小学校低学年児童に発症した喉頭結核を合併した塗沫陽性重症肺結核の一例
A case of progressive pulmonary tuberculosis with laryngeal tuberculosis in a seven-years-old girl

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【要旨】
7 歳女児に発症した塗沫陽性重症肺結核症の一例を経験した．両側全肺野に経気道性及び血行性両機序により散布された広汎な病巣を認め、喉頭結核の合併も確認された．同居祖父結核発症時の接触者検診の不備、有症状受診例に対する診断の遅れ（“Doctor's delay”）、問診を中心とした学校結核健診の限界などの要因が重なりあって、本症例の重症化に繋がったものと考えた．

【はじめに】
小児結核症例は近年減少する傾向にあるが、未だ年間 120 例前後の新規発症例が報告されており、更なる小児結核対策の充実が望まれる．集団生活を送る小・中学生においても塗沫陽性肺結核症例は少数例ながら報告されており、その診断の遅れは公衆衛生学上も大きな影響が懸念される．今回、我々は両側全肺野に拡がる広汎な病巣を形成した塗沫陽性重症肺結核症の 7 歳女児例を経験した．診断に至るまで長期間にわたって嗄声、咳嗽が持続し喉頭結核の合併も確認された．学童期、特に小学校低学年児童に塗沫陽性肺結核を経験することは極めて稀であり、特に本例のような重症結核症例の報告はこれまでに見られない．症例を報告すると共に重症化に至った本症例の問題点について検討する．

【症例】
［症例］7 歳女児（小学校 1 年生）
主訴：発熱、遷延する湿性咳嗽及び嗄声
予防接種歴：生後 4 ヶ月時、ツベルクリン反応陰性確認後に BCG ワクチン接種済み（接種後副反応は認めず）．三種混合、ポリオ、麻疹、風疹、日本脳炎ワクチンもそれぞれ接種済み．
既往歴及び家族歴：4 歳時に同居祖父が塗沫陰性肺結核（咳嗽、血痰などの症状が約 2 ヶ月間持続）を発症した為、管轄保健所において患児に対する接触者検診が実施された．ツベルクリン反応は強陽性（硬結 15X13mm/発赤 32X28mm、二重発赤あり）であったが、胸部単純写真では発症を示唆する所見を認めなかった．感染源が塗沫陰性であった為、化学予防適応なしと判断され、その後の経過観察も実施されなかった．
現病歴：2004 年 5 月頃より嗄声が出現、近医耳鼻科にて喉頭ファイバー検査を実施され声帯ポリープと診断された．吸入・内服等の治療を受けたが嗄声に改善は見られず、徐々に増悪し入院時まで持続した．同年 11 月から湿性咳嗽や微熱が持続、近医小児科で加療を続けたが症状は改善しなかった．12 月に入って咽頭痛、嚥下困難を伴い、食事摂取量も低下した．2005 年 1 月初めより咳嗽が増強、38℃台の発熱も持続し近医小児科及び耳鼻科での治療を継続した．1 月 12 日近医で実施した血液検査において炎症反応強陽性、高度貧血が判明し前医紹介入院．入院後、経過中初めて撮影された胸部写真にて両側全肺野にびまん性粒状影を認め、喀痰塗沫・PCR 検査より結核感染症と診断．1 月 14 日当院に転院となった．
入院時現症：
体温 37.5℃、経皮酸素飽和度 91%．意識清明、髄膜刺激症状なし．顔色不良で活気なく、眼瞼結膜は貧血様．咽頭発赤を認めたが、扁桃肥大や膿苔付着は見られなかった．胸部聴診上軽度の吸気性狭窄音を聴取するが、ラ音や呼気性狭窄音は認めず、呼吸音減弱も明らかで

なかった.頻呼吸や陥没呼吸などの努力性呼吸パターンも認めなかった.湿性咳嗽が頻発し、高度の嗄声も認めた.頸部及び鎖骨上窩リンパ節腫大や肝脾腫大はなかった.尚、両側上腕にBCG接種痕は確認されなかった.

入院時検査所見（表１）：

血液検査；白血球数の増加、赤沈亢進、CRP強陽性など強い炎症所見を認めた。また、血小板増多、高度の小球性低色素性貧血、低アルブミン血症等の所見も認め、高度の炎症が長期に亘って持続していたことが示唆された.リンパ球表面マーカー検索ではCD4陽性T細胞数の減少、CD4/CD8比の低下を認めた.

髄液検査；細胞増多や蛋白上昇、糖低下など髄膜炎の存在を示唆する所見は認めなかった.

細菌学的検査;喀痰抗酸菌染色塗沫陽性(2+;Gaffky5号相当)、PCR(*M. tuberculosis* complex)陽性、抗酸菌培養検査にて結核菌の発育（30コロニー）を認めた.尚、各種抗結核薬に対する薬剤感受性は良好であった.喀痰から病原性一般細菌は検出されなかった.

ツベルクリン反応；発赤・硬結とも全く認めず、anergyと判断した.

入院時画像所見：

胸部単純写真（写真１）；両側全肺野にびまん性に分布する粒状影～小癒合影を認めた.

