A Small Molecule Deubiquitinase Inhibitor Increases Localization of Inducible Nitric Oxide Synthase to the Macrophage Phagosome and Enhances Bacterial Killing[†]

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Received 17 May 2011/Returned for modification 7 July 2011/Accepted 2 September 2011

Macrophages are key mediators of antimicrobial defense and innate immunity. Innate intracellular defense mechanisms can be rapidly regulated at the posttranslational level by the coordinated addition and removal of ubiquitin by ubiquitin ligases and deubiquitinases (DUBs). While ubiquitin ligases have been extensively studied, the contribution of DUBs to macrophage innate immune function is incompletely defined. We therefore employed a small molecule DUB inhibitor, WP1130, to probe the role of DUBs in the macrophage response to bacterial infection. Treatment of activated bone marrow-derived macrophages (BMM) with WP1130 significantly augmented killing of the intracellular bacterial pathogen *Listeria monocytogenes*. WP1130 also induced killing of phagosome-restricted bacteria, implicating a bactericidal mechanism associated with the phagosome, such as the inducible nitric oxide synthase (iNOS). WP1130 had a minimal antimicrobial effect in macrophages lacking iNOS, indicating that iNOS is an effector mechanism for WP1130-mediated bacterial killing. Although overall iNOS levels were not notably different, we found that WP1130 significantly increased colocalization of iNOS with the *Listeria*-containing phagosome during infection. Taken together, our data indicate that the deubiquitinase inhibitor WP1130 increases bacterial killing in macrophages by enhancing iNOS localization to the phagosome and suggest a potential role for ubiquitin regulation in iNOS trafficking.

Innate immune function is critical for protecting the host against pathogens and is coordinated by complex and diverse cellular mechanisms. Ubiquitination is a mode of posttranslational protein modification that regulates diverse cellular processes, including the innate immune response to infection (7). For example, the ubiquitin system is critical for fine-tuning events central to activation of NF- κ B (3), a central regulator of inflammation, and autophagy (21), a mechanism for capturing and killing intracellular pathogens. In addition, both vacuolar (1, 5) and cytosolic (28, 32) pathogens can be directly targeted for degradation by ubiquitin or ubiquitin-derived molecules. At the mechanistic level, protein ubiquitination involves the conjugation of ~8.5-kDa ubiquitin molecules to lysine residues of the target protein. Ubiquitin linkages are formed via an enzymatic cascade consisting of ubiquitin activators (E1), ubiquitin conjugators (E2), and ubiquitin ligases (E3); the E3 class of enzymes is largest, with more than 600 members, and provides a high level of substrate specificity (8). Like phosphorylation, ubiquitination is a dynamic process, reversed through the action of deubiquitinases (DUBs). In contrast to the large number of E3 ligases, the mammalian proteome contains fewer than 100 DUBs, and compared to E3 ligases, the role of

DUBs in regulating specific cellular processes, such as intracellular defenses, is less understood (19).

Ubiquitin involvement in innate immune defenses suggests that ubiquitin machinery may provide useful targets for anti-infective therapeutics (4). Indeed, recent studies have revealed that inhibitors of ubiquitin machinery may alter the outcome of intracellular infection (28, 33, 38). The small molecule WP1130, also known as desgrasyn (29), shares structural and chemical similarities with the known DUB inhibitors curcumin and dibenzylideneacetone (DBA) (18, 25) and inhibits DUBs both *in vivo* and *in vitro* without altering 20S proteasome activity. *In vitro*, WP1130 decreased the activity of purified DUBs, namely, USP5, USP9x, and UCH-L1, but not UCH-L3, in a dose-dependent manner (18). Since WP1130 inhibits some but not all DUBs, for the purposes of this study, this compound will be described as a “selective” DUB inhibitor. In lymphoma cell lines, WP1130 induced intracellular accumulation of ubiquitinated proteins containing K48- and K63-linked polyubiquitin chains while reducing the cellular pool of monomeric ubiquitin and inhibited polyubiquitin chain disassembly in cell lysates (18).

Here we employed the selective DUB inhibitor WP1130 to perturb ubiquitin dynamics in macrophages during bacterial infection to evaluate the potential contribution of DUBs to innate immune function and host-pathogen interactions. We used the opportunistic human pathogen *Listeria monocytogenes* as a model infection system. *L. monocytogenes* is enclosed within the phagosome following uptake by macrophages and secretes the pore-forming toxin listeriolysin O (LLO) to escape

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[†] Supplemental material for this article may be found at <http://iai.asm.org/>.

