特集●マクロファージの再認識

細菌感染症とマクロファージ

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結核菌などのいわゆる細胞内寄生病原細菌は，Mφに貪食されても殺菌されず，Mφはむしろこれら細菌の増殖の場となる．結核菌の場合は，SK活性とS1P形成を抑制するため，ファゴリソソーム形成に必要な細胞内Ca^{2+}濃度の上昇が抑制されるためである．IFN-γによるマウスMφの殺菌活性の活性化は，NOS2の活性化のほか，p47GTPaseの活性化が重要な役割を果たす．ヒトとマウスのMφの結核菌の殺菌機構は異なり，ヒトMφの結核菌殺菌活性にはNRAMP1の発現の関与やIL-10による活性化が示唆された．

Key words macrophage, phagolysosomal fusion, Lrg47, NRAMP1, *M. tuberculosis*

細菌感染に対する免疫応答は，大きく分けて自然免疫応答と適応免疫応答の2つに分けられる．自然免疫応答（innate immunity）は，生体に生来備わった免疫応答で感染早期に働く．適応免疫応答（adaptive immunity）は，自然免疫応答と異なりそれぞれの病原細菌に対する特異性と記憶を有し，再度同一病原体に出会ったときにそれを思い出し病気が起こるのを効果的に防止する．マクロファージ（Mφ）は，自然免疫応答の中心的細胞であり，直接細菌を認識・貪食し，殺菌する．またMφは，細菌およびその菌体成分に反応して，種々のケモカインやサイトカインを産生し，感染局所への種々の炎症性細胞や免疫担当細胞のリクルートメントを促す．さらに，Mφは，抗原提示細胞として取り込んだ細菌を分解処理して抗原としてT細胞に提示し，適応免疫応答の誘導に関与する．

このように，Mφは，病原細菌に対する宿主の防御の第一線に位置する細胞である．しかし，必ずしもすべての細菌がMφにより殺菌されるわけではなく，結核菌，サルモネラ菌，リステリア菌などのいわゆる細胞内寄生細菌と呼ばれる病原細菌は，Mφに貪食されても殺菌されず，Mφはむしろこれらの細菌の増殖の場となる．これらの病原細菌をMφが殺菌するためには，IFN-γなどによる“Mφの活性化”が必要である．

Mφが細菌を貪食すると多様なシグナル伝達経路が活性化される．それらのシグナル伝達経路は，抗菌活性や抗原提示活性，サイトカイン産生やケモカイン産生と密接に関連し，病原細菌を排除するために協調して作用する．一方，病原細菌にとっては，

The activation of bactericidal mechanisms of macrophages against intracellular bacteria
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あかがわ・きよこ 1968年東京教育大学理学部生物学科卒業．同年国立予防衛生研究所（現国立感染症研究所）結核部入所，75年医学博士，80年国立予防衛生研究所結核部主任研究官，88年同細胞免疫部免疫調節室室長，92年同免疫部免疫工学室室長，98年国立感染症研究所免疫部免疫工学室室長，2003年同免疫部第4室室長．研究テーマ：マクロファージの分化とヘテロジェナイティーに関する研究およびヒトマクロファージにおけるHIV-1と結核菌の増殖制御機構に関する研究．

自然免疫応答や適応免疫応答による感染防御機構から逃れて自己を守るために，それらの始動と発現に中心的役割を果たしているMφ機能を制御することが必須であり，そのため細菌は，Mφのシグナル伝達経路を阻害する多様なストラテジーを有することも明らかになってきた．また，マウスMφとヒトMφは，必ずしも同じ機構で活性化されるとは限らない可能性も示唆された．本稿では，これらの点についての最近の知見を述べる．

●Mφによる細菌の認識と応答：TLRとNOD

1. TLR2応答の多様性

Mφは病原体を迅速に認識する必要がある．そのため，補体や抗体でオプソニン化された病原体の認識にかかわる補体のC3レセプターや抗体のFc部分に対するレセプターのほか，pathogen-associated molecular pattern（PAMP）と呼ばれる微生物に特有の分子構造に対する一連のレセプターを発現している（「マクロファージに発現するレクチン」，p.942を参照のこと）．その一つがToll-like receptor（TLR）であり，現在10種のTLRの存在が知られている．TLR2，TLR4，TLR5は，細菌由来成分を特異的に認識する．TLR4は，グラム陰性菌のリポ多糖（LPS），TLR2は，多くのリポタンパク質，ペプチドグリカン，ミコバクテリア，ミコバクテリア由来リポアラビノマンナン（LAM）などを認識し反応する．TLR2のリガンド認識には，TLR1やTLR6とのヘテロダイマー形成が必要である．またTLR5は，グラム陽性および陰性菌の鞭毛を認識する．細菌由来DNAや非メチル化CpG DNAは，TLR9により認識される．多くのTLRはMφの表面に発現しているが，TLR2は，ファゴソーム膜にも存在することより，細胞外のみならずファゴソーム内の菌体由来成分を認識してシグナルを誘導する．

TLRは，多くの微生物および微生物由来物質の認識にかかわるMφの重要なレセプターとして機能し，NF-κBの活性化，一酸化窒素（NO）産生，そしてIL-1，腫瘍壊死因子α（TNF-α），IL-12，IL-18などのサイトカイン産生を誘導し殺菌を促すとともに，T細胞やナチュラルキラー（NK）細胞の活性化を誘導する．結核菌の19 kDaリポタンパク質は，TLR2を刺激して主要組織適合抗原（MHC）クラスII分子の発現抑制や抗原プロセシングの抑制を誘導する．これらの抑制は，炎症性サイトカイン産生に比べ遅い時期に誘導される．また，結核菌の19 kDaリポタンパク質によるTLR2の刺激は，Mφを活性化し結核菌の殺菌やMφのアポトーシスを誘導する．しかし，ヒトとマウスのMφにおけるTLR2を介した殺菌活性の活性化機構は必ずしも同じではなく，マウスMφでは殺菌のエフェクターはNOで，ヒトMφではNO以外の殺菌因子がエフェクターとして関与する[1]．

2. NOD2とCrohn病

TLRは，細胞表面やファゴソームそしてエンドソームなどの膜に存在するが，細胞質局在型のPAMP認識レセプターとして，NOD1およびNOD2が明らかにされた．NOD1は，グラム陰性菌のペプチドグリカンのジアミノピメリン酸（DAP）のレセプターであり，その最小認識構造は，γ-グルタミル-DAPである．NOD2は，グラム陰性菌およびグラム陽性菌のペプチドグリカンに共通して存在するムラミルジペプチド（MDP）のレセプターである．NOD1とNOD2は高い相同性を有し，ともにC末端にロイシンリッチリピート（LRR），中央にNODファミリーの特徴であるヌクレオチド結合性多量化ドメイン（nucleotide-binding oligomerization domain；NOD），N末端にcaspase recruitment domain（CARD）を有する．LRRで細菌由来リガンド分子を認識し，NODの自己集合化阻害の解除により多量体化し，次いでCARDをもつ下流因子RICK（Rip2）とCARD-CARD相互作用を介して結合し，NF-κBの活性化を誘導する．NOD2は主にMφに発現しており，一方，NOD1は，腸管上皮細胞をはじめ多くの細胞で発現しているが，Mφでの発現は低レベルである．

最近，Crohn病患者の一部で，NOD2の遺伝子変異を認めること，この変異は，MDPに対する不応

答性を示す変異であることが明らかになった[2]．Crohn病は，再燃と緩解を繰り返す原因不明の非乾酪性肉芽腫形成を伴う慢性の炎症性腸疾患であり，単球・Mφの機能異常（TNF-αの異常産生，IL-10感受性の低下，IL-10産生抑制など），$CD4^+$T細胞によるTh1型免疫応答の異常亢進，腸内細菌などの関与などが示唆されているが，詳しい発症機序は不明である．TNF-αの異常産生が病態に深くかかわり，抗TNF-α抗体の投与により病態の改善がみられる．野生型NOD2と異なり変異型NOD2を発現した上皮細胞株Caca2では，サルモネラ菌の生存低下を認めないとする報告もあり[3]，NOD2が殺菌に関与する可能性が示唆される．また，NOD1はアポトーシス誘導因子であるApaf-1（apoptotic protease activating factor-1）との相同性をもとに同定された分子であり，NF-κBの活性化だけでなくアポトーシス誘導分子であるカスパーゼ9（caspase-9）も活性化する．それゆえ，変異NOD2をもつMφは，アポトーシスが誘導されず，TLRを介した炎症性サイトカイン産生が持続する可能性も考えられる．しかし，NOD2の変異がどのようにCrohn病の発症と関係するのか，その機構はまだ不明である．NOD2ノックアウトマウスを用いた解析が待たれる．

Crohn病に関しては，ヒトのCrohn病によく似たウシの炎症性腸炎であるJohne（ヨーネ）病の原因菌である*Mycobacterium paratuberculosis*の感染の関与が古くから示唆されている．また，マクロライド系抗生物質などによる抗菌療法で臨床的改善を認めるという報告も1990年代後半よりいくつか出されている．同じ抗酸菌に属する結核菌による結核もTh1応答優位の肉芽腫性疾患であることを考えると，肺と腸と場所は異なるが，興味深い．わが国でも近年Crohn病の増加がみられ，一方で，ウシのJohne病は1990年代後半以降増加していることも報告されている[4]．直接関連があるか否かは不明であるが，興味深い点である．

●ファゴリソソームの形成と細胞内寄生細菌によるその抑制機構

1．ファゴリソソームの形成機構

細菌がMφに貪食されると，ファゴソームの形成に引き続き，NADPHオキシダーゼの会合と活性化がファゴソーム膜上に起こり，酸素（O_2）が還元されてスーパーオキシドアニオン（$\cdot O_2^-$）が産生され，さらに還元されて，過酸化水素（H_2O_2）やヒドロキシルラジカル（·OH）などの強い殺菌性の活性酸素（ROI）がファゴソーム内に産生される．その後，ファゴソームとリソソームの融合が起こる．リソソームは，酸性ホスファターゼ，カテプシンDをはじめ多くの加水分解酵素群をもち，これらの物質により強力な殺菌活性が誘導される．また，ミエロペルオキシダーゼ（MPO）がファゴソーム内へ入りハロゲン化物の共存下にH_2O_2などに働き，次亜塩素酸（$HOCl^-$）のようなより殺菌活性の強い毒性酸化物が産生される．しかし，好中球に比べMφではMPO活性は弱い．一般の細菌は，この一連のNADPHオキシダーゼの活性化やファゴリソソーム形成によるリソソーム酵素の殺菌作用により殺菌される．しかし，後述するように結核菌などの細胞内寄生細菌は，種々の機構によりこの殺菌作用から逃れている．

図1に示すように，ファゴソームは，エンドソームやリソソームとの"kiss and run"と呼ばれる一連の融合により成熟する．まず，初期エンドソームとの融合により低分子量GTPaseであるRab5やリソソームとの融合に必要なEEA1（early endosomal autoantigen 1）を発現する．その後，後期エンドソームとの融合により，GTPaseであるRab7を発現し，一方Rab5の発現を失う．このファゴソームは，その後リソソームと融合し，リソソームに関連する膜タンパク質LAMP-1（lysosomal associated membrane protein-1）やCD63（lysosomal intrinsic membrane protein 1），カテプシンDなどの加水分解酵素，リゾホスファチジン酸そしてファゴソームの酸性化に関与するvacuolar proton-ATPase（v-

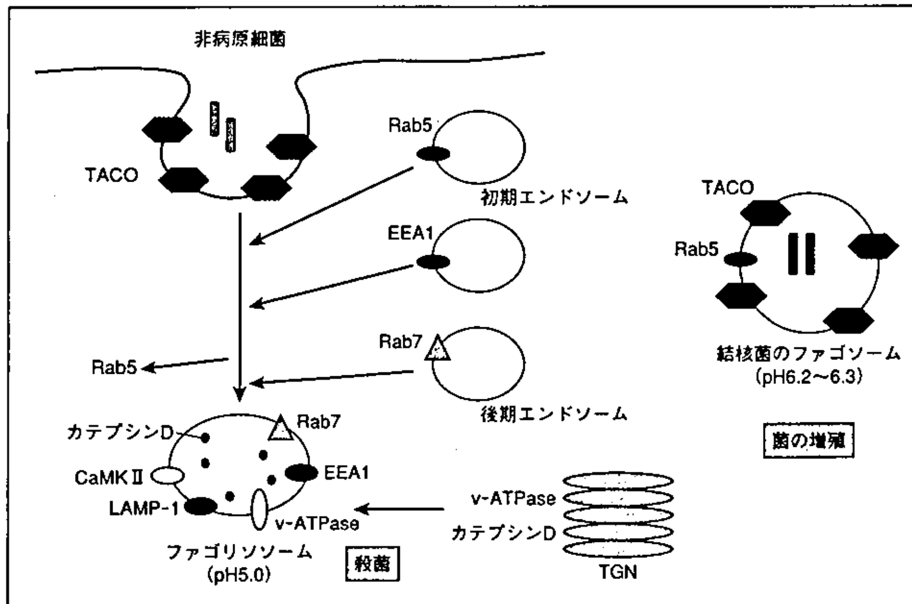

図1 ファゴリソソーム形成機構と結核菌のファゴソーム
略号は本文参照.

