Lipopolysaccharide primes the NALP3 inflammasome by inhibiting its ubiquitination and degradation mediated by the SCF\(^{FBXL2}\) E3 ligase.

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CAPSULE

Background: LPS increases NALP3 levels, but the mechanisms remain unknown.

Results: LPS prolongs the lifespan of NALP3 protein by reducing E3 ligase (SCF^{FBXL2})-mediated ubiquitination.

Conclusion: Pro-inflammatory cytokine release is reduced by a small molecule that restores cellular SCF^{FBXL2} levels.

Significance: We identified a novel pathway of inflammasome priming which may serve as a springboard for future translational studies.

ABSTRACT

The inflammasome is a multi-protein complex that augments the pro-inflammatory response by increasing the generation and cellular release of key cytokines. Specifically, the NALP3 inflammasome requires two-step signaling, priming and activation, to be functional to release the pro-inflammatory cytokines, IL-1β and IL-18. The priming process, through unknown mechanisms, increases the protein levels of NALP3 and pro-IL-1β in cells. Here, we show that lipopolysaccharide (LPS) increases NALP3 protein lifespan without significantly altering steady-state mRNA in human cells. LPS exposure reduces ubiquitin-mediated proteasomal processing of NALP3 by inducing levels of an E3 ligase component, FBXO3, which targets FBXL2, the latter an endogenous mediator of NALP3 degradation. FBXL2 recognizes W73 within NALP3 for interaction and targets K689 within NALP3 for ubiquitin ligation and degradation. A unique small molecule inhibitor of FBXO3 restores FBXL2 levels resulting in decreased NALP3 protein levels in cells and thereby reducing the release of IL-1β and IL-18 in human inflammatory cells after NALP3 activation. Our findings uncover NALP3 as a molecular target for FBXL2 and suggest that therapeutic targeting of the inflammasome may serve as a platform for preclinical intervention.
INTRODUCTION

The inflammasome is a large multi-protein cytosolic complex that consists of three components: a sensor (NALP), an adaptor (ASC), and an effector (pro-caspase 1). Inflammasomes have emerged as key complexes, that when properly assembled, result in the release of interleukin 1β (IL-1β) and interleukin 18 (IL-18) that partake in illnesses characterized by severe inflammation. Some inflammasomes are activated by pore-forming toxins, ATP, or hypoxic cellular injury when these conditions are primed with microbial ligands or endogenous cytokines (1). Once assembled, inflammasomes cleave and activate potent pro-inflammatory cytokines IL-1β and IL-18, which are associated with higher risk and worse outcomes of disorders such as the acute respiratory distress syndrome (ARDS) (2,3). NALP3, the most studied inflammasome thus far, requires two-step signaling to engage in cytokine release: priming and activation. The gram negative bacterial endotoxin lipopolysaccharide (LPS) is known to prime the NALP3 inflammasome by increasing NALP3 protein and pro-IL-1β levels in a dose- and time-dependent fashion in mice (4). However, the molecular mechanisms whereby LPS priming increases NALP3 protein abundance in cells remain elusive. This question is critical because identification of molecular targets that transcriptionally or post-translationally mediate increased NALP3 concentrations in cells could be important in pharmacotherapeutic design to control innate immune responses.

Protein ubiquitinylation is a highly conserved irreversible post-translational modification among vertebrates that governs cellular protein concentrations. The ubiquitin proteasome system (UPS) selectively targets proteins in humans by a series of enzymatic steps requiring a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin E3 ligase. E3 ligases are multi-subunit complexes that confer relatively high specificity to ubiquitination by bringing in close proximity a substrate to an E2 enzyme that facilitates the transfer of an activated ubiquitin moiety from the E2 to the substrate (5). Hundreds of human genes encode RING domain containing E3 ligases, and among these the Skp-Cullin-F box (SCF) family is among the best studied (6). The SCF superfamily regulates diverse biological events including cell growth, proliferation, DNA repair and inflammation (7).

We have previously demonstrated that an SCF subunit, F box protein, F-box O3 (FBXO3), is a pro-inflammatory molecule that is activated in leukocytes by endotoxin (8). FBXO3 ubiquitinates and mediates degradation of another F box protein, F-box L2 (FBXL2); FBXL2 is an anti-inflammatory molecule that mediates degradation of tumor necrosis factor (TNF) receptor associated factors (TRAFs), resulting in a reduction of inflammatory cytokine release. The aim of our study is to investigate how LPS priming increases NALP3 inflammasome protein levels. Here we show that NALP3 is also a target for FBXL2. Moreover, we demonstrate a unique mechanism whereby LPS priming decreases FBXL2-mediated NALP3 ubiquitination and degradation by activating the FBXL2 inhibitor, FBXO3 in cells. A small molecule FBXO3 antagonist, BC-1215, potently reduces the severity of NALP3-mediated inflammation by increasing FBXL2 protein levels, thus antagonizing actions of endotoxin on the inflammasomes.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

Antibodies against FBXL8, anti-mouse IgG and anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against FBXL2 were from Aviva systems biology (San Diego, CA). Antibodies against F-box L7 (FBXL7) and NALP6 were obtained from Abcam (Cambridge, MA, USA). Ubiquitin and IL-1β antibodies were purchased from Cell Signaling (Danvers, MA, USA). NALP3 antibodies were purchased from Cell Signaling (Danvers, MA, USA). GAPDH and β-actin antibodies were obtained from Sigma (Saint Louis, MO, USA). IL-18 human antibodies were purchased from MBL (Woburn,
MA). QuikChange II site-directed mutagenesis kits were purchased from Agilent Technologies (Santa Clara, CA, USA). Pierce Protein A/G Agarose beads were purchased from Thermo Scientific (Rockford, IL, USA). Cycloheximide (CHX), MG132, and leupeptin were from Calbiochem (Darmstadt, Germany). Mouse V5 antibodies, pcDNA3.1D cloning kits, and the pENTR Directional TOPO cloning kits were purchased from Invitrogen. ATP was obtained from Sigma. Miniprep spin kits were obtained from Qiagen (Valencia, CA, USA) and Nucleobond Xtra Maxi prep kits were obtained from Macherey-Nagel (Düren, Germany). In vitro transcription and translation (TnT) kits were from Promega. BC-1215 was synthesized to >98% purity.

