NOD2 pathway activation by MDP or *Mycobacterium tuberculosis* infection involves the stable polyubiquitination of Rip2

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SUMMARY

The Rip2 kinase contains a caspase recruitment domain (CARD) and has been implicated in the activation of the transcriptional factor NF-κB downstream of Toll-like receptors, Nod-like receptors and the T cell receptor. Although Rip2 has been linked to Nod signaling, how Nod-Rip2 proteins mediate NF-κB activation has remained unclear. We find Rip2 required for Nod2-mediated NF-κB activation and to a lesser extent MAP kinase activation. We demonstrate that Rip2 and IKK-γ become stably polyubiquitinated upon treatment of cells with the NOD2 ligand, muramyl dipeptide. We also demonstrate a requirement for the E2 conjugating enzyme Ubc13, the E3 ubiquitin ligase Traf6 and the ubiquitin activated kinase Tak1 in Nod2-mediated NF-κB activation. Rip2 polyubiquitination is also stimulated when macrophages are infected with live Mycobacterium tuberculosis (Mtb), but not when infected with heat killed bacteria. Consistent with our data linking Rip2 to NOD and not Toll-like receptor signaling, Mtb-induced Rip2 polyubiquitination appears MyD88 independent. Collectively, these data reveal that the NOD2 pathway is ubiquitin regulated and that Rip2 employs a ubiquitin-dependent mechanism to achieve NF-κB activation.

INTRODUCTION

Conserved structures on pathogens are recognized by specific host receptors such as Toll-like receptors (TLRs) or nucleotide binding oligomerization domain (NOD)-like receptors (NLRs). In contrast to TLRs which recognize pathogens at the cell surface or within the endosome, NLRs induce innate immune responses by recognizing bacterial products released into the cytosol. Several NLRs, including Nod1 and Nod2 proteins, induce cytokine production by activating the transcription factor NF-κB and by stimulating MAP kinase activation (1). The Nod1 protein recognizes a form of peptidoglycan (PGN) containing the amino acid meso-diaminopimelic acid (IE-DAP) produced by Gram-negative and some Gram-positive bacteria (2). The Nod2 pathway recognizes muramyl dipeptide (MDP) present on most types of PGN (3). Transfection studies have shown that expression of Nod proteins stimulates NF-κB activity and the ability to stimulate NF-κB reporter activity is dependent on the co-expression of the adapter protein Rip2 (4). Yet how Rip2 proteins achieve Nod-mediated NF-κB or MAP kinase activation remains undefined.

Rip2 is a member of the Rip protein family of which there are currently 7 members (5). Like the prototype Rip1, Rip2 contains an N-terminal serine threonine kinase domain followed by an intermediate region and a C-terminal caspase recruitment domain (CARD). Overexpression of Rip2 stimulates NF-κB activation and induces apoptosis and expression of kinase inactive Rip2 activates an NF-κB reporter, suggesting that the kinase activity of Rip2 may not be required for NF-κB activation upon stimulation of the Nod pathway (6). To elucidate function, several groups generated Rip2-deficient mice and stimulated Rip2-deficient macrophages with TLR and NLR ligands. Consistent with our data linking Rip2 to NOD and not Toll-like receptor signaling, Mtb-induced Rip2 polyubiquitination appears MyD88 independent. Collectively, these data reveal that the NOD2 pathway is ubiquitin regulated and that Rip2 employs a ubiquitin-dependent mechanism to achieve NF-κB activation.

We found Rip2 required for Nod2 pathway activation in macrophages, however we did not find Rip2 required for TLR 2, 3 or 4-mediated IKK activation. Similar data have recently been published by Gabriel Nunez and colleagues who find Rip2 required for cytokine production induced through NLRs, but not TLRs (9). We show that treatment of macrophages with the Nod2 ligand MDP stimulates the ubiquitin modification of endogenous Rip2 and IKK-γ. The ubiquitin modification of Rip2 is stable and involves the
conjugation of K63-linked polyubiquitin chains. MDP-induced Rip2 polyubiquitination and NF-κB activation requires the E2 conjugating enzyme Ubc13, the E3 ubiquitin ligase Traf6 and the ubiquitin activated kinase Tak1.