ATPase）などを発現する.

細菌の貪食刺激による細胞内Ca^{2+}濃度の上昇によるカルモジュリン（CaM）の活性化とそれによるCaMキナーゼⅡ（CaMKⅡ）の活性化は，EEA1のファゴソーム膜へのリクルートメントとエンドソームとファゴソームの膜の融合に必要である．EEA1のエンドソームやファゴソームへのリクルートメントには，hvPS34というタイプⅢホスファチジルイノシトール-3キナーゼ（PI3K）により生成されるホスファチジルイノシトール三リン酸（PIP_3）がEEA1のFYVEドメインに結合することが必要である．このPI3Kのファゴソーム膜へのリクルートメントもCa^{2+}結合CaMに依存する．またEEA1は，syntaxin 6とともにトランスゴルジネットワーク（TGN）からリソソームのコンポーネントをファゴソームに運ぶ役割をもつ．この一連の過程に重要な細胞内Ca^{2+}濃度の上昇は，貪食の刺激により活性化されたリピドキナーゼであるスフィンゴシンキナーゼ（sphingosine kinase ; SK）によりスフィンゴシンがリン酸化されて形成されたスフィンゴシン-1-リン酸（S1P）の作用による．S1Pは細胞質のCa^{2+}貯蔵庫からCa^{2+}を放出して，細胞内Ca^{2+}濃度のホメオスタシスを調節している．

2. 結核菌のファゴソームの特徴

細胞内寄生細菌の代表例である結核菌は，スーパーオキシドジスムターゼやカタラーゼなどの抗酸化酵素活性が強く，初期の活性酸素系に対する殺菌抵抗性を示すとともに，ファゴリソソーム形成を抑制することでMϕの殺菌機構に抵抗性を示す．結核菌のファゴソームは，Rab5や菌の増殖に必要な鉄の取込みに関与するトランスフェリンレセプター（TfR）を発現するが，EEA1やRab7を発現しない．またv-ATPaseの発現やpHの低下も認めず，ファゴソーム内での結核菌の生存に優位な状態が形成されている．また，TACO（tryptophan aspartate-containing coat protein）と呼ばれる分子量57,000のアクチン結合タンパク質がファゴソームの周りを取り囲むが，死菌BCGではこの取り囲みは一過性であるのに対し，生菌BCGでは持続的であることより，TACOがファゴソームにとどまることが，一連のファゴソームとエンドソームおよびリソソームとの融合阻害の原因とする報告がなされた．しかし，ヒトMϕを用いた実験からは，生菌BCGでもTACOはファゴソームに一過性にしか認められず，ファゴリソソーム融合の形成抑制に必ずしも関与しないことも示唆されている[5]．

3. 結核菌によるファゴリソソーム形成抑制機構

結核菌生菌の貪食では，結核菌死菌の貪食に比べ細胞内Ca^{2+}濃度の上昇は一過性であり，また，Ca^{2+}結合CaMの量やリン酸化されたCaMKⅡの発現が低い．これは，結核菌がSK活性を阻害し，その結果S1P生成が減少するため細胞内Ca^{2+}濃度の上昇が抑制されるためである[6]．

結核菌は脂質に富むことがその特徴であるが，結核菌などの有毒ミコバクテリア菌の糖脂質成分の一つであるマンノースキャップのLAM（mannose-capped lipoarabinomannan；Man-LAM）は，細胞内Ca^{2+}濃度の上昇を抑制し，Ca^{2+}結合CaM形成の抑制やPI3Kのリクルートメントと活性化を阻害し，EEA1のファゴソームへの結合を抑制する[7]．非病原性ミコバクテリア菌由来のLAMはアラビノースキャップ型（Ara-LAM）であるが，この糖脂質にはそのような抑制作用は認められない．また，非病原性のミコバクテリア菌の貪食では，ファゴリソソーム形成の抑制は認められない．これらのことより，結核菌のファゴリソソーム形成抑制は，結核菌などの有毒ミコバクテリア菌がもつMan-LAMの細胞内Ca^{2+}濃度上昇抑制作用に基づくものである．しかし，Man-LAMがSK活性を直接阻害しているか否かはまだ不明である．また，結核菌によるSK活性の抑制は，生菌のみで認められ死菌では認められない．これは，結核菌生菌感染Mφでは，LAMなどの菌体成分がファゴソームから細胞質に分泌されることが報告されていることと関連するであろう．最近，ファゴソームの周りのアクチンフィラメントの形成がエンドソームなどとの融合に重要なことが示されたが，セラミドやS1Pを添加することで，結核菌感染ファゴソームのアクチンフィラメント形成やその後のファゴリソソーム融合と殺菌を誘導できる[8]．

一方，結核菌を貪食したMφは，p38 MAPキナーゼ（MAPK）の活性化を誘導するが，p38 MAPKの阻害剤で活性化を抑制すると，ファゴソームへのEEA1のリクルートメントとその後のファゴリソソーム融合が起こることから，p38 MAPKの活性化もファゴリソソーム融合の抑制に関与する[9]．

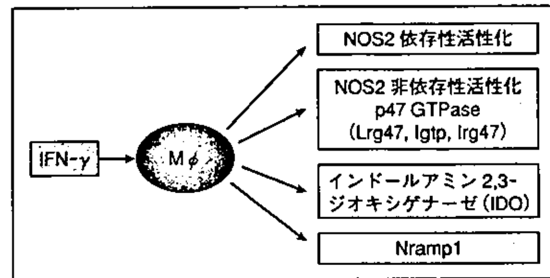

図2　IFN-γによるマウスマクロファージの殺菌機構の活性化

4. IFN-γによるMφの殺菌活性の活性化とLrg47

図2に示すように，古くよりIFN-γによるマウスMφのBCGや結核菌をはじめ多くの細胞内寄生細菌や原虫の殺菌活性の活性化は，誘導型一酸化窒素合成酵素NOS2の誘導と活性化によるNO産生によると考えられてきた．また，IFN-γはトリプトファンやその代謝産物である種々のインドールアミンに$\cdot O_2^-$を添加してインドール環を開環する酵素であるインドールアミン2,3-ジオキシゲナーゼ（IDO）を誘導することで殺菌作用を示すことも知られている．後述するNramp1の発現もIFN-γは増強する．しかし，最近IFN-γによるマウスMφの殺菌活性の活性化に，p47 GTPaseファミリー分子が実は重要であることが知られた[10]．マウスのp47 GTPaseとしては，Lrg47，Igtp，Gtpi，Irg47，Tgtp/Mg21，Iigpの6分子が知られているが，これらの分子は普段は低レベルでしか発現せず，IFN-γ刺激によりその発現が急速に上昇する．IFN-γは，転写因子STAT1とIRF1（IFN regulatory factor 1）の活性化を誘導するが，p47 GTPase発現はSTAT1依存性である．IFN-α/β，LPSもp47 GTPaseの発現を誘導する．

Lrg47，Igtp，Irg47のそれぞれのノックアウトマウスが作製され，それらへの感染実験から，結核菌，サルモネラ菌，リステリア菌などの細胞内寄生細菌の感染にはLrg47が，またトキソプラズマやリーシュマニアなどの原虫感染にはIgtp，Lrg47，Irg47が関与することが明らかになった．Lrg47ノックアウト（$Lrg47^{-/-}$）マウスは，$IFN\text{-}\gamma^{-/-}$マウスや

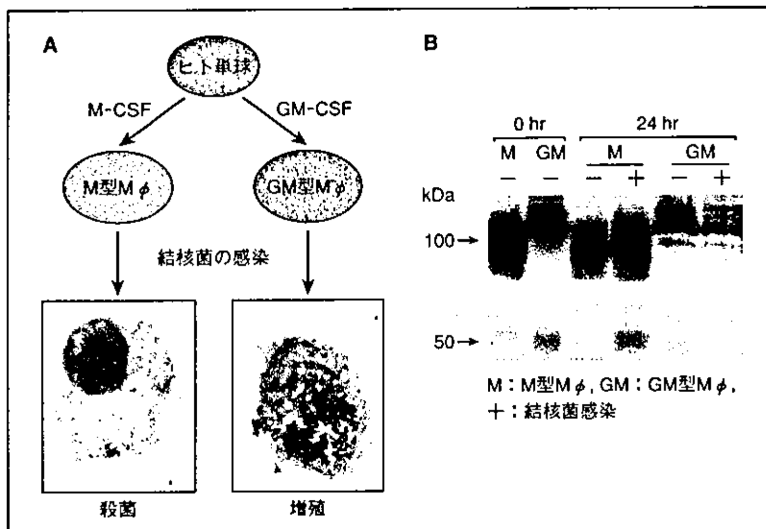

図3 ヒトマクロファージにおける結核菌の殺菌・増殖（A）と結核菌感染によるM型マクロファージにおけるNRAMP1の発現（B）

NOS2$^{-/-}$マウスと同レベルに結核菌に対する感受性が高まるが，NOS2の発現や活性は影響されず正常である[11]．この結果は，NOS2非依存性の系がIFN-γにより活性化され，それが宿主の結核菌感染抵抗性に重要な役割をもつこと，そしてその系に関与する分子がLrg47であることを示している．野生型Mφと異なり，Lrg47$^{-/-}$マウス由来Mφでは，IFN-γによるファゴソームのpH低下を認めない．Lrg47などのp47 GTPaseは小胞体/ゴルジ体に存在すること，またIFN-γ活性化野生型Mφでは結核菌のファゴソームにその存在を認めることより，Lrg47がファゴリソソームの形成をコントロールしていることが示唆されている．また，Lrg47$^{-/-}$マウスへの非定型抗酸菌症の原因菌である*Mycobacterium avium*の感染実験からは，Lrg47がリンパ球のアポトーシス抑制因子としても作用している可能性が示唆されている[12]．

●ヒトMφの結核菌感染感受性とNRAMP1（SLC11A1）

Nramp1（natural resistance-associated macrophage protein 1；Slc11A1）は，マウスのBCG，サルモネラ，リーシュマニアなどの細胞内寄生病原体の感染抵抗性を規定している遺伝子である．*Nramp1*は，Mφおよび好中球に特異的に発現し，エンドソームやリソソームの膜上に存在が認められる，分子量53,000の10回ほど膜を貫通する膜タンパク質をコードしている．169番目のグリシンがアスパラギンへ変換することで抵抗性から感受性に変化する．Nramp1タンパク質の機能として，2価金属のトランスポーターとしてファゴソームからのZn^{2+}，Cu^{2+}，Fe^{2+}，Mn^{2+}などの排出や移入の制御作用が示された[13,14]．Fe^{2+}は細菌の増殖に必要であり，またこれらの2価金属は多くの酵素の補助因子として重要であり，その欠如はファゴソーム内での菌の増殖抑制に関与すると考えられている．また一方で，Fe^{2+}は，Fenton反応によるヒドロキシルラジカル（・OH）の産生に必要であり，・OHは，その後NOと反応して，より殺菌活性の高いperoxynitriteを合成する．それゆえ，Nramp1による2価イオンのトランスポートの制御は，Mφの殺菌活性に重要な役割を果たす．

*NRAMP1*は，マウス*Nramp1*遺伝子のヒトホモログである．マウス*Nramp1*と異なりヒトの*NRAMP1*は，コード部分における遺伝子変異は認められないが，プロモーターの（GT)$_n$部分に多型の存在が知られ，*NRAMP1*の発現が低いアレル2は，ヒトの結核やハンセン氏病などの感染症に対する感受性との関