[‡] Published ahead of print on 12 September 2011.

this degradative compartment (10). Genetic mutants of *L. monocytogenes*, in which LLO is rendered either nonfunctional or highly active (11, 30, 35), enable dissection of the host response to both phagosomal and cytosolic bacteria. We show that the selective DUB inhibitor WP1130 enhances antimicrobial function in macrophages, since WP1130 significantly decreased survival of intracellular bacteria in an inducible nitric oxide synthase (iNOS)-dependent manner by promoting colocalization of iNOS with the *L. monocytogenes*-containing phagosome. Our data highlight the utility of WP1130 for probing the role of the ubiquitin system in the host response to intracellular infection and suggest involvement of deubiquitinases in regulation of iNOS trafficking.

MATERIALS AND METHODS

Bacterial strains and cell culture. *L. monocytogenes* strains 10403S (wild type [WT]), LLO⁻ (DP-L2161) (17), and L461T (DP-L4017) (11) were grown in brain heart infusion (BHI) broth. *L. monocytogenes* cultures were incubated statically at 30°C overnight (11). *Escherichia coli* DH5 α was inoculated into Luria-Bertani (LB) broth and incubated at 37°C overnight with shaking. RAW264.7 macrophages (American Type Culture Collection) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% nonessential amino acids, and 1% HEPES and grown at 37°C in 5% CO₂. Primary bone marrow-derived macrophages (BMM) were obtained from C57BL/6 mice (WT) (Jackson Laboratories) and B6.129P2-Nos2/J mice (*nos2*^{-/-}) (Jackson Laboratories). Briefly, BMM were differentiated in DMEM with 20% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 30% L929 conditioned medium. Cells were cultured at an initial density of 10⁷ cells per 150 mm non-tissue-culture-treated dish for 6 days, with fresh medium added at day 3. At day 6, cells were harvested with cold phosphate-buffered saline (PBS) without calcium or magnesium. Unless otherwise noted, prior to infection assays, BMM were activated overnight with lipopolysaccharide (LPS) (Sigma L6143) and gamma interferon (IFN- γ) (Peprotech 315-05), each at 100 ng/ml.

In vitro inhibitor treatment and infections. RAW264.7 cells were cultured on sterile coverslips at an initial density of 6 $\times$ 10⁶ cells, and BMM were cultured at an initial density of 4 $\times$ 10⁶ cells per 24-well plate. For DUB inhibition studies, macrophages were treated with 5 μ M WP1130 dissolved in dimethyl sulfoxide (DMSO) or DMSO only (vehicle control) or with 10 μ M dibenzylidenacetone (DBA) dissolved in ethanol or ethanol only (vehicle control). DUB inhibitors were added to cells either 1 h prior to (pretreatment) or 1 h after (posttreatment) initiation of infection. In pretreated cells, DUB inhibitors were removed from cell culture medium by washing three times prior to infection. Macrophages were infected with *L. monocytogenes* at a multiplicity of infection (MOI) of 1 unless otherwise noted. At 30 min postinfection (p.i.), infected cells were washed three times with Dulbecco's phosphate-buffered saline (D-PBS) and incubated in medium containing 10 μ g/ml gentamicin. For intracellular growth curves, at indicated times p.i., 3 coverslips containing infected macrophages were removed and individually lysed in 5 ml sterile water, and an aliquot of the lysate was plated on LB agar to enumerate CFU. For the 30-min time point, coverslips were sampled within 5 min of addition of gentamicin.

Protein fractionation and immunoprecipitation. To prepare detergent-soluble and detergent-insoluble cell protein fractions, BMM were lysed in cell lysis buffer (10 mM Tris-HCl, 0.5% Triton X-100, 150 mM NaCl) and centrifuged at 16,000 relative centrifugal force (RCF). Supernatants were used as the Triton X-100-soluble (detergent-soluble) protein fraction. To obtain Triton X-100-insoluble (detergent-insoluble) proteins, SDS-PAGE sample buffer was added to cell pellets, and suspensions were briefly sonicated and heated at 95°C. Detergent-soluble and -insoluble protein fractions were used for SDS-PAGE and immunoblot analysis. For immunoprecipitations, BMM were lysed in denaturing buffer (1% SDS, 250 mM NaCl) and incubated at 65°C for 30 min to disrupt protein-protein interactions. Lysates were briefly sonicated to ensure protein solubility and centrifuged at 16,000 RCF. Supernatants were diluted with cell lysis buffer and immunoprecipitated with anti-iNOS for 4 h at 4°C, followed by overnight incubation with protein A agarose beads. Beads were washed with TBS (25 mM Tris-HCl [pH 8.0], 150 mM NaCl) containing 0.05% Tween and then boiled in 1 $\times$ reducing SDS-PAGE sample buffer (50 mM Tris, 2% SDS, 0.1 M dithiothreitol [DTT], and 0.01% bromophenol blue). The resulting samples were used for SDS-PAGE and immunoblot analysis.

