A Dual Role for Receptor-interacting Protein Kinase 2 (RIP2) Kinase Activity in Nucleotide-binding Oligomerization Domain 2 (NOD2)-dependent Autophagy*§

Craig R. Homer†, Amrita Kabi‡, Noemí Marina-García‡, Arul Sreekumar‡, Alexey I. Nesvizhskii‡, Kourtney P. Nickerson‡‖, Arul M. Chinnaiyan∗§, Gabriel Nuñez†, and Christine McDonald‡‖

From the †Department of Pathobiology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195, the ‡Department of Pathology, Comprehensive Cancer Center, and the **Michigan Center for Translational Pathology, Howard Hughes Medical Institute, University of Michigan Medical School, Ann Arbor, Michigan 48109, the †Department of Molecular and Cell Biology, Alkek Center for Molecular Discovery, Baylor College of Medicine, Houston, Texas 77030, and the ‖Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine, Case Western Reserve University, Cleveland, Ohio 44105

Background: Autophagy is triggered by NOD2 as an anti-bacterial response.
Results: NOD2-stimulated autophagy requires RIP2-dependent activation of p38 MAPK and repression of the PP2A phosphatase in intestinal epithelial cell lines.
Conclusion: RIP2 kinase activity is necessary for anti-bacterial autophagy induction by NOD2.
Significance: These findings provide novel molecular targets for modulation of autophagy as an anti-bacterial response.

Autophagy is triggered by the intracellular bacterial sensor NOD2 (nucleotide-binding, oligomerization domain 2) as an anti-bacterial response. Defects in autophagy have been implicated in Crohn’s disease susceptibility. The molecular mechanisms of activation and regulation of this process by NOD2 are not well understood, with recent studies reporting conflicting requirements for RIP2 (receptor-interacting protein kinase 2) in autophagy induction. We examined the requirement of NOD2 signaling mediated by RIP2 for anti-bacterial autophagy induction and clearance of Salmonella typhimurium in the intestinal epithelial cell line HCT116. Our data demonstrate that NOD2 stimulates autophagy in a process dependent on RIP2 tyrosine kinase activity. Autophagy induction requires the activity of the mitogen-activated protein kinases MEKK4 and p38 but is independent of NFκB signaling. Activation of autophagy was inhibited by a PP2A phosphatase complex, which interacts with both NOD2 and RIP2. PP2A phosphatase activity inhibited NOD2-dependent autophagy but not activation of NFκB or p38. Upon stimulation of NOD2, the phosphorylation activity of the PP2A complex is inhibited through tyrosine phosphorylation of the catalytic subunit in a process dependent on RIP2 activity. These findings demonstrate that RIP2 tyrosine kinase activity is not only required for NOD2-dependent autophagy but plays a dual role in this process. RIP2 both sends a positive autophagy signal through activation of p38 MAPK and relieves repression of autophagy mediated by the phosphatase PP2A.

Autophagy is a cell stress response that causes the encapsulation of cellular contents in multilamellar vesicles for subsequent degradation and recycling (1). It is best known as a starvation response; however, autophagy is also essential for the capture and removal of intracellular bacteria, such as Salmonella typhimurium, Listeria monocytogenes, and Mycobacterium tuberculosis (2). Genetic variants in several autophagy genes are associated with Crohn’s disease (CD), a debilitating and chronic inflammatory bowel disease (3–6). There is a strong link between bacteria and CD pathogenesis; therefore, it has been proposed that ineffective bacterial clearance due to impaired anti-bacterial autophagy is an important contributor to the pathogenesis of this chronic inflammatory disease (2, 6).

The first identified CD risk gene is NOD2 (nucleotide-binding oligomerization domain 2), which encodes an intracellular bacteria sensor involved the innate immune response to bacteria (7). NOD2 detects a conserved component of bacterial peptidoglycan consisting of muramyl dipeptide (MDP). MDP is released from bacteria when the cell wall is fragmented as a part of bacterial killing, as well as during bacterial division, or is co-injected into cells with pathogen effector proteins by type III or IV secretion systems. Upon stimulation by MDP, NOD2 oligomerizes and recruits the receptor-interacting protein 2 kinase (RIP2/RICK/CARD1AK). Activation of RIP2 recruits ubiquitin-modifying enzymes and stimulates protein kinase cascades, resulting in the activation of NFκB and the mitogen-activated kinases (MAPKs) p38, JNK, and ERK1/2. These path-
ways coordinate to regulate inflammatory cytokine production, anti-bacterial killing, and the recruitment of other professional immune response cells. Functional analyses of CD-associated NOD2 variants demonstrate defects in both inflammatory signaling and bactericidal activity in response to MDP (8).

Recent reports have demonstrated that NOD2 stimulates autophagy as an anti-bacterial response and that this process is impaired by CD-associated variants in either NOD2 or the autophagy gene, ATG16L1 (9–11). The exact mechanism behind how NOD2 directs autophagosome formation has not yet been determined. There remains some discrepancy regarding whether this process requires NOD2 signaling (9, 10, 12) or is mediated solely by direct recruitment of autophagic machinery to sites of bacterial invasion (11). In this report, we determine that NOD2-dependent signaling is required for autophagy induction in intestinal epithelial cells and examine which signaling components are required for this process. Our studies illustrate a dual role for RIP2 tyrosine kinase activity in NOD2-dependent autophagy through activation of p38 MAPK and repression of PP2A phosphatase activity.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—MDP was purchased from Bachem (Torrance, CA). Erolitinib (LC Laboratories, Woodburn, MA) was a gift of Derek Abbott (Case Western Reserve University, Cleveland, OH). Antibodies against FLAG (M2), MEKK4 (clone MEKK4-338), Atg1/ULK1 (A7481), or tubulin (clone DM1A) were obtained from Sigma. Antibodies to GAPDH (clone 14C10), phosphorylated NFκB p65 (Ser-536) (clone 93H1), p38 MAPK (catalog no. 9212), phosphorylated p38 (Thr-180/Tyr-182) (catalog no. 9211), and phosphorylated ULK1 (Ser-555) (clone D1H4) were purchased from Cell Signaling (Boston, MA). Anti-LC3B antibody (catalog no. NB100-2220) was purchased from Novus Biologicals (Littleton, CO). Antibodies to RIP2/RICK (H-300), PP2A (1D6), phosphorylated PP2A (Tyr-307) (F-8), PPP2R1A (A-5), and the epitope tags Omni (M-21) and HA (Y-11) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-HA (HA.11) antibody was purchased from Covance (Emeryville, CA). A rat monoclonal antibody to RIP2/RICK (Nick-1) was purchased from Assay Designs (Farmingdale, NY). HRP-conjugated donkey secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**RNA Interference (RNAi) Reagents**—MISSION short hairpin RNA (shRNA) constructs targeting NOD2 (NM_022162.1-2959s1c1), RIP2 (NM_003821.5-2364s21c1), p38 (NM_139012.x-503s1c1), JNK (NM_002752.x-356s1c1), ERK1 (NM_002746.1-876s1c1), ATG16L1 (NM_030803.5-2181s1c1), and nonspecific control (SHC002) were purchased from Sigma. Control siRNA (D-001210-01) and siGENOME SMARTpool siRNA to PPP2R1A (M-101259-02-0005) were obtained from Thermo Scientific (Waltham, MA).

