Diffential Effects of a Toll-Like Receptor Antagonist on Mycobacterium tuberculosis-Induced Macrophage Responses

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We previously showed that viable Mycobacterium tuberculosis (Mtb) bacilli contain distinct ligands that activate cells via the mammalian Toll-like receptor (TLR) proteins TLR2 and TLR4. We now demonstrate that expression of a dominant negative TLR2 or TLR4 proteins in RAW 264.7 macrophages partially blocked Mtb-induced NF-κB activation. Coexpression of both dominant negative proteins blocked virtually all Mtb-induced NF-κB activation. The role of the TLR4 coreceptor MD-2 was also examined. Unlike LPS, Mtb-induced macrophage activation was not augmented by overexpression of ectopic MD-2. Moreover, cells expressing an LPS-unresponsive MD-2 mutant responded normally to Mtb. We also observed that the lipid A-like antagonist E5531 specifically inhibited TLR4-dependent Mtb-induced cellular responses. E5531 could substantially block LPS- and Mtb-induced TNF-α production in both RAW 264.7 cells and primary human alveolar macrophages (AMφ). E5531 inhibited Mtb-induced AMφ apoptosis in vitro, an effect that was a consequence of the inhibition of TNF-α production by E5531. In contrast, E5531 did not inhibit Mtb-induced NO production in RAW 264.7 cells and AMφ. Mtb-stimulated peritoneal macrophages from TLR2- and TLR4-deficient animals produced similar amounts of NO compared with control animals, demonstrating that these TLR proteins are not required for Mtb-induced NO production. Lastly, we demonstrated that a dominant negative MyD88 mutant could block Mtb-induced activation of the TNF-α promoter, but not the inducible NO synthase promoter, in murine macrophages. Together, these data suggest that Mtb-induced TNF-α production is largely dependent on TLR signaling. In contrast, Mtb-induced NO production may be either TLR independent or mediated by TLR proteins in a MyD88-independent manner. The Journal of Immunology, 2001, 166: 4074–4082.

Abstract

Almost one-third of the world’s population is infected with Mycobacterium tuberculosis (Mtb),3 the causative agent of pulmonary tuberculosis. Internalization of Mtb is mediated in part by the complement receptors and the macrophage mannose receptor (1, 2). Macrophage phagocytosis of Mtb is accompanied by activation of the transcription factor NF-κB, secretion of inflammatory mediators (e.g., TNF-α), release of the reactive nitrogen intermediate NO, and secretion of several chemokines (reviewed in Ref. 3). Until recently, the signaling pathways that elicit the production of these mediators have remained unknown. Members of the mammalian Toll-like receptor (TLR) family have been implicated in the activation of macrophages by a variety of chemically diverse bacterial products (reviewed in Ref. 4–6). Both TLR2 and TLR4 have been shown to mediate Mtb-induced intracellular signaling in vitro. Means et al. demonstrated that viable Mtb bacilli contain distinct ligands that activate cells via TLR2 and TLR4 (7), whereas heat-killed Mtb failed to activate cells via TLR4. Several purified mycobacterial ligands have now been identified as TLR2 agonists (4), but the identity of the Mtb TLR4 agonist remains unknown. Mtb binding to macrophages induces both phagocytosis of the bacilli and the activation of innate immune response genes. The potential roles of TLR proteins in both processes has been examined. Underhill et al. demonstrated that TLR2 is selectively associated with macrophage phagosomes that contain yeast particles or zymosan (8). Abs against TLR2 failed to block zymosan uptake, although they did block zymosan-induced TNF-α production. These findings suggest that TLR proteins mediate cellular responses to micro-organisms, but are not required for phagocytosis.

Mtb is an intracellular pathogen that resides mainly within macrophages. Paradoxically, the normal function of macrophages is to engulf and destroy micro-organisms. Mtb possess specific mechanisms to evade destruction by the host macrophage. Phagosomes that contain live Mtb bacilli resist fusion with acidified lysosomes, whereas phagosomes containing heat-killed Mtb fuse freely with lysosomes (9). The ability of live Mtb to inhibit acidification of the phagosome is thought to be a major mechanism for intracellular Mtb survival. However, the exact molecular mechanisms by which the mycobacterial phagosome resists lysosomal fusion remains unknown. Several studies have demonstrated that phagosomes containing live Mtb express early endosomal markers, but lack markers that are specific for later stages of the endocytic pathway (9, 10). An intracellular protein termed TACO (for tryptophan aspartate-containing coat protein) has recently been shown to normally dissociate from phagosomes during the course of phagosome maturation, and this dissociation is required for subsequent lysosomal fusion. Ferrari et al. recently demonstrated that TACO is retained
by phagosomes that contain live Mtb, but not by phagosomes containing heat-killed bacteria (11). These data suggest that Mtb triggers the retention of TACO on the phagosome as a means to prevent phagosome-lysosome fusion and bacterial destruction.

Given the spread of drug-resistant Mtb strains, there is a pressing need to develop treatments that do not rely on new antibiotics. One novel approach would be to develop therapeutics that antagonize TLR proteins. This has been accomplished for one TLR4 agonist, Gram-negative bacterial LPS, and its pharmacophore lipid A. Three lipid A analogs, lipid IVα, Rhodobacter sphaeroides lipid A (RSLA), and E5531 have all been reported to function as LPS antagonists when tested both in vitro and in vivo (12–14). These antagonists were all shown to be selective, because they are all capable of blocking NF-κB activation by LPS, but not NF-κB activation induced by the TLR-independent agonist IL-1β (15–17).

