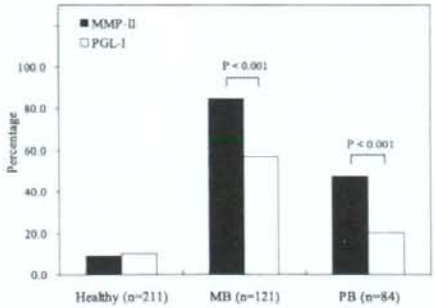

FIG. 2. Comparison of positivity rates of patients as determined by MMP-II and PGL-I ELISA. Black bars show percentages of healthy individuals and patients positive by MMP-II ELISA, and white bars show those for PGL-I ELISA. Statistically significant differences were confirmed by the chi-square test and are indicated as *P* values.

was significantly higher than those for healthy individuals and HHCs.

Monitoring of HHCs. Previous studies suggested the usefulness of PGL-I ELISA in monitoring the effects of leprosy treatment (5, 8, 9, 22). Therefore, we monitored anti-MMP-II antibody titers in patients after treatment and compared them to anti-PGL-I antibody titers. Ninety-two MB and 56 PB patients were monitored. The anti-MMP-II antibody value of approximately 30% of monitored MB patients declined within 1 to 2 years after the start of treatment, in accordance with changes in bacterial index values (data not shown), although approximately 50% of MB patients showed no reduction in ELISA values and 20% of patients showed mild increases in value. Three representative samples of MB patients are shown in Fig. 4. Among PB patients, 18% of the monitored patients had reduced anti-MMP-II antibody titers. On the other hand, anti-PGL-I antibody titers were reduced approximately only 20% in both MB and PB patients during the monitoring period. Therefore, anti-MMP II antibody may reflect the efficacy of treatment similarly to or slightly better than anti-PGL-I antibody in some cases. Furthermore, 9 individuals out of 428

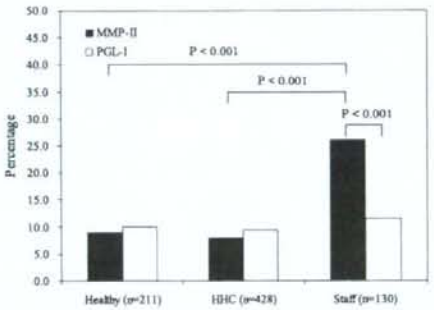

FIG. 3. Positivity rates of HHCs and medical staff members as determined by MMP-II and PGL-I ELISA. Black bars show percentages of HHCs and medical staff members positive by MMP-II ELISA, and white bars show those by PGL-I ELISA. Statistically significant differences were confirmed by the chi-square test and are indicated as *P* values.

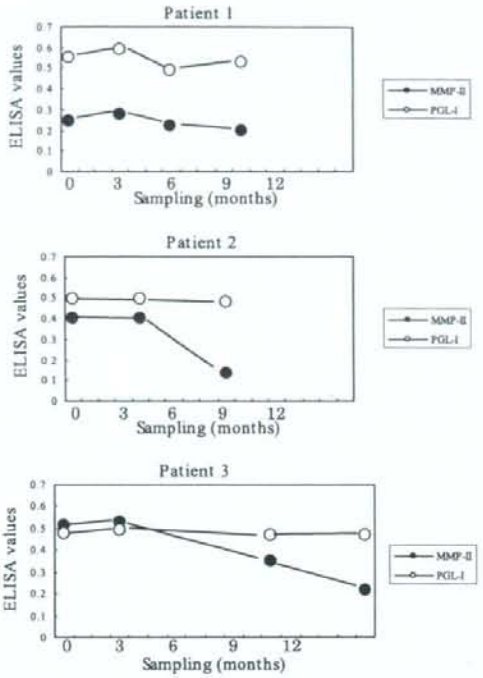

FIG. 4. Monitoring of three MB leprosy patients by MMP-II and PGL-I ELISAs. Three cases of monitored leprosy patients are shown. The closed circles show MMP-II ELISA values, and the open circles show PGL-I ELISA values. Note that the cutoff value for MMP-II is 0.103 and that of PGL-I is 0.452.

HHCs developed leprosy after several years of monitoring. Among the nine cases, two individuals had increasing antibody titers by MMP-II and/or PGL-I ELISA 1 year before manifesting clinical symptoms (data not shown). Patient HHC192 showed a prominent rise in anti-MMP-II antibody values during the asymptomatic period. Both patients developed MB leprosy. The other seven, whose antibody levels did not show an apparent increase during the observation period, developed PB leprosy.

DISCUSSION

Serodiagnosis is the easiest, cheapest, and least invasive diagnostic tool for infectious diseases. Currently, PGL-I is used as a specific antigen for *M. leprae*, but in practice, its sensitivity and specificity are not as high as expected, even though previous studies using stock sera reported that the detection rate for MB patients was more than 80% (1, 3, 4, 7). The present study involving Vietnamese leprosy patients indicated that there is a significant difference between MMP-II ELISA and PGL-I ELISA in detecting both MB and PB leprosy. The positivity rate of anti-MMP-II antibody for MB leprosy was approximately 85%, and that for PB leprosy was 48%; these titers were significantly higher than the titers obtained by PGL-I ELISA (57% and 20%, respectively). The detection rates obtained by

MMP-II ELISA were similar to those for a previous study using stock sera from Japanese leprosy patients (18). However, the positivity rates of anti-PGL-I antibody in the present study were significantly lower than those for the Japanese patients, although the same antigens for both MMP-II and PGL-I were used in the two studies.

There are several possible reasons why the sensitivity of PGL-I ELISA was low in the present study. One reason may be that some healthy Vietnamese individuals have high anti-PGL-I antibody titers. Although we could not conduct further detailed analysis on the subjects, these individuals might be highly exposed to *M. leprae*, and so their B lymphocytes might be repeatedly stimulated with *M. leprae*-derived antigens, including PGL-I. It seems quite difficult to discriminate the healthy individuals from MB or PB leprosy patients by PGL-I ELISA, as shown in Fig. 1. Furthermore, we concluded that a reasonable cutoff point for PGL-I ELISA was an OD_{405} of 0.452, as deduced from Fig. 1 and the ROC values, but this resulted in lower sensitivity. The difference in sensitivity between PGL-I ELISA and MMP-II ELISA may also be due to differences in the biochemical features of the antigens. PGL-I is a glycolipid component, and as such, it might be retained in some infected cells for a long time after the initial exposure (13, 33). This speculation is supported by previous reports showing that healthy individuals residing in areas where leprosy is endemic had high anti-PGL-I antibody titers, and *M. leprae* DNA was recovered by PCR from the nasal swabs of these individuals (31, 32). Also, it has been reported that the usefulness of PGL-I-based tests for early diagnosis is limited, since 7 to 10% of individuals testing positive do not develop the disease (14).

In contrast, MMP-II is a protein antigen and is considered to be one of the immunodominant antigens of *M. leprae* (19). Therefore, in individuals who have been exposed to *M. leprae* but have not developed leprosy, antigen-presenting cells expressing MMP-II might feasibly be eliminated from the body by immune cells such as cytotoxic T lymphocytes and thus lack the ability to produce anti-MMP-II antibodies through antigen-presenting-cell-dependent mechanisms. These speculations seem to be supported by our present observations with sera from patients monitored over time. Anti-MMP-II antibody titers of MB patients declined earlier than PGL-I titers with MDT treatment, indicating the disappearance of MMP-II antigens, while no apparent reduction in PGL-I antigens was observed during the 12 months of observation (Fig. 4). Furthermore, in one case the anti-MMP-II antibody titer increased drastically before manifestation of clinically apparent leprosy (data not shown).

Medical staff members (n = 130) showed a high positivity rate by MMP-II ELISA, compared with healthy individuals or HHCs. These medical staff members were mostly BCG vaccinated, as were the HHCs. Therefore, it seems that BCG vaccination has no effect on anti-MMP-II antibody titers. Although we could not determine a conclusive reason for the high positivity rate, these medical personnel may be repeatedly exposed to *M. leprae* in hospitals. However, we cannot eliminate the possibility that they have produced the antibody in response to exposure to other mycobacteria, since the MMP-II protein is conserved in other pathogenic mycobacterial species, such as *M. tuberculosis* and *M. avium*, though the staff members with high anti-MMP-II antibody titers did not manifest any clinical signs or features indicating infection with other mycobacteria. We tried to perform nested PCR using the *M. leprae*-specific repetitive element for DNA extracted from nasal swabs of some hospital staff members (n = 25). However, because the sampling dates for the serological test and the PCR test were not coordinated, we could not come to a definite conclusion. Nevertheless, we were surprised to find that ≈40% (n = 25) of the nasal swab samples were positive (data not shown). As for tuberculosis, it is said that one-third of the world population is infected with *M. tuberculosis*. The same may be the case with leprosy, although further studies are needed with larger populations, including medical staff members as well as contacts and noncontacts of leprosy.

Taken together, our data indicate that MMP-II ELISA could be useful as a supporting serodiagnostic tool in combination with other clinical diagnostic methods and may also be useful in monitoring disease activity. Furthermore, in this study the correlation between MMP-II and PGL-I was low, with a correlation coefficient among the 205 leprosy patients of only 0.63. If both PGL-I and MMP-II antibodies could be measured simultaneously, the sensitivity of the assay system could be increased. Considering that PGL-I is a sugar antigen (eliciting IgM antibodies) and MMP-II is a protein antigen (eliciting IgG antibodies), assaying for a combination of these antibodies could lead to more-accurate detection of leprosy in the field.