胸部CT（写真２）；両側上～中肺野を中心に気管支・血管に沿って拡がる癒合影、小葉中心性に分布する多数の粒状影～小結節影（周囲に小さな枝を伸ばし"tree in bud"と表現できる陰影も含む)、気管支壁の肥厚を伴った気管支拡張像や小空洞などを認め、経気道散布病巣と判断した。一方、両側下肺野を中心に気道や小葉構造とは無関係にランダムに分布する、比較的サイズの揃ったびまん性小粒状影（径1～3mm程度）を認めた。これらの陰影の一部は血管周囲や胸膜直下に分布しており、血行性に散布した粟粒結核病巣と考えられた。尚、早期播種型粟粒結核症に伴うことが多い縦隔及び肺門部リンパ節腫大は認めなかった。

喉頭ファイバー（写真３）；喉頭蓋から声帯にかけて全周性に発赤を伴い、浮腫状で粗造な粘膜面が確認された.さらに声門下の気管粘膜にも同様な所見が連続して観察され、喉頭結核に合致する所見と判断した.

入院後経過：

胸部画像所見、喉頭ファイバー所見、喀痰検査所見より喉頭結核を合併した塗沫陽性重症肺結核症と診断し抗結核剤４剤（Isoniazid; INH、Rifampicin; RFP、Pyrazinamide ; PZA、Streptomycin; SM）による治療を開始した.当初は細菌感染の合併も考慮し抗菌薬（Meropenem）静脈内投与も併用した.入院後３日間は38℃を越える発熱を認めたが、その後は解熱し炎症反応、貧血、低蛋白血症も順次正常化した.湿性咳嗽も徐々に軽減し、治療経過中に強い呼吸苦が出現することはなかった.しかし、治療開始２週後より吸気性喘鳴、嗄声が増強、治療に伴う喉頭病変初期悪化の可能性を考慮し全身性にステロイド投与（Methylprednisolone 2mg/kg/日、以後漸減）を併用した.その後は吸気喘鳴、嗄声ともに軽減した.

入院２週目以降は喀痰塗沫、培養検査共に陰性化、胸部画像所見も徐々に改善傾向を示し(写真４、写真５)、PZA（２ヶ月)、SM（３ヶ月）治療を終え2005年４月退院した.

以後、外来治療を継続し計12ヶ月間のINH、RFP内服治療を終了した.現在、ごく軽度の嗄声は持続しているが、呼吸苦も見られず日常生活に制限を受けることなく元気に学校生活を送っている.呼吸機能検査上中等度の拘束性呼吸障害（%FVC 60%前後）及び末梢気道閉塞性パターン（$\%V_{25}$ 40～50%）を認めている.また、治療終了時に再検したツベルクリン反応は陽性（発赤20mm)、リンパ球表面マーカー検索もそのsubsetに異常を認めなかった.成長曲線では乳児期より平均的な身長発育を認めていたが、5歳６ヶ月頃より成長速度の鈍化(2～3cm/年）が明らかとなり入院時まで持続した.治療開始後は再度良好な身長発育（7cm/年）を認め、徐々にキャッチ・アップしている.

患児を中心とした接触者検診：

患児の姉（13歳)・兄（10歳)・父母、近隣に暮らし頻回に接触のある従弟（７歳)・従妹（５歳、１歳）については当院において家族検診を実施、患児の姉、兄、従妹（１歳）がツ反強

陽性を示し感染例として化学予防の対象となった(画像的検索において発症は確認されず).また、管轄保健所により患児の通学する小学校児童を対象とした接触者検診が実施されたが感染例と判断された児童はなかった.

【考察】

今回報告した症例は、①小学校低学年児童に発症した塗沫陽性重症肺結核症例、さらに②喉頭結核病変を合併した、という二つの点において極めて稀な症例である.

近年、本邦における小児結核症例は年間 120 例前後へと減少し、新登録塗沫陽性小児肺結核患者数は 2000 年以降年間 10 例未満の少数例で推移している.特に 5〜9 歳の年齢層での塗沫陽性肺結核患者数は毎年 0〜1 例と稀である.この年齢層において本例のように両側全肺野に拡がる広汎な病巣を形成した重症肺結核を経験することは極めて稀であり、過去 20 年間の本邦の文献に同様な症例報告は確認されなかった.また、小児における喉頭結核症例の報告も世界的にも極めて少なく[1)、2)、3)、4)]、本邦では 1986 年以降現在まで江口らの粟粒結核に伴う学童例の報告[5)]が見られるのみである.喉頭結核はその進展経路により 1)進行した活動性肺結核病巣から経気管的に喉頭粘膜へ直接侵入し二次的に病巣を形成するもの、及び 2)血行性、リンパ行性に進展し粟粒結核の部分症状として現れるもの、の二つのタイプに分類される[6)].本例はその胸部CT所見より経気道性及び血行性の両機序により肺野病変が進展拡大したものと判断したが[7)]、喉頭病変についてはその喉頭ファイバー所見、即ち喉頭のみならず声門下気管粘膜にも連続した病変を認めたことより前者の進展様式が強く疑われた.

一般に小児結核症例では塗沫陽性例が少なく、また咳嗽も無いか軽度であることがほとんどであり、周囲への感染源となることは極めて稀である.しかしながら、Curtisら[8)]は空洞を有する 9 歳の塗沫陽性肺結核症例において患児を中心に多数の感染例が確認された事例を報告し、小児であっても空洞を有する肺結核や喉頭結核症例は周囲への感染源として極めて重要であり、症例を中心とした慎重な接触者検診の実施が必要である、と述べている.本症例も長期に亘って湿性咳嗽が持続した空洞を伴う塗沫陽性肺結核例、喉頭結核合併例であり、集団感染事例への進展が懸念された.管轄保健所の指導のもと、患児を中心とした接触者検診が実施され、患児の姉、兄、従妹 3 人が感染例として化学予防対象とされたが、通学していた小学校では幸いにも感染・発症例は 1 例も検出されなかった.

では、なぜ本症例では両側全肺野にびまん性に拡がる広汎な病巣が形成され、高度の嗄声や吸気性喘鳴を伴う喉頭結核病巣を呈するまで重症化してしまったのであろうか?

