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Fang-Mei Chang

Sara M. Reyna

Jose C. Granados

Sung-Jen Wei

Wendy Innis-Whitehouse

*See next page for additional authors*

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**Authors**

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# Inhibition of Neddylolation Represses Lipopolysaccharide-induced Proinflammatory Cytokine Production in Macrophage Cells

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Fang-Mei Chang<sup>‡</sup>, Sara M. Reyna<sup>‡§</sup>, Jose C. Granados<sup>‡</sup>, Sung-Jen Wei<sup>‡¶</sup>, Wendy Innis-Whitehouse<sup>‡</sup>, Shivani K. Maffi<sup>¶||</sup>, Edward Rodriguez<sup>‡</sup>, Thomas J. Slaga<sup>¶</sup>, and John D. Short<sup>‡¶||</sup>

From the <sup>‡</sup>Regional Academic Health Center, Medical Research Division, University of Texas Health Science Center at San Antonio, Edinburg, Texas 78541, the Departments of <sup>§</sup>Medicine/Division of Diabetes and <sup>¶</sup>Pharmacology, University of Texas Health Science Center, San Antonio, Texas 78229, and the <sup>||</sup>Department of Molecular Medicine, University of Texas Health Science Center, San Antonio, Texas 78245

**Background:** Lipopolysaccharides (LPSs) up-regulate proinflammatory cytokines in macrophages, partly through a NF- $\kappa$ B-dependent process.

**Results:** Blocking neddylation, which helps regulate NF- $\kappa$ B, represses LPS-induced up-regulation of proinflammatory cytokines.

**Conclusion:** Neddylation plays a role in the up-regulation of NF- $\kappa$ B-regulated proinflammatory cytokines produced by macrophages in response to LPS.

**Significance:** Inhibition of neddylation represents a novel and effective method for the prevention of LPS-induced proinflammatory cytokines.

Cullin-RING E3 ligases (CRLs) are a class of ubiquitin ligases that control the proteasomal degradation of numerous target proteins, including I $\kappa$ B, and the activity of these CRLs are positively regulated by conjugation of a Nedd8 polypeptide onto Cullin proteins in a process called neddylation. CRL-mediated degradation of I $\kappa$ B, which normally interacts with and retains NF- $\kappa$ B in the cytoplasm, permits nuclear translocation and transactivation of the NF- $\kappa$ B transcription factor. Neddylation occurs through a multistep enzymatic process involving Nedd8 activating enzymes, and recent studies have shown that the pharmacological agent, MLN4924, can potently inhibit Nedd8 activating enzymes, thereby preventing neddylation of Cullin proteins and preventing the degradation of CRL target proteins. In macrophages, regulation of NF- $\kappa$ B signaling functions as a primary pathway by which infectious agents such as lipopolysaccharides (LPSs) cause the up-regulation of proinflammatory cytokines. Here we have analyzed the effects of MLN4924, and compared the effects of MLN4924 with a known anti-inflammatory agent (dexamethasone), on certain proinflammatory cytokines (TNF- $\alpha$  and IL-6) and the NF- $\kappa$ B signaling pathway in LPS-stimulated macrophages. We also used siRNA to block neddylation to assess the role of this molecular process during LPS-induced cytokine responsiveness. Our results demonstrate that blocking neddylation, either pharmacologically or using siRNA, abrogates the increase in certain proinflammatory cytokines secreted from macrophages in response to LPS. In addition, we have shown that MLN4924 and dexamethasone inhibit LPS-induced cytokine up-regulation at the transcriptional level, albeit through different molecular mechanisms. Thus, neddylation represents a novel molecular process in macrophages that can be targeted to prevent and/or treat the LPS-induced up-reg-

ulation of proinflammatory cytokines and the disease processes associated with their up-regulation.

Macrophage cells, which are part of the mononuclear phagocyte system, are key players in the innate immune response where they act as cellular sentinels to detect invading pathogens (1, 2). In addition to performing phagocytosis, macrophages are a major source of secreted mediators, including inflammatory cytokines that help regulate host defense (2). These secreted mediators subsequently act as autocrine and paracrine factors that influence neighboring cells within the body, and macrophages have been shown to play a role in various disease processes (*i.e.* infertility, fibrosis, metabolic syndrome, and liver cancer) through dysregulation of these secreted mediators (1, 3–5).

The up-regulation of secreted inflammatory cytokines by macrophages occurs upon the selective recognition of microorganisms by pattern recognition receptors such as the Toll-like receptor (TLR)<sup>2</sup> proteins, which interact with pathogen-associated molecular patterns that are conserved in bacterial species (*e.g.* lipopeptides, lipopolysaccharides, or Flagellin) (6, 7). Perhaps the best characterized example of this microbial recognition by TLRs is the recognition of lipopolysaccharides (LPS) from Gram-negative bacteria by TLR4. The interaction between LPS and TLR4 signals through interleukin-1 receptor-

<sup>1</sup> To whom correspondence should be addressed. Tel.: 956-393-6420; Fax: 956-393-402; E-mail: shortj@uthscsa.edu.

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<sup>2</sup> The abbreviations used are: TLR, Toll-like receptor; AP-1, activator protein-1;  $\beta$ -TrCP,  $\beta$ -transducin repeat-containing protein; Cul1, Cullin1; CRL, Cullin ring ligase; Dex, dexamethasone; GR, glucocorticoid receptor; I $\kappa$ B, inhibitor of NF- $\kappa$ B; IL-6, interleukin-6; MDM, monocyte-derived macrophage; MKP-1, mitogen-activated protein kinase phosphatase 1; Nedd8, neural precursor cell expressed, developmentally down-regulated 8; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PMA, phorbol 12-myristate 13-acetate; Ubc12, ubiquitin-conjugating enzyme E2M.

associated kinases, which subsequently signal through mitogen-activated protein kinase kinase 6 (MAPKK6) and the inhibitor of nuclear factor- $\kappa$ B kinase (7, 8). MAPKK6 and inhibitor of nuclear factor- $\kappa$ B kinase activation culminate in the activation of activator protein-1 (AP-1) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcription factors, respectively (7, 8), resulting in the transcriptional up-regulation of several proinflammatory cytokines (7, 8). Previous studies have shown that this LPS-induced increase in proinflammatory cytokines from macrophage cells can be inhibited by glucocorticoid agonists such as dexamethasone (Dex) (9).

The NF- $\kappa$ B transcription factor is comprised of homodimers or heterodimers of the Rel family of proteins, which includes p50, p52, cRel, p65 (RelA), and RelB, and regulation of NF- $\kappa$ B transcriptional activity occurs through a series of complex molecular processes (10). Under normal conditions, NF- $\kappa$ B subunits interact with inhibitor of NF- $\kappa$ B (I $\kappa$ B) proteins (I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , and I $\kappa$ B $\epsilon$ ) in the cytosol, allowing sequestration of NF- $\kappa$ B from DNA targets in the nucleus (10, 11). However, LPS stimulation, similar to other pathogen-associated molecular patterns, causes phosphorylation and ubiquitin-mediated degradation of I $\kappa$ B proteins, thereby promoting nuclear translocation and activation of NF- $\kappa$ B in macrophages and other cell types (6, 7, 11–14).

This ubiquitin-mediated degradation of I $\kappa$ B proteins is regulated by an ubiquitin E3 ligase complex for I $\kappa$ B comprised of multiple proteins, including Cullin1 (Cul1), S-phase kinase associated protein 1, and an F-box protein known as  $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP) (15, 16). This complex, when associated with the RING (really interesting new gene)-type E3 ubiquitin ligase, is referred to as the Cullin-RING ligase 1 <sup>$\beta$ -TrCP</sup> (CRL1 <sup>$\beta$ -TrCP</sup>) complex (15, 17, 18). The ability of the CRL1 <sup>$\beta$ -TrCP</sup> complex to ubiquitinate target proteins, including I $\kappa$ B, is enhanced by the covalent attachment of a neural precursor cell expressed, developmentally down-regulated 8 (Nedd8) polypeptide to the Cul1 protein (19).