**Plasmids**—NOD2 expression plasmids as well as the NFκB and CMV β-gal reporters have been described previously (13). The kinase-dead (KD) mutant of MEKK4 (pCMV5-MEKK4KD), dominant negative mutants of IKKα and IKKβ (pFLAG-IKKα DN and pEBG-IKKβ DN), and the wild type and T95M point mutant of RIP2 (pcDNA3-Omni-RIP2 and pcDNA3-Omni-RIP2 T95M) were kind gifts of Derrick Abbott (14). Kinase-dead mutants of p38 (pCMV-p38 KD), JNK (pCE4-JNK KD), and ERK1 (pCE4-ERKKD) were gifts of Melanie Cobb (University of Texas Southwestern Medical Center, Dallas, TX). The IκBα superrepressor mutant (S32A/S36A) (pBabe-IκBα-SR, Addgene plasmid 15291) was created by William Hahn (Harvard Medical School, Boston, MA) (15) and purchased from Addgene (Cambridge, MA). Wild type PP2Ac (pEF4C-HA-PP2A), a phosphatase-dead mutant of PP2Ac (pEF4C-HA-PP2Amut), wild type PPP2R1A (pEF4C-FLAG-PPP2R1A), and an amino-terminal deletion mutant of PPP2R1A that lacks binding to PP2Ac (pEF4C-FLAG-PPP2R1AΔN) were kind gifts of Daniel Krappmann (Institute of Toxicology, Nuremberg, Germany) (16).

**Cell Culture and Transfection**—HCT116 and HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (FBS; Lonza, Allendale, NJ). HCT116 cell lines stably expressing shRNAs were generated by lentiviral infection, followed by selection with 0.5 μg/ml puromycin for 2 weeks. Transient transfection of HCT116 cells was performed by Polyfect (Qiagen, Valencia, CA) or nucleofection with Kit V (Lonza) according to the manufacturer’s instructions and assayed 48 h post-transfection. HEK293T cells were transfected using Polyfect (Qiagen) or calcium phosphate as described previously (10, 13) and assayed 24–48 h post-transfection.

**Immunofluorescence**—HCT116 shRNA cell lines were plated onto glass coverslips for 48 h. Cells were stimulated with 20 μg/ml MDP for 4 h, fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS), and permeabilized in 0.4% Triton X-100, PBS. Cells were washed with PBS, blocked in 2% FBS, PBS, and incubated with anti-LC3B antibody (1:200; catalog no. 2775, Cell Signaling Technology) in 2% FBS, PBS overnight at 4 °C. Cells were subsequently stained with goat anti-rabbit-Alexa488 antibody (1:1,000; catalog no. A11034, Invitrogen) and mounted on slides with Vectashield plus DAPI (Vector Laboratories, Burlingame, CA). Samples were visualized by confocal microscopy using a ×40 objective lens on a Leica TCS-SP spectral laser-scanning confocal microscope equipped with a Q-Imaging Retiga EXi cooled CCD camera and Image-Pro Plus Capture and Analysis software (Media Cybernetics, Silver Spring, MD). LC3+ vesicles were scored in z-stack overlays from at least four separate fields and quantitated in an automated fashion using a customized visual basic Image-Pro Plus macro.

**ImmunobLOTS**—For analysis of phosphosignaling events and protein expression levels, cells were washed twice in PBS and then lysed in Ginger buffer (310 mM Tris, pH 6.8, 25% glycerol, 5% SDS, 715 mM β-mercaptoethanol, 125 mg/ml bromphenol blue) on ice. The resulting lysate was separated by SDS-PAGE, and proteins were transferred to a PVDF membrane. Membranes were blocked in 5% milk, Tris-buffered saline, 0.1% Tween 20 and then probed with primary antibodies overnight at 4 °C. Blots were developed by enhanced chemiluminescence (Millipore, Billerica, MA).

**Gentamycin Protection Assays**—An overnight culture of *Salmonella enterica serovar typhimurium* SL1344 was diluted 1:7
and grown at 30 °C for 1 h and then added to cells at a multiplicity of infection of 10 for 30 min. Cells were washed twice with PBS, and then DMEM supplemented with 10% FBS and 50 μg/ml gentamycin (Sigma) was added for 1 h. Cells were lysed in 50 μl of lysis buffer (PBS, 0.1% Triton X-100), and dilutions were plated in duplicate onto Luria broth plates. After growth overnight at 30 °C, colonies recovered were counted, and colony-forming units/well were calculated (10). Statistical significance between groups was determined by using a two-tailed t test. Results were considered significant when p was ≤ 0.05.

**Immunoprecipitation-coupled Mass Spectral Screen** — Identification of NOD2-interacting proteins by immunoprecipitation of NOD2 complexes from MDP-stimulated HEK293T cells stably expressing low levels of FLAG-NOD2 followed by liquid chromatography-coupled tandem mass spectrometry has been described in detail previously (17).

**NFκB and p38 MAPK Reporter Gene Assays** — Luciferase reporter gene assays to measure NFκB or p38 MAPK activity were performed in HCT116 or HEK293T cells as described previously (17).

**Cytokine Secretion Assays** — Cells were plated in triplicate and stimulated for 18 h with MDP (100 ng/ml). Interleukin-8 (IL-8) levels secreted into the cell culture medium were determined by the QuantiKine human IL-8 immunoassay (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**RESULTS**

The Kinase Activity of RIP2 Is Required for MDP-stimulated Autophagy Induction — The autophagic process is highly conserved and can be broken down into discrete steps (1). First, an isolation membrane nucleates in a process initiated by the ULK1 (uncoordinated-51-like kinase) complex, which subsequently activates a type III phosphatidylinositol 3-kinase (PI3K) complex. The isolation membrane is then elongated under the control of two ubiquitin-like conjugation systems composed of autophagy-related gene (ATG) proteins. The first system is composed of ATG7 and ATG10, which assist formation of an elongation complex containing ATG5, ATG12, and ATG16L1. The ATG5-ATG12-ATG16L1 complex localizes to the isolation membrane and aids in the recruitment of microtubule-associated protein light chain 3 protein (LC3), which is modified from a cytosolic form (LC3-I) to a lipid-conjugated form (LC3-II) and inserted into the forming autophagosomal membrane. The autophagosome then fuses with a lysosome, resulting in the degradation of autophagosomal contents by lysosomal enzymes.