The Nod2 pathway has been implicated in the innate immune response to *Mycobacterium tuberculosis* (Mtbc) infection, as Nod2-deficient macrophages are impaired in their cytokine response to infection with Mtbc (10). Moreover, a quantitatively small fraction of the transcriptional changes induced by Mtbc infection of macrophages can be attributed to TLR2/4- or MyD88-dependent pathways (11). Collectively, these studies suggest nonredundant roles for NOD2 and TLR in the innate immune recognition of Mtbc. We find that infection with live, but not heat killed, virulent strains of Mtbc stimulates Rip2 polyubiquitination. Therefore, we hypothesize that Nod proteins recognize Mtbc components that are translocated into the cytosol and consistent with this model, Rip2 polyubiquitination induced by live Mtbc infection appears MyD88 independent, implicating NLRs in the cytosolic recognition of Mtbc.

**EXPERIMENTAL PROCEDURES**

*Isolation of Macrophages and Mycobacterium tuberculosis infection.* To isolate peritoneal activated macrophages, wild type and rip2-/- mice (gift of Dr. Vishva M. Dixit, Genentech, Inc. South San Francisco, California) were injected with 3ml of thioglycolate by intraperitoneal injection. Three days later, macrophages were isolated by gentle flushing of the peritoneal cavity. Peritoneal macrophages were left unstimulated or treated with MDP or purified LPS (Sigma). NF-κB activation was measured using phospho-IκBα antibodies (Cell Signaling Technology), p38 MAP kinase and JNK1/2 activity was measured using anti-phospho-p38 and phospho-JNK1/2 antibodies (Cell Signaling Technology), respectively. Bone marrow derived macrophages for *Mycobacterium tuberculosis* (Mtbc) infection were harvested from wild type or MyD88-deficient mice as described in (12). Mtbc (strain H37Rv) was cultivated in 7H9 broth to exponential phase and washed thoroughly in phosphate buffered saline prior to infection. Bacterial clumps were removed by passing the washed suspension through a 5µM syringe filter. “Heat-killed” bacteria were inactivated by heating to 80°C for 30 minutes. Macrophages were infected for 1 or 2 hours after which filtered cell lysates were immunoprecipitated with anti-Rip2 antibody (Santa Cruz) or immunoblotted with anti-phospho-IκB-α antibody (Cell Signaling Technology).

*Plasmids and Constructs.* The full-length Rip2 constructs was provided by Dr. Vishva M. Dixit (Genentech, South San Francisco, California). The wild type NOD2 construct has been described previously (13). The HA-ubiquitin, HA-K48-only ubiquitin and HA-K63-only ubiquitin constructs were generously provided by Dr. Zhijan James Chen (University of Texas Southwestern, Dallas, TX).

*Antibodies, siRNA and Reagents.* Anti-Rip2 (Rabbit), anti-Nemo (Rabbit), anti-Traf2 (Rabbit), anti-Traf6 (Mouse), anti-Ubiquitin (Mouse), anti-Omnin (Rabbit), anti-Tak1 (Rabbit), anti-p38 (Goat) and anti-JNK (rabbit) antibody were obtained from Santa Cruz Biotechnology. Anti-phospho-IκB-α, anti-phospho-p38 and anti-phospho-JNK antibodies were obtained from Cell Signaling Technology. Anti-UBC13 antibody was obtained from Zymed, anti-FLAG, and anti-β-actin antibody were obtained from Sigma and anti-HA antibody was obtained from Boehringer Mannheim. Human Traf2 and Traf6 siRNA and mouse Tak1 siRNA were obtained from Santa Cruz Biotechnology. MDP was obtained from InvivoGen. Mouse TNF-α were obtained from Sigma. LPS derived from *Escherichia coli* strain 0111.B4 was purchased from Sigma, dissolved in deoxycholate, and re-extracted with phenol/chloroform as described in (14).

*Cell Culture, DNA transfection and SiRNA transfection.* HEK293T cells and the mouse
RAW 264.7 macrophage cell lines were obtained from ATCC and were grown in DMEM containing 10% FBS. For all transfection studies, HEK293T cells were plated at 5x10^5 cells/plate on 60mm plates. Constructs were transfected to HEK293T cells using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. siRNAs were transfected to HEK293T cells using Lipofectamine 2000 (Invitrogen). Mouse control and Tak1 specific siRNAs were transiently transfected into the mouse RAW 264.7 macrophage cell line using a two step transfection procedure and Lipofectamine 2000 (Invitrogen).

**Generation of Ubc13 stably knock down RAW264.7 cell lines.** The mouse macrophage cell line RAW264.7 was infected with lentiviruses containing shRNA sequences specific for Ubc13 (Open Biosystems) and selected with 2.5 µg/ml puromycin. Selected puromycin resistant pools of cells were left unstimulated or treated with MDP and Rip2 polyubiquitination assayed by immunoprecipitation with anti-Rip2 antibody (Santa Cruz) followed by immunoblotting with anti-ubiquitin antibodies. The expression of the E2 conjugating enzyme Ubc13 in the lentiviral infected pools was determined by immunoblotting with an anti-Ubc13 antibody (Zymed).

