マクロファージに侵入した菌はファゴソーム内で生存増殖する。通常、ファゴソームの成熟が進むと、ファゴソームはリソソームと融合してファゴリソソームが形成される。このファゴリソソーム内の環境は結核菌にとっても殺菌的である。しかし、結核菌はファゴリソソーム融合を阻害することで、細胞内寄生性を発揮しており、これは結核菌がマクロファージ内で生存するための最も重要な機序である。最近、この抑制機序に、結核菌細胞壁に豊富に存在するリポ多糖体成分であるlipoarabinomannan（LAM）が重要な役割を果たしていることが報告されている。Kangらは、病原性の強い株に存在するMannose-capped LAMと細胞表面のマンノースレセプターとの結合が、ファゴリソソーム融合阻害に関与することを示した[3]。また、その機序としてVergneらは、ファゴソームとリソソームの融合にはphosphatidylinositol 3-kinase (PI3K)により合成されるファゴソーム膜上のphosphatidylinositol 3-phosphate (PI3P)が重要であるが、LAMのシグナルはPI3Kのファゴソームへの動員を抑制するため、ファゴソーム上のPI3Pの合成が抑えられることで発揮されることを示した[4]。さらにVergneらは、結核菌が産生するlipid phosphataseであるSapMがPI3Pの脱リン酸化を引き起こすことで、ファゴリソソーム融合を抑制することも明らかにした[5]。これらに加えて、Scherrらは、結核菌が真核細胞のプロテインキナーゼ類似のタンパク質であるプロテインキナーゼG（PknG）を産生することを示した。さらに彼らは、このPknG欠損変異株の感染では、ファゴリソソームが形成され、菌が殺菌処理され易いことを示し、PknGが結核菌によるファゴリソソーム融合阻害に関与することを明らかにした[6]。さらに、Jayachandranらは、マクロファージに存在するcoronin 1が結核菌を含むファゴソームに集積し、Ca2+依存性フォスファターゼであるcalcineurinを活性化することで、ファゴリソソーム融合が阻害されることを示した[7]。今のところ、coronin 1をファゴソーム上に維持するための菌側因子については明らかにされていないが、それが菌の病原性において重要な役割を果たすことは間違いない。またMacGurnらは、結核菌の分泌装置であるESX-1変異株の感染では、感染後のファゴリソソーム融合が阻害されないことを明らかにした[8]。ESX-1を構成する遺伝子群は、菌の病原性に重要なRD1領域に存在する。この領域にはESX-1より分泌される重要なT細胞抗原であるearly secreted antigenic target-6（ESAT-6）とculture filtrate antigen 10kD（CFP-10）が存在すが[9]、しかし、これらの欠損変異株を感染させてもファゴソームリソソーム融合が生じるため、これら因子のファゴリソソーム融合阻害への関与は否定的である。しかし、ESX-1を介して分泌される因子はESAT-6/CFP-10以外にも報告されおり、それらの中にファゴリソソーム融合阻害に関与する因子が存在することが示唆される。このように、結核菌はマクロファージのファゴソーム内で生存増殖を行う細胞内寄生戦略をとるが、ファゴソームの成熟過程を阻害するためには、エフェクター分子を細胞質内に放出する必要がある。このため、結核菌の分泌装置は、グラム陰性菌が有する分泌装置と同様に、菌が感染を成立させるための重要な役割を果たしているものと考えられている。

2．アポトーシスの抑制

マクロファージに結核菌弱毒株（H37Ra）を感染させると、アポトーシスが誘導されるが、カスパーゼ阻害剤を添加してアポトーシスの誘導を抑制すると、菌の細胞内増殖が改善される。一方、結核菌強毒株（H37Rv）の感染ではH37Ra感染と比較して、アポトーシスが抑制され、菌は細胞内で増殖することが示されている。この結果は、アポトーシスが菌の細胞内生存に影響することを示すものである。また、結核菌由来19kDaリポタンパク質のToll-like receptor 2（TLR2）を介した認識が細胞のアポトーシスを誘導することから、感染後に誘導されるアポトーシスは結核菌に対するひとつの宿主初期防御反応と捉えることができる。しかし、病原性の強い菌は、抗アポトーシス因子であるMcl-1の発現を誘導することでアポトーシスを抑制することが示されている[10]。結核菌の有するアポトーシス抑制活性は、菌にとって増殖の場を確保するという意味で重要な機序であり、細胞内寄生を可能にするために必須であると考えられる。

最近、結核菌を感染させたマクロファージにオー

トファジーを誘導すると、細胞内菌数が減少することが示された。また、マクロファージを interferon-γ (IFN-γ) で刺激した場合には、LRG 47 (p47 GTPase) の誘導を介してオートファジー機構が活性化される。その結果、菌を含むファゴソームはオートファゴソームと融合し、さらにリソソームと融合してオートリソソームが形成され、菌が殺菌処理されることが示されている[11]。オートファジーは、細胞の恒常性維持に必要と考えられてきた機構であるが、この結果は、オートファジーが結核菌の細胞内殺菌にも関与することを示すものである。しかし、結核菌は感染後にオートファジーの誘導に必要な PI3K 活性を抑制することで、オートファジーの誘導を抑制するものと考えられ、この抑制機序を解除するために IFN-γ による刺激が必要となる。

マクロファージや DC の機能制御

マクロファージや DC の感染局所への動員、あるいは結核菌貪食後のマクロファージの活性化は結核菌に対する初期防御反応だけでなく、特異的免疫応答の誘導においても重要である。マクロファージや DC は細胞表面の TLR を介して結核菌を認識する。TLR2 は LAM、フォスファチジルイノシトールマンノシド (phosphatidylinositolmannoside, PIM)、あるいは 19kDa リポタンパク質を認識する。また、易熱性結核菌体抗原は TLR4 により識別され、炎症性サイトカインが産生される。一方、結核菌はこれら TLR からのシグナル伝達を修飾する能力があることが最近明らかとなってきた。結核菌の主要な T 細胞抗原である ESAT-6 は、これまでその病原性における機能は明らかにされていなかったが、Pathak らは、ESAT-6 が TLR2 に結合すると Akt キナーゼが活性化され、その結果 TLR のアダプター分子である MyD88 と IRAK4 の会合が阻害されるため、TLR を介したシグナルが抑制されることを示した[12]。また、結核菌由来熱ショックタンパク質 hsp70 が、DC の分化を阻害するとともに、DC からの抗炎症性サイトカインである IL-10 産生を亢進させることが示された[13]。さらに、病原性の強い結核菌や BCG の LAM はその先端にマンノース残基が付加しており (Man-LAM)、非結核性抗酸菌の LAM とは構造的に異なる。この Man-LAM は DC 上に存在する DC-SIGN (DC-specific intracellular adhesion molecule-3 grabbing nonintegrin) を介してセリン / スレオニンキナーゼである Raf-1 の活性化を誘導する。活性化された Raf-1 は NF-κB p65 サブユニットのアセチル化を誘導し、その結果、TLR4 を介した刺激により IL-10 産生が亢進することが示されている[14]。また、この Man-LAM は DC の分化を阻害することも明らかにされている[15]。このように、結核菌の菌体成分はマクロファージや DC の機能を修飾することができる。さらに、これら糖脂質やタンパク成分は菌体より遊離して exosome の形で細胞外に放出され、近接した細胞に取り込まれることが報告されている[16, 17]。同様の過程は、アポトーシスにおいて観察され、アポトーシス小胞によって運ばれた菌体成分は効率よく抗原提示される (cross priming) ことが示されている。しかし、exosome を介して細胞外に放出された成分が同様に抗原提示されるのか、あるいは取り込んだ細胞の機能に影響を及ぼすのかについては不明であり、今後明らかにしていかなければならない。

防御免疫成立後の結核菌の抵抗性

感染の進行に伴い、感染病巣部には肉芽腫が形成される。肉芽腫内部は酸素分圧が低く、偏性好気生菌である結核菌には非常に苛酷な環境と考えられる。しかし、結核菌はその代謝系を環境に適応したものに切り替えて、感染を成立させるものと考えられている。McKlenney らは、結核菌が持続感染を成立させるためにはイソクエン酸リアーゼ (isocitrate lyase, ICL) が重要な役割を果たすことを示した[18]。ICL は脂質を材料とした糖の生合成経路、グリオキシル酸サイクルの酵素の一つである。肉芽腫内に存在する結核菌の ICL 活性が菌の生存に重要であるということは、菌が酸素分圧の低い環境では脂質を炭素源として利用することを示すものである。その他、熱ショックタンパクの一種である α -crystallin や nitrate reductase、または Eis (Enhanced intracellular survival) や KatG など多くの因子が細胞内増殖や持続感染に関与することが報告されており、菌が宿主体内で長期間

生存するためには、複雑なメカニズムが働いていると思われる。

一方、結核菌の活発な増殖が認められる組織では、ネクローシスに陥った細胞が多数認められる。これまでの解析から、細胞内で増殖した菌が感染を拡大するため、菌は感染細胞のネクローシスを誘導することが示されている。このネクローシス誘導には結核菌ゲノム上のRD1領域が重要な役割を果たしており、この領域に存在する遺伝子産物が、ミトコンドリア膜障害に関与し、その結果として細胞内ATP濃度が減少するため、細胞がネクローシスに陥ることが示されている[19]。また、結核菌感染初期には、増殖に必要な細胞内環境を維持するため、菌はカスパーゼ9の活性化を誘導してネクローシスを抑制することが示唆されている[20]。このような結核菌による感染マクロファージの細胞死の制御が、生体内での生存増殖に寄与するところは大きいと考えられる。

おわりに

結核菌はヒトを宿主として共生することに最も成功した微生物の一つであり、ヒトへの感染の歴史は紀元前にまで遡ることができる。一方、結核菌の有する宿主防御免疫の回避機構については、今のところ断片的にしか明らかにできていない。しかし、1998年に結核菌のゲノム配列が明らかにされ、遺伝子発現の網羅的な解析が可能になって以来、この研究分野において多くの成果が発表されている。今後さらなる研究成果の蓄積が、結核菌の病原性の解明だけでなく、新たな予防ワクチンの基礎となり、結核の撲滅に結びつくものと期待される。

文　献

1) Kohwiwattanagun J, Kawamura I, Fujimura T, Mitsuyama, M: Mycobacterial mammalian cell entry protein 1A (Mce1A)-mediated adherence enhances the chemokine production by A549 alveolar epithelial cells. Microbiol Immunol 51: 253-261, 2007.

2) Aoki K, Matsumoto S, Hirayama Y, Wada T, Ozeki Y, Niki M, Domenech P, Umemori K, Yamamoto S, Mineda A, Matsumoto M, Kobayashi K: Extracellular mycobacterial DNA-binding protein 1 participates in mycobacterium-lung epithelial cell interaction through hyaluronic acid. J Biol Chem 279: 39798-39806, 2004.

3) Kang PB, Azad AZ, Torrelles JB, Kaufman TM, Beharka A, Tlbesar E, DesJardin LE, Schlesinger LS: The human macrophage mannose receptor directs *Mycobacterium tuberculosis* lipoarabinomannan-mediated phagosome biogenesis. J Exp Med 202: 987-999, 2005.

4) Vergne I, Chua J, Deretic V: Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade. J Exp Med 198: 653-659, 2003.

5) Vergne I, Chua J, Lee HH, Lucas M, Belisle J, Deretic V: Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 102: 4033-4038, 2005.

6) Walburger A, Koul A, Ferrari G, Nguyen L, Prescianotto-Baschong C, Huygen K, Klebl B, Thompson C, Bacher G, Pieters J: Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. Science 304: 1800-1804, 2004.

7) Jayachandran R, Sundaramurthy V, Combaluzier B, Mueller P, Korf H, Huygen K, Miyazaki T, Albrecht I, Massner J, Pieters J: Survival of mycobacteria in macrophages is mediated by coronin 1-dependent activation of calcineurin. Cell 130: 37-50, 2007.

8) MacGurn JA, Cox JS: A genetic screen for *Mycobacterium tuberculosis* mutants detective for phagosome maturation arrest identifies components of the ESX-1 secretion system. Infect Immun 75: 2668-2678, 2007.

9) Ize B, Palmer T: Mycobacteria's export strategy. Science 313: 1583-1584, 2006.

10) Sly LM, Hingley-Wilson SM, Reiner NE, McMaster WR: Survival of *Mycobacterium*

tuberculosis in host macrophages involves resistance to apoptosis dependent upon induction of antiapoptotic Bcl-2 family member Mcl-1. J Immunol 170: 430-437, 2003.

11) Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V: Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. Cell 119: 753-766, 2004.

12) Pathak SK, Basu S, Basu KK, Banerjee A, Pathak S, Bhattacharyya A, Kaisho T, Kundu M. Basu J.: Direct extracellular interaction between the early secreted antigen ESAT-6 of *Mycobacterium tuberculosis* and TLR2 inhibits TLR signaling in macrophages Nature Immunol. 8: 610-618, 2007.

13) Motta A, Schmitz C, Rodrigues L, Ribeiro F, Teixwira C, Detanico T, Bonan C, Zwichey H, Bonorino C: *Mycobacterium tuberculosis* heat-shock protein 70 impairs maturation of dendritic cells from bone marrow precursors, induces interleukin-10 production and inhibits T-cell proliferation in vitro. Immunol 121: 462-472, 2007.

14) Gringhuis SI, den Dunnen J, Litjens M, van Het Hof B, van Kooyk Y, Geijtenbeek TB: C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-κB. Immunity 26: 605-616, 2007.

15) Dulphy N, Herrmann JL, Nigou J, Rea D, Boissel N, Puzo G, Charron D, Lagrange PH, Toubert A: Intermediate maturation of *Mycobacterium tuberculosis* LAM-activated human dendritic cells. Cell Microbiol 9: 1412-1425, 2007

16) Beatty WL, Ullrich HJ, Russell DG: Mycobacterial surface moieties are released from infected macrophages by a constitutive exocytic event. Eur J Cell Biol 80: 31-40, 2001.