The molecular requirements for NOD2-dependent autophagy induction have not been clearly elucidated. *S. typhimurium* is an intracellular bacteria targeted by autophagy, and the clearance of this bacterium is often used to monitor autophagy (18). We have previously demonstrated that MDP stimulation during infection enhances *Salmonella* killing in a NOD2- and ATG16L1-dependent manner (10). To further dissect the essential components of NOD2-dependent autophagy, we examined the requirement for RIP2 (a kinase required for NOD2 signaling), ULK1 (a kinase involved in autophagy initiation), and Beclin-1 (a component of the PI3K complex) in this process by RNAi knockdown in the human intestinal epithelial cell line HCT116 (Fig. 1, A, B, and J, and supplemental Fig. 1). Our data demonstrate that expression of ULK1, Beclin-1, or ATG16L1 is required for enhancement of *Salmonella* killing by MDP in gentamycin protection assays (Fig. 1A and supplemental Fig. 1A). MDP-enhanced bacterial killing was also ablated when expression of NOD2 or RIP2 was inhibited by RNAi (Fig. 1, A and B). The role of these proteins in MDP-stimulated autophagy was confirmed in additional assays. The formation of LC3+ vesicles in response to MDP was dependent on both NOD2 and RIP2 expression as determined by quantification of confocal micrographs (Fig. 1, D and E). Likewise, an MDP-stimulated increase in LC3-II levels was dependent on NOD2 and RIP2 expression (Fig. 1, G and H). These findings suggest that NOD2-dependent autophagy is dependent on the intersection of NOD2 signaling with an autophagic cascade initiated by ULK1.

RIP2 has been described to promote NLR-dependent signaling, acting as both a kinase and a protein scaffold (7, 14). To investigate whether RIP2 kinase activity is required for NOD2-dependent autophagy induction, we treated HCT116 cells with erlotinib, a newly described RIP2 tyrosine kinase inhibitor (14). We observed that erlotinib pretreatment of HCT116 cells blocked MDP-enhanced *Salmonella* killing in gentamycin protection assays (Fig. 1C). Additionally, erlotinib pretreatment impaired both MDP-stimulated LC3+ puncta formation and LC3-II accumulation in HCT116 cells (Fig. 1, F and J). These findings demonstrate a requirement for RIP2 tyrosine kinase activity in MDP-induced autophagy.

**NOD2 Induces Autophagy in a p38 MAPK-dependent Manner** — In response to bacterial infection or exposure to MDP, NOD2 recruits RIP2 and activates two major signaling pathways, NFκB and the MAPKs: p38, JNK, and ERK1/2 (7). NFκB signaling involves activation of IκB kinases (IKKα and IKKβ), which phosphorylate the NFκB-inhibitory molecule, IκBα, initiating its degradation and subsequent nuclear translocation of NFκB. However, NOD2-dependent autophagy was not altered when dominant negative mutants of IKKα and -β (IKKα DN and IKKβ DN) or a non-phosphorylatable mutant of IκBα (IκBα-SR) were expressed in HCT116 cells (Fig. 2A). Although these dominant negative molecules were effective at blocking NFκB activity in reporter gene assays (supplemental Fig. 2, A–D), the lack of effect of these constructs in either gentamycin protection assays (Fig. 2A) or LC3-II immunoblots (Fig. 2C) clearly demonstrates that NFκB activation is dispensable for NOD2-mediated autophagy.

Next, we examined components of the MAPK signaling cascade for their role in NOD2-mediated autophagy. The MAPK pathway involves the cell type-specific activation of several mitogen-activated protein kinase kinase kinases (MEKKs), which stimulate signaling cascades, resulting in activation of p38, JNK, and ERK1/2. MDP-enhanced *Salmonella* killing in HCT116 cells was blocked by RNAi-mediated knockdown of MEKK4 and p38 MAPK but unaffected by knockdown of JNK, ERK1, or NFκB p65 (Fig. 2B and supplemental Fig. 2, E–G). The requirement of p38 MAPK expression for MDP-stimulated autophagy was confirmed in LC3-II immunoblots (Fig. 2C). These results suggest that autophagy induction by NOD2 in...
intestinal epithelial cells is specifically dependent on p38 MAPK.

Additionally, we examined whether the kinase activity of p38 MAPK was required for NOD2-dependent autophagy. In HCT116 cells, MDP-stimulated Salmonella killing and p38 activity was suppressed by expression of a dominant negative kinase-dead mutant of p38 in gentamycin protection and reporter gene assays (Fig. 2, D and E). The dominant negative kinase-dead p38 mutant also suppressed MDP-induced LC3-II in immunoblots (Fig. 2F).

To further confirm the requirement of p38 kinase activity in NOD2-dependent autophagy, we treated cells with the kinase inhibitor SB203580. SB203580 was originally described as a specific p38 MAPK inhibitor but has now been demonstrated to inhibit both p38 MAPK and RIP2 kinase activities (19). To differentiate between the two inhibitory actions of this drug, we...
assessed *Salmonella* killing in HEK293T cells expressing either RIP2 or a RIP2 mutant (T95M) that contains a point mutation in the ATP binding pocket that interferes with the binding of both SB203580 and erlotinib but does not affect RIP2 kinase activity (14, 19). We observed that expression of either RIP2 or RIP2 T95M increased *Salmonella* killing (Fig. 2G and supplemental Fig. 2H). Treatment of RIP2-expressing cells with either SB203580 or erlotinib inhibited this effect. However, in RIP2 T95M-expressing cells, only SB203580 inhibited *Salmonella* killing, indicating that p38 kinase activity is required in addition to RIP2 kinase activity.

We further linked p38 activation in our system to autophagy through assessing whether p38 expression in itself is sufficient to induce *Salmonella* killing via autophagy. In a manner similar to other kinases, overexpression of p38 results in autophagosome phagophores and activation (20). When p38 was overexpressed in HCT116 cells, we observed an increase in *Salmonella* killing similar to the effects of MDP treatment (Fig. 2H). Knockdown of ULK1 expression by RNAi blocked both MDP-enhanced killing and the increased killing by p38 overexpression (Fig. 2H), indicating that p38 expression alone in HCT116 cells enhances bacterial killing in a process dependent on autophagy. These findings demonstrate that NOD2 activates autophagy in a process dependent on RIP2 tyrosine kinase activity and mediated by the kinases MEKK4 and p38 but independent of NFκB signaling.