**Immunoprecipitation and Western Blot Analysis.** For all immunoprecipitation and ubiquitination assays, cell lysates were prepared in radioimmune precipitation assay (RIPA) buffer (150mM NaCl, 50mM Tris-HCl (pH 7.5), 1% NP40, 0.25% deoxycholate, 0.1% SDS, 1mM EDTA), supplemented with a protease mixture inhibitor (Roche Applied Science) and 5mM N-ethylmaleimide (Sigma), immunoprecipitated with anti-Rip2 or anti-NEMO antibody (Santa Cruz), Polyubiquitinated Rip2 or NEMO/IKK-γ proteins were detected by immunoblotting with an anti-ubiquitin antibody (Santa Cruz). To examine endogenous protein interactions, cells were lysed in an endogenous lysis buffer (0.5 M Tris (pH 7.6), 0.5 M NaCl, 0.1 M EDTA, 1% Triton X-100, 0.5 M NaF, and 0.5 M sodium pyrophosphate) supplemented with a protease mixture inhibitor (Roche Applied Science), immunoprecipitated with anti-Rip2 antibody, and then immunoblotted with either anti-Tak1 or anti-Traf6 antibody (Santa Cruz Biotechnology). Expression of epitope-tagged, transfected proteins or endogenous proteins was detected by immunoblotting with corresponding antibodies.

**RESULTS**

The CARD domain kinase Rip2 has been implicated in signaling from multiple TLRs including TLR 2, 3 and 4, the T cell receptor and the cytosolic NOD1/2 pathways. To understand how Rip2 mediates such diverse signals, we isolated macrophages from Rip2-deficient mice and stimulated the cells with the NOD2 ligand muramyl dipeptide (MDP) or with purified LPS to stimulate the TLR4 pathway. We found that MDP and LPS induced NF-κB, p38 MAP kinase and JNK1/2 activation when wild type macrophages were stimulated (Figure 1A). Rip2-deficient macrophages also respond upon LPS stimulation, but appear impaired in their ability to mediate NF-κB activation when treated with MDP (Figure 1A). Similarly, the MDP-induced phospho-p38 and phospho-JNK1/2 activity was consistently impaired but not abrogated, suggesting that Rip2 contributes to MAP kinase activation but is not absolutely required (Figure 1A and 1B). This data suggests that Nod-mediated MAP kinase activation may involve other CARD domain containing adapter proteins. One possibility may be CARD9, as Card9-deficient mice exhibit defects in p38 MAP kinase and JNK activation in Nod stimulated cells.

We hypothesized that Nod mediated IKK activation may involve the ubiquitin modification of Rip2 or IKK-γ/NEMO and tested this possibility by stimulating the mouse macrophage cell line RAW 264.7 with the Nod2
ligand MDP and assayed for the presence of polyubiquitinated Rip2 or NEMO/IKK-γ. We observed a ligand-dependent induction of Rip2 and NEMO/IKK-γ polyubiquitination and interestingly, Rip2 polyubiquitination appears to precede the ubiquitin modification of NEMO/IKK-γ. Nod2 pathway activation by MDP appears to induce the stable modification of Rip2 and IKK-γ, suggesting that Rip2 and IKK-γ may be conjugated by K63-linked polyubiquitin chains. To test this possibility, HEK293 cells were transfected with epitope-tagged versions of Rip2 and NOD2 in the presence of wild type or mutant forms of ubiquitin where only the lysine(s) at positions 63 or 48 are available for conjugation. In this setting, Rip2 appeared preferentially modified by K63-linked polyubiquitin chains and this activity appeared dependent on the presence of NOD2 (Figure 2B). These data suggest that activation of the NOD2 pathway stimulates the stable K63-linked polyubiquitination of Rip2.

