Multi-omic Data Integration Links Deleted in Breast Cancer 1 (DBC1) Degradation to Chromatin Remodeling in Inflammatory Response

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This study investigated the dynamics of ubiquitinated proteins after the inflammatory stimulation of RAW 264.7 macrophage-like cells with bacterial lipopolysaccharide. Ubiquitination is a common protein post-translational modification that regulates many key cellular functions. We demonstrated that levels of global ubiquitination and K48 and K63 polyubiquitin chains change after lipopolysaccharide stimulation. Quantitative proteomic analysis identified 1199 ubiquitinated proteins, 78 of which exhibited significant changes in ubiquitination levels following stimulation. Integrating the ubiquitinome data with global proteomic and transcriptomic results allowed us to identify a subset of 88 proteins that were targeted for degradation after lipopolysaccharide stimulation. Using cellular assays and Western blot analyses, we biochemically validated DBC1 (a histone deacetylase inhibitor) as a degradation substrate that is targeted via an orchestrated mechanism utilizing caspases and the proteasome. The degradation of DBC1 releases histone deacetylase activity, linking lipopolysaccharide activation to chromatin remodeling in caspase- and proteasome-mediated signaling. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.026138, 2136–2147, 2013.

Ubiquitination is a versatile protein post-translational modification related to many key cellular functions. Protein ubiquitination is a reversible process in which the addition of ubiquitin units to protein substrates is mediated by the coordinated action of three ubiquitin ligases (E1, E2, and E3) (1) and the removal of these subunits by deubiquitinases (DUBs) (2). The functional roles of ubiquitination have been associated with the modification type; for example, monoubiquitination, involving proteins with single ubiquitin units, has been shown to play a role in cellular trafficking and the regulation of gene expression, and polyubiquitination (PolyUb), through either the N-terminus (linear) or one of the lysine residues (K6, K11, K27, K29, K33, K48, or K63), has different functional roles. As examples, K48 PolyUb works as a marker for proteasome- and autophagy-mediated protein degradation, whereas K11 plays a role in recycling proteins from the cell cycle, and K63 and linear PolyUbs are associated with cell signaling events (1).

Ubiquitination also plays an important role in host-pathogen interactions. For example, ubiquitination is a key element in innate and adaptive immune responses, such as in inflammation, autophagy, and antigen processing and presentation (3). One mechanism includes microbe-derived molecules called pathogen-associated molecular patterns that are recognized by toll-like receptors (TLRs) and trigger signaling cascades inside host immune cells (4). Upon the recognition of pathogen-associated molecular patterns by TLRs, kinase IRAK1 is phosphorylated by another kinase, IRAK4, and associates with E3 ubiquitin ligase TRAF6, which polymerizes K63 PolyUb chains (3, 4). These PolyUb chains serve as a scaffold to bring Tat-associated kinase 1 and IκB kinase (IKK) complexes into proximity, which enables Tat-associated kinase 1 to phosphorylate the beta subunit of the IKK complex, which further phosphorylates the inhibitory component of an NF-κB complex, IκB. Phosphorylated IκB is then poly-

1 The abbreviations used are: DBC1, deleted in breast cancer 1; DAVID, Database for Annotation, Visualization and Integrated Discovery; DUB, deubiquitinase; FBS, fetal bovine serum; HDAC, histone deacetylase; IKK, IκB kinase; iTRAQ, isobaric tag for relative and absolute quantification; LPS, lipopolysaccharide; NEM, N-ethylmaleimide; PolyUb, polyubiquitination; TLR, toll-like receptor.
ubiquinated with K48-linked chains and is targeted for proteasome degradation, releasing NF-κB to activate the transcription of cytokines and chemokines (4). In other instances, pathogens can express proteins that interfere with host ubiquitination pathways. For example, the pathogen Salmonella enterica serovar Typhimurium (S. Typhimurium) interferes with these pathways by expression and secreting four known E3 ubiquitin ligases (SopA, SspH1, SspH2, and StpP) and two known DUBs (SseL and AvrA) into host cells (5). Of these proteins, SseL was shown to deubiquitinate IκB, which prevents it from being degraded by the proteasome and leads to inhibition of NF-κB activation (6). Despite critical insights, exactly how pathogens coordinately affect, evade, or even hijack the host ubiquitination pathway remains an open question.