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今月の主題　結核

トピックス

結核ワクチン研究の現状と展望

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臨　床　検　査
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トピックス

結核ワクチン研究の現状と展望

松本壮吉[1)]/小林和夫[2)]

〔KEYWORDS〕 結核，細菌感染症，ワクチン

1. 結核(症)の現状

世界の年間死亡総数の約1/4を占める感染症において，結核は感染症の死因で後天性免疫不全症候群(AIDS)に次ぐ第二位で，全感染症による死亡者数の約1/7を占める．世界保健機関の統計(2008年5月23日現在)によると2005年の結核患者発生数は881.1万人，死亡者数が157.7万人である．AIDS患者における結核死亡を考慮した場合，毎年約200万人が結核によって死亡している．このように現在でも結核は甚大な健康被害を招来している．

結核には菌の感染後即発症する一次結核と，潜伏期を経て発症する二次結核がある(図1)．わが国を含め，結核の低—中蔓延地域における成人肺結核の多くは二次結核である．結核菌は現在人類の1/3(20億人)に潜伏感染しており，既感染者の5～10%が終生の間に二次結核を発症する．ヒト免疫不全ウイルス(HIV)感染は内因性再燃を加速し，HIV-結核菌重複感染者の約10%が毎年結核を発症する．したがって，結核ワクチン開発においては，感染暴露前(pre-exposure vaccine)のみならず，感染暴露後(治療的)ワクチン(post-exposure vaccine)の開発が希求される．

2. 結核ワクチンの歴史とBCG

結核菌は，1882年にRobert Kochによって同定されたグラム陽性桿菌である．当時，Kochは結核菌の培養濾液に予防効果があると信じた．こ

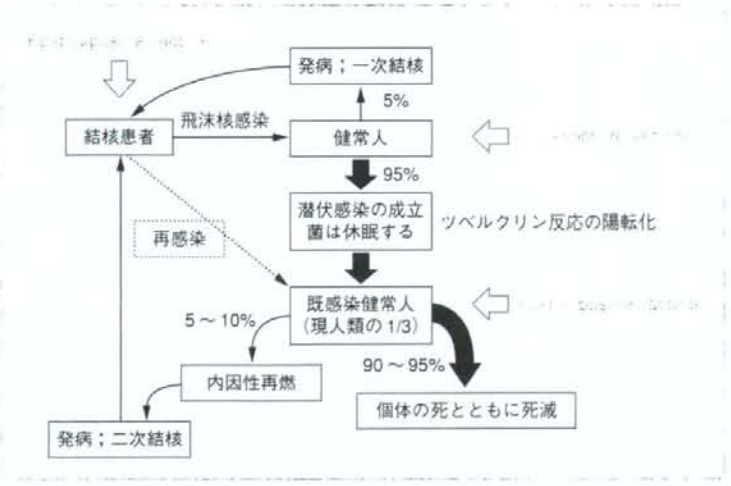

図1　結核菌の伝播と結核の発症
結核患者由来の飛沫が空中にて乾燥し菌を含んだ飛沫核となり，健常人もしくは既感染者(再感染)の肺胞に届いて感染が成立する．この時，感染者の5%未満が一時結核を発症する．残る95%は発症しないが，菌は生体から排除されずに潜伏感染が成立する．既感染者は現人類の1/3にのぼる．既感染者の5～10%が終生の間に結核を発病する(二次結核)．また，HIVの感染は二次結核発症率を顕著に上昇させる．初感染時の感染や発病を抑制するpre-exposure vaccineと既感染者の発症を予防する．もしくは免疫介入療法に用いるpost-exposure vaccineの両方が結核ワクチンに開発において求められる．

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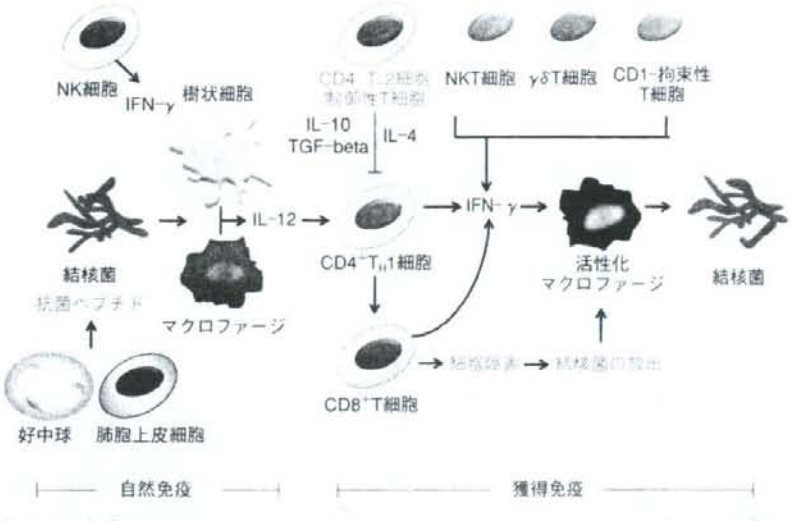

図2　結核菌感染と宿主応答

肺に侵入した結核菌は，肺胞マクロファージに貪食されるか，Ⅱ型肺胞上皮細胞に感染する．感染初期には好中球の浸潤があり，Ⅱ型肺胞上皮細胞とともに抗菌ペプチドによって結核菌を攻撃する．またNK細胞はIFN-γを生産し，細胞性免疫の誘導を促す．マクロファージや樹状細胞が結核菌抗原をIL-12の存在下において提示することで，CD4陽性T細胞はT_H1細胞に分化する．T_H1細胞はIFN-γを生産し，マクロファージを活性化することで結核菌の増殖停止や殺傷を促す．T_H1細胞以外にも，CD8陽性T細胞，NKT細胞，γδT細胞，CD1-拘束性T細胞もIFN-γを生産しマクロファージを活性化する．これを抑制するのが，T_H2細胞や制御性T細胞である．CD8陽性T細胞は細胞傷害性を有するキラー細胞でもあり，活性化マクロファージによる結核菌の再貪食を誘導する．

れが現在，結核菌など抗酸菌感染の診断に用いられているツベルクリンの起源である．

一方，Louis Pasteurらが当時確立したワクチン開発法(すなわち，自然発生的な弱毒病原体の取得)をPasteur研究所のCalmetteとGuérinが実践し，牛型結核菌Nocard株を13年間230代に渡って継代培養を行った結果，弱毒菌株を得た．これが現行の結核ワクチンbacillus Calmette-Guérin(BCG)の原型である．日本で接種されているBCG Tokyo 172は，1924年に志賀潔がPasteur研究所から持ち帰った菌株に由来する[1]．結核に対する予防効果は，BCG接種で得られるのに対し，ツベルクリン接種では得られないことから防御免疫は生菌免疫でのみ獲得されるとの考えが定着する．

現行ワクチンBCGは乳幼児結核(全身播種性結核や髄膜結核)の予防に効果(70～80%)が認められている．しかしながら，成人型肺結核の予防効果は疑問視されている．また，HIV感染者にBCGを含む生ワクチンの接種は有害事象を惹起する可能性があり，原則禁忌である．このような現状は，肺結核に有効，かつ，安全な新規ワクチンの必要性を示唆している

3. 自然免疫と結核

免疫賦活物質であるアジュバントは自然免疫の活性化物質であり，特に成分ワクチンの開発に欠かせない．自然免疫はマクロファージや樹状細胞のパターン認識受容体の活性化を介して活性化され，T細胞への円滑な抗原提示を促すことで獲得免疫の発動と免疫記憶を誘導する(**図2**)．しかしながら，結核菌は樹状細胞の活性化をC型レクチンを介して抑制する機構を有している．また，結核菌菌体成分は自然免疫の賦活化において最も主要なレセプターToll-like receptor(TLR)4をほとんど活性化しない．これらは結核菌の巧妙な寄生戦略の一端を示すものであり，ワクチン開発において憂慮すべき問題である．一方，結核菌の菌体成分は，TLR2やTLR9(それぞれリポ蛋

白質とCpG-DNA)を刺激し，これらの受容体は結核の防御に重要な役割を果たすことが明らかとなっている．

4. 獲得免疫と結核

結核菌は感染後対数的に増殖するが，健常宿主においては獲得免疫(特に，マクロファージとT細胞から構成される細胞性免疫)の発動により増殖は阻止される．この防御免疫の主役を担う細胞がCD4陽性の1型ヘルパーT(T_H1)細胞である(**図2**)．活性化されたT_H1細胞はエフェクターT細胞に分化してinterferon-γ(IFN-γ)[3]を産生し菌の増殖抑制や殺菌を促す．ワクチン効果の主体はこのエフェクターT細胞が病原体の駆逐による抗原消失後，記憶T細胞に分化し長期間の免疫記憶が成立することで形成される．しかしながら，結核菌は潜伏感染して宿主から排除されることがないため，多くのT_H1細胞がエフェクター細胞のまま次第に死滅してしまう．BCGも生体内に持続感染するため記憶T細胞の誘導能に乏しく，この機構が成人接種者における効果の減衰にかかわっている．

T_H1細胞以外にも，免疫記憶を担うCD8陽性細胞傷害性T細胞や結核菌糖脂質を認識するCD1拘束性T細胞も感染防御に重要な役割を果たす．他方，interleukin 4(IL-4)を産生するT_H2細胞や制御性T細胞(Treg)は防御免疫の抑制にかかわる記憶T細胞である．細胞内寄生菌である結核菌に対し，抗体など液性免疫の防御的役割はマイナーとされる．

5. 結核ワクチン開発の現状

成人型肺結核に対する有効で安全な結核ワクチンの成功は未了であるが，①遺伝子組み換えBCG，②組み換え弱毒結核菌，③成分ワクチン，④DNAワクチンやウイルスベクター組み換えワクチンなど，世界的に結核ワクチン研究・開発が進行中である[4]．以下に抜粋して紹介する(**表**)．

1) 組み換えBCG

BCGに特定の防御抗原や免疫賦活分子を発現させる，もしくはBCGが欠失した結核菌抗原を再度入れ戻すことでBCGを改良する試みである．Antigen 85Bなどの抗原や，IL-2, IFN-γなどのサイトカインを発現させたBCGが作成されているが，特に注目すべきはIL-15を組み入れたBCGであろう[5]．IL-15は記憶CD8陽性T細胞の維持にかかわり，防御免疫の持続を可能にするかもしれない．

BCGは，region of deleted 1(RD1)領域を欠いているため，抗原提示細胞内でほとんどの菌体抗原はファゴゾーム内にとどまっている．結果として十分なCD8陽性T細胞を活性化することができない．Kaufmannらは，低pHでファゴゾーム膜を障害するリステリアの毒素をウレアーゼの欠失したBCGに発現させた組み換えBCG, rBCG Δ UreC：Hly＋を作成した．rBCG Δ UreC：Hly＋はCD4陽性細胞とともにCD8T細胞の活性化を促し，BCG親株を超える効果のあることが判明している[6]．

