-parameter flow cytometry enables analysis of these cytokine-producing antigen-specific T cells ex vivo. Using this technology, it was proposed that polyfunctional T cells that produce multiple cytokines are associated with protective immunity (18), and T-cell polyfunctionality has been analyzed in individuals with HIV and *M. tuberculosis* double infection (19,20). These studies suggested that the number of *M. tuberculosis*-specific T cells producing a combination of IFN-γ, IL-2, and/or TNF-α may be correlated with the level of *M. tuberculosis* protection; however, evidence that these polyfunctional T cells are directly associated with protection is still lacking. Furthermore, no previously published study has evaluated polyfunctional T cells, including subsets of $CD4^+$ T cells, with a wide range of *M. tuberculosis* antigens such as latent phase proteins.

In this study, we postulated that the profiles of multiple T cell cytokines produced in response to latency-associated antigens vary at distinct clinical stages of *M. tuberculosis* infection and provide diagnostic information that supplements the ESAT-6 and CFP-10 profiles detected by conventional IGRAs. Multicolor flow cytometric analyses of the five $CD4^+$ T cell cytokine responses, such as IFN-γ, IL-2, IL-10, IL-13, and IL-17, to several *M. tuberculosis*-specific antigens, including latency-associated antigens, in active TB (on-treatment), non-active TB (recovery stage and contact cases), and healthy control cases are presented.

MATERIALS AND METHODS

Study subjects: Twelve *M. tuberculosis*-infected cases, including seven cases of TB diagnosed by sputum smear or PCR analysis and five cases with a history of close contact with active TB, were recruited from Tokyo National Hospital (Tokyo, Japan). The characteristics of the *M. tuberculosis*-infected cases, all of which were QuantiFERON TB (QFT) test positive, are shown in Table 1. At the time of evaluation, two of the seven TB cases were receiving on-going treatment with anti-TB drug therapy and five were in the recovery stage after completion of anti-TB drug therapy. One contact case was receiving prophylactic anti-TB treatment.

Table 1. Characteristics of *M. tuberculosis*-infected cases

Case no.	Sex	Age	Anti-TB therapy (duration after treatment)	QFT test	Others
1	male	25	ongoing (6 month)	positive	
2	male	58	none (contact)	positive	
3	male	79	none (contact)	positive	
4	male	53	prophylaxis (contact)	positive	
5	female	82	ongoing (<1 month)	positive	
6	male	31	previous (>6 years)	positive	
7	male	76	previous (>2 years)	positive	
8	male	79	previous (>1 year)	positive	
9	female	36	none (contact)	positive	
10	male	77	previous (>4 years)	positive	HIV+
11	female	46	none (contact)	positive	
12	male	95	previous (>1 year)	positive	

Eight healthy controls with no history of TB infection or exposure, including two males and six females with a mean age of 32.1 years, were recruited from Nagasaki University (Nagasaki, Japan). A blood sample from one of the laboratory staff, who had a previous history of being highly reactive to many mycobacterial antigens without displaying symptoms, was used as a positive control. All the samples included in the present study were obtained with informed consent and with ethical approval from the Institute of Tropical Medicine Nagasaki University Joint Ethics Committee as well as from the research and ethical committees of the National Institute of Infectious Diseases (Tokyo, Japan).

Reagents: Staphylococcal enterotoxin B (SEB) and purified protein derivatives (PPD) of *M. tuberculosis* were purchased from Sigma-Aldrich (St. Louis, Mo., USA) and Japan BCG Laboratory (Tokyo, Japan), respectively.

ESAT-6, CFP-10, Acr, methylated HBHA (mHABA), and methylated MDP-1 (mMDP-1) were recombinant protein products from *Escherichia coli*. The vectors expressing ESAT-6, CFP-10, HBHA, and MDP-1 were produced by a PCR-based approach using a bacterial chromosome. Each PCR product containing the relevant coding region was designed to allow the expression of a C-terminal, 6× histidine-tagged variant of the recombinant protein following ligation into the pET-21b vector (Toyobo, Osaka, Japan). Recombinant *M. tuberculosis* proteins were purified using Ni-NTA columns (1 ml bed volume) (QIAGEN, Germantown, Md., USA), according to the manufacturer's instructions. The endotoxins were excised from recombinant *M. tuberculosis* proteins using EndoTrap columns (Profos AG, Regenburg, Germany), according to the manufacturer's instructions. Residual endotoxin levels

were determined using a Limulus amebocyte lysate test (Lonza, Walkersville, Md., USA) and were found to be below 0.5 EU/10 μg protein. Chemical methylation of the lysine residues in recombinant mHBHA and mMDP-1 was performed as described previously (21).

The following fluorescently labeled monoclonal antibodies (mAbs) were used in this study: anti-CD3-APC-Cy7 (HIT3a), anti-IFN-γ-PE-Cy7 (4S.B3), anti-IL-10-PE (JES3-9D7), anti-IL-17-Alexa Fluor 700 (BL168), anti-TNF-α-PerCP-Cy5.5 (MAb11) (Biolegend, San Diego, Calif., USA), anti-CD4-Pacific Blue (OKT4), anti-IL-2-APC (MQ1-17H12), and anti-IL-13- FITC (PVM13-1) (eBioscience, San Diego, Calif., USA). Where necessary, the relevant isotype control mAb was used. Cell viability was assessed using the LIVE/DEAD kit (Invitrogen, Carlsbad, Calif., USA). Brefeldin-A (BFA) was purchased from Sigma-Aldrich.

In vitro culture: Peripheral blood mononuclear cells (PBMCs) were isolated from 10 ml of heparinized blood by density gradient centrifugation using Lymphoprep solution (AXIS-Shield, Oslo, Norway). After two washes with PBS, cells were resuspended in R10 medium, consisting of RPMI 1640 (Wako Junyaku Co., Tokyo, Japan) supplemented with 10% FBS, penicillin/streptomycin (Invitrogen), and 2 mM L-glutamine. A total of 0.5 to 1 $\times$ 10^6 PBMCs in 200 μl of R10 medium were plated into each well of a 96-well round bottom culture plate. Cultures were incubated with no antigen (medium only) or medium containing SEB (16.7 μg/ml), PPD (25 μg/ml), ESAT-6 and CFP-10 (0.9 μg/ml each), Acr (1.4 μg/ml), mHBHA (1.8 μg/ml), or mMDP-1 (7.5 μg/ml). All antigen stimulations were performed in the presence of BFA (1 μg/ml). The cells were incubated overnight (14–16 h) at 37°C in a 5% CO_2 incubator.

Flow cytometry: Following overnight incubation, the culture plate was centrifuged and the supernatant was removed. This was followed by the addition of 20 μl of a previously titrated surface marker cocktail (CD3 and CD4) and LIVE/DEAD reagent. The plate was vortexed and incubated for 30 min at 4°C. After washing with PBS, the cells were permeabilized, fixed with FACS permeabilizing solution (BD Bioscience, San Jose, Calif., USA), and incubated for 20 min at 4°C. The cells were washed three times with Perm/wash solution (BD Bioscience), and 20 μl of a previously titrated anti-cytokine mAbs cocktail containing IFN-γ, IL-2, IL-10, IL-13, and IL-17 was then added. After 30-min incubation at 4°C, the cells were washed and acquired using a FACS Canto II flow cytometer (BD Bioscience). FACS data were reanalyzed using FlowJo software, version 8.8.7 (TreeStar, San Carlos, Calif., USA).

Statistical analysis: Group medians and distributions were analyzed using Wilcoxon matched-pairs signed-rank tests and Mann-Whitney *U* tests. All analyses were performed using GraphPad Prism software, version 5 (San Diego, Calif., USA). A *P* value $<0.05\%$ was considered significant.

RESULTS

Assessment of IFN-γ-producing *M. tuberculosis*-specific T cells in fresh and frozen PBMCs: T cells stimulated with recombinant *M. tuberculosis*-related antigens were analyzed by flow cytometry. Because the majority of the Japanese population is immunized with BCG in early childhood, many individuals produce PPD-reactive memory T cells. Interestingly, one asymptomatic volunteer was found to be highly reactive to both PPD and the *M. tuberculosis*-specific antigens, ESAT-6 and CFP-10. This volunteer was a 60-year-old laboratory technician who had worked in a microbiology laboratory for almost 40 years and was therefore at a high risk of *M. tuberculosis* exposure; however, this individual had no history of TB symptoms or signs of respiratory diseases. PBMCs from this individual were used to determine the concentration of *M. tuberculosis*-related antigens required to maximize the T cell response without causing cell toxicity. Because fresh PBMC samples were not always available, levels of several cytokines from both fresh and frozen PBMC samples stimulated with a mixture of ESAT-6 and CFP-10 were compared. Both fresh and frozen PBMCs were stimulated with an ESAT-6/CFP-10 mixture (1.5 μg each) overnight. T cell surface staining with CD3, CD4, and CD8, followed by intracellular staining, was then performed using a multicolor flow cytometer and mAbs targeting IL-2, TNF-α, MIP-1β, and IL-17. The frequencies of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in fresh PBMCs (Fig. 1A) were approximately 2% and 3.8%, respectively. These frequencies were reduced by more than 50% in the frozen samples (Fig. 1B). The frequency of MIP-1β-producing $CD8^+$ T cells was decreased from 2.79 + 0.81% (3.6% total) in fresh samples (Fig. 1A) to 1.48 + 0.35% (1.83% total) in frozen samples (Fig. 1B). However, the frequencies of IL-2, TNF-α, and MIP-1β-producing $CD4^+$ T cells were similar in fresh and frozen PBMCs. These results strongly suggest that IFN-γ-producing T cells are susceptible to degradation by freeze-thaw procedures. Therefore, subsequent studies were performed using PBMCs obtained from fresh blood samples.

