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13. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
14. Northern and Southern analysis using human DNA and RNA (Clontech) were performed as described (*4*).
15. Rabbits were immunized with peptides corresponding to the cytoplasmic domain of either human DAP10 (PAQEDGKVYINMPGRG) (Anaspec, San Jose, CA) or human NKG2D (KKSDFSTRWQKQR) (Research Genetics, Birmingham, AL) (*13*). Antisera were affinity-purified with the immunizing peptide. Specificity was determined by testing the affinity-purified antibodies by immunoprecipitation and protein immunoblot analysis on Ba/F3 cells transfected with human DAP10 and NKG2D (or both), compared with preimmune sera or control antibodies.
16. Cells were labeled with ^{125}I and solubilized [1% digitonin, 0.12% Triton X-100, 150 mM NaCl, 20 mM triethanolamine, 0.01% NaN_3 (pH 7.8), and protease inhibitors] (*4*). Lysates were incubated (2 hours, 4°C) with Pansorbin (Calbiochem) coated with clg, affinity-purified anti-DAP10, and affinity-purified anti-NKG2D. For immunoprecipitation with mouse mAb, Pansorbin was precoated with rabbit antibody to mouse Ig (Sigma) and mouse NKG2D mAb 5C6, anti-Flag mAb M2 (Sigma), or clg. Immunoprecipitates were washed in buffer [5 mM CHAPS, 50 mM tris, 150 mM NaCl (pH 8.0), and protease inhibitors] (*4*).
17. A cDNA containing the human CD8 leader segment, followed by the Flag epitope (DYKDDDDK), and joined to the extracellular, transmembrane, and cytoplasmic segments of human DAP10, was subcloned into the pMX-neo retroviral vector (*4*, *13*). Human NKG2D cDNA (*8*) was subcloned into pMX-puro. The D-A transmembrane Flag-DAP10 mutant cDNA with an A (GCC) substituted for D (GAT) and the R-L transmembrane NKG2D mutant cDNA with an L (CTT) substituted for R (CGT) were generated by PCR mutagenesis. Retroviruses were generated using the Phoenix packaging cell (*4*). Ba/F3 cells were infected and drug-selected, and transfectants were isolated by flow cytometry (*4*).
18. Phosphorylated or unphosphorylated biotinylated peptides of the cytoplasmic domain of DAP10 (PAQEDGKVYINMPGRG) were incubated with lysates from NKL, precipitated with avidin-agarose, and washed [1% NP-40, 50 mM tris, 150 mM NaCl (pH 7.8), and protease inhibitors] (*4*, *13*). Immunoprecipitates were analyzed by protein immunoblot using either anti-p85 or anti-Shc (specificity control) (UBI, Lake Placid, NY).
19. NKL and NKG2D-Flag-DAP10$^+$ Ba/F3 cells were incubated with 100 mM pervanadate and lysed (0.875% Brij 97, 0.125% NP-40, 150 mM NaCl, 10 mM tris, and protease and phosphatase inhibitors) (*4*). Antigens precipitated with clg, Flag mAb M2, or NKG2D mAb 5C6 were analyzed by protein immunoblot using horseradish peroxidase (HRP)–conjugated phosphotyrosine mAb 4G10 (UBI) or antiserum to p85 (UBI).
20. We thank M. Robertson, J. P. Houchins, C. Turck, G. Nolan, and T. McClanahan for reagents; J. Katheiser, G. Burget, and M. Andonian for graphics; J. Cupp, E. Callas, and D. Polakoff for flow cytometry; and D. Gorman and D. Liggett for sequencing. DNAX is supported by Schering Plough. S.B. and T.S. are supported by NIH grant AI30581.

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Host Defense Mechanisms Triggered by Microbial Lipoproteins Through Toll-Like Receptors

Hans D. Brightbill,[1]* Daniel H. Libraty,[4]* Stephan R. Krutzik,[1] Ruey-Bing Yang,[6] John T. Belisle,[7] Joshua R. Bleharski,[1] Michael Maitland,[8] Michael V. Norgard,[9] Scott E. Plevy,[10] Stephen T. Smale,[1,2,5] Patrick J. Brennan,[7] Barry R. Bloom,[8,11] Paul J. Godowski,[6] Robert L. Modlin[1,2,3]†

The generation of cell-mediated immunity against many infectious pathogens involves the production of interleukin-12 (IL-12), a key signal of the innate immune system. Yet, for many pathogens, the molecules that induce IL-12 production by macrophages and the mechanisms by which they do so remain undefined. Here it is shown that microbial lipoproteins are potent stimulators of IL-12 production by human macrophages, and that induction is mediated by Toll-like receptors (TLRs). Several lipoproteins stimulated TLR-dependent transcription of inducible nitric oxide synthase and the production of nitric oxide, a powerful microbicidal pathway. Activation of TLRs by microbial lipoproteins may initiate innate defense mechanisms against infectious pathogens.