17) Beatty WL, Russell DG: Identification of mycobacterial surface proteins released into subcellular compartments of infected macrophages. Infect Immun 68: 6997-7002, 2000.

18) McKinney JD, zu Bentrup KH, Munoz-Elias EJ, Miczak A, Chen B, Chan WT, Swenson D, Sacchettini JC, Jacobs WR Jr, Russell DG: Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature 406: 735-738, 2000.

19) Kaku T, Kawamura I, Uchiyama R, Kurenuma T, Mitsuyama M: RD1 region in mycobacterial genome is involved in the induction of necrosis in infected RAW264 cells via mitochondrial membrane damage and ATP depletion. FEMS Microbiol Lett 274: 189-195, 2007.

20) Uchiyama R, Kawamura I, Fujimura T, Kawanishi M, Tsuchiya K, Tominaga T, Kaku T, Fukazawa Y, Sakai S, Nomura T, Mitsuyama M: Involvement of caspase-9 in the inhibition of necrosis of RAW 264 cells infected with *Mycobacterium tuberculosis*. Infect Immun 75: 2894-2902, 2007.

Advances in understanding of the virulence mechanism of *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis (MTB) is an intracellular pathogen that has evolved strategies to enable growth in macrophages. The bacterium is able to inhibit fusion of phagosomes with lysosomes through secretion of some bacterial components and modulation of host intracellular signaling pathways. Furthermore, it has been shown that phagositosed MTB is killed within macrophages after treatment with IFN- γ in vitro. However, virulent MTB is capable of surviving in macrophages in vivo and persists in host even after acquired immunity has developed. These data suggest that MTB has developed a sophisticated immune evasion mechanism. In this issue, the strategies of MTB for intracellular survival and immune evasion, which have been unraveled so far, are shown and the mechanisms are discussed.

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Critical Involvement of Pneumolysin in Production of Interleukin-1α and Caspase-1-Dependent Cytokines in Infection with *Streptococcus pneumoniae* In Vitro: a Novel Function of Pneumolysin in Caspase-1 Activation▽

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Pneumolysin is a pore-forming cytolysin known as a major virulence determinant of *Streptococcus pneumoniae*. This protein toxin has also been shown to activate the Toll-like receptor 4 (TLR4) signaling pathway. In this study, a mutant *S. pneumoniae* strain deficient in pneumolysin (Δ*ply*) and a recombinant pneumolysin protein (rPLY) were constructed. Upon infection of macrophages in vitro, the ability to induce the production of interleukin-1α (IL-1α), IL-1β, and IL-18 was severely impaired in the Δ*ply* mutant, whereas there was no marked difference in the induction of tumor necrosis factor alpha (TNF-α) and IL-12p40 between the wild type and the Δ*ply* mutant of *S. pneumoniae*. When macrophages were stimulated with rPLY, the production of IL-1α, IL-1β, and IL-18 was strongly induced in a TLR4-dependent manner, whereas lipopolysaccharide, a canonical TLR4 agonist, hardly induced these cytokines. In contrast, lipopolysaccharide was more potent than rPLY in inducing the production of TNF-α, IL-6, and IL-12p40, the cytokines requiring no caspase activation. Activation of caspase-1 was observed in macrophages stimulated with rPLY but not in those stimulated with lipopolysaccharide, and the level of activation was higher in macrophages infected with wild-type *S. pneumoniae* than in those infected with the Δ*ply* mutant. These results clearly indicate that pneumolysin plays a key role in the host response to *S. pneumoniae*, particularly in the induction of caspase-1-dependent cytokines.

Streptococcus pneumoniae is a gram-positive bacterium that causes bacterial pneumonia, otitis media, bacterial meningitis, and septicemia (32). Due to the severe disease burden and mortality, the emergence of drug-resistant clinical isolates (14, 36), the lack of a universally effective vaccine (17, 39), and an increase in the number of immunocompromised patients, it is increasingly important to understand the pathogenic processes of pneumococcal disease in order to develop novel therapeutic modalities and an effective vaccine.

Pneumolysin (PLY), a 53-kDa protein toxin produced by virtually all clinical isolates of *S. pneumoniae*, has been regarded as a key virulence factor of this bacterium (7) and as one of the candidates for vaccine development against pneumococcal infection (21). PLY is one of the cholesterol-dependent cytolysins (24) that are known to form ring- or arc-shaped pores on cholesterol-containing membranes and whose activity is blocked by free cholesterol (40, 50). PLY is a multifunctional protein toxin that causes cytolysis and induces complement activation and the production of cytokines and nitric oxide (5, 9, 18, 33, 43). However, the mechanisms by which PLY affects the host defense are only partly understood.

In mammals, the sensing of microbial components by innate immune cells, such as macrophages and dendritic cells, is initiated by the recognition of conserved and unique pathogen-derived structures via pattern recognition molecules, such as Toll-like receptors (TLRs) (31). TLRs, transmembrane proteins belonging to the Toll/interleukin-1 (Toll/IL-1) receptor family (16), mediate host immune responses by inducing proinflammatory cytokines and costimulatory molecules through the activation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) after recognition of specific agonists (19). Among several TLRs, TLR4 plays a critical role in the recognition of lipopolysaccharide (LPS), a major component of the outer membranes of gram-negative bacteria, in cooperation with LPS-binding protein, CD14, and MD2 (1, 25, 51, 53), and plays a critical role in the host defense against gram-negative pathogens (8, 37). Several recent studies have shown that the association of TLR4 with PLY (and other cholesterol-dependent cytolysins) initiates an intracellular signaling cascade resulting in the activation of NF-κB (20, 27, 41, 49, 51). For pneumococcal infection, Malley et al. (27) reported an important role of TLR4 in the host defense with the finding that C3H/HeJ mice, which display mutant nonfunctional TLR4, were more susceptible than wild-type (wt) mice to invasive disease after pneumococcal colonization in the nasopharynx. Moreover, a protective role of TLR4 in the pneumococcal pneumonia model was also reported (8). Thus, it appears that PLY, a TLR4 agonist in this gram-positive pathogen (27), is involved in the induction of a host protective response against *S. pneumoniae*.

Several reports have shown that proinflammatory cytokines, such as IL-1, tumor necrosis factor alpha (TNF-α), IL-6, and IL-18, play protective roles against pneumococcal infection (22, 26, 48, 54, 55). Moreover, gamma interferon has been

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demonstrated to protect mice against infection with *S. pneumoniae* by promoting the accumulation of neutrophils in the infected lung (34). In addition, exogenous administration of IL-12 improved the innate defense against *S. pneumoniae* in the lung by inducing gamma interferon production (23). However, the involvement of PLY in the production of various cytokines induced in pneumococcal infections has yet to be clarified.

In the present study, we constructed an in-frame deletion mutant with a mutation in the *S. pneumoniae ply* gene and a recombinant protein of PLY to analyze the precise role for PLY in the host cytokine response to *S. pneumoniae*. Using an in vitro model of infection, we compared the levels of various proinflammatory cytokines secreted from macrophages, the cells comprising the front line of host defense and the innate immune response, after infection with the wt or *ply*-deficient mutant strain of *S. pneumoniae*. This study revealed a unique function of PLY in the induction of caspase-1-dependent cytokine production that could not be observed with LPS, a canonical TLR4 ligand.

TABLE 1. Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5′–3′)
IL-1α-F	CTCTAGAGCACCATGCTACAGAC
IL-1α-R	TGGAATCCAG-GGGAAACACTG
IL-1β-F	AAGCTCTCCACCTCAATGGACAG
IL-1β-R	CTCAAACTCCAC-TTTGCTCTTGA
IL-18-F	ACTGTACAACCGCAGTAATACGG
IL-18-R	AGTGAACATTACAGATTTATCCC
TNF-α-F	GGCAGGTCTACTTTGGAGTCATTGC
TNF-α-R	ACATTCGAGGCTCCAGTGAATTCCA
IL-6-F	GAGGATACCACTCCCAACAGACC
IL-6-R	AAGTGCATCATCGTTGTTCATACA
IL-12p40-F	TCCGGAGTAATTTGGTGCTTCACA
IL-12p40-R	GCAAGAGACACAGTCCTGGG
GAPDH-F	TGCCCAGAACATCATCCCTG
GAPDH-R	AACACGGAAGGCCATGCCAG
PLY-F	CGATGGATCCTATGGCAAATAAAGCAGTAA
PLY-R	ACGCGGTACCCTAGTCATTTTTCTACCTTAT
P1	ACACGGATCCTCAACAGGCACTCATCCACA
P2	GCGCAAGCTTGGAGAATGCTTGCGAC AAAA
P3	GCGCAAGCTTAAATCAGCCGTGGTTGGACT
P4	ACACGGATCCCGCAAAGCCCTTTTTCTAGC

MATERIALS AND METHODS

Experimental animals. wt female C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 background TLR4 gene knockout (TLR4 KO) mice were purchased from Oriental Bioservices (Kyoto, Japan) and maintained in a specific-pathogen-free environment for use at 7 to 8 weeks of age. All animal experiments were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine.

Bacterial strains and growth conditions. A serotype 2 strain of *Streptococcus pneumoniae* D39 was purchased from the National Collection of Type Cultures (NCTC 7466; Central Public Health Laboratory, London, United Kingdom). *S. pneumoniae* was grown on tryptic soy agar (Difco Laboratories, Detroit, MI) with 5% (vol/vol) defibrinated sheep blood (Nacalai Tesque, Kyoto, Japan) and in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (THY) at 37°C and 5% CO_2 and subsequently stored at −80°C in THY plus 10% glycerol. For the preparation of bacterial stocks for macrophage stimulation, pneumococci were grown overnight on blood agar plates at 37°C and 5% CO_2. Colonies were inoculated into the THY medium, grown until mid-logarithmic phase (optical density at 600 nm [OD_{600}] = 0.5), and centrifuged at 6,000 × *g* for 15 min. The bacterial pellet was suspended in phosphate-buffered saline (PBS) and stocked at −80°C. The concentration was determined by viable cell counting on blood agar plates.

Construction of *ply* deletion mutant. A deletion mutant of *S. pneumoniae* D39 for the PLY gene (*ply*) was constructed by using homologous recombination-based allelic exchange. To generate the *ply* deletion, the upstream (733 bp) and downstream (692 bp) flanking regions of *ply* were PCR amplified from D39 genomic DNA, using the primer sets P1/P2 and P3/P4, respectively (primer sequences are given in Table 1). Primers P1 and P4 carried one BamHI site, and P2 and P3 carried HindIII sites in their 5′ ends. Amplified fragments were digested with HindIII and ligated. The resulting fusion gene product was amplified by PCR using primers P1 and P4, digested with BamHI, and then ligated with BamHI-digested vector DNA (pTN-E18EM) (Amp^r Em^r). Plasmid pTN-E18EM is a pUC18-derived vector carrying ampicillin and erythromycin resistance genes and the multiple cloning site of pUC18. The erythromycin resistance gene (*emrC*) was amplified from plasmid pE194 by PCR and inserted into pUC18.

Transformation into *S. pneumoniae* and selection of *ply* deletion mutant. To carry out the transformation of the recombinant plasmid, frozen stocks of *S. pneumoniae* were thawed and diluted 1:20 in competence medium (tryptic soy broth [Difco], pH 8.0, 10% glycerol, 0.16% bovine serum albumin, 0.01% $CaCl_2$) containing competence-stimulating peptide 1 (100 ng/ml; Invitrogen, Carlsbad, CA). *S. pneumoniae* D39 was preincubated for 20 min at 37°C and 5% CO_2 and then incubated for 1 h with approximately 1 μg of DNA. The cells were plated on blood agar containing erythromycin, and transformants were obtained. For the selection of the *ply* deletion mutant, transformants were grown in THY medium without antibiotics and plated on blood agar without antibiotics, and then colonies were plated on replica plates with or without erythromycin. Erythromycin-sensitive colonies were selected, and *ply*-negative mutants were confirmed by PCR to have an absence of *ply*, using primers PLY-F and PLY-R (Table 1), and the presence of the upstream and downstream sequences of the *ply* gene. The absence of PLY in the *ply* deletion mutant was verified by Western blotting using a monoclonal antibody against PLY (NovoCastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom).

Production and purification of rPLY. Full-length recombinant PLY (rPLY) was prepared as described previously (4). Briefly, the *ply* gene was cloned into the pQE-31 vector (Qiagen, Hilden, Germany), and the recombinant vector was transformed into *Escherichia coli* SG13009 (Qiagen) harboring a pREP4 plasmid, which contains *lacI* and kanamycin resistance genes. rPLY was produced in *E. coli* cells as a six-His-tagged protein by incubation of the transformants with 2 mM isopropyl-β-D-thiogalactopyranoside (Nacalai Tesque) at 25°C for 6 h. The *E. coli* cells were then harvested by centrifugation, incubated with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 1 mg/ml lysozyme, 200 U DNase I, pH 8.0), and disrupted by vortexing with 0.1-mm zirconia-silica beads (Bio-Spec Products, Inc., Bartlesville, OK). rPLY was then purified from the soluble fraction by use of a nickel-nitrilotriacetic acid column (Qiagen) under native conditions according to the manufacturer's instructions. Contaminating LPS was extensively removed using a Detoxi-Gel endotoxin-removing gel (Pierce Chemical Co., Rockford, IL). The level of LPS in the rPLY preparation was determined by the *Limulus* Color KY test (Wako Pure Chemical Industries, Osaka, Japan) and was found to be <0.4 pg/ml when the preparation was suspended in PBS at a protein concentration of 1 μg/ml. The purity was analyzed by Coomassie brilliant blue staining and immunoblotting using an anti-His-tag monoclonal antibody (penta-His antibody; Qiagen) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

To inhibit its cytolytic activity, rPLY was treated with 20 μg/ml of cholesterol for 30 min on ice (35). Heat-treated PLY and LPS were prepared by heating the aqueous stock suspensions, in 50% glycerol-water (vol/vol) for PLY and in PBS for LPS, in a boiling water bath for 60 min (27).