本症例では診断時ツ反陰性、CD4 陽性 T 細胞数減少など細胞性免疫能減弱を示唆する所見を認めたが、過去に BCG 感染を含む易感染性を示唆するエピソードはなく、抗結核剤治療開始後は順調に治癒傾向が見られ、治療終了時にはツ反は再度陽転、リンパ球サブセットも正常化した.これらより診断時には重症結核に伴う続発性細胞性免疫不全状態にあったが、T 細胞の欠陥や INF-γ及び IL-12 レセプター変異を伴う原発性免疫不全の存在は否定的と判断した.

本症例の経過を振り返り、①同居祖父結核発症時の接触者検診の不備、②有症状受診例に対する診断の遅れ=“Doctor's delay”、③問診を中心とした学校結核健診の限界、などの要因が重なり合った結果、本症例の重症化に繋がったものと考察した.

①同居祖父結核発症時の接触者検診の不備

患児が 4 歳時、連日濃厚な接触を有する同居祖父が咳嗽、血痰などの症状を伴って肺結核を発症し管轄保健所において接触者検診が実施された.二重発赤を伴うツ反強陽性(発赤径 32mm)が明らかとなったが、旧厚生省化学予防適応基準に沿った判断により化学予防の対象とはされなかった(BCG既接種で塗沫陽性患者との接触があった場合、ツ反発赤径 30mm 以上で化学予防対象となるが本例では感染源が塗沫陰性であった).また、初回検診時に胸部単純写真のチェックを受けた後は定期的な検診実施を指示されることなく放置されてい

た．喀痰塗沫検鏡の結果を問わず、呼吸器症状を有する結核患者と頻回かつ濃厚な接触を有する幼児において二重発赤を伴うツ反強陽性が判明した場合には、感染成立の可能性を強く疑って化学予防を開始する、或いは発症の可能性も考慮に入れ定期的な経過観察を行う、などの慎重な対応が必要であったのではなかろうか．雉本ら[9]や高松ら[10]はそれぞれの小児結核症例に関する検討のなかで、結核発症例のうち約 3 割が旧厚生省基準の化学予防適応外に相当し、同基準を機械的に適用することにより化学予防が必要な症例の相当数が見逃される危険性を指摘している．BCG接種歴やツ反結果のみを機械的に当てはめるのでなく、それぞれの症例において感染源の状況（排菌状況や症状、有症状期間）、感染源との接触頻度、検診対象児の年齢などを含む感染成立のリスクを丁寧に評価した上で感染（＝化学予防適応）の有無を判断する態度が必要であろう．また、感染の可能性が疑われるケースについては常に発症の可能性を念頭においた慎重なフォロー・アップ（概ね接触判明後 2 年間に亘る画像的検索）を継続することも重要である．本ケースでは初回検診結果の機械的解釈、さらにその後の慎重な経過観察体制の欠如が重症化へ繋がった可能性は否定できない．

また、さらに接触者検診の精度を向上させる為、結核菌特異抗原刺激によるINF-γ測定検査（QuantiFERON® -TB2G）の導入に関して検討することも必要であろう．小児結核症例診断における本検査の有効性は既に報告されている[11]が、今後小児潜在感染例診断における有用性の検討、さらに小児を対象とした接触者検診のおける本検査適応及び結果解釈に関するガイドラインの作成も強く望まれる．

②有症状受診例に対する診断の遅れ＝“Doctor's delay”

本症例では診断される半年以上前より嗄声や咳嗽などの呼吸器症状が遷延し、頻回に医療機関を受診していた．しかしながら、適切な診断、治療開始が遅れ、結果として多量の排菌を伴う重症肺結核、喉頭結核へと進展し、高度の貧血や低蛋白血症、成長障害を伴う状態に至った．即ち、患者側の医療機関受診の遅れ（”Patient's delay”）ではなく、医療機関における診断の遅れ（”Doctor's delay”）が重症化に繋がった．遷延する呼吸器症状を主訴とする症例で、その原因が明らかでない、或いは初期治療に対して抵抗性で改善傾向が乏しい場合には胸部聴診所見や発熱の有無にかかわらず早期に画像的評価を実施する姿勢が必要である．また、診察に際しては予防接種歴や家族歴を含む問診を徹底、再確認することも重要である．

③問診を中心とする学校結核健診の限界

小児結核症例の減少を受けて2003年春より学校における結核健診の方法が大きく変更され、それまでの結核予防法に基づくツベルクリン反応による定期健診は中止され、学校保健法による定期健診の中に結核に関する問診が導入された．即ち、結核に関する治療歴・予防内服歴・家族歴、結核高蔓延地域での最近の居住歴、長く続く咳や痰などの呼吸器症状の有無、BCG 接種歴などに関する問診結果を教育委員会に設置された結核対策委員会において検討し、精検の要否を判断する方法へと変更された．

本症例において学校結核健診は有効に機能したのであろうか？本症例では 2004 年春（小学校入学時）の問診票に家族の結核既往歴が記載されていたにもかかわらず、レントゲン検査を含む精検対象とされなかった．結核に関する治療歴、予防内服歴、家族歴に関する問診結果が要検討例に該当していても保健所からの情報により適切に事後管理がされている、或いは発病のおそれなし、等と判断されれば精検対象から外されてしまう．同様に有症状例であっても医療機関を受診し検査・治療中であれば要精検例から除外されることとなる．

学校結核健診方法の変更により、これまでツ反結果のみから適応ありと判断されていた過剰な化学予防対象例が著減した一方で、学校健診により発見される患者数も減少しており[12]新たに導入された健診方法の有効性（特にその感度）について再度検討する必要があろう．