Nedd8 is a highly conserved ubiquitin-like protein that is covalently attached to target proteins, including Cullin proteins, in a process called neddylation (17, 20, 21). Neddylation occurs in a multistep enzymatic process in which Nedd8 is transferred from Nedd8 activating enzymes onto target proteins in an ATP-dependent manner (15, 22). Recent studies have shown that the pharmacological agent MLN4924 can potentially inhibit Nedd8 activating enzymes, thereby prohibiting the transfer of Nedd8 onto target proteins (23). Furthermore, MLN4924 has been shown to increase phosphorylated I $\kappa$ B $\alpha$  in B-cells and myeloid leukemia cells and to reduce the expression of several NF- $\kappa$ B target genes in B-cells (24, 25).

In this study, we have analyzed the effect of MLN4924 on LPS-induced up-regulation of cytokines in macrophage cells. We have also compared the molecular mechanisms by which both MLN4924 and Dex inhibit LPS-induced cytokine up-regulation in macrophages. Although both MLN4924 and Dex blocked increased cytokine transcription and secretion in response to LPS, only MLN4924 inhibited I $\kappa$ B degradation and the subsequent nuclear translocation and activation of the NF- $\kappa$ B transcription factor. Last, we have used siRNA to deplete Nedd8 or the Nedd8-conjugating enzyme known as

ubiquitin-conjugating enzyme E2M (or Ubc12) to determine whether Nedd8 or the process of neddylation plays a role in LPS responsiveness in macrophages. Depletion of either Nedd8 or Ubc12 abrogated LPS-induced cytokine secretion.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Differentiation**—The human monocytic leukemia cell line, THP-1, was cultured in Roswell Park Memorial Institute 1640 media (RPMI: ATCC) containing 10% fetal bovine serum (FBS) (Invitrogen) and 0.05 mM 2-mercaptoethanol (Invitrogen). Differentiation was achieved by the addition of 100 ng/ml of PMA (Promega) for 48 h. The mouse monocyte/macrophage cell line, RAW 264.7, was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% FBS. Both THP-1 and RAW 264.7 cells were obtained from ATCC.

Human peripheral blood mononuclear cells were isolated from the blood of healthy donors that was purchased from the Gulf Coast Regional Blood Center (Houston, TX). Peripheral blood mononuclear cells were isolated according to a protocol derived from Lonza Group Ltd. Briefly, blood was centrifuged ( $400 \times g$  for 40 min) using a Ficoll Paque Plus (GE Healthcare) density gradient, and peripheral blood mononuclear cells were then removed from the gradient and plated in growth media consisting of RPMI containing 0.05 mM 2-mercaptoethanol and 10% human AB serum (Fisher). After 2–3 h, nonadherent cells (lymphocytes) were removed, and adherent cells (monocytes) were cultured in growth medium overnight. The following day, the supernatant and easily detachable cells, containing monocytes, were collected and the remaining adherent cells (*e.g.* fibroblasts) were discarded. The harvested monocytes were then placed into differentiation media consisting of growth medium containing 50 ng/ml of recombinant human monocyte-colony stimulating factor (R&D Systems) for 8 days. Differentiation media was replaced every 2–3 days. All cells were cultured at 37 °C with 5% CO<sub>2</sub>.

**Cytokine Detection**—Differentiated THP-1 cells, RAW 264.7 cells, and human monocyte-derived macrophages (MDMs) were grown on 24-well dishes at  $1 \times 10^6$ ,  $2 \times 10^5$ , and  $1.2 \times 10^6$  cells/well, respectively. Cells were pre-treated with vehicle (DMSO), MLN4924 (Sequoia Research Products Ltd.), or Dex (Sigma) at the indicated dosages for 2 h. After 2 h, cells were treated with 100 ng/ml of *Escherichia coli*-derived LPS (Sigma) for 6 additional hours, and supernatants were collected and stored at –80 °C until analysis. IL-6 and TNF- $\alpha$  from the culture media of cells were measured and quantified using a Bio-Plex 200 system along with either the human or mouse Bio-Plex Pro Cytokine Assay Kits (Bio-Rad) according to the manufacturer's instructions.

**Cell Viability Assay**—THP-1 cells (7,000 cells/well) were seeded on a 96-well plate and differentiated with 100 ng/ml of PMA for 48 h. Differentiated cells were then treated as indicated for either 24 or 48 h. After treatment, cell viability was measured and quantified using the CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay (MTS Assay: Promega) according to the manufacturer's instructions. The cell viability of all treatment groups were normalized to nontreated cells.

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**Quantitative RT-PCR**—THP-1 cells ( $3 \times 10^6$  cells/well) were seeded on a 6-well plate and differentiated with 100 ng/ml of PMA for 48 h. Differentiated cells were treated with LPS in the absence or presence of MLN4924 or Dex, as indicated. RNA was isolated from treated cells using TRIzol reagent (Ambion) according to the manufacturer's instructions. cDNA was then synthesized from 1  $\mu$ g of RNA using the iScript Reverse Transcription Supermix Kit (Bio-Rad). Validated RT-PCR primers specific for human IL-6 (forward, TTCGGTCCAGTTGCCTTCTC; reverse, GAGGTGAGTGGCTGTCTGTG), human TNF- $\alpha$  (forward, CCTGCCCAATCCCTTTATT; reverse, CCCTAAGCCCCCAATTCTCT), or human Actin (forward, GAGCCTCGCCTTTGCCGATCCG; reverse, CACATGCCG-GAGCCGTTGTGCGA) were used to quantify mRNA levels using cDNA (4 ng) and the iTaq SYBR Green Supermix with ROX Kit (Bio-Rad). Real-time PCR was carried out using an AB7900HT Fast Real-Time PCR System (Applied Biosystems), and IL-6 and TNF- $\alpha$  mRNA levels were calculated using the  $\Delta\Delta C_T$  method according to the manufacturer's instructions.

**Western Blotting and Subcellular Fractionation**—THP-1 cells ( $3 \times 10^6$  cells/well) were seeded onto 6-well dishes and differentiated with 100 ng/ml of PMA for 48 h. Differentiated cells were treated with LPS in the absence or presence of MLN4924 or Dex, as indicated. After treatment, cells were lysed using SDS lysis buffer (0.5% SDS and 50 mM Tris-HCl, pH 8.0) containing 2.5 mM sodium pyrophosphate (Fisher), 1 mM  $\beta$ -glycerophosphate (Sigma), 1 mM  $\text{Na}_3\text{VO}_4$  (Sigma), 1 mM PMSF (Sigma), 1 mM DTT (Fisher), 1 mM NaF (Fisher), and 1 $\times$  Complete<sup>®</sup> protease inhibitor mixture (Roche Applied Science). Protein concentrations were determined using the Pierce BCA Protein Assay (Thermo Scientific), and equal amounts of protein were separated by electrophoresis and subsequently transferred to nitrocellulose membranes. Membranes were analyzed using the following antibodies: Nedd8 (19E3), I $\kappa$ B $\alpha$  (L35A5), and phosphorylated I $\kappa$ B $\alpha$  (Ser-32/36) (5A5) were from Cell Signaling; Cull1 (H-213) and Ubc12 (L-34) were from Santa Cruz; actin antibody was from Sigma. Primary antibodies were detected using secondary antibodies conjugated to horseradish peroxidase (Santa Cruz).

For subcellular fractionation, differentiated THP-1 cells ( $10 \times 10^6$  cells/dish on 10-cm dishes) were treated, and the nuclear and cytosolic fractions were isolated using the Nuclear Extraction Kit (Panomics) according to the manufacturer's instructions. The extracted fractions were then analyzed by Western blotting with antibodies against NF- $\kappa$ B/p65 (Cell Signaling), lactate dehydrogenase (Millipore), and lamin A/C (Santa Cruz).

**Immunofluorescence Microscopy**—THP-1 cells (70,000 cells/well) were plated onto Nunc Lab-TekII 8-chamber slides (Fisher Scientific) and treated with PMA (100 ng/ml) for 48 h. Differentiated cells were treated with  $\text{Me}_2\text{SO}$ , 500 nM MLN4924, or 100 nM Dex for 2 h. Cells were then treated with 100 ng/ml of LPS for 1 h. After treatment, cells were fixed in 4% paraformaldehyde for 30 min and then permeabilized with 1% Triton X-100 for 10 min at room temperature. Fixed and permeabilized cells were stained using an anti-NF- $\kappa$ B p65 (C22B4) primary antibody (Cell Signaling) and a secondary antibody conjugated with Alexa Fluor<sup>®</sup> 488 (Invitrogen). Cells were

mounted using VectaShield mounting medium containing DAPI (Fisher Scientific), and a set of Z-series images were visualized using an Olympus FV1000 confocal microscope with a  $\times 40$  objective: numerical aperture = 1.30 along with a 2.5 electronic zoom (Olympus). Standard inbuilt ultraviolet diode laser settings for DAPI (excitation 405 nm/emission 461 nm) and 488 nm argon laser settings (excitation 488 nm/emission 520 nm) were used. Laser exposure for image acquisition was attenuated to minimize photobleaching and also set using appropriate iso-controls, and microscope settings were kept identical for each treatment group.