Isolation and stimulation of peritoneal macrophages. Peritoneal exudate cells were collected from C57BL/6 wt and TLR4 KO mice 3 days after an intraperitoneal injection of 4% thioglycolate medium (Eiken Chemical, Tokyo, Japan). Cells were cultured in 48-well plates (2×10^5 cells per well) in medium consisting of RPMI 1640 (Gibco-BRL Life Technologies, Rockville, MD) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco) for 2 h at 37°C and 5% CO_2. After removal of nonadherent cells, the adherent cells were used as peritoneal macrophages and infected with wt *S. pneumoniae* and its isogenic *ply* deletion mutant (Δ*ply*) at a multiplicity of infection (MOI) of 10 in gentamicin-free medium. To inhibit the growth of bacteria in the medium, 100 μg/ml of gentamicin (Gibco) was added to the cultures 8 h after infection, when the largest number of bacteria were associated with cells, and the cells were incubated for an additional 16 h. Supernatants were collected and stored at −80°C until they were assayed for cytokines. Similarly, peritoneal macrophages were stimulated with rPLY, LPS (from *E. coli* O55:B5; Sigma-Aldrich, St. Louis, MO), or Pam_3CSK_4 (Invivogen, San Diego, CA). In some experiments, polymyxin B

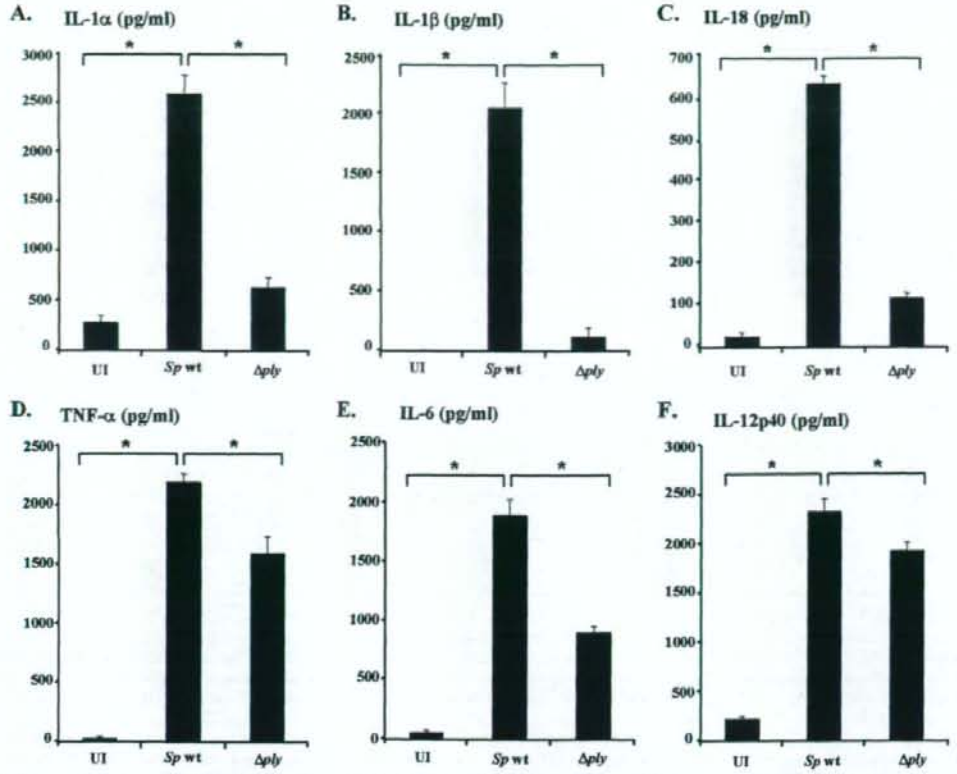

FIG. 1. Pneumolysin is essential for the secretion of IL-1α, IL-1β, and IL-18 in response to *Streptococcus pneumoniae*. Peritoneal macrophages were left uninfected (UI) or infected with wt *S. pneumoniae* (*Sp* wt) or the *ply* mutant (Δ*ply*) at a macrophage/bacterium ratio of 1:10 for 8 h. Cells were cultured for an additional 16 h in the presence of gentamicin (100 μg/ml), and culture supernatants were then collected. The amounts of IL-1α (A), IL-1β (B), IL-18 (C), TNF-α (D), IL-6 (E), and IL-12p40 (F) were determined using ELISAs specific for each cytokine. The results are representative of three similar experiments. The data are the means ± standard deviations for three determinations. *, $P < 0.05$ for uninfected cells compared to wt *S. pneumoniae*-infected cells and for wt *S. pneumoniae*-infected cells compared to Δ*ply* mutant-infected cells.

(PMB; Nacalai Tesque), z-VAD-fmk (Peptide Institute, Osaka, Japan), and z-YVAD-cmk (R&D Systems, Minneapolis, MN) were added to the cultures 30 min before stimulation with rPLY or LPS.

Reverse transcription-PCR. Total cellular RNA was extracted from peritoneal macrophages by using Nucleospin RNA II (Macherey-Nagel, Düren, Germany). Total RNA (0.2 μg) was treated with RNase-free DNase (Promega, Madison, WI) to eliminate contaminating DNA and then subjected to reverse transcription using random primers (Invitrogen) and ReverTra Ace (Toyobo, Osaka, Japan). PCR was performed using KOD-Plus DNA polymerase (Toyobo) under the following PCR conditions: 94°C for 15 s, 60°C for 30 s, and 68°C for 30 s. The reaction was extended by incubation at 68°C for 7 min. The samples were amplified for 28 to 30 cycles. The most appropriate number of amplification cycles for each cytokine was determined by preliminary experiments. PCR products were analyzed in 2% agarose gels. Primer sequences used for the amplification of specific genes by reverse transcription-PCR are shown in Table 1.

Cytokine measurement. Levels of secreted cytokines in culture medium were determined by two-site sandwich enzyme-linked immunosorbent assay (ELISA). ELISA kits for IL-1α and IL-1β were purchased from BD Biosciences (San Diego, CA). IL-6, TNF-α, and IL-12p40 kits were obtained from eBioscience (San Diego, CA), and an IL-18 kit was obtained from MBL (Nagoya, Japan). All samples were assayed according to the respective manufacturer's instructions.

Western blot analysis. For Western blotting, cells were lysed in 2× SDS-PAGE sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue) and subjected to brief ultrasonication and boiling for 5 min. The lysates were resolved by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Antibodies specific for mouse IκBα, p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), and phospho-ERK1/ERK2 (Thr202/Tyr204) were purchased from Cell Signaling Technology (Danvers, MA) and were used as recommended by the manufacturer.

Caspase-1 activation assay. To determine the activation of caspase-1, peritoneal exudate cells were seeded in 24-well tissue culture plates at 5×10^5 cells per well. After removal of nonadherent cells, the adherent cells were incubated with 30 μM biotinylated YVAD-cmk (Alexis Biochemicals, San Diego, CA) for 1 h. The cells were then either left unstimulated or stimulated with rPLY or LPS for 3 h at different concentrations. In the case of LPS plus ATP stimulation, cells were primed with LPS for 2.5 h and subsequently stimulated with 1 mM ATP (Amersham) for 30 min. Similarly, peritoneal macrophages were infected with wt *S. pneumoniae* and the Δ*ply* mutant at an MOI of 10 for 3 or 6 h. Cells were then washed with PBS three times and lysed with 1 ml lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 12,000 ×*g* for 1 min to remove cell debris. To adjust the amount of lysate recovered from each well, 10 μl of cleared lysate was subjected to Western blotting using a specific antibody for β-actin (Sigma), and the rest of the lysate was used for pull-down assay for detecting the active form of caspase-1. Activated caspase-1 (bound to biotinylated YVAD-cmk) was

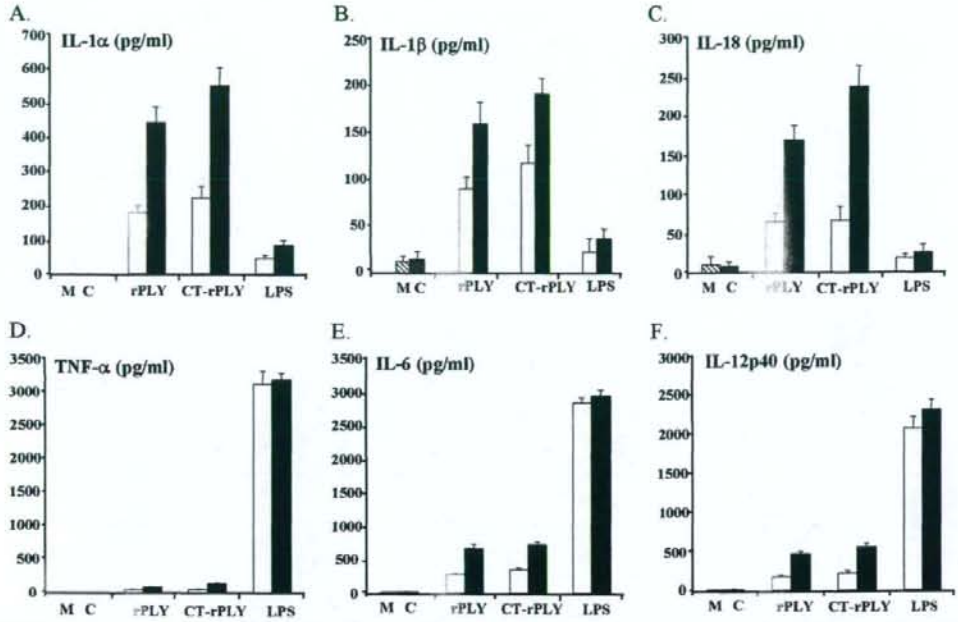

FIG. 2. Pneumolysin is a strong inducer of IL-1α, IL-1β, and IL-18 production. Peritoneal macrophages were left unstimulated (M), treated with cholesterol only (C), or stimulated with rPLY, cholesterol-treated rPLY (CT-rPLY), or LPS at a concentration of 0.1 μg/ml (white bars) or 1 μg/ml (black bars) for 24 h, and culture supernatants were then collected. The levels of cytokines in supernatants were determined by ELISA. The results are representative of three similar experiments. The data are the means ± standard deviations for three determinations.

concentrated with tetrameric avidin resin (Promega) and detected by Western blotting using anti-caspase-1 polyclonal antibody (MBL).

Statistical analysis. For comparisons between two groups, the Mann-Whitney U test was used, and statistical significance was determined as a P value of <0.05. Multigroup comparisons of mean values were conducted by the Kruskal-Wallis test and the Games-Howell post hoc test ($P < 0.05$) after the confirmation of homogeneity of variances among the groups by using Bartlett's test.

RESULTS

Role of PLY in cytokine production by macrophages infected with *S. pneumoniae* in vitro. To evaluate the involvement of PLY in inducing proinflammatory cytokines in response to *S. pneumoniae*, peritoneal macrophages from C57BL/6 mice were infected with live wt *S. pneumoniae* or the Δ*ply* mutant, and the levels of cytokines in supernatants were assayed. wt *S. pneumoniae* potently induced IL-1α, IL-1β, and IL-18, whereas the Δ*ply* mutant was unable to induce these cytokines, indicating a critical role of PLY in inducing IL-1α, IL-1β, and IL-18. In contrast, no marked difference was observed between the two strains in the ability to induce TNF-α, IL-6, and IL-12p40, suggesting a minor role of PLY in induction of these cytokines (Fig. 1). The impaired ability of the Δ*ply* mutant to induce cytokine production was not due to any enhanced cytotoxicity, as the release of lactate dehydrogenase from macrophages infected with the mutant never exceeded the level induced by wt *S. pneumoniae* (data not shown).

PLY strongly induces IL-1α, IL-1β, and IL-18. To confirm whether the *ply* gene product was responsible for the difference in cytokine induction between the *ply*-positive wt and *ply*-deficient mutant strains, we next examined the profiles of various cytokines induced by rPLY stimulation. Although it was difficult to determine the exact amount of PLY released from wt *S. pneumoniae* in our in vitro infection system, we tried to estimate the amount based on a previous report on the relationship between bacterial number and the amount of PLY protein (18). The amount of PLY released from the infection dose of wt *S. pneumoniae* used in this study was calculated to be 0.04 μg/ml. In a study by Malley et al. (27), rPLY was used at concentrations of 0.1 μg/ml to 10 μg/ml to determine TLR4-dependent cytokine-inducing activity. Taking these findings into consideration, we used rPLY in the present study at cytolytic (1 μg/ml) and sublytic (0.1 μg/ml) concentrations, which caused 53.0% and 5.1% release of lactate dehydrogenase from macrophages, respectively. Both doses of rPLY exhibited a stronger activity to induce IL-1α, IL-1β, and IL-18 than did LPS (Fig. 2) and TLR2 ligands, such as Pam_3CSK_4 (data not shown). In contrast, the ability of rPLY to induce IL-6, IL-12p40, and TNF-α was significantly lower than that of LPS. A treatment of 1 μg/ml or 0.1 μg/ml of rPLY with cholesterol resulted in the reduction of the cytolytic activity determined by lactate dehydrogenase release assay, by 95.3% and 100%, respectively. However, the cytokine-inducing activity was not affected by cholesterol pretreatment, even after the abolishment of cytolytic activity. Both cholesterol-treated rPLY and untreated rPLY induced similar levels of all cytokines tested (Fig.