2) 組み換え弱毒結核菌

結核菌の弱毒株を作成して，より病原体そのものに近い抗原で免疫することが効果的なワクチンの作成に繋がるとの考えがある．結核菌そのものを使用するため少なくともゲノム上離れた二種の遺伝子を欠失させ病原性の回帰を阻止している．結核菌H37Ra株の病原性の消失に強くかかわる二成分制御系分子のPhoP[7]やビタミンB5の合成酵素(PanC, PanD)[8]を欠失させた結核菌株の臨床試験が始まっている．

3) 成分ワクチン

成分ワクチンは生ワクチンに比べ安全性に優れ，HIV感染者にも対応可能である．加えて，抗原は投与後しばらくして消失するために，記憶T細胞を誘導しやすい利点がある．一方，免疫原性は生菌ワクチンに劣るため一般的にアジュバントや追加免疫を必要とする．現行のアジュバントの多くが体液性免疫の賦活を念頭に開発されてきたため，細胞性免疫の誘導に優れるアジュバントの開発も必要である．

ワクチン抗原は当初，分泌蛋白質を標的として行われた．これは生菌免疫の効果が，分泌する蛋白質に依存するとの考えによる．防御免疫を誘導する結核菌分泌蛋白質は，Antigen 85B(α抗原)をさきがけとして，Antigen 85 complex, ESAT6, MPT51, MPT64, HBHA, Mtb32などが同定されている．しかしながら，Dnak, Mtb39, HSP65, MDP1[9]など，非分泌性蛋白質にも防御免疫を誘導する抗原が多数同定されている．蛋白質成分ワ

表　現在開発中の主な結核ワクチン

ワクチン	施設・施行者	備考
組み換え BCG		
rBCG-Ag85B-IL15	九州大学・吉開ら	Antigen 85B と IL-15 を BCG より発現.
rBCG30	カリフォルニア大学・Horwitz ら	Antigen 85B を BCG より発現. Phase1 済み.
BCG::RD1	パスツール研究所・Cole ら	結核菌の RD1 領域を BCG に入れ戻したもの.
rBCGΔUreC：Hly+	マックスプランク研究所・Kaufmann ら	本文参照. Phase1 済み.
組み換え結核菌		
M. tuberculosis mc²6030	ニューヨーク大学・Jacobs ら	panCD と RD1 領域を欠失させた結核菌.
M. tuberculosis PhoP	ニューヨーク大学・Jacobs ら	PhoP を欠失させた結核菌.
その他の生菌ワクチン		
組み換えリステリア	浜松医科大学・小出ら	リステリアに Antigen 85A, 85B, MPT51 を発現させたもの.
成分ワクチン		
Mtb72f	Corixa 社・Reed ら	Mtb39 と Mtb32 の融合蛋白質. Phase1 済み.
Hybrid-1	Statens Serum Institutes Andersen ら	Antigen 85B と ESAT6 の融合蛋白質. Phase1 済み.
HyVac-4	Statens Serum Institutes Andersen ら	Antigen 85B と TB10.4 の融合蛋白質.
DNA やウィルスベクターを利用したワクチン		
HSP65DNA	英国国立医学研究所・Lowrie ら	ライ菌由来 *HSP60* 遺伝子を用いた DNA ワクチン.
HVJ-liposome/HSP65 DNA＋IL-12 DNA	近畿中央病院・岡田ら	結核菌由来 HSP65 と *IL-12* 遺伝子をリポソームに封入した DNA ワクチン.
MVA85A	オックスフォード大学・Hill ら	ワクシニアウィルスを用いて Antigen 85A を発現させたもの.
Aeras-402	Aeras 社	アデノウイルスベクターを用いて Antigen 85A, 85B, TB10.4 を発現させたもの.

クチン開発においては，Mtb72f[10]，Hybrid1，HyVac-4 など，複数の抗原をハイブリッドさせることでより強い免疫応答を惹起できる融合蛋白質ワクチンも作成され試験中である．

一方，脂質抗原が CD1 分子拘束性の T 細胞の分化を促すことが判明している[11]．結核菌細胞壁の 40% は脂質であり，結核菌感染においては脂質抗原に対する免疫応答が活発である．脂質抗原は蛋白質に比べ生産効率や操作性に劣ることから，ワクチンへの応用は現在のところ低調であるが，特にアジュバントとしての利用価値は高い．将来のワクチン設計において脂質抗原も加えて検討すべきと考えられる．

4）DNA ワクチンやウイルスベクター組み換えワクチン

抗原そのものを接種するのでなく，蛋白質抗原の遺伝子を発現ベクターやウイルスベクターに導入し生体内で発現させる手法である．これらのワクチンは，これまでヒトでの実績がなく安全性を慎重に検討しなければならないが，内在性抗原として蛋白質を提示するため CD8 陽性細胞傷害性 T 細胞の誘導に優れる．また，DNA 取り扱い技術の発達により簡便かつ安価にワクチンを作成できる．一方，生体内における DNA の分解を防ぐ必要があり，DNA ワクチンをリポソームに封入したり[12]，病原性を失活させたウイルス粒子を用いることで対応している．現在，結核菌の主要防御抗原やサイトカインを発現するワクチンが作成され検討されている（**表**）．

6. 今後の展望

人類の 1/3 に結核菌が潜伏感染している．天然ワクチンの接種者である結核菌既感染者に再感染が生じるように，結核は“二度がかり有り”の慢性疾患である．これまでのワクチンが著効を示してきたのは，天然痘や麻疹に代表されるような“二度がかり無し”の急性疾患のみである．従来

のワクチン開発戦略のみでは結核ワクチンの開発は困難であり，安易な抗原の組み合わせや一時的な免疫応答の惹起のみでは最終的な成功に至らぬことは明白である．加えて，ワクチンの評価は成人の肺結核に効果の乏しい native BCG を実験対照として用いているため，評価系自体にも問題がある．ヒトの一次結核と二次結核，それぞれの病態を表現するモデルを確立し，検討することが重要と考えられる．

一方，"ヒト" に立ち返れば，結核菌に感染しても終生発病を免れるヒトが約 90% である事実は，優れたワクチンの開発が可能であることを示している．結核菌既感染者における "菌の増殖を制御する機構" の解明はワクチン開発に寄与するであろう．"二度がかり有り" の慢性疾患に対して有効・安全なワクチンを作成することは，これまでに人類が成しえていない大きな挑戦である．今後，免疫理論と実践の蓄積により，結核ワクチン開発は成し遂げられるものであろう．

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「臨床検査技師教育　発祥の地」記念碑の設立 その経緯，臨床検査技師教育の歴史と未来

東京文化医学技術専門学校は2006年3月31日閉校し，同年4月1日より東京文化短期大学臨床検査学科に引き継がれた．1952年に臨床検査の重要性を認識して東京文化短期大学に医学技術研究室が設置されたのが日本の臨床検査教育の始まりとされている．1958年に衛生検査技師法，1959年から衛生技師学校(養成所)が設立され，教育が本格化された．

新渡戸文化学園(前東京文化学園：4月より名称変更)は，初代校長であった新渡戸稲造博士の建学の精神である，愛情のある心(heat)と，実践に活用できる柔軟な頭脳(head)，そして医療チームの一員として一生懸命に働く実行力，行動力(hands)(3H精神)を，学園のモットーとして臨床検査の新しい道を開拓してきた．

臨床検査技師教育発祥の学園として，キャンパス内の新渡戸稲造博士銅像の隣に，2008年5月31日，医学技術専門学校・短期大学移行記念式典を機に河合忠博士(国際臨床病理センター所長/自治医科大学名誉教授)揮毫による「臨床検査技師教育　発祥の地」という言葉が記された記念碑を建立した．

移行記念式典には臨床検査教育，教育病院実習および学会関係者の来賓代表として，河合忠博士，矢冨裕東京大学医学部附属病院検査部長，三村邦裕日本臨床検査教育協議会理事長，石井暢昭和大学名誉教授，本間伊佐子元校長が出席された．

医学技術専門学校はこれまで3,000名の有為な人材を医療の場に送り出してきたが，臨床検査技師養成校のリーダーとして，短期大学への移行後も，時代のニーズの即した人材の育成を目指して行きたい．

Potent Antimycobacterial Activity of Mouse Secretory Leukocyte Protease Inhibitor[1]

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Secretory leukocyte protease inhibitor (SLPI) has multiple functions, including inhibition of protease activity, microbial growth, and inflammatory responses. In this study, we demonstrate that mouse SLPI is critically involved in innate host defense against pulmonary mycobacterial infection. During the early phase of respiratory infection with *Mycobacterium bovis* bacillus Calmette-Guérin, SLPI was produced by bronchial and alveolar epithelial cells, as well as alveolar macrophages, and secreted into the alveolar space. Recombinant mouse SLPI effectively inhibited in vitro growth of bacillus Calmette-Guérin and *Mycobacterium tuberculosis* through disruption of the mycobacterial cell wall structure. Each of the two whey acidic protein domains in SLPI was sufficient for inhibiting mycobacterial growth. Cationic residues within the whey acidic protein domains of SLPI were essential for disruption of mycobacterial cell walls. Mice lacking SLPI were highly susceptible to pulmonary infection with *M. tuberculosis*. Thus, mouse SLPI is an essential component of innate host defense against mycobacteria at the respiratory mucosal surface. ***The Journal of Immunology*****, 2008, 180: 4032–4039.**

Mycobacterium tuberculosis is a top killer among bacterial pathogens and is responsible for 2 million deaths annually. The emergence of AIDS and development of multidrug-resistant *M. tuberculosis* have increased the incidence of tuberculosis, and it has now become a serious problem. Therefore, the host defense mechanisms against *M. tuberculosis* have been intensively investigated and important roles of T cell-mediated adaptive immunity are now well established (1, 2). In addition, functional characterization of TLRs has recently indicated the importance of innate immunity in infection with *M. tuberculosis* (3, 4). Macrophages and dendritic cells are the major effectors of TLR-mediated antimycobacterial immune responses, because they produce a variety of proinflammatory cytokines and have the capacity of phagocytosis. However, during *M. tuberculosis* infection, epithelial cells in the respiratory tract as well as alveolar macrophages are the first targets for invasion by *M. tuberculosis*. Therefore, these epithelial cells are expected to play roles in preventing mycobacterial infection by establishing physical barriers and producing proinflammatory and antimicrobial mediators (5).