IFN-γ and IL-2 responses of $CD4^+$ T cells to *M. tuberculosis*-related antigens: The IFN-γ and IL-2 responses of $CD4^+$ T cells to various *M. tuberculosis*-related antigens in the 12 *M. tuberculosis*-infected cases and eight healthy controls were investigated by flow cytometry. Double-positive (IFN-γ and IL-2) $CD4^+$ T cells were detected following PPD and ESAT-6/CFP-10 stimulation; the frequency of these double-positive cells was lower for ESAT-6/CFP-10 stimulation than that for PPD stimulation (Fig. 2A). $CD4^+$ T cell responses to other *M. tuberculosis*-related antigens, namely Acr, mHBHA, and mMDP-1, were also investigated (Fig. 2A). In *M. tuberculosis*-infected cases, the IFN-γ response of $CD4^+$ T cells to all *M. tuberculosis*-related antigens tested was significantly higher than that of control cells exposed to medium alone (Fig. 2B). In contrast, the IFN-γ responses to *M. tuberculosis*-related antigens in healthy controls were not statistically significant (Fig. 2B). The latter finding remained the same even if analysis was confined to individuals with a high $CD4^+$ T cell response to PPD. These results indicate that the *M. tuberculosis*-related antigens used in this study can stimulate *M. tuberculosis*-reactive or mycobacteria-reactive $CD4^+$ T cells. Interestingly, IL-2-producing $CD4^+$ T cells were detectable in the absence of antigen in three *M. tuberculosis*-infected cases; however, none were detected in healthy controls (Fig.

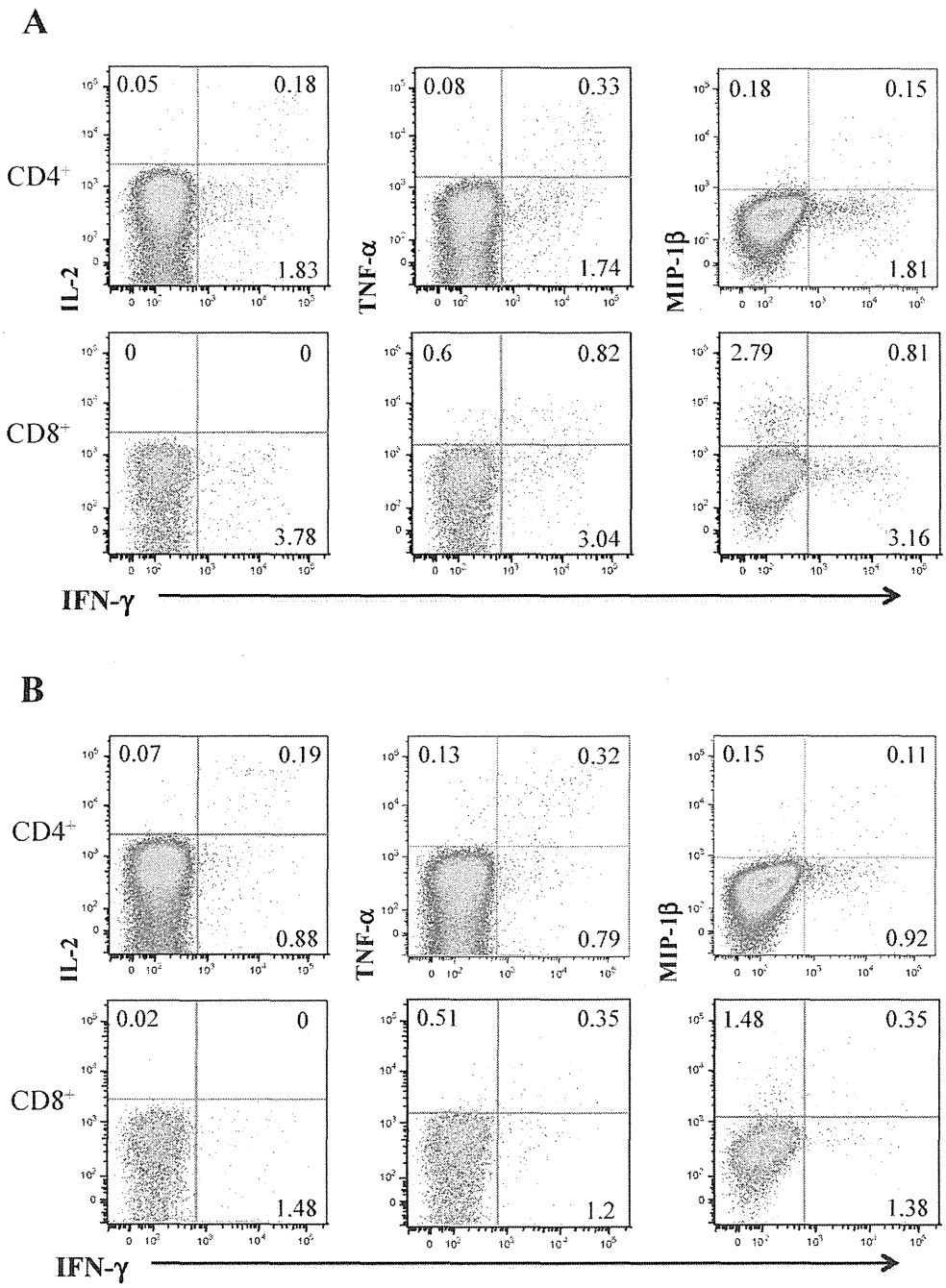

Fig. 1. Flow cytometric analyses of fresh and frozen PBMCs following ESAT-6/CFP-10 stimulation. (A) Fresh PBMCs. (B) Frozen PBMCs. PBMCs were prepared from one asymptomatic high responder. Upper plots display $CD3^+CD4^+$ T cells, and lower plots display $CD3^+CD8^+$ T cells. The plots display the percentage of cells producing IFN-γ and either IL-2, TNF-α, or MIP1-β. Values in the lower right quadrant represent the percentage of IFN-γ-producing cells; values in the upper left quadrant represent the percentage of IL-2, TNF-α, or MIP1-β-producing cells; and values in the upper right quadrant represent the percentage of cells producing both IFN-γ and the relevant cytokine.

2C). Despite this non-specific response, the IL-2 response to PPD in *M. tuberculosis*-infected cases was significantly higher than that of media-exposed control cells ($P = 0.0425$). In healthy controls, a slight IL-2 response to PPD was detected; however, the response was not significant (Fig. 2C). Further analysis of the IL-2 response to mHBHA in *M. tuberculosis*-infected cases grouped by clinical status revealed a significant IL-2 response in recovery stage TB cases compared with that in control cases ($P = 0.0225$, Fig. 2D) and contact cases ($P = 0.0119$, Fig. 2D). Interestingly, two distinct groups were identified, one of which had a high IL-2 response and the other had a low IL-2 response in on-treatment and recovery stage TB cases. This finding suggests that the IL-2-producing $CD4^+$ T cells that are stimulated by mHBHA may indicate ongoing *M. tuberculosis* replication.

Use of polyfunctional $CD4^+$ T cells producing both IFN-γ and IL-2 as an indicator of TB: The polyfunctionality and corresponding high mean fluorescence in-

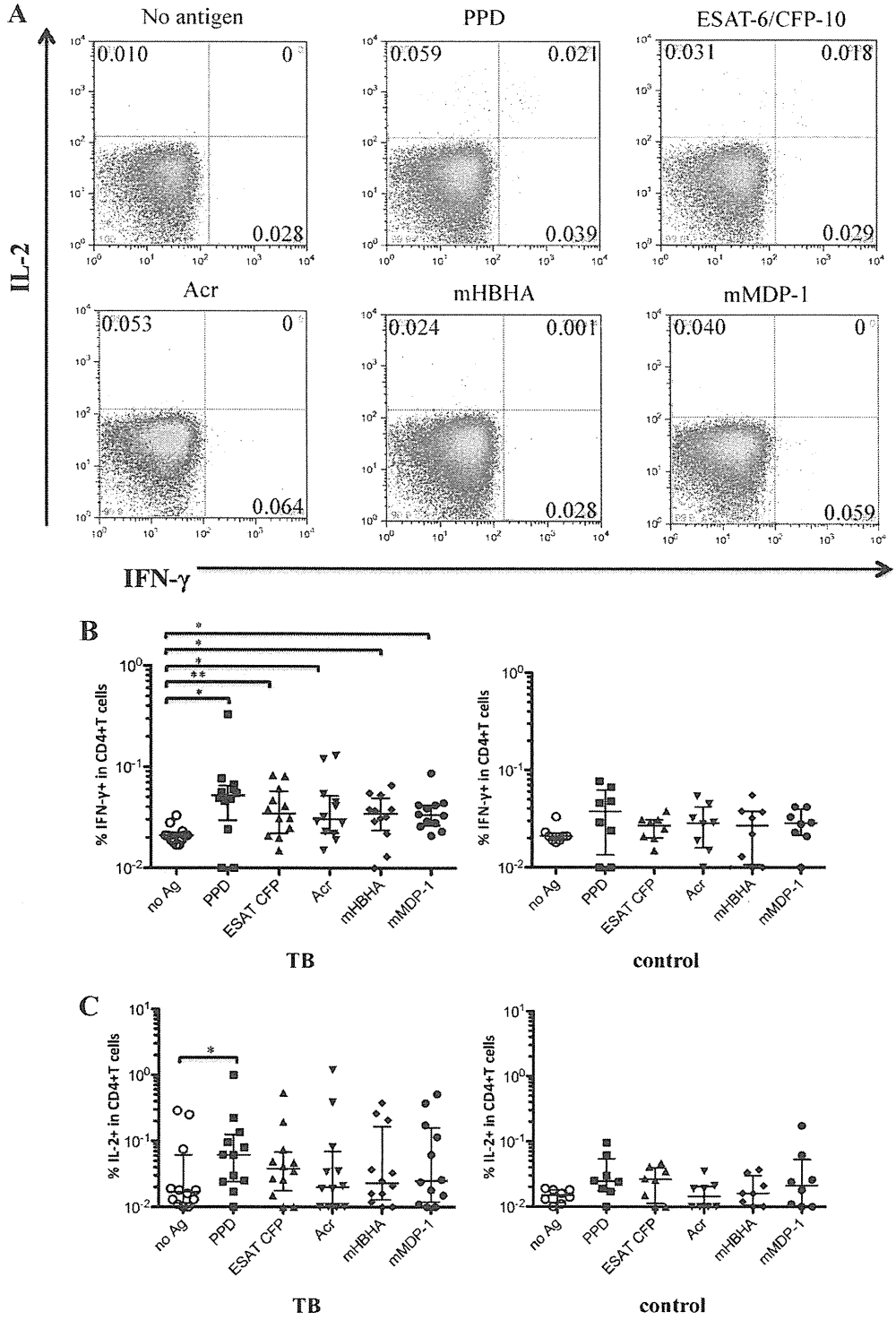

Fig. 2. IFN-γ and IL-2 responses of CD4$^+$ T cells to *M. tuberculosis*-related antigens. (A) Representative flow cytometry results showing IFN-γ and IL-2 responses of CD3$^+$ CD4$^+$ T cells to various *M. tuberculosis*-related antigens. Values in the lower right quadrant represent the percentage of IFN-γ-producing cells, values in the upper left quadrant represent the percentage of IL-2-producing cells, and values in the upper right quadrant represent the percentage of cells producing both IFN-γ and IL-2. IFN-γ (B) and IL-2 (C) responses to antigens in *M. tuberculosis*-infected cases (left) and healthy controls (right). The percentages of IFN-γ-producing CD4$^+$ T cells are shown. The long horizontal line represents the median, and the vertical line represents the interquartile range. The differences between control cells exposed to culture medium only (no antigen) and cells exposed to each antigen were assessed using the Wilcoxon matched-pairs signed rank test ($^{**}P < 0.01$, $^{*}P < 0.05$). (D) IL-2 responses to mHBHA in *M. tuberculosis*-infected cases, grouped according to the disease status as on-treatment TB cases ($n = 2$), recovery stage TB cases ($n = 5$), and contact cases ($n = 5$). Responses of control cases ($n = 8$) are also shown. The differences between each set of samples were assessed using the Mann-Whitney test ($^{*}P < 0.05$).