連が，また発現が高いアレル3はリウマチなどの自己免疫疾患の発症との関連が数多く報告されている[14]．

筆者らは，図3Aに示すように，ヒト単球よりマクロファージコロニー刺激因子（M-CSF）で分化誘導したM型Mφと顆粒球マクロファージコロニー刺激因子（GM-CSF）で誘導したGM型Mφに有毒結核菌H37Rvを感染させると，M型Mφでは殺菌が，GM型Mφでは菌の増殖を認めることを見出した[15]．M型Mφは，結核菌感染によりNRAMP1タンパク質の発現増強を認めたが（図3B），GM型Mφでは発現が抑制されることから，NRAMP1タンパク質の発現と結核菌の増殖抑制との関連が示唆された．上記したように，マウスMφはIFN-γで結核菌殺菌活性の活性化を誘導できるが，ヒトMφでは誘導できないことが数多く報告されている．筆者らの場合も，IFN-γではGM型Mφの結核菌の増殖抑制や殺菌を誘導できない．しかし，GM型MφをIL-10で処理すると菌の増殖を抑制し，NRAMP1タンパク質発現も誘導された．現在のところ，ヒトMφにおけるNRAMP1タンパク質の発現が，直接結核菌の殺菌活性と関連するかどうかは明らかでない．しかし，変異型Nramp1を発現するマウスMφ細胞株に野生型のNramp1を発現させサルモネラ菌を感染させると，IFN-γで活性化しなくてもファゴリソソーム融合が起こり殺菌されることが報告されている．筆者らの実験において，NRAMP1を発現するMφのファゴリソソーム融合がどのようになっているのか，今後の課題である．

ヒトの樹状細胞でもIL-10は結核菌の増殖を抑制することが報告されている[16]．一般的に，IL-10はIFN-γやLPSによるMφの活性化を抑制することから，抑制性サイトカインとして知られてきた．しかし，IL-10は必ずしもMφの機能に対して抑制的に作用するわけでなく，M-CSFによる単球からのMφの分化，Fcレセプターの発現，活性酸素産生能などの増強を誘導し，またNK細胞の活性増強作用も有する[17, 18]．IL-10は，むしろ自然免疫応答におけるMφやNK細胞の活性化に実は重要な役割を果たしているのかもしれない．

Mφにおける細胞内寄生細菌に対する殺菌機構およびその活性化機構はかなり明らかにされた．また，病原細菌およびその菌体由来成分によるそれらの活性阻害機構も少しずつ明らかになってきた．これらの研究の進歩は，それらをターゲットにした新たな抗菌活性をもつ薬剤の開発にも有用と思われる．しかし，本稿にも述べたように，マウスMφとヒトMφではかなり異なる面もあることから，今後はヒトMφにおける殺菌機構やその活性化機構の詳細を明らかにすることも大事であろう．また，生体においては，細菌感染症の病態の発現や防御機構におけるMφの役割や機能は，T細胞をはじめとする免疫応答や炎症に関与する種々の細胞との関係により成り立っていることから，それらの点についてもよりいっそうの解明が必要であろう．

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Pulmonary Collectins Enhance Phagocytosis of *Mycobacterium avium* through Increased Activity of Mannose Receptor[1]

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Collectins, including surfactant proteins A (SP-A) and D (SP-D) and mannose binding lectin (MBL), are the important constituents of the innate immune system. *Mycobacterium avium*, a facultative intracellular pathogen, has developed numerous mechanisms for entering mononuclear phagocytes. In this study, we investigated the interactions of collectins with *M. avium* and the effects of these lectins on phagocytosis of *M. avium* by macrophages. SP-A, SP-D, and MBL exhibited a concentration-dependent binding to *M. avium*. The binding of SP-A to *M. avium* was Ca^{2+}-dependent but that of SP-D and MBL was Ca^{2+}-independent. SP-A and SP-D but not MBL enhanced the phagocytosis of FITC-labeled *M. avium* by rat alveolar macrophages and human monocyte-derived macrophages. Excess mannan, zymosan, and lipoarabinomannan derived from the *M. avium*-intracellular complex, significantly decreased the collectin-stimulated phagocytosis of *M. avium*. Enhanced phagocytosis was not affected by the presence of cycloheximide or chelation of Ca^{2+}. The mutated collectin, $SP\text{-}A^{E195Q,\ R197D}$ exhibited decreased binding to *M. avium* but stimulated phagocytosis to a level comparable to wild-type SP-A. Enhanced phagocytosis by cells persisted even after preincubation and removal of SP-A or SP-D. Rat alveolar macrophages that had been incubated with SP-A or SP-D also exhibited enhanced uptake of ^{125}I-mannosylated BSA. Analysis by confocal microscopy and flow cytometry revealed that the lung collectins up-regulated the cell surface expression of mannose receptor on monocyte-derived macrophages. These results provide compelling evidence that SP-A and SP-D enhance mannose receptor-mediated phagocytosis of *M. avium* by macrophages. ***The Journal of Immunology,* 2004, 172: 7592–7602.**

Pulmonary surfactant is a complex mixture of lipids and proteins that functions to keep alveoli from collapsing at expiration (1). Surfactant proteins A (SP-A)[3] and D (SP-D) are important constituents of lung surfactant (2) and are synthesized and secreted by alveolar type II cells (3). SP-A and SP-D belong to the collectin subgroup of C-type lectin superfamily along with mannose-binding lectin (MBL) and conglutinin (4). The lung collectins are now recognized to be important modulators of innate immunity. Lung collectins can bind to a wide spectrum of microorganisms (5, 6) and interact with macrophages (7–9) and enhance phagocytosis of a variety of microorganisms, including *Staphylococcus aureus* (10), HSV type I (11), type A *Haemophilus influenzae* (12), and *Klebsiella* (13). The lung collectins have been shown to have a direct antimicrobial effect on Gram-negative bacteria by increasing membrane permeability (14). Direct fungicidal effects of SP-A and SP-D have also been recently described (15). Transgenic mice with null alleles for SP-A and SP-D exhibit reduced bacterial clearance and elevated proinflammatory cytokine secretion in response to microbial challenge (16–19). In vitro studies have revealed that SP-A modulates the cellular response to LPS by interaction with CD14 (20) and inhibits peptidoglycan- or zymosan-induced TNF-α secretion by direct interaction with Toll-like receptor 2 on macrophages (21, 22). Lung collectins have also been shown to modulate lung inflammation by interacting with signal inhibitory regulatory protein α and calreticulin/CD91 (23). These studies indicate that SP-A and SP-D play crucial roles in the regulation of pulmonary inflammation and host defense of the lung.

Mycobacterium avium is a member of group III nontuberculous mycobacteria and a facultative intracellular pathogen similar to *Mycobacterium tuberculosis* (*M.tb*). Attention has been focused on *M. avium* because the bacteria infects patients suffering from AIDS. The cell wall envelope of *M. avium* consists of large amounts of fatty acids (mycolic acids) which are covalently linked to an arabinogalactan-peptidoglycan cell wall core and a number of highly unusual lipids and glycolipids, including lipoarabinomannan (LAM), phenolic glycolipids, and glycopeptidolipids (24–26). During the early phase of the infection, *M. avium* stimulates secretion of cytokines including TNF-α, IL-6, IL-12, and IL-10 (27) and subsequent IFN-γ production by NK cells. Recent studies have demonstrated that Toll-like receptor 2 mRNA is induced following infection of murine macrophages with *M. avium* (28), and SP-A decreases *M. avium*-elicited NO production by IFN-γ-primed murine alveolar macrophages (29). *M. avium* enters and

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[3] Abbreviations used in this paper: SP-A, surfactant protein A; SP-D, surfactant protein D; MBL, mannose-binding lectin; wt, wild type; MDM, monocyte-derived macrophage; LAM, lipoarabinomannan; LM, lipomannan; MAC, *M. avium*-intracellular complex; *M.tb*, *Mycobacterium tuberculosis*; MR, mannose receptor; CRD, carbohydrate recognition domain; CR, complement receptor.

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can survive in macrophages, although the mechanism of entry into host cells is poorly understood.

M.tb is also a intracellular bacterial pathogen. Both SP-A and SP-D bind *M.tb*, but the effects of these proteins on the *M.tb* uptake are different (30, 31). SP-A directly interacts with macrophages and enhances phagocytosis of the virulent Erdman strain of *M.tb* by up-regulation of mannose receptor (MR) activity (30), and enhances MR expression on monocyte-derived macrophages (MDMs) (32). In contrast, SP-D exhibits reduced adherence of *M.tb* to MDMs (33, 34).

It is very difficult to manage *M. avium* infection because of the ineffective nature of the cell-mediated immune response by immunosuppressed hosts and the intrinsic drug resistance of the bacteria. Since the interaction of *M. avium* with the collectins and its uptake into macrophages are not well understood, we examined these processes. The purposes of this study were to determine 1) the direct binding of the collectins to *M. avium*, 2) the effect of these proteins on the phagocytosis of *M.avium* by mononuclear phagocytes, and 3) the role of the proteins in altering phagocytic receptor activity. Our findings demonstrate that SP-A, SP-D, and MBL bind to *M. avium* and that SP-A and SP-D but not MBL enhance the phagocytosis of *M. avium* by macrophages through up-regulating MR activity.

Materials and Methods

Collectins

Human SP-A and SP-D derived from bronchoalveolar lavage fluid of an alveolar proteinosis patient were purified by affinity chromatography on mannose-Sepharose 6B, followed by gel filtration as described previously (35, 36). Rat SP-A and SP-D were also purified from the lung lavage fluids of Sprague Dawley rats that had been given intratracheal instillation of silica (37) by the method described elsewhere (35, 38). Recombinant wild-type (wt) rat SP-A, a mutant SP-A (SP-A$^{E195Q, R197D}$), and recombinant rat MBL (mannose-binding protein A) were expressed by the baculovirus-insect cell expression system and purified as described previously (39, 40).

Isolation of rat alveolar macrophages

Alveolar macrophages were isolated from bronchoalveolar lavage fluids of Sprague Dawley rats by lavaging the lungs with pyrogen-free saline (Otsuka Pharmaceutical, Tokyo Japan) and sedimenting the cells at 150 × *g* for 10 min.

Isolation of human macrophages

Human MDMs were isolated from whole blood buffy coats obtained from healthy volunteers (Hokkaido Red Cross Blood Center, Sapporo, Japan) according to the method of Ferguson et al. (33). Mononuclear cells were isolated from heparinized blood on Ficoll gradients and the MDM fraction was purified by adherence. The MDMs were cultured in Teflon wells (Savillex, Minnetonka, MN) for 5 days in the presence of 10% pooled AB$^+$ human serum (Sigma-Aldrich, St. Louis, MO).

Growth and preparation of bacteria

M. avium was obtained from sputum of an infected patient. The bacteria were cultured for 14 days in Mycobroth (Kyokuto Pharmaceutical, Tokyo, Japan) and were then heat-killed at 90°C for 5 min. The bacterial suspension was centrifuged at 2500 × *g* and the pellet was resuspended in PBS. The concentration of bacteria was adjusted at 0.4–1.0 × 10^8 CFU/ml by measuring absorbance at 600 nm and the suspension was stored at −80°C until its use.

Labeling of M. avium *with FITC*

FITC- labeling of heat-treated *M. avium* was performed by a method based on that described by Tino and Wright (41). Briefly, a 1 ml-suspension of *M. avium* in 0.1 M sodium carbonate (pH 9.0) was mixed with 1 μl of FITC (10 mg/ml in DMSO; Molecular Probes, Eugene, OR) and incubated for 1 h in the dark, at room temperature, with gentle agitation. FITC-labeled bacteria were washed by centrifugation in PBS and diluted to 1.0 × 10^9 CFU/ml and stored at −80°C.

Extraction of crude LAM

Extraction of LAM from the *M. avium*-intracellular complex (MAC) was performed according to the method of Khoo et al. (42). Briefly, cells (10 g wet weight) were suspended in PBS containing 2% (v/v) Triton X-114 (PBS/Triton X-114) and were disrupted by probe sonication. The suspension was centrifuged at 27,000 × *g* for 1 h at 4°C. The cell pellet was suspended with PBS/Triton X-114 and centrifuged as described above. The combined supernatant was placed at 37°C to initiate biphase formation and centrifuged at 12,000 × *g* for 15 min at room temperature. The aqueous layer was re-extracted and the detergent layer was washed with PBS. The final detergent layers were precipitated with 9 volumes of acetone. The acetone precipitates were dried under a stream of nitrogen and partitioned between phenol and water. The aqueous layer containing LAM and lipomannan (LM) was freeze dried after dialysis against water. The final fraction is referred to as crude LAM.