**Identification of PPP2R1A as a Novel NOD2-interacting Protein**—Having established that NOD2 mediates autophagy through p38, we wished to identify specific regulators of this process. We had previously performed an immunoprecipitation-coupled mass spectrometry screen to identify NOD2-interacting proteins from MDP-stimulated HEK293 cells stably expressing low levels of FLAG-tagged NOD2 protein (17). Analysis of the screen results for enzymes described to modulate both p38 and autophagic activity resulted in the identification of a candidate regulator. This candidate was the protein phosphatase 2 regulatory subunit A α protein (PPP2R1A), a scaffolding component of the protein phosphatase 2A (PP2A) complex (supplemental Fig. 3). PP2A is a ubiquitous serine/threonine phosphatase with a myriad of functions in growth, cell death, and cancer (21). PP2A primarily exists as a heterotrimer consisting of a catalytic subunit (PP2Ac), a scaffolding A subunit (PPP2R1A or PPP2R1B), and a regulatory B subunit, which confers specificity. A smaller fraction of PP2A exists as a heterodimer of PP2Ac and the α4 subunit. PP2A has demonstrated roles in both starvation-induced autophagy (22–24) and p38 modulation (25–29), suggesting that PP2A may also regulate anti-bacterial autophagy mediated by NOD2.

Initially, we confirmed the interaction between NOD2 and PPP2R1A in co-immunoprecipitation assays using epitope-tagged expression constructs in HEK293T cells. To preserve signaling complex stoichiometry, we expressed HA-RIP2 in addition to combinations of Omni-NOD2, FLAG-PPP2R1A, and HA-PP2Ac. When cell lysates were immunoprecipitated for NOD2 using an Omni antibody, complexes containing NOD2, RIP2, PPP2R1A, and PP2Ac were observed (Fig. 3A). We confirmed an endogenous interaction of PPP2R1A with RIP2 by co-immunoprecipitation from HCT116 cells (Fig. 3B) as well as an endogenous interaction of RIP2 with PP2Ac (Fig. 3C). These endogenous interactions were not significantly altered in immunoprecipitation assays from MDP-stimulated cells. These findings demonstrate a novel interaction between the NOD2-RIP2 complex and a PP2A phosphatase heterocomplex mediated through the scaffold protein PPP2R1A.

**Characterization of PPP2R1A as a Negative Regulator of NOD2-mediated Autophagy**—The role of PPP2R1A in the PP2A phosphatase complex is to provide a scaffold for recruitment of the catalytic and regulatory subunits and to direct PP2Ac to specific targets (21, 30). We assessed if alterations in PPP2R1A expression or function affected MDP-stimulated autophagy in HCT116 cells. MDP-stimulated autophagy was increased in cells when PPP2R1A expression was knocked down by RNAi, as measured by LC3-II immunoblots (Fig. 4A). Reduction of PPP2R1A expression also increased bacterial killing in gentamycin protection assays with decreased *Salmonella* survival in cells treated with PPP2R1A RNAi but not with control RNAi (Fig. 4B). This enhancement of killing occurred in the absence of exogenous MDP stimulation and was not further increased by MDP treatment. Because MDP is released during the natural course of *Salmonella* infection, we examined whether the enhancement of *Salmonella* killing by PPP2R1A RNAi knockdown in the absence of exogenous MDP stimulation was due to enhanced NOD2 activity. When NOD2 activity was blocked by expression of a dominant negative NOD2 construct (NOD2 D291N), the PPP2R1A RNAi-enhanced *Salmonella* killing was ablated (Fig. 4C). These results indicate that NOD2-dependent killing of *Salmonella* is negatively regulated by PPP2R1A.

These findings are complemented by the results obtained with a dominant negative mutant of PPP2R1A that lacks the
amino-terminal PP2Ac interaction domain (PPP2R1A/H9004N) (16). We observed a significant increase in Salmonella killing in the presence and absence of exogenous MDP treatment when PPP2R1A/H9004N was expressed in HCT116 cells (Fig. 4, D and E). These results are similar to what was observed with PPP2R1A knockdown (Fig. 4B). The increase in Salmonella killing by PPP2R1AΔN expression required the expression of NOD2, p38, and ATG16L1 because RNAi-mediated knockdown of
the PP2Ac mutant was dependent on NOD2, p38, and ATG16L1 expression because RNAi-mediated knockdown of these genes blocked this effect (Fig. 5F). These results indicate that a PP2A phosphatase complex consisting of PP2Ac and PPP2R1A is a selective inhibitor of NOD2-triggered anti-bacterial autophagy.

**PP2A Inhibits MDP-stimulated Autophagy Downstream of the MAPK p38**—Next, we determined if PP2A inhibited NOD2 signaling globally or specifically targeted processes required for autophagy induction. Therefore, we examined whether overexpression of PP2Ac affected NOD2-dependent activation of NFκB or p38 MAPK by reporter gene assays in NOD2-expressing HEK293T cells. In contrast to the dramatic effects on MDP-induced autophagy (Fig. 5, A and C), PP2Ac overexpression had no effect upon either MDP-stimulated NFκB or p38 activity (Fig. 6, A and B), suggesting a role downstream of p38 activation. We confirmed the effect of PP2Ac on events downstream of p38 through measurement of IL-8 secretion in response to MDP. The MAPK p38 has been demonstrated to be critical for IL-8 production in response to inflammatory stimuli (31). Overexpression of PP2Ac blocked IL-8 secretion in response to MDP stimulation (Fig. 6C), suggesting that PP2Ac does not affect NOD2 activation of NFκB and MAPK pathways but rather modulates a target of p38 activity involved in autophagy induction and IL-8 secretion.

**PP2Ac Is Inactivated upon Activation of RIP2 Kinase by MDP Treatment**—Because PP2Ac phosphatase activity inhibits MDP-induced autophagy, we investigated whether PP2A activity was altered by MDP treatment. PP2Ac activity is tightly controlled by post-translational modifications of the carboxyl terminus, which include phosphorylation on threonine 304 and tyrosine 307 as well as carboxymethylation of leucine 309 (30, 32, 33). The best characterized regulatory site is phosphorylation of Tyr-307, which has been reported to reduce PP2Ac activity by up to 90% (32). We assessed whether MDP treatment altered tyrosine phosphorylation of PP2Ac on Tyr-307 by immunoblot using a phosphospecific antibody to PP2Ac Tyr-307. We observed a significant increase in Tyr-307 phosphorylation upon MDP treatment of HCT116 cells (Fig. 7A). Induction of PP2Ac phosphorylation was dependent on RIP2 expression because RNAi-mediated knockdown of RIP2 ablated MDP stimulated phosphorylation of PP2Ac Tyr-307 (Fig. 7A). RIP2 kinase activity was also found to be required for this modification because pretreatment of HCT116 with erlotinib similarly blocked MDP-stimulated phosphorylation of Tyr-307 (Fig. 7B). However, RIP2 does not appear to directly phosphorylate PP2Ac because results from in vitro kinase assays were negative (supplemental Fig. 4). We also tested whether PPP2R1A expression was required for MDP-induced phosphorylation of PP2A on Tyr-307. We found that MDP-induced phosphorylation of Tyr-307 was prevented by RNAi knockdown of PPP2R1A (Fig. 7C). These results indicate that upon MDP stimulation, the phosphatase activity of the PP2Ac-PPP2R1A complex is inhibited through the phosphorylation of PP2Ac on Tyr-307 in a process dependent on the kinase activity of RIP2.