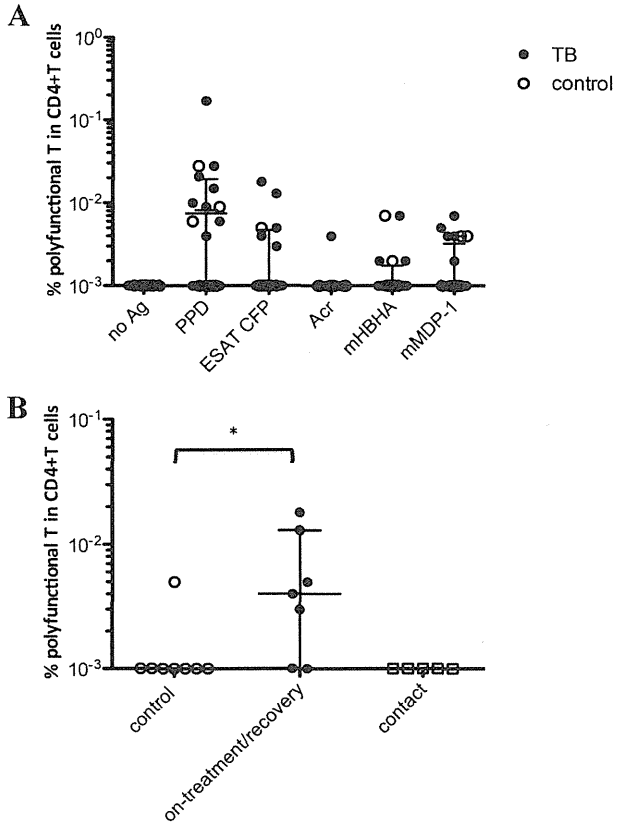

Fig. 3. Responses of polyfunctional CD4+ T cells to *M. tuberculosis*-related antigens. (A) Percentages of polyfunctional (secreting both IFN-γ and IL-2) CD4+ T cells following stimulation by various *M. tuberculosis*-related antigens. Filled circles represent the *M. tuberculosis*-infected cases, and open circles represent the healthy controls. (B) Percentages of polyfunctional CD4+ T cells following stimulation by ESAT-6/CFP-10; *M. tuberculosis*-infected cases are grouped according to the disease status as either TB (on-treatment/recovery; $n = 7$) or contact cases ($n = 5$). Responses of control cases ($n = 8$) are also shown. The differences between control cells exposed to culture medium only and grouped *M. tuberculosis* cases were assessed using the Mann-Whitney test ($^*P < 0.05$). (A, B) The long horizontal line represents the median, and the vertical line represents the interquartile range.

tensity of T cells that produce multiple cytokines are highly correlated with protection in various infections and vaccination (26). In this study, polyfunctional CD4+ T cells producing both IFN-γ and IL-2 were detected following all *M. tuberculosis*-related antigen stimulations. No polyfunctional CD4+ T cells were detectable without antigen stimulation. Although the overall frequency of polyfunctional CD4+ T cells following PPD stimulation was higher in the *M. tuberculosis*-infected cases than that in the healthy controls, three of the eight healthy controls showed a high frequency of polyfunctional CD4+ T cells (Fig. 3A). The difference between the frequency of polyfunctional CD4+ T cells in *M. tuberculosis*-infected cases and healthy controls was not significant for the other *M. tuberculosis*-related antigen stimulations (Fig. 3A); however, when the seven on-treatment and recovery stage *M. tuberculosis*-infected cases were analyzed separately from the five *M. tuberculosis* contact cases, the frequency of polyfunctional CD4+ T cells following ESAT-6/CFP-10 stimulation was significantly higher in the on-treatment and recovery stage groups than that in the control group (Fig. 3B; $P = 0.0365$). These results suggest that the polyfunctional CD4+ T cell response to this set of *M. tuberculosis*-specific antigens and not the IFN-γ response alone (Fig. 2B) has a diagnostic value for the detection of on-treatment and recovery stage TB cases.

IL-17A responses to some *M. tuberculosis*-related antigens are associated with active or latent *M. tuberculosis* infection: Compared with control cells exposed to culture medium, the percentage of CD4+ T cells producing IL-17A was slightly but significantly increased by PPD and mHBHA stimulation ($P = 0.0117$ and 0.0233, respectively) (Fig. 4A). This increase was observed only in *M. tuberculosis*-infected cases and not in healthy controls (data not shown). Furthermore, when the on-treatment and recovery stage cases were analyzed separately from the contact cases, the IL-17A responses induced by mHBHA (Fig. 4B) and mMDP-1 (Fig. 4C) were significantly higher ($P = 0.0127$ and 0.0237, respectively) than those in healthy controls in the on-treatment and recovery stage groups only. Overall, these data suggest that an increased frequency of IL-17A-producing CD4+ T cells is a supportive indicator of *M. tuberculosis* infection.

The frequency of IL-10-producing CD4+ T cells is reduced following TB therapy: IL-13-producing CD4+ T cells were not detected in the subjects included in this study. In *M. tuberculosis*-infected cases, the IL-10 responses to ESAT-6/CFP-10, mHBHA, and mMDP-1 were significantly higher than the response to culture medium alone ($P = 0.0413$, 0.0231, and 0.0144, respectively) (Fig. 5A). Notably, the frequency of Acr-stimulated IL-10-producing CD4+ T cells in the recovery stage TB cases was significantly lower than that in the contact cases ($P = 0.0362$) (Fig. 5B). This trend needs to be confirmed with a larger cohort of TB cases.

DISCUSSION

In this study, we performed cross-sectional analysis of cytokine profiles in CD4+ T cells from different clinical stages of TB infection, including on-treatment (active TB), recovery stage, and contact cases (both as non-active TB), following stimulation by PPD, ESAT-6/CFP-10, and other latency-associated *M. tuberculosis* antigens. We demonstrated here that the IFN-γ response alone was unable to distinguish between TB cases and healthy controls, even after exposure to a new set of latency-related *M. tuberculosis* antigens. However, when TB cases were grouped by distinct clinical stages of TB infection, significant differences between the recovery stage TB group and control group and between the recovery stage TB group and contact group were observed in the IL-2 response to mHBHA (Fig. 2D), and significant differences were observed between the recovery stage TB group and control group in the IL-17 response to mHBHA and mMDP-1 (Fig. 4B and 4C). Similarly, the number of *M. tuberculosis*-specific polyfunctional T cells producing both IFN-γ and IL-2 was also significantly different from that of the control group in ESAT-6/CFP-10 stimulation, which is consistent with previous findings (22). Thus, although further studies are necessary, our results indicate that cytokine

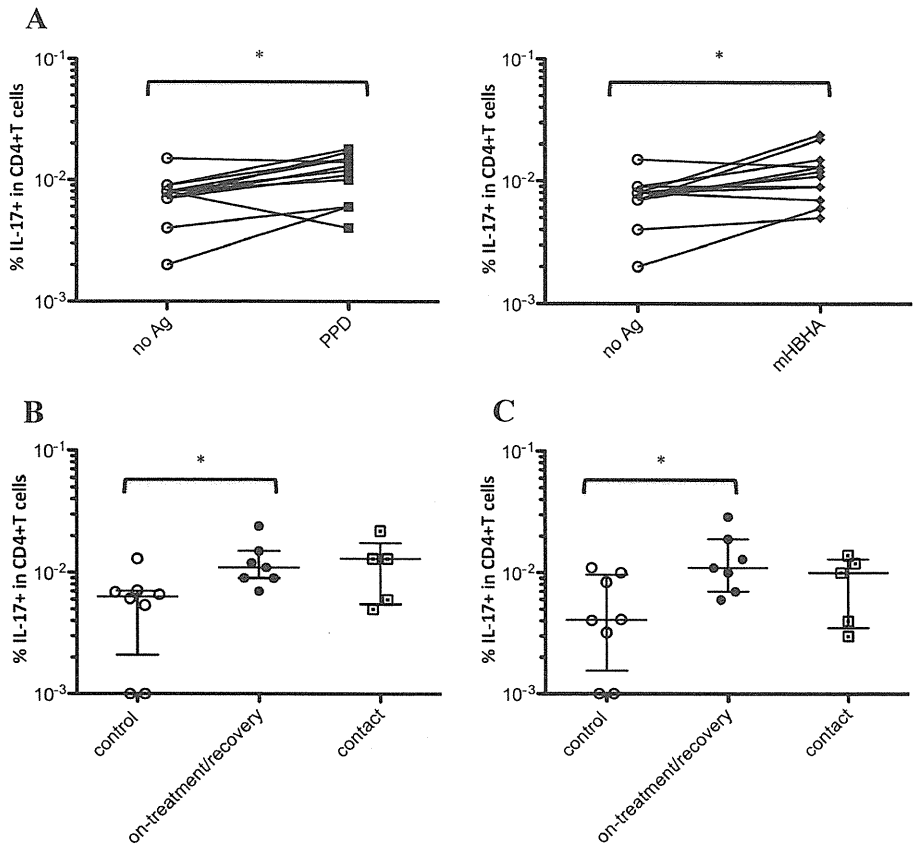

Fig. 4. IL-17 responses of $CD4^+$ T cells to *M. tuberculosis*-related antigens. (A) IL-17 responses to culture medium alone (no antigen), PPD, and mHBHA in *M. tuberculosis*-infected cases. The differences between control cells and each antigen were assessed using the Wilcoxon matched-pairs signed rank test ($^*P < 0.05$). (B, C) IL-17 responses to mHBHA (B) and mMDP-1 (C) in TB cases grouped according to the disease status as TB (on-treatment/recovery; $n = 7$) or contact cases ($n = 5$). Responses of control cases ($n = 8$) are also shown. The differences between control cells and each *M. tuberculosis* group were assessed using the Mann-Whitney test ($^*P < 0.05$). The long horizontal line represents the median and the vertical line represents the interquartile range.

profiles of various *M. tuberculosis*-related antigen-specific T cells can be useful parameters to assess TB status.