Interestingly, we previously demonstrated that RSLA could also block signaling by a TLR2 agonist, the mycobacterial glycolipid lipoarabinomannan (18). Together, these data suggest that certain lipid A structural antagonists are capable of blocking TLR-dependent activation by molecules that are chemically dissimilar to LPS.

We have now extended these findings using the well-characterized synthetic lipid A-like antagonist, E5531, to block TLR4-dependent signaling. Here we show that E5531 is capable of inhibiting some Mtb-induced macrophage responses, namely apoptosis and TNF-α secretion. In contrast, E5531 did not block Mtb-induced NO production. Additional data demonstrate that Mtb-induced NO production by macrophages is a TLR-independent response.

Materials and Methods

Cell cultures

The RAW 264.7 murine macrophage (CCL-61) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). RAW 264.7 macrophages, CHO fibroblasts, and CHO-derived cell lines were cultured as we have previously described (19). The CHO-derived cell lines 3E10 (CHO/CD14/ELAM-CD25), 3E10/TLR2, and 3E10/TLR4 were generated and cultured as previously described (16, 19, 20). These 3E10/TLR cells expressed FLAG-tagged human TLR proteins. Clones that expressed similar levels of TLR proteins, measured by flow cytometry using an anti-FLAG Ab, were selected for further study. Furthermore, the TLR2- and TLR4-expressing cell lines were generated from the same CHO/CD14 parental clones; therefore, each line also expresses the same levels of CD14. A mutant 3E10 line that lacks functional MD-2 was generated by chemical mutagenesis as previously described (19). This line (designated 7.19) was found to be LPS unresponsive, yet fully responsive to exogenous IL-1β. Sequence analysis of the MD-2 cDNA from these cells revealed a single point mutation in a highly conserved region resulting in a C95Y amino acid exchange. All medium components were <10 pg/ml final concentration as measured by Limulus amebocyte lysate kit (BioWhittaker, Walkersville, MD).

In experiments using 3E10 cells, which contain a stably transfected CD25 reporter gene under the control of the NF-κB-dependent ELAM-1 promoter, CD25 expression was measured by flow cytometry as previously described (19). Data were collected using CellQuest software (Becton Dickinson, Mountain View, CA) and expressed as either mean channel fluorescence or the ratio (fold activation) of the percentage of CD25+ cells in unstimulated and stimulated cell populations (gated to exclude the lowest 5% of cells based on mean fluorescence). The 95% confidence limit for nonspecific fluorescence was established using isotype control Abs. Each experiment was repeated at least three times.

Reagents

Mtb (strain H37Ra, ATCC 25177) and Mycobacterium bovis bacillus Calmette-Guérin (BCG; ATCC 35734) were purchased from the ATCC. Bacterial cultures were grown in LPS-free Middlebrook 7H9 medium supplemented with Tween 80 and ADC (Difco, Detroit, MI) at 37°C under biosafety level 3 conditions. Protein-free, phenol/water-extracted LPS (purified from Escherichia coli K235) was prepared as described previously (21, 22). E5531 was obtained from Eissai Research Institute (Eissai Research Institute, Andover, MA). Arabinosyl-capped mycobacterial lipoarabinomannan, purified from a rapidly growing avirulent Mycobacterium species (ArLAM), was provided by Dr. John Belisle (Colorado State University, Fort Collins, CO). Preparation of a soluble H37Ra-conditioned culture filtrate, termed soluble tuberculosis factor (STF), was previously described (7). Levels of contaminating LPS in the ArLAM and STF preparations were measured using a quantitative Limulus lysate assay (BioWhittaker) and were <10 pg/ml final concentration in all experiments. Recombinant human TNF-α was purchased from BioSource (Camarillo, CA). Stauruspore was purchased from Sigma (St. Louis, MO).

Animals

C3H/10tIl and C3H/Het 1 mice (female, 5–7 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Chinese and Syrian hamsters (female, 8–10 wk old) were obtained from Cytogen Research and Development (Boston, MA). All mice and hamsters were housed within 2 wk of receipt. Mice and hamsters were maintained in a laminar flow hood in cages fitted with polyester filter hoods and fed standard lab food and acid water at libitum. Peritoneal macrophages were elicited by ip injection of 10 ml of sterile fluid thioglycolate medium (3%). Four days after injection peritoneal macrophages were recovered from mice or hamsters by instilling and withdrawing 10 ml of sterile serum-free DMEM medium from the peritoneal cavity. Peritoneal macrophages were allowed to adhere for 2 h to the culture dishes, the nonadherent cells were removed, and the adherent macrophages were cultured for 3–4 days before use.

