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Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model

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Abstract

We have developed two novel tuberculosis (TB) vaccines: a DNA vaccine combination expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) by using the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ) and a recombinant BCG harboring the 72f fusion gene (72f rBCG). These vaccines provide remarkable protective efficacy in mouse and guinea pig models, as compared to the current by available BCG vaccine. In the present study, we extended our studies to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis, to evaluate the HSP65 + IL-12/HVJ and 72f rBCG vaccines. Vaccination with HSP65 + IL-12/HVJ as well as 72f rBCG vaccines provided better protective efficacy as assessed by the Erythrocyte Sedimentation Rate, chest X-ray findings and immune responses than BCG. Most importantly, HSP65 + IL-12/HVJ resulted in an increased survival for over a year. This is the first report of successful DNA vaccination and recombinant BCG vaccination against *M. tuberculosis* in the monkey model.
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Keywords: HSP65 DNA + IL-12 DNA vaccine; Tuberculosis; Monkey

1. Introduction

Tuberculosis (TB) is a major global threat to human health, with more than 3 million people dying each year from *M. tuberculosis* (TB) infections. The only tuberculosis vaccine currently available is an attenuated strain of *M. bovis* BCG (BCG), although its efficacy against adult TB disease remains controversial. Therefore, we have recently developed two novel TB vaccines: a DNA vaccine combination expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) by using the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ) and a recombinant BCG harboring the 72f fusion gene (r72f BCG). The former vaccine was 100-fold more efficient than BCG in the elimination of *M. tuberculosis* in mice by the induction of CTL [9].

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Researchers have recognized that a nonhuman primate model of TB will be able to provide critical information for vaccine development. However, several TB vaccine candidates who appear to protect better than BCG against virulent *M. tuberculosis* in mice, have rarely been tested in the nonhuman primate model because of cost and limited facilities.

In the present study, we evaluated the protective efficacy of HSP65 + IL-12/HVJ and r72f BCG in the cynomolgus monkey model, which is an excellent model of human tuberculosis [1]. These vaccines provided a strong prophylactic effect in monkeys challenged with *M. tuberculosis* as we have seen previously in mice.

2. Materials and methods

DNA vaccines encoding *M. tuberculosis* HSP65, mouse IL-12 and guinea pig IL-12 were encapsulated with HVJ-liposomes [2]. Groups of animals (mice and guinea pigs) were vaccinated intramuscularly with HVJ-liposome DNA vaccines. CTL activity was assessed by ^{51}Cr-release and IFN-γ activity [3,4]. A total of 16 cynomolgus monkeys were housed in a BL 3 animal facility of the Leonard Wood Memorial. Groups of animals were vaccinated three times with either the HVJ-liposome combination with HSP65 DNA plus human IL-12 DNA (HSP65 + hIL-12/HVJ: 400 μg i.m.), r72f BCG (1×10^6 CFU i.d.), BCG Tokyo (1×10^6 CFU i.d.) or saline. One month after the third vaccination, monkeys were challenged with the *M. tuberculosis* Erdman strain (5×10^2) by intratracheally instillation, Erythrocyte Sedimentation Rate (ESR), body weight, chest X-ray, immune responses, DTH reaction against PPD and survival periods were examined during 14 months [1].

3. Results

Mice vaccinated with HSP65 + mIL-12/HVJ had significantly reduced numbers of CFU [5] in the lungs, liver and spleen as compared with mice vaccinated with BCG [9]. CTL activity correlated with the protective efficacy of vaccination. The fusion protein Mtb72f (Mtb39 + Mtb32) vaccine was developed by Skeiky et al. [6]. To improve its vaccine efficacy, a recombinant BCG harboring the 72f fusion gene (r72f BCG) was generated [7]. The ELISPOT assay showed that r72f BCG induced a greater number of IFN-γ producing T-cells than BCG in the mouse model. In the guinea pig model, r72f BCG as well as HSP65 + gpIL-12/HVJ provided better protection against the pulmonary pathology caused by pulmonary challenge with TB than BCG vaccination (data not shown).

The purpose of this study was to evaluate two TB vaccines we have developed in a nonhuman primate model of *M. tuberculosis* infection. To this end, a total of 16 monkeys were vaccinated either with HSP65 + hIL-12/HVJ, r72f BCG, BCG or saline, followed by TB challenge by intratracheally instillation. Table 1 shows survival periods of vaccinated monkeys after TB challenge. All four monkeys in the control (saline) group died of TB infection within 8 months. In contrast, three and two monkeys from the 72f rBCG and HSP65 + hIL-12/HVJ groups, respectively, were alive more than 14 months post-infection (the termination period of the experiment). Survival periods of the remaining monkeys in the both groups were much longer than those of saline control group. In addition, both HSP65 + hIL-12/HVJ and r72f BCG significantly improved ESR and chest X-ray findings (Table 2). Body weights of the HSP65 + hIL-

Table 1
Survival of cynomolgus monkeys immunized with HVJ-liposome/HSP65 DNA + IL-12 DNA vaccine and recombinant 72f BCG vaccine

Vaccination	Total monkeys	Survival	Dead	% Survival
HVJ-liposome/HSP65 DNA + IL-12 DNA	4	2	2	50
Recombinant 72f BCG	4	3	1	75
BCG Tokyo	4	2	2	50
Saline	4	0	4	0

Cynomolgus monkey (4 monkeys/group) were immunized three times (every 3 weeks) with (1) HVJ-liposome/ HSP65 DNA + IL-12 DNA vaccine, (2) r72f BCG vaccine, (3) BCG Tokyo and (4) saline as control group as described in Section 2. One month after last immunization, M.TB (Erdman strain 5×10^2) was challenged by intratracheally instillation. Survival was studied more than 14 months.

Table 2
Improvement of Erythrocyte Sedimentation Rate (ESR) in the cynomolgus monkeys immunized with HVJ-liposome/HSP65 DNA + IL-12 DNA vaccine and recombinant 72f vaccine

Vaccination	ESR (nm/h)	Mean ± S.D.	Statistical significance *P*-value compared to saline group (Student *t*-test)
HVJ-liposome/HSP65 DNA + IL-12 DNA	2	3.5 ± 1.9	<0.01
	6		
	4		
	2		
Recombinant 72f BCG	3	6.75 ± 8.9	Not significant
	1		
	20		
	3		
BCG Tokyo	22	11.25 ± 11.3	Not significant
	2		
	20		
	1		
Saline	50	29.75 ± 18.1	
	14		
	15		
	40		

Cynomolgus monkey (4 monkeys/group) were immunized and challenged as described in Table 1. Elevation of Erythrocyte Sedimentation Ratio (ESR) of all monkeys was evaluated every month and maximum values of ESR in each monkey were shown.