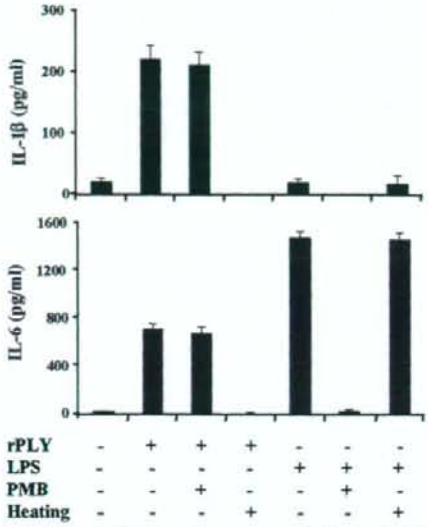

FIG. 3. Effects of PMB and heating on rPLY-induced IL-1β and IL-6 production. Peritoneal macrophages were stimulated with cholesterol-treated rPLY or LPS for 24 h, and the culture supernatant was then collected. The amounts of IL-1β (A) and IL-6 (B) in the culture supernatant were measured by ELISA. The effects of the addition of 0.5 μg of PMB/ml and heating at 100°C for 1 h were examined. The results are representative of three similar experiments. The data are the means ± standard deviations for three determinations.

2), indicating that cytokine-inducing activity and cytolytic activity of PLY may be dissociated. The absence of any stimulating or toxic activity was confirmed in the medium containing cholesterol alone.

To eliminate the possibility that cytokine induction by rPLY was due to contamination of LPS from *E. coli*, we added PMB to the cell culture before stimulation with rPLY and LPS. PMB did not affect rPLY-induced production of IL-1β or IL-6 (Fig. 3) as well as IL-1α, IL-18, TNF-α, and IL-12p40 (data not shown), whereas the same amount of PMB completely abolished the production of cytokines induced by LPS. Furthermore, when rPLY was heated at 100°C for 60 min, the ability to induce these cytokines was completely abrogated. Based on these findings, the possibility of contaminating LPS-induced cytokine production was ruled out.

Supplementation with rPLY enhanced IL-1α, IL-1β, and IL-18 production by macrophages infected with the Δ*ply* mutant. Although rPLY demonstrated a significantly higher activity in inducing IL-1α, IL-1β, and IL-18 than did LPS (Fig. 2), the levels were significantly lower than those induced by infection of macrophages with wt *S. pneumoniae* (Fig. 1). To examine whether high levels of these cytokines induced upon infection with wt *S. pneumoniae* were due to simultaneous stimulation with PLY and other bacterial components, we infected macrophages with the Δ*ply* mutant in the presence of rPLY. Costimulation with rPLY and the Δ*ply* mutant induced a greatly enhanced production of IL-1α, IL-1β, and IL-18, which was comparable to that induced by wt *S. pneumoniae* (Fig. 4). In the absence of rPLY, an increase in the dose of the

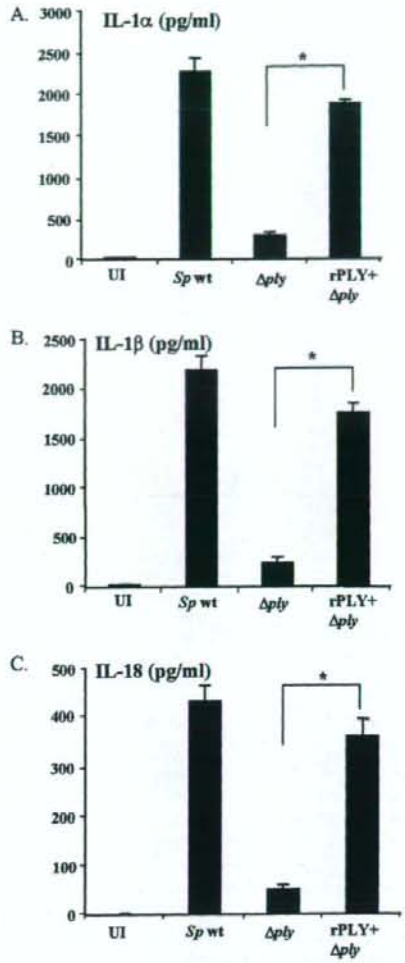

FIG. 4. Costimulation of rPLY with the Δ*ply* mutant significantly increased IL-1α, IL-1β, and IL-18 production by the Δ*ply* mutant. Peritoneal macrophages were left uninfected (UI) or infected with wt *S. pneumoniae* (*Sp* wt), the Δ*ply* mutant, or rPLY (1 μg/ml) plus the Δ*ply* mutant at a macrophage/bacterium ratio of 1:10. The amounts of IL-1α (A), IL-1β (B), and IL-18 (C) in culture supernatants were determined by ELISAs specific for each cytokine. The results are representative of three similar experiments. The data are the means ± standard deviations for three determinations. *, $P < 0.05$ for the Δ*ply* mutant versus rPLY plus the Δ*ply* mutant.

Δ*ply* mutant up to an MOI of 50 did not result in such an enhancement (data not shown). These findings clearly indicate that the PLY-dependent cytokine response is enhanced by other stimuli from bacterial cells but that PLY, not any other bacterial components, is solely responsible for the induction of IL-1α, IL-1β, and IL-18 production, which requires the activation of cleaving enzymes, including caspase-1. However, costimulation with rPLY and purified TLR agonists, such as LPS or Pam_3CSK_4, resulted in no marked enhancement of the

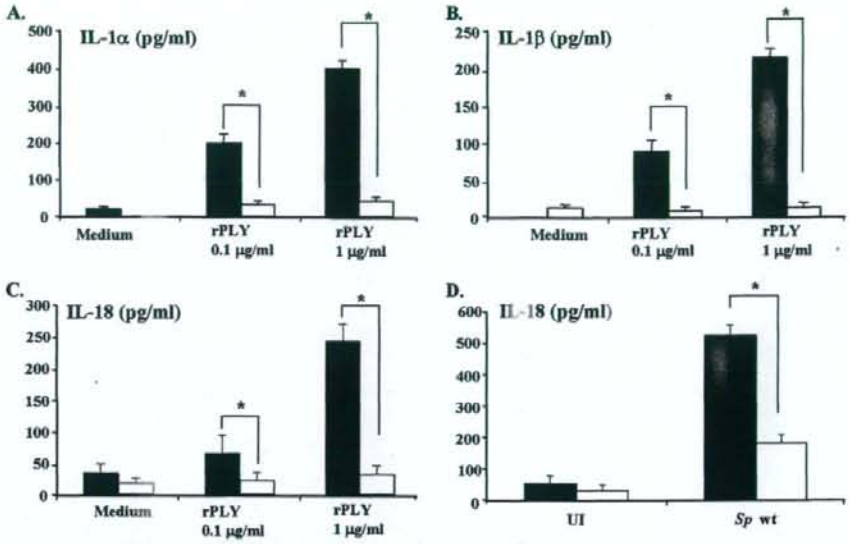

FIG. 5. TLR4-dependent induction of inflammatory cytokines by PLY. Peritoneal macrophages from C57BL/6 wt mice (black bars) and TLR4 KO mice (white bars) were stimulated with cholesterol-treated rPLY (A to C) or infected with wt *S. pneumoniae* at a macrophage/bacterium ratio of 1:10 (D) for 24 h, and the culture supernatants were then collected. The amounts of IL-1α, IL-1β, and IL-18 in culture supernatants were determined by ELISA. The results are representative of three similar experiments. The data are the means ± standard deviations for three determinations. Asterisks indicate that the value is significantly different from that of wt cells ($P < 0.05$).

production of IL-1α, IL-1β, and IL-18 (data not shown), suggesting that the mechanism of enhancement might be more complex than just a simultaneous stimulation with rPLY and TLR agonists from *S. pneumoniae*.

Critical role of TLR4 in IL-1α, IL-1β, and IL-18 production induced by PLY. In a previous study, it was shown that PLY induces TNF-α and IL-6 production through TLR4 (27). To investigate whether TLR4 is also required for the production of IL-1α, IL-1β, and IL-18 induced by rPLY, we compared the levels of these cytokines produced by macrophages from C57BL/6 background TLR4-deficient mice and wt mice in response to rPLY. In addition to that of TNF-α and IL-6, the production of IL-1α, IL-1β, and IL-18 induced by rPLY was dependent on TLR4, as these cytokines were produced only by wt macrophages upon stimulation with rPLY (Fig. 5A to C). The production of IL-18 induced by infection with wt *S. pneumoniae* was also critically dependent on the presence of TLR4, suggesting that production of the cytokine in response to *S. pneumoniae* is due to the recognition of PLY via TLR4 (Fig. 5D). Because the production of IL-1α, IL-1β, and IL-18 is regulated both transcriptionally and posttranscriptionally, we next examined the involvement of TLR4 in the rPLY-induced gene expression of these cytokines. rPLY induced or up-regulated the expression of IL-1α, IL-1β, and IL-18 in macrophages from wt mice but not in those from TLR4 KO mice (Fig. 6). These results indicate that TLR4 plays a role in the production of IL-1α, IL-1β, and IL-18 induced by rPLY, at least at the gene expression level. In addition, the difference between rPLY and LPS in inducing the production of IL-1α, IL-1β, and IL-18 resulted from a posttranscriptional process, not the ability to induce gene expression, because LPS induced gene expression of these cytokines at levels comparable to those induced by rPLY.

Activation of TLR4 downstream signals by rPLY. As shown in Fig. 2 and 6, there was dissociation between the gene expression and production of TNF-α after stimulation of macrophages with rPLY. Moreover, the levels of rPLY-induced gene

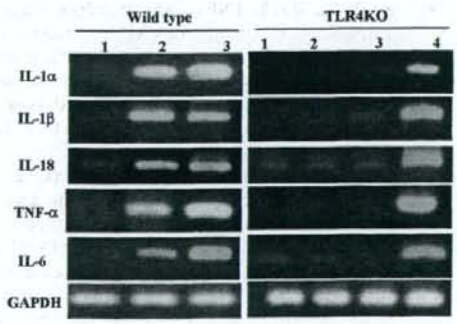

FIG. 6. Expression of mRNAs for various cytokines induced by rPLY was TLR4 dependent. Peritoneal macrophages of C57BL/6 wt and TLR4 KO mice were stimulated with rPLY, LPS, and Pam_3CSK_4 (1 μg/ml) for 6 h. Total RNA was extracted and subjected to reverse transcription-PCR for detection of cytokine mRNAs for IL-1α, IL-1β, IL-18, TNF-α, IL-6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lanes: 1, PBS; 2, rPLY; 3, LPS; 4, Pam_3CSK_4. Representative results are shown. Similar results were obtained in three separate experiments.

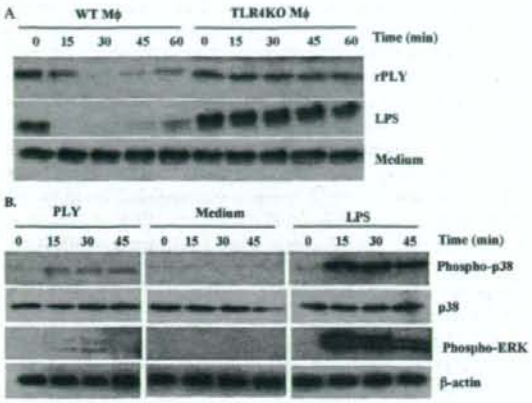

FIG. 7. Activation of TLR4 downstream signals by rPLY. Peritoneal macrophages from C57BL/6 (WT) and TLR4 KO mice were stimulated with medium alone, rPLY, or LPS (1 μg/ml). (A) At the indicated times, cell lysates were collected and IκBα degradation was analyzed by Western blotting. (B) The cell lysates described in panel A were then subjected to Western blotting using antibodies specific to p38, phospho-p38, phospho-ERK, and β-actin. The results are representative of at least three independent experiments.

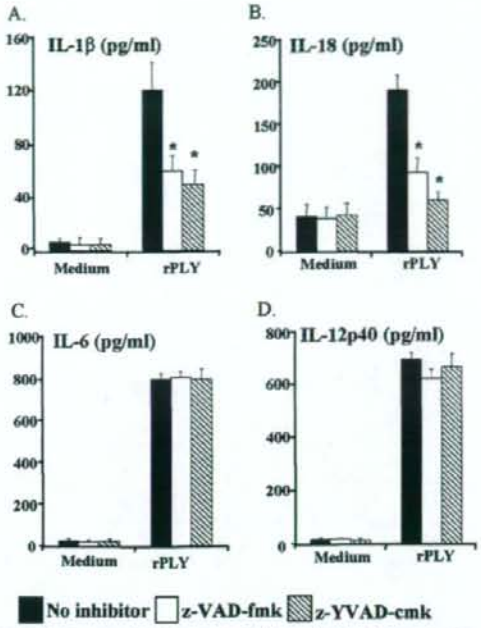

FIG. 8. Pneumolysin-induced IL-1β and IL-18 production is caspase-1 dependent. Peritoneal macrophages were stimulated with cholesterol-treated rPLY (1 μg/ml) in the presence or absence of a broad-spectrum caspase inhibitor (z-VAD-fmk [30 μM]) and a caspase-1-specific inhibitor (z-YVAD-cmk [30 μM]) for 24 h, and the culture supernatant was collected. The amounts of IL-1β (A), IL-18 (B), IL-6 (C), and IL-12p40 (D) were determined by ELISA. The results are representative of three separate experiments. The data are the means ± standard deviations for three or four determinations. *, $P < 0.05$ compared to no-inhibitor treatment.

expression and production of TNF-α and IL-6 were significantly lower than those induced by LPS (Fig. 2 and 6). These findings may be due to some difference in the events downstream of TLR4 ligation with rPLY or LPS. Therefore, we first examined the proteolysis of IκBα, a regulator of NF-κB, to estimate the activation of NF-κB. Both rPLY and LPS induced the degradation of IκBα, but some differences were observed in its strength and kinetics. The LPS-induced degradation of IκBα was observed as early as 5 min after stimulation (data not shown), whereas that induced by rPLY took place later (Fig. 7A). The degradation of IκBα was not observed in TLR4-deficient macrophages after stimulation with both rPLY and LPS. Next, the activation of MAPKs was compared. LPS strongly induced the phosphorylation of p38 MAPK and extracellular signal-regulated kinases (ERKs). In contrast, rPLY induced only weak phosphorylation of p38 MAPK and hardly induced ERK phosphorylation (Fig. 7B). These results indicate that the activation of the signaling process after sensing by TLR4 differs between stimulation with rPLY and LPS, which may account for the different profiles of the cytokine response to stimulation with these two TLR4 agonists.