SDS-PAGE and immunoblotting. Protein samples were separated by SDS-PAGE and transferred to nitrocellulose. Blots were blocked in TBS-Tween and 5% nonfat dry milk and probed with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody. Blots were developed using ECL Plus chemiluminescence reagent (GE Healthcare), and band density was analyzed using the Image J software program.

Cell viability assay. The CellTiterFluor assay (Promega) was performed to assess macrophage viability following exposure to WP1130. Briefly, RAW 264.7 macrophages were treated with 5 μ M WP1130, DMSO, or 0.1% Triton X-100 (a detergent control that reduces cell viability by compromising plasma membrane integrity) for 1, 5, or 8 h at 37°C with 5% CO₂. Following the respective treatments, the cell-permeant, fluorogenic peptide substrate glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC) was added to macrophages, and cells were incubated for 30 min at 37°C with 5% CO₂. Fluorescent signal, which is proportional to living cells, was measured at 380/505 nm using a spectrofluorometer.

Nitrite assay. Cellular nitrite levels were assayed as an indicator of total iNOS activity using the Measure-iT high-sensitivity fluorescent nitrite assay kit (Invitrogen). BMM were plated on 24-well plates and activated overnight (unless otherwise indicated) with LPS and IFN- γ (100 ng/ml each). Cells were treated with 5 μ M WP1130 or DMSO for 1 h, followed by infection (where indicated) with *L. monocytogenes* or *E. coli* DH5 α at an MOI of 10. As a positive control, uninfected cells were treated with phorbol myristate acetate (PMA) (1 μ g/ml) for 1 h. At 1 h p.i., cells were washed with D-PBS and lysed in water. Cell lysates were immediately assayed for nitrite according to the manufacturer's protocol. Fluorescence values were determined at 365/450 nm using a spectrofluorometer.

Confocal microscopy. BMM were grown on coverslips in a 6-well plate at a concentration of 5 $\times$ 10⁶ cells/plate and infected at an MOI of 5 as described above. At indicated times p.i., coverslips were removed and fixed in 4% paraformaldehyde in TBS. Cells were washed 3 times with TBS plus 0.1% Triton X-100 (TBS-TX) and blocked for 10 min with TBS-TX plus 1% bovine serum albumin (BSA). Primary antibody in TBS-TX was added to the cells for 1 h. Cells were washed 3 times with TBS-TX and incubated with secondary antibody for 1 h. Coverslips were washed 3 times with TBS-TX and mounted with Pro-Long Gold Antifade reagent (Invitrogen). Samples were analyzed with a VisiTech Infinity3 array scanning confocal microscope using a 100 $\times$ objective and MetaMorph quantitative image analysis software. Colocalization of iNOS with phagosomes was determined by scoring at least 50 randomly chosen bacterium-containing phagosomes per treatment for presence and intensity of iNOS within a 1- μ m perimeter around the bacterium. Cells in which the intensity of phagosome-associated iNOS was greater than that of cytoplasmic iNOS were scored as positive for iNOS-phagosomal colocalization. The percentage of total cellular iNOS associated with phagosomes was determined by scoring at least 35 randomly chosen bacterium-containing phagosomes from cells with four or fewer phagosomes. Colocalization data were obtained from three independent experiments.

Antibodies and reagents. The antiubiquitin monoclonal antibody (MAb) (FK2) was obtained from BioMol International, the anti-iNOS MAb from BD Biosciences, and the anti-*Listeria* antibody from BD Diagnostic Systems. The following additional reagents were obtained: DBA (Sigma), MG132 (Tocris Bioscience), recombinant mouse IFN- γ (Peprotech), LPS (Sigma-Aldrich), PMA (Fisher BioReagents), and the Measure-iT high-sensitivity nitrite assay kit (Invitrogen).

Statistical analysis. Data were analyzed using Student's paired two-tailed *t* test. *P* values of <0.05 were considered significant, and all error bars represent standard deviations.