Our studies suggest that NOD2-induced NF-κB activation may be mediated by the ubiquitin conjugation of Rip2 and IKK-γ and the transfection studies suggest that K63-linked polyubiquitin chains may stably modify both proteins. Therefore, we were interested in testing whether MDP-induced NF-κB activation and Rip2/IKK-γ polyubiquitination require expression of the E2 conjugating enzyme Ubc13, known to conjugate K63-linked polyubiquitin chains. To address a requirement for Ubc13, we infected the MDP responsive mouse macrophage cell line RAW 264.7 with lentiviruses expressing shRNAs specific for the Ubc13 enzyme. These stable pools of RAW 264.7 macrophages were impaired in their ability to phosphorylate IκBα in response to MDP or TNF-α (data not shown). Rip2 polyubiquitination was detected in RAW 264.7 macrophages expressing a control shRNA, however, treatment with the Nod2 ligand MDP failed to stimulate Rip2 polyubiquitination when the Ubc13-deficient cells were stimulated (Figure 3A and not shown). The level of Rip2 polyubiquitination and MDP-induced NF-κB activation correlated with the reduction of Ubc13 expression achieved in the RAW 264.7 pools.

In transfected cells, Rip2 has been shown to interact with Traf2, 5 and 6 proteins (6), suggesting that one of these Traf proteins may modify Rip2 upon Nod2 pathway stimulation. To identify the E3 ubiquitin ligase responsible for the modification of Rip2, we transfected HEK293 cells with epitope-tagged versions of NOD2 and Rip2 and tested whether MDP- or NOD2-dependent Rip2 polyubiquitination is observed in cells where Traf2 or Traf6 expression was reduced. In HEK293 cells transfected with NOD2 and stimulated with MDP, Rip2 polyubiquitination was detected, as expected (Figure 3B). Similarly, in cells where Traf2 expression is reduced via transfection of siRNAs specific for Traf2, MDP stimulated Rip2 polyubiquitination and this modification appeared NOD2-dependent. In contrast, Rip2 polyubiquitination was reduced in MDP-stimulated HEK293 cells in which Traf6 expression was reduced (Figure 3B), thereby implicating Traf6 as a potential Rip2 E3 ubiquitin ligase in the Nod2 pathway. We also examined whether MDP or NOD2 pathway activation stimulates an association between Rip2 and Traf6. Using co-immunoprecipitation, MDP treatment stimulated interaction between endogenous Rip2 and Traf6 between 30 and 60 min following MDP treatment. Consistently, polyubiquitinated Rip2 proteins are detected at similar time periods following MDP treatment (Figure 2A).

To further test a requirement for Traf6, we attempted to knock down the expression of Traf6 in the RAW 264.7 mouse macrophage cell line and then stimulated the cells with IL-1 or MDP. As expected, no IL-1-induced NF-κB activation is detected in the Traf6 knocked down cells, however, the control shRNA treated cells activate NF-κB upon IL-1 treatment. MDP-induced NF-κB activation is clearly impaired in cells where Traf6 expression is...
reduced, suggesting that Traf6 is required for optimal NF-κB responses to MDP (Figure 3D). These data are consistent with a model whereby Traf6 modifies Rip2 upon activation of the Nod2 pathway, raising the possibility that this enzyme may be targeted in human inflammatory disease.

Our data suggest that Rip2 polyubiquitination may contribute to NOD2-mediated NF-κB activation by recruiting the ubiquitin activated kinase Tak1. Therefore, we tested whether Rip2 interacts with Tak1 and whether MDP stimulates an endogenous Rip2/Tak1 interaction. A Rip2/Tak1 interaction could be readily demonstrated in transfected cells (Figure 4A) and an endogenous Rip2/Tak1 interaction was detected following MDP treatment (Figure 4B), during a time period when a Rip2/Traf6 interaction was also observed (Figure 3C). Collectively, these data suggest that MDP stimulation or NOD2 pathway activation stimulates the formation of a Rip2/Ubc13/Traf6/Tak1 complex that results in the stable ubiquitin modification of Rip2.

These data predict that MDP-induced NF-κB activation will be dependent on the expression of the ubiquitin-dependent kinase Tak1. We reduced Tak1 expression level in the RAW 264.7 macrophage cell line and tested its effects on MDP- and TNF-induced NF-κB activation. Consistent with the transfection studies, NF-κB activation was reduced in both the MDP- and TNF-stimulated Tak1 ‘silenced’ cells, whereas it remained unaffected when the control cells were stimulated with either MDP or TNF-α (Figure 4C). These data led us to propose that Tak1 is critical for NOD2 signaling to NF-κB. Thus, stimulation of the NOD2 pathway results in the recruitment and subsequent ubiquitin modification of Rip2 by an Ubc13/Uev1a and Traf6 complex. Polyubiquitinated Rip2 then signals the recruitment and activation of the Tak1 kinase.