In this study, we investigated global protein ubiquitination dynamics in host immune cells induced by S. Typhimurium lipopolysaccharide (LPS), a potent pro-inflammatory pathogen-associated molecular pattern. We used a RAW 264.7 macrophage-like cell line that retains many common functions of primary macrophages (7) as our model host immune cells. The results from this investigation demonstrate that global levels of protein ubiquitination change after LPS stimulation and that this change modulates the activity of DUBs. We also took advantage of previously published transcriptomics and proteomics data that, when integrated with our ubiquitinome data, allowed us to identify proteins degraded in response to LPS stimulus. We show that one of these targeted proteins, a histone deacetylase (HDAC) inhibitor known as deleted in breast cancer 1 (DBC1), provides a signaling mechanism that controls chromatin remodeling in an inflammatory response.

EXPERIMENTAL PROCEDURES

Cell Culture, Treatment, and Deubiquitinase Assay—A RAW 264.7 murine macrophage-like cell line (American Type Culture Collection) was grown at 37 °C and 5% CO₂ atmosphere in DMEM (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml)-streptomycin (100 μg/ml). Cells were seeded at a density of 2 x 10⁵ cells/dish in 150-mm dishes for pull-down experiments, or 1.5 x 10⁵ cells/well in white 96-well plates for DUB activity. Confluent cells were pretreated for 15 min with 20 μM N-(benzoyloxy-carbonyl)leucinylleucinylleucinal (Z-LLL, Calbiochem) or 40 μM Z-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD(O-Me)-FMK) (Enzo Life Sciences, Farmingdale, NY) and stimulated with 100 ng/ml LPS as described elsewhere (8). Cell pellets were resuspended in 1 ml extraction buffer (50 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 μM sucrose, 10% glycerol, 0.4% triton X-100, 10 mM NEM, 2 mM Tris (2-carboxyethyl) phosphine (TCEP), 10 mM sodium butyrate and protease inhibitor mixture), incubated for 10 min on ice, and centrifuged for 5 min at 6500 x g at 4 °C. The supernatant containing the cytoplasmic/membrane fraction was collected and centrifuged at 16,000 x g for 20 min at 4 °C to remove other organelles. The pellet containing the nuclear fraction was washed with 1 ml extraction buffer without triton X-100 and centrifuged for 5 min in 6500 x g at 4 °C. Purified nuclei were lysed with 1 ml hypotonic buffer (3 mM EDTA, 10 mM NEM, 2 mM TCEP, 10 mM sodium butyrate and protease inhibitor mixture), rotated for 30 min at 4 °C, and centrifuged for 5 min at 6500 x g at 4 °C. The supernatant containing the nuclear soluble proteins was collected, and the pellet that contained DNA-binding proteins was rotated for 30 min at 4 °C with solubilization buffer (50 mM Tris-HCl, pH 8.0, 2.5 mM NaCl, 0.1% Triton X-100, 10 mM NEM, 2 mM TCEP, 10 mM sodium butyrate and protease inhibitor mixture). The sample was then centrifuged for 20 min at 16,000 x g, and the supernatant was combined with the nuclear soluble proteins. Extracted samples were quantified via BCA assay, precipitated with 10% trichloroacetic acid, and then washed with cold acetone prior to Western blot analysis.

