The CATERPILLER Protein Monarch-1 Is an Antagonist of Toll-like Receptor-, Tumor Necrosis Factor α-, and Mycobacterium tuberculosis-induced Pro-inflammatory Signals

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Abstract

The CATERPILLER (CLR, also NOD and NLR) proteins share structural similarities with the nucleotide binding domain (NBD)-leucine-rich repeat (LRR) superfamily of plant disease-resistance (R) proteins and are emerging as important immune regulators in animals. CLR proteins contain NBD-LRR motifs and are linked to a limited number of distinct N-terminal domains including transactivation, CARD (caspase activation and recruitment), and pyrin domains (PyD). The CLR gene, Monarch-1/Pypaf7, is expressed by resting primary myeloid/monocytic cells, and its expression in these cells is reduced by Toll-like receptor (TLR) agonists tumor necrosis factor (TNF) α and Mycobacterium tuberculosis. Monarch-1 reduces NFκB activation by TLR-signaling molecules MyD88, IRAK-1 (type I interleukin-1 receptor-associated protein kinase), and TRAF6 (TNF receptor (TNFR)-associated factor) as well as TNFR signaling molecules TRAF2 and RIP1 but not the downstream NFκB subunit p65. This indicates that Monarch-1 is a negative regulator of both TLR and TNFR pathways. Reducing Monarch-1 expression with small interference RNA in myeloid/monocytic cells caused a dramatic increase in NFκB activation and cytokine expression in

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Plants have several classes of disease-resistant genes (R genes) that enable host defense to a wide variety of pathogens. A major class of R genes encodes proteins that contain an N-terminal Toll-like receptor (TLR)2/IL-1 receptor or leucine zipper domain followed by a nucleotide binding domain (NBD) and a C-terminal leucine-rich repeat (LRR). In mammals the class II transactivator and NOD1/NOD2 proteins were initially identified as proteins with a similar arrangement of NBD and LRR domains. More recently, our laboratory identified a 20+ member family of genes in humans that we called the CATERPILLER (CARD, transcription enhancer, r(purine) binding, pyrin, lots of leucine repeats) gene family (1). Subsequently, others described a similar family and designated it as NOD (2, 3), whereas others described a subgroup with a pyrin domain designated as the NALP, PAAD/PAN, and PYPAF families (3–6). By definition, all CLR proteins contain NBD-LRR motifs and are linked to a limited number of distinct N-terminal domains including transactivactation, CARD (caspase activation and recruitment), and pyrin domains (PyD) (1). A great majority of CLR proteins contain a PyD, originally defined as a domain within the PYRIN protein that has been linked to familial Mediterranean fever (MEFV) (7). The PyD has recently been characterized as a member of the death domain-fold superfamily (8). This report describes the function of one such PyD containing NBD-LRR protein, Monarch-1.

A strong clue that CLR proteins are likely to be critical regulators of the immune response, inflammation, and host response to pathogens is the genetic linkage of several CLR gene products to susceptibility to autoinflammatory and immunodeficiency disorders. For example, mutations in MHC2TA are linked to a severe immunodeficiency Bare Lymphocyte Syndrome (9); mutations in NOD2/CARD15 are associated with a subpopulation of patients with Crohn disease and Blau syndrome (10–13). Mutations in the CIAS1/Pypaf1/NALP3 gene are associated with a variety of clinical autoinflammatory syndromes, including familial cold autoinflammatory syndrome, chronic infantile neurological cutaneous and articular syndrome, or neonatal-onset multisystem inflammatory disease and Muckle-Wells syndrome (14–18). Their important role in bacterial infection is underscored by a number of recent studies showing that the NOD1 protein mediates recognition of a peptidoglycan derived primarily from Gram-negative bacteria (19, 20), whereas NOD2 mediates the recognition of muramyl dipeptide (19, 21). These findings support the provocative idea that this family of proteins constitutes
intracellular sensors of bacterial products and that mutations within these genes lead to a
dysregulated inflammatory response.

In addition to the role of CLR proteins as intracellular cytoplasmic mediators, TLRs in
mammals have rapidly emerged as predominant molecules by which the innate immune system
senses and responds to microbial pathogens (22, 23). There are 13 TLRs that recognize an array
of microbial products derived from bacteria, viruses, and fungi (24–26). TLR signal
transduction is initiated by stimulation followed by the formation of an intracellular signaling
complex with adapter proteins, the predominant one being MyD88 (27). An early step in TLR
signaling is the recruitment of the serine/threonine kinase, IRAK-1, to activated receptor
complexes. IRAK-1 activation is regulated by sequential phosphorylation events (28).
Hyperphosphorylation of IRAK-1 is important for TLR signal transduction as it results in a
decreased affinity for the TLR receptor complex and enables the association of IRAK-1 with
the TRAF6 complex, leading to activation of NFκB and its functional sequelae (29).

We have recently characterized a CLR gene designated as Monarch-1 (30), also known as
Pypa7 (6). Monarch-1 is expressed primarily by cells of the myeloid lineage, including
monocytes and granulocytes. This study shows that Monarch-1 interferes with IRAK-1
function, resulting in the repression of TLR signaling, and thus, represents a novel negative
regulator of inflammatory responses.

EXPERIMENTAL PROCEDURES

Reagents
The TLR2 agonist, the synthetic lipoprotein S-[2,3-bis-(palmitoyloxy)-(2-RS)-propyl]-N-
palmitoyl-(R)-Cys-(S)-Ser-Lys-4-OH trihydrochloride (Pam3Cys) (ECM) was used at a final
concentration of 100 ng/ml. Initially, lipopolysaccharide (LPS) derived from Escherichia
coli 026:B6 (Sigma-Aldrich) was used at a final concentration of 200 ng/ml; however, for most
of the experiments protein-free, phenol/water-extracted E. coli K235 LPS prepared by the
method of McIntire et al. (31) was used to preclude the contribution of non-TLR4 contaminants
that are often found in commercial LPS preparations (32).