Although MDP stimulates NOD2-mediated NF-κB activation and mutations in the CARD15 gene are associated with human inflammatory disease, MDP has not, as yet, been shown to bind to the NOD2 protein (15). Hence, we were interested in testing whether infection with live bacteria stimulates the ubiquitin modification of Rip2. The cytosolic NOD2 pathway has been implicated in the cytokine response to Mycobacterium tuberculosis (Mtb) infection in vitro and recent studies reveal important, nonredundant roles for NOD2 and TLRs in vivo in the recognition of Mtb (16,17). To test whether Mtb infection stimulates Rip2 polyubiquitination, we infected bone marrow derived macrophages with a virulent strain of Mtb (H37Rv) or with a heat-killed bacteria and assayed for polyubiquitinated Rip2 proteins at 1 and 4 hours post infection. Infection with live Mtb stimulated Rip2 polyubiquitination, whereas heat killing destroyed the ability of Mtb to induce the modification of Rip2 (Figure 5A).

TLR2/4 have been implicated in the innate immune recognition of Mtb and published studies place Rip2 downstream of TLR2/4 (7,8,18). Thus, it was important to test whether Mtb-induced Rip2 polyubiquitination was TLR-dependent. To test this idea, we isolated wild type and MyD88-deficient macrophages and left the cells uninfected or infected them with Mtb. Rip2 polyubiquitination was observed in Mtb-infected wild type and MyD88-deficient macrophages, suggesting that Mtb recognition is TLR independent (Figure 5B). We also examined Rip2 polyubiquitination in macrophages lacking both TLR2 and TLR4 to directly test the contribution of the TLR4, MyD88 independent pathway, but observed no differences in the ability of Mtb infection to stimulate Rip2 polyubiquitination (Figure 5B). Although it remains unclear precisely how the cytosolic NOD2 pathway is activated by Mtb infection, several observations suggest that the Mycobacterial phagosome may be permeable to some compounds (19-21), suggesting a mechanism whereby Mtb products are released...
into the cytosol where they stimulate Rip2 recruitment and polyubiquitination.

**DISCUSSION**

Our work demonstrates that Rip2 primarily mediates NF-κB activation but is not required for MAP kinase activation in MDP-stimulated peritoneal macrophages. MDP treatment of macrophages or infection with the human pathogen *Mycobacterium tuberculosis* stimulates Rip2 polyubiquitination, suggesting that Rip2 polyubiquitination may be required for NF-κB activation by NLRs. Our work also implicates the E2 conjugating enzyme Ubc13, the E3 ubiquitin ligase Traf6 and the ubiquitin-activated kinase Tak1 in Nod2-mediated NF-κB activation. In collaboration with Abbott and Cantley, we also recently showed that IKK-γ is ubiquitin modified at lysines 285 and 399 in NOD2 transfected cells (22). These transfection studies are limited however, as the NOD2 pathway is activated in these studies not by ligand or by bacterial infection, but rather by NOD2 or Rip2 protein overexpression. In the work herein, macrophages are stimulated with ligand (MDP) or infected with the intracellular pathogen *Mtb* and the endogenous Rip2 and IKK-γ proteins examined for evidence of polyubiquitination. In the work herein, macrophages are stimulated with ligand (MDP) or infected with the intracellular pathogen *Mtb* and the endogenous Rip2 and IKK-γ proteins examined for evidence of polyubiquitination. Collectively, both studies provide strong evidence that the NOD2 pathway is ubiquitin regulated and that IKK-γ and Rip2 polyubiquitination is mediated by an Ubc13/Traf6/Tak1 complex. Abbott et al. also suggest that in contrast to wild type NOD2, expression of the Crohn’s disease (CD) associated *NOD2* allele L2007insC fails to stimulate IKK-γ polyubiquitination (22). Yet NOD2 L2007insC is impaired in its ability to stably interact with Rip2 ((13) and Yang and Kelliher, unpublished data), suggesting that Rip2 and potentially polyubiquitinated Rip2 stimulates IKK-γ recruitment and its subsequent ubiquitination. These findings also raise the intriguing possibility that human inflammatory diseases like Crohn’s Disease or Blau syndrome (BS) may reflect deregulated ubiquitin mediated signaling. This hypothesis need be further addressed by directly examining Rip2 and IKK-γ proteins in the relevant cell types isolated from CD and BS patients.

Although we observed a defect in MDP-induced NF-κB activation in Rip2-deficient macrophages, MDP-induced p38 MAP kinase or JNK1/2 activation appears only modestly affected. These data suggest that other CARD containing adapter proteins may contribute to NOD2-mediated MAP kinase activation. One possibility may be CARD9, that is expressed in the mouse RAW 264.7 macrophage cell line and whose expression has been shown to preferentially activate the p38 MAP kinase and JNK1/2 pathways. Consistent with this model, MDP-induced MAP kinase responses and cytokine production are impaired in CARD9-deficient cells, whereas MDP-induced NF-κB activation appears unaffected (23).