Plasmids

The NF-κB-dependent ELAM-Luc reporter plasmid and the human FLAG-tagged TLR2 and TLR4 expression plasmids were previously described (7). The mutant murine TLR2 dominant negative (DN) expression plasmid contains a FLAG-tagged TLR2 fusion cDNA, which lacks the C-terminal 13 aa of the TLR2 coding sequence; this cDNA was generated by PCR and subcloned into the 5′/NheI/3′/XhoI site of pcDNA3.1 (Invitrogen, Carlsbad, CA). The murine TLR4-DN expression plasmid containing a FLAG-tagged mutant TLR4 cDNA (P712H) was subcloned into the 5′/NolⅠ/3′/SalI site of the pFLAG-CMV-1 expression vector and was previously described (17). A murine MyD88 cDNA fragment was generated by RT-PCR using mRNA purified from RAW 264.7 cells and PCR primers based on the N- and C-terminal sequences of murine MyD88 (GenBank accession no. P22366). A cDNA fragment containing only the C-terminal half of MyD88 (aa 146–296) plus a synthetic N-terminal methionine codon was cloned into the expression plasmid pcDNA3.1/Hyg. The murine MD-2 expression plasmid was a gift from Dr. Kensuke Miyake (Sagra Medical Systems, Sagra, Japan) and was previously described (23). The hamster MD-2 expression plasmid was cloned from CHO-K1 cells (see footnote 4). The luciferase reporter plasmid pGL3/mTNF-α (1059) contains 1064 bp of the murine TNF-α promoter and was previously described (24). The luciferase reporter plasmid pGL3/mTNF-α-1059 Luc contains 1064 bp of the murine TNF-α promoter and was previously described (25). Plasmid DNA was prepared using Qiagen (Valencia, CA) plasmid DNA purification columns, DNA was eluted from the columns using LPS-free buffers, and contaminating LPS levels were <10 pg/ml.

Transient transfections

Transient transfections of RAW 264.7 and CHO-K1 cell lines were performed using SuperFect reagent (Qiagen) according to the manufacturer’s instructions and as previously described (26). Each DNA mixture (3 μg of total DNA) was prepared individually, and each condition was performed in triplicate. DNA mixtures were incubated with the cells for 2–3 h, whereupon the cells were washed and cultured in fresh medium containing serum. On the following day, individual wells were left untreated or were stimulated with Mtb, LPS, STF, or ArLAM as indicated in the text and figures. Cells were then incubated for an additional 5 h before harvesting and lysis. Luciferase assays were performed using the Luciferase Assay System (Promega) according to the manufacturer’s instructions. An equal amount of total protein from each lysate was assayed for luciferase activity as measured by light emissions in a Monolight 3010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI) for 20 s. Luciferase activity is expressed as the average total relative light units subtracted from background ± SD. Background luciferase activity was established by assaying lysates from mock-transfected cells.
Measurement of TNF-α and NO levels

TNF-α protein levels in the culture supernatants were measured by sandwich ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Samples were assayed in triplicate, and the data were expressed as the average ± SD. Recombinant human and mouse TNF-α standards were obtained from R&D Systems. The data were recorded and analyzed using the SOFTmax version 2.01 software program (Molecular Devices, Menlo Park, CA). The detection limit was 4 pg/ml for TNF-α. As an indirect indicator of NO production by macrophages, nitrite levels were measured in culture supernatants using the Greiss reagent assay (27). Samples were assayed in triplicate, and the data were expressed as the average ± SEM. The detection limit was 2 μM for nitrite.

Analysis of macrophage apoptosis

Human alveolar macrophages (AMb) were plated at a density of 4 × 10⁴ cells/well in 96-well microtiter plates. After 2 days portions of the cells were stimulated with Mtb (five bacilli per macrophage), in the absence or the presence of E5531 (1 or 10 μg/ml). Four hours later the medium was removed and replaced with fresh medium. After 5 days the supernatant was removed, the adherent macrophages were resuspended in 200 μl of lysis buffer, and the lysate was centrifuged at 200 × g for 10 min. These culture supernatant contain mono- and oligonucleosomes released by apoptotic cells, and an Ag-capture ELISA was used to measure the levels of histone-associated DNA fragments (Cell Death Detection ELISA Plus; Roche, Mannheim, Germany) present in the cell lysates according to the manufacturer’s instructions. Samples were assayed in triplicate, and the data are expressed as the average ± SEM.

Statistical analysis

Statistical analysis was performed using SuperANOVA (version 1.11) software from Abacus Concepts (Berkeley, CA). This computer program applied one-way ANOVA with multiple comparisons on different sample datasets. A 95% confidence level was used for all statistically analyzed data. Statistically significant data are indicated in the figures by an asterisk, and the corresponding p values are listed in the figure legends.