Secretory leukocyte protease inhibitor (SLPI)[3] is a 12-kDa secreted protein composed of two cysteine-rich whey acidic protein (WAP) domains (also called WAP four-disulfide core (WFDC) domains) (6–8). It was originally identified in seminal fluid and is produced by secretory cells in the genital, respiratory, and lacrimal glands as well as dermal keratinocytes (9–13). SLPI is a potent inhibitor of serine proteases, such as neutrophil elastase and cathepsin G, and has therefore been proposed to protect tissues from protease-mediated damage at sites of inflammation (14, 15). Indeed, SLPI was subsequently shown to mediate wound healing (16, 17). Further studies have revealed that SLPI has additional functions. For example, it possesses an antimicrobial activities against Gram-negative and Gram-positive bacteria, fungi, and viruses, including HIV (18–20). In addition to SLPI, several other serine protease inhibitors containing a single WAP domain, such as Eppin, Elafin, SWAM1, and SWAM2, also possess antimicrobial activities against Gram-negative and Gram-positive bacteria (8, 21, 22). Thus, serine protease inhibitors possessing WAP domains exhibit antimicrobial activities. However, the precise mechanisms by which these serine protease inhibitors exert their antimicrobial activities remain elusive. More recently, SLPI was found to mediate anti-inflammatory responses. Briefly, SLPI is induced in monocytes and macrophages in response to inflammatory stimuli mediated by TLRs (23) and subsequently suppresses TLR-dependent production of inflammatory mediators in macrophages by modulating NF-κB activity (23–25). Consistent with these findings, SLPI-deficient mice are highly sensitive to TLR4 ligand (LPS)-induced endotoxin shock with increased production of IL-6 (26). Thus, SLPI has diverse functions and its precise roles need to be investigated more carefully

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[3] Abbreviations used in this paper: SLPI, secretory leukocyte protease inhibitor; WAP, whey acidic protein; WFDC, WAP four-disulfide core; qPCR, quantitative PCR; BALF, bronchoalveolar lavage fluid; BCG, bacillus Calmette-Guérin; FLICA, 5-(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester; NPN, 1-*N*-phenylnaphthylamine; AEC, alveolar epithelial cell.

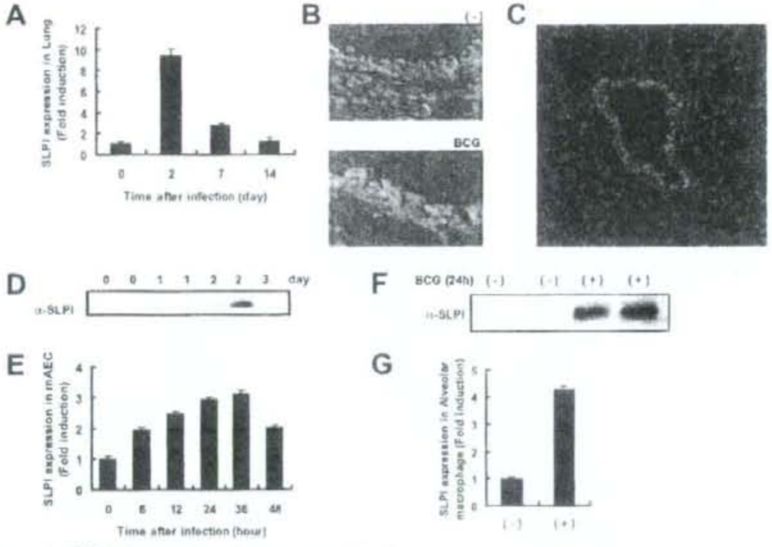

FIGURE 1. Expression of SLPI during mycobacterium infection. *A*, Wild type mice were intratracheally infected with BCG (4 × 10[?] CFU). At the indicated periods, total RNA was extracted from the lungs. SLPI mRNA expression was analyzed by quantitative real time RT-PCR. Data are shown as the relative mRNA levels normalized by the corresponding 18S rRNA level. *B* and *C*, At 2 days after intratracheal infection with BCG, lung tissue sections were stained with an anti-SLPI Ab (red) and 4',6-diamidino-2-phenylindole (blue) and visualized by fluorescence microscopy. *D*, BALF was collected at the indicated periods after BCG infection. Mouse SLPI protein expression was analyzed by Western blotting with an anti-SLPI Ab. Data obtained from two independent mice (0, 1, and 2 days) are indicated. *E*, AEC were incubated with the same number of BCG for the indicated periods. SLPI mRNA expression was analyzed by quantitative real time RT-PCR. Data are shown as the relative mRNA levels normalized by the corresponding 18S rRNA level. *F*, AEC were incubated with the same number of BCG. Culture supernatants were collected before (−) and after 24 h of infection (+) and subjected to Western blot analysis using an anti-SLPI Ab. Data obtained from two independent cell clones are shown. *G*, Alveolar macrophages were collected from uninfected wild type mice, cultured with or without BCG for 48 h, and then analyzed for their SLPI mRNA expression by quantitative real time RT-PCR. The results are presented as the mean ± SD.

In this study, we investigated the roles of murine SLPI in the context of host defenses against mycobacteria, since SLPI expression is greatly induced in macrophages and the lungs during mycobacterial infection (27). Recombinant SLPI inhibited mycobacterial growth at a lower concentration than that required to inhibit bacterial growth. Inhibition of mycobacterial growth was mediated by increased permeability of the mycobacterial membrane. Mutation of cationic residues in the WAP domains of SLPI resulted in loss of its antimycobacterial activity. Furthermore, SLPI-deficient mice were highly susceptible to pulmonary infection with *M. tuberculosis*. These findings demonstrate that SLPI is a potent antimycobacterial molecule.

Materials and Methods

Cells and bacteria

M. tuberculosis strain H37Ra (ATCC 25177; American Type Culture Collection) and *M. tuberculosis* strain H37Rv (28) were grown in Middlebrook 7H9 ADC medium for 2 wk and stored at −80°C until use. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG; Tokyo strain) was purchased from Kyowa Pharmaceuticals. *Salmonella* [illegible] were provided by the Research Institute for Microbial Diseases (Osaka University). For each experiment, the dose was confirmed by plating an aliquot of the injected bacterial suspension. Isolation and immortalization of type II alveolar epithelial cells from the lungs of transgenic H-2K[b]-tsA58 mice were performed as previously described (29) with some modifications.

Immunohistochemistry

Lungs were [illegible] with PBS and [illegible] (Sakura, Tokyo, Japan). [illegible] cold acetone for 10 min, dried, rehydrated with PBS, and blocked with PBS containing 20 mM HEPES, 10% FBS, and 1 μg of Fc blocking mAb (2.4G2; BD Pharmingen). Next, the sections were sequentially incubated with a biotinylated anti-mouse SLPI Ab (R&D Systems) and Alexa Fluor 594-conjugated streptavidin (Molecular Probes). The nuclei were stained with 4',6-diamidino-2-phenylindole (Molecular Probes). After washing with PBS, the sections were analyzed by confocal microscopy (Zeiss).

Western blot analysis

Samples were boiled for 5 min in reducing SDS-PAGE sample buffer and then subjected to SDS-PAGE. The separated proteins were transferred to a 0.45-μm pore polyvinylidene fluoride membrane (Millipore). After blocking with 5% milk, the membrane was incubated with the above described biotinylated anti-mouse SLPI Ab (0.2 μg/ml) and a streptavidin-HRP complex (1/10,000 dilution; R&D Systems). The bound Abs were detected by the SuperSignal reagent (Pierce).

Quantitative real-time RT-PCR

After isolation of total RNA with the TRIzol reagent (Invitrogen Life Technologies), 1 μg of the RNA was treated with RQ1 DNase (Promega) and then reverse transcribed using M-MLV murine leukemia virus reverse transcriptase (Promega) and Random Primers (Toyobo). Gene expression was quantified with an Applied Biosystems PRISM 7000 sequence detection system using TaqMan Universal PCR Master Mix (Applied Biosystems). To determine the relative expression level of each sample, the corresponding 18S rRNA expression level was measured as an internal control. The primer and probe sequences for SLPI were as follows: quantitative PCR forward primer [illegible] 5'-[illegible]-3'; reverse primer [illegible] 5'-[illegible]-3'; [illegible]

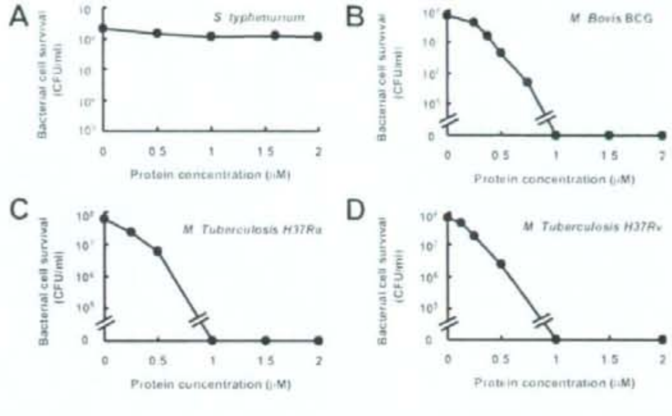

FIGURE 2. Mouse recombinant SLPI inhibits in vitro BCG and *M. tuberculosis* growth. *A*, *S. typhimurium* (5×10^7 CFU/ml) were incubated with SLPI for 2 h and plated on LB agar plates. *B–D*, BCG (*B*), *M. tuberculosis* H37Ra (*C*), or *M. tuberculosis* H37Rv (*D*; 5×10^7 CFU/ml) were incubated with increasing concentrations of recombinant mouse SLPI for 24 h and then plated on 7H10 agar plates.