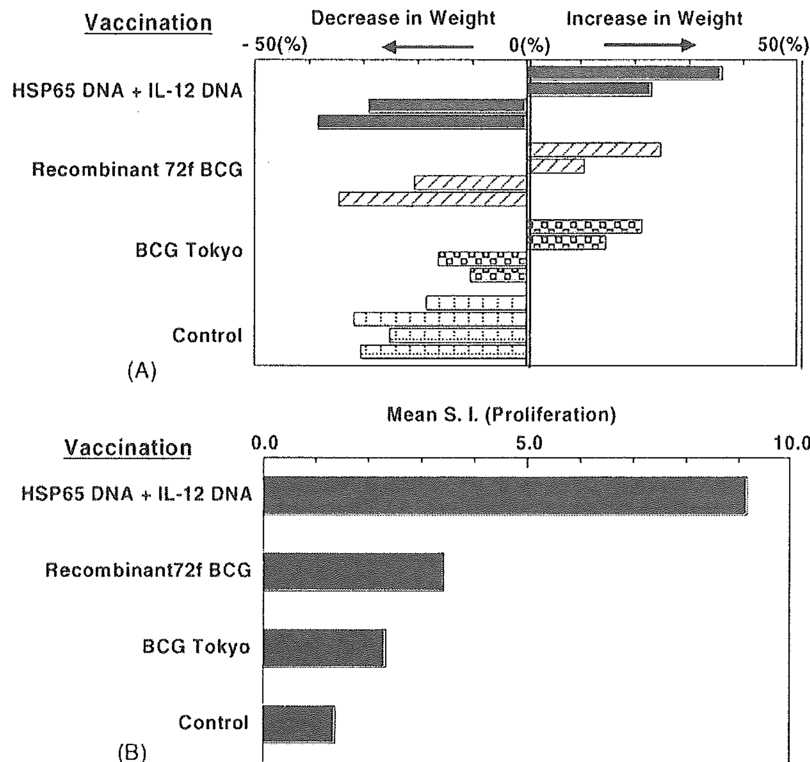

Fig. 1. (A) Increase in body weight: the prophylactic effect of novel vaccines (HSP65 DNA + IL-12 DNA, recombinant 72f BCG) on *M. tuberculosis* infection of cynomolgus monkeys. Percent of increase or decrease in body weight of monkeys immunized with (1) HSP65 DNA + IL-12 DNA (■), (2) recombinant 72f BCG (▨), (3) BCG Tokyo vaccines (▩) and (4) saline (control) (▤) and challenged with *M. tuberculosis*, compared to the weight of pre-immunized monkeys. (B) Lymphocyte proliferation activity (LPA) against recombinant HSP65 protein in the peripheral blood (whole blood) from the cynomolgus monkeys immunized with novel vaccines and challenged with *M. tuberculosis*. Peripheral blood lymphocytes (whole blood) 4 weeks after TB challenge were cultured with 10 μg/ml of recombinant HSP65 antigen in a 96-microwell plate for 5 days at 37 °C and then pulsed with 1 μCi of [3H] thymidine per well for the final 16–18 h of incubation. Results are expressed as a stimulation index (S.I.) and compared to the pre-immune LPA from the same monkey.

12/HVJ group also increased significantly, as compared to saline control group (Fig. 1A). IL-2 and IFN-γ production were augmented in the two groups vaccinated with HSP65 + hIL-12/HVJ and r72f BCG (data not shown). Furthermore, proliferation of PBL was strongly enhanced in the group vaccinated with HSP65 + hIL-12/HVJ in response to HSP65 protein 4 weeks after TB challenge (Fig. 1B). Taken together, these results clearly demonstrate that both HSP65 + hIL-12/HVJ and r72f BCG could provide protective efficacy against *M. tuberculosis* in the cynomolgus monkey model.

4. Discussion

HSP65 + hIL-12/HVJ vaccine as well as r72f BCG vaccine exerted the significant prophylactic effect against TB, as indicated by: (1) prolongation of survival for over a year, (2) improvement of ESR and chest X-ray findings, (3) increase in the body weight and (4) augmentation of immune responses, in a cynomolgus monkey model which closely mimics human TB disease. It is very important to evaluate the long survival period in a monkey model, as human TB is a chronic infection disease. Furthermore, the decrease in the body weight of TB patients with TB is usually accompanied by progress of TB disease. Suppression of IFN-γ production, CTL activity and T-cell proliferation has also been observed in patients with TB [8].

Our results with the HSP65 + hIL-12/HVJ vaccine in the cynomolgus monkey model should provided a significant rational for moving this vaccine into clinical trials. In fact, the 72f fusion protein vaccine entered Phase I testing after its evaluation in cynomolgus monkeys in Leonard Wood Memorial [4] by Reed and Skeiky. Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig, and monkey) to accumulate essential data on the HVJ-liposome DNA vaccine in anticipation of a Phase I clinical trial.

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ゆえに結核病棟内での再感染が判明した事実をもとに，国内外の文献の考察も含め，宿主側の因子，菌側の因子および結核菌の再曝露程度により，外来性再感染が普遍的に起こり得ることを，分子疫学的解析により実証した。低蔓延国に近づいている本邦では，外来性再感染は結核入院病棟を中心に起こるので，これを意識した入院患者・職員への感染防止対策が必要であることを各演者は警告した。

1. 多剤耐性結核の再感染

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はじめに

わが国の結核医療は，長年にわたってそのほとんどが隔離入院治療という形で行われてきた。しかし，結核病棟が特に他の一般病棟と比べて特別な感染対策が施されていたわけではなく，空気感染防止のための空調管理設備を備えた病室が整備され始めたのもごく近年のことである。結核病室の多くは大部屋であり，感受性結核患者と耐性結核患者が同室となることも多かった。これは，①結核患者が新たに他の結核菌の感染を受ける（再感染）ことは稀である，②耐性結核菌は変異菌であるので毒力は弱い，という漠然とした認識があったからと考えられる。すなわち，感受性結核患者が耐性結核菌の外来性再感染を受けることはまずあり得ないと想定されていたのである。

しかし近年分子疫学の進歩により結核の再感染発病を確実に証明することが可能となり，耐性結核菌による再感染発病が起こり得ることも報告されている。ここでは，われわれが経験した多剤耐性結核菌による再感染発病と考えられる2事例について概説し，今後の結核感染対策のあり方について考えてみたい。

事　例　1

本事例は多剤耐性結核の院内集団感染事例である。初発患者Aは56歳の男性で，平成12年3月発症の初回多剤耐性結核患者である。発症時の分離結核菌の薬剤感受性検査でisoniazid（INH），rifampicin（RFP）を含む多剤に既に耐性を示しており，近医入院にて化学療法を施行されるも大量排菌持続していた。平成14年6月に他患者とのトラブルのため当院転院となる。

当院転院までの約2年間における患者Aの接触者から後に5名の多剤耐性結核患者が発生し，5名の分離菌株はRFLP分析により患者Aの菌株と同一であると考えられた。うち3名は特に基礎疾患のない若年女性であった。他の2名は63歳男性と53歳男性であり，基礎疾患として肺気腫，糖尿病を有していた。2名とも全剤感受性肺結核にて入院加療を受けており，入院中にのみ患者Aと接触歴があった。2名とも感受性肺結核治癒後に多剤耐性肺結核を発症している。従って感受性結核罹患中に多剤耐性結核菌の再感染を受けたと考えられる。なお，2名とも感受性肺結核罹患時の分離菌は保存されておらず，RFLP分析は行えなかった。本事例の患者は6名全員HIV陰性であった。

事　例　2

本事例は当院で経験した多剤耐性結核菌による再感染発病事例である。患者Xは特に基礎疾患を有さない28歳男性で，平成13年1月より全剤感受性結核にて当院入院し化学療法を行った。入院中の一時期，多剤耐性肺結核に罹患していた患者Yと同室であった。順調に排菌陰性化して退院し，化学療法にて治癒に至ったが，その後，平成16年6月に再発し，そのときの検出菌の薬剤感受性検査ではINH，RFP，ethambutol（EB），streptomycin（SM）を含む多剤に対して耐性を示していた。RFLP分析を行ったところ，再発時の検出菌は初回治療時の検出菌とはパターンが異なっており，患者Yの検出菌と同一パターンであった。すなわち，感受性結核治療中に多剤耐性結核菌の再感染を生じて，後に多剤耐性結核による再発を生じたと考えられた。なお，患者XもHIV陰性であった。

多剤耐性結核菌のクラスター解析

2001年から2004年までに当院で分離した多剤耐性結核菌株115株を対象に，RFLP法，spoligotyping法により解析を行った。RFLP法では48株（42%）が10群のクラスターを形成していた。5株以上からなる大きなクラスターが3群あり，クラスターa（12株），クラスターb（11株），クラスターc（7株）とした。事例1の株はクラスターc，事例2の株はクラスターaに属していた。spoligotyping法でクラスターa，クラスターbはBeijing strainと判定されたが，クラスターcはBeijing strainではなかった。

多剤耐性結核は，一般にはその多くが不十分な治療による耐性の誘導が原因と考えられているので，クラスター形成率は低くなることが予想される。しかし，今回の検討ではクラスター形成率は42%であった。また，大きなクラスターを形成するクラスターa，b，cの株は，広く蔓延する強毒株であることが示唆された。

再発時に多剤耐性を示した結核における再感染の頻度

当院において，いったん結核にて化学療法を行い治癒した後，少なくとも排菌陰性期間が6カ月以上持続した後に多剤耐性結核を発症した例につき，前後の菌株が入手できた8症例に対してRFLP分析を行った。8例中6例は前後の菌株のRFLPパターンが一致し内因性再燃であると考えられたが，残り2例（事例2を含む）はパターンが異なり再感染発病であると考えられた。この2例の再発時の耐性菌はクラスターa（事例2）とクラスターcに属する大クラスター形成株であった。