**Cell Culture**

Human monocyte U937 and THP1 cells were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human erythroleukemia K562 cells, human bronchial epithelial A549 cells, Beas2B cells, mouse lung epithelial (MLE-12) cells, and human cervical epithelial HeLa cells were obtained from ATCC (Manassas, VA, USA). Human primary macrophages and their culture medium were purchased from Celprogen (San Pedro, CA, USA). Bronchoscopic was performed to collect bronchoalveolar lavage fluid after obtaining informed consent. Human primary alveolar macrophages from the fluid were cultured in culture medium (Celprogen) as previously described (9). The study was approved by the University of Pittsburgh Institutional Review Board. DMEM and RPMI 1640 medium were obtained from Gibco (Life Technologies, Grand Island, NY) supplemented with fetal bovine serum. CHX treatment was carried out at a concentration of 40 µg/mL at varying time points in 0% FBS medium, with or without MG132 (20 µg/mL) or leupeptin (20 µg/mL).

**Immunoprecipitation and Immunoblotting**

Cells were lysed with lysis buffer (0.05% Triton X-100 in PBS and 1:1000 protease inhibitor mixture), and sonicated. After centrifugation at 13,200 rpm for 10 min, cell lysates (containing 2 mg of protein) was incubated and rotated with 5 µg of anti-ubiquitin, anti-NALP3 or FBXL2 antibody at 4 °C for 3 h and then incubated with 30 µL of protein A/G-agarose beads overnight. The next day, the beads were spun down and washed with lysis buffer three times. The washed beads were mixed with SDS-PAGE loading dye prior to SDS-PAGE and immunoblot analysis. Immunoblotting was performed as described previously (10). We precipitated extracellular media with trichloroacetic acid, and subsequently measured cytokine levels by immunoblotting.

**RT-PCR**

RNA was isolated from cells using RNeasy Mini Kits (Qiagen) per the protocol provided. Isolated RNAs were immediately converted to cDNA using High Capacity RNA-to-cDNA Kits (Life Technologies, Grand Island, NY) after their concentrations were measured. Real-time PCR assays were performed using SYBR® Select Master Mix for CFX (2X) (Life Technologies, Grand Island, NY) per the protocol provided in C1000 Thermal Cycler (BioRad, Hercules, CA).

**Cloning and Mutagenesis**

Human NALP3 cDNA was cloned into a pcDNA3.1D/V5-His vector. Site-directed mutagenesis of lysine to arginine residues was carried out via a PCR-based approach using the QuikChange II XL kit from Agilent Technologies (Santa Clara CA, USA) and appropriate primers. Primers used for lysine to arginine mutagenesis were K689R forward (5′-CATAACATGCCCAGGGAGGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAAGAG-3′) and reverse (5′-TGTGCCCTCCTTCACATGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA-3′).
3') and reverse (5'-
CTCTCCTCTCCCTGGCATGTTATG-3');
K696R forward (5'-
GAAGAGGAGGAAAGGAAGGCCGA
CACCTTG-3') and reverse (5'-
CAAGGTGTGCCCTCCTCTTCTCCTCTCC
TCTTC-3'); K86R forward (5'-
GAGGACCTTTATGAGGAGGAAAAAGA
GATGAG-3') and reverse (5'-
CTCATCTCTTTTGTCTCTCTATAAGGT
CTCTC-3'); and K742R forward (5'-
GGTCCTCAGCAGCAACCAGAGGCTGGTG
GAGCTGGACCTGAG-3') and reverse (5'-
CTCATCTCTTTTGTCTCCTCTATAAGGT
CTCTC-3'). Generated mutants were sequence-
confirmed (10).

Transfection
2.5×10^6 MLE cells were suspended in 100 µL of
20 mM HEPES and mixed with 4 µg of DNA in
a cuvette. Cells were nucleofected on the T-013
protocol using an Amaxa Nucleofector II device
(Basel, Switzerland). Immediately following
nucleofection, 0.5 mL of 10% HITES medium
was added to the cuvette and the cell solution
was plated in 1.5 mL of 10% HITES culture
medium in a six-well plate. Cells were allowed
to grow until 50% confluency prior to half-life
experiments (48-72 h). For HeLa cells, the
PolyJet™ DNA In Vitro Tranfection Reagent
was used following SignaGen's protocols
(Rockville, MD). Cells were then treated with 40
µg/mL of CHX at different time points for half-
life studies and were harvested in lysis buffer
using a rubber policeman for subsequent
immunoblot analysis. Small interfering RNA
(siRNA) to FBXL2, purchased from Integrated
DNA Technologies (Coralville, IA, USA), was
introduced into primary human macrophages
using GenMute™ siRNA Transfection Reagent
based on SignaGen’s protocols.

In vitro ubiquitination conjugation assay
The details of this assay were described
previously (11). Briefly, the ubiquitination of
NALP3 was performed in a volume of 25 µL
containing 50 mM Tris (pH 7.6), 5 mM MgCl₂,
0.6 mM DTT, 2 mM ATP, 1.5 ng/µL E1, 10
ng/µL Ubc5, 10 ng/µL Ubc7, 1 µg/µL ubiquitin
(Calbiochem), 1 µM ubiquitin aldehyde, 4-16
µL purified Cullin1, Skp1, Rbx1, and in vitro-
synthesized FBXL2. Reaction products were
processed for NALP3 immunoblotting.