**Luciferase Assays**—RAW 264.7 cells were transfected with 2  $\mu$ g of pNF- $\kappa$ B-MetLuc2-Reporter plasmid (Clontech) using an Amaxa Nucleofector II apparatus along with the Amaxa Cell Line Nucleofector V Kit (Lonza) in accordance with the manufacturer's protocol. After transfection, cells were plated onto a 24-well dish ( $1.5 \times 10^5$  cells/well) and pre-treated with vehicle, MLN4924, or Dex as indicated for 2 h. Cells were then stimulated with LPS (100 ng/ml) for 24 h, and luciferase activity was measured using the Ready-To-Glow<sup>™</sup> NF $\kappa$ B Secreted Luciferase Reporter System according to the manufacturer's protocol (Clontech). Luciferase activity in the culture media, which was detected and measured using a SpectraMaxM5 plate reader (Molecular Devices), was normalized to the total protein concentration of the corresponding whole cell lysates.

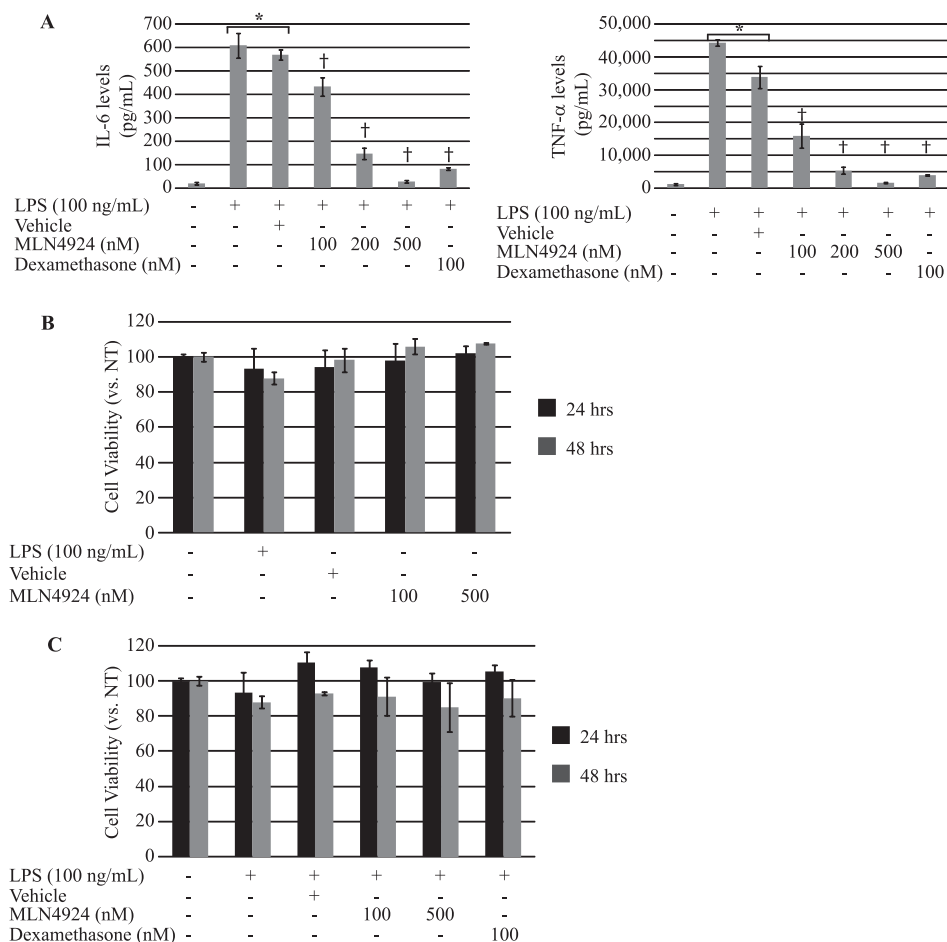
**RNA Interference**—RAW 264.7 cells ( $2.5 \times 10^6$  cells/sample) were transfected with 3  $\mu$ g of control siRNA-A, mouse Nedd8 siRNA, or mouse Ubc12 siRNA (Santa Cruz) using an Amaxa Nucleofector II apparatus along with the Amaxa Cell Line Nucleofector V Kit (Lonza) according to the manufacturer's instructions. After transfection, cells were plated onto 6-well dishes or split between two wells of a 12-well dish for 12 h. After 12 h, cells were left untreated or treated with 100 ng/ml of LPS for 6 h. 6-Well dishes were lysed and analyzed by Western blotting, and the media from 12-well dishes were analyzed for IL-6 and TNF- $\alpha$  levels.

**Statistical Analyses**—All statistical analyses were performed using SigmaPlot software (Systat Software, Inc.), and *p* values were calculated using the Student's *t* test.

## RESULTS

**MLN4924 Inhibits LPS-induced Proinflammatory Cytokines from Macrophage Cells at Sublethal Doses**—THP-1 monocytes can be converted into macrophage-like cells by the addition of PMA to culture media, and this conversion is a physiological model for differentiation of monocytes to macrophages *in vivo* (26–28). LPS treatment of these PMA-differentiated THP-1 cells has been shown to stimulate production of proinflammatory cytokines, including TNF- $\alpha$  and IL-6 (29). To determine whether MLN4924 could inhibit the effects of LPS on cytokine expression and secretion in THP-1 cells, we analyzed IL-6 and TNF- $\alpha$  protein levels in the culture media of PMA-differentiated THP-1 macrophage-like cells. MLN4924 significantly inhibited the LPS-induced increase in IL-6 and TNF- $\alpha$  cytokine levels in a dose-dependent manner when used at dosages of 100 to 500 nM (Fig. 1A). This effective dose was similar to the inhibition of LPS-induced cytokine production when using Dex, a known anti-inflammatory agent, which significantly

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**FIGURE 1. MLN4924 inhibits LPS-induced IL-6 and TNF- $\alpha$  secretion from THP-1 macrophage-like cells at sublethal doses.** *A*, quantification of IL-6 (*left*) and TNF- $\alpha$  (*right*) cytokine levels from PMA-differentiated THP-1 cells that were treated with LPS in the presence or absence of MLN4924 or Dex at the indicated doses for 6 h. Each *bar* indicates cytokine levels from cells that were treated in triplicate, and data are represented as mean  $\pm$  S.D. (a representative experiment is shown;  $n > 3$ ). *B* and *C*, cell viability of THP-1 macrophage-like cells treated with LPS or MLN4924 (*B*), or LPS in combination with MLN4924 or Dex (*C*), at the indicated doses. Each *bar* represents cells that were treated in duplicate and data are represented as mean  $\pm$  S.D. \*,  $p < 0.05$  when compared with nontreated cells; †,  $p < 0.05$  when compared with LPS-treated cells in the presence or absence of vehicle.

inhibited LPS-stimulated cytokines at a dose of 100 nM in these cells (Fig. 1A).