考　察

近年RFLPをはじめとする分子疫学的手法の進歩により結核の再感染発病について幅広い検討がなされている。当初はHIV感染者での報告が相次ぎ，再感染発病の宿主側の危険因子としてHIV感染が注目されたが，その後HIV陰性者を含めて様々な状況下での再感染発病事例が報告された。伊藤はこれまでの報告の分析により，かつて考えられていたほど再感染発病は稀なものではなく，宿主側の因子，菌側の因子および曝露程度により普遍的に起こり得ることを指摘している[1]。

今回の事例1では，2年間に基礎疾患をもたない若年女性3人が発病し，また，2人の中高年男性が再感染を受けて発病している。また，事例2では基礎疾患をもたないHIV陰性若年男性が再感染を受けて発病している。以上よりこの2事例の菌は強毒菌であったことがうかがわれる。いずれも大きなクラスターを形成する菌であったこともその裏付けとなる。

かつて動物実験でカタラーゼ活性を欠くINH耐性菌の増殖が感受性菌に比べて劣ることが示されたことから，変異株である耐性菌は感受性菌に比べて毒力が弱いと漠然と信じられてきた。しかし，今回われわれが経験したように，多剤耐性結核菌といえども再感染発病を引き起こす病原性の高い菌も存在する。それでは，病原性を規定するものは何であろうか？ NiemannやNarvskayaもHIV陰性者における多剤耐性結核再感染事例を報告しており[2][3]，いずれも菌はBeijing strainであった。欧米では，集団感染や再感染発病の原因となる強毒菌としてBeijing strainが関与しているとの報告が多い[4]。しかし，わが国や中国ではもともと半数以上がBeijing strainである[5]。一方，事例1の菌はBeijing strainではなかった。結局，Beijing strainであることも必ずしも決め手とはならず，現時点で菌の病原性を決定するのは困難であると言わざるを得ない。あえて言えば，クラスター解析で大きなクラスターを形成する菌が強毒菌であると言えるかもしれない。

多剤耐性結核の再感染は，結核の感染対策上大きな影響を与える。多剤耐性結核菌による再感染が起こり得，しかもどの菌が再感染し得るか予測することが不可能な以上，すべての排菌陽性耐性結核患者は感受性結核患者と同室に収容すべきではない。さらに，初回耐性結核の可能性も考えると，感受性不明の排菌陽性結核患者は全員陰圧個室収容が望ましい。CDCの結核院内感染防止ガイドラインではこの点を考慮に入れ，薬剤感受性パターンが同一であると判明し有効な化学療法が行われている場合に限り患者同士を同室にしてよいとしている[6]。わが国の現状では，これを守るのはインフラの面からもコストの面からもきわめて困難である。しかし，結核患者の減少，在院日数の短縮化により結核病棟の稼働率が下がっていく中で，思い切った対策の転換を考慮する必要があるのではないだろうか。多剤耐性結核は，その医療にかかる金銭的・時間的コストの膨大さ，さらに，院内感染が生じたときの社会的なインパクトの大きさなどを考慮に入れると，その発生防止に最善の対策が講じられるべきである。

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The 80th Annual Meeting Mini-Symposium

EXOGENOUS RE-INFECTION IN TUBERCULOSIS

Chairperson: Toshiaki TSUCHIYA

Abstract Patients infected by tuberculosis (TB) had been thought to never experience exogenous re-infection. However, exogenous re-infection in HIV-positive patients is well known. Thanks to the introduction of histopathological examination, analysis of similarities in drug-resistance patterns and epidemiological surveys of genetic phage typing for TB infection, we have begun to understand that even people with a normal immune system can experience re-infection.

Recent advances in the techniques of restriction fragment length polymorphism (RFLP) and spoligotyping allow determination of similarities in tubercle bacilli, revealing a high ratio of exogenous re-infection.

In this mini-symposium, Dr. Kazunari Tsuyuguchi reported cases of nosocomial multidrug-resistant tuberculosis (MDRTB) infection, as exogenous re-infection, at 3 tuberculosis hospitals in the Osaka area. Although the virulence of MDRTB as a variant strain has generally been regarded as weaker than that of drug-sensitive strains, he reported even non-Beijing strain MDRTB, which displays strong virulence, could possess possible infectiosity with a 42% ratio of clustering formation and 2 of 8 patients with MDRTB exhibiting exogenous re-infection, as analyzed by RFLP.

Dr. Hideo Ogata reported the actual condition of exogenous re-infection, having cited a large number of reports at home and abroad. In his report he indicated that even among hosts without serious hypoimmunity, re-infection rate is high in high-prevalence countries. Conversely, endogenous TB reactivation is high in low-prevalence countries. As Japan has become a low-prevalence country, endogenous reactivation might be seen in TB wards.

Dr. Katsuhiro Kuwabara reported on his study about exogenous re-infection of *Mycobacterium avium*, which represented resident flora in the environment, using IS*1245* RFLP analysis. He demonstrated that re-infection and multiple infections were frequently observed in *M. avium* infection.

Dr. Tomoshige Matsumoto finally added that about 90% of patients with recurrence in the Osaka area exhibit endogenous reactivation, as found using molecular epidemiologic analysis of bacterial strains from initially treated and retreated patients. Compared with reports from other countries, the ratio of exogenous re-infection in Japan is lower than elsewhere. Thanks to the public health service about TB, sources of TB infection are not present, so patients with TB do not experience exogenous re-infection, he concluded. He also discussed the variable number of tandem repeats (VNTR)-typing method that has been taking the place of the IS*6110* RFLP.

In this mini-symposium referring to molecular epidemiological analyses and reports from Japan and overseas, we showed that depending on factors involving hosts, parasites and the density of TB re-exposure, the possibility of universal exogenous nosocomial re-infection exists. Each presenter alerted us to the fact that as exogenous re-infection occurs mainly in TB inpatient wards, prevention of TB infection is crucial for inpatients and medical staff in Japan as a low-prevalence country.

1. Exogenous re-infection by multidrug-resistant tuberculosis: Kazunari TSUYUGUCHI, Shiomi YOSHIDA, Katsuhiro SUZUKI, Masaji OKADA, Mitsunori SAKATANI (NHO Kinki-chuo Chest Medical Center)

We describe three recurrent cases of multidrug-resistant (MDR) tuberculosis (TB) nosocomially re-infected with MDRTB strain during treatment for drug-sensitive TB. The first and the second patients, both of whom were middle-aged heavy smoker men, were associated with the outbreak caused by non-Beijing MDRTB strain. The third patient was a immunocompetent young man and the isolated strain was Beijing MDRTB strain. All the patients were HIV-seronegative. We conclude that exogenous re-infection by

MDRTB can occur on various situations. These results underscore the importance of placing MDRTB patients separately from drug-sensitive TB patients.

2. Reviews of the exogenous re-infection in tuberculosis: Hideo OGATA (Fukujuji Hospital, JATA)

In Japan, they have thought that a tubercular relapse is based on endogenous reactivation in almost all cases. However, there are many studies which prove exogenous re-infection using tuberculin test or drug susceptibility test. The technique of developed strain typing contributed exogenous re-infection to clarifying greatly in a real proof and its frequency in recent years.

3. Multiple and repeated polyclonal infections in patients with *Mycobacterium avium* lung diseases: Katsuhiro KUWABARA (NHO Nishi-Niigata Chuo National Hospital)

The routes of transmission and environmental reservoirs of *Mycobacterium avium* infections have been unclear. IS*1245* based RFLP analysis showed genetic diversity of *Mycobacterium avium* clinical isolates and the relation between clinical subtype and polyclonal infection. Our study demonstrates that polyclonal infections are common in *Mycobacterium avium* lung diseases, especially nodular bronchiectasis type. In addition, not only simultaneous polyclonal infections but also repeated polyclonal infections were observed in some patients. The knowledge of polyclonal infection will lead to better understanding of *Mycobacterium avium* pathogenesis and epidemiology.