**RIP2 Mediates NOD2-dependent Autophagy**

The PP2Ac-PPP2R1A Phosphatase Complex Regulates NOD2-mediated Autophagy—Previous studies have shown that PP2A modulates autophagy both positively and negatively, depending on the cell type and stimulus used (22–24). Our data indicate that the PPP2R1A subunit of PP2A regulates MDP-stimulated autophagy, so we assessed the requirement of the catalytic subunit (PP2Ac) in this process. Overexpression of PP2Ac in HCT116 cells blocked MDP-stimulated bacterial killing but not enhanced killing stimulated by rapamycin in gentamicin protection assays (Fig. 5A). PPP2R1A expression was also required for PP2Ac inhibition of NOD2-mediated *Salmonella* killing because depletion of PPP2R1A by RNAi knockdown prevented PP2Ac blockade of MDP-enhanced killing (Fig. 5B). PP2Ac was also demonstrated to inhibit MDP-stimulated LC3-II levels in immunoblots (Fig. 5C).

Next, we investigated the requirement of PP2A phosphatase activity for modulation of NOD2-dependent autophagy. Expression of a phosphatase-dead PP2Ac mutant (PP2Ac Mt) enhanced MDP-stimulated LC3-II levels and increased *Salmonella* killing in the presence and absence of MDP stimulation (Fig. 5, C and D). This increase in *Salmonella* killing stimulated by the PP2Ac mutant was dependent on NOD2, p38, and ATG16L1 expression because RNAi-mediated knockdown of these genes blocked this effect (Fig. 5F). These results indicate that a PP2A phosphatase complex consisting of PP2Ac and PPP2R1A is a selective inhibitor of NOD2-triggered anti-bacterial autophagy.

**PP2A Inhibits MDP-stimulated Autophagy Downstream of the MAPK p38**—Next, we determined if PP2A inhibited NOD2 signaling globally or specifically targeted processes required for autophagy induction. Therefore, we examined whether overexpression of PP2Ac affected NOD2-dependent activation of NFκB or p38 MAPK by reporter gene assays in NOD2-expressing HEK293T cells. In contrast to the dramatic effects on MDP-induced autophagy (Fig. 5, A and C), PP2Ac overexpression had no effect upon either MDP-stimulated NFκB or p38 activity (Fig. 6, A and B), suggesting a role downstream of p38 activation. We confirmed the effect of PP2Ac on events downstream of p38 through measurement of IL-8 secretion in response to MDP. The MAPK p38 has been demonstrated to be critical for IL-8 production in response to inflammatory stimuli (31). Overexpression of PP2Ac blocked IL-8 secretion in response to MDP stimulation (Fig. 6C), suggesting that PP2Ac does not affect NOD2 activation of NFκB and MAPK pathways but rather modulates a target of p38 activity involved in autophagy induction and IL-8 secretion.

**PP2Ac Is Inactivated upon Activation of RIP2 Kinase by MDP Treatment**—Because PP2Ac phosphatase activity inhibits MDP-induced autophagy, we investigated whether PP2A activity was altered by MDP treatment. PP2Ac activity is tightly controlled by post-translational modifications of the carboxyl terminus, which include phosphorylation on threonine 304 and tyrosine 307 as well as carboxymethylation of leucine 309 (30, 32, 33). The best characterized regulatory site is phosphorylation of Tyr-307, which has been reported to reduce PP2Ac activity by up to 90% (32). We assessed whether MDP treatment altered tyrosine phosphorylation of PP2Ac on Tyr-307 by immunoblot using a phosphospecific antibody to PP2Ac Tyr-307. We observed a significant increase in Tyr-307 phosphorylation upon MDP treatment of HCT116 cells (Fig. 7A). Induction of PP2Ac phosphorylation was dependent on RIP2 expression because RNAi-mediated knockdown of RIP2 ablated MDP stimulated phosphorylation of PP2Ac Tyr-307 (Fig. 7A). RIP2 kinase activity was also found to be required for this modification because pretreatment of HCT116 with erlotinib similarly blocked MDP-stimulated phosphorylation of Tyr-307 (Fig. 7B). However, RIP2 does not appear to directly phosphorylate PP2Ac because results from in vitro kinase assays were negative (supplemental Fig. 4). We also tested whether PPP2R1A expression was required for MDP-induced phosphorylation of PP2A on Tyr-307. We found that MDP-induced phosphorylation of Tyr-307 was prevented by RNAi knockdown of PPP2R1A (Fig. 7C). These results indicate that upon MDP stimulation, the phosphatase activity of the PP2Ac-PPP2R1A complex is inhibited through the phosphorylation of PP2Ac on Tyr-307 in a process dependent on the kinase activity of RIP2.
RIP2 Mediates NOD2-dependent Autophagy

**DISCUSSION**

Autophagy is a crucial innate immune response for the clearance of intracellular pathogens (2). Previous studies have linked genetic variants of autophagy genes and NOD2 to defects in the development of new therapeutics for CD and chronic inflammatory diseases, such as tuberculosis and leprosy.

The exact mechanism behind how NOD2 directs autophagosome formation is still under examination, and there has been some discrepancy regarding whether this process requires NOD2 signaling (9, 10, 12) or is mediated solely by direct recruitment of autophagic machinery to sites of bacterial invasion (11). Our data as well as data from other groups (9, 10, 12) suggest that NOD2 function is involved in clearance of several bacteria (M. tuberculosis, M. leprae, S. typhimurium, and L. monocytogenes (7, 34)). A more complete understanding of the mechanisms underlying the induction of autophagy may have a significant effect on development of new therapeutics for CD and chronic bacterial diseases, such as tuberculosis and leprosy.

Several reports examining NOD2-dependent autophagy demonstrate a requirement for RIP2 expression (9, 10, 12), but the role of RIP2 kinase activity in this process was unknown. Early analyses of NOD1 and NOD2 signaling indicated that RIP2 functioned primarily as a scaffolding protein to induce the proximity of signaling molecules for the initiation of NFκB signaling (35). This was further supported by reports demonstrating that the polyubiquitination of RIP2 required for the recruitment of TAK1 is independent of RIP2 kinase activity (36, 37).