Primary Cell Isolation and Stimulation
Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (American Red
Cross) using lymphocyte separation media (ICN, Costa Mesa, CA). For adherent cell
purification, cells were plated at 1 × 10^8 cells/150 mM plate and allowed to adhere for 2 h at
37 °C. Non-adherent cells were removed by washing three times with phosphate-buffered
saline and replaced with fresh media. Granulocytes were isolated from the red blood cell pellet
after lymphocyte separation media purification after ammonium, chloride, potassium buffer
red blood cell lysis. Granulocytes were plated at 2 × 10^7 cells/150 mM plate. Cells were
stimulated for 1 h with 200 ng/ml commercial LPS from E. coli (LPS 026:B6; Sigma-Aldrich)
or, where noted, protein free, phenol/water-extracted E. coli LPS K235 prepared as described
in McIntire et al. (31), 1 μg/ml commercial peptidoglycan (Sigma), or 100 ng/ml Pam3Cys
(ECM).
Expression Plasmids

The complete open reading frame of Monarch-1 was cloned into pcDNA-3 (Invitrogen) as described (26). Expression plasmids and reporter vectors included HA-MyD88 (33), HA-IRAK-1, HA-TRAF6 (34), 3×AP-1 luc (35), HA-TRAF2, Myc-RIP1, p65, NFκB luc (36), and ELAM-luciferase. Monarch-1 truncation mutants were amplified by PCR and cloned into the pcDNA3.1 V5/HIS vector following the manufacturer’s recommendations (Invitrogen).

Tissue Culture Cells and Conditions

HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal calf serum, 5 mM L-glutamine, and streptomycin-penicillin. Undifferentiated THP-1 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and streptomycin-penicillin. All cells were grown at 37 °C with 5% CO₂.

Luciferase Reporter Gene Assays

For transfections 2 × 10⁵ HEK293T cells were plated in 12-well plates followed by transfection using FuGENE 6 transfection reagent (Roche Applied Science) following the manufacturer’s recommended protocol. For analysis of TLR receptor signaling molecule induction of NFκB activity, cells were co-transfected with 250 ng of pNFκB-luc, 100, 250, or 500 ng of pcDNA3-HA vector, or HA-tagged Monarch-1, and 250 ng of HA-MyD88, FLAG-IRAK-1 HA-TRAF6, p65, HA-TRAF2, Myc-RIP1 or stimulated with 10 ng/ml TNFα to induce NFκB reporter activity. For IL-6 gene reporter luciferase assays, 1 × 10⁴ HEK293T cells were plated in 12-well plates and transfected with 10 ng of IL-6-luc and 10, 20, 50, and 100 ng of pcDNA3-HA vector or HA-tagged Monarch-1. DNA concentrations were consistent for each transfection. At 24 h post-transfection, cells were lysed in 1× reporter lysis buffer (Promega), and luciferase assays were performed per the manufacturer’s instructions.

TLR Reporter Assays

HEK293T cells (2 × 10⁵ cells/well in a 12-well plate) were co-transfected for 3 h with pFLAG-huTLR4 (7.5 ng/well), pCDNA3-huCD14 (300 ng/well), pEFBOS-HA-huMD-2 (3 ng/well), or Monarch-1 (0–500 ng/well) together with the NFκB reporter (500 ng/well) and pCMV1-βgal (100 ng/well). The final DNA quantity was adjusted to 1.5 μg/well, each with the pCDNA-HA vector using Super-Fect transfection reagent (Qiagen Inc.). Cells were recovered for 20 h, washed, and stimulated with phenol purified LPS at different concentrations for 5 h. Cells were lysed and β-galactosidase (Tropix, Galacto-Light system), and luciferase (Promega, luciferase assay system) activities were analyzed.

Transfection, Immunoprecipitation, and Western Analysis

HEK293T cells were transfected with the indicated plasmid cDNA using FuGENE 6. Cells were lysed in buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 8, 50 mM NaF, 2 mM EDTA, plus a protease inhibitor mixture (Roche Applied Science). Protein concentrations were determined by Bradford assay (Bio-Rad), and equivalent amounts of cellular extract were used in subsequent immunoprecipitations. Immunoprecipitates were
washed four times in lysis buffer and eluted by boiling in Laemmli sample buffer. Samples were fractionated by SDS-PAGE and transferred to nitrocellulose.

Immunoblots were probed with the indicated primary antibodies and visualized by enhanced chemiluminescence (Pierce): anti-HA 3F10 (Sigma), mouse monoclonal anti-HA 12CA5 and anti-HA3F10-HRP (Roche Applied Science), anti-IRAK-1 C-20 and anti-actin-HRP (Santa Cruz), or anti-FLAG M2-HRP (Sigma). Anti-V-5 was obtained from Invitrogen.

Generation of Antibodies Specific for Human Monarch-1 and Expression Analysis

Polyclonal antisera against human Monarch-1 was generated by immunization of rabbits with keyhole limpet hemocyanin-conjugated Monarch-1 peptide (RGQREDLVRDTPPGC). The IgG from the polyclonal antiserum was purified with protein G-Sepharose affinity chromatography utilizing the manufacturer’s protocols. A mouse monoclonal Monarch-1 antibody was generated from mice immunized with hexahistidine-tagged Monarch-1 (amino acids 1–593) expressed in E. coli and purified on nickel nitrilotriacetic-agarose (Qia-gen) using denaturing conditions described in the manufacturer’s protocol. THP-1 cells stimulated with purified LPS for the indicated time points were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) supplemented with Complete protease inhibitor mixture (Roche Applied Science). Protein concentrations were determined by the Bradford assay (Bio-Rad), and equivalent amounts of cellular extract were used in subsequent immunoprecipitations. Immunoprecipitation of Monarch-1 was accomplished utilizing the rabbit antiMonarch-1 peptide IgG. To control for immunoprecipitation efficiency and recovery, a rabbit polyclonal antibody for actin (I19, Santa Cruz) was included in the immunoprecipitation reaction. Monarch-1 and actin immune complexes were precipitated with protein A-agarose beads. Immunoprecipitates were washed 2 times in lysis buffer and eluted by boiling in 60 μl of Laemmli sample buffer. Samples were fractionated by SDS-PAGE and transferred to nitrocellulose. Immunoblots were probed with mouse monoclonal anti-Monarch-1 and HRP-conjugated anti-actin antibody (C11, Santa Cruz) and visualized by enhanced chemiluminescence (Pierce).

RNA Preparation and Real-time PCR

Total RNA was isolated according using Trizol reagent (Invitrogen) following the manufacturer’s protocol. Real-time PCR was performed with the TaqMan sequence detection system (Applied Biosystems): Monarch-1 forward 5′-AGAG-GACCTGGTGAGGGATAC-3′, reverse 5′-CTTCCAGAAGGCAT-GTTGAC-3′, probe 5′-CCCGTCCTCACTTGGGAACCA-3′, IL-6 forward 5′-GGTACATCTCAGGGCATCT-3′, reverse 5′-CTCTTTGCTGCTTTC-3′, probe 5′-TGTTACTCTTGTTACA-TGTCTCCTTTTCTCAGGGCT-3′; 18 S forward 5′-GCTGCCTGGCA-CCAGACTT-3′, reverse 5′-CGGCTACCACATCCAAGG-3′, probe 5′-CAAATTACCCACTCAGGACC-3′. All results are normalized to 18 S ribosomal RNA internal controls and are expressed in relative numbers.