Binding of collectins to M. avium

Rat SP-A was iodinated according to the method of Bolton and Hunter (43) using Bolton-Hunter reagent (Amersham Biosciences, Arlington Heights, IL). The specific radioactivity was typically 200–400 cpm/ng, and >85% of the radioactivity was precipitated by treatment with 10% (w/v) trichloroacetic acid. The binding of ^{125}I-SP-A to *M. avium* was examined using microtiter wells. The bacterial suspensions (1.0 × 10^6 CFU in 40 μl of PBS/well) were put onto the wells, dried under a vacuum, and then fixed by incubating the wells with PBS containing 0.25% glutaraldehyde. After blocking with PBS (pH 7.4) containing 100 mM glycine, nonspecific binding to the wells was blocked with 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 2% (w/v) BSA (blocking buffer). The indicated concentration of ^{125}I-SP-A (50 μl/well) in blocking buffer was added to the wells with bacteria and incubated for 1 h at 37°C. The wells were washed three times with blocking buffer and the ^{125}I-SP-A specifically bound to the bacteria was solubilized in 200 μl of 0.1 M NaOH. The radioactivity of ^{125}I-SP-A recovered was finally determined by using a gamma counter.

Binding experiments were also performed using specific Abs to detect the recombinant SP-As, SP-D, and MBL. SP-A, SP-D, or MBL (50 μl/well) in blocking buffer was incubated with *M. avium* coated onto the microtiter wells. The wells were washed with PBS (pH 7.4) containing 0.1% (v/v) Triton X-100 (buffer A). Subsequently, either HRP-labeled anti-rat SP-A IgG (1/250), or anti-rat SP-D IgG (1/50) or anti-rat MBL IgG in PBS (pH 7.4) containing 0.1% (v/v) Triton X-100 and 3% (w/v) skim milk were added and incubated for 1 h at 37°C. After washing the wells with buffer A, the peroxidase reaction was performed using *o*-phenylenediamine as a substrate. The binding of the collectins to *M. avium* was quantified by measuring absorbance at 492 nm.

In some experiments, the wells were incubated with the indicated concentrations of the collectins in the absence or the presence of 5 mM EDTA to determine Ca^{2+}-dependent binding. Where indicated, unlabeled rat SP-A (20–200 μg/ml), crude LAM (0.1–100 μg/ml) from MAC or 50 μg/ml mAbs or mouse control IgG were added as selective inhibitors of specific binding.

Phagocytosis of M. avium *by macrophages*

Rat alveolar macrophages (2.0 × 10^5) were incubated with FITC-labeled *M. avium* (1.0 × 10^7 CFU) in 200 μl of HBSS (Life Technologies, Grand Island, NY) in the absence or the presence of the indicated concentrations of the collectins for 30 min at 37°C. The assay was stopped by the addition of ice-cold PBS to the macrophage-bacteria suspension. The bacteria that had not been associated with the cells were separated from cell-associated bacteria by centrifugation at 150 × *g*. The cell pellet was washed three times with PBS. The cells were next suspended with 5 μl of 25 μg/ml ethidium bromide in PBS and pipetted onto a slide. The number of macrophages with or without intracellular (green fluorescent) bacteria were counted in at least 100 macrophages per slide for duplicate samples using fluorescence microscopy at ×200 magnification. The results were expressed as the percentage of total macrophages that contained intracellular bacteria. In some experiments, the phagocytosis assay was performed in the absence or the presence of 2 mM EDTA, 4 mg/ml mannan, 50 μg/ml crude LAM, or 10–100 μg/ml zymosan. To determine whether new protein synthesis was involved in collectin-enhanced phagocytosis, 10 μg/ml cycloheximide (Sigma-Aldrich) was preincubated with the macrophages for 60 min at 37°C before the addition of the collectin and the bacteria.

The analysis of phagocytosis was also conducted using MDMs. MDMs were plated onto glass coverslips in 24-well plates (Falcon; Costar, Cambridge, MA) for 2 h at 37°C (2.0 × 10^5/coverslip). The wells were washed with RPMI 1640 and then incubated with FITC-labeled *M. avium* (1.0 ×

10^7 CFU) in 200 μl of HBSS in the absence or presence of the indicated concentrations of human SP-A, rat SP-D, recombinant rat SP-A, or recombinant rat MBL for 1 h at 37°C in a tissue culture incubator. After the incubation, the cells were washed with PBS, stained with ethidium bromide, and *M. avium* phagocytosis was evaluated as described above.

Uptake of ^{125}I-mannosylated BSA by rat alveolar macrophages

Mannosylated BSA was iodinated according to the method of Bolton and Hunter (43) using Bolton-Hunter reagent (Amersham Biosciences). The specific radioactivity ranged from 51 to 57 cpm/ng, and >96% of the radioactivity was precipitated by treatment with 10% (w/v) trichloroacetic acid. Rat alveolar macrophages (2×10^5/well) were seeded onto 48-well plates (Falcon) for 2 h in RPMI 1640 containing 10% (v/v) FCS (RPMI 1640/FCS). The medium was removed and each monolayer was washed with RPMI 1640/FCS. Next, rat SP-A (0–20 μg/ml) or SP-D (5 μg/ml) in RPMI 1640 containing 1 mg/ml BSA (RPMI 1640/BSA) was incubated with the cells for 1 h at 37°C. After the incubation with the collectins, the cells were washed with RPMI 1640/BSA. Subsequently, the indicated concentration of ^{125}I-mannosylated BSA (200 μl/well) in RPMI 1640/BSA was added, followed by further incubation for 30 min at 37°C. The monolayers were then washed with 50 mM Tris (pH 7.4) containing 0.15 M NaCl and 2 mg/ml BSA. A final wash was performed with 50 mM Tris (pH 7.4) containing 0.15 M NaCl, and the macrophages were removed from the wells by dissolution in 0.1 M NaOH. The amount of ^{125}I-mannosylated BSA associated with the cells was determined using a gamma counter.

Immunostaining of the MR and fluorescence microscopy

MDMs seeded onto glass coverslips were incubated with or without human SP-A (20 μg/ml) or human SP-D (5 μg/ml) for 1 h at 37°C. After the incubation, the cells were washed with PBS, and the cell monolayers were fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature. The cells were then washed with 50 mM PIPES buffer (pH 7.2) containing 100 mM NaCl, 1 mM EGTA, 2 mM $MgCl_2$, and 0.5% (w/v) BSA (buffer B) and were incubated with monoclonal anti-human MR Ab (clone 19.2, 1/100; BD PharMingen, San Diego, CA) in buffer B for 30 min at room temperature. The cells were then washed with buffer B and further incubated with Alexa 488-conjugated anti-mouse IgG (1/500; Molecular Probes) for 45 min at room temperature. The cells were finally washed with buffer B, sealed in the presence of Vectorshield Antifade (Vector Laboratories, Burlingame, CA), and examined using a laser microscope (LSM510; Zeiss, Tokyo, Japan) with a ×63 oil planapochromatic lens (NA 1.4).

Binding of collectins to crude LAM isolated from MAC

Crude LAM was isolated from MAC as described above and was electrophoresed and transferred onto the nitrocellulose membrane. The membrane was overlayed and incubated with 5 μg/ml rat SP-A, SP-D, or BSA for 3 h at room temperature after blocking the membrane with buffer A containing 3% (w/v) skim milk. The protein binding to LAM and LM was detected by Ab to each protein followed by the incubation with HRP-labeled anti-rabbit IgG.

The binding of SP-D to crude LAM was also performed by using crude LAM (1 μg/well) coated onto microtiter wells. The wells were incubated with the indicated concentrations of rat SP-D in the presence of 5 mM $CaCl_2$ or 5 mM EDTA for 3 h at 37°C. The SP-D binding to crude LAM was detected by anti-SP-D Ab.

Flow cytometry

MDMs (1.0×10^6) were incubated with or without 20 μg/ml human SP-A, human SP-D, or MBL. After washing the cells with PBS, they were fixed with 1% (w/v) paraformaldehyde. The cells were then washed and incubated on ice for 45 min with PE-conjugated mouse anti-human MR mAb (clone 19.2; BD PharMingen) or PE-conjugated mouse IgG1, κ monoclonal Ig isotype control (clone MOPC-31C; BD PharMingen) in PBS containing 2% FCS (v/v) and 0.1% (w/v) sodium azide. The cells were finally washed and analyzed by using FACSCalibur and CellQuest software (BD Biosciences).

Phagocytosis of zymosan

Rat alveolar macrophages (2.0×10^5) were incubated at 37°C for 30 min with or without 20 μg/ml rat SP-A or 5 μg/ml rat SP-D in the absence or presence of 2 mM EDTA or 4 mg/ml mannan. The cell suspension was further incubated with FITC-conjugated zymosan A (Molecular Probes) at 37°C for 30 min. The cells were then washed with PBS, stained with ethidium bromide, and the phagocytosis was evaluated as described above.

Uptake of FITC-conjugated mannosylated BSA by rat alveolar macrophages

Rat alveolar macrophages (1.0×10^5/well) were seeded onto 96-well plates (Matrix Technologies, Hudson, NH) and incubated for 3 h in RPMI 1640 containing 10% (v/v) FCS. After the incubation, the cells were washed with the medium and were incubated at 37°C for 30 min with the indicated concentrations of FITC-conjugated mannosylated BSA (Sigma-Aldrich) in the absence or presence of rat SP-A (20 μg/ml) or rat SP-D (5 μg/ml) in HBSS containing 1 mg/ml BSA. In some experiments, 2 mM EDTA was included in the incubation buffer. The cells were then washed three times with 50 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and 2 mg/ml BSA and the final wash was performed by using Tris buffer without BSA. The fluorescence intensities of FITC-mannosylated BSA associated with the cells were measured at 485 nm (excitation) and 528 nm (emission) using Synergy HT and KC4 software (Bio-Tek Instruments, Watford Herts, U.K.). Specific uptake of mannosylated BSA was calculated by subtracting the fluorescence intensity of the FITC-labeled protein binding to the wells without cells from total fluorescence intensity.

Binding of FITC-conjugated mannosylated BSA and FITC-labeled M. avium *to rat alveolar macrophages*

Rat alveolar macrophages (5.0×10^5) were incubated with or without rat SP-A (20 μg/ml) at 4°C for 4 h in HBSS containing 1 mg/ml BSA. The cell suspension was washed three times with PBS and incubated at 4°C for 1 h with FITC-mannosylated BSA (5 μg/ml) or FITC-labeled *M. avium* (5.0×10^6 CFU) in HBSS containing 1 mg/ml BSA. After washing the cells with PBS, the cell suspension was resuspended with 200 μl of PBS containing 1% (w/v) paraformaldehyde and put onto 96-well plates (Matrix Technologies). The fluorescence intensities of FITC-mannosylated BSA or FITC-labeled *M. avium* binding to the cells were finally determined using Synergy HT and KC4 software (Bio-Tek Instruments) as described above.

Results

SP-A and SP-D bind M. avium *with high affinity.*

We first examined the binding of SP-A to *M. avium* coated onto microtiter wells by using ^{125}I-labeled rat SP-A. ^{125}I-SP-A bound to *M. avium* in a concentration-dependent manner (Fig. 1*A*). The Scatchard plot analysis (Fig. 1*A*, *inset*) gives an approximate dissociation constant of 32.6 ± 2.34 nM ($n = 3$, mean ± SE), assuming that the molecular mass of SP-A is 0.64 MDa (2). Eighty-six nanograms of SP-A was capable of binding to 10^6 CFU of bacteria. Inclusion of EDTA in the binding buffer almost completely abolished the binding of SP-A to *M. avium* (Fig. 1*B*). Unlabeled SP-A competed well with ^{125}I-labeled SP-A for the binding to *M. avium* (Fig. 1*C*), indicating the specific binding of SP-A to *M. avium*. One hundred-fold excess unlabeled SP-A inhibited ^{125}I-SP-A binding by 86.81 ± 2.52% ($n = 3$, mean ± SE). To further characterize the SP-A binding to *M. avium*, we examined the effect of crude LAM containing LAM and LM upon the interaction. LAM and LM are major cell wall-associated lipoglycans, which were isolated from MAC. Excess LAM failed to attenuate the binding of SP-A to *M. avium* (Fig. 1*D*), indicating that LAM is not a ligand for SP-A on *M. avium*. This was consistent with the result obtained from a ligand blot showing that no SP-A protein was detected on LAM and LM that were transferred onto the nitrocellulose membrane (Fig. 2*C*). The effect of anti-rat SP-A mAbs on the rat SP-A binding to the bacteria was also examined (Fig. 1*E*). Ab 1D6 completely blocked the SP-A binding to *M. avium* although Ab 6E3 decreased its binding to a significant extent. In contrast, control mouse IgG or anti-human SP-A mAb, PE10, did not affect the binding of rat SP-A to *M. avium*. Since the epitopes for Abs 1D6 and 6E3 are localized at the carbohydrate recognition domain (CRD) and the neck domain of rat SP-A, respectively (44), these data suggest that SP-A binds to *M. avium* through its CRD. This result is also consistent with the Ca^{2+} dependence of binding since most CRD interactions require Ca^{2+}.