Results

TLR2-DN and TLR4-DN mutants block Mtb-induced NF-κB activation in macrophages

We previously reported that live Mtb bacilli can activate CHO fibroblasts and RAW 264.7 macrophages via TLR2 or TLR4, but not TLR1 (7). These TLR-dependent responses were induced by distinct secreted and cell-associated bacterial products. Furthermore, this activation did not require either membrane-bound or soluble CD14. Here we sought to determine the relative contributions of TLR2 and TLR4 to total macrophage activation by Mtb. As shown in Fig. 1, overexpression of a TLR2-DN protein in RAW 264.7 macrophages blocked activation by the TLR2 agonist AraLAM, but not the TLR4 agonist LPS. Similarly, overexpression of a TLR4-DN protein in these cells specifically blocked LPS-induced activation, but not AraLAM-induced activation. Overexpression of the TLR2-DN or TLR4-DN proteins partially blocked Mtb-induced cellular activation. The TLR2-DN protein alone consistently blocked 70–80% of total Mtb-induced cellular activation. This is similar to the level of blocking observed by other investigators using a TLR2-DN protein to block stimulation of RAW 264.7 cells with heat-killed Mtb (8). In contrast, the TLR4-DN protein alone blocked only 30–40% of Mtb-induced cellular activation. Because both DN proteins were maximally overexpressed in the macrophages, these results suggest that TLR2 mediates a relatively higher proportion of the cellular response to Mtb compared with TLR4. Furthermore, coexpression of both DN proteins blocked virtually all Mtb-induced NF-κB activation (Fig. 1). Together, these data suggest that TLR2 and TLR4 are responsible for virtually all Mtb-induced NF-κB activation.

TLR4 protects mice from lethal mycobacterial infection

The gene responsible for the LPS hyposensitiveness phenotype of the C3H/HeJ mouse was identified as tlr4 (28–30). The C3H/HeJ strain of mice possesses a mutant TLR4 protein that contains a missense mutation within the tlr4 coding sequence (P712H), and these mice are functionally TLR4 deficient for LPS signaling. We sought to determine whether these mice were abnormally susceptible to mycobacterial infection compared with normal C3H/OuJ mice. C3H/OuJ and C3H/HeJ mice were injected with 3 × 10⁶ CFU of M. bovis BCG into their peritoneal cavity. Survival was measured 3 days postinfection. M. bovis BCG was selected for this experiment because, like Mtb, these mycobacteria activate cells via both TLR2 and TLR4 (data not shown). We found that 9 of 20 C3H/HeJ mice died, while only 1 of 20 C3H/OuJ mice died. These data suggest that TLR4 serves a protective function in the host response to mycobacterial infection.

Role of MD-2 expression in Mtb-induced NF-κB activation

A novel protein called MD-2 was recently demonstrated to associate with TLR4 and to be necessary for TLR4-dependent cellular activation by LPS and Taxol (23, 31). We determined whether MD-2 was required for Mtb-induced cellular activation via TLR4. We transiently cotransfected RAW 264.7 cells with the NF-κB-dependent reporter plasmid and a murine MD-2 expression plasmid. We then compared the responses of these cells to different TLR agonists. We found that overexpression of MD-2 in RAW 264.7 macrophages enhanced cellular responsiveness to the TLR4 agonist LPS by 2- to 3-fold, whereas it did not enhance cellular responsiveness to the TLR2 agonist AraLAM (Fig. 2A). Furthermore, MD-2 overexpression did not enhance the capacity of live Mtb to activate RAW 264.7 macrophages. To directly determine whether MD-2 was required for TLR4-dependent cellular activation by Mtb, we used a mutant 3E10 (CHO/CD14) cell line that expresses a nonfunctional form of MD-2. These mutant cells were transiently cotransfected with an NF-κB-dependent luciferase reporter plasmid and a hamster TLR4 expression plasmid. A portion of the cells was also transfected with an expression plasmid encoding a function MD-2 protein. As shown in Fig. 2B, the MD-2 mutant 3E10 line was incapable of activation by LPS in the absence of ectopically expressed functional MD-2. In contrast, this
activation. The NF-κB stimulated with LPS (100 ng/ml), AraLAM (1 μg/ml), or Mtb (H37Ra, 5 bacilli per RAW 264.7 cell) for 5 h. MD-2 mutant 3E10 (CHO/CD14) cells were transiently transfected with the NF-κB-dependent ELAM-Luc reporter plasmid, an expression plasmid encoding hamster TLR4, and an expression plasmid encoding functional hamster MD-2. The next day, a portion of the cells was stimulated with LPS (100 ng/ml) or Mtb (H37Ra, five bacilli per CHO cell) for 16 h. Cells were then harvested, and luciferase activity was measured as described in the text. All transfection experiments were performed in triplicate and repeated at least three times, and a single representative experiment is shown. Data are expressed as the average luciferase values from a single experiment (subtracted for background) ± SD.

Figure 2. MD-2 does not enhance LAM- and Mtb-induced NF-κB activation. A. RAW 264.7 macrophages were transiently transfected with the NF-κB-dependent ELAM-Luc reporter plasmid and an expression plasmid encoding murine MD-2. The next day, a portion of the cells were stimulated with LPS (100 ng/ml), AraLAM (1 μg/ml), or Mtb (H37Ra, 5 bacilli per RAW 264.7 cell) for 5 h. B. MD-2 mutant 3E10 (CHO/CD14) cells were transiently transfected with the NF-κB-dependent ELAM-Luc reporter plasmid, an expression plasmid encoding hamster TLR4, and an expression plasmid encoding functional hamster MD-2. The next day, a portion of the cells was stimulated with LPS (100 ng/ml) or Mtb (H37Ra, five bacilli per CHO cell) for 16 h. Cells were then harvested, and luciferase activity was measured as described in the text. All transfection experiments were performed in triplicate and repeated at least three times, and a single representative experiment is shown. Data are expressed as the average luciferase values from a single experiment (subtracted for background) ± SD.