以上、本症例が重症化するに至った 3 つの要因は、小児結核の予防及び早期診断に向けて特に重点を置いて取り組むべき重要な方策である．即ち、小児結核症例は身近な親族から強い感染を受けて発症するケースが多く、周囲に感染源が判明した場合には迅速かつ徹底した

接触者検診を実施し、慎重な事後処置を行うことが非常に大切である．また、小児結核の発見機会としては家族検診（約 50%）に次いで医療機関受診（約 25%）が多く[13]、有症状受診に対する適切な対応も非常に重要である．長期に遷延する呼吸器症状を主訴とする症例に対しては結核感染症の可能性も念頭に置き早期に適切な画像的評価を行うことが必要である．さらに、10 歳代の新登録患者数は近年横這いで推移しており、学校結核健診の必要性は決して低下していない．健診問診結果を慎重に検討し、要精検例に対して精度の高い精密検査を実施することも重要である．また、効率的、かつ精度の高い学校健診を行う為に、現在の学校結核健診制度に関する批判的な検討を行うことも必要であろう．

【結語】

7 歳女児に発症した塗沫陽性重症肺結核症の一例を経験した．両側全肺野に広汎に分布する多彩な病巣を認め、さらに喉頭結核病変の合併も確認された．肺野病変はその CT 所見より経気道性及び血行性の両機序により全肺野に散布したものと判断した。診断に至るまで長期間に亘って咳嗽や嗄声、発熱等の症状が持続し、頻回に医療機関を受診していた．患児を中心とする接触者検診では家族内に 3 例の潜伏感染例を認めたが、幸いにも通学する小学校における感染・発症例は認めなかった．同居祖父結核発症時の接触者検診の不備、有症状受診例に対する診断の遅れ（“Doctor’s delay”）、問診を中心とした学校結核健診の限界、などの要因が重なりあって、本症例の重症化に繋がったものと考えた．未だ年間 120 例前後の小児結核新規発症例が報告され、小児の親の世代に当たる 20 歳代、30 歳代の結核登録患者数も横這いで推移しており、小児結核を取り巻く環境は未だ油断できる状況にはない．小児結核の予防及び早期診断の為、乳児期早期からの BCG 接種徹底に加え、小児の特殊性を考慮に入れた精度の高い接触者検診の実施、有症状受診例に対する早期の画像検査を含む適切な対応、学校結核健診要精検例に対する慎重な対応、などの対策徹底が強く望まれる．

謝辞：本症例の画像所見につき御教授頂きました当院放射線科游逸明先生、古市健治先生、呼吸器内科佐藤敦夫先生に深謝致します。

Key Words
小児結核・肺結核・喉頭結核・接触者検診・Doctor’s delay

表1．入院時検査所見

WBC	12300	/μl
Stab	8.5	%
Seg	70.0	%
Mono	7.5	%
Baso	0.5	%
Lym	13.5	%
RBC	454X10⁴	/μl
Hb	8.4	g/dl
MCV	61.5	fl
MCH	18.6	pg
Plt	69.2X10⁴	/μl
ESR(1hr)	97	mm
TP	6.1	g/dl
Alb	2.8	g/dl
AST	22	IU/l
ALT	11	IU/l
LDH	299	IU/l
BUN	3.0	mg/l
Cr	0.25	mg/l

Fe	3	μg/d
Ferritin	76.0	ng/l
CRP	20.13	mg/d
IgG	1360.0	mg/l
IgA	234.0	mg/l
IgM	96.7	mg/l
CH50	50.7	U/ml
Mycoplasma Ab	X320	
β-D-glucan	<4.76	pg/l
CD3+	30.3	%
CD3+CD4+	14.4	%
CD3+CD8+	13.6	%
CD4/CD8 ratio	1.06	

髄液一般検査

細胞数	0/3	
Protein	11	mg/l
Glucose	52	mg/l
ADA	4	IU/l

細菌検査

喀痰）

抗酸菌塗沫	(2+)
PCR (*M..tuberculosis* complex)	(+)
培養（小川）	(+);30コロニー
薬剤感受性（各種抗結核剤）	良好

髄液）

抗酸菌塗沫	(-)
PCR (*M..tuberculosis* complex)	(-)
培養	(-)

ツベルクリン反応　0X0/0X0mm;(-)