Caspase-1 dependency of PLY-induced IL-1β and IL-18 production. Intracellular IL-1β and IL-18 are not secreted from cells as active forms until they are cleaved by caspase-1. To understand whether PLY-induced macrophage production of IL-1β and IL-18 is due to the activation of caspase-1, the effects of caspase inhibitors were examined. Peritoneal macrophages were pretreated with the caspase-1-specific inhibitor z-YVAD-cmk or the broad-spectrum caspase inhibitor z-VAD-fmk. Both inhibitors effectively reduced the secretion of IL-1β and IL-18 in the culture supernatant, suggesting that caspase-1 activation was induced by rPLY stimulation (Fig. 8). Nonspecific effects of the two inhibitors could be ruled out because the production of IL-6 and IL-12p40 was unaffected by either inhibitor.

Activation of caspase-1 by PLY. Since the production of IL-1β and IL-18 induced by rPLY was dependent on caspase-1, to confirm the PLY-induced caspase activation, we first tried to detect the active form of caspase-1 in macrophages stimulated with rPLY by means of Western blotting using a polyclonal antibody against the cleaved p20 fragment of caspase-1. This approach was not successful, however, because the amount of p20 fragment inside cells was too small, probably due to a rapid secretion of the active form soon after the cleavage (28). For this reason, we concentrated intracellular activated caspase-1 by a pull-down method using biotinylated YVAD-cmk, which binds to the active form of caspase-1, as previously described, with slight modification (29), and then applied the samples to Western blotting. The activated form of caspase-1 could be detected in macrophages stimulated by both cholesterol-treated rPLY and untreated rPLY (Fig. 9A). Caspase-1 activation was induced sufficiently with even a low dose (0.1 μg/ml) of rPLY, whereas 1 μg/ml LPS was not capable of inducing caspase-1 activation. As previously reported (47), an additional stimulation with ATP was required for the activation of caspase-1 in macrophages stimulated with LPS (Fig. 9B). To

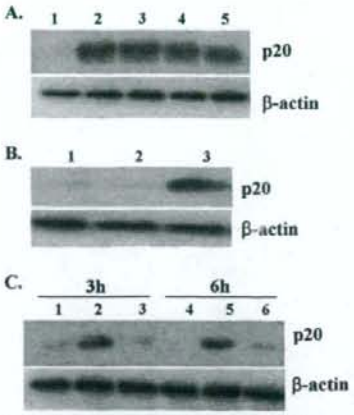

FIG. 9. Activation of caspase-1 by rPLY and *Streptococcus pneumoniae* infection. Peritoneal macrophages were incubated with biotinylated YVAD-cmk (30 μM) for 1 h and subsequently stimulated with rPLY, cholesterol-treated rPLY, or LPS for 3 h, using different concentrations of rPLY or LPS. Activated caspase-1 was precipitated using streptavidin beads. Precipitates were subsequently analyzed for the presence of active caspase-1 (p20 subunit) by Western blotting using a caspase-1 antibody (A and B). (A) Lanes: 1, unstimulated; 2, 1 μg/ml rPLY; 3, 0.1 μg/ml rPLY; 4, 1 μg/ml cholesterol-treated rPLY; 5, 0.1 μg/ml cholesterol-treated rPLY. (B) Lanes: 1, unstimulated; 2, 1 μg/ml LPS; 3, LPS plus ATP (1 mM). (C) Similarly, peritoneal macrophages were incubated with biotinylated YVAD-cmk (30 μM) for 1 h and infected with wt *S. pneumoniae* and the Δ*ply* mutant at an MOI of 10, and cell lysates were collected at the indicated time points. Activated caspase-1 was detected as mentioned above. Lanes 1 and 4, uninfected cells; lanes 2 and 5, wt *S. pneumoniae*-infected cells; lanes 3 and 6, Δ*ply* mutant-infected cells. For all panels, results are representative of at least three separate experiments.

rule out the contamination of our rPLY preparations with ATP, the level of ATP was determined using the Enliten ATP assay system (Promega). The ATP level in 1 μg/ml of rPLY was <17.9 pM, and this concentration of ATP never enhanced caspase-1 activation in LPS-stimulated macrophages (data not shown). These results suggest that rPLY induces the activation of caspase-1 without any other additional stimuli and that this ability is independent of its cytolytic activity (Fig. 9A). When macrophages were infected with wt *S. pneumoniae*, caspase-1 activation was strongly induced, whereas the Δ*ply* mutant was unable to induce it effectively (Fig. 9C), suggesting that PLY is also required for the activation of caspase-1 in macrophages infected with *S. pneumoniae*.

DISCUSSION

In the present study, we examined the role of PLY in the induction of various proinflammatory cytokines in response to *S. pneumoniae* infection. Among a number of different cytokines induced in macrophages infected with *S. pneumoniae*, the production of IL-1α, IL-1β, and IL-18 was exclusively dependent on the presence of the *ply* gene, as clearly shown using an in-frame deletion mutant of *ply*. In contrast, deletion of *ply* did not result in any severe change in the ability of *S. pneumoniae* to induce the production of TNF-α, IL-6, and IL-12. This finding strongly implied that the PLY protein itself is an effective ligand that activates caspase-1, which is required for the maturation of IL-1β and IL-18. This possibility was confirmed by the fact that the rPLY protein alone did induce the production of these cytokines. The reduced production of IL-1α, IL-1β, and IL-18 observed in macrophages infected with the Δ*ply* mutant recovered when it was supplemented with rPLY, to a level comparable with that in macrophages infected with wt *S. pneumoniae*. Taken together, these findings clearly show that the profile of the *S. pneumoniae*-induced cytokine response strongly depends on PLY and that PLY is unique in terms of activity for inducing caspase-1 activation, which is not observed with LPS.

Various bacterial ligands are recognized by several TLRs, with a strict specificity of each TLR and the pattern of each ligand. TLR4, which plays an essential role in LPS recognition, is also reported to be involved in the recognition of PLY (27, 51) and other related bacterial cytolysins, including anthrolysin (41) and *Listeria*-derived cytolysins (20). In the present study, using rPLY, we demonstrated that TLR4 was essential for the PLY-induced production of IL-1α, IL-1β, and IL-18. Interestingly, in spite of their similar and exclusive dependence on TLR4, rPLY and LPS induced quite different profiles of cytokine production. This suggested that PLY and LPS differentially activated the downstream signaling cascades. Indeed, there was some difference in the timing and duration of NF-κB activation between macrophages stimulated with rPLY and LPS. Furthermore, PLY only weakly activated ERK and p38 MAPK, whereas LPS strongly activated these downstream molecules. Because both NF-κB and MAPKs contribute to the induction of various cytokines, the different modes of activation of these signaling molecules might account for the difference observed in cytokine-inducing activity between PLY and LPS. For example, the production of TNF-α is regulated by NF-κB and MAPKs during and after transcription. ERK is involved particularly in the posttranscriptional transport of TNF-α mRNA from the nucleus to the cytoplasm in response to LPS (15). Although the exact reason that rPLY induced just a small amount of TNF-α is not yet clear, it is possible that TNF-α production was down-regulated posttranscriptionally, because rPLY clearly induced the gene expression of TNF-α but hardly activated ERK.

Although both LPS and PLY are TLR4 agonists, the precise interaction of each ligand with TLR4 seems to be different. Malley et al. (27) showed that MD2, which associates with TLR4 and plays a critical role in the cytokine response to LPS, was not required for PLY-dependent activation of NF-κB. Furthermore, Srivastava et al. (51) demonstrated that there is a specific and strong physical interaction between TLR4 and PLY by using a solid-phase binding assay. It is likely that cofactors required for LPS sensing via TLR4, such as MD2, LPS-binding protein, and CD14, are not so important for the recognition of PLY by TLR4. Sa15-21, which is an anti-TLR4 monoclonal antibody, was reported to bind to the N-terminal leucine-rich repeat of TLR4 and to act as an agonistic antibody. This antibody strongly induced NF-κB activation but modestly induced TNF-α production in vivo and in vitro (2). Therefore, it is conceivable that the observed difference in the activation of the downstream signaling cascade and the overall difference in the profile of cytokine production may result from the different interaction between TLR4 and LPS or PLY.

LPS directly binds to MD2 associated with the extracellular domain of TLR4 and then induces clustering of TLR4/MD2, which leads to the activation of two main signaling pathways downstream of TLR4, namely, the MyD88/Toll–IL-1 receptor (TIR) domain-containing adaptor protein (TIRAP)-dependent pathway and the TIR domain-containing adaptor inducing beta interferon (IFN-β) (TRIF)/TRIF-related adaptor molecule (TRAM)-dependent pathway (46). The MyD88/TIRAP-dependent pathway is involved preferentially in the induction of proinflammatory cytokines, whereas the TRIF/TRAM-dependent pathway is involved in the induction of IFN-β rather than proinflammatory cytokines (3). Although it remains unclear whether TLR4/MD2 clustering occurs in response to PLY, our data suggested that MyD88 was required for the rPLY-induced expression of proinflammatory cytokines. A finding that rPLY did not induce the expression of IFN-β (data not shown) implied that PLY may activate the MyD88/TIRAP-dependent but not the TRIF/TRAM-dependent pathway via its binding to TLR4. It has been reported that the integrity of lipid rafts is essential for the cellular response to LPS and that TLR4 is recruited to lipid rafts after stimulation with LPS (52). Do TLR4/MD2 clustering and/or recruitment of TLR4 to lipid rafts occur upon stimulation with PLY? Are lipid rafts required for the TLR4-dependent cytokine response to PLY? Which part of TLR4 interacts with PLY, and does binding result in some conformational change of TLR4 which leads to activation of the downstream signals? Although there are a number of questions to be addressed, a PLY-induced, noncanonical pathway may provide a new insight into the study of TLR signaling, especially that via TLR4.

An interesting finding of this study is that the caspase-1-dependent cytokines, IL-1β and IL-18 were strongly induced by stimulation with rPLY but hardly induced by LPS alone. Our study confirmed that PLY-induced production of IL-1β and IL-18 was caspase-1 dependent, and the active form of caspase-1 was detected in macrophages stimulated with rPLY. The question of whether TLR4 is involved in the activation of caspase-1 arose. To clarify this point, we tested the involvement of TLR4 in PLY-induced caspase-1 activation. When macrophages were stimulated with rPLY at a sublytic concentration (0.1 μg/ml), our result suggested a requirement for TLR4 in caspase-1 activation, but TLR4 dependency was not observed when cells were stimulated with rPLY at a lytic concentration (1 μg/ml) (data not shown). Our assumption is that both TLR4-dependent and -independent pathways might be involved in PLY-induced activation of caspase-1 and that TLR4 might potentiate the activation of caspase-1. The decrease in intracellular K^+ level induced by danger signals and toxins, such as ATP and nigericin, results in the enhancement of caspase-1 activation (44). Furthermore, a cytolytic concentration of PLY caused a K^+ efflux without inhibiting the activity of Na^+,K^+-ATPase (11). It is therefore possible that a PLY-formed pore may mediate K^+ efflux and consequently modulate the caspase-1 processing pathway. However, the ability of rPLY to induce the production of IL-1β and IL-18 was not affected, even when its pore-forming activity was blocked by cholesterol pretreatment. Moreover, rPLY-induced activation of caspase-1 was not abolished by cholesterol pretreatment. Because cholesterol pretreatment completely blocked the cytolytic activity of 0.1 μg/ml rPLY and this concentration of cholesterol-treated rPLY clearly induced caspase-1 activation and the production of caspase-1-dependent cytokines, pore formation does not appear to be essential for the induction of caspase-1 activation by PLY. Nonetheless, we cannot completely rule out the possibilities that pore-forming activity of rPLY might remain at a level under the detection limit of the lactate dehydrogenase release assay, even after cholesterol treatment, and that the level of pore formation might be sufficient to mediate K^+ efflux that can induce caspase-1 activation. In a future study, we plan to clarify whether the PLY-formed pore is essentially involved in the activation of caspase-1 by using a truncated rPLY that completely lacks cytolytic activity. Indeed, a noncytolytic mutant of PLY has been reported to retain its ability to activate TLR4 signaling and to induce IL-6, TNF-α, and IFN-γ (5, 27), and therefore such a mutant protein would be useful for this line of investigation. PLY is a multifunctional protein and its mechanisms for cytolysis have been investigated, but how this cytolysin induces caspase-1 activation remains to be clarified.

Both IL-1 and IL-18 have been reported to play a key role in infections caused by *S. pneumoniae*. Zwijnenburg et al. (55) demonstrated that endogenous IL-1 is essential for an adequate host defense in pneumococcal meningitis, as reflected by impaired bacterial clearance and reduced survival of IL-1 receptor-deficient (IL-1R$^{-/-}$) mice. Similarly, in a murine pneumococcal pneumonia model, IL-1R$^{-/-}$ mice showed an impaired early host defense (45). Studies have also shown that IL-18 has a protective role in the early immune response in a murine pneumococcal pneumonia model by promoting bacterial clearance from the lung and delaying the progression to a systemic infection (26), although the contribution of an inflammatory response to the detrimental effect was also reported for a meningitis model (56). In the present study, PLY was identified as an essential factor that contributes to IL-1α, IL-1β, and IL-18 production in response to *S. pneumoniae*.