Rat SP-D also exhibited concentration-dependent and saturable binding to *M. avium* (Fig. 2*A*). However, unlike SP-A, inclusion of

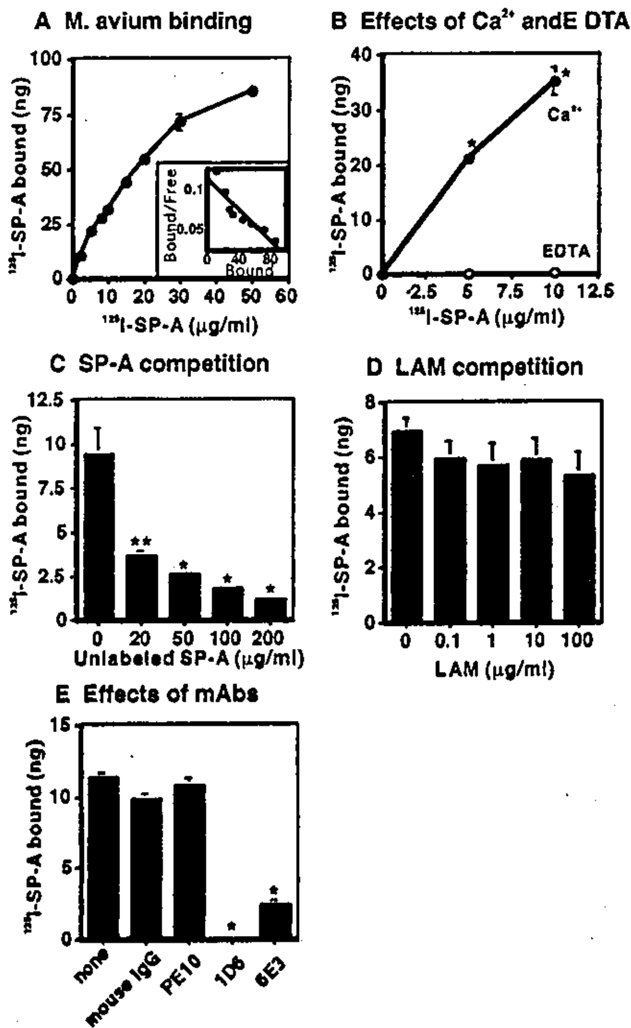

FIGURE 1. SP-A binds *M. avium* with high affinity and LAM is not a competitor for the interaction. *A* and *B*, The indicated concentrations of ^{125}I-rat SP-A were incubated at 37°C for 1 h with *M. avium* (10^6 CFU/well) coated onto microtiter wells in the presence of 5 mM $CaCl_2$ (●) or 5 mM EDTA (○). The radioactivity bound to the wells was determined using a gamma counter as described in *Materials and Methods*. The results represent the specific binding calculated by subtracting the amounts of the labeled protein binding to the wells without the bacteria from total binding. The data shown are the mean ± SE of three experiments. *, $p < 0.01$ when compared with the binding in the presence of EDTA. The *inset* indicates a Scatchard plot of the binding data. *C* and *D*, ^{125}I-rat SP-A (2 μg/ml) was incubated at 37°C for 1 h with *M. avium* coated onto microtiter wells in the presence of the indicated concentrations of unlabeled SP-A (*C*) or crude LAM (*D*) isolated from MAC. The amount of ^{125}I-SP-A binding to *M. avium* was determined as described above. The data shown are the mean + SE of three experiments. *, $p < 0.01$ and **, $p < 0.05$ when compared with the values obtained without unlabeled SP-A. *E*, ^{125}I-rat SP-A (2 μg/ml) was incubated at 37°C for 1 h with *M. avium* coated onto microtiter wells in the absence or presence of 50 μg/ml control mouse IgG, anti-human SP-A mAb PE10, anti-rat SP-A mAbs 1D6, and 6E3. The amount of ^{125}I-SP-A binding to *M. avium* was determined as described above. The data shown are the mean + SE of three experiments. *, $p < 0.01$ when compared with the binding in the presence of control mouse IgG.

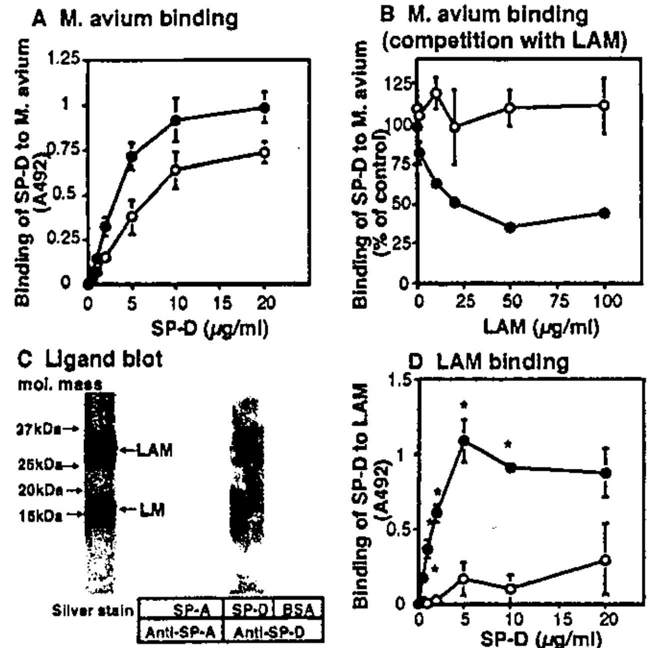

FIGURE 2. SP-D binds *M. avium* with high affinity and exhibits Ca^{2+}-dependent and Ca^{2+}-independent interactions that determine sensitivity to LAM competition. *A*, The indicated concentrations of rat SP-D were incubated with *M. avium* (10^6 CFU/well) coated onto microtiter wells at 37°C for 1 h in the presence of 5 mM $CaCl_2$ (●) or 5 mM EDTA (○). The binding of SP-D to *M. avium* was detected using anti-SP-D IgG as described in *Materials and Methods*. The data shown are the mean ± SE of three experiments. *B*, Five micrograms per milliliter rat SP-D was incubated with *M. avium* in a buffer containing 5 mM $CaCl_2$ (●) or 5 mM EDTA (○) in the presence of the indicated concentrations of crude LAM isolated from MAC. The binding of SP-D to *M. avium* was determined as described above. The results are expressed as percentages of the binding in the absence of LAM. The data shown are the mean ± SE of three experiments. *C*, Ligand blot analysis of crude LAM. Crude LAM isolated from MAC was electrophoresed and transferred onto the nitrocellulose membrane. LAM and LM were detected with silver stain (*left lane*). The membrane was also overlayed with 5 μg/ml rat SP-A, SP-D, or BSA. The protein binding to LAM and LM was detected by Ab to each protein followed by the incubation with HRP-labeled anti-rabbit IgG. *D*, Crude LAM (1 μg/well) coated onto microtiter wells was incubated with the indicated concentrations of rat SP-D in the presence of 5 mM $CaCl_2$ (●) or 5 mM EDTA (○). The SP-D binding to LAM was detected by anti-SP-D Ab as described above. The data shown are the mean ± SE of three experiments. *, $p < 0.01$ when compared with the binding in the presence of EDTA.

EDTA in the binding buffer failed to abolish the binding of SP-D to *M. avium* (Fig. 2*A*), indicating that the majority of the SP-D binding to *M. avium* is Ca^{2+} independent. Excess crude LAM attenuated the SP-D's binding to *M. avium* in the presence of Ca^{2+} but failed to inhibit its binding in the presence of EDTA (Fig. 2*B*). Analysis by ligand blot and microtiter well binding revealed that SP-D but not SP-A binds to LAM and LM (Fig. 2*C*), and that the binding of SP-D to crude LAM coated onto microtiter wells is Ca^{2+} dependent (Fig. 2*D*). These results indicate that SP-D can bind to LAM and LM on *M. avium* in the presence of Ca^{2+}, but suggest that it is also capable of binding to molecules other than LAM and LM when Ca^{2+} is chelated.

SP-A and SP-D enhance the phagocytosis of M. avium *by macrophages*

We next examined the effects of SP-A and SP-D on the phagocytosis of *M. avium* by macrophages. Rat alveolar macrophages (2×10^5) were incubated with FITC-labeled *M. avium* for 30 min in the absence or presence of rat SP-A or SP-D (Fig. 3, *A* and *B*). Both SP-A and SP-D enhanced the phagocytosis of *M. avium* by rat alveolar macrophages in a concentration-dependent manner. SP-A at 20 μg/ml increased the number of cells phagocytosing the bacteria from 13 to 32% of the population. SP-D at 5 μg/ml also

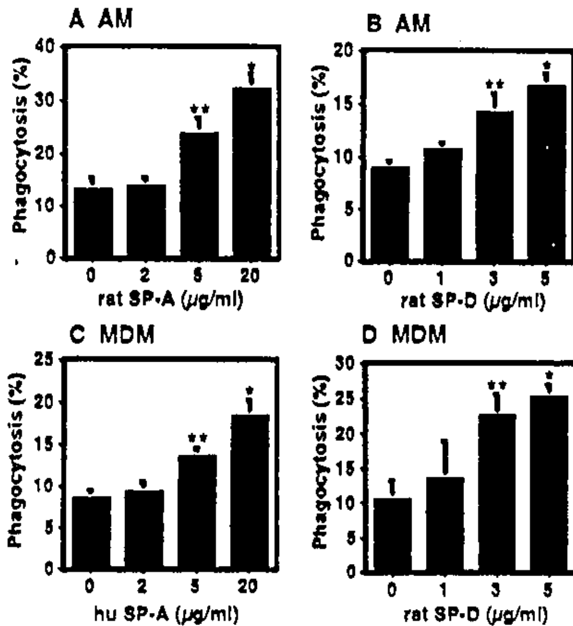

FIGURE 3. SP-A and SP-D enhance the phagocytosis of *M. avium* by macrophages. *A* and *B*, Rat alveolar macrophages (2×10^5) were incubated with FITC-labeled *M. avium* (1×10^7 CFU) at 37°C for 30 min in the absence or presence of the indicated concentrations of rat SP-A (*A*) or rat SP-D (*B*). After washing the cells to remove unbound bacteria, the cells were suspended with ethidium bromide solution and the numbers of macrophages with or without intracellular bacteria were counted. The results are expressed as percentage of macrophages containing intracellular bacteria in total macrophages counted (percent phagocytosis) as described in *Materials and Methods*. The data shown are the mean + SE of three experiments. *, $p < 0.01$ and **, $p < 0.05$ when compared with the phagocytosis without SP-A or SP-D. *C* and *D*, Human MDMs plated onto glass coverslips (2×10^5) were incubated with FITC-labeled *M. avium* (1×10^7 CFU) at 37°C for 1 h in the absence or presence of the indicated concentrations of human SP-A (*C*) or rat SP-D (*D*). The percent phagocytosis was determined as described above. The data shown are the mean + SE of three experiments. *, $p < 0.01$ and **, $p < 0.05$ when compared with the phagocytosis without SP-A or SP-D.

increased the uptake of *M. avium* from 9 to 16% of the population. When 200 ng/ml LPS was incubated with alveolar macrophages in the presence of FITC-labeled *M. avium*, the uptake of *M. avium* was not augmented. In addition, the endotoxin-free SP-A, that had been treated with polymyxin B in the presence of octyl-β-D-glucoside (20) augmented *M. avium* phagocytosis at a level comparable to nontreated SP-A, 32.2 ± 5.6% (mean ± SE, $n = 3$) and 32.2 ± 2.7% for polymyxin-treated SP-A and nontreated SP-A, respectively. These results suggest that the effects of SP-A and SP-D are not due to the endotoxin contamination in the protein preparations. We also examined the effects of lung collectins on *M. avium* phagocytosis by human MDMs. Coincubation of human SP-A or rat SP-D with MDM monolayers and FITC-labeled *M. avium* exhibited a concentration-dependent stimulation of *M. avium* phagocytosis (Fig. 3, *C* and *D*). Human SP-A at 20 μg/ml increased the uptake of *M. avium* from the background level of 8% to the stimulated level of 18%. Rat SP-D at 5 μg/ml augmented the phagocytosis from 10 to 25%.