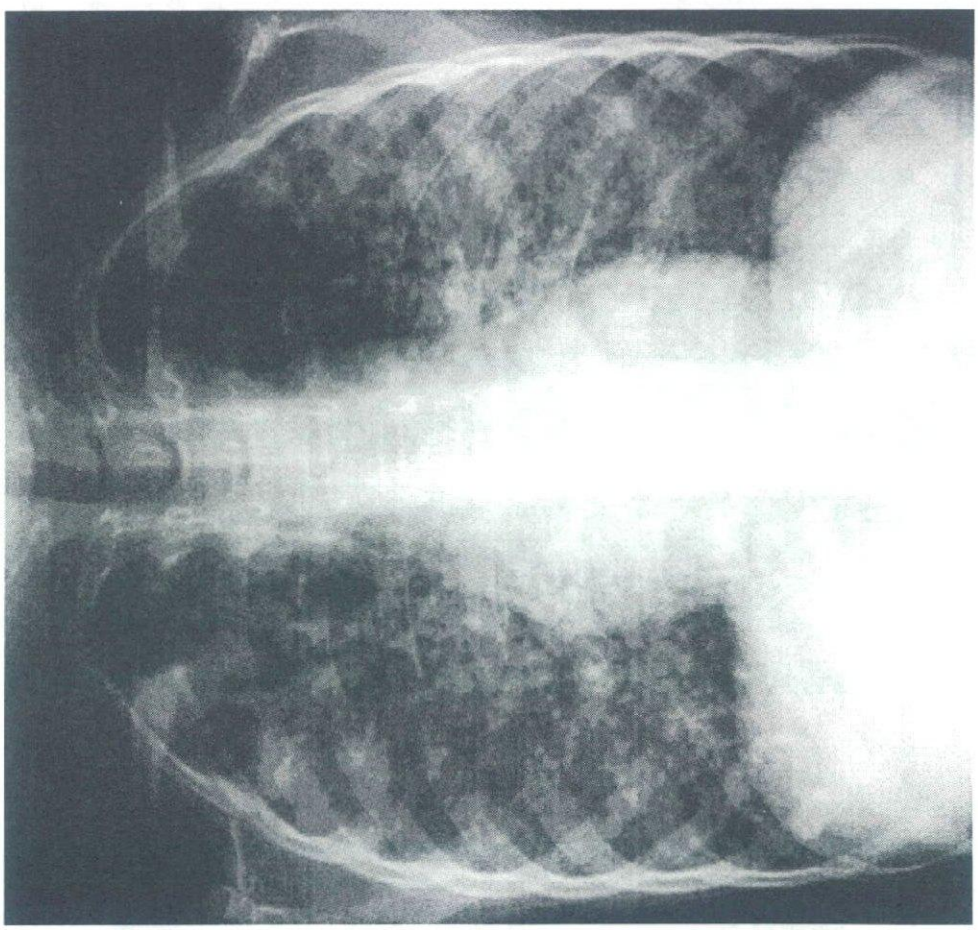

図1

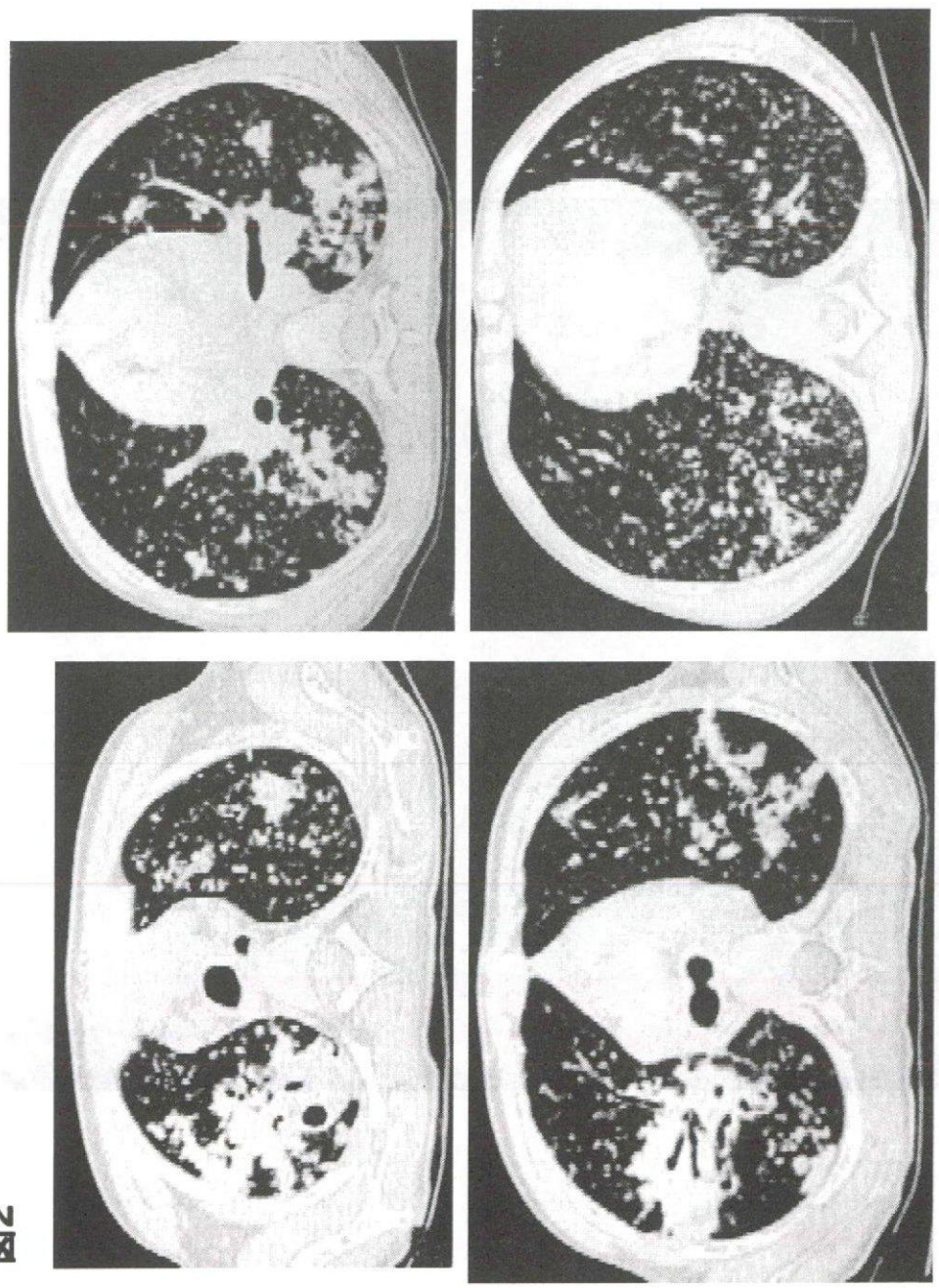

図2

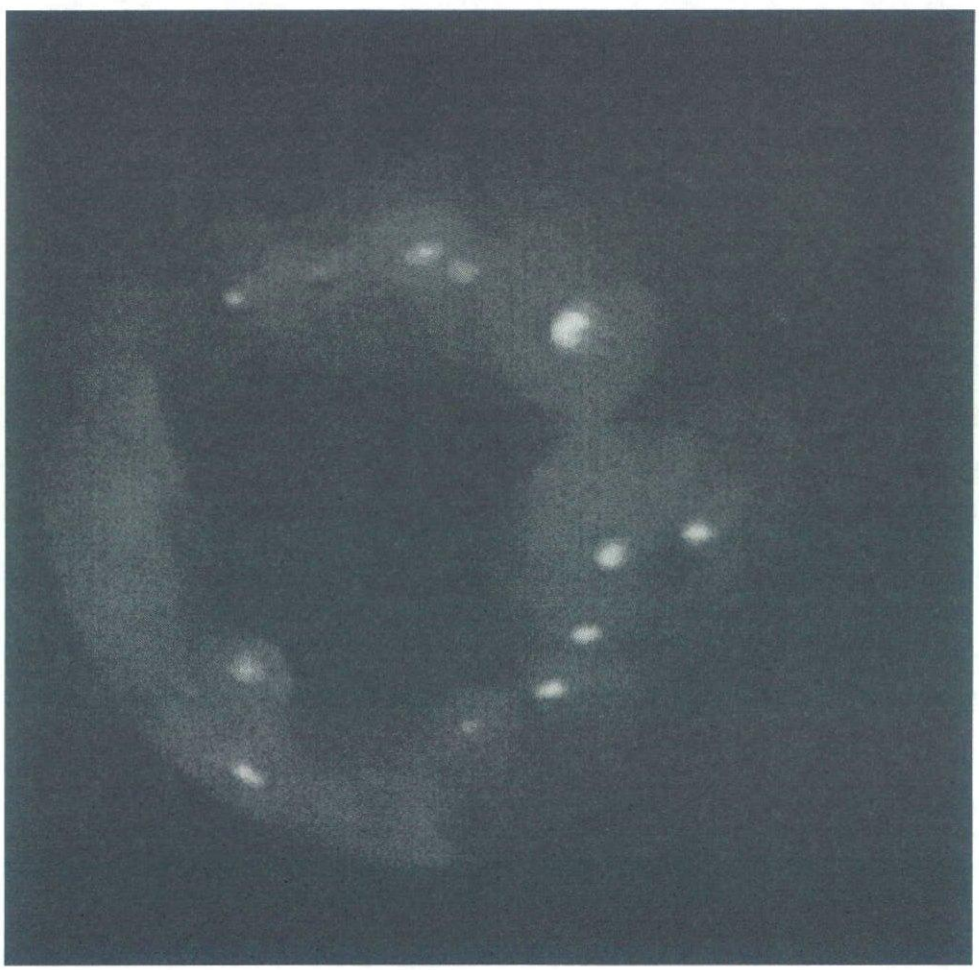

図3