Monarch-1-specific Small Interference RNA (siRNA) Construction

Wild-type and mutant human Monarch-1 short hairpin RNAs were stably expressed in the human THP-1 monocyte cell line by infection of the HSPG retrovirus containing short hairpin
RNA transcription cassettes driven by the H1 RNA promoter and a separate cassette containing green fluorescent protein driven by the phosphoglycerate kinase promoter. The targeted sequences are GTCCATGCTGGCACA-CAAG, and the mutant sequence is GTCCATGCTAACACACAAG (siRNA#1), and GGACATCAACTGTGAGAGG, and the mutant sequence is GGACATCGGCTGTGAGAGG (siRNA#2).

**Promoter-Reporter Retroviral Plasmids**

pHermes-κBluc was derived from pHRSpuro-GUS (a gift of H. Blau, Stanford University), a retroviral vector bearing a self-inactivating 3′ long terminal repeat (LTR) that lacks promoter and enhancer elements (37). A fragment of pGL3basic (Promega) bearing the transcription silencer, multicloning site, firefly luciferase-coding sequence was inserted into pHRSpuro-GUS. This yielded pHermes-luc. pHermes-κBluc was constructed by inserting the 5× NFκB response element and TATA box from pNFκBluc (Stratagene) into pHermes-luc. Transduction with pHermes-κBluc retrovirus results in the integration of a synthetic promoter-reporter gene into the host genome that has no retroviral LTR-driven transcription, because the self-inactivating 3′ LTR, which lacks promoter and enhancer elements, determines the sequences of both the 5′ and 3′ LTRs of the integrated retroviral genome. Reporter luciferase retroviral supernatants were generated as described and used for infection of THP-1 cells. Cells were stimulated for 6 h with LPS followed by luciferase assay (Promega) as per the manufacturer’s recommendations.

**Retroviral Infections**

The A293T cell line were co-transfected with pHSPG-667 (siRNA#1) or pHSPG-1504 (siRNA#2). Monarch-1-specific siRNA vectors or pHermes-κBluc and co-transfected with pVSV-G (38) and Pgag-pol (38) in 60-mm dishes. Retroviral supernatants (3 ml/well) were collected 48 and 72 h later, clarified by centrifugation, and used to infect 10^6 THP-1 cells by spinning for 3 h at 2500 rpm at 22 °C. Infection was repeated twice on successive days. For Monarch-1-specific siRNAs, THP-1 cells were purified to greater than 95% green fluorescent protein-positive using fluorescence-activated cell sorting and used in experiments as pools.

**ELISA**

THP-1 Monarch-1 siRNA cell populations were stimulated with purified K235 LPS at 200 ng/ml for 24 h followed by analysis of IL-6 protein expression. The IL-6 cytokine levels in cell supernatants were analyzed by sandwich ELISA using antibody pairs and protocols recommended by R&D Systems. The sensitivity of the assays was 16 pg/ml IL-6.

**Mycobacterium tuberculosis Infection**

THP-1 cells were infected with *M. tuberculosis* strain H37Rv. *M. tuberculosis* was grown to early log phase in Middlebrook 7H9 with oleic acid, albumin, dextrose, catalase media. Colony-forming units/ml were determined by plating on Middlebrook 7H10 with oleic acid, albumin, dextrose, catalase medium from actively growing cultures A = 2.5 × 10^8. Bacilli were washed in 0.5% Tween 80, phosphate-buffered saline. Briefly, 2 × 10^6 THP-1 cells were seeded in 6-well, flat-bottom cell culture plates 1 day before infection and incubated at 37 °C. Bacteria were added at a multiplicity of infection of 10 for THP-1 cells. Infection was evaluated by
measuring colony-forming units. THP-1 cells were collected at the time points indicated; RNA was made using Trizol reagent (Invitrogen) and evaluated for Monarch-1 or IL-6 expression by real-time PCR.

Cytokine Bead Array

THP-1 Monarch-1 siRNA#1 cell populations were stimulated with purified E. coli K235 LPS at 200 ng/ml, with Pam3Cys at 100 ng/ml or with TNFα at 20 ng/ml for 24 h followed by analysis of cytokine protein expression. The cytokine levels in cell supernatants were analyzed by Human Inflammation Cytokine Bead Array using antibody pairs and protocols recommended by R&D Systems.

RESULTS

Expression of Monarch-1 Is Decreased by TLR Signaling in PBMC

Monarch-1 is found primarily in cells of the myeloid and monocytic lineage (6, 30) that represent a first line of defense against invading microorganisms. These cell types can discriminate among different pathogens via the capacity of their TLRs to sense pathogen-associated molecular patterns (39). This causes a very rapid defensive response including the up-regulation of inflammatory and antimicrobial genes. Because of the highly restricted expression of Monarch-1 to cells of the innate immune system (30), we hypothesized that activation of cells via TLRs may influence the expression level of Monarch-1. To test this, human primary adherent PBMC or granulocytes were isolated and exposed to TLR agonists. After 1 h of treatment with LPS from E. coli, which activates TLR4, or peptidoglycan, which activates TLR2, Monarch-1 expression was significantly reduced in both cell types (Fig. 1, A and B). Because of the concern of contaminants in commercial preparations of TLR agonists, highly purified LPS was prepared by phenol purification (32). Pam3Cys, the pure synthetic agonist for TLR2, was used in concert to specifically activate TLR2. Analysis of Monarch-1 expression in granulocytes stimulated with phenol-purified LPS and synthetic Pam3Cys for 1 h confirmed that Monarch-1 expression is down-regulated after TLR activation (Fig. 1C).

Monarch-1 expression is down-regulated upon exposure of PBMC to TNFα (30). To compare the level of down-regulation to that observed for LPS, human PBMC were stimulated for 1 and 6 h with phenol LPS or TNFα. A similar reduction of Monarch-1 mRNA was observed at 1 h for all treatments (Fig. 1D). At 6 h post-stimulation Monarch-1 expression was further down-regulated by LPS and TNFα stimulation. This suggests that Monarch-1 is reduced to similar levels after stimulation of human PBMC with LPS or TNFα.