Mutant cell line was fully capable of activation by Mtb, and expression of ectopic functional MD-2 did not affect the responses of these cells to the bacilli. Together, these data suggest that MD-2 may not be required for cellular activation by Mtb.

E5531 blocks LPS- and Mtb-induced NF-κB activation

Because both TLR2 and TLR4 mediate cellular activation by Mtb, we sought to determine the relative contribution of TLR4 to Mtb-induced macrophage activation. Because blocking anti-murine TLR Abs were not available, we used the lipid A-like LPS-antagonist E5531 to block TLR4-dependent signaling (14, 15). We hypothesized that E5531 could block cellular activation via TLR4 regardless of the activating ligand based on our earlier finding that another lipid A-like antagonist, RSLA, blocked macrophage activation by both LPS and AraLAM (18). RAW 264.7 macrophages were stimulated for 5 h in the absence and the presence of E5531. As shown in Fig. 3A, E5531 blocked 90–100% of LPS-induced NF-κB activity, but only 30–40% of Mtb-induced NF-κB activity in these murine macrophages. A similar level of blocking was observed using the TLR4-DN mutant, as shown in Fig. 1, again suggesting that TLR4 mediates a lower relative proportion of the cellular responses to Mtb (as judged by NF-κB activation) compared with TLR2. Interestingly, the specificity of E5531 for TLR4-dependent signaling was demonstrated by the fact that it did not block NF-κB activation by the TLR2 agonist AraLAM (Fig. 3A).

Thus, E5531 appears to block only TLR-4 dependent signaling, whereas RSLA appears to block both TLR2- and TLR4-dependent signaling (18).

The inability of E5531 to block macrophage activation by AraLAM did not exclude the possibility that other mycobacterial TLR2 agonists might be blocked by E5531. We subsequently used a CHO/CD14 reporter cell line (3E10) (16, 20) to determine whether E5531 could block any Mtb-induced TLR2-dependent signaling. We found that E5531 blocked virtually all Mtb-induced NF-κB activity in macrophages. A portion of the cells was stimulated with LPS (100 ng/ml), AraLAM (1 μg/ml), or Mtb (H37Ra, five bacilli per RAW 264.7 cell) in the presence or the absence of E5531 (1 μg/ml) for 5 h. These reporter lines contain a stably transfected ELAM-CD25 reporter gene and express human CD25 on their surface as a consequence of NF-κB activation. Stimulated cells were stained with a PE-labeled anti-CD25 mAb, and surface expression of CD25 was measured by flow cytometry. Data are expressed as the ratio (fold activation) of the percentage of CD25+ cells in unstimulated and stimulated cell populations that were gated to exclude the lowest 5% of cells based on mean FL1 fluorescence. Statistical significance of stimulated responses, compared with E5531 cotreatment, was measured by ANOVA (p < 0.001) and is indicated by an asterisk.

Figures 2 and 3. E5531 blocks LPS- and Mtb-induced NF-κB activation. A. RAW 264.7 macrophages were transiently transfected with the NF-κB-dependent ELAM-Luc reporter plasmid. A portion of the cells was stimulated with LPS (100 ng/ml), AraLAM (1 μg/ml), or Mtb (H37Ra, five bacilli per RAW 264.7 cell) in the presence or the absence of E5531 (1 μg/ml) for 5 h. Cells were then harvested, and luciferase activity was measured as described in the text. All transfection experiments were performed in triplicate and repeated at least three times, and a single representative experiment is shown. Data are expressed as average luciferase values from a single experiment (subtracted for background) ± SD. B. The CHO/CD14, CHO/CD14/TLR2, and CHO/CD14/TLR4 reporter cell lines were stimulated with Mtb (H37Ra, five bacilli per CHO cell) in the presence or the absence of E5531 (1 μg/ml) for 16 h. These reporter lines contain a stably transfected ELAM-CD25 reporter gene and express human CD25 on their surface as a consequence of NF-κB activation. Stimulated cells were stained with a PE-labeled anti-CD25 mAb, and surface expression of CD25 was measured by flow cytometry. Data are expressed as the ratio (fold activation) of the percentage of CD25+ cells in unstimulated and stimulated cell populations that were gated to exclude the lowest 5% of cells based on mean FL1 fluorescence. Statistical significance of stimulated responses, compared with E5531 cotreatment, was measured by ANOVA (p < 0.001) and is indicated by an asterisk.

E5531 blocks Mtb-induced human alveolar macrophage apoptosis

The data presented above demonstrate that E5531 has the capacity to specifically block Mtb-induced NF-κB activation in macrophages. We also wanted to determine whether E5531 could block...
cellular responses that are known to be part of the innate immune response against Mtb. We previously proposed that macrophage apoptosis contributes to the host defense against Mtb infection (32) and hypothesized that this may be a TLR-dependent response. Both TLR4 and TLR2 have been previously implicated in macrophage activation and apoptosis. TLR4-deficient C3H/HeJ macrophages have been shown to be resistant to LPS-induced cell death (33). In addition, soluble bacterial lipoproteins have been demonstrated to induce TLR2-dependent macrophage apoptosis (34, 35). Therefore, we sought to determine whether TLR4 was necessary, but not sufficient, for Mtb-induced macrophage apoptosis (32). Thus, our data suggested that E5531 might block Mtb-induced cell death by blocking Mtb-induced TNF-α production. This possibility was tested by providing the Mtb-infected AMφ with exogenous recombinant human TNF-α to determine whether E5531 could still block cell death. As shown in Fig. 4B, addition of exogenous TNF-α abrogated the capacity of E5531 to block Mtb-induced AMφ apoptosis. Thus, E5531 may block cell death by inhibiting TLR4-dependent Mtb-induced TNF-α production by AMφ. In control experiments we also confirmed that addition of recombinant TNF-α alone was not sufficient to induce AMφ apoptosis. Staurosporine was used as a positive control to induce cell death in this assay.