Besides causing disease, mycobacteria have long been recognized for having powerful immunologic adjuvant activity, augmenting both cell-mediated and humoral immune responses. In 1972, a study of the mechanism of mycobacterial adjuvants demonstrated the induction of "soluble mediators," now known to be cytokines, which mediated the augmentation of immune responses (*1*). One cytokine induced by mycobacteria is IL-12 (*2*), a powerful signal for the generation of T helper type 1 lymphocyte (T_H1) responses (*3*) required to eliminate intracellular pathogens (*4*), including *Mycobacterium tuberculosis* (*5*). Furthermore, individuals with mutations in the IL-12 receptor (IL-12R) have increased susceptibility to mycobacterial infection (*6*). We therefore investigated the mycobacterial products that induce IL-12 as well as the mechanism responsible for its induction.

Mycobacterium tuberculosis $H_{37}Rv$ was gamma-irradiated and lysed by mechanical disruption, subcellular fractions were generated (*7*) and tested for the capacity to induce IL-12 with a human monocyte line, THP-1 (*8*). Other than the *M. tuberculosis* lysate, the soluble cell wall–associated proteins (SCWPs) contained most of the IL-12 p40–inducing capacity, consistent with the known adjuvant activity of mycobacterial cell walls (Fig. 1A). The combined cytosolic and membrane protein fraction, the culture filtrate, the lipoglycan from *M. tuberculosis*, and the mycolyl arabinogalactan peptidoglycan complex were less potent on a per weight basis (*9*).

To identify the cell wall–associated proteins responsible for IL-12 p40 release, we fractionated the SCWP preparation by gel filtration chromatography and preparative isoelectric focusing and monitored the bioactivity by measuring IL-12 p40 production (*10*). Subsequent separation of the bioactive fraction by SDS-PAGE indicated four prominently stained proteins: three bands at 17, 21.5, and 39 kD and a doublet at 60 to 70 kD (Fig. 1B). After these regions were transferred to nitrocellulose, they were solubilized (*11*) and used to stimulate THP-1 cells. The band migrating to 21.5 kD induced the highest levels of IL-12 p40 release, followed by the 39-kD band, with the two other regions inducing little activity (Fig. 1C). By using monoclonal antibodies to known *M. tuberculosis* antigens for protein immunoblot analysis, we identified the 21.5-kD band as the 19-kD lipoprotein antigen of *M. tuberculosis*

[1]Department of Microbiology and Immunology, and the [2]Molecular Biology Institute, Divisions of [3]Dermatology and [4]Infectious Disease, Department of Medicine, [5]Howard Hughes Medical Institute, University of California Los Angeles School of Medicine, Los Angeles, CA 90095, USA. [6]Genentech Incorporated, South San Francisco, CA 94080, USA. [7]Department of Microbiology, Colorado State University, Fort Collins, CO 80523, USA. [8]Albert Einstein College of Medicine and Howard Hughes Medical Institute, Bronx, NY 10461, USA. [9]University of Texas Southwestern Medical Center, Dallas, TX 75235, USA. [10]Immunobiology Center, Mount Sinai School of Medicine, New York, NY 10029–6574, USA. [11]Office of the Dean, Harvard School of Public Health, Boston, MA 02115, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: rmodlin@mednet.ucla.edu

and the 39-kD band as the *M. tuberculosis* 38-kD lipoprotein antigen (PstS homolog) (*12*). Purified 19- and 38-kD lipoproteins of *M. tuberculosis* induced IL-12 p40 release, with the 19-kD lipoprotein greater than one log more potent than whole *M. tuberculosis* and the 38-kD lipoprotein (Fig. 1D). The ability of the 19-kD lipoprotein to induce IL-12 was independent of tumor necrosis factor–α (TNF-α) release, because neutralizing antibodies to TNF-α (anti-TNF-α) blocked by less than 10% the induction of IL-12 p40 (*13*). The 19-kD lipoprotein also stimulated IL-12 p70 release from normal human monocytes (*14*). Thus, the *M. tuberculosis* 19-kD lipoprotein is a major inducer of IL-12, a cytokine that can amplify T_H1 and cytolytic T cell responses and hence contribute to the adjuvant activity of mycobacteria.

We investigated the mechanism by which the 19-kD lipoprotein induces IL-12 by using an IL-12 p40 promoter CAT reporter transiently transfected into the mouse macrophage cell line RAW 264.7 (*15, 16*). The 19-kD lipoprotein induced IL-12 p40 promoter activity in a dose-dependent manner and at a level comparable to lipopolysaccharide (LPS) (Fig. 2A). Previous studies indicated that LPS induction of IL-12 p40 promoter activity is dependent on both C/EBP and nuclear factor kappa B (NF-κB) sites (*15, 17*). The ability of both the 19-kD lipoprotein and LPS to induce IL-12 p40 promoter activity was reduced in substitution mutants of the C/EBP and NF-κB sites, but was not affected by a mutation in a PU.1-binding site (Fig. 2B).