Immunoprecipitation—RAW 264.7 cells grown in 150-mm plates to confluence as described above were harvested in 3.5 ml/plate Tris lysis buffer (20 mM Tris-Cl, 5 mM EDTA, pH 7.5, 150 mM NaCl and 1% Triton X-100) supplemented with protease inhibitor mixture and 10 mM sodium butyrate. Cells were extracted via sonication for 30 s at 100% amplitude and a pulse of 0.5 and rotated for 1 h at 4 °C. The cell extract was then centrifuged for 15 min at 10,000 x g at 4 °C, and the supernatant was collected and rotated for 2 h at 4 °C with 2 μg anti-DBC1 (rabbit polyclonal #5693, Cell Signaling, Danvers, MA) or anti-histone H4 lysine 12 acetylation (H4K12Ac) (rabbit polyclonal A-4029 Epigentek, Farmingdale, NY). A control without antibody was run in parallel. 50 μl of protein G-conjugated magnetic beads (MagNA Bind, Thermo) was added to the lysate and rotated for 1 h at 4 °C. The beads were then washed three times with Tris lysis buffer, and the immunoprecipitated proteins were eluted with Laemmli sample buffer via incubation for 10 min at 70 °C prior to the Western blot analysis.

Western Blots—Extracted proteins were separated using SDS-PAGE and transferred onto PVDF membranes. Membranes were stained with 0.1% Ponceau S in 5% acetic acid to verify that equal amounts of proteins were loaded. Western blots were performed with the following antibodies: monoclonal anti-K63 (Aupb3), monoclonal anti-K48 (Aupb2), polyclonal anti-ubiquitin (all three from Millipore, Billerica, MA), polyclonal anti-H4K12Ac, polyclonal anti-DBC1, and polyclonal anti-histone H4 (39270, Active Motif, Carlsbad, CA). Blots were developed with ECL reagent (Pierce) and visualized in a FluorChem Q imaging system (Alpha Innotech, Santa Clara, CA).

Fluorescence Microscopy and Image Analysis—RAW 264.7 cells were grown on 35-mm glass bottom culture dishes (WillCo Wells, Amsterdam, The Netherlands) and treated with 100 ng/ml LPS as described above. Then cells were washed with PBS and fixed in 100% methanol for 2 min. After fixing, cells were washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and blocked with 3% BSA in PBS for 1 h. Primary antibody against DBC1 was incubated at 1:800 dilution, with rocking, overnight at 4 °C. After the cells had been washed four times with PBS, each sample was incubated with anti-rabbit conjugated 488 nm Alexa Fluor (Invitrogen) at 1:1000 dilution and two drops (per 2 ml total) of SlowFade...
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Gold with DAPI (Invitrogen) for two hours at room temperature while protected from light. Samples were then rinsed with PBS a total of five times and stored at 4 °C, protected from light, until imaged. Secondary antibody controls were treated the same, except for omission of the anti-DBC1 primary. For co-localization analysis, each sample was dually probed with antibodies against DBC1 (mouse monoclonal 3G4, Cell Signaling) and H4K12Ac at dilutions of 1:800 and 1:400, respectively. After washing, each dish was incubated with appropriate secondary conjugated 488 and 647 nm Alexa Fluor antibodies. All dishes were also incubated with SlowFade Gold with DAPI.

Immunolabeled cells were imaged with a Zeiss LSM 710 inverted confocal microscope using a 63× oil immersion objective. DBC1 was imaged using the 488-nm line of an argon laser (green channel), H4K12Ac was imaged using a 633-nm diode laser, and DAPI was imaged using a 405-nm diode laser (blue channel). Multiple dual-channel three-dimensional stacks were acquired for each sample with a 113 × 113 μm horizontal size and a z range covering the entire cell volume. The sampling density in x, y, z was optimized to satisfy the Nyquist sampling criterion for each individual channel. Image processing was done using Velocity Image Analysis software (PerkinElmer Life Sciences). For each three-dimensional stack, volume segmentation in the DAPI channel was used to define the cell nuclei, and nuclear DBC1 for individual cells was calculated as the sum of the fluorescence intensity in the green channel. Partially imaged cells

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late that this phenomenon might be due to an increase in DUB activity, autophagy, and/or slowing down of ubiquitin conjugation (discussed further below). A completely different phenomenon was observed for K63 PolyUb when cells were pretreated with MG132: an initial reduction in the levels of K63 PolyUb and then a gradual increase in late time points (Figs. 1E and 1F, supplemental Fig. S1). Collectively, these results suggest that ubiquitination levels change in response to LPS stimulation and that the dynamics of these changes are not solely dependent on or modulated by proteasome activity.