In vitro protein binding assays
FBXL2 protein was immunoprecipitated from 1
mg cell lysate using FBXL2 antibody and
coupled to protein A/G agarose resin. FBXL2
beads were then incubated with in vitro
synthesized products (50 µL) expressing
NALP3-V5 mutants. After washing, the proteins
were eluted and processed for NALP3-V5
immunoblotting.

Enzyme-linked immunosorbent assay (ELISA)
Quantikine ELISA kits were purchased from
R&D systems (Minneapolis, MN, USA) to
measure human IL-1β and human IL-18 levels.
The quantitative assays were performed
following protocols provided by the
manufacturer using cell culture supernatants
after LPS/ATP exposure with or without BC-
1215.

Statistical Analysis
Descriptive statistics were reported with mean ±
standard deviation (SD) or standard error (SE) as
indicated. As our sample sizes of each
experimental group were all less than 10 which
limits ability to study a normal sample
distribution, we employed appropriate non-
parametric methods such as a Mann-Whitney U
test and a Kruskal-Wallis equality-of-
populations rank test to compare multiple groups
within and between experiments. Also, a
Wilcoxon-type test for trend was used where
appropriate to check trends in data significance.
Using these methods provided conservative
analysis to determine statistical significance. All
analyses were performed using Stata Statistical
Software: Release 10.1 (StataCorp. 2007.
College Station, TX: StataCorp LP).
RESULTS

LPS priming up-regulates steady-state NALP3 protein levels by prolonging its half-life.

Human monocyte U937 cells express NALP3 as evidenced by immunoblotting with a NALP3 antibody. NALP3 protein levels increased in a dose- and time-dependent fashion with LPS exposure, while NALP6, another NOD-like receptor did not (Fig. 1A and 1B). There were modest, if any, increases in mRNA expression of NALP3 with LPS exposure (Fig. 2A and 2B), and the changes were not statistically significant. These data suggest that LPS up-regulates NALP3 protein mainly through post-translational mechanisms. Thus, we measured the half-life of NALP3 protein both at baseline and after LPS exposure, by blocking new protein synthesis with CHX. The half-life of NALP3 is approximately 4 h in an unchallenged condition, and prolonged to >6 h after LPS exposure (Fig. 2C and 2D). NALP3 degradation was reduced with inclusion of MG132 in the medium, but not with a lysosomal inhibitor, leupeptin, indicating the degradation of NALP3 occurs via the UPS rather than the lysosome (Fig. 2E and 2F).

FBXL2 mediates NALP3 ubiquitination and degradation, an effect inhibited by LPS.

To confirm that NALP3 is degraded through the UPS, we transfected MLE cells with either control plasmids or HA-ubiquitin plasmids. High-level ubiquitin cellular expression correlated with lower levels of NALP3 protein in cells (Fig. 3A). NALP3-ubiquitin binding, measured by co-IP, was decreased by 40% after LPS exposure when densitometrically controlled for input loading (Figs. 3B and 3C). Hence NALP3 is ubiquitinated, an effect reduced by LPS.

Because our prior studies demonstrated that FBXO3 targets FBXL2 for its disposal in cells, and an FBXO3 inhibitor reduces IL-1β levels after LPS treatment (8), we tested the hypothesis that FBXL2 targets NALP3 to mediate its degradation. Indeed, the ubiquitination of NALP3 is increased after expression of FBXL2. As shown in Fig. 4A, ectopic expression with FBXL2 plasmid in MLE cells decreases levels of NALP3. Further, NALP3 protein levels in both MLE cells and primary human macrophages were increased by using one of the several lentivirally delivered short hairpin RNAs (shRNAs) or small interfering RNAs (siRNAs) to FBXL2 (Fig. 4B). To determine if FBXL2 interacts with NALP3, we performed co-IP studies showing that FBXL2 binds NALP3 selectively as it does not associate with the NALP6 inflammasome (Fig. 4C). When V5-NALP3 was overexpressed in cells, in co-IP studies it was associated with FBXL2 but not other F-box proteins, FBXL7 or FBXL8. In vitro ubiquitin assays demonstrated that FBXL2 robustly catalyzes NALP3 ubiquitination (Fig. 4D). The binding between NALP3 and FBXL2 was also decreased after LPS exposure, as was NALP3 ubiquitination (Fig. 4E and 4F). These observations suggest that NALP3 is a molecular target for FBXL2 mediated degradation, a process impaired by endotoxin.

K689 is a NALP3 ubiquitination acceptor site.

Protein ubiquitination involves generation of an isopeptide bond between an acceptor lysine on a target protein (e.g. NALP3) and the C-terminal glycine of the incoming ubiquitin moiety. Human NALP3 protein has 68 K residues. Using the UbPred software (12), potential ubiquitination sites in NALP3 protein were predicted among the K residues: One with high probability (K689), five with medium probability (K93, K192, K324, K430, and K696), and six residues with low probability (K86, K194, K437, K510, K742, and K958) (Fig. 5A). We cloned the human NALP3 cDNA into a pcDNA3.1D/V5-His vector, and generated mutants substituting a arginine at each of the predicted K residues within NALP3 protein. MLE cells were transfected with wild-type and mutant plasmids, and were exposed to CHX to measure the half-life of ectopically expressed NALP3. The K689R NALP3 mutant has an extended half-life (>6 h) in cells compared to wild-type NALP3 resembling effects of LPS exposure on wild-type NALP3 (Fig. 2C, Figs. 5B and 5C); other expressed
mutants did not exhibit a significantly prolonged lifespan in cells (Fig. 5D). Co-transfection of NALP3 mutant plasmids with or without FBXL2 plasmid decreased ectopically expressed wild-type NALP3 protein and a variant, K696R. However, levels of the K689R NALP3 mutant were not decreased with overexpressed FBXL2 (Fig. 5E). In vitro ubiquitin assays confirmed that the K689R NALP3 mutant is not ubiquitinated in comparison to wild-type or another mutant NALP3 (Fig. 5F). Thus, K689 is likely a major ubiquitin acceptor residue within human NALP3 protein.