Given that the 19-kD lipoprotein induced monocyte IL-12 in a manner analogous to LPS, we reasoned that the cell surface receptor that transduces the signal for the 19-kD lipoprotein may be identical to that for LPS. Because Toll-like receptors (TLRs) have been reported to activate monocyte cytokine production (*18*), to bind LPS, and to transduce the proper signal for LPS-stimulated gene activation in monocytes (*19*), we hypothesized that the 19-kD lipoprotein could induce IL-12 through TLRs. TLR family members are transmembrane proteins containing repeated leucine-rich motifs in their extracellular portions, similar to other pattern

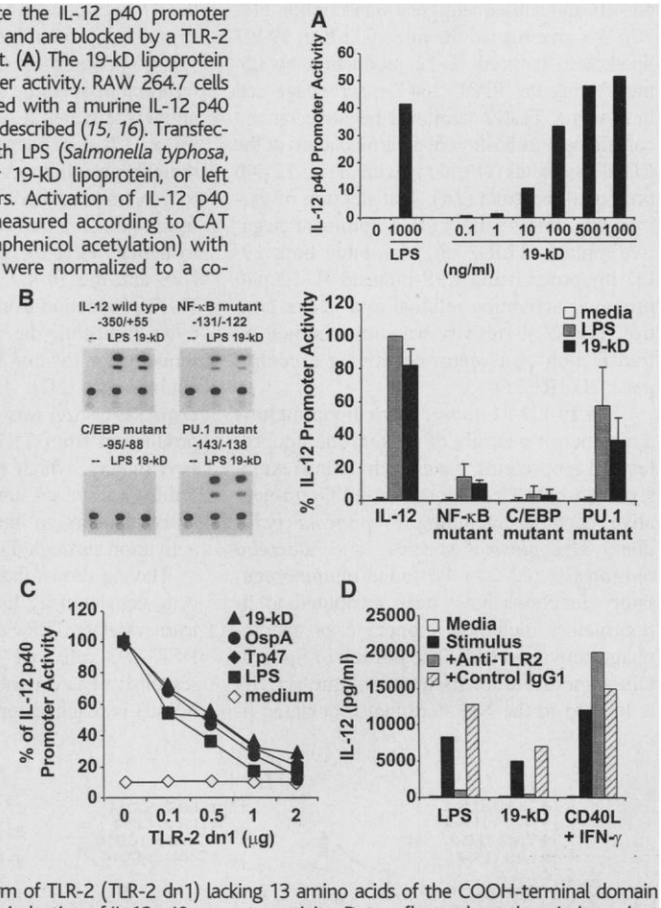

Fig. 2. Lipoproteins induce the IL-12 p40 promoter through NF-κB and C/EBP and are blocked by a TLR-2 dominant negative mutant. (**A**) The 19-kD lipoprotein induces IL-12 p40 promoter activity. RAW 264.7 cells were transiently transfected with a murine IL-12 p40 promoter CAT reporter as described (*15, 16*). Transfectants were stimulated with LPS (*Salmonella typhosa*, Sigma, St. Louis, MO) or 19-kD lipoprotein, or left unstimulated for 24 hours. Activation of IL-12 p40 promoter activity was measured according to CAT activity (percent chloramphenicol acetylation) with a phosphorimager. Data were normalized to a co-transfected β-galactosidase construct for transfection efficiency. Data are representative of three experiments. No stimulation was observed with a control CAT reporter plasmid that lacked the IL-12 p40 promoter sequence. (**B**) IL-12 p40 promoter mutations in the NF-κB (−131/−122) and C/EBP (−95/−88) sites blocked LPS (gray, 5 μg/ml) and 19-kD lipoprotein (black, 5 μg/ml) stimulated promoter activation. RAW 264.7 cells were transiently transfected as described above with NF-κB, C/EBP, and PU.1 mutant constructs, and promoter induction levels were measured by CAT assay (*15, 16*). (**C**) A mutant form of TLR-2 (TLR-2 dn1) lacking 13 amino acids of the COOH-terminal domain inhibits LPS and lipoprotein induction of IL-12 p40 promoter activity. Data reflect at least three independent experiments and are reported as a percentage of antigen-stimulated IL-12 p40 promoter activity cotransfected with a vector control. Lipoproteins and lipopeptides were prepared as described (*31*). (**D**) Monoclonal antibody to TLR-2 blocks LPS- and lipoprotein-induced, but not CD40L trimer–induced, IL-12 p40 production from human adherent monocytes (*28, 38, 41*).