**Global Quantitative Analysis of Ubiquitinated Proteins**—Having determined that global ubiquitination levels change upon LPS treatment, we further investigated ubiquitination levels of individual proteins using quantitative proteomic analysis. RAW 264.7 cells were treated for 0, 15, 120, and 240 min with LPS, as protein ubiquitination at these time points exhibited the most evident differences as determined via Western blot analysis (Fig. 1, supplemental Fig. S1). Ubiquitin-modified proteins in these samples were captured either with a ubiquitin-binding domain, Dsk2-UBA, that was conjugated to agarose or with agarose beads alone (control pull-down) and then digested with trypsin. To ensure the quality of our affinity purifications, we first tested the reproducibility of the pull-downs and the binding capacity of the beads; both showed
satisfactory results (supplemental Fig. S2). Quantitative measurements were enabled by labeling the peptides with isobaric tags for relative and absolute quantification (iTRAQ). Labeled peptides were combined into two 8-plex iTRAQ sets. The first set contained the four time points captured with control and Dsk2 beads, and the second set contained pull-downs with Dsk2 beads only. The first set was used to eliminate proteins that bound non-specifically to the beads, whereas the second set provided biological replicates for the quantitative analysis (Fig. 2A). Peptides from both iTRAQ sets were fractionated by means of high-pH reverse-phase chromatography and analyzed using LC–tandem mass spectrometry. After the spectra had been searched against the mouse International Protein Index database, a total of 1789 non-redundant proteins were identified from the two experiment sets (supplemental Tables S1 and S2). After the proteins that bound directly to the beads had been filtered out (2-fold Dsk2/control intensity ratio at the peptide level), 1199 ubiquitinated proteins were identified (Fig. 2B, supplemental Table S3). Interestingly, well-known monoUb proteins such as histones were identified, which supports the application of our strategy for analysis of both mono- and polyubiquitinated proteins (Fig. 2C, supplemental Table S3). A function-enrichment analysis using DAVID (15) revealed 23 pathways in which ubiquitinated proteins were overrepresented by more than 2-fold relative to the genome background ($p \leq 0.1$). Most of these functions were related to basic functions in the cells, such as ribosome activity, glycolysis/gluconeogenesis, and the cell cycle; however, two of them (Fc-gamma receptor mediated phagocytosis and leukocyte transendothelial migration) were more specific for macrophage biology (supplemental Table S4).

Quantitative analysis considered the 1057 ubiquitinated proteins present in both sets of iTRAQ experiments. Of these, 78 proteins were found to be differently abundant via Z-test, including 42 down-regulated and 36 up-regulated ones (Figs. 3A and 3B). The function-enrichment analysis showed only one pathway for the down-regulated ones (spliceosome, 12-fold enriched, $p = 0.02$), and another for the up-regulated ubiquitinated proteins (ribosomes, 51-fold enriched, $p = 8 \times 10^{-25}$) (Figs. 3C and 3D). Although not annotated into specific enriched pathways, several of the differentially ubiquitinated proteins were previously shown to be involved in the immune response, with a few of them increased in abundance (Traf1, Ripk3, Itm2b, Sfn2, and cytokines IL1b and Scye1) and others decreased (Csf1r, Phb2, and Impdh2) (16–24).
LPS Stimulation Leads to an Increase in DUB Activity—An interesting protein observed among the differentially ubiquitinated substrates was ataxin-3, which is a DUB involved in cell homeostasis and stress response (25). The DUB activity of ataxin-3 is amplified upon ubiquitination (25). Because ubiquitination levels of ataxin-3 were augmented in our ubiquitinomic analysis (Fig. 3B), we wondered whether global levels of DUB activity might not be altered after LPS stimulation, which could explain why the levels of ubiquitination decreased at later time points (Fig. 1). Thus, we measured DUB activity after stimulation with LPS in cells that were pretreated with or without MG132. When cells were stimulated with LPS alone, total cellular DUB activity increased at 240 min (Fig. 4), which explains (at least in part) why global ubiquitination levels decreased at this same time point (Fig. 1). In agreement with previous reports (25), proteasome inhibition increased the levels of ubiquitination (Fig. 1) and also led to the activation of DUB function (Fig. 4). Furthermore, the combination of MG132 and LPS treatments led to an additive effect on DUB activity at 120 min (Fig. 4). These observations show that DUB is activated upon LPS stimulation and suggest the involvement of ubiquitination in this process.