Bronchoalveolar lavage fluid (BALF)

Mice were intratracheally administered 4×10^7 CFU of BCG suspended in 30 μl of PBS. BALF was collected at the indicated periods. To obtain alveolar macrophages, BALF was centrifuged at 2000 × g for 2 min and the pellet was resuspended in RPMI 1640 containing 4% FBS. The cell count of alveolar macrophages was ~1×10^5 cells/mouse. To eliminate contamination by bacteria, alveolar macrophages were cultured with 50 U/ml penicillin and 50 μg/ml streptomycin for 16 h, washed five times, and infected with 5×10^5 CFU/well of BCG without penicillin and streptomycin.

Preparation of recombinant SLPI protein and variants

PCR-amplified mouse SLPI cDNA fragments were inserted into pGEX-6P-1 (Amersham Biosciences). pGEX-6P-1 containing mouse SLPI cDNA was transformed into *Escherichia coli* Rosetta gami B (DE3). Expression of GST-SLPI fusion proteins was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactoside, and the expressed fusion proteins were purified using glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions. The purified proteins were incubated with PreScission Protease (Amersham Biosciences) at 4°C for 16 h to cleave the GST tag and then purified with glutathione Sepharose 4B.

Antibacterial activity

Mid-log phase *Salmonella typhimurium* were diluted with PBS containing 1% Luria-Bertani (LB) to give ~5×10^7 CFU/ml. A final volume of 250 μl was used to examine the antibacterial activities of proteins. After incubation for 2 h, *S. typhimurium* were plated onto LB agar plates. Colonies were counted (CFU/ml) after overnight incubation at 37°C.

Antimycobacterial activity

M. tuberculosis and BCG were grown in Middlebrook 7H9-ADC medium at 37°C with vigorous agitation. After 7 days of incubation, rapidly growing mycobacteria were harvested by centrifugation and adjusted to 5×10^7 CFU/ml in 7H9-ADC medium. After incubation of the mycobacteria with the indicated concentrations of proteins for 24 h at 37°C, serial 20-fold dilutions were conducted in PBS. Aliquots (50 μl) of the dilutions were plated on Middlebrook 7H10 agar plates and incubated at 37°C for 21–28 days. Colonies were counted (CFU/ml) at intervals until no new colonies appeared.

Protein binding assay

SLPI and BSA were labeled with 5 (and 6)-carboxyfluorescein N-hydroxysuccinimide ester (FLUOS; Roche Diagnostics) as described previously (30). Briefly, 100 μg of SLPI or BSA was mixed with 0.096 mg of FLUOS in 1 ml of PBS for 2 h at room temperature. Nonreacted FLUOS was separated by gel filtration using a Sephadex G-25 column (Amersham Biosciences). The labeled SLPI or BSA was then incubated with BCG, and the OD at 600 nm was adjusted to 0.2. After 30 min of incubation at 37°C, BCG were washed three times with 7H9 medium containing 0.05% Tween 80 [illegible]

Scanning electron microscopy

After culture with or without 1 μM SLPI for the indicated times, BCG cultures were fixed with 5% glutaraldehyde, postfixed with 1% osmium tetroxide, dehydrated with ethyl alcohol, treated with isoamyl acetate to replace the alcohol, dried with liquid CO_2 in a critical point apparatus (HCP-2; Hitachi), and coated with Pt-Pd by ion sputtering (Hitachi) in ion distilled water. The specimens were analyzed using S-4700 scanning electron microscope (Hitachi) operated at 10 kV.

Outer membrane permeabilization assay

The ability of proteins to permeabilize the outer membranes of BCG was investigated using 1-N-phenylnaphthylamine (NPN; Wako Pure Chemical Industries) as described previously (31). Briefly, BCG were suspended in 5 mM HEPES (pH 7.4) containing 10 μM NPN to an OD at 590 nm of 0.15. After incubation at 37°C for 30 min, proteins were added and the fluorescence of NPN was monitored. The excitation wavelength used was 340 nm, and the emission wavelength was 425 nm. The experiment was conducted at 37°C.

Generation of $Slpi^{-/-}$ *mice*

The *Slpi* gene was isolated from genomic DNA extracted from embryonic stem cells (E14.1) by PCR using TaKaRa LA *Taq*. The targeting vector was constructed by replacing a 1.2-kb fragment containing exons 2–4 with a neomycin resistance gene cassette (*neo*) driven by the PGK promoter and inserting a HSV thymidine kinase into the genomic fragment for negative selection. After transfection of the targeting vector into embryonic stem cells, colonies resistant to both G418 and ganciclovir were selected and screened by PCR and Southern blotting. Homologous recombinants were microinjected into blastocysts of C57BL/6 female mice and heterozygous F_1 progenies were intercrossed to obtain $Slpi^{-/-}$ mice.

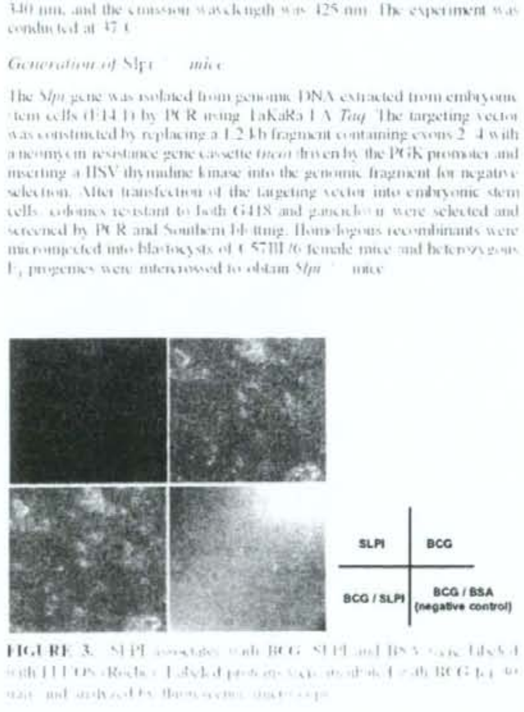

FIGURE 3. SLPI associates with BCG. SLPI and BSA were labeled with FLUOS (Roche). Labeled proteins were incubated with BCG for 30 min and analyzed by fluorescence microscopy.

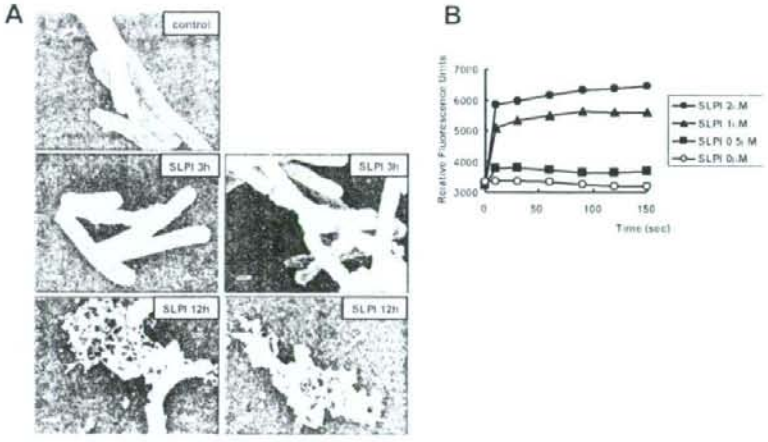

FIGURE 4. SLPI disrupts the BCG cell membrane. *A*, BCG was incubated with or without SLPI for the indicated periods and observed with scanning electron microscopy. *B*, The indicated concentrations of SLPI were added to a BCG suspension containing NPN, and the NPN fluorescence was monitored for the indicated periods. Representative data of three independent experiments are shown.

Slpi$^{-/-}$ mice were backcrossed to C57BL/6 mice for five generations, and *Slpi*$^{-/-}$ and their wild-type littermates from these intercrosses were used for experiments at 6–8 wk of age. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kyushu University.

In vivo infection

For intratracheal infection, 4×10^5 CFU of *M. tuberculosis* suspended in 30 μl of sterile PBS were administered intratracheally. For i.v. infection, 4×10^5 CFU of *M. tuberculosis* suspended in 100 μl of sterile PBS were administered i.v. At 3 wk after infection, homogenates of the lungs and spleen were plated on 7H10 agar plates. For histological examination, 1×10^7 CFU of *M. tuberculosis* suspended in 30 μl of sterile PBS were administered intratracheally. At 5 days after infection, the lungs were fixed in 4% formalin, embedded in paraffin, cut into sections, and stained with H&E.

Results

SLPI expression in the lungs of BCG-infected mice

To assess the roles of SLPI in mycobacterial infection, we first analyzed SLPI expression in the lungs of mice intratracheally infected with *M. bovis* BCG. Total RNA was extracted from the lungs after 2, 7, and 14 days of infection and analyzed for SLPI mRNA expression by real-time qPCR (Fig. 1*A*). Expression of SLPI mRNA was increased by ~9-fold after 2 days of infection, but decreased thereafter. Next, we analyzed pulmonary cell types expressing SLPI by immunohistochemical analysis (Fig. 1, *B* and *C*). SLPI was detected in bronchial epithelial cells before BCG infection (Fig. 1*B*, *upper micrograph*). After 2 days of BCG infection, increased amounts of SLPI expression were observed, and mainly localized at the apical side of bronchial epithelial cells (Fig. 1*B*, *lower micrograph*). This prompted us to investigate whether SLPI was secreted into the alveolar space after BCG infection. Accordingly, BALF was collected from BCG-infected mice and analyzed for SLPI protein expression by Western blotting (Fig. 1*D*). SLPI was not detected in BALF from uninfected mice. After 2 days of BCG infection, SLPI was abundantly detected in BALF from infected mice, indicating that SLPI was secreted into the alveolar space during the early phase of mycobacterial infection. In addition to bronchial epithelial cells, SLPI was expressed in cells of the alveolar area (Fig. 1*C*). Therefore, we isolated type II alveolar epithelial cells (AEC) and alveolar macrophages and analyzed their SLPI expression levels after BCG infection. Since AEC are difficult to culture in vitro, we took advantage of transgenic mice harboring a temperature-sensitive mutation of the SV40 large T-antigen gene under the control of an IFN-γ-inducible H-2K^b promoter element (32, 33). Using these mice, we successfully established AEC lines expressing surfactant protein C (data not shown).