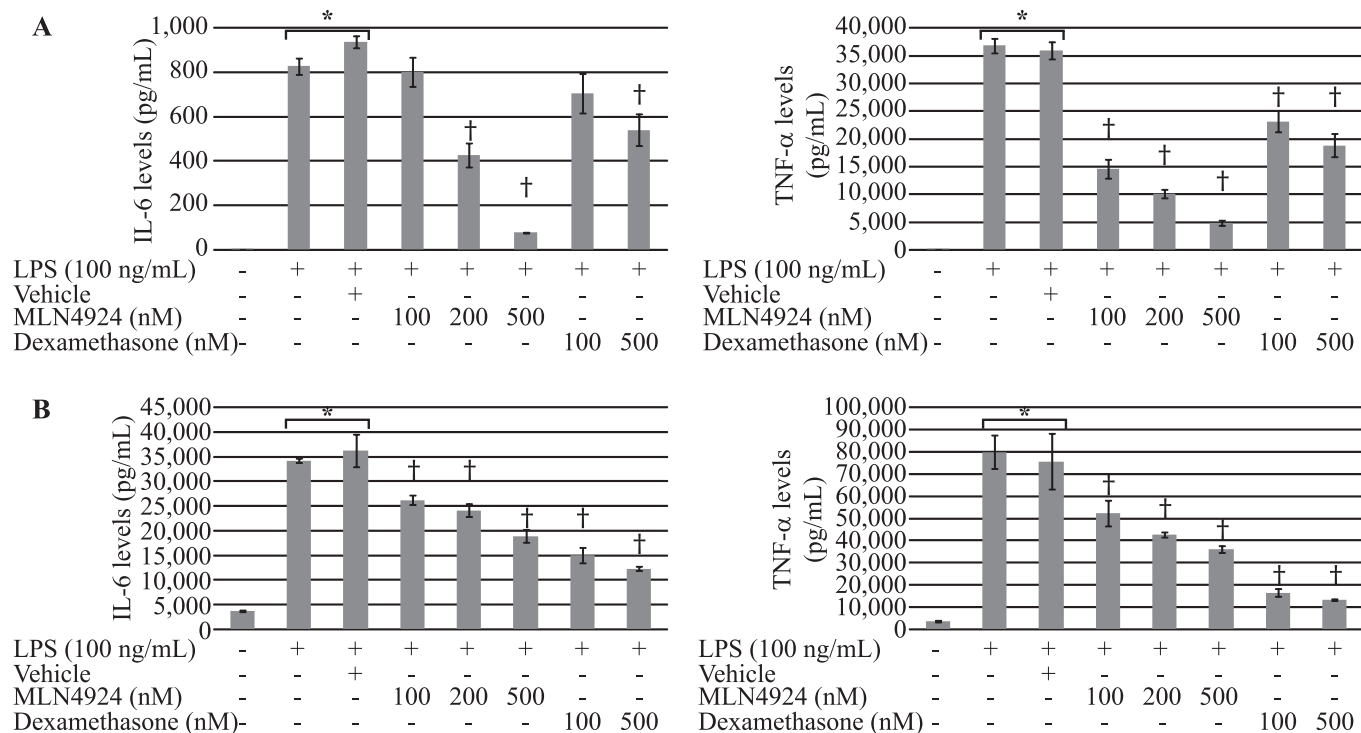
Previous studies have shown that MLN4924 reduces viability of acute myeloid leukemia cell lines at dosages greater than 100 nM (25). Therefore, we sought to determine whether MLN4924 reduced proinflammatory cytokine levels simply by reducing macrophage cell viability. Treatment of THP-1 macrophage-like cells with either MLN4924 alone or MLN4924 in combination with LPS for 24 or 48 h had no effect on cell viability (Fig. 1, *B* and *C*). Likewise, treatment of THP-1 macrophage-like cells with Dex along with LPS had no effect on THP-1 cell viability. Taken together, these data indicate that MLN4924, like Dex, is capable of inhibiting LPS-induced up-regulation of IL-6 and TNF- $\alpha$  at sublethal doses in differentiated THP-1 cells.

To assess whether the effect of MLN4924 on LPS-induced cytokine production was specific to THP-1 cells or whether this effect took place in other types of macrophage cells, we analyzed the effect of MLN4924 on LPS-induced IL-6 and TNF- $\alpha$  cytokine levels in a mouse monocyte/macrophage cell line, RAW 264.7, and human peripheral blood MDM cells. Similar to previous reports, the MDMs used for this study readily adhered to tissue culture plates after 8 days in culture and

expressed a cell surface protein, PM-2K, that is expressed on macrophages but not monocytes (data not shown) (30, 31). Similar to THP-1 macrophage-like cells and similar to previous studies (29), LPS induced the up-regulation of both IL-6 and TNF- $\alpha$  in RAW 264.7 cells and MDMs. More importantly, treatment of either RAW 264.7 cells or MDMs with MLN4924 or Dex ablated the LPS-induced effects on cytokine production (Fig. 2). These data indicate that MLN4924 and Dex inhibit LPS-induced cytokine production in macrophage cells.

**MLN4924 Inhibits LPS-induced Transcriptional Activation of Proinflammatory Cytokines**—Both IL-6 and TNF- $\alpha$  expression have previously been shown to be regulated by LPS at the level of transcription (32, 33). These data combined with the fact that MLN4924 inhibited LPS-induced up-regulation of IL-6 and TNF- $\alpha$  cytokine levels without causing cytotoxicity in macrophage cells (Fig. 1) led us to speculate that MLN4924 was blocking LPS-induced transcriptional activation of IL-6 and TNF- $\alpha$ . To test this, IL-6 and TNF- $\alpha$  mRNA levels were assessed in THP-1 macrophage-like cells that had been treated with LPS in the presence or absence of MLN4924. Similar to previous studies, LPS treatment led to increased levels of both IL-6 and TNF- $\alpha$  mRNAs (32, 33); however, both MLN4924

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**FIGURE 2. MLN4924 prevents LPS-induced IL-6 and TNF- $\alpha$  secretion from RAW 264.7 cells and human blood monocyte-derived macrophages.** Quantification of IL-6 (left) and TNF- $\alpha$  (right) cytokine levels from RAW 264.7 cells (A) and human monocyte-derived macrophages (B) that were treated with LPS in the presence or absence of MLN4924 or Dex at the indicated doses for 6 h. Each bar indicates cytokine levels from cells that were treated in triplicate and data are represented as mean  $\pm$  S.D. (a representative experiment is shown;  $n > 3$ ). \*,  $p < 0.05$  when compared with nontreated cells; †,  $p < 0.05$  when compared with LPS-treated cells in the presence or absence of vehicle.

and Dex inhibited LPS-stimulated IL-6 and TNF- $\alpha$  mRNA levels (Fig. 3). Additionally, MLN4924 inhibited LPS-mediated increases in IL-6 and TNF- $\alpha$  mRNAs in a dose-dependent manner (Fig. 3). These data suggest that both MLN4924 and Dex inhibit LPS-induced transcriptional activation of certain proinflammatory cytokines in macrophage cells.

**MLN4924 and Dexamethasone Differentially Affect Neddylation and I $\kappa$ B Degradation in Macrophage Cells**—The ability of MLN4924 and Dex to similarly inhibit LPS-stimulated cytokine production in macrophage cells suggested to us that these two drugs have overlapping molecular targets. Because MLN4924 has been characterized as an inhibitor of neddylation in other cell types (23–25, 34), we first assessed whether MLN4924 and/or Dex could inhibit this molecular process in macrophages. Not surprisingly, treatment of THP-1 macrophage-like cells with MLN4924 in the presence of LPS for a time period that correlated with the effect of this drug on LPS-induced cytokine production (6 h) revealed that MLN4924 was capable of reducing neddylation in THP-1 macrophage-like cells (Fig. 4A). This reduction in neddylation was detected using a Nedd8 antibody that is capable of detecting a Nedd8-conjugated protein band at  $\sim 90$  kDa and is thought to be neddylated Cullin protein (Fig. 4A, top panel) (23–25, 34). In addition, the MLN4924-mediated reduction of neddylated Cullin1 was also detected using an anti-Cullin1 antibody (Fig. 4A, middle panel), which is capable of detecting a protein doublet at  $\sim 90$  kDa, with neddylated Cullin1 above the non-neddylated form of Cullin1 (35). In contrast, Dex did not block the process of neddylation in macrophage cells, as measured with either of the antibodies described above (Fig. 4A).