Another possible mechanism for inhibition of both NF-κB activation and apoptosis by E5531 by its blocking Mtb binding or uptake, and thus cellular activation. This hypothesis was directly tested by determining whether E5531 could block the binding of with M. bovis BCG that constitutively express green fluorescence protein (GFP) to human AMφ in vitro. Human AMφ were incubated with 10 BCG-GFP bacilli/macrophage in the absence or the presence of E5531 (1–10 μg/ml). The next day, the cells were washed twice with PBS, and internalization of BCG-GFP by human AMφ was quantified by manual counting using a fluorescence microscope. We observed that 28 ± 5% of the human AMφ contained BCG-GFP after 16 h of infection. Inclusion of 1–10 μg/ml of E5531 had no effect on the percentage of BCG-GFP-positive macrophages (data not shown). Thus, E5531 does not block binding or uptake of Mtb. Together, these data suggest that E5531 blocks Mtb-induced apoptosis indirectly via its ability to block TNF-α production.

E5531 inhibits LPS- and Mtb-induced TNF-α production, but not NO production

We next sought to directly test the possibility that E5531 blocked Mtb-induced TNF-α production by macrophages. We concomitantly measured the production of NO by these macrophages in the absence and the presence of E5531. While murine macrophages produce high levels of NO in response to LPS and IFN-γ, human macrophages are not similarly responsive. However, high levels of NO can be produced by human AMφ stimulated in vitro with live Mtb (27, 36). We stimulated RAW 264.7 cells and primary human AMφ with Mtb in the presence and the absence of E5531. One day after stimulation, culture supernatants were collected and assayed for the production of both TNF-α and nitrite. Nitrite (NO₃⁻) is a stable catabolite of NO, and the level of nitrite in culture supernatants is a good indirect indicator of NO production by macrophages. E5531 substantially blocked Mtb-induced TNF-α production in both RAW 264.7 murine macrophages (Fig. 5, A and C) and human AMφ (Fig. 6A). In contrast, NO production was not affected by E5531 in either Mtb-stimulated RAW 264.7 murine macrophages (Fig. 5, B and D) or in human AMφ (Fig. 6B) regardless of the concentration of E5531 used. Thus, Mtb-induced TNF-α and NO production were differentially affected by the TLR4-specific antagonist E5531.

TLR2 and TLR4 are not necessary for Mtb-induced NO production

The data reported above suggest that TLR4 is not necessary for Mtb-induced NO production, but do not exclude the possibility that other TLR proteins mediate this response. For example, the purified 19-kDa lipoprotein Ag from Mtb has been shown to activate the iNOS promoter in transiently transfected murine macrophages in a TLR2-dependent manner (37). Thus, we used macrophages from TLR-deficient animals to compare the roles of TLR2 and TLR4 in Mtb-induced NO production (Fig. 7). Primary

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**FIGURE 4.** E5531 blocks Mtb-induced TNF-dependent alveolar macrophage apoptosis. A, Primary human AMφ were plated at a density of 4 x 10⁴ cells/well in 96-well microtiter plates. After 3 days portions of the cells were stimulated with Mtb (H37Ra, five bacilli per AMφ) in the absence or the presence of E5531 (1 μg/ml). Four hours later the medium was removed and replaced with fresh medium. At this time, cells that had received E5531 were again treated with fresh E5531. On days 2 and 4 the medium was removed and replaced with fresh medium plus E5531 where appropriate. On day 5 apoptosis was measured using an Ag capture ELISA to detect histone-associated DNA fragments. The statistical significance of Mtb-stimulated responses compared with responses to E5531 cotreatment was measured by ANOVA (p < 0.001) and is indicated by an asterisk. B, Human AMφ were plated at a density of 4 x 10⁴ cells/well in 96-well microtiter plates. After 3 days portions of the cells were stimulated with Mtb (H37Ra, five bacilli per AMφ) in the absence or the presence of E5531 (1 μg/ml) or recombinant human TNF-α (100 U/ml). Four hours later the medium was removed and replaced with fresh medium. At this time portions of the cells were again treated with E5531 (1 μg/ml), TNF-α (100 U/ml), or staurosporine (2 μM). On days 2 and 4 the medium was removed and replaced with fresh medium plus E5531 where appropriate. On day 5 apoptosis was measured as described above.
murine peritoneal macrophages from normal C3H/OuJ and TLR4-deficient C3H/HeJ mice and primary hamster peritoneal macrophages from normal Syrian and TLR2-deficient Chinese hamsters were stimulated with Mtb (five bacilli per macrophage) for 16 h. Culture supernatants were then assayed for nitrite production. We found that macrophages lacking either TLR2 or TLR4 produced identical levels of nitrite in response to Mtb compared with control cells. Together, these data demonstrate that TLR2 and TLR4 are not necessary for Mtb-induced NO production. Furthermore, these findings suggest that mycobacterial TLR2 agonists do not significantly contribute to NO production by macrophages stimulated by live Mtb bacilli in vitro.