RESULTS

WP1130 decreases intracellular viability of *L. monocytogenes* in macrophages. WP1130 acts as a DUB inhibitor in lymphoma, myeloma, and epithelial cell lines (18), but its activity on innate immune effector cells has not been tested. To determine whether WP1130 exhibited similar DUB-inhibitory properties in macrophages, we treated uninfected RAW264.7 macrophages with 5 μ M WP1130 and extracted detergent-soluble and detergent-insoluble fractions. In treated macrophages, ubiquitinated proteins accumulated in a rapid, time-dependent manner in the detergent-insoluble fraction (Fig. 1A). Similar results were observed in murine primary bone marrow-derived macrophages (BMM) following WP1130 treatment (data not

FIG. 1. WP1130 increases bacterial killing in macrophages. (A) Uninfected RAW 264.7 cells were treated with 5 μ M WP1130 for the indicated time interval. Lysates were separated into detergent-soluble and detergent-insoluble fractions and immunoblotted for distribution of ubiquitinated proteins and actin (as a loading control). (B) RAW 264.7 cells were treated with 5 μ M WP1130 or DMSO for 1 h prior to infection with *L. monocytogenes* (MOI = 1). Gentamicin (10 μ g/ml) was added at 30 min p.i. to kill extracellular bacteria, and intracellular bacteria were enumerated at 30 min, 1 h, 2 h, 5 h, and 8 h p.i. (C) Uninfected RAW 264.7 macrophages were incubated for 1, 5, or 8 h in cell culture medium containing 5 μ M WP1130, DMSO, or 0.1% Triton X-100 at 37°C with 5% CO₂. After specified treatments, the CellTiterFluor assay (Promega) was performed to assess cell viability. Fluorescent signal, which is proportional to living cells, was measured at 380/505 nm. Asterisks indicate significant differences in cell viability between Triton X-treated cells and cells exposed to all other treatments (**, $P < 0.01$). (D) RAW 264.7 cells were pretreated with 5 μ M WP1130, 10 μ M DBA, DMSO, or ethanol (vehicle controls for WP1130 and DBA, respectively) for 1 h prior to infection with *L. monocytogenes* (MOI = 1). Gentamicin (10 μ g/ml) was added at 30 min p.i. as for panel B, and intracellular bacteria were enumerated at 5 h p.i.

shown). Increased ubiquitination of cellular proteins in treated macrophages is consistent with the function of WP1130 as a DUB inhibitor. To determine whether WP1130 could alter the outcome of bacterial infection, RAW264.7 peritoneal macrophages were treated with 5 μ M WP1130 for 1 h prior to infection (pretreatment) with *L. monocytogenes*. WP1130 pretreatment caused a significant decrease in intracellular *L. monocytogenes* as early as 1 h postinfection, which lasted throughout the 8-h duration of the experiment (Fig. 1B). The observed decrease in intracellular bacteria could not be attributed to an effect of WP1130 on bacterial uptake, since there were similar numbers of intracellular bacteria in control and treated cells at 30 min p.i. (Fig. 1B), both in the presence and absence of gentamicin (see Fig. S1 in the supplemental material). Moreover, there was no effect of WP1130 on *in vitro* growth of *L. monocytogenes* (see Fig. S2) or on macrophage viability (Fig. 1C). We observed no antimicrobial effect when WP1130 was added 1 h after initiation of infection (see Fig. S3), suggesting that WP1130 affects survival of *L. monocytogenes* at an early stage of infection. The antimicrobial phenotype associated with WP1130 treatment might have resulted from effects on targets other than DUBs. However, treatment of macrophages with dibenzylideneacetone (DBA), a known DUB inhibitor with structural similarities to WP1130 (18, 25), also enhanced *L. monocytogenes* killing (Fig. 1D), further in-

dicating that DUBs may be involved in regulating macrophage antimicrobial activity.

WP1130 enhances antimicrobial capacity of the phagosome.

During the first hours of *L. monocytogenes* intracellular pathogenesis, bacteria must first survive phagosomal defenses to then escape from the phagosome into the host cytosol, where replication and actin nucleation occur (36). Our observation that WP1130 enhanced bacterial killing when added prior to, but not at, 1 h p.i. indicated that WP1130 likely affected bacterial viability prior to phagosomal escape. We evaluated the influence of WP1130 on phagosome function by infecting RAW264.7 macrophages with bacterial strains that differ in ability to escape from the phagosomal compartment: *L. monocytogenes* LLO⁻, *E. coli* DH5 α , and *L. monocytogenes* LLO L461T. *L. monocytogenes* LLO⁻ is a mutant lacking the pore-forming toxin that facilitates phagosomal escape (30), and *E. coli* DH5 α is a nonpathogenic bacterium also incapable of escaping the phagosome. In contrast, *L. monocytogenes* LLO L461T expresses LLO with a threonine substitution for leucine 461, a mutation that results in increased hemolytic activity (11, 35) and more rapid vacuolar escape (31). We observed decreased survival of *L. monocytogenes* LLO⁻ (Fig. 2A) and *E. coli* DH5 α (Fig. 2B) in WP1130-treated macrophages relative to results for controls ($P < 0.01$). However, *L. monocytogenes* WP1130 did not alter survival of L461T (Fig. 2C). The bacte-