Identification of Proteins Targeted to Degradation in Response to LPS Stimulation—Previous studies have shown that proteasome activity is increased in macrophages in response to LPS treatment (26). This increase in proteasome activity was shown to be important in antigen presentation, pro-inflammatory signaling, and gene expression (27). Thus, we aimed to identify proteins that would be targeted to proteasome degradation after LPS stimulation. To achieve this goal, we retrieved the protein abundances from a recent publication of our ubiquitination targets (7). Because the focus was on degradation targets, we considered only proteins that exhibited a 1.5-fold reduction after LPS treatment and had a false discovery rate of < 0.05 as determined by Z-test.

Fig. 3. RAW264.7 cells were stimulated with LPS for 0 (unstimulated control), 15, 120, and 240 min. Then ubiquitinated proteins were captured with agarose-conjugated Dsk2 UBA domain, digested with trypsin, and labeled with iTRAQ prior to proteomic analysis. A, B, heat maps show significantly (false discovery rate < 0.05 as determined by Z-test) down- (A) and up-regulated (B) ubiquitinated proteins. C, D, functional annotation of down- (C) and up-regulated (D) ubiquitinated proteins. The annotation was performed with DAVID and manually curated using KEGG and Uniprot databases. The asterisks indicate the enriched functions (p < 0.05) by DAVID analysis.

Fig. 4. Deubiquitinase activity in LPS-stimulated cells. RAW264.7 cells were pre-treated or not for 15 min with MG132 and then stimulated with LPS for 0 (unstimulated control), 15, 30, 60, 120, and 240 min. LPS stimulation was done in a regressive order for all cells having the same exposure time to MG132. DUB activity was measured based on luminescence using a DUB-Glo kit. The asterisks represent significant increases (p < 0.05 as determined by t test) in the activity relative to the unstimulated control. RLU – relative luminescence unit.
We integrated our proteomics data with those obtained in a parallel microarray experiment (7).

Fig. 5 depicts selected examples of the proteome and transcriptome data of proteins targeted to proteasome degradation after LPS stimulation, and they can roughly be classified into three groups. Group 1 consists of five proteins whose abundances were mainly driven by gene expression, as protein level changes were highly synchronized with RNA levels (Fig. 5, supplemental Table S5). Group 2 contains 14 entries in which protein abundances were regulated by both protein degradation and gene expression. This group was characterized by a rapid decrease in protein abundance, probably due to protein degradation, and abundance levels remained low at late time points, which might be associated with a decrease in transcription levels (Fig. 5, supplemental Table S5). Group 3 is composed of 74 proteins whose abundances were reduced by LPS treatment but diverged from RNA levels, indicating that the abundance changes were mainly driven by degradation rather than gene expression (Fig. 5, supplemental Table S5).

To gain insight into the main cellular functions involved, we performed an analysis to identify pathways enriched with the proteins that are putatively targeted to degradation under LPS stimulation. No pathways were identified for the first two groups of proteins; however, group 3 had four pathways (ribosome, spliceosome, regulation of actin cytoskeleton, and glycolysis/gluconeogenesis) enriched in proteins whose levels appeared to be diminished by protein degradation (data not shown). Although the four pathways were previously shown to be regulated by ubiquitination (28–31), the function of this modification during LPS stimulation is still poorly understood.