Binding and uptake of M. avium *by MBL*

Another collectin homologue, MBL, was also examined for *M. avium* binding and macrophage uptake of the bacteria. Recombinant rat MBL exhibited a concentration-dependent binding to *M. avium* (Fig. 4*A*). Its binding was not inhibitable by the presence of EDTA, indicating the Ca^{2+}-independent binding of MBL to the bacteria. Excess crude LAM did not significantly inhibit the MBL binding to *M. avium* (Fig. 4*B*). When MDMs were incubated with FITC-labeled *M. avium* in the presence of 20 μg/ml MBL, the uptake of the bacteria was not significantly increased (Fig. 4*C*), whereas the presence of the pulmonary collectins in the same experiment significantly increased phagocytosis of the bacteria.

Characterization of SP-A- or SP-D-stimulated phagocytosis of M. avium

To characterize SP-A- or SP-D-stimulated phagocytosis of *M. avium*, the phagocytosis assays were conducted under various conditions. Inclusion of 2 mM EDTA instead of $CaCl_2$ did not block

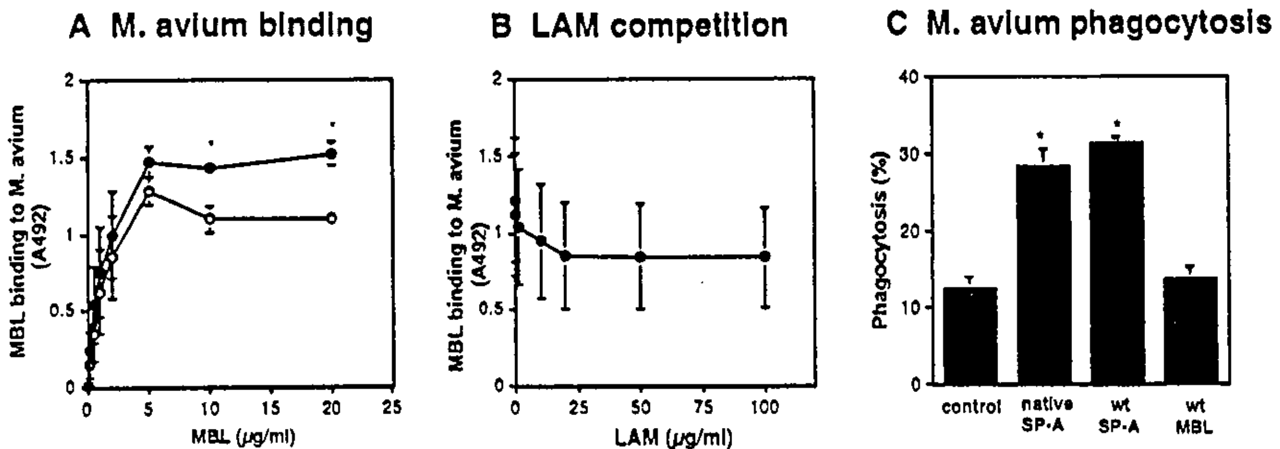

FIGURE 4. MBL binds *M. avium* with high affinity but fails to stimulate its phagocytosis by macrophages. *A*, The indicated concentrations of recombinant rat MBL were incubated with *M. avium* (10^6 CFU/well) coated onto microtiter wells at 37°C for 1 h in the presence of 5 mM $CaCl_2$ (●) or 5 mM EDTA (O). The binding of MBL to *M. avium* was detected using anti-MBL IgG as described in *Materials and Methods*. The data shown are the mean ± SE of three experiments. *B*, Five micrograms per milliliter rat MBL was incubated with *M. avium* in the presence of the indicated concentrations of crude LAM isolated from MAC. The binding of MBL to *M. avium* was determined as described above. The data shown are the mean ± SE of three experiments. *C*, MDMs (2×10^5) were incubated with FITC-labeled *M. avium* (1×10^7 CFU) at 37°C for 30 min in the absence or presence of 20 μg/ml native rat SP-A, wt SP-A, or wt MBL. After washing the cells to remove unbound bacteria, they were suspended in an ethidium bromide solution and the macrophages with or without intracellular bacteria were counted. The results are expressed as percentage of macrophages containing intracellular bacteria in total macrophages counted (percent phagocytosis) as described in *Materials and Methods*. The data shown are the mean + SE of three experiments. *, $p < 0.01$ when compared with the control phagocytosis.

the SP-A-enhanced phagocytosis of *M. avium* by rat alveolar macrophages (Fig. 5*A*). Since the binding of SP-A to *M. avium* is blocked in the presence of EDTA (Fig. 1*B*), the results indicate that the SP-A-stimulated phagocytosis of *M. avium* can occur independently of its binding to the bacteria. EDTA also failed to block the SP-D-stimulated uptake of *M. avium* (Fig. 5*D*).

We next investigated the *M. avium* binding and uptake by rat SP-A mutant (SP-$A^{E195Q,\ R197D}$), which exhibits altered carbohydrate binding specificity such that the rank order affinity for galactose and mannose is the converse of that for wt rat SP-A (39). Native and wt rat SP-As exhibited a concentration-dependent and a saturable binding to *M. avium* coated onto microtiter wells (Fig. 6*A*). SP-$A^{E195Q,\ R197D}$, however, showed significantly decreased binding to *M. avium*. We also examined the effect of this mutant on the phagocytosis of *M. avium*. SP-$A^{E195Q,\ R197D}$ as well as native and wt rat SP-As significantly stimulated the phagocytosis of *M. avium* by rat alveolar macrophages (Fig. 6*B*). Taken together with the results obtained from EDTA experiments (Figs. 1*B* and 5*A*), these results indicate that SP-A enhances *M. avium* phagocytosis, independent of its binding to the bacteria.

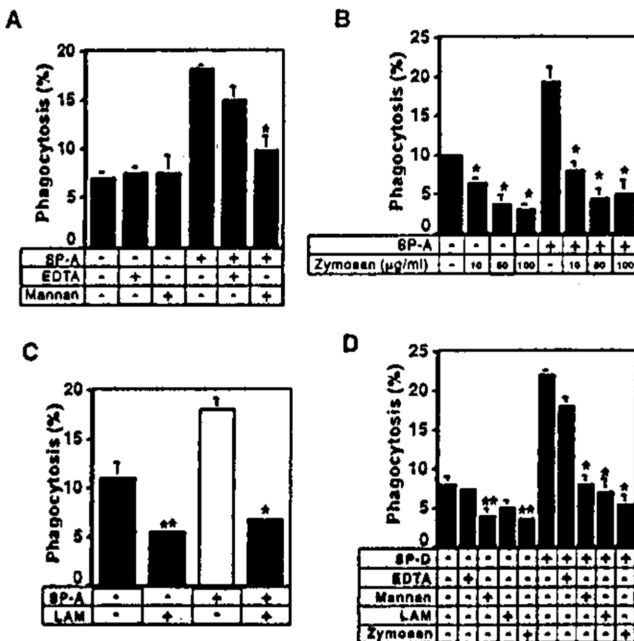

FIGURE 5. Mannan, zymosan, and LAM block the pulmonary collectin-induced increase in *M. avium* phagocytosis. *A–C*, Rat alveolar macrophages (2×10^5) were preincubated with or without 20 μg/ml rat SP-A in the absence or presence of 2 mM EDTA or 4 mg/ml mannan (*A*), 0–100 μg/ml zymosan (*B*), or 50 μg/ml crude LAM (*C*) at 37°C for 30 min. FITC-labeled *M. avium* (1×10^7 CFU) was then added into the cell suspension, and the mixture was further incubated at 37°C for 30 min. After washing the cells to remove unbound bacteria, the cells were suspended with ethidium bromide solution and the numbers of macrophages with or without intracellular bacteria were counted. The results are expressed as percentage of macrophages containing intracellular bacteria in total macrophages counted (percent phagocytosis) as described in *Materials and Methods*. The data shown are the mean + SE of three experiments. *, $p < 0.01$ when compared with the phagocytosis in the absence of the competitors. *D*, Rat alveolar macrophages (2×10^5) were preincubated with or without 20 μg/ml rat SP-D in the presence or absence of 2 mM EDTA, 4 mg/ml mannan, 10 μg/ml zymosan, or 50 μg/ml crude LAM at 37°C for 30 min. FITC-labeled *M. avium* (1×10^7 CFU) was then added into the cell suspension, and the mixture was further incubated at 37°C for 30 min. The percent phagocytosis of *M. avium* was determined as described above. The data shown are the mean + SE of three experiments. *, $p < 0.01$ and **, $p < 0.05$ when compared with the incubation without competitors.

The effects of anti-rat SP-A mAbs were also investigated. Rat SP-A enhanced the phagocytosis of *M. avium* even in the presence of anti-rat SP-A mAbs 1D6 and 6E3 (Fig. 6*C*). The Abs were used at concentrations (50 μg/ml) previously shown to alter CRD-dependent activity of SP-A (44, 45). Since Ab 1D6 almost completely blocked the binding of rat SP-A to *M. avium* (Fig. 1*E*), the results support the conclusion obtained from the experiments with EDTA and SP-$A^{E195Q,\ R197D}$, indicating that SP-A enhances *M. avium* phagocytosis by alveolar macrophages, independent of bacterial binding.

Thus, we determined whether the preincubation of macrophages with the lung collectins stimulated *M. avium* phagocytosis. Native and wt rat SP-As and SP-$A^{E195Q,\ R197D}$ augmented the phagocytosis of *M. avium* by alveolar macrophages (Fig. 7*A*), even after the cells were washed to remove the proteins. Preincubation of SP-D with alveolar macrophages also stimulated *M. avium* uptake to a level comparable to that obtained by coincubation of SP-D and the bacteria with the phagocytes (Fig. 7*B*). These results indicate that interaction of the collectins with macrophages elicits the stimulation of *M. avium* phagocytosis. In addition, Ab experiments suggest that the CRDs or the neck domain of SP-A may not be involved in the SP-A-macrophage interaction. The effect of cycloheximide on the phagocytosis was also examined. SP-A and SP-D retained the activity of stimulating phagocytosis of *M. avium* even in the presence of 10 μg/ml cycloheximide (Fig. 7*C*), suggesting that new protein synthesis is not involved in the stimulatory effects of lung collectins on *M. avium* phagocytosis.

We next examined the effects of the ligands for macrophage MR on the *M. avium* phagocytosis. When 4 mg/ml mannan was included, the stimulatory effect of SP-A on *M. avium* uptake was significantly attenuated (Fig. 5*A*). Consistently, zymosan significantly inhibited the SP-A-stimulated phagocytosis in a concentration-dependent manner (Fig. 5*B*). Crude LAM derived from MAC at 50 μg/ml also significantly blocked the SP-A stimulatory effect on *M. avium* uptake (Fig. 5*C*). Since mannan, LAM, and zymosan are ligands for the macrophage MR (46–48), these data support the idea that SP-A enhances MR-mediated phagocytosis of *M. avium* by macrophages. The effects of mannan, LAM, or zymosan on the SP-D-enhanced *M. avium* phagocytosis were also examined (Fig. 5*D*). The MR ligands of mannan, LAM, and zymosan significantly inhibited the SP-D-mediated phagocytosis. These results clearly show that the lung collectins, SP-A and SP-D, stimulate the MR-mediated phagocytosis of *M. avium* by macrophages.