In the human monocytic cell line, THP-1, Monarch-1 expression was also down-regulated by exposure to purified LPS for 1 h, and a further reduction was observed at 3 h (Fig. 1E). Additionally, Monarch-1 protein level, measured by immunoblot analysis with antibodies raised against recombinant Monarch-1, was decreased in THP-1 cells after exposure to Pam3Cys over a 6-h time period with respect to untreated cells (Fig. 1F). It was necessary to immunoprecipitate Monarch-1 protein followed by immunoblotting, as endogenous Monarch-1 has been difficult to detect in cell lysates. Taken together these data indicate that Monarch-1 expression is down-regulated after exposure of cells to TLR agonists. This suggests
that down-regulation of Monarch-1 may be required for the normal defensive response to microorganisms.

**Monarch-1 Down-regulates TLR- and TNF-induced NFκB Activation**

The decrease in Monarch-1 expression after TLR and TNFα stimulation suggested that the down-regulation of Monarch-1 mRNA expression may be necessary for an antimicrobial response to occur. If this were the case, Monarch-1 would be predicted to exert inhibitory effects on steady-state cells and to initially dampen potentially overzealous pro-inflammatory activities that are activated by TLR agonists or by microbial infection. To test this hypothesis, we co-transfected the HEK293T epithelial cell line with expression vectors for TLR4, MD-2, and CD14 to reconstitute the TLR4 receptor complex. The ELAM-luciferase reporter, which can be activated by LPS-induced NFκB translocation, was co-transfected together with varying amounts of the Monarch-1 expression vector. Cells were treated with LPS, and ELAM-luciferase reporter activity was measured. Transfection of increasing amounts of Monarch-1 reduced LPS-stimulated ELAM reporter activation in a dose-dependent manner (Fig. 2A). The inhibitory effect of Monarch-1 was observed at both low and high concentrations of LPS but may be suboptimal because of the strong signal provided by over-expression of exogenous TLR4, MD-2, and CD14. To bypass the requirement for overexpression of all three components to reconstitute an LPS-responsive receptor complex, we examined the effect of Monarch-1 on the ability of the TLR signaling proteins MyD88, IRAK-1, and TRAF6 as well as the NFκB subunit p65 to transactivate the NFκB luciferase reporter. This reporter consists of three copies of the NFκB binding site linked to a luciferase gene. MyD88, IRAK-1, TRAF6, and p65 overexpression caused a dramatic induction of NFκB activation (Fig. 2B). Co-transfection of a Monarch-1 expression plasmid resulted in decreased NFκB activity induced by MyD88, IRAK-1, and TRAF6 but not by p65. This indicates Monarch-1 functions upstream of p65. In the experiments below we show that Monarch-1 interferes with IRAK-1 function. Another report3 shows that Monarch-1 also interferes with the downstream NFκB-inducing kinase, which explains the inhibition of TRAF6-activated NFκB by Monarch-1. This is consistent with the data in Fig. 2A, which measured the affect on an LPS-responsive receptor complex. These data indicate that the observed capacity of Monarch-1 to diminish TLR signaling is mediated through its effect on MyD88 and/or IRAK-1 and TRAF6 but not at the level of p65, resulting in reduced NFκB activity.

To determine whether the negative regulatory effect of Monarch-1 is restricted to the TLR pathway, we next sought to investigate if Monarch-1 could regulate the TNFR pathway. HEK293T cells were transfected with different amounts of Monarch-1 expression vector and either stimulated with TNFα or co-transfected with expression vectors for TNFα signaling molecules, TRAF2 or RIP1 (Fig. 2C). Overexpression of Monarch-1 resulted in decreased NFκB activity induced by TNFα, TRAF2, or RIP1. Monarch-1 did not suppress the activation of an HLA-DR luciferase reporter, suggesting that the effects are specific. These data suggest that Monarch-1 down-regulates both TLR and TNFR pathways.

3 J. Lich, submitted for publication.
Monarch-1 Associates with IRAK-1 and Down-regulates IRAK-1 Activation

One mechanism by which Monarch-1 could reduce TLR signaling protein-induced NFκB activity may be through its association with the signaling complex. It is currently believed that once MyD88 is recruited to the TLR4 complex, and it in turn recruits IRAK-4 to a region between the MyD88 TIR and death domain and IRAK-1 to its death domain (40). IRAK-4 is believed to phosphorylate IRAK-1, leading to autophosphorylation and hyperphosphorylation of IRAK-1 (41). To investigate the mechanism of reduced TLR signaling by Monarch-1, HEK293T cells were co-transfected with expression vectors for Monarch-1 and the TLR signaling proteins MyD88 or IRAK-1 followed by immunoprecipitation. Immunoprecipitation of FLAG-tagged IRAK-1 was performed by using anti-FLAG antibody followed by immunoblotting for HA-tagged Monarch-1 using an anti-HA antibody. Monarch-1 was found to co-precipitate with IRAK-1, indicating that these proteins interact in situ (Fig. 3A). In contrast, no association was detected between Monarch-1 and MyD88 in experiments in which HA-tagged MyD88 was immunoprecipitated using anti-HA antibody, and immunoblots were probed with anti-FLAG antibody to detect FLAG-tagged Monarch-1 (Fig. 3B). Upon IRAK-1 overexpression, both native and phosphorylated forms of IRAK-1 could be visualized by Western analysis, providing a convenient surrogate for IRAK-1 activation in the absence of extracellular stimuli (Fig. 3C, lane 3) (28). Because IRAK-1 activation is regulated by sequential phosphorylation events (28), it is possible to distinguish between the species that is first phosphorylated by IRAK4 (resulting in the appearance of a single band that migrates more slowly than native IRAK-1 during SDS-PAGE) (40, 41) and the hyperphosphorylated species that is the consequence of autophosphorylation (28). The appearance of hyperphosphorylated IRAK-1 was nearly eliminated by the presence of Monarch-1, suggesting inhibition of IRAK-1 autophosphorylation (Fig. 3C, lane 2, band III). In contrast, the faster migrating protein bands (Fig. 3C, bands I and II, respectively) corresponding to both native and IRAK4-phosphorylated IRAK-1 were not affected, demonstrating specificity for the inhibition of hyperphosphorylation.