It is therefore possible that PLY-dependent caspase-1 activation and subsequent production of IL-1β and IL-18 protect the host against pneumococcal infection. The present finding that IL-18 production induced by *S. pneumoniae* was dependent on TLR4 may account for the increased susceptibility to *S. pneumoniae* of mice that lack functional TLR4 (27). A previous report demonstrated that the interaction between PLY and TLR4 resulted in the induction of caspase-dependent host cell apoptosis in mice infected with *S. pneumoniae* (51), although several reports demonstrated that apoptosis-like cell death induced by *S. pneumoniae* or PLY in various types of cells is independent of caspases (6, 10, 12). In that report (51), it appeared that *S. pneumoniae*-induced apoptosis contributed to the host defense, because administration of the broad-spectrum caspase inhibitor z-VAD-fmk to mice increased the mortality rate after pneumococcal colonization in the nasopharynx. Because PLY-induced IL-18 production was also inhibited by z-VAD-fmk in our study, IL-18 production in addition to apoptosis may participate in the host defense mechanism induced by the TLR4-PLY interaction.

IL-1 and IL-18 are proinflammatory cytokines that can induce inflammatory reactions and are involved in the development of systemic and local inflammatory illnesses, including arthritis, asthma, sepsis, pneumonia, and meningitis caused by various agents (13, 30, 38, 42, 56). From our finding that PLY,

which is a toxin highly associated with the pathogenicity of *S. pneumoniae*, plays an essential role in the activation of caspase-1 and subsequent induction of IL-1 and IL-18 production upon *S. pneumoniae* infection, it is thus suggested that the PLY-dependent cytokine response comprises part of the pathophysiological mechanism, in addition to cytolytic activity and other functions of the cytolysin as a virulence factor. Indeed, the absence of IL-1R or IL-18 affected the histopathology of the lungs and brains in murine models of *S. pneumoniae* infection (26, 55). Analysis of infection models using mice deficient for caspase-1 or both IL-1R and IL-18 would elucidate the significance of PLY-dependent cytokines in the aspects of host defense and disease progression. For better control of the diseases caused by *S. pneumoniae* infection, more detailed investigations into the virulence of this pathogen, host defense mechanisms, and their influences on pathophysiology are required. A full understanding of the functions and physiological roles of PLY may pave the way for the control of harmful cytokines during pneumococcal infection.

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REFERENCES

1. **Akashi, S., S. Saitoh, Y. Wakabayashi, T. Kikuchi, N. Takamura, Y. Nagai, Y. Kusumoto, K. Fukase, S. Kusumoto, Y. Adachi, A. Kosugi, and K. Miyake.** 2003. Lipopolysaccharide interaction with cell surface Toll-like receptor4–MD-2: higher affinity than that with MD-2 or CD14. J. Exp. Med. **198:**1035–1042.
2. **Akashi-Takamura, S., T. Furuta, K. Takahashi, N. Tanimura, Y. Kusumoto, T. Kokayashi, S. I. Saitoh, Y. Adachi, T. Doi, and K. Miyake.** 2006. Agonistic antibody to TLR4/MD2 protects mice from acute lethal hepatitis induced by TNF-alpha. J. Immunol. **176:**4244–4251.
3. **Akira, S., and K. Takeda.** 2004. Toll-like receptor signaling. Nat. Rev. Immunol. **4:**499–511.
4. **Baba, H., I. Kawamura, C. Kohda, T. Nomura, Y. Ito, T. Kimoto, I. Watanabe, S. Ichiyama, and M. Mitsuyama.** 2001. Essential role of domain 4 of pneumolysin from *S. pneumoniae* in cytolytic activity as determined by truncated proteins. Biochem. Biophys. Res. Commun. **281:**37–44.
5. **Baba, H., I. Kawamura, C. Kohda, T. Nomura, Y. Ito, T. Kimoto, I. Watanabe, S. Ichiyama, and M. Mitsuyama.** 2002. Induction of gamma interferon and nitric oxide by truncated pneumolysin that lacks pore-forming activity. Infect. Immun. **70:**107–113.
6. **Bermpohl, D., A. Halle, D. Freyer, E. Dagand, J. S. Braun, I. Bechmann, N. W. Schroder, and J. R. Weber.** 2005. Bacterial programmed cell death of cerebral endothelial cells involves dual death pathways. J. Clin. Investig. **115:**1607–1615.
7. **Berry, A. M., A. D. Ogunniyi, D. C. Miller, and J. C. Paton.** 1999. Comparative virulence of *Streptococcus pneumoniae* strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. Infect. Immun. **67:**981–985.
8. **Branger, J., S. Knapp, S. Weijer, J. C. Leemans, J. M. Pater, P. Spleelman, S. Florquin, and T. van der Poll.** 2004. Role of Toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. Infect. Immun. **72:**788–794.
9. **Braun, J. S., R. Novak, G. Gao, P. J. Murray, and J. L. Shenep.** 1999. Pneumolysin, a protein toxin of *Streptococcus pneumoniae*, induces nitric oxide production from macrophages. Infect. Immun. **67:**3750–3756.
10. **Braun, J. S., R. Novak, P. J. Murray, C. M. Eischen, S. A. Sustin, G. Kroemer, A. Halle, J. R. Weber, E. I. Tuomanen, and J. L. Cleveland.** 2001. Apoptosis-inducing factor mediates microglial and neuronal apoptosis caused by pneumococcus. J. Infect. Dis. **184:**1300–1309.
11. **Cockeran, R., A. J. Theron, H. C. Steel, N. M. Matlola, T. J. Mitchell, C. Feldman, and R. Anderson.** 2001. Proinflammatory interactions of pneumolysin with human neutrophils. J. Infect. Dis. **183:**604–611.
12. **Colino, J., and C. M. Snapper.** 2003. Two distinct mechanisms for induction of dendritic cell apoptosis in response to intact *Streptococcus pneumoniae*. J. Immunol. **171:**2354–2365.
13. **Dinarello, C. A.** 1996. Biologic basis for interleukin-1 in disease. Blood **87:**2095–2147.
14. **Doern, G. V., A. B. Brueggemann, H. Huynh, E. Wingert, and P. Rhomberg.** 1999. Antimicrobial resistance with *Streptococcus pneumoniae* in the United States, 1997-98. Emerg. Infect. Dis. **5:**757–765.
15. **Dumitru, C. D., J. D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J. H. Lin, C. Patriotis, N. A. Jenkins, N. G. Copeland, G. Kollias, and P. N. Tsichlis.** 2000. TNF-α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell **103:**1071–1083.
16. **Dunne, A. O., and A. J. Luke.** 2003. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. Science STKE **170:**1–17.
17. **French, N., J. Nakiyingi, L. M. Carpenter, E. Lugada, C. Watera, K. Moi, M. Moore, D. Antvelink, D. Mulder, E. N. Janoff, J. Whitworth, and C. F. Gilks.** 2000. 23-Valent pneumococcal polysaccharide vaccine in HIV-1-infected Ugandan adults: double-blind, randomised and placebo controlled trial. Lancet **355:**2106–2111.
18. **Houldworth, S., P. W. Andrew, and T. J. Mitchell.** 1994. Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes. Infect. Immun. **62:**1501–1503.
19. **Inohara, N., and G. Nunez.** 2003. NOD-LRRs: intracellular proteins involved in inflammation and apoptosis. Nat. Rev. Immunol. **3:**371–382.
20. **Ito, Y., I. Kawamura, C. Kodha, K. Tsuchiya, T. Nomura, and M. Mitsuyama.** 2005. Seeligeriolysin O, a protein toxin of *Listeria seeligeri* stimulates macrophage cytokine production via Toll-like receptors in a profile different from that induced by other bacterial ligands. Int. Immunol. **17:**1597–1606.
21. **Jedrzejas, M. J.** 2001. Pneumococcal virulence factors: structure and function. Microbiol. Mol. Biol. Rev. **65:**187–207.
22. **Jones, M. R., B. T. Simms, M. M. Lupa, M. S. Kogan, and J. P. Mizgend.** 2005. Lung NF-kappa B activation and neutrophil recruitment require IL-1 and TNF receptor signaling during pneumococcal pneumonia. J. Immunol. **175:**530–535.
23. **Keer, S., S. L. Salmon, S. A. Lotz, and D. W. Metzger.** 2007. Interleukin-12 promotes gámma interferon-dependent neutrophil recruitment in the lung and improves protection against respiratory *Streptococcus pneumoniae* infection. Infect. Immun. **75:**1196–1202.
24. **Kelly, S. J., and M. J. Jedrzejas.** 2000. Structure and molecular mechanism of a functional form of pneumolysin: a cholesterol-dependent cytolysin from *Streptococcus pneumoniae*. J. Struct. Biol. **132:**72–81.
25. **Latz, E., A. Visintin, E. Lien, K. A. Fitzgerald, B. G. Monks, E. A. Kurt-Jones, D. T. Golenbock, and T. Espevik.** 2002. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the Toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. J. Biol. Chem. **277:**47834–47843.
26. **Lauw, F. N., J. Branger, S. Florquin, P. Speelman, S. J. van Deventer, S. Akira, and T. van der Poll.** 2002. IL-18 improves the early antimicrobial host response to pneumococcal pneumonia. J. Immunol. **168:**372–378.
27. **Malley, R., P. Henneke, S. C. Morse, M. J. Cieslewicz, M. Lipsitch, C. M. Thompson, E. Kurt-Jones, J. C. Paton, M. R. Wessels, and D. T. Golenbock.** 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc. Natl. Acad. Sci. USA **100:**1966–1971.
28. **Martinon, F., K. Burns, and J. Tschopp.** 2002. The inflamasome: a molecular platform triggering the activation of inflammatory caspases and processing of proIL-1 beta. Mol. Cell **10:**417–426.
29. **Martinon, F., L. Agostini, E. Meylan, and J. Tshopp.** 2004. Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflamasome. Curr. Biol. **14:**1929–1934.
30. **Matsui, K., H. Tsutsui, and K. Nakanishi.** 2003. Pathological role for IL-18 in inflammatory arthritis. Expert Opin. Ther. Targets **7:**701–724.
31. **Medzhitov, R.** 2001. Toll-like receptors and innate immunity. Nat. Rev. Immunol. **1:**135–145.
32. **Mitchell, T. J., J. E. Alexander, P. J. Morgan, and P. W. Andrew.** 1997. Molecular analysis of virulence factors of *Streptococcus pneumoniae*. Soc. Appl. Bacteriol. Symp. Ser. **26:**62S–71S.
33. **Mitchell, T. J., P. W. Andrew, F. K. Saunders, A. N. Smith, and G. J. Boulnois.** 1991. Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. Mol. Microbiol. **5:**1883–1888.
34. **Nakamatsu, M., N. Yamamoto, M. Hotta, C. Nakasone, T. Kinjo, K. Miyagi, K. Uezu, K. Nakamura, M. Taniguchi, Y. Iwakura, M. Kaku, J. Fujita, and K. Kawakami.** 2007. Role of interferon-gamma in Valpha 14+ natural killer T cell-mediated host defense against Streptococcus pneumoniae infection in murine lungs. Microbes Infect. **9:**364–374.
35. **Nomura, T., I. Kawamura, K. Tsuchiya, C. Kohda, H. Baba, Y. Ito, T. Kimoto, I. Watanabe, and M. Mitsuyama.** 2002. Essential role of interleukin-12 (IL-12) and IL-18 for gamma interferon production induced by listeriolysin O in mouse spleen cells. Infect. Immun. **70:**1049–1055.
36. **Novak, R., B. Henriques, E. Charpentier, S. Normark, and E. Tuomanen.** 1999. Emergence of vancomycin tolerance in *Streptococcus pneumoniae*. Nature **399:**590–593.
37. **O'Brien, A. D., D. L. Rosenstreich, I. Scher, G. H. Campbell, R. P. Mac-**