SP-A and SP-D enhance macrophage MR activity

Since SP-A and SP-D enhance MR-mediated phagocytosis of *M. avium* by direct interaction with macrophages as shown above, we investigated whether these proteins alter MR activity by using ^{125}I-mannosylated BSA as a ligand for MR. Rat alveolar macrophages were preincubated in the absence or presence of 20 μg/ml rat SP-A or 5 μg/ml rat SP-D for 1 h. After washing, the cells were further incubated with the indicated concentrations of ^{125}I-mannosylated BSA for 30 min. The uptake of mannosylated BSA by rat alveolar macrophages was concentration dependent (Fig. 8*A*). Both SP-A and SP-D increased the uptake of mannosylated BSA when compared with that without collectin preincubation. SP-A enhanced mannosylated BSA uptake by 94 ± 28% (mean ± SE, $n = 3$, $p < 0.02$) at 10 μg/ml ^{125}I-mannosylated BSA, when compared with the control. SP-D had an almost identical effect. When rat alveolar macrophages were incubated with FITC-labeled mannosylated BSA in the presence of collectins and EDTA, the fluorescence intensities of the proteins associated with the cells were almost completely diminished (Fig. 8*B*). These results are consistent with the characteristics of MR sharing homology with C-type lectins

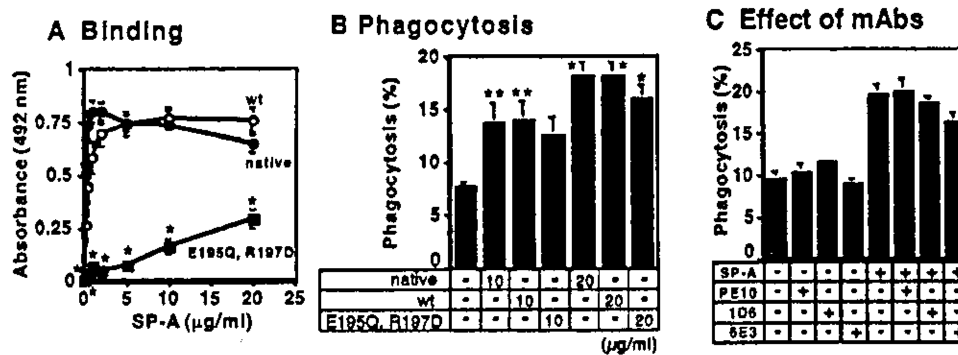

FIGURE 6. SP-A structural mutants and anti-SP-A Abs reveal that *M. avium* binding and enhanced macrophage phagocytosis are different processes. *A*, *M. avium* (10^6 CFU/well) was coated onto microtiter wells and incubated with the indicated concentrations of rat native SP-A (●) or recombinant wt SP-A (○) or SP-A$^{E195Q, R197D}$ (■) at 37°C for 1 h. The binding of SP-A to *M. avium* was detected using anti-SP-A IgG as described in *Materials and Methods*. The data shown are the mean ± SE of three experiments. *, $p < 0.01$ when compared with the binding of wt SP-A at each concentration. *B*, Rat alveolar macrophages (2×10^5) were incubated with FITC-labeled *M. avium* at 37°C for 30 min in the absence or presence of the indicated concentrations of rat native SP-A (native) or wt SP-A (wt) or SP-A$^{E195Q, R197D}$ (E195Q, R197D). The percent phagocytosis of *M. avium* was determined as described in *Materials and Methods*. The data shown are the mean + SE of three experiments. *, $p < 0.01$ and **, $p < 0.05$ when compared with the incubation without SP-As. *C*, Rat SP-A (20 µg/ml) was preincubated with 50 µg/ml anti-rat SP-A mAb 1D6, or 6E3, or control mAb PE10 at 37°C for 1 h, and the mixture was further incubated with rat alveolar macrophages and FITC-labeled *M. avium* at 37°C for 30 min. Phagocytosis of *M. avium* was evaluated as described in *Materials and Methods*. The data shown are the mean + SE of three experiments.

(49). Taken together, these data demonstrate that SP-A and SP-D directly interact with macrophages and up-regulate MR activity.

However, the uptake of FITC-labeled zymosan by alveolar macrophages was not completely blocked by the presence of mannan or EDTA, although it was significantly attenuated (Fig. 8*C*). In addition, SP-A and SP-D failed to augment the uptake of zymosan in the absence of mannan or EDTA. These results suggest that additional mechanisms may be involved in the phagocytosis of zymosan in addition to the MR-mediated phagocytosis.

Alveolar macrophages were first preincubated with 20 µg/ml SP-A and were then washed as described in Fig. 8*A* legend. In our previous report (20), only 0.5 ng/ml unbound SP-A was found to be detectable in the medium after the cells were incubated with 20 µg/ml ^{125}I-SP-A, washed, and replaced with fresh medium, indicating that most of the collectin had been removed by washing.

In addition, the binding experiments with FITC-labeled mannosylated BSA and FITC-labeled *M. avium* to rat alveolar macrophages were performed at 4°C after the cells were preincubated with 20 µg/ml rat SP-A at 4°C, washed, and replaced with the medium containing mannosylated BSA or *M. avium*. The fluorescence intensity of mannosylated BSA that bound to the cell surface with or without SP-A treatment was 1.91 ± 0.07 (mean ± SE, n = 3) or 1.19 ± 0.24, respectively. The fluorescence intensity of *M. avium* that bound to the cell surface with or without SP-A treatment was 11.43 ± 1.69 (mean ± SE, n = 3) or 12.26 ± 3.06, respectively. These results indicate that the amounts of mannosylated BSA or *M. avium* binding to the SP-A-treated cells are not different from those binding to the untreated cells when the cells were preincubated with SP-A at 4°C. Thus, it is unlikely that the increased association of mannosylated BSA shown in Fig. 8*A* is

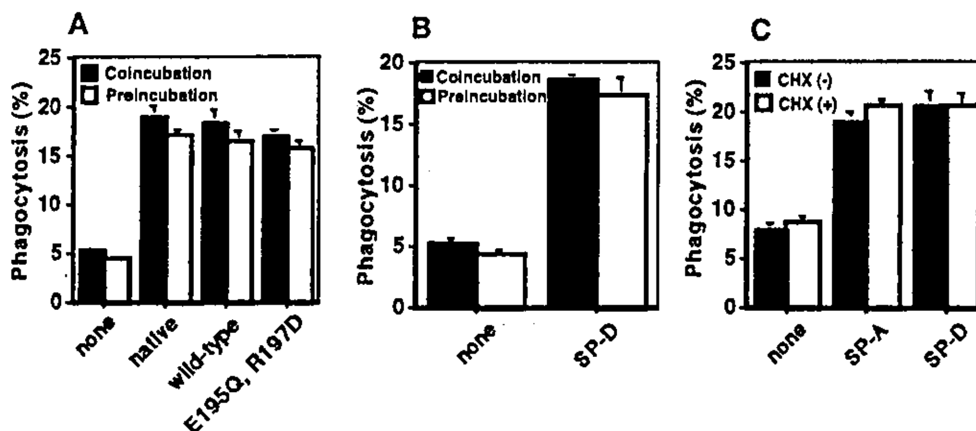

FIGURE 7. Preincubation of macrophages with pulmonary collectins is sufficient to enhance phagocytosis of *M. avium*. *A* and *B*, Rat alveolar macrophages (2×10^5) were preincubated with rat native SP-A, recombinant wt SP-A or SP-A$^{E195Q, R197D}$ (*A*), or rat SP-D (*B*) at 37°C for 1 h, and the medium containing the collectins was removed and the cells were washed before adding FITC-labeled *M. avium*. The percent phagocytosis was determined as described in *Materials and Methods*. The phagocytosis assays were also performed using coincubation of the cells with FITC-labeled *M. avium* and the collectins. The data shown are the mean + SE of three experiments. *C*, Cycloheximide does not decrease SP-A- or SP-D-stimulated phagocytosis of *M. avium*. Ten micrograms per milliliter cycloheximide was preincubated with alveolar macrophages at 37°C for 1 h, and the mixture was further incubated with FITC-labeled *M. avium* in the absence or presence of rat SP-A or rat SP-D for 30 min. The percent phagocytosis was determined as described in *Materials and Methods*. The data shown are the mean + SE of three experiments.

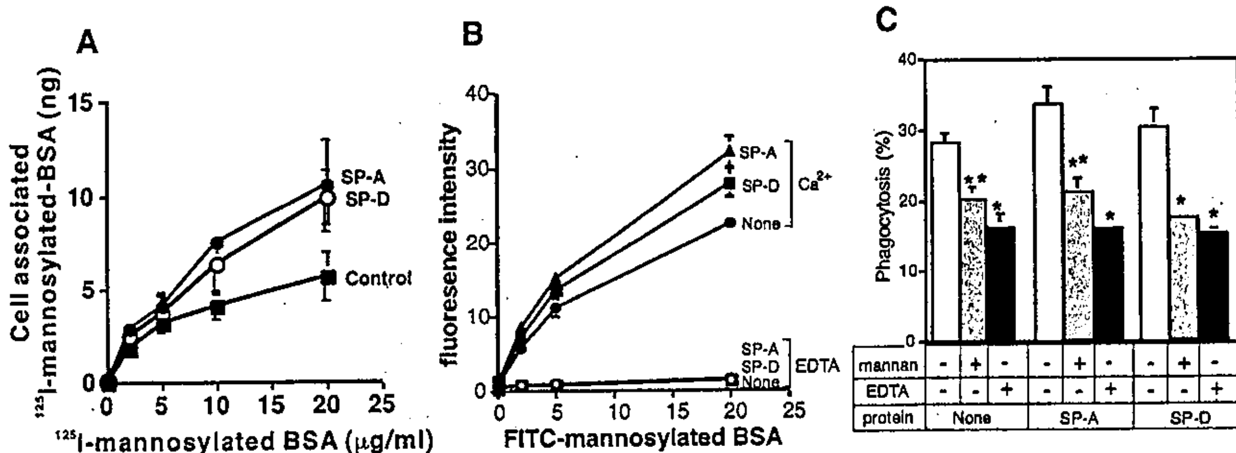

FIGURE 8. SP-A and SP-D enhance the uptake of ^{125}I-mannosylated BSA by rat alveolar macrophages. *A*, Rat alveolar macrophages (2 × 10^5/well) were preincubated in the absence (■) or presence of 20 μg/ml rat SP-A (●) or 5 μg/ml rat SP-D (○) at 37°C for 1 h. After the incubation, the cells were washed and further incubated with the indicated concentrations of ^{125}I-mannosylated BSA for 30 min. The radioactivity associated with the cells was determined using a gamma counter as described in *Materials and Methods*. The data shown are the mean ± SE of three experiments. *B*, Rat alveolar macrophages (1 × 10^5/well) were incubated at 37°C for 30 min with the indicated concentrations of FITC-mannosylated BSA in the absence or presence of rat SP-A (20 μg/ml) or rat SP-D (5 μg/ml). In some experiments 2 mM EDTA was included in the incubation buffer. The fluorescence intensity of FITC-mannosylated BSA associated with the cells was measured at 485 nm (excitation) and 528 nm (emission). Specific uptake of mannosylated BSA was calculated by subtracting the fluorescence intensity of the FITC-labeled protein binding to the well without cells from total fluorescence intensity as described in *Materials and Methods*. The data shown are the mean ± SE of three experiments. *C*, Phagocytosis of zymosan. Rat alveolar macrophages (2 × 10^5) were incubated at 37°C for 30 min with or without 20 μg/ml rat SP-A or 5 μg/ml rat SP-D in the absence or presence of 2 mM EDTA, or 4 mg/ml mannan. The cell suspension was further incubated with FITC-conjugated zymosan A at 37°C for 30 min. The phagocytosis was evaluated as described in *Materials and Methods*. The data shown are the mean + SE of three experiments. *, $p < 0.01$ and **, $p < 0.05$ when compared with the experiments without mannan or EDTA.

due to increased binding of the mannosylated BSA to the collectins that bound to the cell surface of macrophages.

SP-A and SP-D increase cell surface localization of MR on MDMs

We next examined whether SP-A and SP-D enhance MR expression on the MDM cell surface. Human MDMs were first incubated in the absence or presence of 20 μg/ml human SP-A or 5 μg/ml human SP-D for 1 h. After washing, the cells were fixed in paraformaldehyde and immunostained with anti-MR mAb and then analyzed using a confocal microscope. Both SP-A and SP-D up-regulated expression of the MR on MDMs when compared with control (Fig. 9), demonstrating that SP-A and SP-D up-regulate MR expression on MDMs. We further assessed the cell surface expression of MR on MDMs by flow cytometry. MR was constitutively expressed on cell surfaces of MDMs (Fig. 10, gray line). After exposure of SP-A and SP-D, cell surface expression of MR on MDMs was enhanced (Fig. 10, *B* and *C*, solid black line). These results are consistent with those obtained from the uptake experiments with ^{125}I-mannosylated BSA. The data confirm a previous study (32) indicating the MR up-regulation by SP-A and demonstrate for the first time that SP-D increases the MR activity. MBL failed to increase the MR expression on MDMs (Fig. 10*A*), consistent with the finding that this collectin did not stimulate *M. avium* phagocytosis (Fig. 4). Taken together, these results support the idea that lung collectins enhance *M. avium* phagocytosis by increased MR activity on macrophages.