Presence of the NBD Domain Is Associated with Reduced IRAK-1 Activation

Common to all CLR proteins, Monarch-1 consists of three distinct domains. To determine the domain(s) responsible for IRAK-1 association, truncation mutants were constructed that consisted of the N-terminal pyrin domain and the C-terminal LRR domain alone or in combination with the NBD. The analysis of NBD alone was not possible because this domain appeared to be very unstable in the absence of either PyD or LRR (not shown). These truncation mutants (shown in Fig. 4A) were transfected along with recombinant IRAK-1 into HEK293T cells. Protein complexes were then immunoprecipitated with anti-IRAK-1-specific antibodies. These experiments revealed that IRAK-1 association is dependent upon the presence of the NBD region in the presence of either PyD or LRR. Both PyD-NBD and NBD-LRR interacted with IRAK-1 (Fig. 4C). Neither the pyrin domain (Fig. 4B) nor the LRR region (Fig. 4C) alone formed molecular complexes with over-expressed IRAK-1. Incongruent with this, the analysis of IRAK-1 in cellular lysate demonstrated that the reduction of IRAK-1 hyperphosphorylation (Form III) was most dramatic in the presence of the NBD-LRR mutant followed by the PyD-NBD mutants (Fig. 4D).
Monarch-1 Associates with IRAK-1 and Prevents Its Activation in THP-1 Cells

The previous experiment utilized HEK293T cells. To determine whether Monarch-1 negatively regulates IRAK-1 activation in a more physiologically relevant system, human monocytic THP-1 cells that stably expressed HA-tagged Monarch-1 (THP-Mon) were produced (Fig. 5). Endogenous IRAK-1 containing protein complexes were immunoprecipitated with an IRAK-1-specific antibody, and Western blots were probed with anti-HA to detect co-precipitating Monarch-1. Complex formation between IRAK-1 and Monarch-1 was induced after Pam3Cys treatment (Fig. 5B, top panel, lanes 2–4). In contrast, no Monarch-1 was detected in IRAK-1 immunoprecipitates derived from resting cells, indicating a dependence upon TLR signaling (Fig. 5B, top panel, lane 1). Notably, when these Western blots were re-probed to detect immunoprecipitated IRAK-1, sharply reduced levels of hyperphosphorylated IRAK-1 were detected in THP-Mon cells as compared with their empty vector controls (compare the second panel, Fig. 5, A and B). This supports the data derived from transient transfection experiments in HEK293T cells and confirms a role for Monarch-1 in reducing the accumulation of hyperphosphorylated IRAK-1 in monocytes. These results demonstrate that Monarch-1 associates with IRAK-1 upon TLR stimulation and inhibits the accumulation of hyper-phosphorylated IRAK-1. Together these data provide a mechanism by which Monarch-1 inhibits TLR induced NFκB activation.

Endogenous Monarch-1 Functions as a Negative Regulator in Myeloid Cells

The observation that Monarch-1 expression is rapidly decreased after TLR stimulation and that Monarch-1 is a negative regulator of inflammatory gene activity suggest that Monarch-1 may be involved in reducing the inflammatory response to pathogens. Specifically, it may be necessary for innate immune cells to reduce Monarch-1 expression in response to pathogens to allow for an increase in inflammatory gene induction. If this hypothesis were correct, the ablation of Monarch-1 expression in myeloid cells would be predicted to result in increased TLR-induced activation signals. To investigate this, Monarch-1-specific siRNAs were generated to reduce endogenous Monarch-1 expression in the THP-1 myeloid cell line. Because of the difficulty in transfecting human myeloid/monocytic cell lines in general and THP-1 cells specifically, a retroviral based vector was used to express Monarch-1-specific siRNAs (Fig. 6A). Two sets of siRNAs specific for distinct regions of the Monarch-1 transcript (designated as siRNA#1 and siRNA#2) were utilized to control for potential nonspecific affects of siRNA. As further controls, corresponding mutant siRNA with two mutated nucleotides (designated as mutMon#1 and mutMon#2) were generated to ensure that the observed effects of siRNA are specific. Monarch-1 short hairpin RNAs were inserted into the pHSPG retroviral vector. Transcription of siRNAs were driven by the H1 RNA gene promoter situated immediately upstream of the insertion site in the 3’ LTR. A phosphoglycerate kinase promoter that drives transcription of the enhanced green fluorescent protein permitted transduced cells to be separated by fluorescence-activated cell sorting. Therefore, the THP-1 monocytic cell line was transduced with the siRNA-containing constructs and their corresponding controls. Sorted enhanced green fluorescent protein-positive cells were maintained in bulk culture and used to investigate endogenous Monarch-1 function. Expression of Monarch-1 in the four cell samples were assessed by real-time PCR (Fig. 6B). Compared with their controls, both siRNA#1 and siRNA#2 achieved greater than 70% reduction in endogenous Monarch-1.
expression. To assess the level of NFκB activity in the Monarch-1 siRNA cells, the siRNA cells and their mutant controls were transduced with a retrovirus that contained the luciferase reporter driven by the NFκB-responsive promoter element 5× NFκB response element and TATA box from pNFκBluc (Stratagene). Cells were stimulated with phenol-purified LPS derived from E. coli K235 for 6 h followed by analysis of NFκB luciferase activity. If Monarch-1 were a negative regulator of pro-inflammatory signaling, ablation of the negative regulatory activity of Monarch-1 would result in an increase in NFκB activity. Indeed, reduced Monarch-1 expression achieved by the siRNA resulted in a >1000-fold increase in NFκB luciferase reporter activity compared with the control mutant siRNA (Fig. 6C). Basal levels of NFκB reporter activity were higher in the Monarch-1 siRNA cells than in their mutant control cells, suggesting that endogenous Monarch-1 functions to suppress NFκB activity in the absence of stimulation. Monarch-1 siRNA#2 cells stimulated with LPS had increased NFκB activity, although the levels were less dramatic (data not shown). These data suggest that endogenous Monarch-1 functions to dampen NFκB activation.

Monarch-1 Is a Negative Regulator of IL-6 Cytokine Expression in Response to TLR Agonists

The experiment shown above supports the previous hypothesis that Monarch-1 functions as a negative regulator of TLR4-induced NFκB activity as assessed by an NFκB reporter assay. To further investigate the function of Monarch-1 in mediating the inflammatory response to pathogens, we tested whether endogenous Monarch-1 could regulate the IL-6 gene induced by TLR activation. Both siRNA#1 and siRNA#2 THP-1 cells were stimulated with E. coli K235 LPS for 6 h followed by analysis of IL-6 mRNA expression by real-time PCR. Stimulation of both siRNA#1 and siRNA#2 (Mon-1) THP-1 cells resulted in a 4-fold increase in IL-6 mRNA expression compared with mutant controls (mutMon-1) (Fig. 7A). To investigate if the increased IL-6 expression were also observed at the protein level, supernatants from LPS-stimulated Monarch-1 siRNA#1 and siRNA#2 THP-1 cells were analyzed for IL-6 cytokine expression by ELISA. Consistent with the mRNA data, both Monarch-1 siRNA-containing lines exhibited increased IL-6 protein expression compared with their mutant controls (Fig. 7B). These data suggest that endogenous Monarch-1 functions as a negative regulator of IL-6 cytokine expression in response to TLR stimulation.