**TLR proteins mediate activation of the TNF-α promoter, but not the iNOS promoter**

The findings presented above suggest that Mtb-induced NO production is not mediated by either TLR2 or TLR4. This raises the possibilities that 1) Mtb-induced NO production may be mediated by other TLR proteins; and 2) Mtb-induced NO production is the consequence of a TLR-independent mechanism. To discriminate between these possibilities we determined whether a MyD88-DN protein could block Mtb-induced NO production. We postulated that such a MyD88-DN mutant would block cellular activation via any TLR protein, because all known TLR proteins require MyD88 for intracellular signaling (38–40). RAW264.7 macrophages were cotransfected with a MyD88-DN expression plasmid plus one of three reporter plasmids. The luciferase reporter plasmids used were under control of the NF-κB-dependent ELAM promoter, the murine TNF-α promoter, and the murine iNOS promoter. The lipid A-like LPS antagonist E5531 was capable of substantially blocking Mtb-induced NF-κB activation and TNF-α production in both RAW 264.7 murine macrophages and primary human AMs. In CHO/CD14/TLR4 cells, E5531 blocked virtually all Mtb-induced NF-κB activation. Be-cause these cells do not express functional TLR2 and TLR4, E5531 blocked Mtb-induced NO production in the iNOS promoter. In contrast, overexpression of the MyD88-DN mutant failed to block Mtb-induced activation of the iNOS promoter. These data suggest that Mtb-induced TNF-α activation is a TLR-dependent process, whereas Mtb-induced NO production is TLR independent (Fig. 9). Alternatively, Mtb-induced NO production may be mediated by TLR proteins in an MyD88-independent manner.

**Discussion**

Our previous data demonstrated that viable Mtb are capable of activating NF-κB via both TLR2 and TLR4 in macrophages (7). Here we examined the relative contributions of TLR2 and TLR4 to Mtb-induced cellular responses. The lipid A-like LPS antagonist E5531 was capable of substantially blocking Mtb-induced NF-κB activation and TNF-α production in both RAW 264.7 murine macrophages and primary human AMs. In CHO/CD14/TLR4 cells, E5531 blocked virtually all Mtb-induced NF-κB activation. Because these cells do not express functional TLR2 and TLR4, E5531 blocked Mtb-induced NF-κB activation. The inhibitory activity of E5531 was specific for TLR4 because it did not block NF-κB activation
induced by TLR2 agonist AraLAM. Moreover, E5531 did not affect activation of CHO/CD14/TLR2 cells by Mtb, suggesting that additional mycobacterial TLR2 ligands were not blocked by the antagonist. E5531 also blocked Mtb-induced human AMø apoptosis, an effect that was secondary to the inhibition of TNF-α production. Lastly, we found that the capacity of E5531 to block Mtb-induced TNF-α production was not shared by all Mtb-inducible macrophage products. Mtb-induced NO production was not blocked by E5531, did not depend on the presence of TLR2 or TLR4, and may be mediated by a TLR-independent mechanism. Together, these data demonstrate E5531 specifically blocks TLR4-dependent Mtb-induced cellular responses. Because Mtb are not known to possess glycolipids that are chemically similar to LPS, our data suggest three potential mechanisms for E5531 antagonism. First, E5531 may block the assembly of functional TLR4 receptor complexes, rather than simply competing for binding of an Mtb ligand to TLR4. Second, E5531 may compete for binding to a common binding site on TLR4 with a mycobacterial TLR4 agonist. Third, E5531 may block a distinct TLR protein that functions in association with TLR4 to mediate responsiveness to LPS and mycobacterial ligands. The recent finding that another lipid A-like antagonist can block Taxol-induced cellular activation via TLR4 (41) is consistent with our findings, but does not favor any of these possible mechanisms. Regardless of the mechanism of E5531 action, this antagonist has proven to be a useful and selective tool for inhibiting TLR4 signaling by both LPS and chemically unrelated TLR4 agonists. It is interesting to note that CD14 (7) and MD-2 (this report), which greatly augment LPS and taxol stimulation via TLR4, do not appear to be required for Mtb-mediated activation in vitro. Our studies do not address the possibility that these TLR4 coreceptors modulate macrophage responses to Mtb TLR4 agonists in vivo. Interestingly, the β₂ integrin CD11b/CD18 appears to function as a TLR4 coreceptor in macrophages stimulated with taxol (42). The ability of CD11b/CD18 (also known as CR3) to mediate binding of Mtb to macrophages has been well described (1), but its potential role in modulating TLR4-dependent cellular activation by Mtb has not been clearly defined.