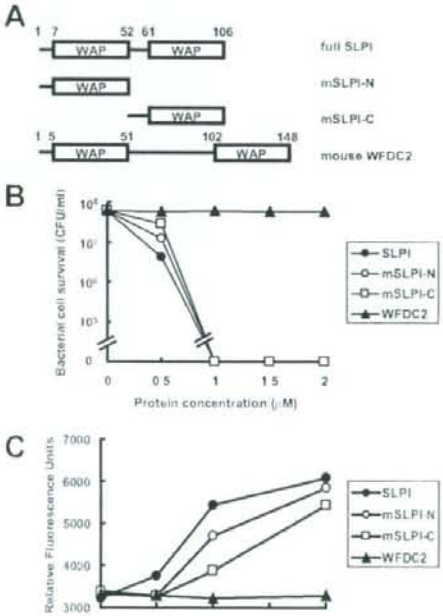

FIGURE 5. A single WAP domain in SLPI is sufficient to inhibit BCG growth. *A*, The deletion mutant constructs mSLPI-N and mSLPI-C lack the C-terminal and N-terminal portions, respectively. White boxes denote WAP domains. *B*, BCG (5×10^5 CFU/ml) was incubated with increasing concentrations of the deletion mutants (mSLPI-N and mSLPI-C) or WFDC2 for 24 h and then plated on 7H10 agar plates. *C*, The indicated concentrations of the deletion mutants (mSLPI-N and mSLPI-C) or WFDC2 were added to BCG suspensions containing NPN. The peak of NPN fluorescence within 150 s was plotted. Representative data of three independent experiments are shown.

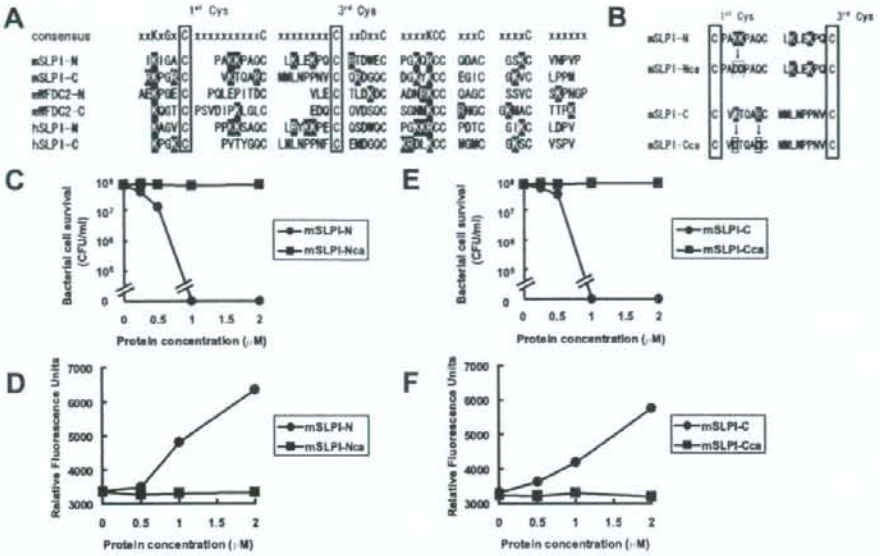

FIGURE 6. Cationic amino acids are responsible for the antimycobacterial activity of SLPI. *A*, Comparison of the WAP domain of SLPI with the WAP domains of other proteins. The consensus amino acid sequence of the WAP domain is shown at the *top* of the protein sequences. Black- and gray-boxed amino acids indicate cationic and anionic amino acids, respectively. Two conserved cysteine residues (first cysteine and third cysteine) are boxed. *B*, Amino acid sequences of the mSLPI-N (mSLPI-Nca) and mSLPI-C (mSLPI-Cca) mutants. *C* and *E*, BCG (5×10^7 CFU/ml) was incubated with increasing concentrations of mSLPI-Nca (*C*) and mSLPI-Cca (*E*) for 24 h and then plated on 7H10 agar plates. *D* and *F*, The indicated concentrations of mSLPI-Nca (*D*) and mSLPI-Cca (*F*) were added to BCG cultures containing NPN. The peak of NPN fluorescence within 150 s was plotted.

AEC were infected with BCG and analyzed for SLPI mRNA expression (Fig. 1*E*). SLPI mRNA expression was gradually induced after BCG infection and peaked after 36 h of infection. AEC have the ability to secrete several effector molecules into the alveolar space. Therefore, we analyzed the SLPI protein levels in culture supernatants from BCG-infected AEC by Western blotting (Fig. 1*F*). SLPI protein was not detected in supernatants from uninfected AEC, but was clearly detected in supernatants after 24 h of BCG infection. Next, isolated alveolar macrophages were infected with BCG and analyzed for SLPI mRNA expression (Fig. 1*G*). BCG infection resulted in an increase in SLPI mRNA expression. Taken together, mycobacterial infection induces the production and secretion of SLPI into the alveolar space by bronchial and type II alveolar epithelial cells as well as alveolar macrophages in the lung.

SLPI-mediated inhibition of mycobacterial growth

Several previous reports have described antimicrobial activities of SLPI against Gram-positive bacteria, Gram-negative bacteria, HIV, and fungi (18–20). However, SLPI needs to be present at high concentrations (>10 μM) for effective inhibition of microbial growth, particularly *S. typhimurium* and *E. coli* (18, 34). Indeed, addition of 2 μM recombinant mouse SLPI only moderately decreased the growth of *S. typhimurium* (Fig. 2*A*). In sharp contrast to the mild inhibition of *S. typhimurium* growth, addition of lower concentrations of mouse SLPI to BCG cultures dramatically reduced the number of CFU (Fig. 2*B*). Growth of BCG was almost completely inhibited by the addition of 1 μM SLPI. A similar inhibitory effect was observed on the growth of *M. tuberculosis* H37Ra and H37Rv (Fig. 2, *C* and *D*). These findings indicate that SLPI has a more potent antimicrobial activity against mycobacteria than against *S. typhimurium*.

Disruption of the BCG cell wall structure by SLPI

Next, we investigated the mechanism of the antimycobacterial activity of SLPI. First, fluorescence-labeled SLPI was incubated with BCG and analyzed by confocal laser microscopy (Fig. 3). BCG and labeled SLPI were colocalized, suggesting that SLPI becomes associated with BCG. We then examined the morphological effects of SLPI on BCG. BCG was incubated with or without SLPI and analyzed by scanning electron microscopy (Fig. 4*A*). BCG exposed to SLPI for 3 h showed pronounced surface blebbing. After 12 h of incubation, many of BCG were collapsed and few live BCG had rough and irregular membrane surfaces. Next, BCG was subjected to an outer membrane permeabilization assay using a fluorescent dye that is weakly fluorescent in aqueous environments but becomes strongly fluorescent in the hydrophobic environment within the cell membrane (Fig. 4*B*). Addition of SLPI caused rapid increases in fluorescence in a dose-dependent manner. These results suggest that SLPI directly associates with mycobacteria, and disrupts the cell wall structure.

Critical role of cationic amino acids in SLPI in its antimycobacterial activity

We next investigated the critical domain involved in the antimycobacterial activity of SLPI. SLPI has two WAP domains (Fig. 5*A*). Several serine protease inhibitors possessing a single WAP domain, such as Eppin, Elafin, SWAM1, and SWAM2, have antimicrobial activities against bacteria such as *E. coli* and *Staphylococcus aureus* (8, 21, 22). To investigate whether each of the WAP domains of mouse SLPI is sufficient to exert antimycobacterial activity, two deletion mutants of SLPI, mSLPI-N and

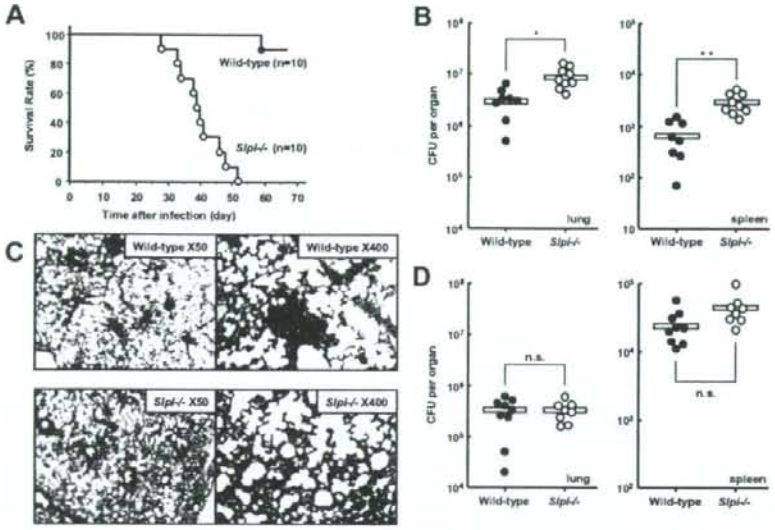

FIGURE 7. *Slpi*$^{-/-}$ mice are highly susceptible to *M. tuberculosis* infection. *A*, *M. tuberculosis* (4×10^5 CFU) were intratracheally infected into wild-type and *Slpi*$^{-/-}$ mice and their survival was monitored. *B*, *M. tuberculosis* (4×10^5 CFU) were intratracheally infected into wild-type and *Slpi*$^{-/-}$ mice. At 3 wk after infection, homogenates of the lungs and spleen were plated on 7H10 agar plates and the CFU titers were counted. Symbols represent individual mice and bars represent the mean of CFU numbers. Statistical analyses were performed using Student's *t* test: *, $p < 0.005$ and **, $p < 0.0005$, significant difference between wild-type and *Slpi*$^{-/-}$ mice. *C*, H&E staining of representative lung tissues from wild-type and *Slpi*$^{-/-}$ mice on day 5 after intratracheal infection with *M. tuberculosis*. *D*, *M. tuberculosis* (4×10^5 CFU) were i.v. infected into wild-type and *Slpi*$^{-/-}$ mice. At 3 wk after infection, homogenates of the lungs and spleen were plated on 7H10 agar plates, and the CFU titers were counted. Symbols represent individual mice and bars represent the mean of CFU numbers. Statistical analyses were performed using Student's *t* test: n.s., Not significant.