Monarch-1 Is a Negative Regulator of IL-6 Cytokine Expression in Response to M. tuberculosis Infection

The above observation was obtained with a pure TLR agonist. To assess if this observation could be extended to a clinically relevant live human pathogen, cells were infected with M. tuberculosis. M. tuberculosis, the causative agent of human tuberculosis, is the most prevalent and deadly bacterial infectious disease worldwide and affects one-third of the world population (42). Incomplete understanding of the molecular nature of protective immune responses has hampered the development of more effective vaccines and therapies. M. tuberculosis leads to the induction of inflammatory cytokines through the activation of TLRs (43). To determine whether exposure of cells to M. tuberculosis could regulate Monarch-1 expression, THP-1 cells were infected with the virulent H37Rv strain of M. tuberculosis for the indicated time points (Fig. 8A). Monarch-1 expression was reduced after exposure of cells to live M. tuberculosis for 2 h, and the expression was nearly abolished after 4 h of exposure. This further
suggests that down-regulation of Monarch-1 may be required for the normal defensive response to microorganisms. Because IL-6 is an important response of the innate immune cells to infection by *M. tuberculosis* (43), we determined if exposure of Monarch-1 siRNA cells to live bacilli would also result in increased IL-6 cytokine expression. Monarch-1 siRNA THP-1 cells were infected with the virulent *M. tuberculosis* followed by analysis of IL-6 cytokine expression by real-time PCR (Fig. 8B). The bacilli caused a dramatic enhancement of IL-6 production, but cells containing siRNA#1 or siRNA#2 had much higher expression of IL-6 cytokine compared with both wild-type THP-1 cells and the mutant control siRNA.

**Monarch-1 Is a Negative Regulator of TLR2-, TLR4- and TNFR-mediated Cytokine Secretion**

To investigate more broadly the function of Monarch-1 in mediating the inflammatory response to TLR agonists as well as TNFα, we investigated whether endogenous Monarch-1 could regulate pro-inflammatory genes in addition to IL-6. We also investigated if Monarch-1 could mediate inflammatory responses by other TLR agonists. Taking into consideration that Monarch-1 inhibits IRAK-1 function, a downstream signaling protein common to both the TLR2 and TLR4 pathway, we stimulated THP-1 Monarch-1 siRNA cells with *E. coli K235* LPS (TLR4) and with the synthetic TLR2 agonist, Pam3Cys, in three separate experiments. To investigate the role of endogenous Monarch-1 in regulating the TNFR pathway, we also stimulated these cells with TNFα. Stimulation by Pam3Cys in general resulted in higher cytokine levels than that observed after LPS stimulation. Supernatant from stimulated cells was analyzed using the Human Inflammation Cytokine Bead Array (Bio-Rad). In Monarch-1 siRNA cells we observed increased levels of IL-6, IL-1β, TNFα and a slight increase in IL-8 when compared with mutMon-1 control cells (Fig. 9A) upon stimulation with Pam3Cys. Among LPS-treated Monarch-1 siRNA containing cells, an increase in IL-6 and IL-1β was observed. TNFα and IL-8 were expressed at similar levels in Monarch-1 siRNA cells compared with the mutant control (Fig. 9B). However, TNFα production in LPS-treated THP-1 cells was low, and the data should not be over-interpreted. Finally, in response to TNFα, Monarch-1 siRNA cells produced higher levels of IL-1β with a modest increase in IL-8 compared with control cells (Fig. 9C). These data support the observation that Monarch-1 broadly down-regulates TLR signaling by a TLR2 agonist. It also caused a more specific down-regulation of IL-6 and IL-1β by a TLR4 agonist. These findings also support the observation that Monarch-1 is a negative regulator of the TNFR receptor pathway. In a separate set of experiments using microarray analysis we determined that other genes were not affected by Monarch-1 including lymphotoxin α, lymphotoxin β, TGFβ1, CCL1 (data not shown). Taken together, these date imply that Monarch-1 is a broad inhibitor of both TLR and TNFR signal transduction pathways.

**DISCUSSION**

The inflammatory response to infection represents a two-edged sword, whereas a proper pro-inflammatory response is necessary to contain infectious microorganisms and foreign antigens, an overzealous response is detrimental to the host (44). The impact of the latter is clearly demonstrated by the 660,000 cases of sepsis per year with a 20% mortality rate (45). Although an array of positive regulators of inflammation has been discovered, relatively few negative feedback modulators have been identified. The SOCS (suppressor of cytokine signaling)
molecules represent a major class of negative regulators that are generally induced by stimulants, including TLR agonists (44). Like SOCS, IRAK-M is also induced by TLR agonists. Studies using the inactive kinase IRAK-M-deficient mice reveal that IRAK-M is a negative regulator of TLR signaling and acts by inhibiting the dissociation of IRAK-1/IRAK-4 from the TLR4/MyD88 complex, resulting in blocking of downstream NFκB activation (46). An alternatively spliced form of MyD88, called MyD88s, binds to TLRs but cannot interact with IRAK-4. As a result, phosphorylation of IRAK-1 does not occur, and NFκB activation is abrogated (40, 47). The cytokine-inducible zinc finger protein called A20 and A20-like proteins including ZNF216 and the zinc finger protein TIZ have been found to inhibit IL-1/TLR4-mediated activation of NFκB (48). Recently, several CLR proteins are shown to negatively affect cell activation pathways (49–51). In this report we show that Monarch-1 is also a negative regulator of pro-inflammatory responses. However, it is the first to exhibit an inhibitory effect on the TLR signaling pathway induced by MyD88/IRAK-1/TRAF6 and specifically on IRAK-1 activation.