The cytosolic Nod2 pathway has been implicated in innate immune responses to *Streptococcus pneumoniae*, *Listeria monocytogenes* and *Mycobacterium tuberculosis* (*Mtb*) (10,24,25). Rip2-deficient mice have been shown to be susceptible to *Listeria* infection (7), however the contribution of Rip2 to the innate immune response to *Mtb* has not yet been examined. We demonstrate that *Mtb* infection results in the ubiquitin modification of Rip2. Rip2 polyubiquitination is observed in macrophages infected with virulent strains of *Mtb*, but is not observed if the bacilli are killed prior to infection (with heat, formalin or streptomycin)(Figure 5A and data not shown). These data indicate that Rip2 polyubiquitination and Nod2 pathway “activation” requires bacterial viability and suggests that *Mtb* components may gain entry into the host cell cytosol to stimulate the Nod2/Rip2 pathway.

Although Rip2 and NLRs have been implicated in the innate immune recognition of *Listeria monocytogenes*, we did not observe
Rip2 polyubiquitination following *Listeria* infection (data not shown). It remains unclear why *Listeria* infection did not stimulate Rip2 polyubiquitination, but recent work by Girardin and colleagues suggests that the *Listeria monocytogenes* genome encodes a peptidoglycan N-deacetylase (*pgdA*) gene and inactivation of the bacterial *pgdA* gene results in reduced bacterial growth and a massive host IFN-β response (26). Thus, our inability to detect Rip2 polyubiquitination in *Listeria*-infected macrophages may reflect the ability of the bacteria to deacetylate peptidoglycan and thereby avoid NLR recognition.

In summary, this study provides genetic and biochemical evidence that Nod2-mediated bacterial recognition is mediated by the stable ubiquitin modification of Rip2, raising the possibility that the enzymes responsible for the ubiquitin modification of Rip2 (i.e. the E3 ubiquitin ligases and deubiquitinases) may serve as potential therapeutic targets in certain infectious diseases and potentially in chronic inflammatory disease.

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FIGURE LEGENDS

Figure 1. Rip2-deficient macrophages are impaired in their response to NOD2 ligand MDP, but respond normally to the TLR4 ligand, LPS. A. Rip2 is required for NF-κB activation in response to MDP or NOD2 pathway stimulation. Macrophages from wild type and rip2-/− mice were grown to confluence on 6 well plates and treated with 10μg/ml MDP or 10ng/ml LPS for the indicated times. The IKK and p38 MAPK activity were measured by immunoblotting with anti-phospho-IκBα and anti-phospho-p38 MAP kinase antibody (Cell signaling). Total p38 protein level was measured by immunoblotting with anti-p38 antibody (Santa Cruz). B. MDP-induced p38 MAP kinase and JNK1/2 activation is modestly impaired in the absence of Rip2. Macrophages from wild type and rip2-/− mice were grown to confluence on 6 well plates and treated with 10μg/ml MDP or 10ng/ml LPS for the indicated periods of time. The JNK MAPK activity was measured by immunoblotting with anti-phospho-JNK antibody (Cell signaling). Total JNK protein level was measured by immunoblotting with anti-JNK antibody (Santa Cruz).
Figure 2. NOD2 pathway activation stimulates the stable K63-linked polyubiquitination of Rip2. A. MDP treatment stimulates the ubiquitin modification of endogenous Rip2 and NEMO/IKK-γ. The RAW 264.7 macrophage cell line was left untreated or treated with the NOD2 ligand, MDP (10µg/ml) for the indicated times. Cells were lysed, and an equal amount of total protein was immunoprecipitated with anti-Rip2 or anti-NEMO antibody (Santa Cruz). To detect ubiquitinated proteins, immunoprecipitated proteins were immunoblotted with an anti-ubiquitin antibody (Santa Cruz). Total immunoprecipitated Rip2 or NEMO/IKK-γ protein was measured by immunoblotting with anti-Rip2 or anti-NEMO antibodies (Santa Cruz). B. NOD2 expression stimulates the stable, K63-linked polyubiquitination of Rip2. Omnitagged NOD2 and FLAG-tagged Rip2 constructs were expressed in HEK293T cells in the presence of HA-tagged K63-only or HA-tagged K48-only forms of ubiquitin. Rip2 was immunoprecipitated with anti-Rip2 antibody (Santa Cruz), and western blots were performed with the indicated antibodies. Expression levels of both Flag-Rip2 and Omni-NOD2 was measured by immunoblotting total cell lysates with anti-Flag or anti-Omni antibodies.