Special commentaries: Consideration of exogenous re-infection of tuberculosis in Osaka. Japan, by using molecular epidemiologic tools: Tomoshige MATSUMOTO (Osaka Prefectural Medical Center for Respiratory and Allergic Diseases)

By using IS*6110* RFLP, we showed that 9.5% of TB recurrence was caused by re-infection in the middle-eastern area of Osaka Prefecture, Japan. The molecular typing tools are now being applicable not only to epidemiological but also to clinical fields by an introduction of PCR-based method, such as Variable Numbers of Tandem Repeats (VNTR) typing. We showed some examples about usefulness of the clinical application of molecular epidemiology, using VNTR.

Key words: Exogenous re-infection, Endogenous reactivation, Multidrug-resistant tuberculosis, Nosocomial infection, *Mycobacterium avium* infection, Molecular epidemiology.

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小児科

インフルエンザ罹患に伴うライ様症候群・急性壊死性脳症

Q インフルエンザ罹患に伴うライ様症候群、急性壊死性脳症の発生機序と予防策について。

（神奈川県 Y）

A ライ様症候群とは「ライ症候群に臨床生化学的所見は酷似しているが、肝所見で病理組織所見がライ症候群とは異なるもの」と定義される。脳浮腫と肝障害がみられるという点がライ症候群に共通している。ライ様症候群の肝所見は必ずしも非炎症性ではなく、細胞浸潤、壊死など多様性がある。実際の医療現場では、最近では発生が減少しているライ症候群よりもライ様症候群のほうが遭遇する機会が多く、重要である。

ライ様症候群の原因はインフルエンザ、水痘、ヘルペスなどのウイルス感染症、脂肪酸酸化障害などの代謝異常、バルプロ酸などの薬剤など、多岐にわたっている。発症機序はミトコンドリア機能を一次的もしくは二次的に障害することが想定されている。すなわち、ホパンテン酸カルシウム、バルプロ酸、マルゴサ油などの薬剤や化学物質ではミトコンドリア機能を障害して発症に至ることが知られているし、メフェナム酸やジクロフェナクなどもライ様症候群の発症に関与している可能性がある。

また、脂肪酸代謝異常で絶食により低血糖を引き起こし、容易に意識障害に陥り、急性脳症を発症することが知られている。この場合は絶食によりグリコーゲンの枯渇化が進展するにもかかわらず、脂肪酸の酸化障害のために、容易にエネルギーの供給不足状態に陥りやすいことと関連している。

予防としては、これらのライ様症候群と関連があると想定されている薬剤の使用を中止もしくはできるだけ控えることであり、脂肪酸代謝異常などでは絶食時間を短くすることや、炭水化物の摂取を早めに心がけることであろう。ウイルス感染症では有効なワクチンが利用できる場合には、積極的に予防接種を受けることであろう。

急性壊死性脳症はわが国で提唱された疾患概念で、日本を含む東アジアに多発し、小児の急性脳症のサブタイプといえる。六カ月〜一歳六カ月の乳幼児が罹患しやすく、発症前には発熱を伴うウイルス感染が必発である。中でもインフルエンザが最も多く、その他、突発性発疹症、ロタウイルス等が知られている。脳の病理所見では浮腫性壊死が観察される。

発症機序については、サイトカインの関与が想定されており、事実、各種サイトカインの増加が知られている。ウイルス感染後、半日〜三日で発症することが多く、意識障害、痙攣、嘔吐で発症する場合が大半である。ライ症候群とは異なり、高アンモニア血症、低血糖、高ピルビン酸および乳酸血症の頻度が低いことから、急性壊死性脳症の発症機序にミトコンドリア障害が関与している可能性は低いと考えられる。いずれにしても、本症はインフルエンザ流行に関連し、多発することが知られており、インフルエンザ対策が何よりの予防であろう。

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STAT1欠損症による免疫不全の病態

Q 免疫不全症の一つSTAT (signal tranducer and activator of transcription) 1欠損症について。インターフェロン (IFN) -α, β, γの反応を悪くし、非定型抗酸菌症の感染を起こしやすくするようであるが。

（東京都 F）

A まず、STAT1の生理学的意義を解説する。STAT1はシグナル伝達と転写活性化の二

つの機能を有する蛋白で、IFN-α/β、IFN-γ、上皮成長因子（EGF）、血小板由来成長因子（PDGF）、インターロイキン6（IL-6）などによりチロシンの部分がリン酸化されることにより活性化され、二量体、三量体などを形成して転写を促進する[1]。

図1に示すように、IFN-γからの刺激の場合、STAT1は主にホモダイマーであるGAF（IFN-γ-activated factor）を形成し、GAS（IFN-γ-activated site）に結合し、転写を促進する。また、IFN-α/βからの刺激の場合、STAT1は主にSTAT2、IRF9（IFN regulatory factor 9）と結合し、ISGF3（IFN-stimulated gene factor 3）を形成する。ISGF3はISRE（IFN-stimulated gene response element）に結合し転写を活性化する。

図1　IFN-α/βとγのシグナル伝達経路の概略

図2　IL-12/IFN-γを介する免疫

また、図2のように、STAT1はIFN-γの刺激により活性化され、腫瘍壊死因子α（TNF-α）、IL-12などの遺伝子発現を促進し、抗酸菌の排除に重要な役割を果たす。

①STAT1部分欠損症

片側のアリルのみのSTAT1の七六一番目のアミノ酸がリジンからセリンに変化した変異の場合、異常なSTAT1のため七〇一番目のチロシンのリン酸化が起きず、転写が促進されない。しかし、この場合、三量体のISGF3は正常に形成され、IFN-α/βのシグナル伝達には異常が生じないため、マイコバクテリア易感染性（BCG播種、非定型抗酸菌症）は起きるが、ウイルスに対する易感染性は生じていない[2]。

②STAT1完全欠損症

両側のアリル変異の場合、GAFとISGF3両方の異常が生じ、マイコバクテリアとウイルス両方に易感染性が生じている。STAT1完全欠損症の患者では、1例がBCGの播種と再発性単純ヘルペスI型脳炎を起こし死亡、もう1例もBCG播種の後、ウイルス様疾患で死亡している[3]。

STAT1はEGF、PDGFなど他のサイトカインのシグナル伝達にも働くが、他のサイトカイン欠損に関連する症状は出現していない。また、STAT1は細胞周期、アポトーシスとも関連し、腫瘍抑制因子としての可能性が考えられているが[1]、ヒトSTAT1完全欠損症と癌との関連は明らかではない。

以上のようにSTAT1欠損症

は、IFN-γ経路の異常により抗酸菌への易感染性を示す疾患群（IL-12, IL-12レセプター、IFN-γレセプター1&2欠損症）に新しい疾患として加わった。これらの疾患は、一般には抗酸菌のみならず、リステリア、サルモネラ、レジオネラなど細胞内寄生性細菌に対しても易感染性を示す[4]。

動物モデルの研究で、STAT1完全欠損マウスでは、ヒトと同様に、IFN-αやIFN-γに反応せず、ウイルス、細胞内寄生性細菌に対して易感染性を示した。しかし、他のサイトカインに対する反応性は正常で、成長・発達には異常を来していない。また、STAT1完全欠損マウスでは悪性腫瘍も発生している[5]。

以上のように、ヒトの原発性免疫不全症の研究により、さまざまな免疫分子の生理学的意義が明らかとなった。

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Novel roles of osteopontin and CXC chemokine ligand 7 in the defence against mycobacterial infection

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Summary

Granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced human monocyte-derived macrophage (GM-Mϕ) or macrophage CSF (M-CSF)-induced human monocyte-derived Mϕ (M-Mϕ) are distinct in terms of the resistance to *Mycobacterium tuberculosis*. To elucidate the role of molecules involved in the functional differences between these Mϕs, we investigated the gene expression profiles using microarray. After culture of $CD14^+$ monocytes with CSFs, Mϕs were cultured with or without bacillus Calmette–Guérin (BCG) (GM-Mϕ-BCG and M-Mϕ-BCG). The gene expression profiles from these cells were compared. Chemokines highly expressed in M-Mϕs were selected and evaluated for anti-mycobacterial activity and superoxide production. *FN1* and *FCGR2B* were the most up-regulated genes in GM-Mϕ and M-Mϕ, respectively. After stimulation with BCG, three chemokine genes (*Osteopontin* (*SPP1*), *CXC chemokine ligand 7* (*CXCL7*) and *CC chemokine ligand 11* (*CCL11*)) were highly expressed in M-Mϕ-BCG when compared to those in GM-Mϕ-BCG. A significantly increased resistance to *M. tuberculosis* H37Ra was observed after the stimulation of GM-Mϕ with SPP1 or CXCL7. Superoxide production levels of SPP1- or CXCL7-stimulated GM-Mϕs were higher than those of GM-Mϕs without stimulation. These results indicate that both SPP1 and CXCL7 might have a role in the resistance against mycobacteria, at least in part, through augmenting reactive oxygen intermediate production in Mϕs.