**NALP3 W73 is a molecular recognition site for FBXL2 interaction.**

We next investigated the FBXL2 binding site within NALP3. Several deletion mutants of NALP3 were constructed and cloned in a pcDNA3.1D V5/HIS/TOPO vector (Fig. 6A). Through progressive mapping we determined that FBXL2 binds the N-terminal region of NALP3, spanning residues 70 to 80 (Fig. 6B and 6C). An alanine scan within this region suggested W73 is a critical residue for FBXL2 binding (Fig. 6D). We tested the half-life of a NALP W73A mutant and a control W68A mutant using CHX treatment. W73A exhibited delayed turnover, with an extended half-life from 4 h to >6 h compared to wild-type or a mutant (W68A) NALP3 protein (Fig. 6E).

Further, to evaluate the biologic role of these NALP3 proteins in cells, we exposed cells to the FBXO3 inhibitor, BC-1215 (8), that in principle should increase FBXL2 levels thereby reducing abundance of its cellular targets such as NALP3. Indeed, exposure of cells to BC-1215 after cellular transfection of NALP3 wild-type, NALP3 W68A, or NALP3 W73A plasmids revealed that the W73A protein was resistant to BC-1215 driven (FBXL2-mediated) NALP3 degradation (Fig. 6F). Finally we co-expressed NALP3 wild-type or NALP3 W73A plasmids with FBXL2 and observed that the W73A variant is resistant to degradation (Fig. 6G). These results indicate that W73 is a key molecular dock site for FBXL2 interaction and its substitution with Ala confers proteolytic resistance within NALP3.

**An FBXO3 inhibitor attenuates LPS induction of NALP3 protein.**

FBXO3 is a pro-inflammatory E3 ligase subunit that eliminates the anti-inflammatory E3 ligase subunit, FBXL2 (8). Hence, our model suggests that LPS increases FBXO3 protein levels that in turn decreases FBXL2 levels (11). The net effect of LPS in this model is increased NALP3 protein levels. We have previously used in silico modeling to design, synthesize, and test a new genus of small-molecules targeting the ApaG motif within the C-terminus of FBXO3. In preclinical studies we observed that BC-1215 binds the F-box protein and significantly decrease the severity of inflammation in multiple murine models (11). Here, our results suggest an FBXO3 ─ FBXL2 ─ NALP3 inflammasome → IL-18 and IL-1β pathway. We validated this pathway by testing BC-1215 in human monocytes. First we verified that BC-1215 in a concentration-dependent manner increases FBXL2 levels in human monocytic U937 cells (Fig. 7A). In a relatively selective manner, NALP3 protein levels were significantly decreased with BC-1215 treatment with or without LPS exposure in human monocyte U937 and THP1 cells (Fig. 7B). BC-1215 destabilizes NALP3 protein following LPS exposure in a dose- and time-dependent fashion without significant changes in steady-state mRNA expression of NALP3 (Fig. 7C and 7D). The prolonged half-life of NALP3 protein after LPS-priming is effectively decreased with BC-1215 treatment to baseline levels without LPS-priming (Fig 7E). In the presence of LPS, BC-1215 did not decrease ectopically overexpressed K689R mutant NALP3 protein levels harboring a modified ubiquitin acceptor residue, compared to wild-type or a mutant (K696R) NALP3 protein (Fig. 7F). The secretion of IL-1β and IL-18, the downstream products of the NALP3 inflammasome, was also decreased with BC-1215 treatment in human inflammatory cell lines including THP1 and K562 cells (Fig. 7G). The ratio of active IL-1β versus pro-IL-1β was also decreased with BC-1215 in a time-dependent fashion (Fig. 7H), suggesting the main mechanism to reduce cytokine release is from decreased conversion from premature to mature forms of cytokines. Lastly, we examined ability...
of the FBXO3 small molecule inhibitor on primary human alveolar macrophages after LPS priming for the NALP3 inflammasome. BC-1215 effectively decreased LPS induction of NALP3 protein induction by 44% (by densitometry when adjusted for β actin loading), and also reduced the secretion of IL-1β and IL-18 after a second stimulus, ATP (Fig. 7I and 7J). Collectively, these observations strongly implicate FBXO3 and FBXL2 as molecular inputs to NALP3 cytokine signaling.

**DISCUSSION**

These studies demonstrate a unique mechanism whereby LPS priming prolongs the lifespan of NALP3 protein in cells. LPS stabilizes the NALP3 protein by inhibiting its FBXL2-mediated poly-ubiquitination and degradation, presumably by limiting its interaction with the inflammasome: SCF^FBXL2^ complex. We also identified K689 as a putative ubiquitin acceptor site and W73 as an FBXL2 binding site within the NALP3 protein. The expression of plasmids encoding mutation at these molecular sites in cells resulted in an extended half-life of NALP3 protein despite co-expression of FBXL2 or treatment with a potent FBXO3 inhibitor. Hence our data support a FBXO3 — FBXL2 — NALP3 inflammasome → cytokine pathway that was validated by observing decreased NALP3 protein and associated cytokines with treatment of an FBXO3 inhibitor. The results raise opportunities for therapeutic targeting of upstream inflammasome regulators to reduce inflammation in a variety of cytokine-driven disorders such as ARDS.