FIG. 2. WP1130 enhances bacterial killing in the macrophage phagosome. (A to C) RAW 264.7 cells were pretreated with 5 μ M WP1130 or DMSO for 1 h prior to infection with *L. monocytogenes* LLO⁻ (A), *E. coli* DH5 α (B), or *L. monocytogenes* LLO L461T (C) (MOI = 1). Gentamicin (10 μ g/ml) was added 30 min p.i., and intracellular bacteria were enumerated at 2 h and 5 h p.i. Asterisks indicate significant differences in the numbers of intracellular bacteria between WP1130-treated and control cells (*, $P < 0.05$; **, $P < 0.01$). Data are representative of at least 3 independent experiments.

ricidal effect of WP1130 on vacuolar bacteria but not on bacteria that quickly escape into the cytoplasm suggests that WP1130 enhances the antimicrobial activity of the phagosome. This is further supported by WP1130-mediated delayed phagosomal escape (see Fig. S4 in the supplemental material) and the reduced rate of intracellular replication of *L. monocytogenes* WT (Fig. 1B) but not *L. monocytogenes* L461T (Fig. 2C). Since our data show no phagosome-independent effect of WP1130 on bacterial intracellular replication, altered replication of *L. monocytogenes* WT in WP1130-treated cells may be a consequence of mutagenic effects of the phagosomal environment (27). Macrophage activation induces greater bactericidal capacity within the phagosome (16), and we therefore hypothesized that activated BMM might represent a system to test the influence of WP1130 on specific activation-induced innate defense mechanisms. We compared the effect of

WP1130 pretreatment on *L. monocytogenes* infection in primary BMM activated with lipopolysaccharide (LPS) and gamma interferon (IFN- γ) to infection in resting BMM. Bacterial killing increased significantly in activated BMM following WP1130 pretreatment (Fig. 3A), whereas WP1130 had no impact on bacterial survival in resting BMM (Fig. 3B). We also observed no effect of WP1130 on *L. monocytogenes* infection of nonphagocytic Caco-2 intestinal epithelial cells (see Fig. S5). These data indicate that this compound primarily modulates antilisterial effector mechanisms of phagocytes induced upon macrophage activation.

iNOS is an antimicrobial effector for WP1130 in macrophages. The inducible nitric oxide synthase (iNOS), encoded by the *nos2* gene, is a well-established macrophage antimicrobial defense mechanism. Induced upon macrophage activation, iNOS catalyzes the formation of nitric oxide (NO). NO and the

FIG. 3. Antimicrobial effects of WP1130 require macrophage activation. (A and B) 5 μ M WP1130 or DMSO was added 1 h prior to *L. monocytogenes* infection (MOI = 1) of BMM activated overnight with 100 ng/ml of each LPS and IFN- γ (A) or resting BMM (B). Gentamicin (10 μ g/ml) was added 30 min after infection, and intracellular bacteria were enumerated at 30 min, 1 h, 2 h, 5 h, and 8 h p.i. Asterisks indicate significant differences in intracellular bacteria between WP1130-treated and control cells (*, $P < 0.05$; **, $P < 0.01$). Data are representative of at least 3 independent experiments.

FIG. 4. iNOS-mediated killing of *L. monocytogenes* is enhanced by WP1130. (A and B) WP1130 (5 μ M) or DMSO was added 1 h prior to infection of *nos2*^{+/+} (WT) BMM (A) or *nos2*^{-/-} (iNOS knockout) BMM (B) by *L. monocytogenes* (MOI = 1). Gentamicin (10 μ g/ml) was added at 30 min p.i. Intracellular bacteria were enumerated at 30 min, 1 h, 2 h, 5 h, and 8 h p.i. Asterisks indicate significant differences in intracellular bacteria between WP1130-treated and untreated *nos2*^{+/+} cells (*, $P < 0.05$; **, $P < 0.01$). Data are representative of at least 3 independent experiments.