Later studies also suggested that the only role for RIP2 kinase activity in this process was unknown. Recent reports indicate that RIP2 tyrosine kinase activity is required for MDP-induced signaling and cytokine secretion (14). Our data demonstrate that NOD2 signaling is required for autophagy induction, and RIP2 tyrosine kinase activity plays an essential dual-faceted role in this process. RIP2 tyrosine kinase activity both provides an activating signal through stimulation of MAPK p38 and relieves PP2Ac-PPP2R1A-mediated repression of autophagy (Fig. 8).

The MAPK p38 has been reported to either negatively or positively regulate autophagy dependent on stimulus as well as cell context. Mechanistically, the process by which p38 regulates autophagy is not well understood, with both enzymatic examined and highlights the need to study autophagy in a cell- and stimulus-dependent context.
and transcriptional roles proposed. For example, TLR4 (Toll-like receptor 4)-stimulated autophagy is mediated by p38-dependent expression of the autophagy gene, LRG47 (40). In other reports, it has been proposed that p38 activation and production of reactive oxygen species results in autophagy induction; however, these reports differ on whether autophagy is stimulated by p38-dependent reactive oxygen species generation or activation of p38 by reactive oxygen species (41–43). Alternatively, p38 activity has been suggested to suppress autophagy by mechanisms targeting ATG9 trafficking and activation of mTOR (44, 45). Our studies demonstrate a positive role for p38 in the induction of autophagy in a ULK1-, Beclin-1-, and ATG16L1-dependent manner and independent of NFκB signaling in human intestinal epithelial cells (Fig. 8). This contrasts with recently published results showing an essential role for ERK1/2 in Listeria-stimulated autophagy in mouse macrophages and may reflect differences in anti-bacterial responses of professional immune cells versus epithelial cells. Future studies will focus on defining the mechanism by which p38 kinase activity promotes NOD2-stimulated autophagy in epithelial cells.

Activation of p38 as well as other MAPKs has been shown to be regulated by PP2A (46). This has been most clearly demonstrated in the context of tumorigenesis and cellular transformation, where inhibition of PP2A leads to enhanced MAPK activity and cellular proliferation (29). PP2A targets multiple steps in

**RIP2 Mediates NOD2-dependent Autophagy**

**FIGURE 5.** The phosphatase activity of the PPP2R1A-PP2A complex is required for regulation of MDP-enhanced autophagy. A, gentamycin protection assay performed in HCT116 cells transfected with vector or PP2Ac expression plasmids for 48 h. Cells were treated with medium, MDP (10 μg/ml), or rapamycin (25 μg/ml) during Salmonella infection and assayed in triplicate, with averages ± S.D. (error bars) graphed. ***, p < 0.001. B, gentamycin protection assay performed in HCT116 cells transfected with vector or PP2Ac expression plasmids and control or PPP2R1A siRNA for 48 h. Cells were treated with medium or MDP (10 μg/ml) during Salmonella infection and assayed in triplicate, with averages ± S.D. graphed. **, p < 0.01. C, immunoblot of LC3-II levels from HCT116 cells transfected with empty vector, wild-type PP2Ac (PP2Ac Wt), and phosphatase-dead PP2Ac (PP2Ac Mt) expression constructs and treated with MDP (10 μg/ml) for the indicated times. Membranes were also probed with HA antibody to confirm expression of PP2Ac constructs and GAPDH as a loading control. D, gentamycin protection assay performed in HCT116 cells transfected with vector, PP2Ac Wt, or PP2Ac Mt expression plasmids for 48 h. Cells were treated with medium or MDP (10 μg/ml) during Salmonella infection and assayed in triplicate, with averages ± S.D. graphed. **, p < 0.01. E, gentamycin protection assay performed in HCT116 cells transfected with vector or PP2Ac Mt expression plasmids and control, NOD2, p38, or ATG16L1 shRNA for 48 h. Cells were treated with medium or MDP (10 μg/ml) during Salmonella infection and assayed in triplicate, with averages ± S.D. graphed. ***, p < 0.001.
the MAPK cascade, depending on the subunit composition of the PP2A complex. For example, PP2Ac-α4 inhibits TGF-β-stimulated p38 MAPK activation through dephosphorylation of the upstream MAPK kinase, MEK3 (27). This contrasts with the results of our studies, where we did not observe an effect of PP2A on the activation of p38 MAPK in response to MDP (Fig. 6). Conversely, the PP2Ac-PPP2R1A complex we analyzed appears to specifically target a substrate downstream of p38 involved in both NOD2-dependent autophagy and IL-8 secretion in epithelial cells.

PP2A has been reported to play both positive and negative roles in autophagy induction. This may be a reflection of multiple mechanisms for autophagy induction, which vary for different stimuli, as well as the combinatorial nature of the PP2A complex, where numerous regulatory subunits can combine with the PP2Ac subunit to form dimeric or trimeric complexes (21). For example, a dimeric PP2Ac-α4 complex has been demonstrated to negatively regulate starvation-induced autophagy in a process dependent on ULK1 (24). Likewise, we found that NOD2-triggered autophagy is also dependent on ULK1 and repressed by PP2A, although the complex we identified in our cells was composed of PP2Ac-PPP2R1A. We determined that this PP2A complex interacts with NOD2-RIP2 and becomes tyrosine-phosphorylated on a negative regulatory site (Tyr-307) upon MDP stimulation. Although RIP2 tyrosine kinase activity is required for phosphorylation of PP2Ac, our in vitro kinase assay results suggest that PP2A is not a direct target of RIP2 kinase activity (supplemental Fig. 4). Future studies,
including the identification of the RIP2-activated tyrosine kinase responsible for inhibitory phosphorylation of PP2A and the downstream targets of the PP2A phosphatase, will provide further insight into the molecular signaling mechanisms controlling anti-bacterial autophagy.

Acknowledgments—We thank the members of the Cleveland Clinic IBD Group (Claudio Fiocchi, Carol de la Motte, Jean-Paul Achkar, and Eleni Stylianou) as well as Derek Abbott (Case Western Reserve University) and Neil Warner (University of Michigan) for numerous helpful discussions related to these studies.