**DBC1 Links LPS Stimulation to Chromatin Remodeling**—We next aimed to further study the degradation substrates found through the integration of multiple omics measurements. We carefully selected one protein after an extensive literature search of potential targets and performed experiments to obtain more mechanistic information about its roles. Among the identified degradation substrates after LPS stimulation, KIAA1967, which is also known as DBC1, was of interest to us in that it has been gaining importance in cancer biology, apoptosis, splicing, and chromatin remodeling, but only very limited information was available about its functional role(s) in inflammation (32–36). Thus far, mice lacking DBC1 have been shown to have reduced inflammation, but the mechanism is largely unknown (36). In our data, DBC1 protein levels decreased following LPS stimulation, but its transcript levels remained constant throughout the 24-h period, which suggests that DBC1 abundance might be regulated through degradation instead of expression mechanisms (Fig. 6A). As DBC1 is a histone deacetylase (HDAC) inhibitor and also participates in apoptosis (32–34), we decided to further investigate DBC1 function and the mechanism by which it degrades. A Western blot analysis performed over a time course allowed us to check DBC1 protein levels. Results showed that DBC1 was detected mainly in two bands, one of ~100 kDa, which matches the calculated mass of the full-length poly-
peptide, and a truncated version of ~80 kDa (Fig. 6B, supplemental Fig. S3). Interestingly, the intensity of the 100-kDa band decreased with LPS treatment, whereas the intensity of the 80-kDa band remained nearly constant.

The full-length version of DBC1 has been proposed to be mainly nuclear, whereas the truncated version, which lacks a nuclear localization signal, has been suggested to be cytosolic and to participate in apoptosis (33). To verify this hypothesis, we performed cell fractionation to obtain nuclear and cytoplasmic/membrane enriched fractions. Nuclear DBC1 was mainly observed in the full-length state, and its abundance decreased after LPS treatment, whereas DBC1 in the cytoplasmic fraction was mostly composed of the truncated version, and its abundance level remained unchanged (Figs. 6C and 6D, supplemental Fig. S3). To further support these findings, we performed a quantitative immunofluorescence experiment on DBC1 (Figs. 6E–6H). As expected, the levels of nuclear DBC1 decreased after LPS treatment (Figs. 6F–6H), as observed in the Western blot experiment (Fig. 6D).

These results validated the hypothesis that DBC1 is a degradation target, but at the same time they raised an intriguing mechanistic question: why did nuclear, but not cytosolic, DBC1 change in abundance? Literature reports that in cancer cells treated with tumor necrosis factor-α, DBC1 is processed by caspases, whereby it loses its nuclear localization signal and is released to the cytosol (33). Our ubiquitinome analysis suggests that DBC1 might be targeted to degradation in the proteasome. In order to understand the mechanism of DBC1 degradation, we pretreated cells with proteasome inhibitor MG132 and the global caspase inhibitor Z-VAD-FMK, and we then stimulated the cells with LPS. Because RAW 264.7 cells die after prolonged MG132 treatment, probably as a result of the uncontrolled increase of active caspases that are normally cleared by the proteasome (37, 38), we decided to treat the cells for no longer than 4 h. The inhibition of the proteasome led to a more rapid decrease of both full-length and truncated versions of DBC1, accompanied by the accumulation of some smaller molecular weight bands. Fig. 7 indicates that DBC1 was further processed in the cytosol and then targeted to proteasome degradation. In contrast, treatment with the caspase inhibitor blocked the processing of the full-length version, but the truncated pieces decreased in abundance (Fig. 7), which shows that caspase processing is required in order for DBC1 to locate into the cytosol and be degraded by the proteasome. These results point to a coordinated mechanism for DBC1 degradation: first, full-length DBC1 is processed by caspases, which switches localization from the nucleus to cytosol, and then the truncated forms are targeted to ubiquitination and degraded by the proteasome.