reactive nitrogen species (RNS) derived from it are potent mediators of bacterial destruction within the phagosome (9). We therefore asked whether WP1130 exerted its antimicrobial effects through iNOS by treating *nos2*^{-/-} and *nos2*^{+/+} macrophages with 5 μ M WP1130 for 1 h prior to infection (pretreatment) with *L. monocytogenes*. Bacterial killing was enhanced by WP1130 in wild-type (*nos2*^{+/+}) BMM (Fig. 4A) but not in *nos2*^{-/-} macrophages (Fig. 4B). We also tested an inhibitor of the phagocyte oxidase, GF109203X, for effects on WP1130-dependent microbial killing and did not observe such effects (data not shown). These results indicate that iNOS is an effector mechanism through which the selective DUB inhibitor WP1130 enhances macrophage antimicrobial activity.

WP1130 alters biochemical partitioning of iNOS. Enhanced iNOS-mediated killing in WP1130-treated cells could reflect a difference in iNOS abundance, degradation, activity, or localization. To examine the effect of WP1130 on total cellular levels of iNOS, we evaluated iNOS abundance in detergent-soluble and -insoluble fractions from activated BMM lysates. WP1130 treatment led to a rapid and time-dependent accumulation of iNOS in the detergent-insoluble protein fraction of uninfected BMM (Fig. 5A), with a concurrent decrease of iNOS in the detergent-soluble fraction. Similarly, WP1130 induced iNOS partitioning to the detergent-insoluble fraction in activated BMM infected for 1 h with *L. monocytogenes* (Fig. 5B). Decreased detergent solubility of a protein indicates altered biochemical characteristics that may reflect association with membranes or with proteinaceous structures such as aggregates, aggregates of ubiquitinated proteins (18). Since iNOS is subject to ubiquitination and proteasomal degradation (22), we examined whether WP1130 altered iNOS ubiquitination. Activated BMM were pretreated with WP1130 prior to infection with *L. monocytogenes* for 1 h in the presence and absence of the proteasome inhibitor MG132. We immunoprecipitated iNOS from cell lysates and probed with a ubiquitin-specific antibody by immunoblotting. No reproducible difference in iNOS ubiquitination could be attributed to WP1130 treatment, suggesting that WP1130 likely modulates iNOS indirectly (Fig. 5C). Ubiquitination of iNOS appeared unaffected by MG132, possibly due to the brief duration of proteasomal inhibition. We also assayed for iNOS activity by monitoring production of nitrite in WP1130-treated BMM by a quantitative fluorescence assay. WP1130 induced a modest but statistically significant increase in nitrite levels in both uninfected cells and cells infected for 1 h with *L. monocytogenes* and *E. coli* (Fig. 5D).

These data show that WP1130 alters the biochemical characteristics of iNOS, most likely by an indirect mechanism.

WP1130 treatment increases iNOS localization to the phagosome. Upon stimulation or infection of macrophages, iNOS expression increases and is recruited from the cytoplasm to the phagosome. This subcellular localization likely maximizes the toxic effects of NO and RNS on invading microbes and minimizes collateral damage to the host cell (9). We therefore evaluated the effect of WP1130 on iNOS localization in BMM infected with *L. monocytogenes* LLO⁻ using confocal microscopy. At 30 min p.i., iNOS colocalized with 78% of phagosomes in WP1130-treated macrophages, while only 33% of phagosomes were associated with iNOS in the absence of WP1130 ($P < 0.01$) (Fig. 6A and B). WP1130-treatment also increased the amount of iNOS per phagosome; the percentage of total cellular iNOS associated with phagosomes increased from 4.5% in control cells to 9.2% in WP1130-treated cells (Fig. 6C). An increase in iNOS-phagosomal colocalization was also observed in macrophages treated with the DUB inhibitor DBA (Fig. 6A to C). These data suggest that WP1130 increases iNOS-dependent bacterial killing by enhancing localization of iNOS to the macrophage phagosome and indicate the potential involvement of deubiquitinases in regulating iNOS trafficking.