Keywords: GM-CSF, M-CSF, macrophage, microarray

Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, is one of the most important burdens on human health [1]. Both environmental and genetic factors contribute to the development of the disease, which approximates 10% of the infected subjects [2]. Twin studies provided the evidence that human genetic factors could influence the development of tuberculosis [3]. The genetic basis of susceptibility to mycobacteria has been clarified partly by the recent identification of defects in the molecules of the interferon (IFN)-γ-mediated immune pathway, such as IFN-γ receptors 1 and 2 [4,5], interleukin (IL)-12 receptor-β1 [6], IL-12p40 [7] and STAT1 [8]. In addition, linkage and/or association studies have demonstrated many susceptibility genes, such as *HLA* [9], *NRAMP1* [10], *IFN-G* [11], *TNF-A* [12], *IL-10* [12], *IL-1RA* [13], *MBL* [14], *VDR* [15] and *TLR2* [16].

The immune response against mycobacteria is mounted in a complex process. In the host, mycobacteria dwell chiefly within macrophages (Mϕs). Following activation, Mϕs produce a wide range of cytokines/chemokines and activate T cells. IFN-γ secreted by activated T cells and natural killer (NK) cells is one of the principal Mϕ activating factors, and acts as the central cytokine in the control of mycobacterial infection. Activated T cells stimulate anti-mycobacterial machinery in Mϕs, which includes reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) [17].

Mϕs that play a pivotal role in the mycobacterial infections are heterogeneous in nature, with different phenotypes and functions. They are derived predominantly from peripheral blood monocytes, and differentiate to specific cells in target tissues. Peripheral blood monocytes need colony-stimulating factors (CSFs) such as granulocyte-macrophage (GM)-CSF or macrophage (M)-CSF for their survival and

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differentiation *in vitro*. GM-CSF-induced monocyte-derived macrophage (GM-Mϕ) and M-CSF-induced monocyte-derived macrophage (M-Mϕ) are distinct in their morphology, cell surface antigen expression and function. GM-Mϕ and M-Mϕ show susceptibility and resistance to mycobacteria, respectively [18,19].

To determine novel host resistance or susceptibility genes in mycobacteria infection, we investigated the differences in the gene expression profiles between GM-Mϕ and M-Mϕ with a high-density oligonucleotide microarray containing approximately 30 000 human genes. The expression profiles of each Mϕ subset were analysed with and without the stimulation of bacillus Calmette–Guérin (BCG) (GM- and M-Mϕ-BCG). Our results enlarged the views in the immunological mechanisms against mycobacteria, especially in the roles of several chemokines.

Materials and methods

Mϕ culture

Peripheral blood mononuclear cells (PBMC) were prepared from blood buffy coats of eight different healthy donors separately by density gradient centrifugation using lymphocyte separation medium (Cappel, Aurora, OH, USA). $CD14^+$ monocytes were purified (> 95%) from PBMC using a magnetic cell separation system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), with anti-CD14 monoclonal antibody (mAb)-coated microbeads and an FcR blocking reagent (Miltenyi Biotec). $CD14^+$ monocytes were cultured at a concentration of 5×10^4 cells/100 µl in 96-well tissue culture plates or at a concentration of 5×10^5 cells/2 ml in 6-well tissue culture plates with RPMI-1640 (Invitrogen Japan KK, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD, USA), and antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml; Sigma-Aldrich, St Louis, MO, USA) in an incubator containing 5% CO_2 at 37°C. Cells were stimulated with GM-CSF (100 ng/ml, PeproTech, London, UK) (GM-Mϕ) or M-CSF (75 ng/ml, PeproTech) (M-Mϕ). Optimal conditions were maintained by refreshing the medium and cytokines every 3 days. After 7 days of culture, a fraction of the cells were stimulated with BCG (10 mg/ml, BCG Tokyo 172; Japan BCG Laboratory, Japan) for 3 h (GM- and M-Mϕ-BCG). During BCG stimulation, a culture medium without antibiotics was used. For the analysis of anti-mycobacterial function and superoxide production, GM-Mϕs were stimulated with or without different concentrations of a chemokine: osteopontin (SPP1) (Biogenesis, Poole, UK: 0·02, 0·25 and 2·5 µg/ml), CXCL7 (Sigma-Aldrich: 0·05, 0·15 and 0·5 µg/ml) or CCL11 (Wako, Osaka, Japan: 0·5, 5 and 50 ng/ml) for another 6 days.

Bacterial preparation and infection to Mϕs

M. tuberculosis H37Ra was grown for 1 week in Middlebrook 7H9 liquid medium (Difco, Detroit, USA) at 37°C and aliquots were frozen at −80°C. In each experiment, an aliquot was thawed and grown in 7H9 medium to mid-exponential growth phase. The culture was sonicated (time: 10 s, output: 1, duty: 80%) (Branson Sonifier 250, CT, USA) to disperse bacilli before the infection. Both types of Mϕ layers were exposed to H37Ra for 3 h in a multiplicity of infection ratio of 1 : 1 in triplicate, washed three times and reincubated in the culture medium (RPMI-1640 plus 10% FBS) with antibiotics. After culture, the medium was removed and sterile phosphate-buffered saline was added to each well. The cells in the bottoms of the wells were scraped with a sterile scraper (Techno Plastic Products AG, Transadingen, Switzerland) and then sonicated as mentioned previously. Serial dilutions of the bacterial suspensions were plated on Middlebrook 7H10 agar plates (Difco). Colonies on the agar plates were counted 3 weeks after inoculation.

RNA isolation

Mϕs were harvested at 7 days after culture with CSF, and after further 24 h with BCG stimulation. Total RNA was extracted using RNA Extraction Kit, Isogen (Nippon Gene, Osaka, Japan), according to the manufacturer's instructions. All experiments were performed according to the guidelines of the ethics committee of Kyushu University.

Microarray processing

mRNA was amplified linearly using an Amino Allyl MessageAmp aRNA Kit (Ambion, Austin, TX, USA). In brief, mRNA (1·5 µg) was reverse transcribed to synthesize complementary DNA (cDNA) using an oligo(dT) primer bearing a T7 RNA polymerase promoter. Second-strand synthesis was carried out to make a transcription template. *In vitro* transcription of the cDNA with incorporation of amino allyl UTP was performed to produce multiple copies of amino allyl-labelled anti-sense RNA (aRNA). After purification, amino allyl-labelled aRNA was reacted with N-hydroxy succinimide esters of Cy3 and Cy5 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for the Mϕ samples and a standard control, respectively. Uncoupled dye molecules were removed using Micro Bio-Spin P-30 Tris chromatography columns (Bio-rad, Hercales, CA, USA). Cy3- and Cy5-labelled products were mixed together in the same amounts. After the aRNA was fragmented in a buffer containing 40 mM Tris-acetate, 100 mM CH_3COOK and 30 mM $(CH_3COO)_2Mg.4H_2O$ at 94°C for 15 min, the hybridization buffer ($5 \times$ SSC, 0·5% SDS, $4 \times$ Denhardt's solution, 100 µg/ml salmon sperm DNA, 10% formamide) was added. The hybridization was performed by incubating 60 µl of the product into three Acegene Human oligo chip 30K slides (Hitachi Software Engineering, Yokohama, Japan). Each slide was rinsed with a solution provided by the manufacturer (Hitachi Software Engineering). Two microarray experiments for each Mϕ subset were conducted, using two

RNA mixtures, each one equally combined from four independent cell cultures.