It is known that the clinical treatment of *M. tuberculosis* shifts the single IFN-γ-producing $CD4^+$ T cell response to both a polyfunctional IFN-γ/IL-2 response and a single IL-2 response (23). Therefore, while the dominant IFN-γ response is detectable during the active TB stage, dominant IL-2 responses are more likely to be detected at the non-active (contact and recovery) stage of the disease. Unfortunately, we were unable to address this issue in the present study because only two on-treatment cases were included; therefore, further studies with a larger sample size are warranted. Notably, the IL-2-producing $CD4^+$ T cell response was elevated in some patients prior to *M. tuberculosis* antigen stimulation. Because IL-2 is known to be crucial for the maintenance of Treg cells (24) and proliferation of Th cells (25), we speculated that these IL-2-producing $CD4^+$ T cells are on duty in vivo to regulate inflammatory responses caused by *M. tuberculosis* infection.

Polyfunctional *M. tuberculosis*-specific T cells have a memory function with proliferative capacity (19,26) as well as the ability to produce high quantities of cytokines (27). These functional abilities are necessary to control the propagation of foreign pathogens such as *M. tuberculosis*. Therefore, polyfunctional T cells are expected to be induced in *M. tuberculosis* cases with active bacterial replication, and some studies have reported that polyfunctional T cells are present at higher frequencies in active TB cases than those in healthy controls or latent TB cases (28,29). In the present study, significant levels of ESAT-6/CFP-10-specific polyfunctional $CD4^+$ T cells producing both IFN-γ and IL-2 were detected in TB cases only. An investigation of the TNF-α responses was not performed in this study owing to the color detection limitation of the flow cytometer employed. Recently, a large cohort investigation of ESAT-6- and CFP-10-induced polyfunctional *M. tuberculosis*-specific T cells (producing IFN-γ, IL-2, and TNF-α) in TB patients showed that the TNF-α-producing *M. tuberculosis*-specific $CD4^+$ T cell response is dominant and accurately reflects the active TB state (30). However, because frozen PBMC samples were used in the investigation, IFN-γ-producing $CD4^+$ T cells may have been exclusively lost, as observed in the present study (Fig. 1). Therefore, further studies are warranted to confirm this finding.

$CD4^+$ T cells that secrete IL-17 are considered as Th17 cells (17), which trigger early inflammatory via neutrophil recruitment (31). Because of the methylation at lysine residues, mycobacterial HBHA proteins are resistant to proteolytic degradation by proteases present in bronchoalveolar lavage fluids, and their abundance

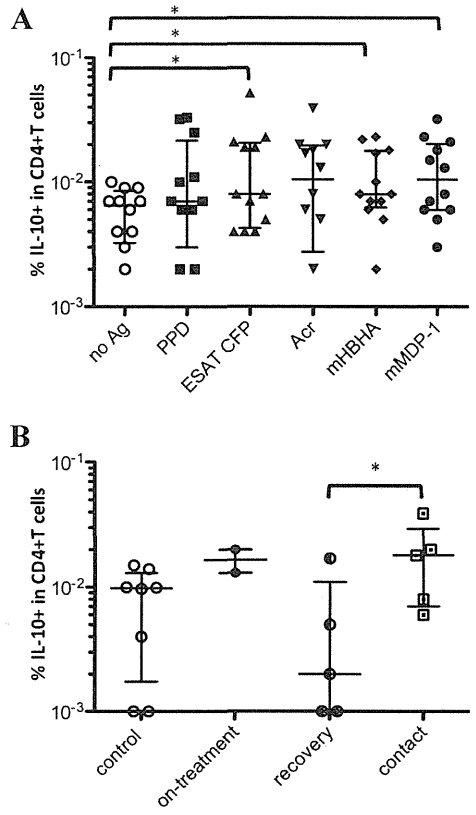

Fig. 5. IL-10 responses of CD4$^+$ T cells to *M. tuberculosis*-related antigens. (A) The percentages of IL-10 secreting CD4$^+$ T cells are shown. The differences between control cells and each antigen were assessed using the Wilcoxon matched-pairs signed rank test ($^*P < 0.05$). (B) IL-10 responses to Acr in *M. tuberculosis*-infected cases grouped according to the disease status as on-treatment TB cases ($n = 2$), recovery stage TB cases ($n = 5$), and contact cases ($n = 5$). Responses of control cases ($n = 8$) are also shown. The differences between each set of samples were assessed using the Mann-Whitney test ($^*P < 0.05$). (A, B) The long horizontal line represents the median and the vertical line represents the interquartile range.

in the cell membrane is believed to aid the attachment of *M. tuberculosis* to alveolar epithelial cells (32). Therefore, we postulated that HBHA proteins are one of the first antigens that the host immune system encounters and that the IL-17 response to HBHA proteins can be detected in a very early phase of *M. tuberculosis* infection or exposure. In the present study, we detected a small but significant number of IL-17A-producing CD4$^+$ T cells in response to HBHA in TB cases. This is compatible with a previous finding that HBHA-specific memory CD4$^+$ T cells are present in vivo (33). To clarify the role of this IL-17 immune response in the course of *M. tuberculosis* infection, it is worth investigating the IL-17 response to HBHA in a cohort of early *M. tuberculosis* exposure population.

The C-terminal domain of HBHA recognizes the lysine-rich domain to be the same as heparin-sulfate receptors, and MDP-1 has a heparin-binding site that resembles HBHA (34). We hypothesized that mMDP-1 could also induce an early host immune response in a similar manner to mHBHA. As expected, significant responses to mMDP-1 were detected in the on-treatment and recovery stage *M. tuberculosis* cases. Therefore, the Th17 response to HBHA and MDP-1 may be a candidate predictive marker of early *M. tuberculosis* infection.

IL-10 functions as an anti-inflammatory cytokine and has been suggested to contribute to the onset of infectious disease by inhibiting other inflammatory cytokines. IL-10 is also known to directly affect macrophages by inhibiting expression of MHC class II and costimulatory molecules (35). A high number of Treg cells, which are a known source of IL-10, are present in human TB granulomas (36). In this study, the IL-10 response was low in recovery stage TB cases and was even lower than that in contact TB cases (Fig. 5B). This result may be due to the migration of IL-10-producing T cells from the blood to local inflammatory sites. Alternatively, *M. tuberculosis*-specific IL-10-producing T cells may have a transient role during early infection and self-antigen-specific IL-10-producing T cells may contribute to halting inflammation (37). The candidate of self-antigen is a dump of *M. tuberculosis* granuloma. Thus, it is considered that the IL-10 response to *M. tuberculosis* antigen simply follows *M. tuberculosis* inflammation and that the IL-10 response may serve as a marker for resolving the disease.

The IL-2 response to mHBHA antigen in recovery stage TB cases was significantly higher than that in healthy controls and contact cases. This finding implies that the IL-2 response to mHBHA is a useful marker of LTBI. Recent analysis of the ratio of HBHA-induced and recombinant ESAT-6-induced IFN-γ responses suggested that latent TB patients can be categorized into three different risk groups (38). Here, we found that the ratio of mHBHA-induced and ESAT-6/CFP-10-induced IFN-γ T cell responses tended to decrease after longer post-treatment durations. Because HBHA molecules are enriched in the *M. tuberculosis* membrane, it is likely that the T cell responses to HBHA accurately reflect the TB burden. On the other hand, ESAT-6 is known to be important for suppressing host immunity by inhibiting macrophage function (39) and may be required when *M. tuberculosis* is present in granulomas. Therefore, the ratio of HBHA-induced to ESAT-6/CFP-10-induced T cell responses may vary at distinct clinical stages of TB.

In conclusion, this study employed various *M. tuberculosis*-related antigens, including a novel methylated MDP-1 antigen, to show that the cytokine profiles of CD4$^+$ T cells differ at each clinical stage of TB. The results indicate that the detection of *M. tuberculosis*-specific polyfunctional T cells reflects the onset of TB. A more accurate prediction of disease onset may be achieved by combining the detection of several cytokines such as IL-10 and IL-17, which are produced in response to various *M. tuberculosis*-related antigens. A combination of this approach with the detection of latent *M. tuberculosis*-related antigens may allow the development of an improved diagnostic test that can more accurately identify *M. tuberculosis*-infected individuals at a higher risk of developing TB and eventually form the basis of a public health measure for controlling *M. tuberculosis* infection. We believe that further analysis of a wide spectrum of T cell cytokine responses to latent *M. tuberculosis*-related antigens will help in the assessment of the disease status of TB patients and the initiation of an early therapeutic inter-

vention.

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Conflict of interest None to declare.