REFERENCES

1. He, C., and Klionsky, D. J. (2009) Regulation mechanisms and signaling pathways of autophagy. Annu. Rev. Genet. 43, 67–93
2. Levine, B., Mizushima, N., and Virgin, H. W. (2011) Autophagy in immunity and inflammation. Nature 469, 323–335
3. Franke, A., McGovern, D. P., Barrett, J. C., Wang, K., Radford-Smith, G. L., Ahmad, T., Lees, C. W., Balschun, T., Lee, J., Roberts, R., Anderson, C. A., Bis, J. C., Bumpstead, S., Ellinghaus, D., Festen, E. M., Georges, M., Green, T., Haritunians, T., Jostins, L., Latiano, A., Mathew, C. G., Montgomery, G. W., Prescott, N. I., Raychaudhuri, S., Rotter, J. I., Schumm, P., Sharma, Y., Simms, L. A., Taylor, K. D., Whiteman, D., Wijmenga, C., Baldassano, R. N., Barclay, M., Bayless, T. M., Brand, S., Bünning, C., Cohen, A., Colomboi, J. F., Cottone, M., Stronati, L., Denson, T., De Vos, M., D'Inca, R., Dubinsky, M., Edwards, C., Florin, T., Franchimont, D., Geary, R., Glas, J., Van Gossum, A., Guthery, S. L., Halfvarson, J., Verspaget, H. W., Hugot, J. P., Karban, A., Laukens, D., Lawrence, I., Lemm, M., Levine, A., Libouille, C., Louis, E., Mowat, C., Newman, W., Panés, J., Phillips, A., Proctor, D. D., Regueiro, M., Russell, R., Rutgeerts, P., Sanderson, J., Sans, M., Seibold, F., Steinhardt, A. H., Stokkers, P. C., Torkvist, L., Ullakko-Ublick, G., Wilson, D., Walters, T., Targan, S. R., Brant, S. R., Rioux, J. D., D'Amato, M., Weersma, R. K., Kugathasan, S., Griffiths, A. M., Mansfield, J. C., Vermeire, S., Duerr, R. H., Silverberg, M. S., Satasi, J., Schreiber, S., Cho, J. H., Annese, V., Hakonarson, H., Daly, M. J., and Parkes, M. (2010) Genome-wide meta-analysis increases to 71 the number of confirmed Crohn’s disease susceptibility loci. Nat. Genet. 42, 1118–1125
4. Hampe, J., Franke, A., Rosenstiel, P., Till, A., Teuber, M., Huse, K., Albrecht, M., Mayr, G., De La Vega, F. M., Briggs, J., Günther, S., Prescott, N. J., Onnie, C. M., Häslar, R., Sipos, B., Fölsch, U. R., Lengaue, T., Platter, M., Mathew, C. G., Krawczak, M., and Schreiber, S. (2007) A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat. Genet. 39, 207–211
5. Rioux, J. D., Xavier, R. J., Taylor, K. D., Silverberg, M. S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barnama, M. M., Datta, L. W., Shugart, Y. Y., Griffiths, A. M., Targan, S. R., Ippoliti, A. F., Bernard, E. J., Mei, L., Nicolae, D. L., Regueiro, M., Schumm, L. P., Steinhardt, A. H., Rotter, J. I., Duerr, R. H., Cho, J. H., Daly, M. J., and Brant, S. R. (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat. Genet. 39, 596–604
6. Kabi, A., Nickerson, K. P., Homer, C. R., and McDonald, C. (2012) Digesting the genetics of inflammatory bowel disease. Insights from studies of autophagy risk genes. Inflamm. Bowel Dis. 18, 782–792
7. Strober, W., and Watanabe, T. (2011) NOD2, an intracellular innate immune sensor involved in host defense and Crohn’s disease. Mucosal Immunol. 4, 484–495
8. Abraham, C., and Cho, J. H. (2006) Functional consequences of NOD2 (CARD15) mutations. Inflamm. Bowel Dis. 12, 641–650
9. Cooney, R., Baker, J., Brain, O., Danis, B., Pichulik, T., Allan, P., Ferguson, D. J., Campbell, B. J., Jewell, D., and Simmons, A. (2010) NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat. Med. 16, 90–97
10. Homer, C. R., Richmond, A. L., Rebert, N. A., Achkar, J. P., and McDonald, C. (2010) ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn’s disease pathogenesis. Gastroenterology 139, 1630–1641, 1641.e1–1641.e2
11. Travassos, L. H., Carneiro, L. A., Ramjeet, M., Hussey, S., Kim, Y. G., Magalhães, J. G., Yuan, L., Soares, F., Chea, E., Le Bourhis, L., Boneca, I. G., Allaoui, A., Jones, N. L., Nuñez, G., Girardin, S. E., and Philpott, D. J. (2010) Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat. Immunol. 11, 55–62
12. Anand, P. K., Tait, S. W., Lamkanfi, M., Amer, A. O., Nunez, G., Pagès, G., Pouyssegur, J., McGarrigle, M. A., Green, D. R., and Kanneganti, T. D. (2011) TLR2 and RIP2 pathways mediate autophagy of Listeria monocytogenes via ERK activation. J. Biol. Chem. 286, 42981–42991
13. Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamakawa, S., and Nunez, G. (2001) Nod2, a Nod1/Apath-1 family member that is restricted to mono- cytes and activates NF-κB. J. Biol. Chem. 276, 4812–4818
14. Tigno-Aranjuez, J. T., Asara, J. M., and Abbott, D. W. (2010) Inhibition of RIP2’s tyrosine kinase activity limits NOD2-driven cytokine responses. Genes Dev. 24, 2666–2677
15. Boehm, J. S., Zhao, J. I., Yao, J., Kim, Y. S., Firestein, R., Dunn, I. F., Sjostrom, S. K., Garraway, L. A., Weremowicz, S., Richardson, A. L., Greulich, H., Stewart, C. I., Mulvey, L. A., Shen, R. R., Ambrogio, L., Hizono, Kishikawa, T., Hill, D. E., Vidal, M., Meyerson, M., Grenier, J. K., Hinkle, G., Root, D. E., Roberts, T. M., Lander, E. S., Polyzak, K., and Hahn, W. C. (2007) Integrative genomic approaches identify IKKBE as a breast cancer oncogene. Cell 129, 1065–1079

RIP2 Mediates NOD2-dependent Autophagy

FIGURE 8. Model of the molecular pathway required for NOD2-dependent autophagy. Essential activating components identified in this study are outlined in black and filled in with dark gray, whereas negative regulators are shown in light gray. Non-essential NOD2 signaling molecules are shown in light gray outlines. Connecting lines ending in balls indicate an inhibitory effect. Dashed lines indicate an indirect link between components.
RIP2 Mediates NOD2-dependent Autophagy