Signal detection and data analysis

Fluorescence signals for approximately 30 000 spots in slides were detected separately by fluorescent image analyser FLA-8000 (Fuji Film, Tokyo, Japan) for Cy3 and Cy5. Hybridization intensities were processed using Arrayvision software version 6·0 (Imaging Research, Ontario, Canada). Signal and background intensities were determined by the median pixel values. Local background values were determined as the average of four background spots around each gene spot. All spots in the image (for both Cy3 and Cy5 signals) were evaluated for a possibility of dusts, to lower the probability of false data in all experiments. GeneSpring version 6·2. (Silicon genetics, Redwood City, CA, USA) was used for data analysis. According to the GeneSpring instruction, normalization of the data was performed using the 'Lowess method' [20]. Spots with dust, or with signal values of which the Cy5 or Cy3 channels were less than three times of background, were excluded.

*Taq*Man real-time quantitative reverse transcriptase-PCR (qRT-PCR)

The same RNA used in the microarray analysis was employed for qRT-PCR. The cDNA was synthesized from the RNA using a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA), as described previously [21]. PCR primers and the target probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from ABI (Applied Biosystems, Foster City, CA, USA) as a *Taq*Man GAPDH control reagent kit. PCR primers and *Taq*Man probes for *FN1* and *FCGR2B* genes were purchased from ABI as assay reagents (Assays-on-Demand™, Gene Expression Products) with the following numbers: Hs00415006_m1 for *FN1* and Hs00414000_m1 for *FCGR2B*, and used according to the instructions of the manufacturer. The qRT-PCR was performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems) [22]. To calculate the relative amount of gene expression, the value of each gene expression was divided by that of the internal control, GAPDH. The analysis was carried out in duplicate samples.

Flow cytometry

Flow cytometric analysis was performed using an EPICS XL (Beckman Coulter, Miami, FL, USA). Multi-colour staining was performed by fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or PE-cyanin 5·1 (PC5)-conjugated mAbs against the following markers: HLA-DR, CD14, CD71, CD44, CXCR2 and appropriate controls (Immunotech, Marseille, France).

Superoxide production assay

Superoxide production by Mϕs was determined as described previously [23]. GM-Mϕs were cultured with or without a chemokine for 7 days. After treatment with trypsin (Invitrogen), cells were harvested, washed and resuspended in Hanks's balance salt solution (HBSS) (Invitrogen) (5×10^4/0·5 ml). They were stimulated with antibody-opsonized zymosan (1 mg/ml, Sigma-Aldrich) at 37°C, and the reaction was terminated by the addition of SOD (50 µg/ml, Sigma-Aldrich). The chemiluminescence was counted for 35 min with an enhancer-containing luminol-based detection system (DIOGENES; National Diagnostics) using a luminometer (Auto Lumat LB953; EG & G Berthold).

Statistical analysis

Data in qRT-PCR, colony forming unit (CFU) counting and superoxide production assays were assessed by Student's *t*-test using SPSS software version 11.

Online supplemental material

The microarray data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series Accession number GSE3408.

Results

Characterization of GM- and M-Mϕ

After culture with GM- and M-CSF for 1 week, peripheral blood monocytes differentiated into GM-Mϕ and M-Mϕ, respectively. These two types of Mϕs showed distinct features in their phenotypes and functions. Although both Mϕs expressed HLA-DR, GM-Mϕs strongly expressed CD71 and M-Mϕs were strongly positive for CD14 (Fig. 1a). M-Mϕs showed a higher resistance to *M. tuberculosis* H37Ra and a higher superoxide production than GM-Mϕs (Figs 2 and 3B), as reported by Akagawa [18].

Comparison of the constitutive gene expression levels between GM- and M-Mϕ, by microarray and quantitative PCR

To identify the molecules involved in the functional differences between GM- and M-Mϕ, we compared the constitutive gene expression profiles in each Mϕ using microarray (Fig. 1b). The 10 most up-regulated genes, which are a result of comparison between these Mϕ, are listed in Table 1. *FN1* and *FCGR2B* were the most up-regulated genes in GM-Mϕs and M-Mϕs, respectively, both of which encode proteins that potentially interact with *M. tuberculosis*. These microarray data were confirmed by qRT-PCR. As shown in Fig. 1c, the

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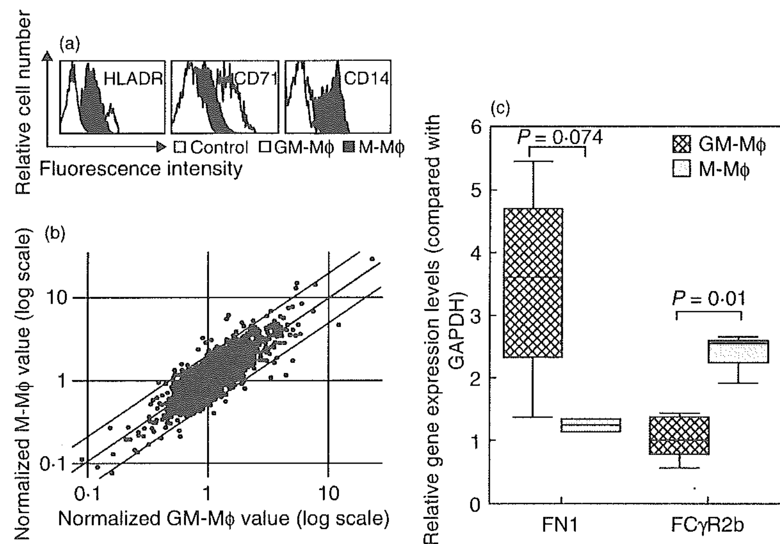

Fig. 1. (a) Phenotypic characteristics of GM-Mϕ and M-Mϕ, generated from human CD14+ monocyte. (b) The scatter-plot between two types of Mϕs in their constitutive states. Each spot is the representative of normalized data in logarithmal scale from the average of two values from each cell type. (c) The qRT-PCR analysis for *FN1* and *FCGR2b* gene expression levels, which were the most up-regulated genes in each Mϕ (Table 1).

expression levels of *FN1* and *FCGR2B* genes were increased in GM-Mϕs and M-Mϕs, respectively, although the difference of *FN1* expression levels did not reach the statistical significance.

Comparison of the gene expression levels between GM- and M-Mϕ with and without BCG exposure by microarray

When we compared the gene expression profiles between GM- and M-Mϕ with and without BCG, *IL-1B* showed the highest expression among BCG-stimulated genes in both Mϕs (Table 2). Also, *SOD2* gene was listed among highly expressed genes in both Mϕs after BCG stimulation (Table 2). Then, we compared the gene expression profiles between GM-Mϕ-BCG and M-Mϕ-BCG (Table 3). *Osteopontin* (*SPP1*) was the most up-regulated gene in M-Mϕ-BCG compared with GM-Mϕ-BCG, suggesting the protective role of *SPP1* in M-Mϕ against mycobacteria. Analysis of genes according to the gene ontology (GeneSpring software) revealed that four HLA-related genes were included in the 10 most up-regulated genes in GM-Mϕ-BCG compared with M-Mϕ-BCG, while three chemokine genes (*SPP1*, *CXC chemokine ligand 7* (*CXCL7*) and *CC chemokine ligand 11* (*CCL11*)) were included in the 10 most up-regulated genes in M-Mϕ-BCG compared with GM-Mϕ-BCG.

Effects of 3 chemokines on the growth of *M. tuberculosis* H37Ra in GM-Mϕ

We selected three chemokine genes (*SPP1*, *CXCL7* and *CCL11*) as the candidate genes that potentially contribute to the protective function of M-Mϕs. To clarify the possible effects of these chemokines on the resistance of M-Mϕs against *M. tuberculosis*, GM-Mϕs were cultured in the presence of different concentrations of one of these chemokines for 6 days, and their protective abilities against *M. tuberculosis* H37Ra were evaluated (Fig. 2). SPP1 or CXCL7-stimulated GM-Mϕs significantly inhibited the growth of H37Ra 6 days after the infection with the organism, while CCL11 stimulation had no effects on it (Fig. 2).