Figure 3. MDP-induced Rip2 polyubiquitination is dependent on the expression of the E2 conjugating enzyme Ubc13 and the E3 ubiquitin ligase Traf6. A. MDP fails to stimulate Rip2 polyubiquitination in Ubc13-deficient RAW 264.7 macrophages. The mouse macrophage cell line RAW 264.7 was infected with lentiviruses containing shRNA sequences specific for Ubc13 or GFP as a control and stable puromycin resistant pools selected. These lines were left untreated or stimulated with MDP (10µg/ml) for the time periods indicated. Cells were lysed, and an equal amount of total protein was immunoprecipitated with anti-Rip2 antibody (Santa Cruz) followed by immunoblotting with an anti-ubiquitin antibody (Santa Cruz). Total immunoprecipitated Rip2 protein was measured by immunoblotting with anti-Rip2 antibody (Santa Cruz). Expression of Ubc13 level was determined by immunoblotting with an anti-Ubc13 antibody (Zymed). B. Rip2 polyubiquitination requires the expression of the E3 ubiquitin ligase Traf6, but not Traf2. Omnitagged NOD2 was expressed in HEK293T cells in the presence of either a control siRNA or siRNA specific for Traf2 or Traf6. Rip2 was immunoprecipitated with anti-Rip2 antibody (Santa Cruz) followed by immunoblotting with anti-ubiquitin to detect polyubiquitinated Rip2 proteins. Expression levels of Omni-NOD2 and Traf2 or Traf6 protein levels was measured by immunoblotting the total cell lysates with anti-Omni, anti-Traf6 and anti-Traf2 antibodies (Santa Cruz). C. MDP stimulates interaction between endogenous Rip2 and Traf6. RAW 264.7 macrophages were left untreated or treated with 10µg/ml MDP for the indicated periods of time. Cells were lysed, and an equal amount of total protein was immunoprecipitated with anti-Rip2 antibody (Santa Cruz) followed by immunoblotting with an anti-Traf6 antibody (Santa Cruz). The total immunoprecipitated Rip2 protein was measured by immunoblotting with anti-Rip2 antibody (Santa Cruz). D. Traf6 expression is required for MDP- and IL-1- induced NF-κB activation. The RAW 264.7 macrophage cell line was transfected with control siRNA or a pool of Traf6 siRNAs. Forty-eight hours later, the cells were left untreated or stimulated with MDP (10µg/ml) for the time periods indicated or with IL-α (5ng/ml) for 10 minutes. NF-κB activation was measured by immunoblotting with a phospho-IκBα antibody (Cell Signaling Technology). Traf6 and β-actin levels were determined by immunoblotting with the indicated antibodies.

Figure 4. Rip2 and the ubiquitin activated kinase Tak1 interact upon MDP stimulation and Tak1 expression is required for MDP-induced IKK activation. A. Rip2 and Tak1
interact in transfected cells. Flag-tagged Tak1 and HA-tagged Rip2 were either co-expressed or expressed alone in HEK293T cells. Tak1 was immunoprecipitated with an anti-Flag antibody and Rip2 detected by immunoblotting with anti-HA antibody or Rip2 was immunoprecipitated with an anti-HA antibody followed by immunoblotting with anti-Flag antibody to detect associated Tak1 proteins. Relative expression of each construct was measured by immunoblotting the total cell lysate with anti-HA or -Flag antibodies. B. MDP stimulates interaction between Rip2 and Tak1. The RAW 264.7 macrophage cell line was left untreated or treated with 10µg/ml MDP for the indicated periods of time. Cells were lysed, and an equal amount of total protein was immunoprecipitated with anti-Rip2 antibody (Santa Cruz) followed by immunoblotting with an anti-Tak1 antibody (Santa Cruz). Total immunoprecipitated Rip2 protein was measured by immunoblotting with anti-Rip2 antibody (Santa Cruz). C. Tak1 expression is required for TNF- and MDP-induced NF-κB activation. Mouse RAW 264.7 macrophages cell line was transfected with control siRNA or a pool of Tak1 siRNAs. Forty-eight hours later, the cells were left untreated or stimulated with MDP (10µg/ml) for the time periods indicated or with TNF-α (10ng/ml) for 10 minutes. NF-κB activation was measured by immunoblotting with a phospho-IκBα antibody (Cell Signaling). Tak1 and β-actin levels were determined by immunoblotting with the indicated antibodies.