The inflammasome has emerged as a critical intracellular multi-protein complex involved with a variety of inflammatory diseases such as ARDS, cryopyrin-associated periodic syndromes (CAPS), gout, and type II diabetes (13). To initiate the release of pro-inflammatory cytokines, NALP3 must first be primed by interacting with pathogen-associated molecular patterns (PAMPs) or host-derived danger signals (danger-associated molecular patterns, DAMPs). Once NALP3 senses second stimuli such as pore-forming toxins or crystals, the NALP3 scaffold recruits ASC adaptor and pro-caspase 1, and assembles into a functional inflammasome. This assembled complex then serves as a platform to activate pro-caspase 1 to caspase 1, which then cleaves pro-IL-1β and pro-IL-18 to their mature forms of IL-1β and IL-18, respectively. The molecular mechanisms whereby NALP3 inflammasomes are activated to release cytokines has been the focus of significant attention as it can provide insight for understanding disease pathogenesis and may ultimately uncover potential therapeutic targets. Initially, NF-kB activation, the traditional priming signal, was thought to induce gene transcription of NALP3 and pro-IL-1β, leading to NALP3 inflammasome activation (4). However, recent studies suggest that assembly of the NALP3 inflammasome to activate caspase-1 is regulated mainly through non-transcriptional processes (14, 15). Further, the final products of the activated NALP3 inflammasome, IL-1β and IL-18, are possibly regulated at post-translational levels suggesting concerted actions of modulators through this mode of regulatory control (16, 17).

Ubiquitination is a universal post-translational modification process in eukaryotic organisms as it serves as a distinct signal for protein disposal or sorting in cells. Further, some E3 ligases and ubiquitin components are activated by endotoxin (8, 18). Nod-like receptors (NLRs) interact with the ubiquitin ligase-associated components such as suppressor of the glycolysis regulation 2 gene that modulate inflammasome activity (19). Studies suggest that the ubiquitination system may play an important role in NALP3 inflammasome activation to release cytokines. For example, deubiquitinases may regulate the activity of caspase-1 and IL-1β secretion via the assembly of inflammasomes (20), although the level of BRCC3, one of the deubiquitinases targeting NALP3, does not correlate with the abundance of NALP3 protein after LPS exposure (21). An E3 ligase, tripartite motif 33, and the linear ubiquitin assembly complex also seem to be involved with NALP3 inflammasome activation (22, 23). In the process of preparing this manuscript, dopamine was recently reported to inhibit the NALP3 inflammasome activation via the dopamine D1 receptor by promoting actions of the E3
ubiquitin ligase, MARCH7 (24). However, to date studies have only focused on the link between ubiquitination and the terminal steps of NALP3 inflammasome activation – assembly of the inflammasome complex and subsequent release of cytokines. Despite evidence that LPS priming increases NALP3 protein levels, the precise molecular mechanisms elucidating how LPS priming up-regulates NALP3 protein levels and whether cytokine release can be altered by manipulating this priming process remains unclear. Here we describe an elegant mechanism by which LPS primes the NALP3 inflammasome by impairing its association with a previously unrecognized NALP3 inhibitor, FBXL2, which in turn extends inflammasome lifespan in cells. We identified that FBXL2 interacts with W73 facilitating ubiquitin ligation to K689 within NALP3 protein. Interestingly a tryptophan within TRAFs1–6 also serves as a critical docking site for FBXL2 interaction (8). Thus, tryptophan may be a common recognition site along with other motifs for FBXL2 recruitment to its substrates.

Previously the unique, selective small-molecule FBXO3 inhibitor, BC-1215, was shown to block IL-1β secretion triggered by LPS (11). The inhibition of FBXO3 resulted in an increase in FBXL2 cellular mass, promoting the degradation of NALP3 protein. Here, the inhibition of NALP3 protein by BC-1215 did not occur at the transcriptional level, but rather at the post-translational level by decreasing NALP3 protein half-life. As the amount of NALP3 protein after LPS priming is reduced with BC-1215 treatment, the absolute amount of activated NALP3 inflammasome complexes is coordinately decreased in cells, which led to a profound reduction of IL-1β and IL-18 release. One unexpected finding here is that prolonged exposure to the FBXO3 inhibitor also inhibited the LPS induction of pro-IL-1β and thus reduced the release of mature IL-1β as limited substrate is available for conversion to mature cytokine. This is probably due to the ability of BC-1215 to inhibit TRAF adaptor proteins (8), leading to the interruption of NF-κB activation that normally induces the pro-IL-1β gene (25). Indeed, shorter exposure to BC-1215 effectively decreased mature IL-1β without significant changes in pro-IL-1β, indicating reduced conversion from premature to mature IL-1β by the FBXO3 inhibitor (Fig. 7H). On the other hand, the reduction of IL-18 secretion by BC-1215 appears to be mediated simply through the inhibited conversion to mature cytokines. Pro-IL-18 is known to be expressed constitutively in cells in the absence of stimuli (17). The change in levels of pro-IL-18 protein after prolonged BC-1215 treatment was relatively small compared to that of pro-IL-1β. However, the reduction of mature IL-18 secretion was much more robust, similar to that of IL-1β, indicating that the mechanism of decreased IL-18 secretion by an FBXO3 inhibitor is mainly achieved by reducing the conversion of pro-IL-18 to mature IL-18 (Fig. 7G). These results are likely secondary to decreased levels of an activated NALP3 inflammasome complex by BC-1215.

In conclusion, our study shows that LPS prolongs the lifespan of the NALP3 protein, likely via reduced ubiquitin-mediated proteasomal processing. We found that SCF^FBXL2 constitutively mediates ubiquitination and degradation of NALP3, an effect disrupted by LPS. Through pharmacological inhibition of FBXO3 that targets disposal of FBXL2 in cells, NALP3 protein levels are profoundly reduced, subsequently decreasing the release of IL-1β and IL-18 in human inflammatory cell lines. Our findings provide valuable mechanistic insights for inflammasome priming, and this may uncover unique opportunities to develop therapeutic strategies to modulate cytokine signaling during inflammatory illness.
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FOOTNOTES

The abbreviations used are: NALP, NACHT, LRR and PYD domains-containing protein; Fbx, F-box; ASC, apoptosis-associated speck-like protein containing a carboxy-terminal caspase-recruitment domain; ARDS, acute respiratory distress syndrome; SCF, Skp-Cullin-F box; IL, interleukin; TNF, tumor necrosis factor; TRAF, TNF receptor associated factors; CHX, cycloheximide; MLE, mouse lung epithelial; UPS, ubiquitin proteasome system; Co-IP, co-immunoprecipitation; CAPS, cryopyrin-associated periodic syndrome; PAMP, pathogen-associated molecular patterns; DAMP, danger-associated molecular patterns.