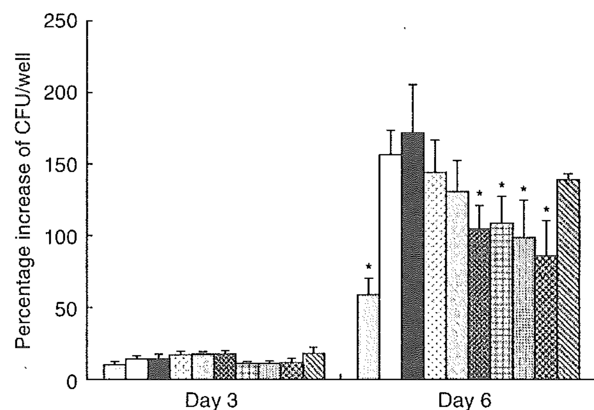

Fig. 2. Inhibition of *M. tuberculosis* H37Ra growth in Mϕs by SPP1 and CXCL7. Mtb colony-forming unit (CFU) assay was performed on days 3 and 6 after H37Ra-Mϕ exposure. M-Mϕ, GM-Mϕ with granulocyte macrophage-colony stimulating factor (GM-CSF) only □ and GM-Mϕ without any cytokines ■ were cultured as controls. GM-Mϕs were stimulated with three different chemokines: for SPP1, 0.02, 0.25 and 2.5 μg/ml; for CXCL7, 0.05, 0.15 and 0.5 μg/ml. Data for CCL11 are shown only for the results using the highest concentration in the experiments (see Methods for details). Mean values and standard deviations of triplicates are shown. *Indicates that *P* value was <0.05 in comparison with GM-Mϕ.

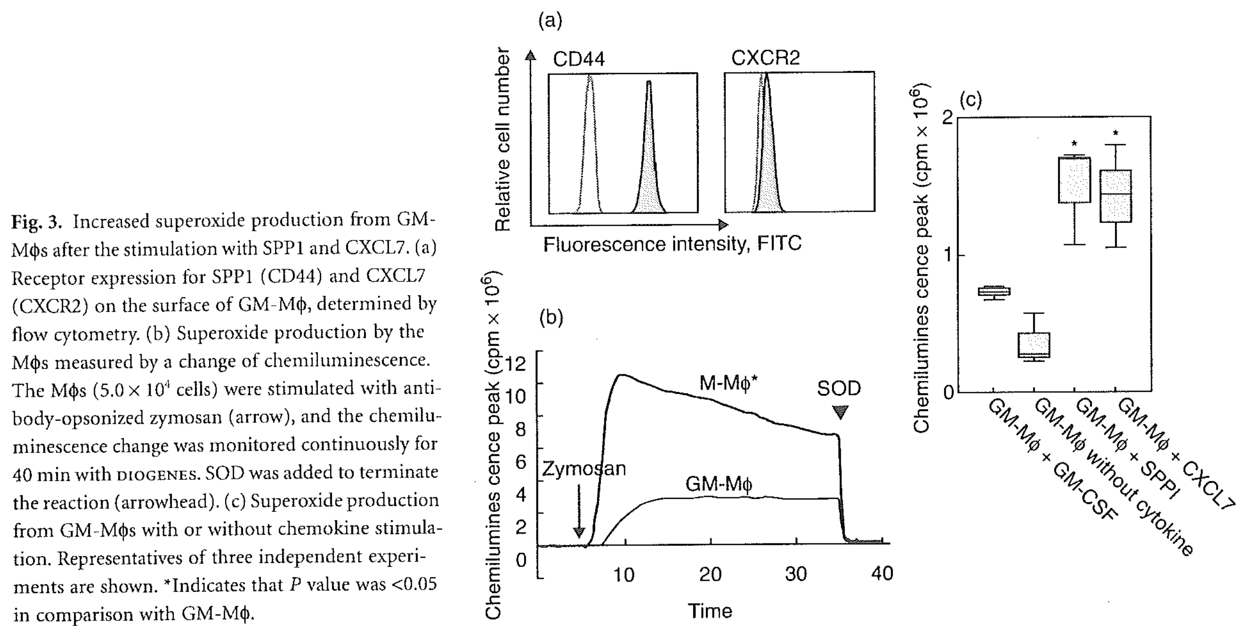

Fig. 3. Increased superoxide production from GM-Mϕs after the stimulation with SPP1 and CXCL7. (a) Receptor expression for SPP1 (CD44) and CXCL7 (CXCR2) on the surface of GM-Mϕ, determined by flow cytometry. (b) Superoxide production by the Mϕs measured by a change of chemiluminescence. The Mϕs (5.0×10^4 cells) were stimulated with antibody-opsonized zymosan (arrow), and the chemiluminescence change was monitored continuously for 40 min with DIOGENES. SOD was added to terminate the reaction (arrowhead). (c) Superoxide production from GM-Mϕs with or without chemokine stimulation. Representatives of three independent experiments are shown. *Indicates that P value was <0.05 in comparison with GM-Mϕ.

Table 1. Gene expression profiles of GM-Mϕ and M-Mϕ. (a) Genes which up-regulated in GM-Mϕ (top 10) compared to those in M-Mϕ; (b) genes which up-regulated in M-Mϕ (top 10) compared to those in GM-Mϕ.

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	FN1	ENSG00000115414	Fibronectin 1	3·81
2	Unknown	ENSG00000079310	ensembl prediction	3·12
3	Unknown	ENSG00000085063	ensembl prediction	2·93
4	CCL7	NM_006273–1	Monocyte chemotactic protein 3 precursor; scya7	2·86
5	AD 158	AL136919–1	Hypothetical protein; dkfzp586j1119	2·83
6	ARPC2	AF116702–1	pro2446	2·56
7	KIAA1838	XM_035688–1	Hypothetical protein xp_035688; loc94580	2·53
8	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	2·53
9	HBD	NM_000519–1	Haemoglobin, delta	2·35
10	ABCC3	AF085692–1	Multidrug resistance-associated protein 3b; mrp3	2·17
(b)				
1	FCGR2B	NM_004001–1	fc fragment of igg, low affinity iib, receptor for (cd32)	4·14
2	Unknown	ENSG00000024862	ensembl prediction	3·90
3	MHC Ag	L34093–1	MHC class ii hla-dq-alpha chain	3·64
4	Unknown	ENSG00000126461	ensembl prediction	3·01
5	C15orf12	AK001830–1	cDNA flj10968 fis clone place1000863 moderately similar to putative mitochondrial 40 s ribosomal protein yhr148w	2·90
6	Unknown	AC069384·3.87217·105230·1	ensembl genscan prediction	2·85
7	Unknown	AP002767·1.52387·73825·2	ensembl genscan prediction	2·84
8	MMP9	NM_004994–1	Matrix metalloproteinase 9 preproprotein	2·82
9	TM7SF1	NM_003272–1	Transmembrane 7 superfamily member 1 (up-regulated in kidney)	2·70
10	Unknown	AC003958·1.1·127834·1	ensembl genscan prediction	2·50

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Table 2. Genes (top 10) whose expression was up-regulated in GM-Mϕ–BCG compared to those in GM-Mϕ (a), and in M-Mϕ-BCG compared to those in M-Mϕ (b).

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	IL1B	NM_000576–1	Interleukin 1, beta	46·66
2	SOD2	NM_000636–1	Superoxide dismutase 2, mitochondrial	13·62
3	MT1G	XM_048213–1	Metallothionein 1 g	10·71
4	CLECSF9	AB024718–1	Macrophage c-type lectin mincle; mincle	6·28
5	CCL1	M57502–1	Secreted protein i-309; scya1	5·96
6	BCL2A1	NM_004049–1	bcl2-related protein a1	5·85
7	AKR1C3	L43839–1	3-alpha-hydroxysteroid dehydrogenase; 3alpha-hsd	5·72
8	Unknown	AC005027·2.1·157073·2	ensembl genscan prediction	5·62
9	GRO1	NM_001511–1	Gro1 oncogene (melanoma growth stimulating activity, alpha)	5·61
10	MT1H	NM_005951–1	Metallothionein 1 h	5·38
(b)				
1	IL1B	NM_000576–1	Interleukin 1, beta	74·62
2	CCL20	NM_004591–1	Small inducible cytokine subfamily a (cys-cys), member 20	59·71
3	ARHGEF1	NM_004706–1	Rho guanine nucleotide exchange factor 1	56·29
4	CCL7	NM_006273–1	Monocyte chemotactic protein 3 precursor	27·49
5	SOD2	NM_000636–1	Superoxide dismutase 2, mitochondrial	25·94
6	IL8	NM_000584–1	Interleukin 8	17·98
7	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	16·33
8	SERPINB2	NM_002575–1	Serine (or cysteine) proteinase inhibitor, clade b (ovalbumin), member 2	15·81
9	TNFAIP6	NM_007115–1	Tumour necrosis factor, alpha-induced protein 6	15·29
10	H1F2	NM_005319–1	h1 histone family, member 2	14·06

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Table 3. Genes (top 10) whose expression was up-regulated in GM-Mϕ–BCG compared to those in M-Mϕ-BCG (a) and in M-Mϕ-BCG compared to those in GM-Mϕ-BCG (b).