Our next step was to investigate possible functions of DBC1 in RAW 264.7 cells. DBC1 was previously shown to bind and inhibit the activity of HDACs such as SIRT1 and HDAC3 (32, 34). Because the levels of nuclear, but not cytosolic, DBC1 decreased after LPS treatment, we reasoned that this stimulus might cause DBC1-dependent chromatin remodeling. We chose histone H4 acetylation at lysine K12 (H4K12Ac) as our model of chromatin remodeling because it has been shown to be involved in the regulation of gene
expression after LPS treatment (39). We first investigated whether DBC1 would interact with acetylated histones and potentially protect them against the activity of HDACs. Immunofluorescence labeling with antibodies against DBC1 and H4K12Ac was performed and showed that although they do not completely overlie, they co-localize at least partially in the nucleus (Fig. 8A). To further investigate whether these proteins interact, a co-immunoprecipitation experiment using an anti-H4K12Ac antibody followed by a Western blot using an anti-DBC1 antibody was performed, and the results showed that acetylated histone H4 and DBC1 interacted in RAW 264.7 cells (Fig. 8B). These findings suggest that DBC1 could be a regulator of histone H4 acetylation. Supporting this hypothesis, a Western blot that targeted the H4K12Ac showed a decrease in the level of this modification in response to LPS over time. Strikingly, the reduction of histone H4K12Ac levels was highly synchronized with the reduction in DBC1 abundance (Figs. 6B and 8C). Given that DBC1 processing was dependent on caspase, we wanted to know whether blocking caspases prevented HDAC activity. Thus we pretreated cells with Z-VAD-FMK and stimulated them with LPS for 4 h. After LPS treatment, the levels of H4K12Ac consistently decreased relative to the unstimulated control. As expected, blocking DBC1 processing with Z-VAD-FMK inhibited the decrease of H4K12Ac (Fig. 8D). Collectively, these results support a model in which LPS stimulation leads to caspase processing followed by ubiquitination and degradation of DBC1, which releases HDAC activity and in turn results in the reduction of H4K12Ac levels, thereby linking LPS signaling to chromatin remodeling (Fig. 9).

DISCUSSION

Despite a number of studies showing that the dynamics of protein ubiquitination has a central function in innate immunity (3, 26, 40), exactly how pathogens modulate the host ubiquitination pathway remains an open question. Here, we investigated global protein ubiquitination dynamics in the RAW 264.7 macrophage-like cell line induced by S. Typhimurium LPS. Our data showed that global levels of ubiquitination change after LPS activation. Interestingly, pre-treatment of cells with MG132, a potent proteasome inhibitor, prior to LPS stimulation showed that not only the proteasome but also DUBs have a major impact on global ubiquitination levels. This finding is in line with recent results suggesting that the DUBs A20, CYLD, USP4, and USP7 can counteract ubiquitin ligase activities and regulate TLR signaling in activating NF-κB (41, 42). Thus, we also investigated the RNA and protein levels of these enzymes. RNA levels of A20 rapidly
increased after LPS stimulation, whereas the transcripts of the other three enzymes remained mostly unchanged (supplemental Fig. S5). Whereas the protein abundance of USP4 rapidly increased after LPS stimulation, USP7 showed the opposite effect (supplemental Fig. S5). Considering only transcript and protein levels, A20 and USP4 seem to be the main regulators of ubiquitination in RAW 264.7 cells activated by LPS, at least in the time frame of this study (0 to 24 h).

Our analysis also shows that Ataxin-3, a DUB activated by ubiquitination (25), was significantly more ubiquitinated upon LPS treatment, which suggests that DUB activity might be higher in cells treated with LPS. Indeed, DUB activity is increased after LPS stimulation, and this activity is further potentiated by inhibiting the proteasome. Our findings also indicate that LPS-triggered regulation of DUBs appears to be more complex than previously thought and involves the expression of enzymes, as well as post-translational modification events. The regulation of NF-κB-related DUBs by post-translational modifications is not new; CYLD was previously shown to be regulated by phosphorylation and by releasing TRAF2 ubiquitination (43).