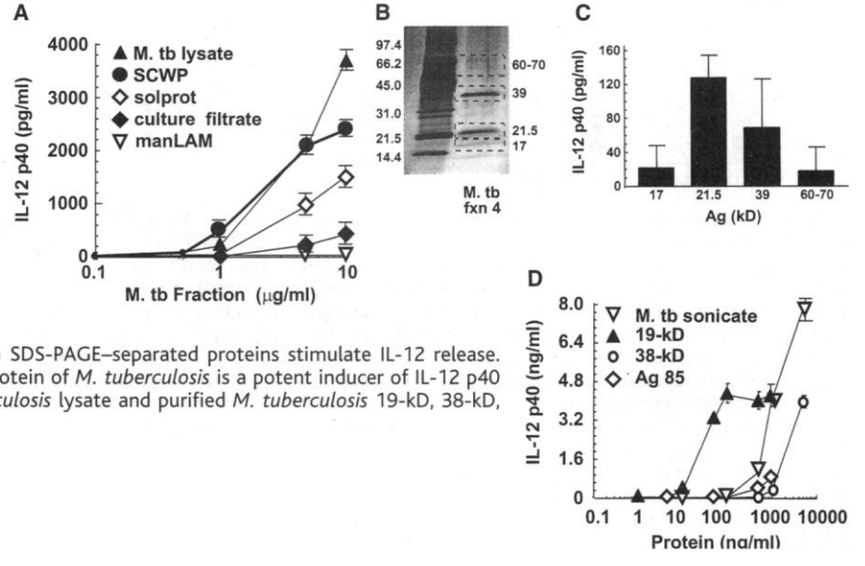

Fig. 1. A 19-kD *M. tuberculosis* lipoprotein induces IL-12 from monocytes. (**A**) Ability of *M. tuberculosis* subcellular fractions to stimulate IL-12 release from monocytes. The detergent-soluble cell wall–associated subcellular fraction (SCWP) retains most of the *M. tuberculosis* IL-12 p40–inducing capacity, as determined with the THP-1 human monocyte cell line (*8*). Values are expressed as mean ± SEM of duplicate determinations. Subcellular fractions of *M. tuberculosis* were isolated as previously described (*7*). Solprot, combined cytosolic and membrane fraction; manLAM, lipoglycan from *M. tuberculosis*. (**B**) SDS-PAGE of *M. tuberculosis* proteins from isoelectric focusing fraction 4 (*10*). Molecular size markers are in the left lane, and sizes are indicated in kilodaltons. (**C**) SDS-PAGE–separated proteins stimulate IL-12 release. Methods are described in (*13*). (**D**) The 19-kD lipoprotein of *M. tuberculosis* is a potent inducer of IL-12 p40 release. THP-1 cells were stimulated with *M. tuberculosis* lysate and purified *M. tuberculosis* 19-kD, 38-kD, and Ag85 complex proteins (*12*).

recognition proteins of the innate immune system. TLR proteins also contain a cytoplasmic domain that is homologous to the signaling domain of the IL-1 receptor and can activate a signaling pathway that includes activation of NF-κB and subsequent gene transcription (*19, 20*). We investigated the role of TLR in 19-kD lipoprotein–induced IL-12 production by cotransfecting the RAW 264.7 macrophage cell line with a TLR-2 dominant negative mutant containing a truncation of 13 amino acids at the COOH-terminus (*19*), along with the IL-12 p40 promoter construct (*16*). Transfection of various amounts of the TLR-2 dominant negative mutant (TLR-2 dn1) inhibited both 19-kD lipoprotein–and LPS-induced IL-12 p40 promoter activation relative to a vector control (Fig. 2C). Activity was not inhibited by transfection of a vector containing a control gene, IL-1R (*21*).

The 19-kD *M. tuberculosis* lipoprotein is a member of a family of prokaryotic lipoproteins. Lipoproteins have been found extensively in both Gram-positive and Gram-negative bacteria, including *Treponema pallidum*, *Mycoplasma* species, and *Borrelia burgdorferi* (*22–24*). Profound immunoregulatory functions have been attributed to lipoproteins, including monocyte or macrophage activation (*25*). The portion of lipoprotein responsible for its immunologic activity is located in the NH_2-terminal triacylated lipopeptide region. Removal of this lipid element rendered the parent product nonactivating, and synthetic lipopeptides could activate B cells and macrophages (*23, 24, 26*). Studies of the *B. burgdorferi* OspA lipoprotein and the 47-kD lipopeptide of *T. pallidum* demonstrated lipoprotein induction of IL-12 mRNA (*24, 27*). We found that OspA and the NH_2-terminal lipopeptide of the *T. pallidum* 47-kD antigen activated IL-12 p40 promoter activity by a TLR-dependent mechanism (Fig. 2C), thereby providing evidence that TLRs serve to recognize a diverse family of microbial lipoproteins. A monoclonal antibody specific to human TLR-2 (*28*) blocked the ability of LPS and the 19-kD lipoprotein to stimulate IL-12 production from primary human monocytes, indicating the crucial role for TLR-2 in monocyte activation by these microbial molecules (Fig. 2D). Because the deacylated OspA (d-OspA) was unable to activate IL-12 production from THP-1 cells (*29*), the fatty acyl moiety, which is genetically and structurally conserved among microbial lipoproteins, appears to be crucial for monocyte activation through TLRs.

Having shown that TLRs are necessary for gene activation by lipoproteins, we sought to learn whether TLRs are also sufficient. Using HEK 293 cells, we transfected the NF-κB–responsive ELAM enhancer, because activated NF-κB is required for IL-12 p40 promoter activity. HEK 293 cells do not express TLR-2, nor could they be activated by LPS or microbial lipoproteins, as determined by examination of NF-κB induction (Fig. 3A) (*19, 30*). In contrast, in stable transfectants of HEK 293 cells expressing TLR-2, microbial lipoproteins induced NF-κB in a dose-dependent manner and at levels comparable to those induced by LPS. Activation by lipoproteins was enhanced by cotransfection with a CD14 expression vector to levels analogous to LPS induction and consistent with the role of CD14 in facilitating lipoprotein activity (*30–32*) (Fig. 3B). Activation through TLR-2 by lipoproteins was dependent on fatty acyl moieties because deacylated forms of OspA and *T. pallidum* lipopeptide (d-Tp47) had no activity (Fig. 3C). Although the data indicate that TLR-2 can mediate gene activation by microbial lipoproteins, the data do not preclude a contributory role for other TLR family members (*33*).