Discussion

This study reveals that three collectins, SP-A, SP-D, and MBL, are capable of binding *M. avium*, but that the mechanisms of binding are different. The binding of SP-A to *M. avium* is Ca^{2+} dependent, while most of the binding by SP-D and MBL is Ca^{2+} independent. In addition, the binding of SP-D, but not SP-A or MBL, to *M. avium* is inhibited by excess LAM and LM isolated from MAC. The inhibition of SP-D binding to *M. avium* by LAM plus LM requires Ca^{2+}. Thus, SP-D may bind to LAM and LM on *M. avium* in the presence of Ca^{2+}, but SP-A and MBL recognize molecules other than LAM and LM. Since crude LAM does not compete with SP-D for the binding to *M. avium* in the presence of EDTA, it is likely that this lectin interacts with molecules other than LAM and LM when Ca^{2+} is chelated. Although SP-A has been reported to bind to LAM isolated from *M.tb* (31), this study shows that SP-A failed to bind to LAM and LM isolated from MAC. LAM derived from *M. avium* has most of its arabinan termini capped with single mannose residues instead of the more common dimannoside as established for LAM from *M.tb.* (42). The structural differences between *M. avium* LAM and *M.tb.* LAM may explain different binding specificities of SP-A to LAM.

Competition experiments reveal that LAM derived from MAC fails to compete for SP-A binding to *M. avium* but blocks *M. avium* phagocytosis. Inclusion of EDTA, which abolishes the binding of SP-A to *M. avium*, does not affect the stimulatory effect of this collectin on *M. avium* phagocytosis. Recombinant rat SP-$A^{E195Q, R197D}$, which exhibits the reduced binding to *M. avium*, still potently enhances the phagocytosis. In addition, anti-rat SP-A mAb 1D6, which completely blocks the SP-A binding to *M. avium*, fails to inhibit SP-A-stimulated phagocytosis. Collectively, these findings demonstrate that SP-A binding to *M. avium* does not parallel the collectin-mediated stimulation of *M. avium* phagocytosis. This conclusion is consistent with the results obtained from experiments, in which preincubation of lung collectins with macrophages enhances *M. avium* phagocytosis. These results support the idea that direct interaction of SP-A and SP-D with the macrophages induces the stimulated phagocytosis.

In this study, both SP-A and SP-D enhance the phagocytosis of *M. avium* by alveolar macrophages and MDMs. The phagocytosis of mycobacteria has been shown to be mediated by a variety of

FIGURE 9. SP-A and SP-D increase cell surface localization of MR on MDMs. MDMs were incubated in the absence (*A*) or presence of 20 μg/ml human SP-A (*B*) or 5 μg/ml human SP-D (*C*) for 1 h. After the incubation, the cells were washed and fixed, immunostained with anti-MR mAb (clone 19.2), and analyzed using a confocal microscope as described in *Materials and Methods*.

phagocytic receptors including complement receptor (CR) 1, CR3, CR4, MR, and scavenger receptors (46, 50–52). Because the inclusion of mannan, zymosan, or *M. avium* LAM blocks the stimulatory effects of the collectins on *M. avium* uptake, the proteins have been proposed to stimulate MR-mediated phagocytosis. This is consistent with the results that lung collectins up-regulate the MR activity. In addition to the previous study describing the up-regulation of MR by SP-A (32), we now show that SP-D also induces cell surface expression of MR on macrophages. The up-regulation of MR activity has been confirmed by uptake experiments with mannosylated BSA using the collectin-treated macrophages. Since the presence of cycloheximide did not inhibit the collectin-stimulated phagocytosis of *M. avium*, new protein synthesis is not involved in the effects by SP-A and SP-D. In addition, the stimulatory effects of *M. avium* uptake are specific for the lung collectins because MBL failed to stimulate *M. avium* phagocytosis.

Analysis by confocal microscopy and flow cytometry has revealed that lung collectins enhance cell surface expression of MR (Figs. 9 and 10). These proteins also stimulate the uptake of mannosylated BSA by alveolar macrophages, which is completely diminished in the presence of EDTA (Fig. 8, *A* and *B*). These results are consistent with the idea that lung collectins enhance MR activity, since EDTA is a potent inhibitor of MR (49). In contrast, the uptake of zymosan is partially EDTA-resistant although some of the uptake is attenuated in the presence of EDTA (Fig. 8*C*). Likewise, the collectin-stimulated phagocytosis of *M. avium* is attenuated but is not completely blocked by EDTA (Fig. 5), suggesting the additional mechanisms may be involved in the phagocytosis of zymosan and mycobacterium in addition to the MR-involved mechanism. Since a variety of phagocytic receptors including MR, CRs, scavenger receptors, glucan receptors, and dectin-1 (50–56) mediate the phagocytosis of mycobacterium and zymosan, it is possible to assume that lung collectin may be involved in other mechanisms in addition to MR-mediated phagocytosis.

The mutant collectin, SP-A$^{E195Q, R197D}$ exhibits decreased binding to *M. avium* but stimulated phagocytosis to a level comparable to wt SP-A (Fig. 6). In addition, enhanced phagocytosis by macrophages persists even after preincubation and removal of lung collectins (Fig. 7). Thus, it is likely that lung collectin does not serve as an opsonin for the phagocytosis of *M. avium*. In contrast, opsonic functions of lung collectins in phagocytosis of HSV, *H. influenzae*, and *Klebsiella pneumoniae* have been shown (11–13). Taken together, these studies support the idea that lung collectins

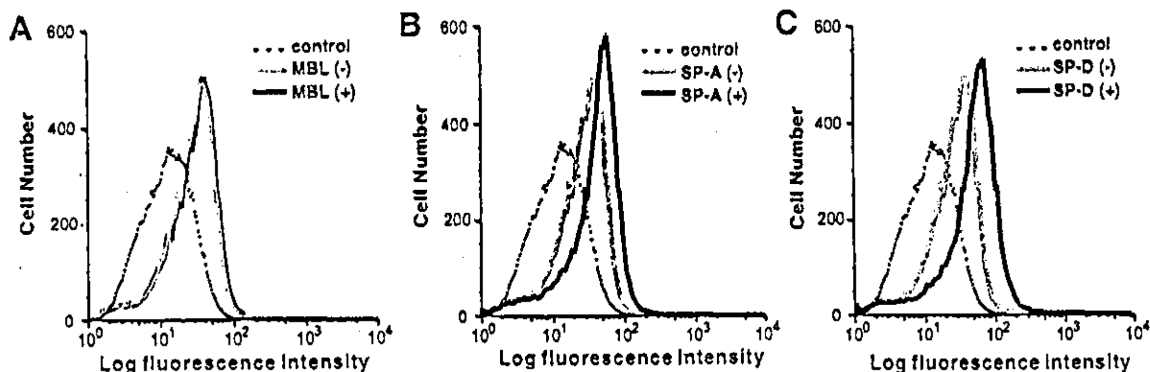

FIGURE 10. SP-A and SP-D but not MBL increase cell surface expression of the MR on MDMs. MDMs (1×10^6) were incubated with or without 20 μg/ml MBL (*A*), 20 μg/ml human SP-A (*B*), or 20 μg/ml human SP-D (*C*) for 1 h. After the incubation, the cells were washed and fixed with 1% paraformaldehyde, and immunostained with PE-conjugated anti-MR mAb or PE-conjugated monoclonal Ig isotype control. The stained cells were analyzed by flow cytometry. The histograms shown are representatives from three experiments. The solid black line shows cytometric analysis of the collectin-treated cells incubated with anti-MR Ab and the gray line shows the untreated cells incubated with anti-MR Ab. The dotted line shows the cells incubated with control IgG.

may stimulate the phagocytosis by two mechanisms, one of which by activating macrophages and the other by serving as an opsonin.

Ferguson et al. (33) have reported that coincubation of *M.tb* with SP-D reduces adherence of the bacteria to macrophages, whereas preincubation of SP-D with the macrophages does not affect bacterial adherence. They have also shown that SP-D binds to LAM on the surface of Erdman *M.tb* via the mannose cap on Erdman LAM and conclude that SP-D-*M.tb* interaction reduces adherence of *M.tb* to monocytes. In this study, both coincubation and preincubation of SP-D with *M. avium* provide consistent results of increased phagocytosis, which is consistent with the result that SP-D-treated macrophages exhibit higher expression of MR. One possible explanation of the difference between these earlier studies and this work is that different binding mechanisms of SP-D between *M.tb* and *M. avium*, due to the structural difference of LAM between these mycobacterium (42), may result in the distinct effects on macrophage interaction. Another possibility may be because of the different assay systems. We have discriminated between the intracellular bacteria and the cell surface-associated bacteria by quenching FITC-labeled *M. avium* with ethidium bromide.

SP-A$^{E195Q, R197D}$ has been shown to be inactive in the regulation of surfactant secretion and lipid uptake by alveolar type II cells and receptor binding on these cells (39). In addition, anti-SP-A Ab 1D6, whose epitope is located at the CRD (44), has previously been reported to block the inhibitory effect of SP-A on lipid secretion from type II cells, the receptor-binding activity of SP-A on type II cells, and the SP-A-stimulated lipid uptake by type II cells (44, 45, 57). These previous studies indicate that the CRD of SP-A is responsible for the SP-A action on alveolar type II cells. In contrast, SP-A$^{E195Q, R197D}$ stimulated *M. avium* phagocytosis by macrophages at a level comparable to that of wt SP-A, although this mutant exhibits significant decreased binding to *M. avium*. Ab 1D6 failed to inhibit the SP-A-enhanced phagocytosis of *M. avium* by alveolar macrophages as shown in this study. Thus, the structural requirement for SP-A-macrophage interaction in *M. avium* uptake is different from that for SP-A-type II cell interaction in regulating lipid secretion and uptake and receptor binding.

One recent study (32) has shown that SP-A up-regulates MR activity on MDMs and that alveolar macrophages from SP-A$^{-/-}$ mice have reduced MR expression relative to SP-A$^{+/+}$. In this study, confocal microscopy has revealed that SP-D as well as SP-A enhances MR activity on MDMs. This finding is also confirmed by the collectin-induced increase in uptake of mannosylated BSA by alveolar macrophages. The up-regulation of MR by lung collectins provides a mechanism for enhanced phagocytosis of *M. avium* since the collectin-stimulated phagocytosis of the bacteria is abolished in the presence of LAM, mannan, or zymosan, which are all ligands for the MR (46–48). Eighty percent of the MR is localized in intracellular vesicles and the protein recycles between the intracellular pools and the cell surface (58, 59). Enhanced expression of MR on MDMs and increased uptake of mannosylated BSA by alveolar macrophages have been observed after 1-h incubation of the collectin with the cells. In addition, the presence of cycloheximide does not affect the SP-A- or SP-D-stimulated *M. avium* phagocytosis. Thus, it is likely that the lung collectins may stimulate the receptor recycling en route to the plasma membrane rather than new receptor synthesis at either the transcriptional or translational level. The precise mechanism by which SP-A and SP-D up-regulate the MR activity remains to be elucidated.

HIV-infected individuals are at risk for opportunistic infections with *M.tb*, *M. avium*, or *Pneumocystis carinii*. Increased recovery of SP-A has been reported in bronchoalveolar lavage fluids from HIV-infected patients with *P. carinii* (60). The increased SP-A level in bronchoalveolar lavage fluids from HIV-infected individuals is closely associated with significant enhancement of *M.tb* attachment to alveolar macrophages and is correlated with the severity of HIV disease (61). The present and other studies (30) have shown that lung collectins mediate enhanced phagocytosis of *M. avium* and *M.tb*. Once internalized, mycobacterium resides in a membrane-bound vacuole that is resistant to lysosomal fusion (49). In addition, SP-A decreases NO production by macrophages infected with *M. avium* (29), which may be a pathway for this bacteria to escape from the bactericidal mechanisms of the host. Thus, lung collectin may play a role in the early phase of *M. avium* infection. Increased SP-A levels in HIV patients, the SP-A-enhanced phagocytosis of *M. avium*, and the SP-A-mediated suppression of NO may explain the increased incidence of mycobacterial infection in HIV-infected individuals.

This study clearly demonstrates that both SP-A and SP-D enhances the phagocytosis of *M. avium* by alveolar macrophages through stimulating the mannose receptor activity. Because *M. avium* can survive in macrophages, the collectin-mediated entry of the bacteria into the host cells may be an important pathway to escape bactericidal killing. In conclusion, this study demonstrates that the collectins bind to *M. avium* and that SP-A and SP-D but not MBL enhance the phagocytosis of *M. avium* by macrophages through up-regulation of MR activity. These findings provide a significant new mechanism by which lung collectins can regulate the host response to *M. avium* infection.

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