Dermott, and S. B. Formal. 1980. Genetic control of susceptibility to *Salmonella typhimurium* in mice: role of the LPS gene. J. Immunol. **124:**20–24.
38. **Okamura, H., K. Nagata, T. Komatsu, T. Tanimoto, Y. Nukata, F. Tanabe, K. Akita, K. Torigoe, T. Okura, S. Fukuda, and M. Kurimoto.** 1995. A novel costimulatory factor for gamma interferon induction found in the livers of mice causes endotoxic shock. Infect. Immun. **63:**3966–3972.
39. **Ortqvist, A., J. Hedlund, L. A. Burman, E. Elbel, M. Hofer, M. Leinonen, I. Lindblad, B. Sundelof, and M. Kalin.** 1998. Randomised trial of 23-valent pneumococcal capsular polysaccharide vaccine in prevention of pneumonia in middle-aged and elderly people. Lancet **351:**399–403.
40. **Palmer, M.** 2001. The family of thiol activated, cholesterol-binding cytolysins. Toxicon **39:**1681–1689.
41. **Park, J. M., V. H. Ng, S. Maeda, R. F. Rest, and M. Karin.** 2004. Anthrolysin O and other gram-positive cytolysins are Toll-like receptor 4 agonists. J. Exp. Med. **200:**1647–1655.
42. **Paterson, G. K., C. E. Blue, and T. J. Mitchell.** 2005. Role of IL-18 in experimental infections with *Streptococcus pneumoniae*. J. Med. Microbiol. **54:**323–326.
43. **Paton, J. C.** 1996. The contribution of pneumolysin to the pathogenicity of *Streptococcus pneumoniae*. Trends Microbiol. **4:**103–106.
44. **Perregaux, D., and C. A. Gabel.** 1994. Interleukin-1 beta maturation and release in response to ATP and nigricin: evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. J. Biol. Chem. **269:**15195–15203.
45. **Rijneveld, A. W., S. Florquin, J. Branger, P. Speelman, S. J. Van Deventer, and T. van der Poll.** 2001. TNF-α compensates for the impaired host defense of IL-1 type I receptor-deficient mice during pneumococcal pneumonia. J. Immunol. **167:**5240–5246.
46. **Saitoh, S., and K. Miyake.** 2006. Mechanism of regulating cell surface expression and activation of Toll-like receptor 4. Chem. Rec. **6:**311–319.
47. **Sanz, J. M., and F. Di Virgilio.** 2000. Kinetics and mechanism of ATP-dependent IL-1β release from microglial cells. J. Immunol. **64:**4893–4898.
48. **Schaaf, B., J. Rupp, M. Muller-Steinhardt, J. Kruse, F. Boehmke, M. Maass, P. Zabel, and K. Dalhoff.** 2005. The interleukin-6-174 promoter polymorphism is associated with extrapulmonary bacterial dissemination in *Streptococcus pneumoniae* infection. Cytokine **31:**324–328.
49. **Schmeck, B., S. Huber, K. Moog, J. Zahlten, A. C. Hocke, B. Opitz, S. Hammerschmidt, T. J. Mitchell, M. Kracht, S. Rosseau, N. Suttorp, and S. Hippenstiel.** 2006. Pneumococci induced TLR- and Rac1-dependent NF-κB-recruitment to the IL-8 promoter in lung epithelial cells. Am. J. Physiol. Lung Cell Physiol. **290:**L730–L737.
50. **Shumway, C. N., and S. J. Klebanoff.** 1971. Purification of pneumolysin. Infect. Immun. **4:**388–392.
51. **Srivastava, A., P. Henneke, A. Visintin, S. C. Morse, V. Martin, C. Watkins, J. C. Paton, M. R. Wessels, D. T. Golenbock, and R. Malley.** 2005. The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. Infect. Immun. **73:**6479–6487.
52. **Triantafilou, M., K. Miyake, D. T. Golenbock, and K. Triantafilou.** 2002. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J. Cell Sci. **115:**2603–2611.
53. **Visintin, A., E. Latz, B. G. Monks, T. Espevik, and D. T. Golenbock.** 2003. Lysines 128 and 132 enable lipopolysaccharide binding to MD-2, leading to Toll like receptor-4 aggregation and signal transduction. J. Biol. Chem. **278:**48313–48320.
54. **Wellmer, A., J. Gerber, J. Ragheb, G. Zysk, T. Kunst, A. Smirnov, W. Bruck, and R. Nau.** 2001. Effect of deficiency of tumor necrosis factor or both of its receptors on *Streptococcus pneumoniae* central nervous system infection and peritonitis. Infect. Immun. **69:**6881–6886.
55. **Zwijnenburg, P. J., T. van der Poll, S. Florquin, K. Takeda, J. J. Roord, and A. M. van Furth.** 2003. IL-1 receptor type-1 gene-deficient mice demonstrate an impaired host defense against pneumococcal meningitis. J. Immunol. **170:**4724–4730.
56. **Zwijnenburg, P. J., T. van der Poll, S. Florquin, S. Akira, K. Takeda, J. J. Roord, and A. M. van Furth.** 2003. Interleukin-18 gene-deficient mice show enhanced defense and reduced inflammation during pneumococcal meningitis. J. Neuroimmunol. **138:**31–37.

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Dependency of Caspase-1 Activation Induced in Macrophages by *Listeria monocytogenes* on Cytolysin, Listeriolysin O, after Evasion from Phagosome into the Cytoplasm[1]

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Listeriolysin O (LLO), an *hly*-encoded cytolysin from *Listeria monocytogenes*, plays an essential role in the entry of this pathogen into the macrophage cytoplasm and is also a key factor in inducing the production of IFN-γ during the innate immune stage of infection. In this study, we examined the involvement of LLO in macrophage production of the IFN-γ-inducing cytokines IL-12 and IL-18. Significant levels of IL-12 and IL-18 were produced by macrophages upon infection with wild-type *L. monocytogenes*, whereas an LLO-deficient mutant (the *L. monocytogenes* Δ*hly*) lacked the ability to induce IL-18 production. Complementation of Δ*hly* with *hly* completely restored the ability. However, when Δ*hly* was complemented with *ilo* encoding ivanolysin O (ILO), a cytolysin highly homologous with LLO, such a restoration was not observed, although ILO-expressing *L. monocytogenes* invaded and multiplied in the macrophage cytoplasm similarly as LLO-expressing *L. monocytogenes*. Induction of IL-18 was diminished when pretreated with a caspase-1 inhibitor or in macrophages from caspase-1-deficient mice, suggesting the activation of caspase-1 as a key event resulting in IL-18 production. Activation of caspase-1 was induced in macrophages infected with LLO-expressing *L. monocytogenes* but not in those with Δ*hly*. A complete restoration of such an activity could not be observed even after complementation with the ILO gene. These results show that the LLO molecule is involved in the activation of caspase-1, which is essential for IL-18 production in infected macrophages, and suggest that some sequence unique to LLO is indispensable for some signaling event resulting in the caspase-1 activation induced by *L. monocytogenes*. *The Journal of Immunology*, 2008, 180: 7859–7868.

Listeria monocytogenes (LM)[3] is a Gram-positive facultative intracellular bacterium that often causes life-threatening infections in immunocompromised hosts, including newborns and elderly people (1–4). The pathogenicity of LM can be attributed to the invasion and subsequent intracellular parasitism in a variety of host cells such as hepatocytes, fibroblasts, and epithelial cells. Professional phagocytes, such as macrophages, are also the major target cells of LM because the pathogen can survive and grow inside macrophages, even once being trapped in phagosomes after phagocytosis. Virulence factors encoded in *Listeria* pathogenicity island 1 are required for the evasion of intracellular bactericidal mechanisms by LM. Among the group of virulence factors, listeriolysin O (LLO), a 56-kDa cytolysin encoded by *hly*, is the most important virulence determinant and plays an essential role in bacterial escape from the phagosome into the cytoplasm where the pathogen multiplies efficiently (5–7).

In mice infected with LM, innate immune cells such as macrophages and dendritic cells are activated to release proinflammatory cytokines, including TNF-α, IL-1, and IL-6. In addition to these proinflammatory cytokines, IL-12 and IL-18, which are IFN-γ-inducing cytokines, are also released from innate immune cells and subsequently induce the production of IFN-γ from NK cells and NK dendritic cells (8, 9). Such an initial IFN-γ response is not only essential for the host defense against primary LM infection but is also important for the establishment of T cell-mediated acquired immunity, which is required for the protection of the host against secondary challenge with LM (10, 11).

In contrast to the established role of IFN-γ for the host defense, the mechanism of IFN-γ induction in the initial stage of infection with LM has been elucidated only partially. On the basis of the fact that an infection with the LM strain lacking LLO, which is incapable of escape into the cytosol and intracellular multiplication, never induces a significant level of IFN-γ response (12, 13), LLO seems to play a critical role in the induction of IFN-γ. Regarding the contribution of LLO to the induction of IFN-γ response, there may be several possibilities. One possibility is that LLO serves just as cytolytic protein and simply enables the bacteria to escape from the phagosomal compartment. Then the recognition of the bacterial ligand(s) by some cytoplasmic pattern recognition receptor in the macrophage cytoplasm, for example, the Nod-like receptor (NLR), may result in the activation of the signaling pathway required for the induction of IFN-γ-inducing cytokines. Another possibility is the direct stimulation of the signaling cascade by LLO itself as an essential ligand after serving as the protein toxin necessary for

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[3] Abbreviations used in this paper: LM, *Listeria monocytogenes*; ILO, ivanolysin O; LI, *Listeria ivanovii*; LDH, lactate dehydrogenase; LLO, listeriolysin O; MOI, multiplicity of infection; NLR, Nod-like receptor; PEC, peritoneal exudate cell; PEST, Pro-Glu-Ser-Thr; z-YVAD-fmk, *N*-benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethyl ketone.

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evasion into the cytoplasm. As LLO is known to modulate various cellular responses (14), it is likely that the LLO molecule itself may induce or enhance the production of IFN-γ by activating macrophages as a bacterial modulin.

Listeria ivanovii (LI) is an animal pathogen and carries a gene cluster that is highly analogous to the *Listeria* pathogenicity island 1 of LM (15). LI produces ivanolysin O (ILO) encoded by *ilo*, a cytolysin that shows ~80% homology with LLO in amino acid sequence (16). Although LI is capable of evasion into and multiplication inside the macrophage cytoplasm like LM, IFN-γ responses after infection with LI in vitro and in vivo were very low as compared with those induced by LM (17). It is therefore unlikely that only the bacterial entry into the macrophage cytoplasm is sufficient for the induction of IFN-γ production. The comparison between LM and LI may not be the best tool to test the second possibility mentioned above as these two species are not isogenic although they belong to the same genus *Listeria*, and there may be some critical difference in the ligands other than the difference between LLO and ILO. To overcome this problem, isogenic LM mutants producing LLO or ILO were constructed by gene complementation of an LLO-deficient LM mutant with *hly* and *ilo*. In our previous study using these isogenic mutants, we examined whether the initial IFN-γ response is due simply to the entry of LM into the macrophage cytoplasm or whether the presence of LLO itself is required. It was found that LLO-producing LM, but not ILO-producing LM, strongly induced the production of IFN-γ on LM infection in vitro and in vivo (11). The results clearly indicated that the LLO molecule is involved by itself in the induction of host IFN-γ response and not by enabling bacterial cells to be delivered into the macrophage cytoplasm.

IFN-γ production in the host as an innate immune response to LM is highly dependent on the release of two major IFN-γ-inducing cytokines, IL-12 and IL-18 (18). In contrast to IL-12 production, which never requires further processing, the release of IL-18 as an active form definitely requires a cleavage of pro-IL-18 by caspase-1 (19). Recent reports have shown that caspase-1 activation is induced efficiently only by LM, which is capable of escaping from the phagosome, but not by the LLO mutant incapable of evasion into the cytosol (20, 21). In a study that emphasized the importance of intracellular parasitism for caspase-1 activation, the cytosolic flagellin of *Salmonella* appeared to be responsible for caspase-1 activation, but no particular component of *Listeria* was identified (22). These findings raised a possibility that LLO itself is one of the candidates for the bacterial molecule responsible for caspase-1 activation.

In this study, we have examined the molecular basis for the induction of IL-12 and IL-18 in macrophages stimulated with LM by using isogenic mutants that differ only in the cytolytic protein, with special reference to the induction of cytokine gene expression and caspase-1 activation.

Materials and Methods

Mice

Female mice of C57BL/6 (normal, TLR4 knockout) strains were purchased from Japan SLC. Caspase-1 knockout C57BL/6 mice were provided by H. Tsutsui (Hyogo Medical College, Hyogo, Japan). Mice were maintained in specific-pathogen-free conditions and used at 7–9 wk of age. All the experimental procedures performed on mice were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine, Kyoto, Japan.

Bacterial strains and growth conditions

The parental wild-type LM strain used in this study was *L. monocytogenes* EGD (serovar 1/2a). Three isogenic mutants, Δ*hly*, Δ*hly*::*hly*, and Δ*hly*::*ilo*, were constructed from wild-type LM using the homologous recombination method and the similarity in the expression level of each cytolysin was shown in a previous study (11). Bacteria were grown overnight in brain-heart infusion broth (EIKEN Chemical) at 37°C with shaking. One volume of the overnight culture was added to 100 volumes of fresh brain-heart infusion medium and cultured further for 5 h. Bacterial cells were washed, suspended in PBS supplemented with 10% glycerol, and stored in aliquots at −80°C. The concentration of bacteria was determined by plating 10-fold serially diluted suspensions on a tryptic soy agar (EIKEN Chemical) plate and counting the number of colonies after cultivation for 24 h.

Cells

Peritoneal exudate cells (PECs) of mice were obtained 3 days after an i.p. injection of 2 ml of thioglycolate medium (EIKEN Chemical). After washing with RPMI 1640, PECs were incubated on culture plates at 37°C for 3 h in culture medium that consisted of RPMI 1640 supplemented with 10% FCS. After incubation, the cells were washed with RPMI 1640 and adherent PECs were used for infection study. Bone marrow cells were obtained from tibiae of mice and then cultured in RPMI 1640 supplemented with 10% FCS, gentamicin (10 μg/ml; Wako Pure Chemical Industries), and recombinant mouse M-CSF (100 ng/ml; R&D Systems) for 5 days. After washing with RPMI 1640, adherent bone marrow-derived macrophages were collected. The cells were plated at 1.5×10^5 cells/well in 96-well microplates or at 2×10^6 cells/well in 6-well microplates for detection of cytokines or active caspase-1, respectively. The cells were infected with bacteria at a multiplicity of infection (MOI) of 1 for 30 min at 37°C.

Immunofluorescence analysis of bacterial escape

Adherent PECs were seeded into a 24-well plate at 5×10^5 cells/well and then infected with bacteria at a MOI of 1 for 30 min at 37°C. Cells were washed three times and cultured for 3 h at 37°C in the presence of 10 μg/ml gentamicin. After several washings, the cells were fixed by 3% paraformaldehyde and incubated overnight at 4°C with a blocking solution that is PBS containing 10% Blocking One (Nacalai) and 0.1% saponin (Nacalai). F-actin formation was visualized by the staining of infected cells with Alexa Fluor 488-phalloidin (Invitrogen), and the bacterial cell was stained by treatment with rabbit anti-*Listeria* polyclonal Ab (ViroStat) in blocking solution at room temperature for 1 h in a dark room and then with Alexa Fluor 594-anti-rabbit IgG Ab (Invitrogen) at room temperature for 1 h in a dark room. Cells were examined under fluorescent microscope for actin cloud or actin tail formation.

Neutralization of cytokines

Neutralization of cytokines in culture was done as reported previously (23, 24). The neutralizing Ab specific for IL-12 (goat, polyclonal) and that for IL-18 (rat, clone 93-10C) were purchased from R&D Systems and Medical & Biological Laboratories, respectively. As control Abs, normal rat IgG (ICN Biomedicals) and normal goat IgG (R&D Systems) were used. After infection with bacteria for 0.5 h, Abs were added to cell culture medium at 5 μg/ml.