Previous studies have also shown that Dex can cause increased I $\kappa$ B $\alpha$  mRNA and protein levels in macrophages (9), and I $\kappa$ B is a known target of CRLs (15). Therefore, we analyzed the effect of MLN4924 and Dex on the degradation of I $\kappa$ B in response to LPS treatment. Similar to previous results in THP-1 cells and other cell types, treatment with LPS led to a reduction in I $\kappa$ B protein levels within 15 min of LPS treatment that was slowly recovered over a 6-h period (Fig. 4B, top left, middle panel) (12, 36, 37). This decrease in I $\kappa$ B coincided with phosphorylation of I $\kappa$ B at serine 32 and serine 36 (I $\kappa$ B<sup>Ser32/Ser36</sup>); LPS treatment of THP-1 macrophage-like cells led to increased levels of phosphorylated I $\kappa$ B<sup>Ser32/Ser36</sup> during the first hour of treatment, and this increase slowly returned to lower levels during the remainder of the 6-h treatment (Fig. 4B, top left, top panel). Treatment of THP-1 macrophage-like cells with MLN4924 at 100 or 500 nM inhibited the LPS-mediated decrease in I $\kappa$ B levels (Fig. 4B, right, middle panels), whereas levels of phosphorylated I $\kappa$ B<sup>Ser32/Ser36</sup> were elevated in MLN4924-treated cells as early as 15 min after LPS treatment and remained elevated for the duration of the 6-h LPS treatment (Fig. 4B, right, top panels). Furthermore, the I $\kappa$ B that was maintained in MLN4924-treated cells exhibited a slightly higher molecular weight when compared with nontreated cells (Fig. 4B, right, middle panels). Surprisingly, Dex treatment of THP-1 macrophage-like cells had no effect on LPS-mediated decreases in I $\kappa$ B or LPS-mediated effects on phosphorylated I $\kappa$ B<sup>Ser32/Ser36</sup> when compared with vehicle-treated cells (Fig. 4B, compare top and bottom groups of panels on left). These data suggest that MLN4924 blocks phosphorylated I $\kappa$ B<sup>Ser32/Ser36</sup> from protein degradation in macrophage cells and indicate that

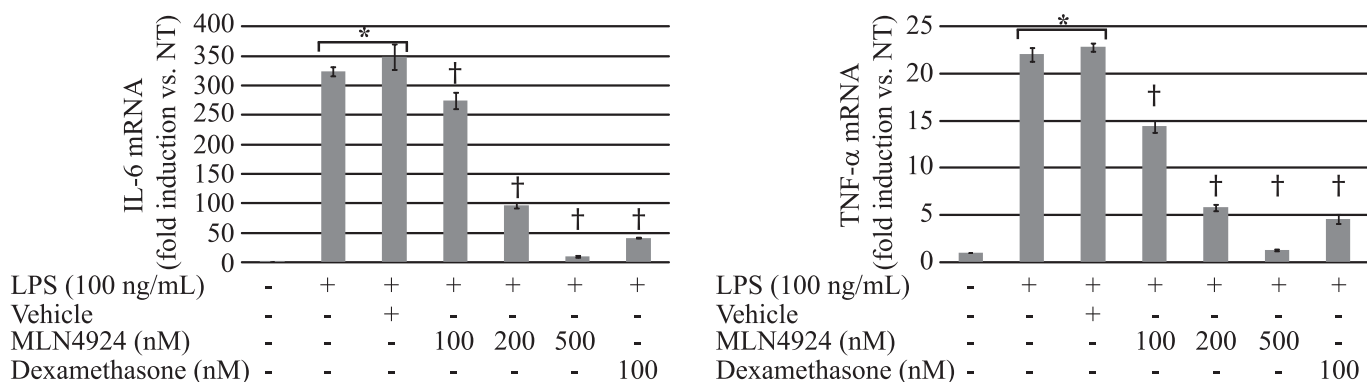


FIGURE 3. **MLN4924 prevents LPS-induced IL-6 and TNF- $\alpha$  mRNA expression in THP-1 macrophage-like cells.** RT-PCR analysis of IL-6 (left) and TNF- $\alpha$  (right) in THP-1 macrophages that were treated with LPS in the presence or absence of MLN4924 or Dex at the indicated doses for 6 h. Each bar indicates cytokine mRNA levels from THP-1 cells that were treated in triplicate, and data are represented as mean  $\pm$  S.D. (a representative experiment is shown;  $n > 3$ ). \*,  $p < 0.05$  when compared with nontreated cells; †,  $p < 0.05$  when compared with LPS-treated cells in the presence or absence of vehicle.

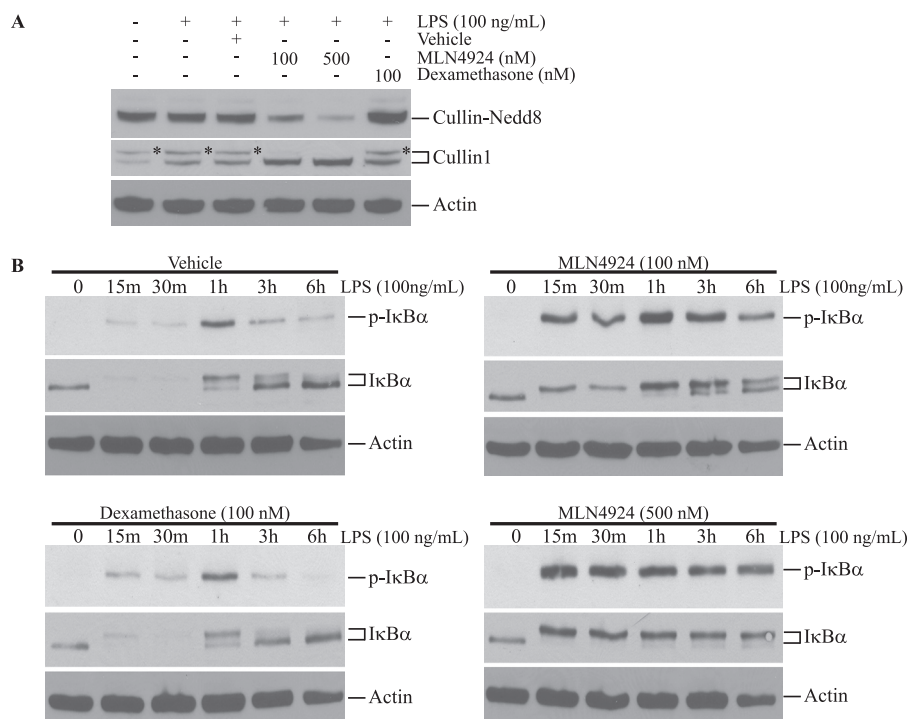


FIGURE 4. **MLN4924 and dexamethasone differentially affect neddylation and I $\kappa$ B degradation in THP-1 macrophage-like cells.** A, Western blot analyses of neddylated Cullin proteins (top panel) and Cullin1 protein (middle panel) using lysates of PMA-differentiated THP-1 cells that were treated with LPS in the presence or absence of MLN4924 or Dex at the indicated doses for 6 h. The asterisks are used to indicate neddylated Cullin1. B, Western blot analyses of phosphorylated I $\kappa$ B $\alpha$  protein levels (top panels) and total I $\kappa$ B $\alpha$  protein levels (middle panels) using lysates of THP-1 macrophage-like cells that were treated with LPS in the presence of vehicle (top left), MLN4924 (top and bottom right), or Dex (bottom left) for the indicated times. Actin protein levels were analyzed as a control for equal loading in both A and B (bottom panels).