mSLPI-C, were generated (Fig. 5*A*). mSLPI-N contained the N-terminal WAP domain, while mSLPI-C contained the C-terminal WAP domain. Both mSLPI-N and mSLPI-C inhibited BCG growth, although their efficiencies were slightly decreased compared with that of full-length SLPI (Fig. 5*B*). Similarly, mSLPI-N and mSLPI-C both induced permeabilization of the outer membrane of BCG with slightly lower efficacies (Fig. 5*C*). These results imply that each WAP domain of mouse SLPI exhibits antimycobacterial activity by disrupting the mycobacterial cell wall structure. WFDC2 is a secreted protein possessing two WAP domains (Fig. 5*A*) (35). However, recombinant mouse WFDC2 had no effect on mycobacterial growth and did not induce permeabilization of the BCG cell membrane, indicating that not all WAP domain-containing proteins have antimicrobial activities (Fig. 5, *B* and *C*). In addition, the N-terminal, but not the C-terminal, WAP domain of human SLPI has been shown to mediate its antimicrobial activities against *E. coli* and *S. aureus* (18). Therefore, we compared the amino acid sequences of the WAP domains of mouse and human SLPI as well as mouse WFDC2 (Fig. 6*A*). The C-terminal regions were conserved among all of the WAP domains. However, the sequences between the first and third cysteine residues were less conserved. In particular, when we examined the sequences between the first and second cysteine residues, we noted that the WAP domains possessing antimycobacterial activities (mSLPI-N, mSLPI-C, and hSLPI-N) contained two or more cationic amino acids, whereas the WAP domains with no antimycobacterial activities (mWFDC2-N, mWFDC2-C, and hSLPI-C) had one or zero cationic acids and instead contained anionic amino acids. Therefore, we produced mSLPI-N (mSLPI-N(a)) and mSLPI-C (mSLPI-C(a)) mutants in which the two cationic amino acids were changed to the anionic amino acid aspartic acid (Fig. 6*B*). Neither mSLPI-N(a) nor mSLPI-C(a) was able to inhibit BCG growth or permeabilize the cell membrane (Fig. 6, *C–F*). These results suggest that the cationic acids of mouse SLPI are responsible for its potent antimycobacterial activities.

High susceptibility of SLPI-deficient mice to M. tuberculosis *infection*

In the next experiment, we assessed the physiological roles of SLPI during mycobacterial infection by generating mice lacking SLPI (*Slpi*$^{-/-}$ mice) via gene targeting (data not shown). First, wild-type and *Slpi*$^{-/-}$ mice were intratracheally infected with *M. tuberculosis* H37Ra, and monitored for their survival (Fig. 7*A*). All *Slpi*$^{-/-}$ mice died within 8 wk of infection at a dose that almost all wild-type mice survived for >9 wk. Next, we counted CFU numbers in the lungs and spleen after 3 wk of infection (Fig. 7*B*). The CFU titers of *M. tuberculosis* in both tissues were higher for *Slpi*$^{-/-}$ mice than that for wild-type mice. The histopathological changes in the lungs after 5 days of *M. tuberculosis* infection were also analyzed (Fig. 7*C*). In wild-type mice, the formation of several small granulomas was observed. In contrast, granulomatous changes were induced to a lesser extent in *Slpi*$^{-/-}$ mice and rather diffuse cell infiltration was observed instead. Next, mice were i.v. infected with *M. tuberculosis*, and the CFU numbers in the lungs and spleen were counted after 3 wk of infection (Fig. 7*D*). The CFU titers were not as dramatically increased in both tissues of *Slpi*$^{-/-}$ mice compared with the corresponding titers in the tissues of wild-type mice, indicating that *Slpi*$^{-/-}$ mice are not highly susceptible to i.v. *M. tuberculosis* infection. Taken together, these findings indicate that *Slpi*$^{-/-}$ mice are highly vulnerable to *M. tuberculosis* infection via the respiratory route.

Discussion

In the present study, we analyzed the roles of mouse SLPI in host defense against mycobacteria. During the early phase of respiratory mycobacterial infection, SLPI was produced and secreted into the alveolar space by bronchial and type II alveolar epithelial cells as well as alveolar macrophages. Recombinant mouse SLPI inhibited the growth of mycobacteria more effectively than it inhibited the growth of Gram-negative bacteria. The SLPI-mediated inhibition of mycobacterial growth was attributable to disruption of the mycobacterial cell wall structure. Furthermore, *Slpi*$^{-/-}$ mice were highly susceptible to pulmonary *M. tuberculosis* infection, highlighting a mandatory role for mouse SLPI in the host defense against *M. tuberculosis* infection. Thus, mouse SLPI is a critical antimycobacterial molecule that acts during the early phase of mycobacterial infection at the respiratory mucosal surface.

Similar structural changes to those observed in SLPI-treated mycobacterial cell walls were induced in several bacteria and *M. tuberculosis* treated with the antimicrobial peptides defensins, which permeabilize microbial membranes (36, 37). We further identified the critical elements for the potent antimycobacterial activity of mouse SLPI. It has been proposed that defensins containing positively charged amino acid residues associate with microorganisms by targeting the surface-exposed negatively charged phospholipid head groups in the microbial membrane (37). Indeed, mutations that change arginine to aspartic acid can attenuate the bactericidal activity of the α-defensin cryptdin-4 (38). Therefore, we supposed that SLPI, which has similar effects on mycobacterial membranes to defensins, also associates with negatively charged mycobacterial membranes through its positively charged amino acid residues. Consistent with this hypothesis, the sequences between the first and second conserved cysteine residues of the WAP domains are not conserved. Moreover, there are several positively charged amino acids (lysine and arginine) in these regions of the WAP domains that possess antimicrobial activities, whereas the regions without any antimicrobial activities contain one or zero positively charged amino acids. Furthermore, structural studies have revealed that the region between the first and second conserved cysteine residues is exposed on the outside of the molecule, thereby enabling this region to associate with microbial membranes (39, 40). Indeed, mutations of the cationic amino acid residues within this region resulted in elimination of the antimycobacterial activity. Thus, mouse SLPI exhibits antimycobacterial activity in quite a similar manner to that of defensins.

In comparison to SLPI, higher concentrations of other serine protease inhibitors containing a WAP domain are required to inhibit microbial growth (8, 21, 22). Recombinant human SLPI is less effective at inhibiting the growth of mycobacteria and *S. typhimurium* (our unpublished data). These differential properties may be attributable to structural differences in the WAP domains, which mediate the antimicrobial activity. SLPI has two WAP domains, whereas other serine protease inhibitors, such as Eppin, Elafin, and SWAMs, have only a single WAP domain. In the case of human SLPI, only the N-terminal WAP domain exhibits antimicrobial activity (18). In addition, only the N-terminal WAP domain of human SLPI contains critical cationic acid residues. The presence of two WAP domains possessing antimicrobial activity may be responsible for the high potency of mouse SLPI for mycobacterial growth inhibition.

Mouse SLPI inhibited mycobacterial growth at profoundly lower concentrations than those required to inhibit the growth of *S. typhimurium* or other microorganisms (18–20). It remains unclear how SLPI becomes more specifically targeted toward mycobacteria. Differential antimicrobial properties against distinct microorganisms have not been reported in the case of defensins. Therefore, SLPI, which has multifunctional properties, may have an unknown strategy for specifically recognizing mycobacteria.

The in vitro findings demonstrating that mouse SLPI inhibits mycobacterial growth were further strengthened by in vivo studies using *Slpi*$^{-/-}$ mice. *Slpi*$^{-/-}$ mice were highly susceptible to pulmonary *M. tuberculosis* infection, but not to i.v. infection. In accordance with this finding, SLPI protein was abundantly detected in the alveolar space after pulmonary BCG infection, but was not detected in sera from mice after i.v. BCG infection (our unpublished data). Therefore, high concentrations of SLPI are supposed to be secreted into the alveolar space during the early phase of respiratory infection with *M. tuberculosis*, thereby promptly killing the mycobacteria before they can invade the lung tissues through the epithelial barrier. Given that mouse SLPI has potent antimycobacterial activities, it would be a good candidate for treatment during the acute phase of *M. tuberculosis* infection and may even be able to be used for the treatment of patients with multidrug-resistant *M. tuberculosis*.

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Disclosures

The authors have no financial conflict of interest.

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Lipocalin 2-Dependent Inhibition of Mycobacterial Growth in Alveolar Epithelium[1]

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Mycobacterium tuberculosis invades alveolar epithelial cells as well as macrophages. However, the role of alveolar epithelial cells in the host defense against *M. tuberculosis* remains unknown. In this study, we report that lipocalin 2 (Lcn2)-dependent inhibition of mycobacterial growth within epithelial cells is required for anti-mycobacterial innate immune responses. Lcn2 is secreted into the alveolar space by alveolar macrophages and epithelial cells during the early phase of respiratory mycobacterial infection. Lcn2 inhibits the in vitro growth of mycobacteria through sequestration of iron uptake. Lcn2-deficient mice are highly susceptible to intratracheal infection with *M. tuberculosis*. Histological analyses at the early phase of mycobacterial infection in Lcn2-deficient mice reveal increased numbers of mycobacteria in epithelial cell layers, but not in macrophages, in the lungs. Increased intracellular mycobacterial growth is observed in alveolar epithelial cells, but not in alveolar macrophages, from Lcn2-deficient mice. The inhibitory action of Lcn2 is blocked by the addition of endocytosis inhibitors, suggesting that internalization of Lcn2 into the epithelial cells is a prerequisite for the inhibition of intracellular mycobacterial growth. Taken together, these findings highlight a pivotal role for alveolar epithelial cells during mycobacterial infection, in which Lcn2 mediates anti-mycobacterial innate immune responses within the epithelial cells. *The Journal of Immunology*, 2008, 181: 8521–8527.