The conclusion that Monarch-1 is a CLR protein with an anti-inflammatory function is most convincingly demonstrated by the use of siRNA in myeloid/monocytic cells. Monarch-1 is a negative regulator of both the TLR and TNFR pathways. Because Monarch-1 is the first CLR protein that interferes with both TLR2 and TLR4 activation, we explored the molecular mechanism by which Monarch-1 interferes with this activation. We show that Monarch-1 blocks TLR signaling by association with IRAK-1 and inhibition of IRAK-1 hyperphosphorylation. IRAK-1 activation is regulated by sequential phosphorylation events involving the initial phosphorylation by IRAK4 and subsequent hyperphosphorylation by IRAK-1 itself. The hyperphosphorylation of IRAK-1 is important for TLR signal transduction because it serves to decrease affinity for the TLR receptor complex, which is required for TRAF6 binding and subsequent downstream signal transduction (28). Our results show that Monarch-1 clearly interferes with the hyperphosphorylation of IRAK-1. Consistent with these findings, mitigated IRAK-1 hyperphosphorylation has been observed in endotoxin-tolerant cells, where LPS exposure no longer stimulates NFκB activation (52, 53). This is thought to be secondary to a failure to recruit MyD88 to the TLR4 receptor complex (53) and the subsequent failure to recruit IRAK-1 appropriately to MyD88 (52). In addition, an inhibitory splice variant of MyD88 has been shown to block phosphorylation of IRAK-1, leading to the inhibition of LPS-induced NFκB activation (40). Thus, phosphorylation of IRAK-1 is emerging as a critical regulatory step in the control of TLR signaling and Monarch-1 is an inhibitor of this important pathway.

An emerging theme among a subgroup of CLRs is that they exhibit negative regulatory function. Among these, CLR16.2 is unique in its down-regulation of T cell receptor-mediated cellular activation of NFκB, AP-1, and NFAT (49). PAN1 down-regulates NFκB and the expression of NFκB regulated genes in a monocytic cell line (50). PYPAF3 down-regulates LPS-stimulated IL-1/β production (51). Both the PAN1 study and the present work relied on the reduction of endogenous CLR by siRNA. Thus, these two reports provide powerful evidence that a subgroup of CLRs represents negative regulators of immune and inflammatory activation.
In conclusion, this work supports the hypothesis that Monarch-1 normally functions as a negative regulator of inflammatory activity in resting monocytic/myeloid cells, but its expression is down-regulated by TLR agonists, TNFα, or whole pathogens. This down-regulation is necessary for proper inflammatory and anti-microbial responses to proceed. The down-regulation of Monarch-1 expression is not specific to M. tuberculosis as we have also observed a decrease of Monarch-1 expression in cells infected with other pathogens (data not shown). Monarch-1 down-regulates LPS-induced NFκB reporter activity in cells co-transfected with TLR4 and in cells co-transfected with the TLR/IL-1 receptor signaling mediators MyD88, IRAK-1, and TRAF6. This inhibition is not specific to the TLR pathway as Monarch-1 also inhibits TNFR signaling mediators such as TRAF2 and RIP1. Monarch-1 is likely to inhibit multiple pathways, although a focus on the TLR pathway in this report shows that it interferes with IRAK-1 hyperphosphorylation. Inhibition of Monarch-1 by siRNA in cell types that naturally express it significantly altered NFκB activation and proinflammatory cytokine synthesis in response to TLR agonists, TNFα, and virulent M. tuberculosis. A greater understanding of the ability of Monarch-1 to modulate the immune response to pathogens including M. tuberculosis will enhance the development of more effective vaccines and therapies. Taken together these data provide compelling evidence that Monarch-1 is a novel negative regulator of TLR- and pathogen-mediated pro-inflammatory gene induction through its interaction with IRAK-1 and its inhibition of IRAK-1 hyperphosphorylation.

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FIGURE 1. TLR activation reduces Monarch-1 expression

A, Monarch-1 expression in human adherent peripheral blood cells stimulated for 1 h with commercial LPS (TLR4 agonist) or commercial peptidoglycan (PGN) (TLR2 agonist). Seven separate cell preparations were used. B, Monarch-1 expression in human peripheral blood granulocytes stimulated with commercial LPS (TLR4 agonist) or commercial peptidoglycan (TLR2 agonist). Three separate cell preparations were used. C, Monarch-1 expression in human peripheral blood granulocytes stimulated with purified phenol LPS (TLR4 agonist) or synthetic Pam3Cys (TLR2 agonist). Two separate cell preparations were used. D, Monarch-1 expression in human adherent peripheral blood cells stimulated with purified phenol LPS (TLR4 agonist) or TNFα for 1 h (black bars) and 6 h (white bars). Data are representative of two separate experiments. E, Monarch-1 expression in the human THP-1 monocyte cell line stimulated with phenol-purified LPS for the indicated time points. Data are the average of three separate experiments. Monarch-1 expression was normalized to the expression of 18 S rRNA and are represented as -fold difference compared with control. Error bars represent the S.E. of separate experiments. F, Monarch-1 protein expression in the human...
THP-1 monocyte cell line stimulated with Pam3Cys for the indicated time points. Lysates were immuno-precipitated (IP) with rabbit anti-Monarch-1 and rat anti-actin followed by immunoblotting (IB) with either mouse anti-Monarch-1 or goat anti-actin-HRP antibodies. Data are representative of two separate experiments.
FIGURE 2. Monarch-1 blocks signaling molecules in the TLR and TNFR pathways

A, HEK293T cells were co-transfected with TLR4, CD14, MD-2, different amounts of Monarch-1, and an ELAM-luciferase reporter construct. After 24 h cells were stimulated with the indicated concentration of purified *E. coli* K235 LPS for 5 h. Results represent the mean ± S.E. of three separate experiments. B, HEK293T cells were co-transfected with either vector alone or expression plasmids for MyD88, IRAK-1, TRAF6, or P65 with/without three concentrations of Monarch-1, and an NFκB luciferase reporter construct. The results represent the mean ± S.E. of three separate experiments. C, HEK293T cells were co-transfected with either vector alone or expression plasmids for TRAF2 or RIP1 or stimulated for 8 h with TNFα with/without three concentrations of Monarch-1 and an NFκB luciferase reporter construct. As a control, cells were co-transfected with an expression plasmid for class II transactivator (*CIITA*) and an HLA-DR luciferase reporter construct plus either vector alone or the highest concentration of Monarch-1 plasmid. The results are representative of four separate experiments.
FIGURE 3. Monarch-1 associates with IRAK-1 and limits the accumulation of hyperphosphorylated forms of the kinase