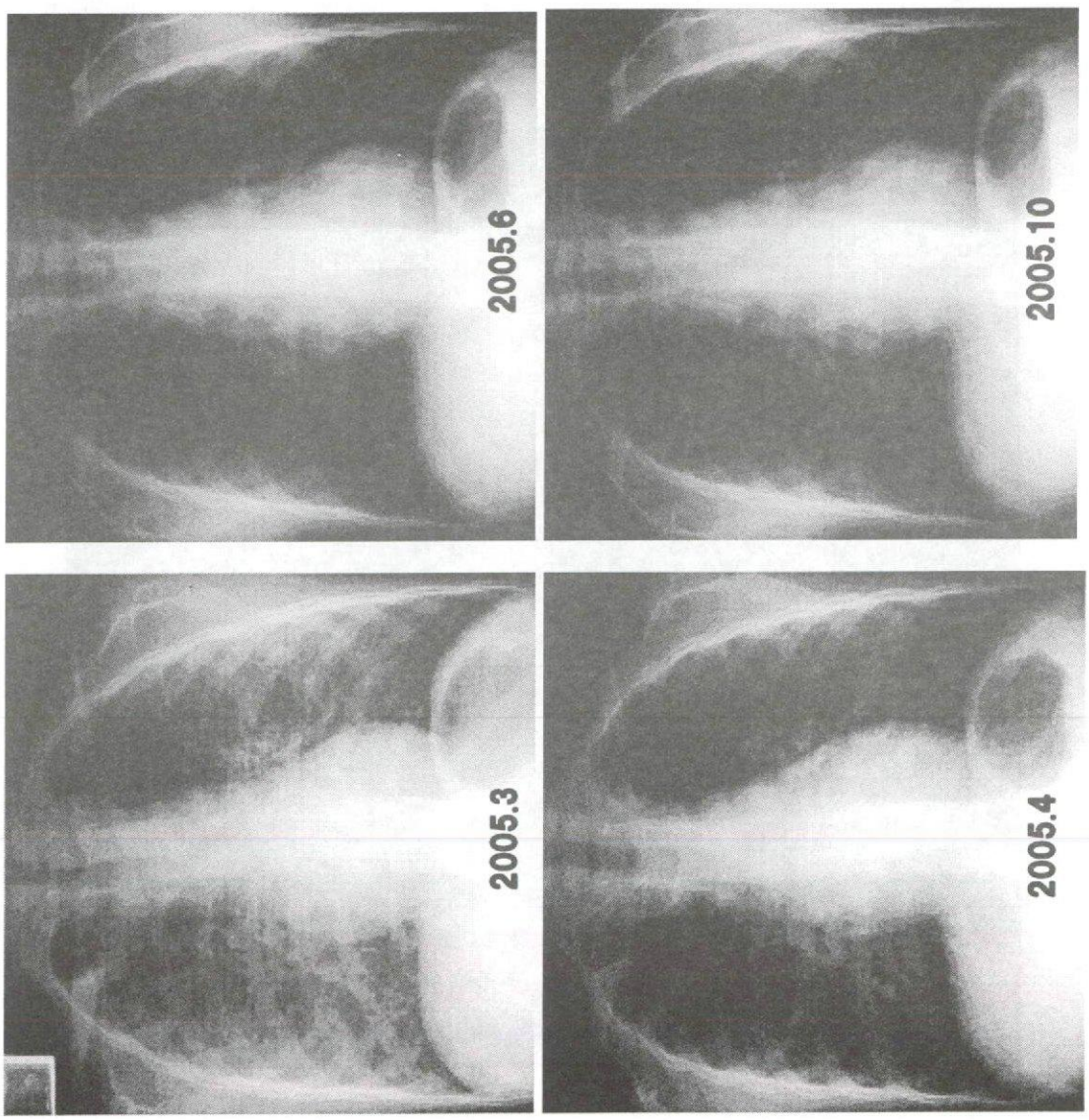

図4

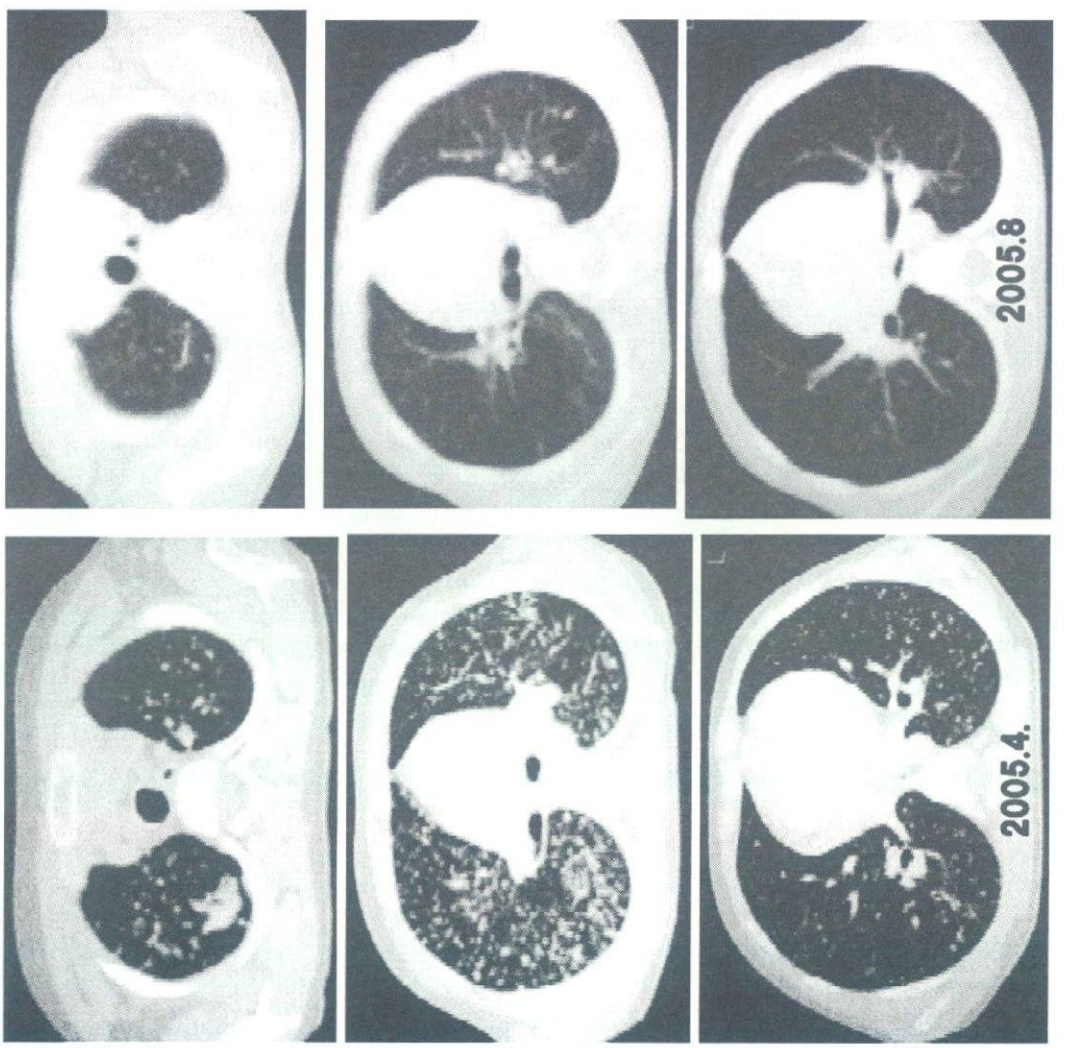

图5

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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

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 × SSC, 0·5% SDS, 4 × 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