REFERENCES

1. Dye, C., Scheele, S., Dolin, P., et al. (1999): Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA, 282, 677-686.
2. Lawn, S.D. and Zumla, A.I. (2011): Tuberculosis. Lancet, 378, 57-72.
3. Horsburgh, C.R., Jr. (2004): Priorities for the treatment of latent tuberculosis infection in the United States. N. Engl. J. Med., 350, 2060-2067.
4. Tuberculosis Research Center, Chennai, India (2003): Association of initial tuberculin sensitivity, age and sex with the incidence of tuberculosis in South India: a 15-year follow up. Int. J. Tuberc. Lung Dis., 7, 1083-1091.
5. Pai, M., Riley, L.W. and Colford, J.M., Jr. (2004): Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review. Lancet Infect. Dis., 4, 761-776.
6. Diel, R., Loddenkemper, R. and Nienhaus, A. (2010): Evidence-based comparison of commercial interferon-gamma release assays for detecting active TB: a meta analysis. Chest, 137, 952-968.
7. Diel, R., Loddenkemper, R. and Nienhaus, A. (2012): Predictive value of interferon-gamma release assays and tuberculin skin testing for predicting progression from latent TB infection to disease state: a meta-analysis. Chest, 142, 63-75.
8. Yuan, Y., Crane, D.D. and Barry, C.E., 3rd. (1996): Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. J. Bacteriol., 178, 4484-4492.
9. Yuan, Y., Crane, D.D., Simpson, R.M., et al. (1998): The 16-kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. Proc. Natl. Acad. Sci. USA, 95, 9578-9583.
10. Menozzi, F.D., Rouse, J.H., Alavi, M., et al. (1996): Identification of a heparin-binding hemagglutinin present in mycobacteria. J. Exp. Med., 184, 993-1001.
11. Locht, C., Hougardy, J.M., Rouanet, C., et al. (2006): Heparin-binding hemagglutinin, from an extrapulmonary dissemination factor to a powerful diagnostic and protective antigen against tuberculosis. Tuberculosis, 86, 303-309.
12. Matsumoto, S., Yukitake, H., Furugen, M., et al. (1999): Identification of a novel DNA-binding protein from *Mycobacterium bovis* bacillus Calmette-Guerin. Microbiol. Immunol., 43, 1027-1036.
13. Matsumoto, S., Furugen, M., Yukitake, H., et al. (2000): The gene encoding mycobacterial DNA-binding protein I (MDPI) transformed rapidly growing bacteria to slowly growing bacteria. FEMS Microbiol. Lett., 182, 297-301.
14. Matsumoto, S., Matsumoto, M., Umemori, K., et al. (2005): DNA augments antigenicity of mycobacterial DNA-binding protein 1 and confers protection against *Mycobacterium tuberculosis* infection in mice. J. Immunol., 175, 441-449.
15. Lawn, S.D., Badri, M. and Wood, R. (2005): Tuberculosis among HIV-infected patients receiving HAART: long term incidence and risk factors in a South African cohort. AIDS, 19, 2109-2116.
16. Diedrich, C.R., Mattila, J.T., Klein, E., et al. (2010): Reactivation of latent tuberculosis in cynomolgus macaques infected with SIV is associated with early peripheral T cell depletion and not virus load. PloS One, 5, e9611.
17. Zhu, J. and Paul, W.E. (2008): CD4 T cells: fates, functions, and faults. Blood, 112, 1557-1569.
18. Seder, R.A., Darrah, P.A. and Roederer, M. (2008): T-cell quality in memory and protection: implications for vaccine design. Nat. Rev. Immunol., 8, 247-258.
19. Day, C.L., Mkhwanazi, N., Reddy, S., et al. (2008): Detection of polyfunctional *Mycobacterium tuberculosis*-specific T cells and association with viral load in HIV-1-infected persons. J. Infect. Dis., 197, 990-999.
20. Sutherland, J.S., Young, J.M., Peterson, K.L., et al. (2010): Polyfunctional CD4(+) and CD8(+) T cell responses to tuberculosis antigens in HIV-1-infected patients before and after antiretroviral treatment. J. Immunol., 184, 6537-6544.
21. Pethe, K., Bifani, P., Drobecq, H., et al. (2002): Mycobacterial heparin-binding hemagglutinin and laminin-binding protein share antigenic methyllysines that confer resistance to proteolysis. Proc. Natl. Acad. Sci. USA, 99, 10759-10764.
22. Sutherland, J.S., Adetifa, I.M., Hill, P.C., et al. (2009): Pattern and diversity of cytokine production differentiates between *Mycobacterium tuberculosis* infection and disease. Eur. J. Immunol., 39, 723-729.
23. Millington, K.A., Innes, J.A., Hackforth, S., et al. (2007): Dynamic relationship between IFN-gamma and IL-2 profile of *Mycobacterium tuberculosis*-specific T cells and antigen load. J. Immunol., 178, 5217-5226.
24. Chen, C.Y., Huang, D., Yao, S., et al. (2012): IL-2 simultaneously expands Foxp3+ T regulatory and T effector cells and confers resistance to severe tuberculosis (TB): implicative Treg-T effector cooperation in immunity to TB. J. Immunol., 188, 4278-4288.
25. Liao, W., Lin, J.X., Wang, L., et al. (2011): Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. Nat. Immunol., 12, 551-559.
26. Day, C.L., Abrahams, D.A., Lerumo, L., et al. (2011): Functional capacity of *Mycobacterium tuberculosis*-specific T cell responses in humans is associated with mycobacterial load. J. Immunol., 187, 2222-2232.
27. Kannanganat, S., Ibegbu, C., Chennareddi, L., et al. (2007): Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. J. Virol., 81, 8468-8476.
28. Young, J.M., Adetifa, I.M., Ota, M.O., et al. (2010): Expanded polyfunctional T cell response to mycobacterial antigens in TB disease and contraction post-treatment. PloS One, 5, e11237.
29. Caccamo, N., Guggino, G., Joosten, S.A., et al. (2010): Multifunctional CD4(+) T cells correlate with active *Mycobacterium tuberculosis* infection. Eur. J. Immunol., 40, 2211-2220.
30. Harari, A., Rozot, V., Enders, F.B., et al. (2011): Dominant TNF-alpha+ *Mycobacterium tuberculosis*-specific CD4+ T cell responses discriminate between latent infection and active disease. Nat. Med., 17, 372-376.
31. Laan, M., Cui, Z.H., Hoshino, H., et al. (1999): Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. J. Immunol., 162, 2347-2352.
32. Temmerman, S., Pethe, K., Parra, M., et al. (2004): Methylation-dependent T cell immunity to *Mycobacterium tuberculosis* heparin-binding hemagglutinin. Nat. Med., 10, 935-941.
33. Loxton, A.G., Black, G.F., Stanley, K., et al. (2012): Heparin-binding hemagglutinin induces IFN-γ+IL-2+IL-17+ Multifunctional $CD4^+$T cells during latent but not active tuberculosis disease. Clin. Vaccine Immunol., 19, 746-751.
34. Aoki, K., Matsumoto, S., Hirayama, Y., et al. (2004): Extracellular mycobacterial DNA-binding protein 1 participates in mycobacterium-lung epithelial cell interaction through hyaluronic acid. J. Biol. Chem., 279, 39798-39806.
35. Moore, K.W., de Waal Malefyt, R., Coffman, R.L., et al. (2001): Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol., 19, 683-765.
36. Rahman, S., Gudetta, B., Fink, J., et al. (2009): Compartmentalization of immune responses in human tuberculosis: few CD8+ effector T cells but elevated levels of FoxP3+ regulatory T cells in the granulomatous lesions. Am. J. Pathol., 174, 2211-2224.
37. Ozeki, Y., Sugawara, I., Udagawa, T., et al. (2010): Transient role of CD4+CD25+ regulatory T cells in mycobacterial infection in mice. Int. Immunol., 22, 179-189.
38. Corbiere, V., Pottier, G., Bonkain, F., et al. (2012): Risk stratification of latent tuberculosis defined by combined interferon gamma release assays. PloS One, 7, e43285.
39. Welin, A., Eklund, D., Stendahl, O., et al. (2011): Human macrophages infected with a high burden of ESAT-6-expressing *M. tuberculosis* undergo caspase-1- and cathepsin B-independent necrosis. PloS One, 6, e20302.

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Whole-Genome Sequence of the Potentially Hypertransmissible Multidrug-Resistant *Mycobacterium tuberculosis* Beijing Strain OM-V02_005

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We report the draft genome sequence of *Mycobacterium tuberculosis* Beijing strain OM-V02_005, which exhibits possible hypertransmissible characteristics among the population of patients with multidrug-resistant tuberculosis in Osaka Prefecture, the largest urban area in western Japan.

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The *Mycobacterium tuberculosis* Beijing strain is a causative agent of tuberculosis with increasing prevalence, and it is assumed to have a considerable impact on resistance to major antituberculosis drugs. We identified the most prevalent cluster (OM-V02) among the population of multidrug-resistant tuberculosis (MDR-TB) patients in Osaka Prefecture, the largest urban area in western Japan (1). To elucidate the underlying genetic characteristics of the transmissibility and drug resistance of this cluster, specifically found in MDR-TB patients, we performed whole-genome sequencing of one representative strain, OM-V02_005.

We sequenced OM-V02_005 genomic DNA on a 454 GS-FLX Titanium Sequencer (Roche) and assembled the reads using Newbler version 2.3. A total of 510,688 reads was generated, with an average read length of 439 bp, yielding a total sequence of 224,340,568 bp.

The assembled sequences contained 134 contigs, and the length of all contigs combined was 4,321,797 bp, with a G+C ratio of 65.5%. The average coverage depth was 52×, the N_{50} contig size was 68,368 bp, the average contig was 32,252 bp long, and the longest contig was 184,273 bp.

Genome annotation was performed using GeneMark.hmm for prokaryotes (http://exon.gatech.edu/gmhmm2_prok.cgi), xBASE (http://www.xbase.ac.uk/annotation/) for coding sequence genes, and tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/) and RNAmmer 1.2 (http://www.cbs.dtu.dk/services/RNAmmer/) for RNA genes. OM-V02_005 has 3,888 coding-sequence genes, 3 rRNAs (in a single operon), and 52 tRNA genes.

We compared the OM-V02_005 sequence with that of other representative *M. tuberculosis* strains: H37Rv (GenBank accession no. AL123456.2), CDC1551 (AE000516), F11 (CP000717), CCDC5079 (CP001641), and CCDC5080 (CP001642). *M. tuberculosis* CCDC5079 is a drug-sensitive strain and CCDC5080 is a multidrug-resistant Beijing strain sequenced in China (2). The reciprocal best-hit BLAST approach revealed that OM-V02_005 shares 90.0%, 92.7%, 90.0%, 92.7%, and 88.7% of its coding sequences with *M. tuberculosis* H37Rv, CDC1551, F11, CCDC5079, and CCDC5080, respectively.

OM-V02_005 has 89 specific genes. Most of these were classified as genes for hypothetical proteins, but genes for both a dehydrogenase enzyme and the Tuf-like elongation factor Tu showed specific sequence alignment between OM-V02_005 and a clinical strain of *M. tuberculosis* T46 isolated in San Francisco from a Filipino patient.