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Figure legends

Figure 1. **LPS priming increases NALP3 protein in human monocyte U937 cells.** (A) Immunoblots for NALP3, NALP6 (negative control) and β-actin in lysates from human monocytes, U937 cells (total 3x10^6 cells), treated with LPS for 16 h as indicated, or (B) treated with LPS (200 ng/ml) for the indicated periods of time. The relative densitometries of NALP3 protein for each immunoblot are shown below. The data represent mean ± S.E. of n=3-5 independent experiments. *P-value; by a nonparametric test for trend.

Figure 2. **LPS priming increases NALP3 protein levels by prolonging its half-life.** Box plots of messenger RNA expression fold-increase of NALP3 in LPS primed human monocytes, U937 cells. (A) Cells were primed with LPS for 16 h as indicated or (B) LPS 200 ng/ml for the indicated periods of time. Data represent n=4 independent experiments. P-value; by a Kruskal-Wallis test. (C) U937 cells were incubated cycloheximide (CHX, (40 µg/ml) for various times with or without LPS priming (200 ng/mL for 16 h). (D) A densitometric plot of NALP3 protein decay versus time of CHX exposure with a best fit line; half-life, ~4 h in an unchallenged condition and >6 h after LPS priming. (E) U937 cells were incubated with CHX (40 µg/mL) and MG132 (20 µg/mL) or leupeptin (20 µg/mL), and immunoblotting for NALP3 was performed. (F) Decay curves over time in the presence of MG132 or leupeptin were best fitted. Data represent mean ± S.D. of n=5-8 independent experiments. *P-value by a Mann-Whitney test comparing two groups at each time point. *; p<0.05, **; p<0.01.

Figure 3. **NALP3 is degraded through the ubiquitin proteasome system.** (A) MLE cells were transfected with either control plasmid or HA-ubiquitin plasmid for 24 h. Cells were collected and assayed for HA, NALP3 and β-actin (loading control) by immunoblotting. (B) A549 cells were incubated with or without LPS (200 ng/mL for 16 h), and cells lysed and used to detect NALP3 or ubiquitin (left, input). In the right upper panel ubiquitin was immunoprecipitated followed by NALP3 immunoblotting showing reduced intensity of several bands (arrows). (C) The relative densitometric intensities of NALP3 bands are shown reflecting reduced association between NALP3 and ubiquitin by 40% after LPS exposure. The bar graph represents mean ± S.D. P-value; by Mann-Whitney test. The data in each panel is representative of at least n=2-3 independent experiments.

Figure 4. **FBXL2 triggers the NALP3 degradation.** (A) MLE cells were transfected with increasing amounts of FBXL2 plasmid for 24-48 h. Cells were collected and assayed for V5 (FBXL2), NALP3, and β-actin by immunoblotting. (B) MLE cells were transfected with several FBXL2 lentiviral short hairpin RNAs (shRNA, 4µg). Human primary macrophages also were transfected with several siRNA to FBXL2 (500 nM) for gene silencing. Shown are protein levels of FBXL2 after transfection of shRNAs or siRNAs and corresponding levels of NALP3 protein. Compared to a control RNA, despite modest reduction of FBXL2 levels increased NALP3 levels in shRNA or siRNA groups were observed. (C) U937 cells were lysed and NALP3 or FBXL2 proteins were co-immunoprecipitated by first i.p. of FBXL2 followed by NALP3, FBXL2, or NALP6 immunoblotting (top blot). In addition, Beas2B cells were transfected with NALP3-V5 prior to V5 i.p. and immunoblotting for NALP3 and various F box proteins (lower blot). IgG was used as a control in i.p. with a 10% input and a upper IgG chain band was detected in the FBXL7
immunoblot. (D) *In vitro* ubiquitination assays show NALP3 is a substrate for FBXL2 mediated ubiquitination. (E) A549 cells were incubated with or without LPS 200 ng/mL for 16 h, and cells lysed. FBXL2 protein was co-immunoprecipitated using i.p. of FBXL2 first followed by NALP3, FBXL2, or β-actin immunoblotting. (F) The relative association between FBXL2 and NALP3 after LPS were quantified using densitometric analysis of bands in (E). The binding capacity between NALP3 and FBXL2 is reduced by ~45-50% after LPS exposure. The data in each panel is representative of at least n=2-3 independent experiments.

Figure 5. **K689 is a NALP3 ubiquitination acceptor site.** (A) Full-length and point-mutant constructs encoding human NALP3 protein were cloned into a pcDNA3.1D/V5- His vector. (B) MLE cells were transfected with either the pcDNA-NALP3 full-length plasmid (4 µg) or point-mutant plasmid for 48 h. Cells were exposed to cycloheximide (CHX, 40 µg/mL) at different time points for a half-life study. Cells were collected and assayed for NALP3 (V5) and β-actin (loading control) by immunoblotting. Representative images are shown. (C) Densitometric plots of NALP3 protein decay versus time of CHX exposure with best fit lines. The half-life was prolonged with a K689R mutant > 6 h (C), while wild-type (WT) full-length protein or other mutants exhibited at t ½ of ~4 h. Data represent mean ± S.E. of 3-4 independent experiments. *p<0.05 (E) MLE cells were co-transfected with FBXL2/empty plasmid (2 µg) and either WT NALP3 plasmid or point-mutant plasmids (2 µg) for 48 h. Cells were collected and assayed for NALP3 (V5) and β-actin (loading control) by immunoblotting. (F) *In vitro* ubiquitination assays show that a K689R NALP3 mutant is less polyubiquitinated compared to WT NALP3 or a K696R mutant. Data in panels (E-F) are representative of n=2-3 independent experiments.