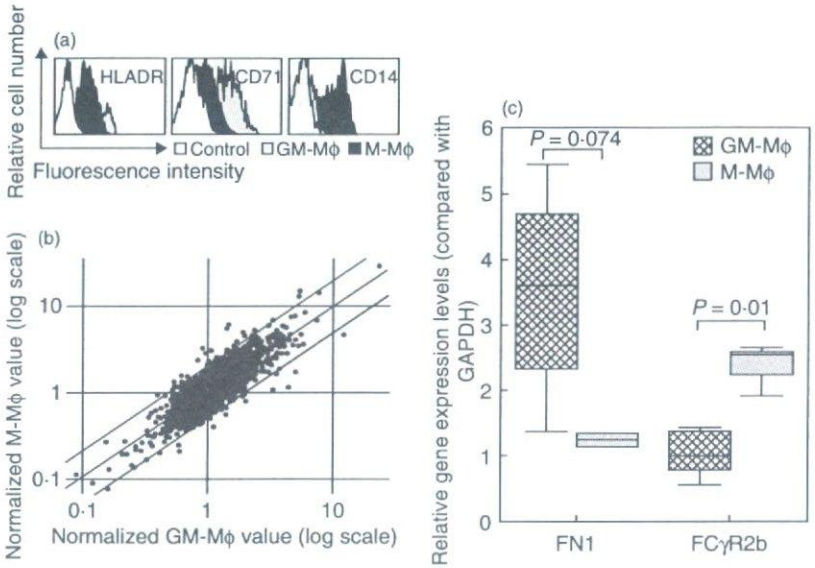

Fig. 1. (a) Phenotypic characteristics of GM-Mϕ and M-Mϕ, generated from human CD14$^+$ monocyte. (b) The scatter-plot between two types of Mϕs in their constitutive states. Each spot is the representative of normalized data in logarithmal scale from the average of two values from each cell type. (c) 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).

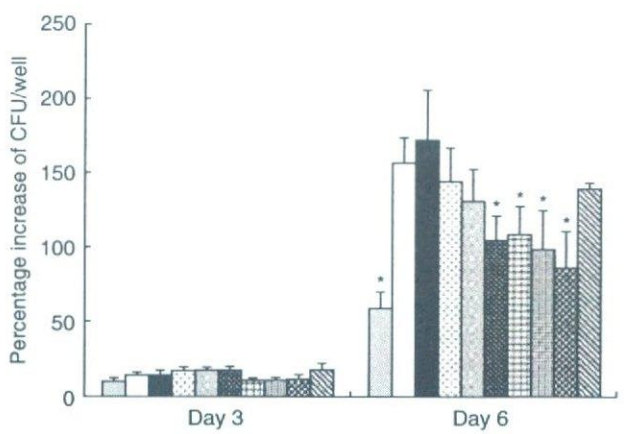

Fig. 2. Inhibition of *M. tuberculosis* H37Ra growth in Mϕs by SPP1 and CXCL7. Mtb colony-forming unit (CFU) assay was performed on days 3 and 6 after H37Ra-Mϕ exposure. M-Mϕ ■, GM-Mϕ with granulocyte macrophage-colony stimulating factor (GM-CSF) only □ and GM-Mϕ without any cytokines ■ were cultured 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.

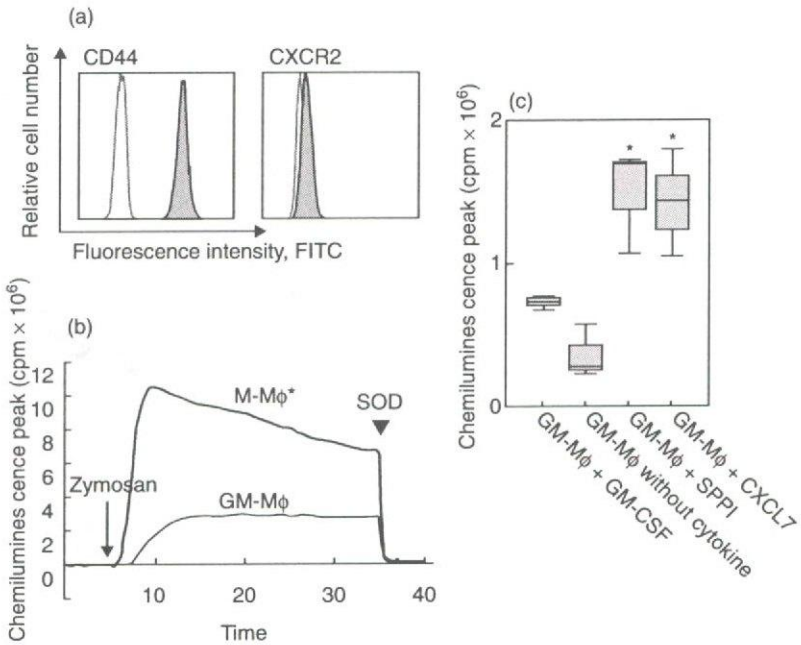

Fig. 3. Increased superoxide production from GM-Mϕs after the stimulation with SPP1 and CXCL7. (a) Receptor expression for SPP1 (CD44) and CXCL7 (CXCR2) on the surface of GM-Mϕ, determined by flow cytometry. (b) Superoxide production by the Mϕs measured by a change of chemiluminescence. The Mϕs (5.0×10^4 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

deficient in the NADPH oxidase complex have a susceptibility to *M. tuberculosis* infection [45]. In humans, patients with chronic granulomatous disease are more susceptible to *M. tuberculosis* [46]. Although our data showed that SPP1 and CXCL7 may play an important role against *M. tuberculosis*, possibly through the up-regulation of superoxide production in Mϕs, it is possible that their anti-mycobacterial effect could be based on other mechanisms.

In summary, our data showed that Mϕs can secret a large array of molecules that induce host defence after the exposure to BCG. Among them, we found new roles of SPP1 and CXCL7 against *M. tuberculosis* in Mϕs. Further analysis of these molecules using iRNA will confirm the role of these chemokines in *M. tuberculosis* infection more clearly.

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