We investigated single nucleotide polymorphisms (SNPs) using SNPs Finder (http://snpsfinder.lanl.gov/). OM-V02_005 has 270 specific SNPs, of which 169 were nonsynonymous mutations, 66 were synonymous mutations, and 26 were mutations in promoter regions. In addition to our previous report of the mutations in *rrs*, *rpoB*, *katG*, *gyrA*, *embB*, and *pncA* (1), mutations were found in *rrl* as I236T and in *gidB* as A413E, respectively. No significant mutations were found in *inhA*, *oxyS*, *iniB*, *iniA*, *iniC*, *furA*, and *rpsL* (Rv0682). Nonsynonymous mutations include regulatory proteins for growth/dormancy regulation (*pknA*, *mprA*, and *rpfA*), intermediary metabolism and regulation (*coaA*, *ribH*, *tal*, and *bfrB*), virulence genes (*clpB and mce3F*), lipid metabolism (*ppsB* and *ppsD*), and cell wall proteins (*lprK/mceIE*, *mmpL1*, *mmpL2*, and *dppA*). The OM-V02_005 sequence data will provide an invaluable resource to elucidate the underlying mechanisms of transmissibility and drug resistance acquisition of *M. tuberculosis*.

Nucleotide sequence accession numbers. The whole genome sequence of OM-V02_005 has been deposited in DDBJ/EMBL/

GenBank under the accession numbers BARZ01000001 through BARZ01000121.

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REFERENCES

1. **Tamaru A, Nakajima C, Wada T, Wang Y, Inoue M, Kawahara R, Maekura R, Ozeki Y, Ogura H, Kobayashi K, Suzuki Y, Matsumoto S.** 2012. Dominant incidence of multidrug and extensively drug-resistant specific *mycobacterium tuberculosis* clones in Osaka Prefecture, Japan. PLoS One 7:e42505. doi:10.1371/journal.pone.0042505.
2. **Zhang Y, Chen C, Liu J, Deng H, Pan A, Zhang L, Zhao X, Huang M, Lu B, Dong H, Du P, Chen W, Wan K.** 2011. Complete genome sequences of *Mycobacterium tuberculosis* strains CCDC5079 and CCDC5080, which belong to the Beijing family. J. Bacteriol. 193:5591–5592.

IMMUNITY & AGEING

SHORT REPORT Open Access

Reactivation of immune responses against *Mycobacterium tuberculosis* by boosting with the CpG oligomer in aged mice primarily vaccinated with *Mycobacterium bovis* BCG

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Abstract

Background: *Mycobacterium bovis* bacillus Calmette Guérin (BCG) vaccine, which has been inoculated to more than one billion people world-wide, has significant effect in preventing tuberculous meningitis and miliary tuberculosis (TB) in neonate and early childhood. However, BCG fails to adequately protect against pulmonary TB and reactivation of latent infections in adults. To overcome this problem, adequate booster is urgently desired in adult who received prior BCG vaccination, and appropriate animal models that substitute human cases would be highly valuable for further experimentation.

Findings: The booster effect of the synthesized CpG oligomer (Oligo-B) on aged mice which had been primarily vaccinated with BCG at the age of 4-week old. The specific Th1 type reaction, production of interferon-γ, in response to TB antigens, purified protein derivatives (PPD) and protection against challenge with *Mycobacterium tuberculosis* (MTB) $H_{37}Rv$ decreased with increasing age and were not observed in 89-week old mice. In order to rejuvenate the Th1 type response against PPD and protection activity against MTB infection, Oligo-B, which is known to augment Th1 responses, was administered as a booster to 81-90-week old mice (late 50's in human equivalent) vaccinated with BCG at 4-week old. The boosting with Oligo-B increased the number of $CD4^+$ $CD44^{high}$ $CD62L^{high}$, central memory type T cell. Furthermore, the Oligo-B boosting rejuvenated the ability of mice to protect against infection with MTB $H_{37}Rv$.

Conclusions: Th1-adjuvant CpG oligo DNA, such as Oligo-B, may be a promising booster when coupled with BCG priming.

Keywords: *Mycobacterium tuberculosis*, BCG, CpG oligomer, Booster, Aging

Introduction

The protective efficacy of BCG vaccine is variable from 0 to 80% in many field trials and uncertain to pulmonary TB in adult [1]. The several reports showed that the effectiveness of prime BCG vaccination would last for around 15–24 years [2,3]. To solve the problem of current BCG vaccine the prime-boost vaccine strategy against TB was investigated strategy [4,5]. In most of the trials in mice, however, intervals between priming and boosting were only 4–8 weeks, which correspond to 2 years in human. Furthermore, the immune response against MTB reaches its peak within several weeks after prime vaccination. Thereby, to evaluate the booster in adult human, it is necessary to investigate the boosting effect in aged animals primed with BCG. The transition of immune system with increasing age has been reported by analyzing the population of T cell subsets

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[6-8]. However, the reports investigating the duration of efficacy of BCG vaccination and the shift of memory type T cell subsets with increasing age are very few. In this study we investigated the efficacy of prime BCG vaccine with aging by analyzing memory T cell subset, immune responses to TB antigens, and also protection activity against MTB infection in animal model. We also evaluated the effect of boosting with Oligo-B to the protective immunity against TB in BCG-primed aged mice.

Materials and methods

1. Bacterial strains and cultures
2. Mice and immunization
   The methods were described in our previous study [9]. The protocol of animal study was approved by the Ethics Committee of Nagoya City University.
3. Whole blood assay
   The whole blood was stimulated with purified protein derivative (PPD) (Japan BCG co., Tokyo, Japan) for 18 h. Then, the supernatant were collected and the amount of interferon gamma (IFN-γ) was measured by enzyme-linked immunosorbent assay (ELISA), using a BD OptEIA^{TM} ELISA set (BD Bioscience, San Jose, CA).
4. Flow cytometric analysis of surface markers
   Splenocytes from young and middle-aged mice were washed by FACS buffer and stained with PE rat anti-mouse CD8a (BD Bioscience, San Jose, CA) and FITC rat anti-mouse CD4 (BD Bioscience, San Jose, CA), then analyzed by FACS. MACS^{TM}(Miltenyi Biotec, Tokyo, Japan) -purified splenocytes $CD4^+$ and $CD8^+$ T cells were stained with PE rat anti-mouse CD44 and FITC rat anti-mouse CD62L (BD Bioscience, San Jose, CA), then cells were analyzed by FACS.
5. Protection assay against infection with MTB
   The precise method was described in our previous study [9].
6. Splenocytes stimulation
   Splenocytes prepared from BCG-immunized with or without Oligo-B (GGGGGGGGGGGG AACGTTGGGGGGGGGGGG) (Nihon Gene Research Laboraroties, Inc., Miyagi, Japan) or Oligo-B negative (GGGGGGGGGGGG ACCGGTGGGGGGGGGGGG) (Nihon Gene Research Laboraroties, Inc., Miyagi, Japan) mice were incubated in a 24-well plate, at a concentration of 5×10^5 cells per well. Cells were stimulated with 10 μg/ml of PPD for 48 h. The productions of IFN-γ in the supernatants of splenocytes were determined by the ELISA set.
7. Statistical analysis
   The methods were described in our previous study [9].

Results

1. The reduction of interferon-γ production from antigen-stimulated T cells of mice immunized with *Mycobacterium bovis* bacillus Calmette Guérin (BCG) with aging
   The difference of the PPD-induced IFN-γ production between unvaccinated and vaccinated mice with BCG at 4-week old was remarkable until 49-week old mice (Figure 1a-1g), however, it was not significant in 57-week and 83-week old mice (Figure 1h and 1i). The PPD-induced IFN-γ production in unvaccinated mice was comparable to that of BCG vaccinated super aged mice (83-week old) (Figure 1i). The immune response to ovalbumin (OVA), non-specific antigen, increased in both unvaccinated and BCG vaccinated 83-week old mice (Figure 2b). These results suggest that the immune responses specific to tuberculosis antigen decreased, and conversely nonspecific immune responses increased with aging and were supported previous studies about immune senescence with aging [6-8].
2. The change of memory type T cell subsets with aging
   We analyzed central type memory T cells (T_{CM}), $CD44^{hi}$ $CD62L^{hi}$, and effector type memory T cells (T_{EM}), $CD44^{hi}$ $CD62L^{lo}$, in both 30-week and 90-week old mice. $CD8^+$ T_{EM} was induced by the immunization with BCG in 30-week mice (Table 1, BCG vaccination; 61.7 ± 0.03 vs un-vaccination; 48.2± 7.95), however, the population of T_{CM} did not change (Table 1, BCG vaccination; 14.7 ± 0.49 vs un-vaccination; 14.0 ± 3.67). Marcela et al. reported that the immunization with BCG failed to induce T_{CM} [10]. The population of both T_{EM} and T_{CM} in $CD8^+$ slightly decreased in BCG-vaccinated 90-week old mice (Table 1, BCG vaccination; 87.51 ± 6.94 vs un-vaccination; 95.90 ± 0.82). These data suggest that the immunization with BCG is not sufficient to induce long term memory type T cells.
3. Boosting with Oligo-B
   Several researches reported that Th1 type responses, such as production of IFN-γ against PPD, were reduced with aging and the immunization with BCG was not sufficient to induce long term memory T cells [10,11]. We have previously reported that CpG oligomer (Oligo-B) activate Th1 response [12] and enhanced the delayed type hypersensitivity against PPD [13]. Therefore, we investigated the boosting effect of Oligo-B on the reactivation of immune