Gene no.	Name	Access	Gene description	Ratio
(a)				
1	HLA-DRA	NM_019111–1	Major histocompatibility complex, class ii	6·86
2	HLA-DMA	NM_006120	Major histocompatibility complex	6·79
3	ID2	NM_002166–1	Inhibitor of dna binding 2, dominant negativ ehelix-loop-helix protein	6·06
4	HLA-DP	S66883–1	Major histocompatibility complex class ii antigen beta chain	5·81
5	HLA-DQA	L34093–1	MHC class II hla-dq-alpha chain	5·30
6	PRG1	NM_002727–1	Proteoglycan 1, secretory granule	4·88
7	RGC32	NM_014059–1	rgc32 protein	4·72
8	TNFSF13B	NM_006573–1	Tumour necrosis factor (ligand) superfamily, member 13b	4·44
9	Unknown	AC026785·3.13728·33112·2	ensembl genscan prediction	4·31
10	Unknown	XM_016170–1	Hypothetical protein xp_016170; loc88021	4·17
(b)				
1	SPP1	NM_000582–1	Secreted phosphoprotein 1 (osteopontin)	22·37
2	Unknown	AC064875·4.1·35064·1	ensembl genscan prediction	19·20
3	CXCL7	NM_002704–1	Pro-platelet basic protein (NAP2, SCYB7, CTAP3, PPBP)	15·08
4	FLJ20033	NM_017629–1	Hypothetical protein flj20033	9·43
5	LOC64182	NM_022359–1	Similar to rat myomegalin	8·82
6	Unknown	BC000845–1	Unknown (protein for image:3457769)	7·01
7	C8B	NM_000066–1	Complement component 8, beta polypeptide	6·78
8	Unknown	BC006174–1	Unknown (protein for image:4053618)	6·73
9	STK4	NM_006282–1	Serine/threonine kinase 4	6·62
10	CCL11	NM_002986–1	Small inducible cytokine subfamily a (cys-cys), member 11 (eotaxin)	6·60

Analysis was performed using GeneSpring version 6·2.
Access indicates GenBank accession number.

Effects of SPP1 and CXCL7 on GM-Mϕs

The expression of cell surface receptors for SPP1 (CD44) and CXCL7 (CXCR2) on GM-Mϕ were confirmed (Fig. 3a). To investigate further the mechanism of increased resistance of SPP1- or CXCL7-stimulated GM-Mϕs against *M. tuberculosis*, superoxide production by Mϕs was investigated. After the stimulation with antibody-opsonized zymosan, M-Mϕs produced a higher amount of superoxide than GM-Mϕ (Fig. 3b). Superoxide production by GM-Mϕs was significantly enhanced after the stimulation with SPP1 or CXCL7 (Fig. 3c). The reaction was terminated by SOD, which inhibits cytochrome *c* reduction (Fig. 3b). These results suggested that increased superoxide production was one of the mechanisms of increased resistance of SPP1- or CXCL7-stimulated Mϕs against *M. tuberculosis*.

Discussion

GM-Mϕs and M-Mϕs show distinct features, although both Mϕs come from the same origin ($CD14^+$ PMNC). It has been reported that GM-Mϕs show a susceptibility to *M. tuberculosis*, while M-Mϕs have a resistance to *M. tuberculosis* with a greater FcγR-mediated phagocytic capacity and a higher capability of ROI production [18].

In our experiment, *FN1* that encode fibronectin (FN) was expressed predominantly in GM-Mϕs compared with M-Mϕs (Table 1a). FN is expressed constitutively in the lung [24]. *M. tuberculosis* binds to the FN by FN attachment proteins on the surface of *M. tuberculosis*. After fibronectin opsonization, *M. tuberculosis* can be phagocytosed easily via complement receptors and integrin receptors [25,26]. Therefore, it is possible that increased FN production led to the enhanced *M. tuberculosis* load into the cells. On the other hand, *FCGR2B* was highly expressed in M-Mϕs compared with GM-Mϕs (Table 1b). It was reported that *FCGR2B* expression levels were increased in peripheral blood monocytes in patients with tuberculosis compared with healthy controls by microarray analysis [27]. In contrast to FcγR1, FcγR2a and FcγR3, FcγR2b is an inhibitory receptor that does not contain immunoreceptor tyrosine-based activation motifs (ITAM) [28]. Therefore, FcγR2b seems to modulate inflammatory responses and inhibits phagocytosis of Mϕs [25]. Further analysis for the role of FcγR2b in *M. tuberculosis* infection would be necessary.

IL-1B and *SOD2* expression levels were up-regulated in both types of Mϕs after the stimulation with BCG (Table 2), which was consistent with the previous report [29]. IL-1β is produced by activated Mϕs following *M. tuberculosis* infections, and is an important mediator of cellular anti-mycobacterial activities [30]. The importance of IL-1 for the generation of protective immunity against mycobacterial infection was clarified using *IL-1*-knock-out mice [31]. Cell wall components of *M. tuberculosis* are known to induce *IL-1B* expression in human monocytes and macrophages [32]. In addition, increased *IL-1B* gene expression was observed in bronchoalveolar lavage cells from tuberculosis patients compared with those from healthy individuals [33]. Mϕ stimulation triggers an oxidative burst and the generation of superoxide anions (O_2^-) and other ROI in Mϕs [34]. SOD2 may play a role in protection of Mϕs against ROI in *M. tuberculosis*-infected Mϕs. None the less, the protective function of M-Mϕs against *M. tuberculosis* in contrast to GM-Mϕs do not seem to be obtained solely by the increased expression of these molecules, because these molecules were also highly expressed in GM-Mϕs.

Three chemokines were included in the 10 most up-regulated genes in M-Mϕ-BCG compared with GM-Mϕ-BCG (Table 3b). In *M. tuberculosis* infections, chemokines contribute to the recruitment of other immune cells, especially of T cells, and the formation and maintenance of granuloma [35]. We also found that GM-Mϕs, which were stimulated with SPP1 and CXCL7, were more bacteriostatic to *M. tuberculosis* than unstimulated GM-Mϕs (Fig. 2). This is the first description that these two chemokines played protective roles against *M. tuberculosis* in humans. After BCG stimulation, the ratio of *SPP1* expression was highest in M-Mϕ compared with GM-Mϕ (Table 3b). SPP1 is a multi-functional protein that is expressed in both alveolar and peritoneal Mϕs [36]. *SPP1* knock-out mice were more susceptible to *M. tuberculosis* with small and immature granuloma formation in their lungs [37]. *M. tuberculosis* infection of primary human alveolar macrophages causes a substantial increase in *SPP1* gene expression [38]. Many investigators recognize SPP1 as a proinflammatory cytokine, which causes cellular adhesion of inflammatory leucocytes. Furthermore, SPP1 promotes chemotaxis and adhesion of human peripheral blood T cells [39] and enhances their IFN-γ production [40]. It is worthy of notice that its expression can be used as a prognostic marker in patients with *M. tuberculosis* infection [41].

CXCL7 is a cleavage product of platelet basic protein with a length of 70 amino acids [42]. In neutrophils, CXCL7 induces an increase of cytosolic calcium concentration, chemotaxis, exocytosis, production of ROI, degranulation and elastase release [42,43]. Although there is a recent report showing that CXCL7 can modulate the synthesis of IL-12 in Mϕs [44], the role of CXCL7 in Mϕs has not been well determined.

In addition, we demonstrated that SPP1 and CXCL7 facilitated the production of superoxide in GM-Mϕs after the stimulation with antibody-opsonized zymosan particles. The high production of ROI in Mϕs following *M. tuberculosis* infections may be compatible with the high expression of SOD2 in microarray results from BCG-stimulated Mϕs, which may play an important role in preventing Mϕ damages induced by ROI. On the other hand, RNI production in GM-Mϕs was not increased after the stimulation with SPP1 or CXCL7 (data not shown). Immunologically activated Mϕs can generate superoxide anion and other ROI [34]. Mice

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