DISCUSSION

We show here that the small molecule selective DUB inhibitor WP1130 promotes colocalization of iNOS with the macrophage phagosome and enhances bacterial killing. WP1130 pretreatment of activated macrophages altered survival of bacteria within the phagosome and induced bacterial killing as early as 1 h p.i. Survival of bacteria within the phagosome in general is compromised by the antimicrobial effectors iNOS and phagocyte NADPH oxidase (phox), producers of NO and superoxide, which couple to form highly reactive peroxynitrite. These reactive species exert direct toxic effects on phagosomal bacteria and also induce DNA mutagenesis that can reduce the viability of future microbial generations (2, 27). Our studies in *nos2*^{-/-} macrophages demonstrated that iNOS is an effector of WP1130-mediated bacterial destruction. WP1130 enhanced iNOS localization to the bacterial phagosome but did not alter overall cellular abundance of iNOS and only modestly affected total iNOS activity. The moderate changes observed in nitrite levels could indicate that WP1130 had either a small effect on total cellular levels of iNOS or a large effect on the small subset

FIG. 5. WP1130 alters iNOS biochemical partitioning. (A) BMM were treated with 5 μ M WP1130 for the indicated time intervals. Lysates were separated into detergent-soluble and detergent-insoluble fractions, and each fraction was probed for iNOS and β -actin (loading control) by immunoblotting. (B) BMM were treated with 5 μ M WP1130 for 1 h prior to infection by *L. monocytogenes* (MOI = 10) (Lm60). Gentamicin (10 μ g/ml) was added at 30 min p.i., and cells were lysed at 1 h p.i. Lysates were separated into detergent-soluble and detergent-insoluble fractions and probed for iNOS and β -actin. Uninf., uninfected. (C) BMM were pretreated with 5 μ M WP1130 or DMSO and infected with *L. monocytogenes* (MOI = 10) for 1 h in the presence and absence of MG132 (10 μ M). iNOS was immunoprecipitated from cell lysates, and immunoprecipitates were probed by immunoblotting with antibodies against iNOS and ubiquitin. (D) Cellular nitrite levels were measured as an indicator of iNOS activity. BMM were treated with 5 μ M WP1130 or DMSO for 1 h prior to infection with *L. monocytogenes* (Lm) or *E. coli* DH5 α (Ec) (MOI = 10). Cells were lysed in water at 1 h p.i., and lysates were assayed for nitrite with the Measure-iT nitrite fluorescent assay (Molecular Probes). Fluorescence, indicating the presence of nitrite, was measured at 365/450 nm. PMA treatment (1 μ g/ml) (PMA) and resting macrophages (Rest.) were used as positive and negative controls, respectively. Asterisks indicate differences in nitrite levels between WP1130-treated and DMSO-treated cells (*, $P < 0.05$). All immunoblots (A, B, and C) are representative of at least three independent experiments. Unless otherwise indicated, all BMM (A to D) were activated overnight with 100 ng/ml of each LPS and IFN- γ .

FIG. 6. DUB inhibitors increase iNOS localization to the phagosome. BMM were treated with 5 μ M WP1130, 10 μ M DBA, or vehicle controls (DMSO and ethanol, respectively) for 1 h prior to infection with *L. monocytogenes* LLO⁻ (MOI = 5). At 30 min p.i., cells were fixed and labeled with anti-*Listeria* (red) and anti-iNOS (green) antibodies. (A) Images were acquired at 30 min p.i. using an Infinity confocal microscope (Visitech International). Arrows indicate colocalization of iNOS with the LLO-phagosome in WP1130- and DBA-treated cells. (B and C) The MetaMorph software program was used to determine the percentage of phagosomes in control or DUB inhibitor-treated cells where iNOS colocalized with *Listeria* (B) and the percentage of total cellular iNOS associated with phagosomes (C) at 30 min p.i. Colocalization of iNOS with phagosomes was determined by scoring at least 50 bacterial phagosomes per treatment, and the percentage of total cellular iNOS associated with phagosomes was determined by scoring at least 35 bacterial phagosomes present within cells containing ≤ 4 phagosomes. Data are averages for 3 independent experiments. Asterisks indicate significant differences between inhibitor-treated and control cells (*, $P < 0.05$; **, $P < 0.01$).

of iNOS associated with the phagosome. Taken together, our data reveal that WP1130 alters the subcellular localization of iNOS and enhances its antimicrobial efficacy, supporting the idea that the action of WP1130 affects the subpopulation of iNOS protein associated with the phagosome. Our findings are consistent with a model in which DUB inhibition increases bacterial killing in macrophages by enhancing the localization of the antimicrobial effector iNOS to the bacterial phagosome.