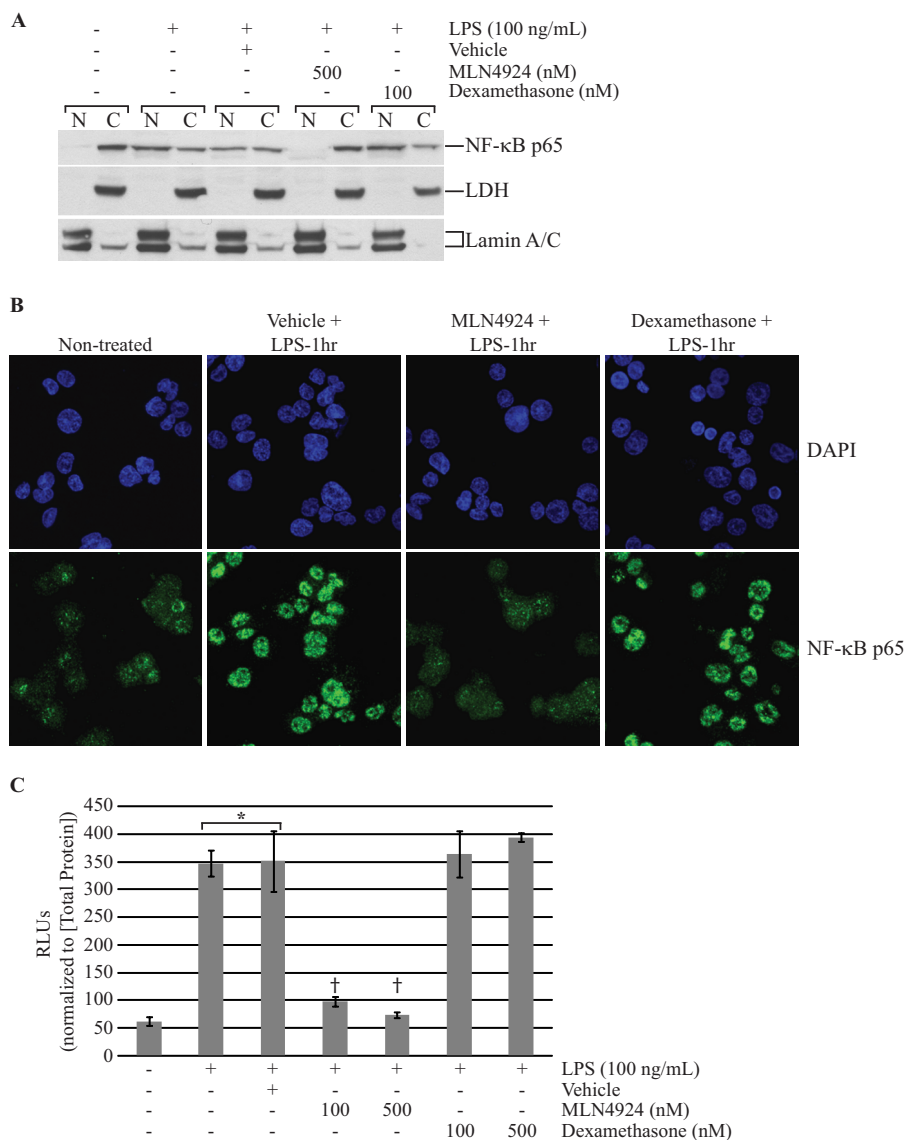
MLN4924 and Dex differentially affect neddylation of Cullin proteins and Cullin-mediated I $\kappa$ B degradation in macrophages.

**MLN4924 and Dexamethasone Differentially Affect NF- $\kappa$ B Nuclear Translocation and Transactivation in LPS-stimulated Macrophage Cells**—To investigate whether MLN4924 prevents LPS-induced NF- $\kappa$ B transcriptional activity, the effect of MLN4924 or Dex on LPS-induced p65 NF- $\kappa$ B nuclear translocation was first assessed by subcellular fractionation or immunocytochemistry in PMA-differentiated THP-1 macrophage-like cells. In nontreated cells, p65 NF- $\kappa$ B was predominantly localized in the cytosolic fraction; however, levels of p65 NF- $\kappa$ B increased in the nuclear fraction after LPS treatment for 30 min (Fig. 5A). This increase of p65 NF- $\kappa$ B protein in the nuclear

fraction was inhibited by pre-treating cells with MLN4924 (500 nM) but not with Dex (100 nM) (Fig. 5A), which are doses that inhibited LPS-induced up-regulation of IL-6 and TNF- $\alpha$  (Fig. 1A). Similarly, after LPS treatment for either 30 min (data not shown) or 1 h (Fig. 5B), prominent nuclear accumulation of p65 NF- $\kappa$ B could be detected by immunocytochemistry, whereas nontreated cells exhibited diffuse cytoplasmic p65 NF- $\kappa$ B staining with no appreciable nuclear accumulation of the protein (Fig. 5B). In THP-1 macrophage-like cells that were pre-treated with MLN4924 before exposure to LPS, p65 NF- $\kappa$ B staining was similar to nontreated cells (Fig. 5B). However, cells pre-treated with Dex and then stimulated with LPS had predominantly nuclear p65 NF- $\kappa$ B staining, similar to that of cells



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**FIGURE 5. MLN4924 and dexamethasone differentially affect NF- $\kappa$ B translocation and transcriptional activity in macrophage cells.** *A*, Western blot analyses of the p65 isoform of NF- $\kappa$ B using nuclear (N) and cytosolic (C) fractions of PMA-differentiated THP-1 cells that had been treated with LPS in the presence or absence of MLN4924 or Dex at the indicated doses for 30 min. Lactate dehydrogenase (LDH) and lamin A/C proteins were analyzed to assess the purity of cytosolic and nuclear fractions, respectively. *B*, immunocytochemistry analysis of p65 NF- $\kappa$ B localization (*bottom panels*) in THP-1 macrophage-like cells that were treated with 100 ng/ml of LPS in the presence or absence of 500 nM MLN4924 or 100 nM Dex for 1 h. Cell nuclei were stained using DAPI (*top panels*), and a representative image of two independent experiments is shown. *C*, quantification of luciferase activity using lysates from RAW 264.7 cells that were transfected with a NF- $\kappa$ B luciferase reporter plasmid and then treated with LPS in the presence or absence of MLN4924 or Dex at the indicated doses for 24 h. Each bar indicates luciferase values (that were normalized to total protein concentration) from cells that were treated in quadruplicate, and data are represented as the mean  $\pm$  S.D. A representative experiment is shown ( $n = 2$ ). \*,  $p < 0.05$  when compared with nontreated cells; †,  $p < 0.05$  when compared with LPS-treated cells in the presence or absence of vehicle. RLU, relative light unit.

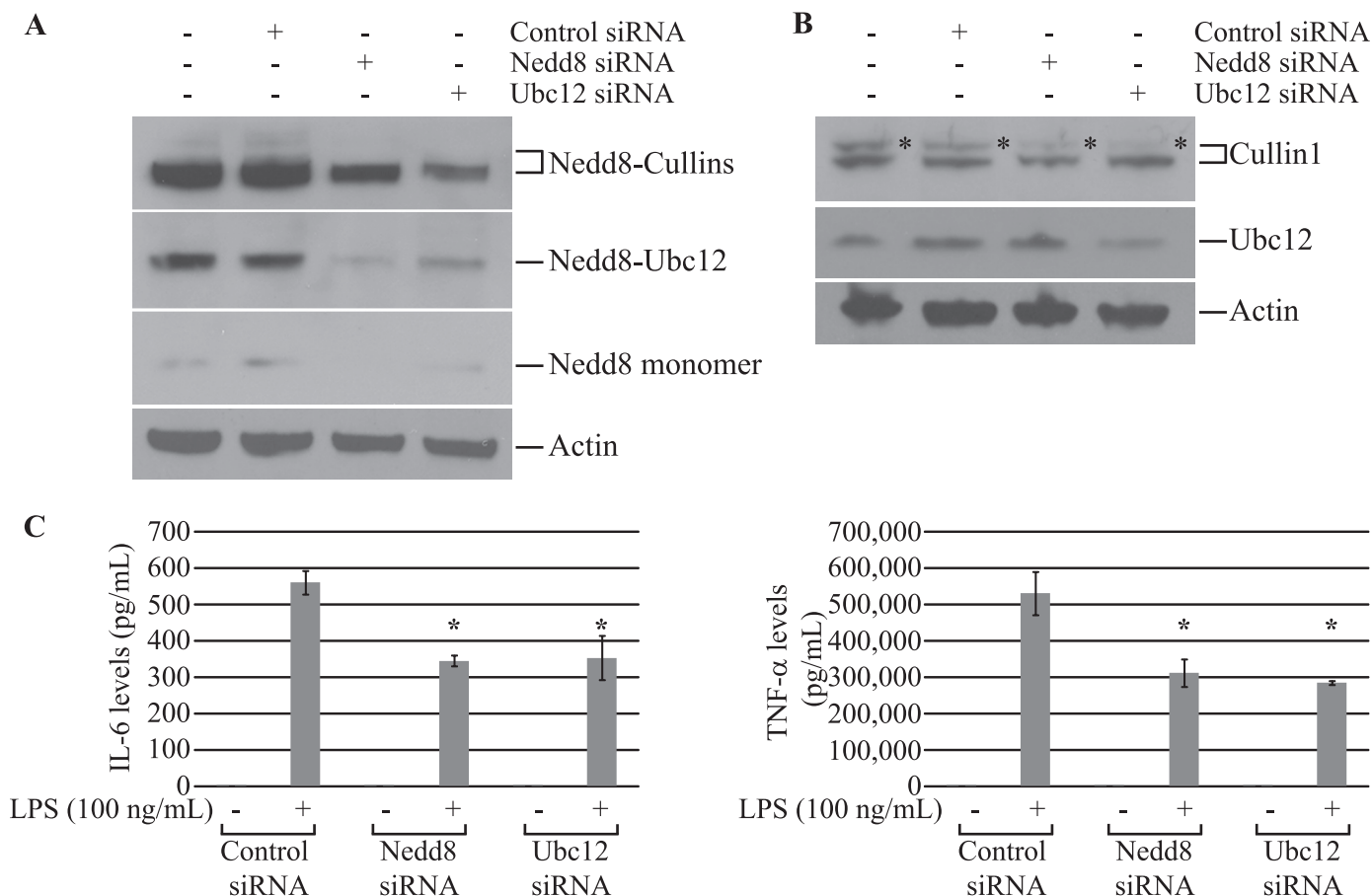
treated with LPS alone (Fig. 5B). Taken together, these data indicate that MLN4924, but not Dex, blocks LPS-induced nuclear accumulation of p65 NF- $\kappa$ B in macrophage cells.