Figure 6. **W73 within NALP3 is required for FBXL2 interaction.** (A) Several deletional or point mutants of NALP3 were designed and cloned into a pcDNA3.1D/V5-HIS vector. (B-D) FBXL2 protein was immunoprecipitated from cell lysates using a FBXL2 antibody and coupled to protein A/G agarose resin. FBXL2 resins were then incubated with *in vitro* synthesized products expressing HIS-V5-NALP3 mutants. After washing, proteins were eluted and immunoblotted for NALP3-V5. (E) HeLa cells were transfected with NALP3 wild-type (WT) or mutant plasmids and the half-life was determined via CHX treatment at 40 µg/mL. (F) NALP3 WT or mutants (4 µg) were transfected into HeLa cells and treated with BC-1215 for 16 h. Cells were then collected and processed for V5 immunoblotting. (G) HeLa cells were co-transfected with NALP3 WT or W73A mutant (2 µg) along with or without FBXL2 plasmid (2 µg). Cells were then collected and immunoblotted for V5 and β-actin.

Figure 7. **An FBXO3 inhibitor reduces NALP3 abundance thereby decreasing cytokine release.** (A) U937 cells were treated with BC-1215 at different concentrations for 16 h. Cells were collected for FBXL2 and β-actin immunoblotting. (B) U937 and THP1 cells (3x10⁶ cells each) were incubated with LPS (200 ng/ml) or BC-1215 (8 µg/ml) for 16 h. Cells were collected and lysed for NALP3, NALP6 (negative control) and GAPDH/β-actin immunoblotting. (C) U937 cells (3x10⁶ cells) were primed with LPS (200 ng/ml) in combination with different concentrations of BC-1215 for 16 h as indicated, or (D) primed with LPS (200 ng/ml for 16 h), and then exposed to BC-1215 (4 µg/ml) in fresh culture medium for the indicated periods of time. Cells were collected to measure NALP3 and β-actin protein (top) and
mRNA levels (below). Box plots of fold-increase of steady-state NALP3 mRNA are shown. Data represent \( n=4 \) independent experiments. \( P \)-value; by Kruskal-Wallis test. (E) U937 cells were incubated in LPS (200 ng/ml) with or without BC-1215 (4 \( \mu \)g/ml) for 16 h. Cells were then exposed to CHX (40 \( \mu \)g/ml) at different time points for a half-life study. Immunoblotting for NALP3 and GAPDH (loading control) was performed. Densitometric plots of adjusted NALP3 protein decay over time in different conditions were best fitted. The half-life of LPS-primed NALP3 protein was reduced with BC-1215 treatment comparable to native conditions. Data represent mean ± S.E of \( n=2 \) independent experiments. (F) MLE cells were transfected either with WT or point-mutant NALP3 plasmids for 48 h. LPS (40 \( \mu \)g/mL) was next added to medium with or without BC-1215 (20 \( \mu \)g/mL) for 18 h. Cells were collected and lysed for NALP3 (V5 tagged) and \( \beta \)-actin (loading control) immunoblotting. Levels of K689R, the point-mutant for a putative ubiquitin acceptor site within the NALP3 protein did not decrease with BC-1215 compared to WT or mutant NALP3. Data in panel (F) is representative of \( n=2 \) independent experiments. (G) THP1 and K562 cells (3x10^6 cells each) were incubated with LPS (200 ng/ml) with or without BC-1215 (8 \( \mu \)g/mL) for 20 h, and then pulsed with ATP (5 mM for 20 min). Culture medium was collected for immunoblotting of pro-IL-1\( \beta \), IL-1\( \beta \), pro-IL-18, and IL-18. (H) THP1 cells (3x10^6 cells each) were incubated with LPS (200 ng/mL) for 20 h and different time periods of BC-1215 (8 \( \mu \)g/mL) as indicated in the same volume of culture medium. Cells were then exposed to ATP (5 mM for 20 min). Culture medium was collected for immunoblotting of pro-IL-1\( \beta \) and IL-1\( \beta \). The ratio of IL-1\( \beta \) versus pro-IL-1\( \beta \) by densitometry was shown in a bar graph (bottom). The data represent mean ± S.D. of \( n=2 \) independent experiments. \( P \)-value; by nonparametric test for trend. (I) Primary human alveolar macrophages were obtained from a healthy volunteer. Human alveolar macrophages (3x10^5 cells) were incubated with BC-1215 (4 \( \mu \)g/mL) for 2 h, and then exposed to LPS (100 ng/mL) for 16 h. Cells were lysed and collected for immunoblotting for NALP3 and \( \beta \)-actin. (J) Primary human alveolar macrophages (3x10^5 cells) from the same subject in (I) were incubated with BC-1215 (4 \( \mu \)g/mL) for 2 h, and then exposed to LPS (100 ng/mL) for 16 h. ATP (5mM) was added for 15 min before collecting cell culture supernatants for ELISA. Data represent mean ± S.D. of duplicate measurements.