Tuberculosis is a worldwide disease caused by infection with *Mycobacterium tuberculosis*. Therefore, the host defense mechanisms against *M. tuberculosis* have been intensively investigated, and important roles of T cell-mediated adaptive immunity have been well established (1, 2). In addition, functional characterization of TLRs has recently indicated the importance of innate immunity in the host responses to infection with *M. tuberculosis* (3, 4). In the TLR-mediated anti-mycobacterial immune responses, macrophages and dendritic cells are major effectors that engulf pathogens and produce a variety of proinflammatory mediators. In respiratory mycobacterial infection, alveolar macrophages are the major targets of invasion. However, several evidences indicate that mycobacteria also interact with epithelial cells in the respiratory tract and invade these cells (5–9). Accordingly, epithelial cells in the lungs are expected to play a role during mycobacterial infection by producing antimicrobial mediators (10).

Lipocalin 2 (Lcn2),[3] also known as neutrophil gelatinase-associated lipocalin, siderocalin, 24p3, or uterocalin, a member of the lipocalin family of proteins that bind to small hydrophobic molecules, is produced by epithelial cells and macrophages (11–16). Lcn2 has been shown to mediate several biological processes, including mammary gland involution, induction of apoptosis, and delivery of iron (12, 17–19). In addition, structural studies have demonstrated that Lcn2 binds to enterobactin-type bacterial siderophores, which facilitate iron uptake by bacteria (16). Subsequent studies revealed that Lcn2 also binds to other types of siderophores, such as carboxy-mycobactin (produced by mycobacteria) and bacillibactin (produced by *Bacillus anthracis*) (20, 21). Lcn2 has been shown to interfere with siderophore-mediated iron uptake in *Escherichia coli* (16). Accordingly, mice deficient in Lcn2 are highly susceptible to infection with *E. coli* (22, 23). Thus, Lcn2 mediates the host defense against *E. coli* infection through sequestration of iron, which is essential for the growth and activity of nearly all bacteria (24).

Mycobacteria replicate within cells, especially in the phagosome of macrophages (25), where iron is limited. Outside host cells, free iron is also limited, because almost all iron ions exist as complexes with host proteins with high affinity for iron, such as transferrin and lactoferrin. To overcome the iron deficiency within the host, some species of mycobacteria, such as *M. tuberculosis* and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), synthesize two type of siderophores, called mycobactin and carboxy-mycobactin (also called exochelin) (26, 27). Mycobactin is hydrophobic, whereas carboxy-mycobactin is hydrophilic. These mycobactins have been shown to remove iron from host iron-binding proteins, such as transferrin and lactoferrin (28). In addition, *M. tuberculosis*

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[3] Abbreviations used in this paper: Lcn2, lipocalin 2; BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; BALF, bronchoalveolar lavage fluid; rLcn2, recombinant Lcn2; SP-C, pro-surfactant protein C; DFO, deferoxamine; AEC, alveolar epithelial cell; CPZ, chlorpromazine.

with mutations in the *mbtB* gene, which lack carboxy-mycobactin and mycobactin, exhibit impaired replication in low-iron medium and within macrophages (27). The mechanisms for the mycobactin-mediated iron acquisition within the phagosome of macrophages have recently been elucidated (29). Because pulmonary epithelial cells are also invaded by mycobacteria, host defense mechanisms that inhibit mycobacterial replication within these cells are expected to exist, however they currently remain unclear.

In the present study, we analyzed the role of Lcn2 in mycobacterial infection. Lcn2, which inhibits mycobacterial growth, was rapidly produced from alveolar macrophages and epithelial cells after mycobacterial infection. Furthermore, analyses using Lcn2-deficient mice revealed a pivotal role of alveolar epithelial cells in mycobacterial infection.

Materials and Methods

Mice

Lcn2$^{-/-}$ and *H-2K*^{b}*-tsA58* transgenic mice have been generated (22, 30) and backcrossed to C57BL/6 for six generations. *Lcn2*$^{-/-}$ and wild-type littermates from intercrosses of *Lcn2*$^{+/-}$ mice were used for experiments at 6–8 wk of age. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kyushu University and Osaka University.

Mycobacteria

M. bovis BCG (Tokyo strain) was purchased from Kyowa Pharmaceuticals. *M. tuberculosis* strains H37Ra (ATCC25177) and H37Rv (ATCC358121) were grown in Middlebrook 7H9-ADC medium for 2 wk and stored at −80°C until use. GFP-expressing BCG, which was generated previously (5), was used for the experiment.

Quantitative real-time RT-PCR

Total RNA was isolated with the TRIzol reagent (Invitrogen), and reverse transcribed using M-MLV reverse transcriptase (Promega) and random primers (Toyobo) after treatment with RQ1 DNaseI (Promega). Quantitative real-time PCR was performed in ABI7300 (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). All data are shown as the relative mRNA levels normalized by the corresponding 18S rRNA level. The primers for 18S rRNA and Lcn2 were purchased from Assays on Demand (Applied Biosystems).

Preparation of alveolar macrophages

Bronchoalveolar lavage fluid (BALF) was collected from uninfected mice. To eliminate contamination by bacteria, the cells were cultured with 50 U/ml penicillin and 50 μg/ml streptomycin for 16 h, and then washed five times to remove nonadherent cells. The resultant adherent cells were used for experiments as alveolar macrophages, because >95% of the adherent cells were CD11b-positive.

Preparation of recombinant Lcn2 (rLcn2) protein

A mouse Lcn2 cDNA fragment was inserted into pGEX6P-2 (GE Healthcare) and transformed into *E. coli* BL21. The expressed GST-Lcn2 fusion proteins were purified using glutathione-Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. The purified proteins were incubated with PreScission Protease (GE Healthcare) to cleave the GST tag, and then purified with Glutathione-Sepharose 4B.

Immunohistochemistry

Lungs were fixed with 4% PFA and frozen in Tissue-Tec OCT compound (Sakura). The sections were incubated with anti-mouse Lcn2 Ab (R&D Systems), anti-pro-surfactant protein C (SP-C) Ab (Chemicon), anti-CD11b Ab (BD Biosciences), or anti-pan cytokeratin Ab (Sigma-Aldrich). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Alveolar epithelial cells were infected with GFP-expressing BCG for 16 h, washed, and incubated with Dextran Conjugates (Cascade Blue; Molecular Probes) and Alexa Fluor 594-labeled rLcn2 for 6 h. rLcn2 was labeled using an Alexa Fluor 594 Protein Labeling Kit (Molecular Probes). The cells were fixed with 4% PFA and analyzed using a confocal microscopy (LSM 510; Carl Zeiss).

Western blot assay

BALF was collected from BCG-infected mice by catheterization techniques into 500 μl of PBS. To normalize BALF samples, we injected the same volume of PBS (500 μl), recovered equal volume, and used them for Western blot analysis. After removal of precipitates, the samples were separated on SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were incubated with anti-mouse Lcn2 Ab. Bound Ab was detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

In vitro mycobacterial growth assay

Mycobacteria were incubated in Middlebrook 7H9-ADC medium with the indicated concentrations of rLcn2 protein for 20 days at 37°C, and were plated on Middlebrook 7H10-OADC agar plates and incubated at 37°C for 30 days. In some experiments, BCG was incubated with the indicated concentrations of deferoxamine mesylate (DFO; Calbiochem), $FeCl_3$ or mycobactin (Kyoritsu Seiyaku) on 7H10-OADC agar plates.

In vivo infection of mycobacteria

Mice were intratracheally infected with *M. tuberculosis* H37Rv (1×10^6 CFU). At 6 wk after infection, homogenates of the lungs and livers were plated on 7H10-OADC agar plates. For histological analyses, the lungs were fixed with 4% PFA at 20 or 5 days after infection, embedded in paraffin, cut into sections, and stained with H&E or by the Ziehl-Neelsen method, respectively.

Establishment of alveolar epithelial cell lines

To establish alveolar epithelial cell lines (AECs) from wild-type and *Lcn2*$^{-/-}$ mice, the mice were crossed with *H-2K*^{b}*-tsA58* transgenic mice, and used for experiments at 4 wk of age. Mouse pulmonary type II AECs were established as previously described (32). The cells were incubated at 33°C and passaged over ten times. The cells were then stained with anti-SP-C Ab to confirm that they were type II alveolar epithelial cells.

In vitro infection of mycobacteria

Wild-type or *Lcn2*$^{-/-}$ derived AECs or alveolar macrophages were incubated with BCG for the indicated periods. To eliminate extracellular BCG, the cells were cultured with 50 μg/ml streptomycin for 1 h, washed three times, and harvested. Lysates of the cells were plated on 7H10-OADC agar plates.

Detection of intracellular growth of mycobacteria

Wild-type and *Lcn2*$^{-/-}$-derived AECs were seeded onto 96-well plates, and infected with BCG for 6 h. To eliminate extracellular BCG, the AECs were cultured with 50 μg/ml streptomycin for 1 h, vigorously washed three times. The cells were pulsed with 37 kBq of [^{3}H]uracil and cultured for 48 h. The cells were harvested on glass fiber filters and the incorporated [^{3}H]uracil was measured using a liquid scintillation counter (Wallac). In some experiments, cytochalasin B (Sigma-Aldrich) or chlorpromazine (CPZ; Calbiochem) was added to the wells at 30 min before the [^{3}H]uracil pulse or rLcn2 addition.

Statistical analysis

Differences between control and experimental groups were evaluated using Student's *t* test or ANOVA plus postboc testing. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Expression of lipocalin 2 in BCG-infected lungs

To assess the role of Lcn2 in mycobacterial infection, we first analyzed the expression of Lcn2 in the lungs of C57BL/6 mice intratracheally infected with BCG. Total RNA was extracted from the lungs at 2, 7, and 14 days after infection, and analyzed for Lcn2 mRNA expression by real-time quantitative PCR (Fig. 1*A*). Expression of Lcn2 mRNA was markedly increased at 2 days after infection and decreased thereafter. Because Lcn2 mRNA expression was shown to be induced in macrophages stimulated with TLR ligands (22), we analyzed whether alveolar macrophages expressed Lcn2 mRNA (Fig. 1*B*). Alveolar macrophages were isolated, infected with BCG, and analyzed for Lcn2 mRNA expression at 2 days