Fig. 9. Proposed model of chromatin changes mediated by DBC1. Upon LPS-stimulation, caspases are activated and process DBC1, which is translocated from the nucleus to the cytosol, releasing HDAC activity. The cytosolic form of DBC1 is further processed by caspases before being ubiquitinated and targeted to proteasome degradation.

Because proteasome activation is a well-documented phenomenon during the stimulation of cells with LPS, we decided to identify potential degradation targets by integrating proteomics and transcriptomics with our data of ubiquitinated proteins. One of the interesting targets of protein degradation was DBC1, which was previously reported to have functions in apoptosis, chromatin remodeling, and spliceosome (32–35); however, limited information was available regarding its roles in inflammation (36). We further showed that this protein is degraded in a synchronized mechanism that involves caspase processing that switches its localization from nuclear to cytosolic, followed by ubiquitination and proteasome degradation. A similar mechanism of protein degradation involving caspases and the proteasome has already been shown to regulate levels of active caspase-3 (38, 44). Cleaved and activated caspase-3 is recognized by the inhibitor of apoptosis class of E3 ubiquitin ligases through the newly formed protein termini. Subsequently, caspase-3 is ubiquitinated and targeted to degradation in the proteasome, preventing the cell from undergoing apoptosis (38, 44).

Considering that DBC1 was previously described as an inhibitor of HDACs (32, 34), we also investigated its function in chromatin remodeling. Histone acetylation, a good example of chromatin remodeling, was shown to be a key element in regulating gene expression in mitogen-activated cells (45–48). Whereas the phosphorylation at serine 10 of histone H3 (H3S10P) by IKK-alpha is related to the activation of gene expression, other post-translational modifications, such as ubiquitination at lysine 119 of histone H2A (H2AK119Ub) and trimethylation at lysine 27 of histone H3 (H3K27Me3), are well-known transcription repressors (49–52). These histone modifications, among others, were proposed to be another checkpoint that aids in the tight regulation of TLR-inducible genes (53). The acetylation of histone H3 and H4 was shown to be another key element in regulating gene expression in mitogen-activated cells (45–48). Unlike H3S10P, H2AK119Ub, and H3K27Me3, the acetylation of histones activates a pool of genes while suppressing another (46, 48). Furthermore, the
treatment of macrophages with trichostatin A, an inhibitor of HDACs, enhances or decreases the expression of selected LPS-activated genes (46, 48). The alteration of gene expression by trichostatin A compromises the ability of mice to fight infections, which was associated with the impairment of phagocytosis and the production of reactive oxygen and nitrogen species (47, 48). Although only limited information is available, it is believed that the regulation of histone acetylation levels in TLR signaling is mainly dictated by the expression of histone acetyltransferases and HDACs (46).

Our study showed that H4K12Ac is decreased when RAW 264.7 cells are treated with LPS. This reduction seems to be regulated by DBC1, as it depends on DBC1 processing by caspases. Thus, the degradation of DBC1 by caspases and the proteasome could be another mechanism of gene expression regulation through chromatin remodeling during an inflammatory response compared with the expression of histone acetyltransferases and HDACs. Interestingly, a decrease in histone H4 acetylation levels was previously shown to lead to reduced inflammation, leading to a tolerance response (39). Furthermore, in support of our findings that DBC1 degradation leads to a decrease in histone H4 acetylation levels, mice lacking DBC1 have already been shown to have a reduced inflammatory response (36).

In summary, we propose DBC1 as another checkpoint for the fine-tuning of gene expression mediated by LPS-induced signaling.

Concluding Remarks—In the present work, we used a systems biology approach that combined state-of-the-art proteomic and transcriptomic technologies to discover new functions of protein ubiquitination in LPS-stimulated cells. The proteomic analysis of ubiquitination targets in conjunction with immunosassays suggested that DUB activities were altered after LPS treatment, which was further confirmed by enzymatic assays. Furthermore, the multi-omic integration followed by cellular and biochemical assays elucidated a role for DBC1 in chromatin remodeling during an inflammatory response.

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