We found that TLR4-deficient C3H/HeJ mice were significantly more sensitive to lethal mycobacterial infection than normal C3H/OuJ mice. This finding supports the hypothesis that TLR4 is necessary for innate host responses to mycobacteria in vivo. Many studies have shown enhanced sensitivity of C3H/HeJ mice to infection by a broad range of infectious agents (reviewed in Ref. 43 and references therein). One recent study using experimental E. coli pyelonephritis demonstrated that the kidneys of C3H/HeJ and C3H/HeN mice were colonized at a similar rate 24 h after infection; however, only the C3H/HeN mice could clear the infection 5 wk postinoculation (44). In contrast, Hopkins et al. revealed that both C3H/HeJ and C3H/OuJ mice were similarly sensitive to E. coli infection, whereas C3H/HeN mice were more resistant to infection compared with the other C3H strains (45). Furthermore, the degree of inflammation in the bladder and kidneys positively correlated with the extent of infection, except for C3H/HeJ mice, which had minimal inflammation despite high infection levels. These findings are consistent with the earliest reports of LPS hypersensitivens in which C3H/HeJ mice failed to respond to i.p. injection of LPS by eliciting a normal inflammatory cell infiltrate into the peritoneal cavity (46). Together, these data suggest that TLR4 is a critical factor in the development of an inflammatory host response to infection, but TLR4 is not the sole determinant of susceptibility to persistent or lethal infection. The comparatively high susceptibility of C3H/HeJ mice to lethal mycobacterial infection may be linked to the TLR4-dependent activation of proinflammatory cytokine and chemokine production in vivo. It has...
been shown that these potent inflammatory mediators play a protective role in host responses to mycobacterial infection (reviewed in Ref. 47), and it is possible that the infected C3H/HeJ mice fail to elicit a protective early inflammatory response in vivo.

Another innate host response that appears to require TLR proteins is Mtb-induced AMβ apoptosis. Macrophage apoptosis has been shown to correlate inversely with the virulence of the mycobacterial species and strain used (48). Macrophage apoptosis has also been proposed to be important in the host defense against Mtb (reviewed in Ref. 49). Apoptosis may be a means to trap live bacilli within apoptotic bodies, thereby reducing the dissemination of Mtb from infected macrophages. Furthermore, Mtb-containing apoptotic cells are probably rapidly phagocytosed and destroyed by uninfected AMβ. This hypothesis is consistent with previous data demonstrating that virulent Mtb strains induce less macrophage apoptosis, thereby promoting survival and ability to replicate within the host (32). Here we demonstrate that Mtb-induced macrophage apoptosis is at least in part a TLR4-dependent process that can be blocked by E5531. The simplest explanation for our findings is that E5531 blocks Mtb-induced macrophage apoptosis as a consequence of its ability to block Mtb-induced TNF-α production. TNF-α production is required to mediate Mtb-induced macrophage apoptosis, and the addition of exogenous TNF-α was sufficient to abrogate the inhibitory activity of E5531. Alternatively, E5531 may block an Mtb-induced proapoptotic pathway, but addition of exogenous TNF-α may circumvent this blockade, thereby promoting apoptosis via a parallel pathway. Whether additional TLR or non-TLR proteins are also involved in other Mtb-induced proapoptotic responses remains to be determined.

One important and unexpected finding arising from our studies was the observation that E5531 could block Mtb-induced TNF-α production, but not NO production. This phenomenon was observed using both a murine macrophage cell line and primary human AMβ. Furthermore, macrophages from animals that lack functional TLR2 or TLR4 produced normal levels of NO when challenged with Mtb in vitro. Subsequently, we demonstrated that a MyD88-DN mutant could block Mtb-induced activation of the TNF-α promoter, but not the iNOS promoter. The rationale for using a MyD88-DN mutant comes from previous studies showing that this adapter protein is required for signaling by all known TLR proteins as well as other related receptors (IL-1R type I, IL-18R) (38–40). Thus, we used the MyD88-DN mutant as a means to block all Mtb-induced TNF signaling. Together, our findings demonstrate that Mtb-induced TNF-α production is a TLR-dependent process, whereas Mtb-induced NO production appears to be a TLR-independent process. Alternatively, Mtb-induced NO production may be mediated by TLR proteins in a MyD88-dependent manner. Both TNF-α and NO are known to play important roles in the host response to Mtb infection. TNF-α-deficient and iNOS-deficient mice are both highly sensitive to lethal Mtb infection compared with normal mice (50–52). Thus, it is interesting that these two critical innate host responses differ in their requirement for TLR proteins. While the significance of this difference in TLR requirement is not clear, our data suggest that inhibition of TNF signaling could leave some innate host responses intact.

Our future studies are aimed at examining the course of Mtb infection in vivo using TLR2 and TLR4 knockout mice. It will be particularly important to determine whether iNOS production and NO-dependent mycobactericidal activities in vivo are affected by the absence of TLR2 or TLR4. TNF-α has been shown to augment IFN-γ-induced NO production by macrophages in vitro, resulting in maximal mycobacterial growth inhibitory activity (53). Thus, the absence of TLR2 and/or TLR4 signaling may have an indirect effect on NO production and mycobacterial growth inhibition. Lastly, the study of naturally occurring TLR mutations in humans may reveal links to disease susceptibility. Arbour et al. recently reported that TLR4 mutations are associated with LPS hyporesponsiveness (54), and it will be interesting to determine whether
such mutations are associated with increased susceptibility to tuberculosis.

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