Although WP1130 was initially characterized as a potential anticancer agent that inhibited DUBs to promote tumor cell apoptosis (18), WP1130 also exhibits characteristics of a DUB inhibitor in macrophages. While we used WP1130 as a tool to characterize mechanisms by which ubiquitin may control innate intracellular defenses, perturbation of key ubiquitinating and deubiquitinating enzymes via small molecule inhibitors such as WP1130 will likely prove useful for development of host-oriented anti-infectives which target host cell processes, rather than pathogens, to limit infection. Selective modulation of host intracellular defenses, such as iNOS, reduces the selective pressure placed on pathogens by conventional antimicrobials and minimizes potential for antimicrobial resistance (13). While specific DUBs targeted by WP1130 have yet to be unambiguously identified in macrophages, our data imply that iNOS trafficking may be subject to ubiquitin regulation. These findings contribute to a growing body of evidence supporting ubiquitin-mediated regulation as a key mechanism governing the antimicrobial function of iNOS. Previous work demonstrated that after 20 h of macrophage activation, iNOS is polyubiquitinated and targeted for proteasomal degradation (22). In addition, the DUB UCH37 promoted degradation of

ubiquitinated iNOS via the 20S proteasome (23). WP1130 is known to target several DUBs, including UCH37, in lymphoma and HEK293 cells (18). However, we found that WP1130-mediated bacterial killing was not dependent on proteasomal activity (see Fig. S3 in the supplemental material), which is inconsistent with a role for proteasome-associated UCH37 in WP1130-dependent antimicrobial function. Instead, our data support a model where WP1130 promotes phagosomal compartmentalization of iNOS rather than altering degradation. WP1130 was also shown to target Usp14 (18), a DUB implicated in regulating the unfolded protein response (26), which may impact processes important for innate immunity, such as protein trafficking and autophagy (15, 20), although its potential role during bacterial infection is unclear. Given the importance of ubiquitin as a regulator of global cellular processes, it is likely that DUBs provide critical control during the host response to infection, although at present the cellular functions of specific DUBs are largely undefined.

Proper compartmentalization of antimicrobial effectors such as iNOS is essential for balancing an effective antimicrobial response with protection of host cell constituents (12). Upon macrophage activation, iNOS is expressed and then trafficked from the cytosol to the vicinity of the phagosome, where NO exerts localized effects (24, 34, 37). Certainly, phagosomal localization of iNOS is critical for restricting RNS-mediated damage to the invading microbe while limiting collateral damage to the host cell. However, the mechanisms by which proteins such as iNOS are targeted to the phagosome during infection are still poorly understood, although a recent report identified the scaffolding proteins EBP50 and coronin as me-

diators of iNOS association with the phagosome (6). Indeed, ubiquitin is known to regulate intracellular sorting of membrane proteins (14), although its specific role in mediating recruitment of phagosomal proteins, or its potential involvement with EBP50 or coronin, is yet to be determined.

Our studies with the DUB inhibitors WP1130 and DBA implicate DUB regulation of iNOS-mediated bacterial destruction within macrophages by specifically enhancing iNOS-phagosomal colocalization. At a broader level, these data reveal the possibility that the ubiquitin cycle may play a pivotal role in modulating the functional phagosome proteome. Defining the specific target(s) for WP1130 that mediates iNOS localization will be critical for determining the mechanism that controls compartmentalization of iNOS during macrophage infection. As a dynamic regulator of cellular processes, the ubiquitin system is likely to be a rich source for identifying targets for therapeutic development (4). Our observation that WP1130 enhances iNOS-mediated bacterial killing suggests involvement of the ubiquitin system in regulating the antimicrobial environment of the phagosome and highlights DUBs as potential drug targets for development of broad spectrum anti-infectives aimed toward enhancing mechanisms of host defense.

ACKNOWLEDGMENTS

We thank Sam Straight of the Center for Live Cell Imaging at the University of Michigan for guidance with confocal microscopy and imaging analysis.

This work in the laboratory of M.X.D.O. was funded by an American Cancer Society Research Scholar Award (114548-RSG-08-075-01-MBC). C.E.W. was funded by University of Michigan start-up funds and by the NIH/NIAID Regional Center of Excellence for Bio-defense and Emerging Infectious Diseases Research (RCE) Program (award 1-U54-AI-057153). Support was also received from the Leukemia Society (award 6278-11 to N.J.D.), the University of Michigan Cancer Center Epstein and Padnos Funds (to N.J.D.), the MICHR Pilot Grant Program (to N.J.D. and H.D.S.), and the University of Michigan College of Pharmacy Ella and Hans Vahlteich Research Fund (to H.D.S.). K.M.B. is a trainee of the University of Michigan Research Training in Experimental Immunology Program (T32AI007413). J.W.P. was a trainee of the University of Michigan Genetics Training Program (T32GM0754) and is also supported by an American Heart Association Predoctoral Fellowship (10PRE3650036).

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