Figure 8. Molecular regulation of NALP3 inflammasome protein stability mediated by F-box proteins. Endotoxin robustly increases NALP3 protein levels in human inflammatory cells and subsequently induces cytokine synthesis and release. The F-box protein FBXL2 serves as a sentinel inhibitor of NALP3 by mediating its polyubiquitination (Ub) through docking at W73 and ubiquitin ligation at K689 resulting in proteasomal degradation in cells. Another E3 ligase component, FBXO3 targets FBXL2 for its ubiquitination and degradation (8). A small-molecule FBXO3 inhibitor, BC-1215, reduces levels of NALP3 protein after LPS priming by restoring FBXL2 levels. BC-1215 subsequently decreases pro-inflammatory cytokine release after the NALP3 inflammasome is activated by a second stimulus.
Figure 1

A

LPS (ng/mL)  0  100  200  1000

NALP3  
NALP6  
β-Actin  

p=0.010

NALP3 Protein

LPS (ng/mL)  0  100  200  1000

B

LPS (h)  0  2  6  12  16  20

NALP3  
NALP6  
β-Actin  

p=0.010

NALP3 Protein

LPS (h)  0  2  6  12  16  20

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Figure 2

(A) NALP3 mRNA expression levels as a function of LPS concentration (ng/mL). P = 0.48

(B) NALP3 mRNA expression levels over time (h). P = 0.12

(C) Western blot analysis of NALP3 and GAPDH proteins under control conditions and LPS 16 h priming. CHX treatment at 0, 2, 4, and 6 h.

(D) NALP3 protein levels over time (h) with LPS+CHX and CHX treatments. Significant differences indicated by **.

(E) Western blot analysis of NALP3 and β-Actin proteins under Mg132 and Leupeptin treatments. CHX treatment at 0, 2, 4, and 6 h.

(F) NALP3 protein levels over time (h) with CHX+MG132 and CHX+leupeptin treatments. Significant differences indicated by *.

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**Figure 3**

**A**

HA-Ubi (μg) 0 2 4 6

HA tag

NALP3

GAPDH

**B**

LPS - +

Input

Ubiquitin IP

NALP3

NALP3

Ubiquitin

IB

Input

Ubiquitin IP

LPS - +

NALP3

NALP3

Ubiquitin

IB

**C**

NALP3:ubiquitin Binding Efficiency (%)

p=0.0369

Control LPS

β-Actin

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Figure 4
Figure 5

A

B

C

D

E

F

NALP3-V5  β-Actin

CHX (h)  0  2  4  6

NALP3-V5  β-Actin

CHX (h)  0  2  4  6

NALP3-V5  β-Actin

CHX (h)  0  2  4  6

NALP3-V5  β-Actin

CHX (h)  0  2  4  6

NALP3-V5  β-Actin

WT  K689R  K742R

FBXL2  -  +  -  +  -  +

SCF  -  +  -  +  -  +

E1/E2  -  +  -  +  -  +

NALP3  +  +  +  +  +  +

FBXL2-V5  -  -  -  -  -  -

β-Actin  -  -  -  -  -  -

NALP3 Protein

CHX (h)  0  2  4  6

NALP3 Protein

CHX (h)  0  2  4  6

NALP3 Protein

CHX (h)  0  2  4  6

NALP3 Protein

CHX (h)  0  2  4  6

NALP3 Protein

CHX (h)  0  2  4  6

NALP3 Protein

CHX (h)  0  2  4  6

NALP3 Protein

CHX (h)  0  2  4  6

NALP3 Protein

CHX (h)  0  2  4  6

NALP3 Protein

CHX (h)  0  2  4  6
### Figure 6

#### A

| WT NALP3 WT | DAPIN | NACHT | Leu Rich Repeat |
|-------------|-------|-------|-----------------|
| C379 NALP3 1-657 | 1 93 220 | 536 | 742 991 |
| C479 NALP3 1-557 | + + + + |
| N100 NALP3 101-1036 | - - - - |
| N93 NALP3 94-1036 | - - - - |
| N80 NALP3 81-1036 | - - - - |
| N70 NALP3 71-1036 | - - - - |
| N60 NALP3 61-1036 | - - - - |
| N40 NALP3 41-1036 | - - - - |
| N20 NALP3 21-1036 | - - - - |
| WT NALP3 WT | 1 65 | 76 220 | 536 742 991 |
| W68A NALP3 W68A | - - - - |
| W73A NALP3 W73A | - - - - |

#### B

**Input**

IB NALP3-V5 WT N100 C479 C379

**IP FBXL2**

IB NALP3-V5 WT N100 C479 C379

#### C

**Input**

IB NALP3-V5

**IP FBXL2**

IB NALP3-V5

#### D

**Input**

IB NALP3-V5

**IP FBXL2**

IB NALP3-V5

#### E

| CHX (h) | 0 | 2 | 4 | 6 |
|---------|---|---|---|---|
| WT NALP3 WT | | | | |
| NALP3-V5 | | | | |
| β-Actin | | | | |
| W68A NALP3 W68A | | | | |
| NALP3-V5 | | | | |
| β-Actin | | | | |
| W73A NALP3 W73A | | | | |
| NALP3-V5 | | | | |
| β-Actin | | | | |

#### F

| BC-1215 (μg/mL) | 0 | 0.1 | 1 | 10 |
|------------------|---|-----|---|-----|
| WT NALP3-V5 | | | | |
| β-Actin | | | | |
| W68A NALP3 W68A | | | | |
| NALP3-V5 | | | | |
| β-Actin | | | | |
| W73A NALP3 W73A | | | | |
| NALP3-V5 | | | | |
| β-Actin | | | | |

#### G

| WT Cont | FBXL2 | W73A Cont | FBXL2 |
|---------|-------|-----------|-------|
| NALP3-V5 | | | |
| FBXL2-V5 | | | |
| β-Actin | | | |
Bacterial Endotoxin

BC-1215

FBXO3

FBXL2

Ub

NALP3

W73

IL-1β and IL-18

Inflammasome
Lipopolysaccharide primes the NALP3 inflammasome by inhibiting its ubiquitination and degradation mediated by the SCFFBXL2 E3 ligase
SeungHye Han, Travis B. Lear, Jake A. Jerome, Shristi Rajbhandari, Courtney A. Snavely, Dexter L. Gulick, Kevin F. Gibson, Chunbin Zou, Bill B. Chen and Rama K. Mallampalli
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