To determine whether the TLR signaling pathway stimulated by microbial lipoproteins could be linked to a known macrophage antimicrobial mechanism, we investigated whether the *M. tuberculosis* lipoprotein could activate gene transcription for inducible nitric oxide

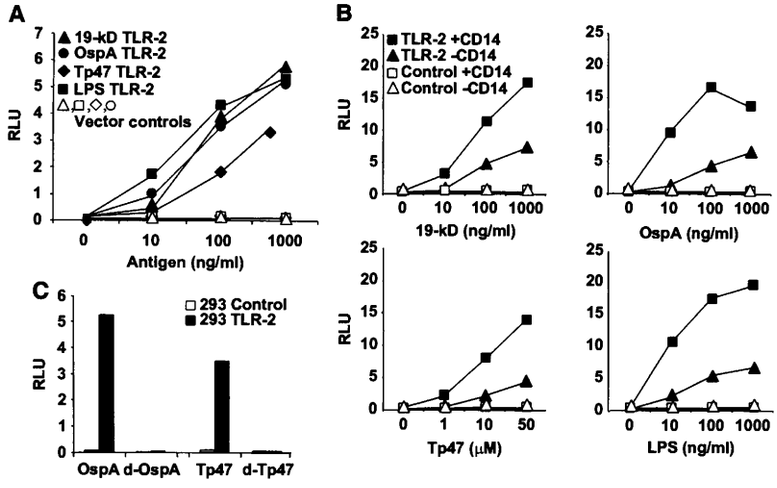

Fig. 3. TLR proteins are sufficient for the induction by lipoproteins of NF-κB activation, and induction is enhanced by the presence of CD14. (**A**) Stable expression of TLR-2 in HEK 293 cells confers lipoprotein responsiveness. HEK 293 TLR-2 and HEK 293 vector control cells were transiently transfected with a luciferase reporter gene driven by the NF-κB responsive enhancer of the E-selectin gene (*19, 30*). Luciferase activity (RLU) was measured with a luminometer (arbitrary units). Tp47 concentrations are corrected by 6.25×10^{-3}. (**B**) CD14 enhances lipoprotein activation of NF-κB through TLR-2. HEK 293 TLR-2 and HEK 293 vector control cells were cotransfected with ELAM-luc (0.5 μg), along with CD14 expression plasmid (1 μg) or vector control (1 μg) (*30*). Twenty-four hours after transfection the transfectants were activated with a titration of LPS or lipoproteins for 6 hours. Activity was measured by luciferase assay. Data are representative of three independent experiments. (**C**) Fatty acyl moieties are required for TLR-dependent activation of NF-κB. The HEK 293 TLR-2 stable clone and HEK 293 control cells were transiently transfected with the ELAM-luciferase construct and activated with the OspA lipoprotein, the Tp47 lipopeptide, or deacylated forms of both antigens (*31*). NF-κB activation was measured by luciferase assay.

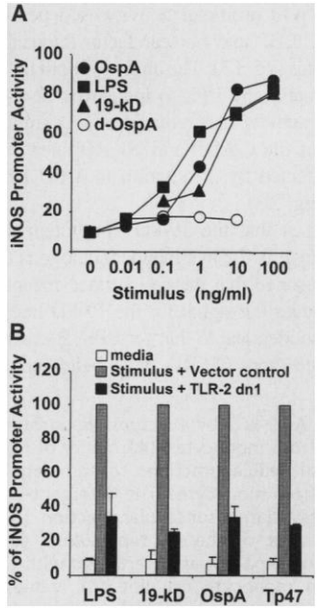

Fig. 4. Microbial lipoproteins induce iNOS gene transcription through TLRs. (**A**) Microbial lipoproteins induce iNOS promoter activity in RAW 264.7 cells. RAW 264.7 cells were transiently transfected with the iNOS promoter construct as described previously (*16*). (**B**) A dominant negative mutant of TLR-2 (TLR-2 dn1) inhibits LPS and lipoprotein induction of iNOS promoter activity. Data reflect at least two independent experiments and are reported as a percentage of antigen-stimulated iNOS promoter activity in cells not transfected with TLR-2 dn1 expression plasmid (*16*). Media controls were comparable between vector control and TLR-2 dn1 transfectants.

synthase (iNOS), given the critical role of iNOS in the production of nitric oxide from macrophages, currently the only effective macrophage mycobactericidal mechanism known in vitro and in vivo (*34*). Analysis of gene-disrupted mice revealed that this mechanism was necessary for protection against *M. tuberculosis* (*35*). The 19-kD and OspA lipoproteins induced iNOS promoter activity in the RAW 264.7 macrophage cell line (*16, 36*). Again, activation was dependent on the fatty acyl moieties, because d-OspA had no activity (Fig. 4A). Cotransfection with the TLR-2 dominant negative mutant inhibited the ability of lipoproteins to induce the iNOS promoter (Fig. 4B), thereby suggesting a role for TLRs in the activation of iNOS by microbial pathogens. Stimulation of monocytes with the 19-kD antigen also induced production of nitric oxide (*37*).