16. Eitelhuber, A. C., Warth, S., Schimmack, G., Düwel, M., Hadian, K., Denk, K., Beisker, W., Shinozaka, H., Kurowski, T., Heissmeyer, V., and Kappmann, D. (2011) Diphosphorylation of Carma1 by PP2A negatively regulates T-cell activation. EMBO J. 30, 594–605
17. Richmond, A. L., Kabi, A., Homer, C. R., Marina-García, N., Nickerson, K. P., Verjovitch, A., Sreekumar, A., Chinnaiyan, A. M., Núñez, G., and McDonald, C. (2012) The nucleotide synthesis enzyme CAD inhibits NOD2 anti-bacterial function in human intestinal epithelial cells. Gastroenterology 142, 1483–1492.e6
18. Birmingham, C. L., Smith, A. C., Bakowski, M. A., Yoshimori, T., and Brumell, J. H. (2006) Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. J. Biol. Chem. 281, 11374–11383
19. Godl, K., Wissing, J., Kurtenbach, A., Habenberger, P., Blencke, S., Gutfroh, H., Salassidis, K., Stein-Gerlach, M., Misio, A., Cotten, M., and Daub, H. (2003) An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. Proc. Natl. Acad. Sci. U.S.A. 100, 15434–15439
20. Huse, M., and Kuriyan, J. (2002) The conformational plasticity of protein kinases. Cell 109, 275–282
21. Virshup, D. M., and Shenolikar, S., (2009) From promiscuity to precision. Protein phosphatases get a makeover. Mol. Cell. 33, 537–545
22. Park, I. H., Yeum, C. E., Chae, G. T., and Lee, S. B. (2008) Effect of rifamycin to inhibit rapamycin-induced autophagy via the suppression of protein phosphatase 2A activity. Immunopharmacol. Immunotoxicol. 30, 837–849
23. Qi, Z., Yang, W., Liu, Y., Cui, T., Gao, H., Duan, C., Lu, L., Zhao, C., Zhao, H., and Yang, H. (2011) Loss of PINK1 function decreases PP2A activity. J. Biol. Chem. 286, 6012–6025
24. Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L., and Elazar, Z. (2007) Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg6. EMBO J. 28, 1749–1760
25. Yuan, H., Perry, C. N., Huang, C., Iwai-Kanai, E., Carreira, R. S., Glembotski, C. C., and Gottlieb, R. A. (2009) LPS-induced autophagy is mediated by oxidative signaling in cardiomyocytes and is associated with cytoprotection. Am. J. Physiol. Heart Circ. Physiol. 296, H470–H479
26. Chen, J., Martin, B. L., and Brautigan, D. L., (1992) Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. Science 257, 1261–1264
27. Lee, J., and Stock, J. (1993) Protein phosphatase 2A catalytic subunit is methyl-esterified at its carboxyl terminus by a novel methyltransferase. J. Biol. Chem. 268, 19192–19195
28. Brooks, M. N., Rajaram, M. V., Azad, A. K., Amer, A. O., Valdivia-Arenas, M. A., Park, J. H., Núñez, G., and Schlesinger, L. S. (2011) NOD2 controls the nature of the inflammatory response and subsequent fate of Mycobacterium tuberculosis and M. bovis BCG in human macrophages. Cell Microbiol. 13, 402–418
29. Inohara, N., Kosaki, T., Lin, J., del Peso, L., Lucas, P. C., Chen, F. F., Ogura, Y., and Núñez, G. (2000) An induced proximity model for NF-κB activation in the Nod1/RICK and RIP signaling pathways. J. Biol. Chem. 275, 27823–27831
30. Abbott, D. W., Yang, Y., Hutt, J. E., Madhavarapu, S., Kellhier, M. A., and Cantley, L. C. (2007) Coordinated regulation of Toll-like receptor and NOD2 signaling by Lys-63-linked polyubiquitin chains. Mol. Cell. Biol. 27, 6012–6025
31. Hasegawa, M., Fujimoto, Y., Lucas, P. C., Nakano, H., Fukase, K., Núñez, G., and Inohara, N. (2008) A critical role of RICK/RIP2 polyubiquitination in Nod1-induced NF-κB activation. EMBO J. 27, 373–383
32. Dong, M., and Park, J. H. (2011) A role for RIP2 in the stability and consequently Nod1 and Nod2-mediated immune responses. J. Biol. Chem. 286, 19183–19188
33. Windheim, M., Lang, C., Peggie, M., Plater, L. A., and Cohen, P. (2007) Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide. Biochem. J. 404, 179–190
34. Xu, Y., Jagannath, C., Liu, X. D., Sharafrkhanee, A., Kolodziejska, K. E., and Eissa, N. T. (2007) Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 27, 135–144
35. Luo, Y., Zou, P., Zou, J., Wang, J., Zhou, D., and Liu, L. (2011) Autophagy regulates ROS-induced cellular senescence via p21 in a p38 MAPK-dependent manner. Exp Gerontol. 46, 860–867
36. Inohara, N., Koseki, T., Lin, J., del Peso, L., Lucas, P. C., Chen, F. F., Ogura, Y., and Núñez, G. (2008) A critical role of RICK/RIP2 polyubiquitination in Nod1/RICK and RIP signaling pathways. J. Biol. Chem. 275, 27823–27831
37. Yang, Y., Hutt, J. E., Madhavarapu, S., Kellhier, M. A., and Cantley, L. C. (2007) Coordinated regulation of Toll-like receptor and NOD2 signaling by Lys-63-linked polyubiquitin chains. Mol. Cell. Biol. 27, 6012–6025
38. Abbott, D. W., Yang, Y., Hutt, J. E., Madhavarapu, S., Kellhier, M. A., and Cantley, L. C. (2007) Coordinated regulation of Toll-like receptor and NOD2 signaling by Lys-63-linked polyubiquitin chains. Mol. Cell. Biol. 27, 6012–6025
39. Dong, M., and Park, J. H. (2011) A role for RIP2 in the stability and consequently Nod1 and Nod2-mediated immune responses. J. Biol. Chem. 286, 19183–19188
40. Windheim, M., Lang, C., Peggie, M., Plater, L. A., and Cohen, P. (2007) Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide. Biochem. J. 404, 179–190
41. Xu, Y., Jagannath, C., Liu, X. D., Sharafrkhanee, A., Kolodziejska, K. E., and Eissa, N. T. (2007) Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 27, 135–144
42. Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L., and Elazar, Z. (2007) Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg6. EMBO J. 26, 1749–1760
43. Yuan, H., Perry, C. N., Huang, C., Iwai-Kanai, E., Carreira, R. S., Glembotski, C. C., and Gottlieb, R. A. (2009) LPS-induced autophagy is mediated by oxidative signaling in cardiomyocytes and is associated with cytoprotection. Am. J. Physiol. Heart Circ. Physiol. 296, H470–H479
44. Cully, M., Genevet, A., Warne, P., Treins, C., Liu, T., Bastien, J., Baum, B., Tapon, N., Levers, S. J., and Downward, J. (2010) A role for p38 stress-activated protein kinase in regulation of cell growth via TORC1. Mol. Cell. Biol. 30, 481–495
45. Mayer-Jaekel, R. E., and Hemmings, B. A. (1994) Protein phosphatase 2A. A “menage a trois”. Trends Cell Biol. 4, 287–291
46. Mayer-Jaekel, R. E., and Hemmings, B. A. (1994) Protein phosphatase 2A. A “menage a trois”. Trends Cell Biol. 4, 287–291