**Figure 5.** Live *Mycobacterium tuberculosis* infection stimulates Rip2 polyubiquitination. A. Live, virulent strains of *Mtb* (Rv) stimulate Rip2 polyubiquitination but heat killed strains fail to stimulate Rip2 polyubiquitination. Wildtype bone marrow-derived macrophages were infected with live virulent (Rv) or heat killed (HK-Rv) strains of *M. tuberculosis* (*Mtb* H37Rv). Polyubiquitinated Rip2 proteins were detected by immunoprecipitating the cell lysates with an anti-Rip2 antibody followed by immunoblotting with an anti-ubiquitin antibody. Immunoprecipitates were immunoblotted with a Rip2 antibody to insure that equal amounts of Rip2 protein were immunoprecipitated. B. *Mtb*-induced Rip2 polyubiquitination is MyD88 and TLR2/4 independent. Wildtype, MyD88/- and TLR2/4/- bone marrow-derived macrophages were left uninfected (-) or infected with live virulent *M. tuberculosis* (H37Rv) for 1 hour. Polyubiquitinated Rip2 proteins were detected by immunoprecipitating the cell lysates with an anti-Rip2 antibody followed by immunoblotting with an anti-ubiquitin antibody.
Fig. 1

A

|          | wt       | rip2 -/-  |
|----------|----------|-----------|
|          | MDP      | LPS       | MDP      | LPS       |
| 0        | 30       | 45        | 60       | 30        | 45        | 60        |
| 90       | 20       | 60        |          |           |           |           |

Anti P-IκBα

Anti P-p38

Anti p38

B

|          | wt       | rip2 -/-  |
|----------|----------|-----------|
|          | MDP      | LPS       | MDP      | LPS       |
| 0        | 30       | 60        | 90       | 20        | 60        |
| 60       | 90       | 20        | 60       | 0         |           |

Anti P-JNK 1/2

Anti JNK 1/2

wt

rip2 -/-
Fig. 2

A

| MDP   | IP: Rip2 | IP: NEMO |
|-------|----------|----------|
| -     | 30 45 60 | - 30 45 60 90 min |

210KDa

111KDa

71KDa

IB: Anti Ub

IP: Anti Rip2
IB: Anti Rip2

IP: Anti Rip2
IB: Anti NEMO

B

|  | FLAG -Rip2 | Omni-NOD2 | HA-K63 ubiquitin | HA-K48 ubiquitin |
|---|-------------|------------|------------------|------------------|
| IP: Anti Rip2 | - | + | - | + |
| IB: Anti Rip2  | - | - | - | + |

IP: Anti Rip2
IB: Anti HA

IP: Anti Rip2
IB: Anti Rip2

Total cell lysate
IB: Anti FLAG
IB: Anti Omni

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**Fig. 3**

**A**

GFP shRNA  UBC151 shRNA  UBC152 shRNA

MCP  0  45  60  90  0  45  60  90  0  45  60  90  90 min

IP: Anti Rip2
IB: Anti Ub

**B**

Omni-NOD2  Traf2 shRNA  Traf6 shRNA

IP: Anti Rip2
IB: Anti Ub

**C**

MDP - 30  60  90  120 (min) input

IP: Anti Rip2
IB: Anti Traf6

**D**

Control siRNA  Traf6 siRNA

MCP - 45  60  90  IL-1 - 45  60  90  IL-1

IP: Anti P-IkBα
IB: Anti Traf6

IP: Anti Rip2
IB: Anti β-actin

IB: Anti Traf6
IB: Anti β-actin
Fig. 4

A

Flag-TAK1
HA-RIP2
- + + +
IP: Anti Flag
IB: Anti HA
IP: Anti Flag
IP: Anti TAK
lysate
IB: Anti HA

IP: Anti HA
IB: Anti Flag

IP: Anti HA
IB: Anti Flag

B

MDP - 30 60 90 120 min
Tak1 siRNA
Control siRNA
IP: Anti Rip2
IB: Anti Tak1
IP: Anti Rip2
IgG

C

Control siRNA
Tak1 siRNA
MDP - 30 45 60 90 TNF - 30 45 60 90 TNF min
IB: Anti p-IκBα
IB: Anti Tak1
IB: Anti β-actin

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Fig. 5
NOD2 pathway activation by MDP or Mycobacterium tuberculosis infection involves the stable polyubiquitination of Rip2
Yibin Yang, Catherine Yin, Amit Pandey, Derek Abbott, Christopher Sassetti and Michelle A. Kelliher

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