Additionally, the effect of MLN4924 or Dex on LPS-induced NF- $\kappa$ B transcriptional activity was assessed in RAW 264.7 cells using a luciferase reporter under control of a promoter containing NF- $\kappa$ B transcription factor binding sites. Luciferase assays revealed that treating transfected cells with LPS caused a 3.7-fold increase in NF- $\kappa$ B transcriptional activity (Fig. 5C). Furthermore, this LPS-induced increase in NF- $\kappa$ B transcriptional activity was significantly inhibited by pre-treatment of cells with either 100 or 500 nM MLN4924 (Fig. 5C). In contrast, pre-treatment with either 100 or 500 nM Dex did not inhibit LPS-

induced NF- $\kappa$ B transcriptional activity in this assay (Fig. 5C), suggesting that MLN4924, but not Dex, inhibits LPS-induced NF- $\kappa$ B transcriptional activity in macrophage cells.

*Neddylation Is Crucial for LPS-induced Cytokine Production in Macrophage Cells*—The ability of MLN4924 to inhibit the effects of LPS suggests that neddylation is crucial for the effects of LPS-induced cytokine production in macrophage cells. To further examine this possibility, we used siRNA to deplete Nedd8 or the Nedd8-conjugating enzyme, Ubc12, in RAW 264.7 cells. As expected, siRNA targeting Nedd8 led to reduced levels of the Nedd8 monomer (Fig. 6A, *second panel from bottom*) and siRNA targeting Ubc12 led to reduced Ubc12 protein levels (Fig. 6B, *middle panel*). Furthermore, depletion of Nedd8

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**FIGURE 6. siRNA-mediated depletion of neddylation inhibits LPS-induced cytokine production in RAW 264.7 cells.** *A*, Western blot analysis of Nedd8 conjugated to Cullin proteins, Nedd8 conjugated to Ubc12, and monomeric Nedd8 using lysates from RAW 264.7 cells that were transfected with the indicated siRNAs (3  $\mu$ g). Actin protein levels were analyzed as a control for equal loading. A representative experiment is shown ( $n > 3$ ). *B*, Western blot analysis of Cullin1 and Ubc12 proteins as described in *A*. *C*, quantification of IL-6 (*left*) and TNF- $\alpha$  (*right*) cytokine levels from RAW 264.7 cells transfected with the indicated siRNAs. Each bar indicates cytokine levels from cells that were transfected with the indicated siRNA in triplicate, and data are represented as mean  $\pm$  S.D. (a representative experiment is shown;  $n > 3$ ). \*,  $p < 0.05$  when compared with control siRNA.

or Ubc12 led to a decrease in Nedd8-conjugated protein bands at  $\sim 90$  and 29 kDa, which correspond to neddylated Cullin and neddylated Ubc12 proteins, respectively (Fig. 6*A*, top two panels) (23–25, 34). In addition, siRNA-mediated depletion of Nedd8 or Ubc12 led to a decrease in neddylated Cullin1 (Fig. 6*B*, top panel) (35).

We next compared LPS stimulation of IL-6 and TNF- $\alpha$  cytokine levels in cells with normal neddylation to cells with depleted Nedd8 or Ubc12 expression. Both IL-6 and TNF- $\alpha$  cytokine levels were readily increased upon LPS treatment of RAW 264.7 cells with control siRNA (Fig. 6*C*); however, this LPS-induced increase of both IL-6 and TNF- $\alpha$  was significantly decreased (30–40%) in cells with siRNA targeting either Nedd8 or Ubc12 (Fig. 6*C*). These data indicate that the siRNA-mediated reduction in neddylation is correlated with the reduction in LPS-induced cytokine levels in macrophages and support the idea that the molecular process of neddylation is crucial for LPS-induced cytokine production in macrophage cells.

### DISCUSSION

Nedd8 and the process of neddylation represent a novel molecular target/process for the inhibition of LPS-induced

proinflammatory cytokine production in macrophages. Furthermore, the use of MLN4924, or other inhibitors of this molecular process, would represent a novel use for these pharmacological agents. MLN4924 is a sulfamated analog of AMP with an aminoindane group at position N6 of the deazapurine base (23), and a recent report has identified another neddylation inhibitor composed of a sulfamated AMP analog containing a *N*-hexyl group located at the same carbon position of the purine base (38). This C6 *N*-alkylated AMP analog inhibited neddylation at  $\sim 10$  nM, which is a similar inhibitory concentration to that of MLN4924 *in vitro* (23, 38). It would be interesting to determine whether this alkylated AMP analog also represses LPS-induced cytokine up-regulation in macrophage cells similarly to MLN4924.

Previous studies have focused on using MLN4924 to suppress the growth of tumor cell lines (*e.g.* myeloid leukemia cell lines, B-cell lymphoma cell lines, and the HCT-116 colon cancer cell line) either *in vitro* or in xenograft assays (23–25), and MLN4924 is currently being tested in Phase 1 clinical trials for both hematological and nonhematological tumors (22). MLN4924 has been reported to induce apoptosis through inhibition of NF- $\kappa$ B signaling or through induction of DNA re-replication via stabilization of the chromatin licensing and DNA

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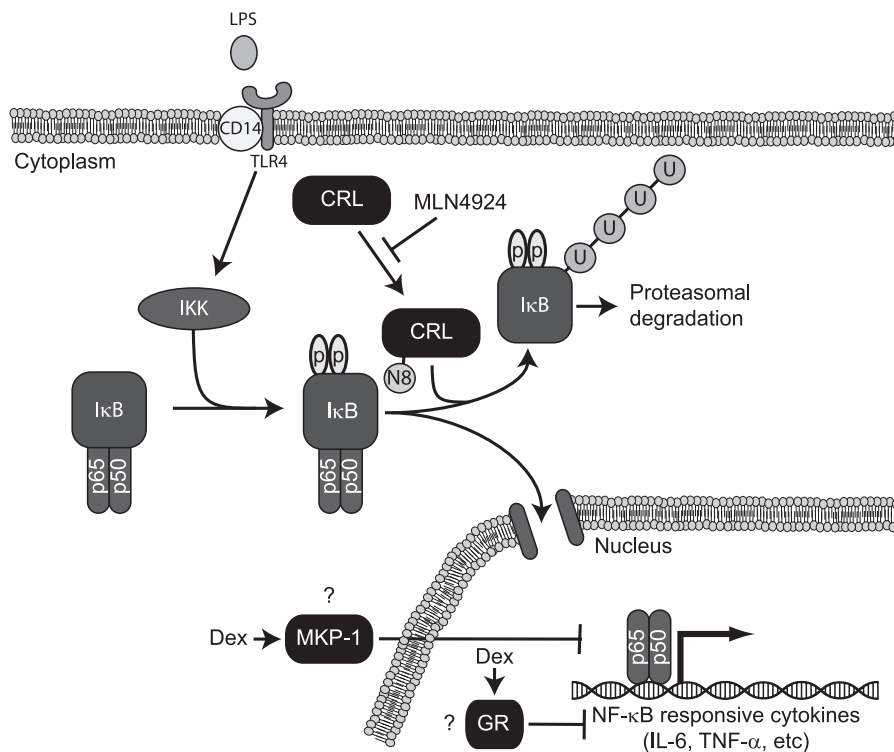


FIGURE 7. Schematic diagram showing the role that Nedd8, and the process of neddylation, plays during LPS-induced cytokine production.

replication factor 1 (Cdt1) protein (23–25, 39). MLN4924 has also been reported to induce cellular senescence in certain cancer cell lines in a p21-dependent manner (39, 40). However, our data indicates that MLN4924 did not have an effect on macrophage cell viability (Fig. 1, B and C), which corresponds to a previous study showing that MLN4924 had a minimal effect on viability of peripheral blood mononuclear cells (25). This is possibly due to macrophage cells being in a predominantly nonproliferative state, where the effects of MLN4924 on Cdt1 or p21 protein levels would not cause apoptosis or senescence.