The presence of Toll in *Drosophila* indicates that Toll proteins represent a host defense mechanism that has been conserved over hundreds of millions of years of evolution. In mammals, TLRs provide the innate immune system with the ability to react to a spectrum of microbial pathogens expressing lipoproteins and lipopolysaccharides. Animals with altered TLRs manifest increased susceptibility to infection (*33*). Our data indicate that TLRs can activate innate immune responses including the generation of NO, a direct microbicidal mechanism, and provide an early signal for induction of IL-12, which functions as a biologic adjuvant amplifying acquired T cell responses to pathogens. Under certain conditions, the TLR signaling pathway can lead to apoptosis of the target cells resulting in down-regulation of the immune response or pathology to the host (*28*). It should be possible, however, to develop strategies to stimulate TLRs in order to activate antimicrobial defense mechanisms and to amplify immune responses to a variety of antigens in vaccines.

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8. THP-1 cells [American Type Culture Collection (ATCC), Manassas, VA] were stimulated with *M. tuberculosis* lysate and various subcellular fractions in a range of concentrations from 0.1 to 10 μg/ml. Cell-free supernatants were collected at 20 hours and assayed for IL-12 p40 by enzyme-linked immunosorbent assay (ELISA) (Pharmingen, San Diego, CA). THP-1 cells were plated at a concentration of 2×10^5 cells/well in 96-well plates. The cells were cultured in the presence of various *M. tuberculosis* preparations at 37°C in a CO_2 incubator with RPMI 1640 and 10% heat-inactivated fetal bovine serum (FBS). Cell-free supernatants were harvested 20 hours later and stored at −20°C until assayed for cytokine concentrations by ELISA as previously described (*38, 39*). The amount of lipopolysaccharide in all preparations was measured quantitatively with a Limulus Amoebocyte Lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be <40 pg per microgram of *M. tuberculosis* fraction or protein, an amount that did not stimulate IL-12 activity by itself.
9. The mycolyl arabinogalactan peptidoglycan complex (mAGP) was also derived from the cell wall pellet after SDS detergent extraction. Most of the IL-12 p40–inducing activity was associated with the SCWP fraction compared with mAGP (see supplementary material available at www.sciencemag.org/feature/data/1040444.shl). Because macrophage phagocytosis of protein-adsorbed particulate suspensions has been reported to induce IL-12 release, we filtered both fractions through a 0.2-μm sterile filter. The SCWP fraction retained most of its IL-12 p40–inducing activity after filtration, whereas the mAGP suspension did not induce IL-12 p40.
10. Cell wall–associated proteins were obtained by suspending the cell wall pellet at a concentration of 1 mg of protein per milliliter of phosphate-buffered saline (PBS) (pH 7.4) containing 2% SDS. The proteins were extracted for 2 hours at room temperature followed by centrifugation at 27,000*g* for 30 min. The supernatant was applied to a Sephacryl S-200 column, and proteins were eluted at a flow rate of 0.5 ml/min with PBS (pH 7.4), 2% SDS. Fractions were pooled on the basis of the relative molecular weight of the products as determined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and SDS was removed. Specific pools of size-fractionated SCWP were further purified by preparative isoelectric focusing. Specifically, the desired fractions were dialyzed against 4 M urea, 10% glycerol and brought to a final volume of 50 ml with the same. NP-40 was then added to a final concentration of 1%, and Pharmalytes pH 5–7 and pH 3–10 were added to a final concentration of 4 and 1%, respectively. This suspension was loaded into a 50-ml Rotofor cell (BioRad Laboratories, Hercules, CA) and electrophoresed with 15-W constant power for 4 hours. Fractions were collected, dialyzed against 100 mM NH_4HCO_3, and stored at −20°C. The fractions were loaded onto a 13.5% preparative SDS-polyacrylamide gel (16 cm by 20 cm by 1.5 mm) and electrophoresed at 20 mA for 1.5 hours. The resolved proteins were visualized by using Coomassie Brilliant Blue R250, excised from the gel, and electroeluted with a BioRad 422 Electro-Eluter with 50 mM NH_4HCO_3. At each step, fractions were tested for their ability to induce IL-12 p40, and the fractions with potent activity were further purified. Rotofor fraction number 4 was electrophoresed under denaturing conditions on a 4 to 20% tris-glycine minigel and transferred to nitrocellulose. Areas containing the four protein bands noted in Fig. 1B were isolated and solubilized as described previously (*11*) and used to stimulate THP-1 cells.
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13. Peripheral blood mononuclear cells were isolated on Ficoll-Paque gradients (Pharmacia LKB Biotechnology, Piscataway, NJ) and cultured (10^6 cells/well) for 2 hours in 96-well plates at 37°C in a CO_2 incubator with RPMI 1640 and 10% heat-inactivated FBS. Nonadherent cells were removed and the medium replaced with the addition of 19-kD lipoprotein at 1, 10, and 100 ng/ml. Neutralizing monoclonal antibody to human TNF-α (Endogen, Cambridge, MA; Biosource, Camarillo, CA) and a mouse isotype control [immunoglobulin G1 (IgG1)] were added to some cultures at 5 and 10 μg/ml. Supernatants were harvested 16 hours later and assayed by IL-12 p40 ELISA (Pharmingen, San Diego, CA). All samples were assayed in duplicate. Previously, it was demonstrated that mycobacteria infection does not induce IL-12 in TNFR1 knockout animals [I. E. Flesch *et al.*, *J. Exp. Med.* **181**, 1615 (1995)].