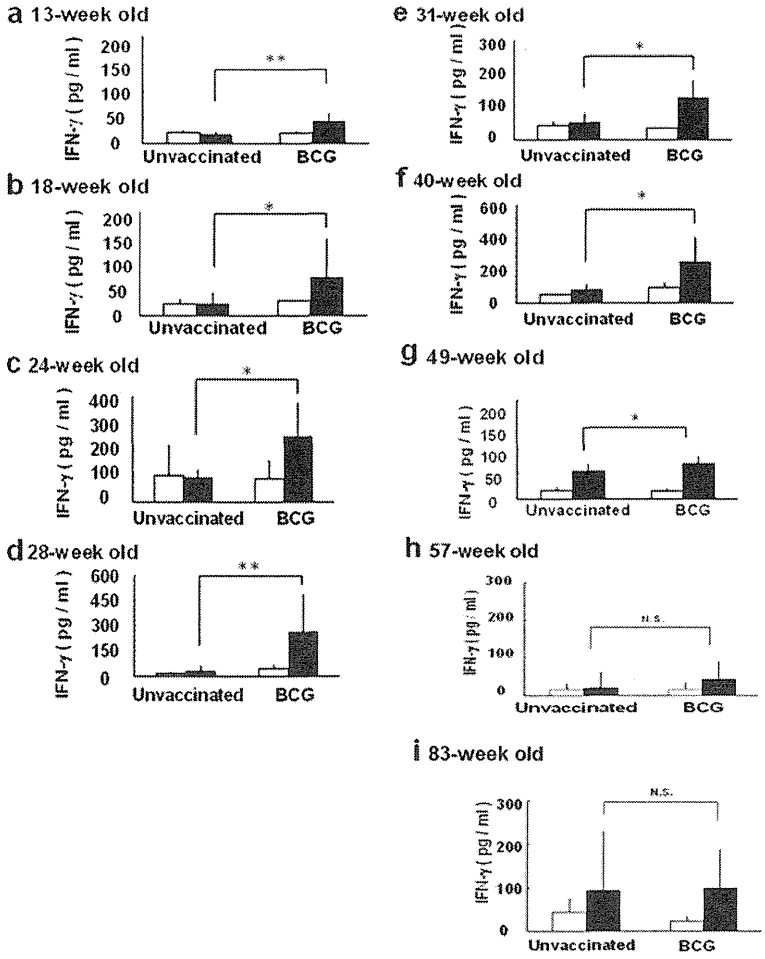

Figure 1 The change of IFN-γ production from whole blood cells stimulated with PPD in mice with increasing age. C57BL/6 mice were subcutaneously vaccinated with BCG (10^6 CFU) or PBS (unvaccinated). Whole-blood obtained from **a**) 13-week old, **b**) 18-week old, **c**) 24-week old, **d**) 28-week old, **e**) 31-week old, **f**) 40-week old, **g**) 49-week old, **h**) 57-week old, **i**) 83-week old was stimulated with purified protein derivative (PPD, solid column) or PBS (open column) and incubated for 24 h. The concentration of interferon-gamma (IFN-γ) from the culture supernatant was measured by ELISA. Results are shown as mean ± SD from groups of 16 animals. ** $p < 0.01$,* $p < 0.05$, N.S.: not significant.

senescence mice. Three times boosting with Oligo-B, but not Oligo-B negative, remarkably augmented the production of IFN-γ from splenocytes stimulated with PPD in the BCG vaccinated mice (Figure 3), and $CD4^+$ memory type T cells were strongly induced by the boosting (Table 1, $CD4^+$ $CD44^{hi}$ $CD62L^{high}$ in 90-week old mice: after boosting; 7.76±3.26 vs before boosting; 2.85 ± 0.67). These results strongly suggest that the boosting with Oligo-B can effectively reactivate the memory T cells developed by primary vaccination.

4. The effect of boosting with Oligo-B on the protectiveness against MTB in aged mice primarily vaccinated with BCG

   The bacterial numbers of MTB $H_{37}Rv$ challenged intravenously were reduced in the spleen and lung by BCG vaccination in 30-week old mice (Figure 4a and 4b). However, at 89-week old, the reduction of challenged MTB number was not significant as compared to unvaccinated control mice (Figure 4c and 4d, open column). After three times boosting of Oligo-B on the 84-week old mice vaccinated with or without the prime BCG vaccination, these mice were challenged with MTB $H_{37}Rv$ intravenously at 90 weeks old. The bacterial numbers decreased in the lungs and spleens from the mice vaccinated prime BCG plus three times boosting with Oligo-B (Figure 4c and 4d, unvaccinated (open column) vs BCG plus OligoB (solid column)). These data indicate that Oligo-B rejuvenates the weakened protective immunity against MTB infection in BCG-primed aged mice.

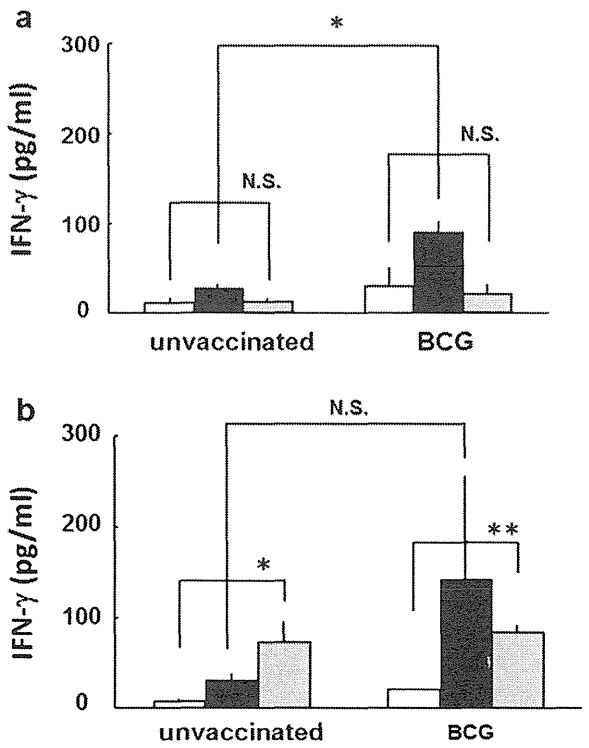

Figure 2 The IFN-γ production from splenocytes stimulated with OVA in BCG vaccinated young and middle-aged mice. Splenocytes were prepared from BCG-vaccinated or non-vaccinated **a**)13-week old, **b**) 89-week old mice, and plated in a 24-well plate, at a concentration of 5×10^5 cells per well. Cells were stimulated with 100 μg/ml of ovalbumin (OVA, gray column), 10 μg/ml of PPD (solid column) or PBS (open column) and incubated for 48 h. Then, supernatants were collected and the concentration of IFN-γ was measured by ELISA. Results are shown by mean ± SD from groups of 3 mice. ** $p < 0.01$, * $p < 0.05$, N.S.: not significant.

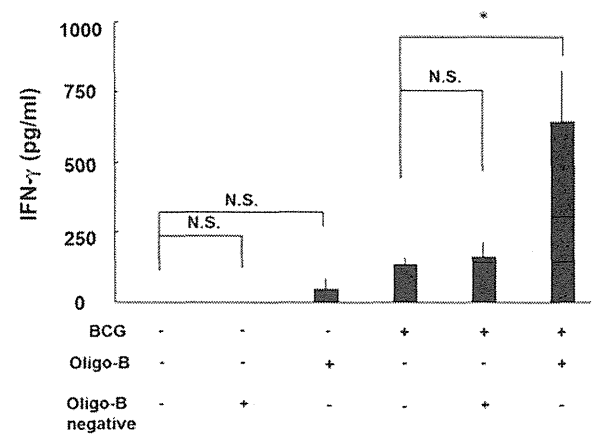

Figure 3 Oligo-B, but not Oligo-B negative augmented the prime BCG vaccination. BCG-vaccinated or unvaccinated 30-week aged mice were subcutaneously injected with 50 μg of booster (Oligo-B or Oligo-B-negative), every 2 weeks. Splenocytes prepared from each mouse 2 weeks after 3-times boosting were incubated in a 24-well plate, at a concentration of 5×10^5 cells per well. Cells were stimulated with 10 μg/ml of PPD (solid column) or PBS (open column) and incubated for 48 h. Then, supernatants were collected and the concentration of IFN-γ was measured by ELISA. Results are shown as mean ± SD from groups of three mice. * $p < 0.05$, N.S.: not significant.

Discussion

In this study, we first analyzed the age related changes of immune responses to MTB antigens, in super aged mice (up to 89-week old) vaccinated with BCG in 4-week old. The IFN-γ production from whole blood cells from C57BL/6 mice immunized with BCG at 4-week old increased up to 13-week old and sustained to 49-week old (Figure 1). These results are consistent with our previous

Table 1 Transition of memory T cells in BCG immunized mice with aging and retrieval effect of the boosting with Oligo-B on memory T cells[$]

Age		Immunization	$CD4^+$		$CD8^+$	
			CD44hi CD62L**		CD44hi CD62L**	
in mice	in human equivarent*	with BCG[&]	low (T_{EM})	high (T_{CM})	low (T_{EM})	high (T_{CM})
30-week	late 10's	+	72.2 ± 0.82	15.6 ± 0.80	61.7 ± 0.03	14.7 ± 0.49
		-	74.5 ± 1.23	13.8 ± 0.35	48.2 ± 7.95	14.0 ± 3.67
81-90-week	late 50's	+	85.75 ± 5.91	2.85 ± 0.67	87.51 ± 6.94	2.07 ± 0.12
		-	93.03 ± 0.56	5.95 ± 0.68	95.90 ± 0.82	3.38 ± 1.94
Boosting with Oligo-B						
30-week	late 10's	+	73.6 ± 1.92	17.3 ± 0.32	53.7 ± 10.3	9.95 ± 0.15
		-	78.1 ± 3.89	14.6 ± 0.88	51.8 ± 0.48	10.2 ± 0.11
81-90-week	late 50's	+	89.12 ± 0.62	7.76 ± 3.26	95.29 ± 0.82	2.66 ± 1.08
		-	85.82 ± 0.01	3.82 ± 0.42	92.07 ± 1.41	3.98 ± 0.12

[$]Splenocytes were prepared from the aged mice indicated in the table and stained with specific antibodies to memory T cells (CD44, CD62L), and then analyzed by a flow cytometer. Precise procedures were described in the Section of Materials and methods.
*The age in human equivalent was applied to survival curve of mice.
[&]Mice were administered transdermally with BCG at 4-week old.
**Effector memory T cell (T_{EM}), $CD44^{hi}$ $CD62L^{lo}$; central memory T cell (T_{CM}), $CD44^{hi}$ $CD62L^{hi}$.
N.D.: not determined.