Instead, we have shown that MLN4924, like the synthetic glucocorticoid Dex, represses LPS-induced TNF- $\alpha$  and IL-6 up-regulation when administered to macrophage cells in the nanomolar range; however, there were noticeable differences when comparing the ability of these two agents to repress LPS-induced cytokine up-regulation. Dex was a more potent inhibitor of LPS-induced cytokine up-regulation in human macrophages when compared with MLN4924, but MLN4924 inhibited LPS-induced cytokine up-regulation more potently in the mouse monocyte/macrophage cell line, RAW 264.7 (Figs. 1A and 2). One potential reason for these differences is that Dex differentially affects its molecular target(s) in human and mouse macrophage cells. Glucocorticoid receptor (GR), which is the primary molecular target of Dex, is comprised of at least five isoforms that originate from a single *Nr3c1* gene (41); these GR isoforms exist as part of a heterocomplex that is rearranged by ligand binding, thereby allowing GR nuclear import and transcriptional modulation of target genes (41). GR responsiveness to glucocorticoids can be modulated by mutations or polymorphisms in the *Nr3c1* gene, differential expression and/or processing of the *Nr3c1* gene, and alteration of the GR heterocomplex (41), and any of these regulatory points could exhibit

species-related differences. Alternatively, MLN4924 could differentially modulate neddylation in human and mouse cells, which would subsequently cause the differences in its ability to repress LPS-induced up-regulation of cytokines in different species.

Glucocorticoids have been postulated to block NF- $\kappa$ B signaling by transrepression of the NF- $\kappa$ B transcription factor (42, 43). However, we have shown that Dex had no detectable effect on LPS-induced NF- $\kappa$ B nuclear translocation in LPS-stimulated THP-1 macrophages or LPS-induced NF- $\kappa$ B transcriptional activation of a NF- $\kappa$ B reporter plasmid in RAW 264.7 cells (Fig. 5). In contrast, Dex was capable of inhibiting LPS-induced up-regulation of two proinflammatory cytokines, IL-6 and TNF- $\alpha$  (Figs. 1 and 2), that are known to be regulated by NF- $\kappa$ B in response to LPS treatment (29). Although the ability of Dex to repress LPS-induced up-regulation of IL-6 and TNF- $\alpha$  was less potent in RAW 264.7 cells than in human macrophages (Figs. 1A and 2), these data suggest that the ability of Dex to induce transrepression is potentially selective for certain gene promoters or it is dependent on additional factors in the promoter regions of target genes other than simply possessing NF- $\kappa$ B response elements. Dex could also inhibit LPS-induced proinflammatory cytokines in macrophages independently of transrepression by activating GR, because GR is the primary molecular target of Dex (43). Alternatively, Dex could function through the mitogen-activated protein kinase phosphatase 1 (MKP-1) protein to inhibit LPS-induced proinflammatory cytokines, because a previous study has shown that the anti-inflammatory effects of Dex were, at least partially, dependent on the expression of MKP-1 in macrophages (44) (Fig. 7).

Glucocorticoids have also been postulated to block NF- $\kappa$ B signaling by inducing I $\kappa$ B expression, thereby retaining NF- $\kappa$ B in the cytosol (42, 43). For example, a previous study has shown that Dex causes an increase in I $\kappa$ B $\alpha$  levels in macrophage cells, which led to an inhibition of LPS-induced down-regulation of I $\kappa$ B $\alpha$  (9). However, we did not observe this inhibition of LPS-induced down-regulation of I $\kappa$ B $\alpha$  in this study (Fig. 4B). One potential reason for this discrepancy is that Crinelli *et al.* (9) examined I $\kappa$ B $\alpha$  degradation at 5 and 12 h after stimulation with LPS in macrophages, whereas we analyzed I $\kappa$ B $\alpha$  levels at much earlier time points ranging from 15 min to 6 h after LPS exposure (Fig. 4B). Although we used different macrophage cell types compared with this previous study (primary human macrophages *versus* PMA-differentiated THP-1 and RAW 264.7 cells), our results indicate that most I $\kappa$ B $\alpha$  degradation in response to LPS treatment takes place within the first half-hour, and I $\kappa$ B $\alpha$  levels are recovered within 6 h after LPS treatment (Fig. 4B). This data are similar to that of other groups that have examined I $\kappa$ B $\alpha$  protein levels in response to LPS treatment (12).

In contrast to Dex, MLN4924 was capable of inhibiting LPS-induced decreases in I $\kappa$ B $\alpha$  protein levels and the subsequent NF- $\kappa$ B translocation and transcriptional activation (Figs. 4 and 5). The notion that MLN4924 represses LPS-induced cytokines through its ability to regulate neddylation and degradation of I $\kappa$ B is strengthened by the fact that siRNA-mediated depletion of Nedd8 or the Nedd8-conjugating enzyme, Ubc12, also represses LPS-induced up-regulation of IL-6 and TNF- $\alpha$  (Fig. 6), both of which are known to contain NF- $\kappa$ B response elements in the promoter regions of their genes (45, 46). Taken together, these data suggest to us that Dex and MLN4924 block LPS-induced cytokine up-regulation through independent molecular mechanisms in macrophage cells (Fig. 7). If this is the case, then it might be interesting to determine whether a combination of Dex and MLN4924 synergistically prevents cytokine up-regulation in response LPS or other pathogen-associated molecular patterns. In addition, targeting neddylation may prove to be an effective alternative in patients with resistance to Dex therapy, because neddylation seems to alter LPS responsiveness prior to or independent of the molecular effects of Dex (Fig. 7).

The inhibition of LPS-induced up-regulation of NF- $\kappa$ B-dependent cytokines by MLN4924 is similar to previous studies in which proteasome inhibitors (*e.g.* MG-132, ALLN, or lactacystin) were shown to repress LPS-induced I $\kappa$ B degradation and up-regulation of certain proinflammatory cytokines (47–49). However, these proteasome inhibitors, in addition to repressing NF- $\kappa$ B signaling in macrophages, were shown to activate the AP-1 transcription factor, presumably by blocking the degradation of interleukin-1 receptor-associated kinase-1 (47, 49). This activation of AP-1 was shown to contribute to LPS-induced cytokines. For example, lactacystin and MG-132 were shown to inhibit the increased TNF- $\alpha$  and IL-8 levels in response to LPS, enhance the ability of LPS to up-regulate IL-10, and to have no effect on LPS-induced IL-6 up-regulation (47). It will be interesting to examine the effects of MLN4924 on other proinflammatory cytokines besides IL-6 and TNF- $\alpha$  and to determine whether or not MLN4924 activates AP-1. How-

ever, we suspect that MLN4924 will not activate AP-1, because interleukin-1 receptor-associated kinase-1 degradation is promoted by its polyubiquitination, which has been reported to be facilitated by either the TNF-receptor associated factor 6 (TRAF6) E3 ligase or the Pellino E3 ligase (50–53). Both TRAF6 and Pellino exist in a multiprotein complex along with interleukin-1 receptor-associated kinase-1 in which the Pellino protein, not Cullin, serves as the scaffold (54). Thus, MLN4924 may be a more selective way of blocking LPS-induced degradation of I $\kappa$ B, and the subsequent increase in proinflammatory cytokines, when compared with proteasome inhibitors.

Last, MLN4924 or other drugs targeting neddylation could provide an alternative preventive or therapeutic strategy for diseases linked to a deregulated immune response, such as fibrosis, chronic liver disease, and liver cancer, metabolic disorders, or septic shock (3, 4, 55–57). Thus, further elucidation of the role that neddylation plays in the response to infectious agents both *in vitro* and *in vivo*, as well as in the development of chronic inflammatory diseases, will be of great importance.

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