14. Adherent monocytes were isolated from 11 tuberculin-negative healthy donors (*13*). Cells were stimulated with the 19-kD lipoprotein at a concentration of 10 ng/ml for 20 hours, and IL-12 p70 amounts were determined by ELISA with monoclonal anti–IL-12 p70 and antibodies 2OC2 and POD-4D6 as described (*39*). Under these conditions the monocytes from 8 out of 11 donors were able to produce measurable IL-12 p70 amounts 3 to 13 times above background levels in the range of 10 to 40 pg/ml. All samples were assayed in duplicate.
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16. All RAW 264.7 cell (ATCC, Manassas, VA) transfections were carried out with the Superfect protocol (Qiagen, Valencia, CA) at a ratio of 1 μg of DNA to 3 μl of Superfect for 2.5 hours. After a PBS wash, cells were divided into two wells and left unactivated or stimulated with LPS or lipoproteins for 24 hours and then harvested for the chloramphenicol acetyltransferase (CAT) assay. In IL-12 p40 and inducible nitric oxide synthase (iNOS) promoter induction studies, RAW 264.7 cells were transiently transfected with either IL-12 p40 (*15*) or iNOS promoter (*36*), CAT reporter plasmid (2 μg), and HSP70–β-Gal construct (0.5 μg) as an internal control. In the TLR-2 dn1 overexpression experiments, increasing doses of TLR-2 dn1 expression plasmid (100 ng, 500 ng, 1 μg, and 2 μg) (*19*) were transfected with the IL-12 p40 promoter construct and β-Gal as an internal control. The amount of expression plasmid was normalized to 2 μg with expression plasmid lacking the TLR-2 dn1 coding sequence (pCDNA3). IL-12 p40 promoter transfectants were either left unactivated or stimulated with LPS, 19-kD lipoprotein, or OspA at 50 ng/ml or Tp47 lipopeptide (*24*) at 10 μM for 24 hours. iNOS promoter transfectants were stimulated with LPS, 19-kD lipoprotein, OspA, or deacylated OspA (d-OspA) from 0.01 to 100 ng/ml, or left unstimulated for 24 hours. The iNOS promoter was also cotransfected with the TLR-2 dn1 construct (1 μg) or vector control (1 μg) and stimulated with LPS at 1 ng/ml, 19-kD lipoprotein and OspA at 5 ng/ml, or Tp47 lipopeptide at 10 μM for 24 hours. IL-12 p40 and iNOS promoter activities were measured according to CAT activity (percent chloramphenicol acetylation) with a phosphorimager. Data were normalized to a cotransfected β-Gal construct for transfection efficiency.
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21. An IL-1R expression construct was cotransfected with the IL-12 p40 promoter in a titration of plasmid (0.1, 0.5, 1, and 2 μg) as was done with the TLR-2 dn1 mutant construct (*16*). Overexpression of IL-1R had no effect on IL-12 p40 promoter activation by LPS or lipoproteins even at the highest levels of expression, suggesting that mere overexpression of a related surface receptor does not have an effect on IL-12 p40 promoter activation.
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29. THP-1 cells were stimulated with LPS, OspA, or d-OspA at 1 μg/ml (*31*). Supernatants were then assayed for IL-12 p40 by ELISA. LPS and OspA induced IL-12 p40 from THP-1 cells in a range of 1000 to 3500 pg/ml, whereas the amount of IL-12 p40 induced by d-OspA was below the level of detection.
30. HEK 293 cells not expressing human TLR-2 (detected with monoclonal anti–TLR-2 or reverse-transcriptase polymerase chain reaction) or HEK 293 clones stably transfected to express human TLR-2 were then further transfected with CD14 and reporter constructs as previously described (*19*). We plated 10^5 cells/well in six-well plates and transiently transfected them the following day with the NF-κB responsive E-selectin (ELAM) gene enhancer luciferase (pGL3) reporter gene (0.5 μg) and a β-galactosidase reporter plasmid (0.5 μg) as an internal control by the Superfect protocol at a 1:3 ratio of DNA (micrograms to Superfect (microliters). Cells were then incubated with the DNA-Superfect mixture for 2 hours and washed, and multiple transfectants were pooled and divided into separate wells for activation by a titration of LPS or lipoprotein stimuli (19-kD lipoprotein, Tp47, and OspA). Twenty-four hours later cells were stimulated for 6 hours then lysed in 200 μl of reporter lysis buffer (Promega, Madison, WI), and 20 μl was used in the luciferase assay. HEK 293 control cells and TLR-2 stable clones were also cotransfected with a CD14 expression plasmid (*19*) (1 μg) or vector control (pCDNA3, 1 μg) with the same protocol.
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37. NO was measured as the concentration of NO_2^- in cell culture supernatants. We plated 10^5 RAW 264.7 cells in a 96-well plate in Dulbecco's modified Eagle medium (DMEM), 10% FCS. After a 1-hour incubation at 37°C, cells were stimulated with media or 19-kD lipoprotein (1 to 1000 ng/ml) for 24 hours. A 100-μl volume of the Greiss reagent (1:1 mix of 0.1% *N*-1-napthylethylenediamine-HCL and 1% sulfanilamide in 2.5% H_3PO_4; Sigma, St. Louis, MO) was added to 100 μl of cell culture supernatant and incubated for 10 min, and the absorbance was read at 560 nm. Sample nitrite concentrations were determined by comparison with a standard curve of $NaNO_2$ (Sigma) (*40*).
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41. Adherent monocytes were isolated as described previously (*14*). Cells were treated with no antibody, mouse anti–human TLR-2 neutralizing monoclonal antibody, or an isotype control mouse IgG1 (10 μg/ml) for 30 min before stimulation with LPS or 19-kD lipoprotein (50 ng/ml) and incubated for 16 hours. Supernatants were then harvested and assayed for IL-12 p40 by ELISA. CD40L- and interferon-γ (IFN-γ)–stimulated adherent monocytes were used to control for the anti–TLR-2 blocking of lipoprotein-induced IL-12. Adherent monocytes were treated with human IFN-γ (100 units/ml; Endogen, Cambridge, MA) for 16 hours to up-regulate CD40 expression. Cells were then treated with antibodies as stated above, then stimulated with soluble CD40L trimer (250 ng/ml; Immunex, Seattle, WA) for 16 hours. Neither antibody nor IFN-γ alone induced IL-12 production.
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Cell Activation and Apoptosis by Bacterial Lipoproteins Through Toll-like Receptor-2