ELISA

Levels of cytokines in culture supernatants were determined by two-site sandwich ELISA as reported previously (11, 25). Briefly, bacteria were added to the cell cultures and incubated at 37°C for 30 min. The infected cells were cultured for an additional 24 h in the presence of 10 μg/ml gentamicin. Culture supernatants were then collected and stored at −80°C until the cytokine measurement. The ELISA kit for TNF-α was purchased from eBiosciences. For the titration of other cytokines, pairs of biotin-labeled and unlabeled mAbs specific to IL-18 (Medical & Biological Laboratories), IL-12p70 (Endogen), and IFN-γ (Endogen) were used.

Quantitative real-time RT-PCR

Total cellular RNA was extracted using NucleoSpin RNA II (Macherey-Nagel), according to the manufacturer's instructions. The collected RNA (0.2 μg) was treated with RNase-free DNase (Promega) to eliminate contaminating DNA before being subjected to reverse transcription using random primers (Invitrogen) and ReverTra Ace (TOYOBO). Quantitative real-time RT-PCR was performed on ABI PRISM 7000 (Applied Biosystems) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Results were analyzed with ABI PRISM 7000 SDS software. The following mouse primer sequences were designed using Applied Biosystems Primer Express software: *tnfα*, 5′-ATGCTGGGACAGTGACCTGG-3′ (forward) and 5′-CCTTGATGGTGGTGCATGAG-3′ (reverse); *il-12p40*,5′-GGATGGAAGAGTCCCCCAAA-3′(forward)and5′-CTGGAAAAGCCAACCAAGC-3′ (reverse); *il-18*, 5′-GAAAGCCGCCTCAAACCTTC-3′ (forward) and 5′-CATTGTTCCTGGGCCAAGAG-3′ (reverse);

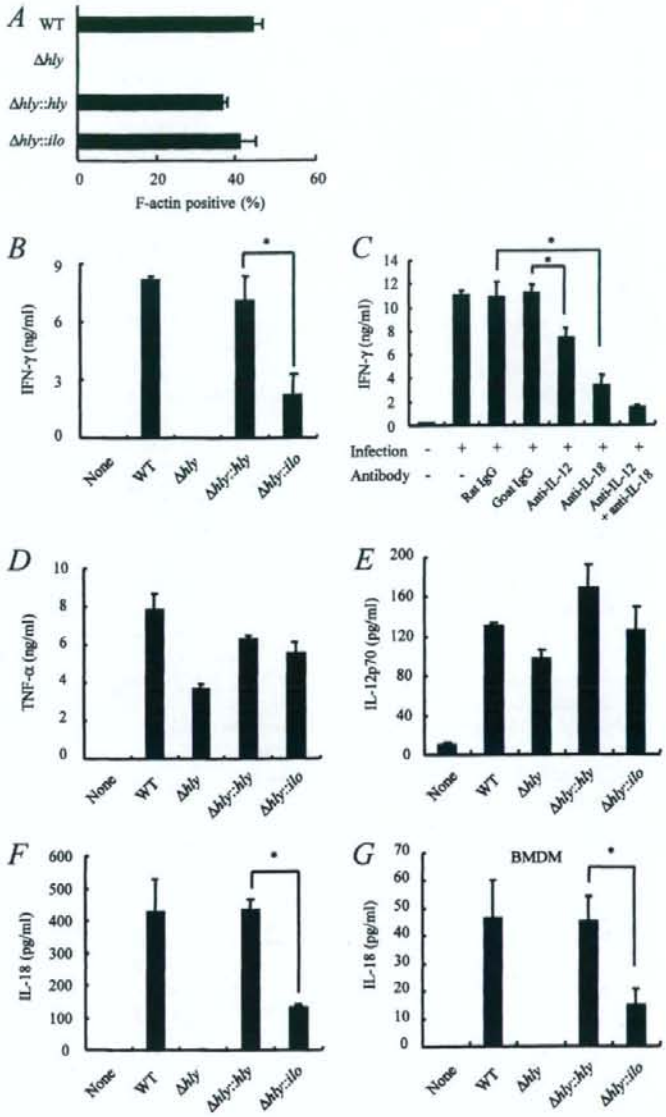

FIGURE 1. LLO- and ILO-expressing LM similarly escape from phagosomes but differently induce IFN-γ and IL-18. Whole PECs (*B* and *C*), adherent PECs (*A* and *D–F*), or bone marrow-derived macrophages (*G*) were infected with each LM strain. *A*, Cells were cultured for an additional 3 h in the presence of gentamicin, then bacteria and F-actin were stained and 300 bacteria were counted. The percentage of bacteria positive for associating F-actin was calculated for each strain. The filled bars represent the mean of three independent wells and the error bars indicate the SD. *B–G*, Cells were cultured for an additional 24 h in the presence (*C*) or absence (*B*, *D–G*) of each neutralizing Ab, and the amounts of each cytokine were then determined by ELISA. Data represent the mean of triplicate assays and SD. Similar results were obtained in three independent experiments. *, $p < 0.01$. WT, Wild type.

caspase-1, 5′-GCCCACTGCTGATAGGGTGA-3′ (forward) and 5′-CCCGGGAAGAGGTAGAAACG-3′ (reverse); and *β-actin*, 5′-GCCCTGAGGCTCTTTTCCAG-3′ (forward) and 5′-TGCCACAGGATTCCATACCC-3′ (reverse). Gene-specific transcript levels were normalized to the amount of β-actin mRNA.

Inhibition of caspase-1

A caspase-1-specific inhibitor, *N*-benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethyl ketone (z-YVAD-fmk), was purchased from R&D Systems. After infection with bacteria for 30 min, this inhibitor dissolved in DMSO was added to cell culture medium at several concentrations. For the control wells, DMSO without inhibitor was added.

Detection of caspase-1

The cells were cultured for several hours at 37°C with gentamicin after infection, with each LM strain at a MOI of 1 for 30 min. After cultivation, supernatants were collected, and cells were lysed in the buffer containing 1% Nonidet P-40 supplemented with 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1.5 μg/ml aprotinin, and 2 mM DTT. Six milliliters of culture supernatants were precipitated with 7 μg of rabbit polyclonal Ab for mouse caspase-1 p10 (Santa Cruz Biotechnology) in the presence of protein G-Sepharose (GE Healthcare). The cell lysates and precipitates were subjected to SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes by electroblotting. The membranes were immunoblotted with anti-caspase-1 Ab or anti-β-actin Ab (Sigma-Aldrich).

Detection of lactate dehydrogenase (LDH) release

Culture supernatants were collected, centrifuged, and transferred to new tubes. LDH activity was measured using an LDH cytotoxicity detection kit (TaKaRa BIO). The percentage of LDH release was calculated by using the following formula: percentage of release = 100 × (experimental LDH release − spontaneous LDH release)/(maximal LDH release − spontaneous LDH release). To determine the maximal LDH release, cells were treated with 1% Triton X-100.

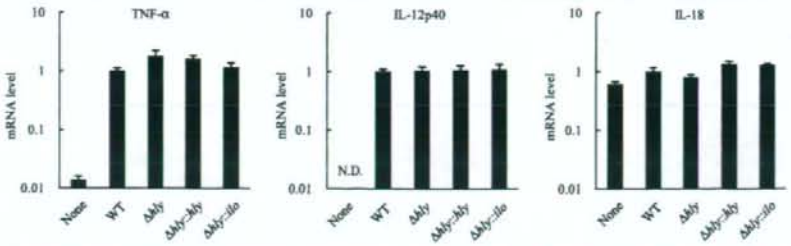

FIGURE 2. Expression of mRNA for various cytokines after infection with LM strains. Adherent PECs were infected with each LM strain. The cells were cultured for an additional 5 h in the presence of gentamicin. Total RNA was extracted and subjected to quantitative real-time RT-PCR for detection of mRNA for TNF-α, IL-12p40, and IL-18. Data represent the mean of triplicate assays and SD. N. D., Not detected; WT, wild type.

Immunofluorescence analysis

Adherent PECs seeded in 24-well microplates at 5×10^5 cells/well were infected with bacteria at a MOI of 1. After cultivation for 21 h, the cells were washed three times with PBS and then fixed in 4% paraformaldehyde. Fragmented DNA was labeled by the TUNEL method using MEBSTAIN Apoptosis Kit Direct (Medical & Biological Laboratories) according to the manufacturer's instructions. Total nucleus was visualized by 4′,6-diamidino-2-phenylindole staining (Dojindo). Bacterial cells were stained by using rabbit anti-*Listeria* polyclonal Ab (VivoStat) and Alexa Fluor 594-labeled anti-rabbit Ig G Ab (Invitrogen). The cells were examined under a fluorescent microscope, and cells positive for each fluorescence were enumerated.

Construction of Δhly::ilo strains expressing full length, truncated, or mutant LLO

An expression vector was constructed by ligation of the *prfA* and *hly* promoter region into the multiple cloning sites of pAT28, which contains a spectinomycin resistance gene, with Ligation High (Toyobo), and then gene fragments of *hly* full length, *hly* $Trp^{492} \rightarrow Ala^{492}$ (W492A), *hly* domain 1–3, and domain 4 were ligated downstream of *hly* promoter. The following primer sequences were used: *prfA*, 5′-cGATGAGCTCTTAATT TAATTTTCCCAAGTAGCAG-3′ (forward) and 5′-ACGCCCCGGGAT GAACGCTCAAGCAGAAG-3′ (reverse); *hly* promoter, 5′-CGATCCCGG GAATGGCCCCCTCCTTTGAT-3′ (forward) and 5′-CGCGGTACCGAT ATCCTTTGCTTCAGTTTG-3′ (reverse); *hly*, 5′-CGATTGCGCATCTGC ATTCAATAAAG-3′ (forward); *hly d4*, 5′-CGATCCCGGGAAAATTAA CATCGATCACTC-3′ (forward); *hly W492A*, 5′-TTGGGAATGGGCGAG AACGGTAA-3′ (forward); *hly*, 5′-GCTCTAGATTATTCGATTGGATTA TCTAC-3′ (reverse); *hly d1–3*, 5′-CGTCTAGATTATGTATAAGCTTT TGAAG-3′ (reverse). The primers for *prfA* were designed to generate restriction sites for *Sac*I and *Sma*I. The primers for *hly* promoter were designed to generate restriction sites for *Sma*I and *Kpn*I/*Eco*RV. The F-primers for *hly* and *hly d4* were designed to generate restriction sites for *Fsp*I and *Sma*I, respectively. The reverse primers for *hly* and *hly d1–3* were designed to generate restriction sites for *Xba*I. The resulting plasmid was introduced into the competent cells of *Δhly::ilo* by electroporation. Transformants were selected on brain-heart infusion agar plates supplemented with spectinomycin (250 μg/ml; Nacalai Tesque). The expression of each LLO molecule produced by transformed *Δhly::ilo* strains was confirmed by Western blotting using anti-LLO Ab, although the Ab showed a weak level of cross-reactivity to ILO. The similarity in the levels of ILO production by these ILO-producing strains carrying the plasmid harboring the *hly* fragment was confirmed by Western blotting using anti-ILO Ab. These polyclonal Abs for LLO or ILO were prepared by hyperimmunization of a normal white rabbit with recombinant LLO or recombinant ILO emulsified in Freund's complete adjuvant.

Statistical analysis

For comparisons between two groups, Student's t test was used when the variances of the groups were judged to be equal by F test. Multigroup comparisons of mean values were made according to the ANOVA and the Fisher's protected least significant difference post hoc test after the confirmation of homogeneity of variances among the groups by using Bartlett's test. Statistical significance was determined as $p < 0.05$.

Results

IL-12- and IL-18-dependent IFN-γ production induced by LM expressing LLO

In the in vitro infection of macrophages prepared from C57BL/6 mice, two *Δhly* strains complemented with *hly* or *ilo* exhibited a similar level of ability to escape from the phagosome of macrophages (Fig. 1*A*). An essential requirement for LLO in the induction of IFN-γ by LM was confirmed by using whole PECs from C57BL/6 mice, the cell population containing IFN-γ-producing cells such as NK cells (Fig. 1*B*). The IFN-γ-inducing ability of wild-type LM that was abolished by the deletion of *hly* could be restored when *Δhly* was complemented with *hly* but not *ilo*. These findings were consistent with our previous report in which PECs from C3H/HeN mice were used (11). In the culture system used in this study, both IL-12 and IL-18 were shown to be important for IFN-γ production induced by LLO-expressing LM, as neutralizing Abs against these two cytokines significantly inhibited the IFN-γ response (Fig. 1*C*).

Gene expression and production of IL-12 and IL-18 in response to LM strains

For the assessment of IL-12 and IL-18 production in response to each LM strain, we used culture supernatants of adherent peritoneal macrophages to rule out the possible influences of the products from other nonadherent cells like IFN-γ. A similar level of production of IL-12p70 was observed in all groups of macrophages infected with any of the LM strains used, indicating that induction of IL-12 is not dependent on the escape of bacteria from phagosome and a sort of cytolysin. Another caspase-independent cytokine, TNF-α, was also produced even by macrophages stimulated with *Δhly* and incapable of evasion into the cytosol (Fig. 1, *D* and *E*). By contrast, the pattern of IL-18 induction by LM strains was quite similar to that of IFN-γ (11). The IL-18-inducing ability of wild-type LM was lost completely by deletion of *hly* (*Δhly*) but was successfully restored by complementation with *hly* (*Δhly::hly*). Interestingly, such a significant level of restoration was not observed when *Δhly* was complemented with *ilo* (*Δhly::ilo*) (Fig. 1*F*). The critical difference in the ability to induce IL-18 production between *Δhly::hly* and *Δhly::ilo* was observed also in bone marrow-derived macrophages (Fig. 1*G*). These data indicate that LLO plays an important role in the induction of IL-18 in infection with LM and that the IL-18 response is dependent not on extraphagosomal evasion mediated by either LLO or ILO, but on the LLO molecule itself.

IL-18 is first synthesized as pro-IL-18, then processed to mature form and secreted from cells in response to appropriate stimuli. To clarify which stage of IL-18 production is stimulated by LLO, we