A, HEK293T cells were transfected with the indicated plasmid DNA. Twenty-four hours later whole cell lysates (WCL) were prepared, and FLAG-IRAK-1-containing protein complexes were immunoprecipitated (IP) with anti-FLAG (Fg) M2-agarose beads and separated by SDS-PAGE. Western blots (IB) were probed with anti-HA (Ha) antibody to detect co-precipitating HA-Monarch-1. p-, phosphorylated. B, cells were transfected with empty vector or expression vectors for FLAG-tagged Monarch-1 and HA-MyD88. MyD88-containing protein complexes were immunoprecipitated with an anti-HA antibody and separated by SDS-PAGE. Western blots were probed with anti-FLAG to detect co-precipitating FLAG-Monarch-1. C, cells were transfected with the indicated plasmid DNA, and 24 h later whole cell lysates were prepared and separated by SDS-PAGE. Western blots were probed with the indicated antibodies. The hyperphosphorylated form of IRAK-1 (marked as III) was greatly reduced by the presence of Monarch-1.
FIGURE 4. The Monarch-1 NBD is required for the association and regulation of IRAK-1 hyperphosphorylation

A, diagram depicting V5-tagged Monarch-1 truncation mutants. B, cells were transfected with expression vectors encoding the V5-tagged PyD of Monarch-1 and HA-IRAK-1. IRAK-1-containing protein complexes were immunoprecipitated (IP) with an anti-IRAK-1 antibody and separated by SDS-PAGE. Western blots (IB) were probed with anti-V5 to detect co-precipitating V5-PyD. C, cells were transfected with expression vectors encoding the indicated V5-tagged truncation mutant of Monarch-1 in addition to HA-IRAK-1. IRAK-1-containing protein complexes were immunoprecipitated with an anti-IRAK-1 antibody and separated by SDS-PAGE. Western blots were probed with anti-V5 to detect co-precipitating V5-tagged Monarch-1 truncation mutants. D, whole cell lysates derived from experiments described in A and B were separated by SDS-PAGE and probed with an anti-IRAK-1 antibody. The hyperphosphorylated form of IRAK-1 (marked as III) was greatly reduced in the presence of the NBD-LRR-containing truncation mutant of Monarch-1.
FIGURE 5. Monarch-1 associates with endogenous IRAK-1 in THP-1 monocytic cells upon TLR stimulation and limits the accumulation of hyperphosphorylated, endogenous IRAK-1

THP-1 cells stably expressing (A) empty vector (THP-HA-EV) or (B) HA-tagged Monarch-1 (THP-HA-Mon) were stimulated with the TLR2 agonist Pam3Cys4 for the indicated times. Endogenous IRAK-1-containing protein complexes were immunoprecipitated (IP) with an anti-IRAK-1 antibody and then fractionated by SDS-PAGE. Western blots were probed with anti-HA to detect co-precipitating HA-Monarch-1 (top panels). These immunoblots (IB) were then stripped and probed with anti-IRAK-1 antibodies (second panels). The hyperphosphorylated form of IRAK-1 was greatly reduced by the presence of Monarch-1. Interestingly, analysis of cellular lysates indicated that the disappearance of the 80-kDa nonphosphorylated form of IRAK-1 occurs more slowly in the presence of Monarch-1. Actin immunoblots were performed to ensure equivalent levels of protein were used among the samples. p-, phosphorylated.

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FIGURE 6. Monarch-1 is a negative regulator of NFκB activity in the THP-1 monocytic cell line
A, plasmid map of Monarch-1 siRNA in the pHSPG retroviral vector. SiRNAs specific for Monarch-1 were developed to block Monarch-1 expression followed by analysis of gene activity. Monarch-1 siRNAs, driven by the H1 RNA promoter, were inserted into the pHSPG retroviral vector within the 3′ LTR. The presence of a phosphoglycerate kinase (PGK) promoter driving enhanced green fluorescent protein (eGFP) allows for selection of transduced cells by fluorescence-activated cell sorting. Two separate Monarch-1 siRNAs and their corresponding mutant, controls were placed in pHSPG vectors. B, analysis of Monarch-1 expression in
Monarch-1 (Mon-1) and control mutant (mutMon-1) siRNA bulk monocyte THP-1 cultures as determined by real-time PCR. THP-1 cells were infected with two different Monarch-1-specific siRNAs, siRNA#1 and siRNA#2, respectively. Expression was normalized to the expression of 18 S mRNA and are represented as -fold over mutant control. The results represent the mean ± S.E. of five separate experiments. C, analysis of NFκB activity in Monarch-1-specific siRNA cultures. Monarch-1 (Mon-1) and control mutant (mutMon-1) siRNA cultures were infected with a retroviral based NFκB luciferase reporter. Cultures were then stimulated with E. coli K235 LPS followed by analysis of NFκB luciferase activity. Expression is represented as -fold over mutant control. Results represent the mean ± S.E. of three separate experiments.
FIGURE 7. Monarch-1 is a negative regulator of IL-6 in monocytic THP-1 cells induced by LPS

Analysis of IL-6 mRNA and protein expression in Monarch-1-specific siRNA cultures. A, two different Monarch-1 (Mon-1) and control mutant (mut-Mon-1) siRNA cultures, siRNA#1 and siRNA#2, were stimulated with E. coli K235 LPS followed by analysis of IL-6 transcript expression by real-time PCR. IL-6 expression was normalized to 18 S RNA and are represented as exponential numbers. Data are a representative of five separate experiments. B, two different Monarch-1 (Mon-1) and control mutant (mutMon-1) siRNA cultures, siRNA#1 and siRNA#2, were stimulated with E. coli K235 LPS followed by analysis of IL-6 cytokine expression by ELISA. Data are representative of three separate experiments. ND, not determined.
FIGURE 8. Monarch-1 is a negative regulator of IL-6 in the THP-1 monocytic cell line infected with virulent *M. tuberculosis*

A, *Monarch-1* transcript expression in the human THP-1 monocytic cell line infected with virulent *M. tuberculosis* (*Mt*) for the indicated time points. Data represent the average of three separate experiments measured by real-time PCR. B, analysis of IL-6 mRNA expression in *Monarch-1*-specific siRNA cultures. THP-1 cells were compared with *Monarch-1* (*Mon-1*) and control mutant (*mut-Mon-1*) siRNA cultures after infection with *M. tuberculosis* followed by analysis of IL-6 expression by real-time PCR. Expression was normalized to 18 S RNA, and data are shown as -fold over unstimulated control. Data are representative of three separate experiments.
FIGURE 9. Monarch-1 is a negative regulator of cytokine induction by TLR4 and TLR2 agonists as well as the TNFR pathway.

Monarch-1 (Mon-1) and control mutant (mutMon-1) siRNA cultures were stimulated for 24 h with Pam3Cys (TLR2 agonist) (A), E. coli K235 LPS (TLR4 agonist) (B), or TNFα (C) followed by analysis of cytokine expression by cytokine bead assay. Results represent the mean ± S.E. of three separate experiments.