Antonios O. Aliprantis,[1]* Ruey-Bing Yang,[2]* Melanie R. Mark,[2] Shelly Suggett,[3] Brigitte Devaux,[3] Justin D. Radolf,[4] Gary R. Klimpel,[5] Paul Godowski,[2] Arturo Zychlinsky[1]†

Apoptosis is implicated in the generation and resolution of inflammation in response to bacterial pathogens. All bacterial pathogens produce lipoproteins (BLPs), which trigger the innate immune response. BLPs were found to induce apoptosis in THP-1 monocytic cells through human Toll-like receptor–2 (hTLR2). BLPs also initiated apoptosis in an epithelial cell line transfected with hTLR2. In addition, BLPs stimulated nuclear factor–κB, a transcriptional activator of multiple host defense genes, and activated the respiratory burst through hTLR2. Thus, hTLR2 is a molecular link between microbial products, apoptosis, and host defense mechanisms.

The innate immune system coordinates the inflammatory response to pathogens. To do so, cells of the innate immune system discriminate between self and nonself by receptors that identify molecules synthesized exclusively by microbes (*1*). These include lipopolysaccharide (LPS), peptidoglycans, lipotechoic acids, and BLPs (*2*). BLPs are characterized by a unique, NH_2-terminal lipo-amino acid, *N*-acyl-*S*-diacylglyceryl cysteine, and are ideal targets for innate immune surveillance because they are produced by all bacteria. Although BLPs are known to activate nuclear factor–κb (NF-κB) (*3*), cytokine production (*4*), and B cell expansion (*5*), it is unclear how the BLP signal is transduced into an intracellular message. Candidate BLP signal transducers are Toll receptors, which are characterized by an extracellular leucine-rich repeat domain and an interleukin-1 (IL-1) receptor type 1–like intracellular signaling domain (*6*). In *Drosophila*, Toll receptors are important for resistance to microbial pathogens (*7*). Toll and TLRs activate homologous signal transduction pathways leading to nuclear localization of NF-κB/Rel–type transcription factors (*8*). Both hTLR2 (*9*, *10*) and murine TLR4 (*11*) (mTLR4) are implicated in the innate response to LPS. mTLR4 does not appear to function analogously in LPS and BLP signaling. A mutation in mTLR4, which renders cells insensitive to LPS, does not abrogate BLP-induced responses in mice (*11*, *12*). Therefore, we investigated the role of hTLR2 in BLP signaling.

A population of human embryonic kidney 293 cells, which do not express hTLR2 (*10*, *13*), were stably transfected with an epitope-tagged hTLR2 (293hTLR2) and tested for the ability to respond to BLP in an NF-κB luciferase reporter gene assay (*14*). Synthetic lipoprotein analogs consisting of a palmitylated version of *N*-acyl-*S*-diacylglyceryl cysteine (Pam_3Cys) and a few COOH-terminal amino acids mimic the immunomodulatory effects of BLP (*15*). The synthetic bacterial lipopeptide, $Pam_3CysSerLys_4$ (sBLP), induced expression of the luciferase reporter gene in 293hTLR2 cells, but not in the parental line (Fig. 1A). Concentrations of sBLP or *Escherichia coli* murein lipoprotein (MLP) as low as 200 pM activated the reporter gene (Fig. 1B). The lipo–amino acid Pam_3Cys and a monoacylated derivative of sBLP (msBLP) generated by base hydrolysis (*16*) did not activate the luciferase reporter gene in 293 or 293hTLR2 cells (Fig. 1, A and B). This is consistent with previous observations that the acyl groups and peptide moieties of sBLP are critical for cell activation (*5*, *17*).

[1]Skirball Institute and Department of Microbiology, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA. [2]Department of Molecular Biology and [3]Antibody Technology Group, Genentech, South San Francisco, CA 94080, USA. [4]Center for Microbial Pathogenesis, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, USA. [5]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: zychlins@saturn.med.nyu.edu