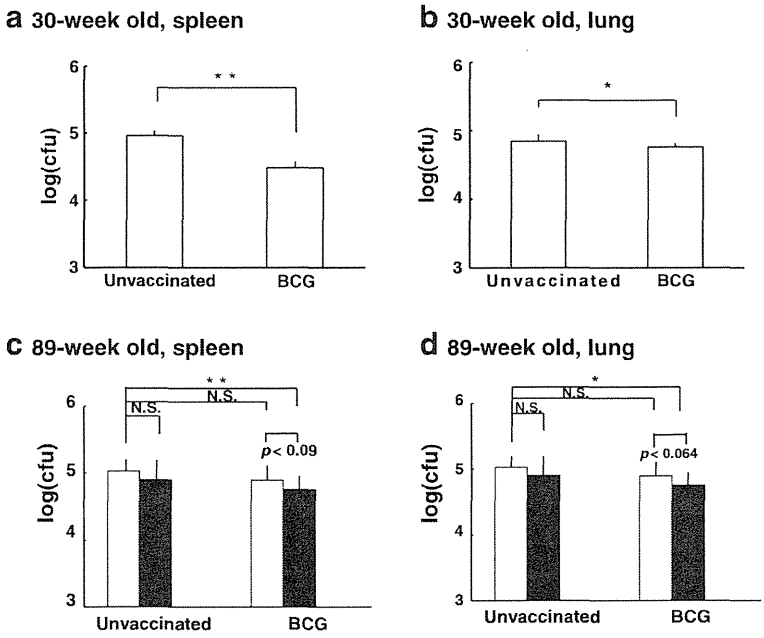

Figure 4 The protective activity against TB in young and middle-aged mice. BCG-vaccinated or unvaccinated mice, young (30-week old) (**a**, **b**) or middle-aged (89-week old) (**c**, **d**), were intravenously challenged with 1×10^5 CFU of M. tuberculosis strain H_3,Rv. Middle-aged mice (**c**, **d**) were non-boosted (open column), 3-times boosted by Oligo-B (solid column). Four weeks after challenge, the bacterial numbers in the spleen (**a**, **c**) and lung (**b**, **d**) were determined by colony assay. Data represent mean ± SD from 8-10 mice. The results of Oligo-B-boosted mice were obtained from 4 mice. ** $p < 0.01$, * $p < 0.05$, N.S.: not significant.

report [14]. We also found the accumulation of $CD8^+$ T cells (data not shown) and increased IFN-γ production from splenocytes stimulated with OVA with increasing age (Figure 2). Kim et al. reported that the non-specific type immune responses increased with increasing age [15]. The productions of tumor necrosis factor-α and IL-6 both in healthy individuals and patients suffering from age related diseases increased with aging [16]. These studies indicate that the age related immunological changes and immune senescence in human are commonly observed in mice, and duration of initial BCG vaccine would be extinguished up to 57-week old.

Next, we investigated the reactivation of the protective immunity against MTB infection in the immune senescent mice by Oligo-B boosting (Figure 3). The CpG oligomer was used as adjuvant conjugated with BCG [17] or MTB antigens, such as Ag85B [18], MPT-51 [19], and MTB culture filtrate proteins [20]. The synthesized CpG oligomer, Oligo-B, induced antigen presentation through toll-like receptor 9 (TLR-9) signaling in plasmacytoid dendritic cells [21] and the production of Th17 cytokines [22], which is known to play an important role in host defense against MTB infection [23]. Therefore, these studies indicated that CpG oligomer is a good inducer of IFN-γ and IL-17 and reactivates acquired immunity. In fact, the production of IFN-γ was reactivated by three times boosting of Oligo-B in the super aged mice (Figure 3), therefore, Oligo-B could generate the number of the memory T cell by IFN-γ and IL-17.

Several studies reported that $CD4^+$ memory T cells were induced by BCG vaccination [24,25]. In our study the number of T_{CM}, $CD44^{hi}$, $CD62L^{hi}$, in $CD4^+$ was highly induced by the boosting with Oligo-B in the immune senescent 81-90-old mice (Table 1, 2.85 ± 0.67 vs. 7.76 ± 3.26) and protection activity against MTB was rejuvenated (Figure 4). These results suggest that Oligo-B could induce T_{CM} in $CD4^+$ which improved protection activity against MTB infection.

In conclusion, this is the first report indicating that Oligo-B boosting can rejuvenates the number of T_{CM} in CD4+ and reactivate the protection immunity against MTB infection in the immune senescent state mice formerly vaccinated with BCG. This report also provides basic information to explore the prime-booster strategy for preventing TB in adult.

Abbreviations

TB: Tuberculosis; MTB: *Mycobacterium tuberculosis*; BCG: *Mycobacterium bovis* bacillus Calmette Guérin; IFN: Interferon; IL: Interleukin; CFU: Colony forming unit; PPD: Purified protein derivatives.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TT, JM, SY SI, YO, SM and KO designed and planned the research. KT, TT, MM, TH, YM, and SI performed the collection of serum and cytokine analysis. TT,

KT, TH, YM and NI performed infectious experiments and counting colonies of bacilli form infected organs. KT and YM performed FACS analysis. JM and SY supplied the BCG vaccine. JM, SI and SY supplied Oligo B. All authors read and approved the final manuscript.

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References

1. WHO: *BCG (Tuberculosis)*. 2011. http://www.who.int/biologicals/areas/vaccines/bcg/Tuberculosis/en/.
2. Hart PD, Sutherland I: **BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life.** *Br Med J* 1977, **2**:293–295.
3. WHO: **BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life.** *Bull World Health Organ* 1972, **46**:371–385.
4. Brooks JV, Frank AA, Keen MA, Bellisle JT, Orme IM: **Boosting vaccine for tuberculosis.** *Infect Immun* 2001, **69**:2714–2717.
5. Brennan MJ, Clagett B, Fitzgerald H, Chen V, Williams A, Izzo AA, Barker LF: **Preclinical evidence for implementing a prime-boost vaccine strategy for tuberculosis.** *Vaccine* 2012, **30**:2811–2823.
6. Broux B, Markovic-Plese S, Stinissen P, Hellings N: **Pathogenic features of CD4(+)CD28(−) T cells in immune disorders.** *Trends Mol Med* 2012, **18**:446–453.
7. Haynes L, Lefebvre JS: **Age-related Deficiencies in Antigen-Specific CD4 T cell Responses: Lessons from Mouse Models.** *Aging Dis* 2011, **2**:374–381.
8. Lee N, Shin MS, Kang I: **T-cell biology in aging, with a focus on lung disease.** *J Gerontol A Biol Sci Med Sci* 2012, **67**:254–263.
9. Ito T, Takii T, Maruyama M, Hayashi D, Wako T, Asai A, Horita Y, Taniguchi K, Yano I, Yamamoto S, Onozaki K: **Effectiveness of BCG vaccination to aged mice.** *Immun Ageing* 2010, **7**:12.
10. Henao-Tamayo MI, Ordway DJ, Irwin SM, Shang S, Shanley C, Orme IM: **Phenotypic definition of effector and memory T-lymphocyte subsets in mice chronically infected with Mycobacterium tuberculosis.** *Clin Vaccine Immunol* 2010, **17**:618–625.
11. Lindenstrom T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, Seder RA, Rosenkrands I, Andersen P: **Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells.** *J Immunol* 2009, **182**:8047–8055.
12. Yamamoto S, Yamamoto T, Shimada S, Kuramoto E, Yano O, Kataoka T, Tokunaga T: **DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth.** *Microbiol Immunol* 1992, **36**:983–997.
13. Maeyama J, Komiya T, Takahashi M, Isaka M, Goto N, Yamamoto S: **The mucosal adjuvanticity of the oligodeoxynucleotides containing a non-methylated CpG motif on BCG and diphtheria toxoid.** *Vaccine* 2009, **27**:1166–1173.
14. Ozeki Y, Hirayama Y, Takii T, Yamamoto S, Kobayashi K, Matsumoto S: **Loss of anti-mycobacterial efficacy in mice over time following vaccination with Mycobacterium bovis bacillus Calmette-Guerin.** *Vaccine* 2011, **29**:6881–6887.
15. Kim YT, Goidl EA, Samarut C, Weksler ME, Thorbecke GJ, Siskind GW: **Bone marrow function I. Peripheral T cells are responsible for the increased auto-antiidiotype response of older mice.** *J Exp Med* 1985, **161**:1237–1242.
16. Bruunsgaard H, Pedersen BK: **Age-related inflammatory cytokines and disease.** *Immunol Allergy Clin North Am* 2003, **23**:15–39.
17. Freidag BL, Melton GB, Collins F, Klinman DM, Cheever A, Stobie L, Suen W, Seder RA: **CpG oligodeoxynucleotides and interleukin-12 improve the efficacy of Mycobacterium bovis BCG vaccination in mice challenged with M. tuberculosis.** *Infect Immun* 2000, **68**:2948–2953.
18. Ballester M, Nembrini C, Dhar N, De Titta A, De Piano C, Pasquier M, Simeoni E, van der Vlies AJ, McKinney JD, Hubbell JA, Swartz MA: **Nanoparticle conjugation and pulmonary delivery enhance the protective efficacy of Ag85B and CpG against tuberculosis.** *Vaccine* 2011, **29**:6959–6966.
19. Silva BD, Da Silva EB, Do Nascimento IP, Dos Reis MC, Kipnis A, Junqueira-Kipnis AP: **MPT-51/CpG DNA vaccine protects mice against Mycobacterium tuberculosis.** *Vaccine* 2009, **27**:4402–4407.
20. Da Fonseca DM, Silva CL, Wowk PF, Paula MO, Ramos SG, Horn C, Marchal G, Bonato VL: **Mycobacterium tuberculosis culture filtrate proteins plus CpG Oligodeoxynucleotides confer protection to Mycobacterium bovis BCG-primed mice by inhibiting interleukin-4 secretion.** *Infect Immun* 2009, **77**:5311–5321.
21. Krieg AM: **CpG motifs in bacterial DNA and their immune effects.** *Annu Rev Immunol* 2002, **20**:709–760.
22. Ito T, Schaller M, Hogaboam CM, Standiford TJ, Sandor M, Lukacs NW, Chensue SW, Kunkel SL: **TLR9 regulates the mycobacteria-elicited pulmonary granulomatous immune response in mice through DC-derived Notch ligand delta-like 4.** *J Clin Invest* 2009, **119**:33–46.
23. Khader SA, Cooper AM: **IL-23 and IL-17 in tuberculosis.** *Cytokine* 2008, **41**:79–83.
24. Ancelet L, Rich FJ, Delahunt B, Kirman JR: **Dissecting memory T cell responses to TB: Concerns using adoptive transfer into immunodeficient mice.** *Tuberculosis (Edinb)* 2012, **92**:422–433.
25. Nambiar JK, Pinto R, Aguilo JI, Takatsu K, Martin C, Britton WJ, Triccas JA: **Protective immunity afforded by attenuated, PhoP-deficient Mycobacterium tuberculosis is associated with sustained generation of CD4+ T-cell memory.** *Eur J Immunol* 2012, **42**:385–392.

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