Proteomic Investigation of the Time Course Responses of RAW 264.7 Macrophages to Infection with *Salmonella enterica*[†]

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To investigate the extent to which macrophages respond to *Salmonella* infection, we infected RAW 264.7 macrophages with *Salmonella enterica* serotype Typhimurium and analyzed macrophage proteins at various time points following infection by using a global proteomic approach. A total of 1,006 macrophage and 115 *Salmonella* proteins were identified with high confidence. Most of the *Salmonella* proteins were observed in the late stage of the infection time course, which is consistent with the fact that the bacterial cells proliferate inside RAW 264.7 macrophages. The peptide abundances of most of the identified macrophage proteins remained relatively constant over the time course of infection. Compared to those of the control, the peptide abundances of 244 macrophage proteins (i.e., 24% of the total identified macrophage proteins) changed significantly after infection. The functions of these *Salmonella*-affected macrophage proteins were diverse, including production of antibacterial nitric oxide (i.e., inducible nitric oxide synthase), production of prostaglandin H₂ (i.e., cyclooxygenase 2), and regulation of intracellular traffic (e.g., sorting nexin 5 [SNX5], SNX6, and SNX9). Diverse functions of the *Salmonella*-affected macrophage proteins demonstrate a global macrophage response to *Salmonella* infection. Western blot analysis not only confirmed the proteomic results for a selected set of proteins but also revealed that (i) the protein abundance of mitochondrial superoxide dismutase increased following macrophage infection, indicating an infection-induced oxidative stress in mitochondria, and (ii) in contrast to infection of macrophages by wild-type *Salmonella*, infection by the *sopB* deletion mutant had no negative impact on the abundance of SNX6, suggesting a role for SopB in regulating the abundance of SNX6.

Macrophages play dual roles in controlling *Salmonella enterica* serotype Typhimurium-mediated systemic infection in susceptible mice. In naive mice, macrophages are directly involved in controlling the morbidity and mortality of the infected mice. Macrophages in vaccinated mice also serve as immune effectors to facilitate the clearance of *S. Typhimurium* and recovery from infection (47). Importantly, *S. Typhimurium* strains unable to replicate inside macrophages usually fail to cause systemic infection (15). For these reasons, *S. Typhimurium*-infected macrophages are often used to elucidate the molecular mechanisms underlying the interactions between macrophage and intracellular pathogens.

Once it is taken up by macrophages, *S. Typhimurium* resides in a membrane-bound compartment called the *Salmonella*-containing vacuole (SCV), in which virulent *S. Typhimurium* strains are able to control SCV biogenesis. The type III secretion system (T3SS) of *Salmonella* pathogenicity island 2 is directly involved in regulating SCV biogenesis in macrophages.

The T3SS apparatus is a needle-like structure that physically connects the cytoplasm of *S. Typhimurium* and host macrophage cells, which permits direct translocation of bacterial effector proteins into the cytosol of host cells. One of the major functions of these translocated *S. Typhimurium* effectors is to block the fusion of SCV with the cellular compartments containing antibacterial activities, including lysosomes and vesicles with functional phagocyte NADPH oxidase or inducible nitric oxide synthase (iNOS). As an integral part of the macrophage defense mechanisms, the lysosomes contain the proteins and peptides that disrupt key processes and/or structural components of *S. Typhimurium* cells, while phagocyte NADPH oxidase and iNOS generate antibacterial reactive oxygen and nitrogen species, respectively. By preventing the delivery of these antibacterial proteins and peptides into the SCV, *S. Typhimurium* cells are able to evade macrophage defense mechanisms and proliferate inside the SCV (for reviews, see references 1, 10, 17, 22, and 40 to 42). *S. Typhimurium* infection activates different macrophage signal transduction pathways, some of which (e.g., those mediated by mitogen-activated protein kinase [MAPK] and MyD88) are directly involved in the production of iNOS or in the activation of phagocyte NADPH oxidase (24, 32, 44).

Despite the advances made in understanding macrophage-*S. Typhimurium* interactions, it is still unclear to what extent macrophages respond to *S. Typhimurium* infection. The results of transcriptome analyses of macrophage response to *S. Typhimurium* infection were equivocal. By using Atlas mouse cDNA

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expression arrays that contained 588 mouse partial cDNAs, Rosenberger et al. found that up to 77 macrophage mRNAs (i.e., 13% of the total cDNAs tested) changed in abundance at 4 h postinfection (hpi) of RAW 264.7 murine macrophages by *S. Typhimurium* strain SL 1344, which included those of proinflammatory cytokines, transcriptional factors, and receptors (33). These results indicate that *S. Typhimurium* infection affects the abundance of significant numbers of macrophage mRNAs. In contrast, Detweiler et al. observed that the abundance of only 68 macrophage mRNAs (i.e., close to 0.3% of the 22,571 human cDNAs tested) changed significantly at 4 h following the infection of human U-937 macrophages by strain SL 1344 (13), which suggests that the infection has only a limited impact on the macrophage transcriptome. By analyzing the RAW 264.7 macrophage proteins that were coisolated with *S. Typhimurium* strain 14028 cells by a global proteomic approach, we previously found that, compared to those detected at 0 hpi, 230 macrophage proteins (i.e., 61% of the total macrophage proteins identified) were detected only at 2, 4, and/or 24 hpi, which demonstrates that the infection significantly increases the macrophage proteins that are coisolated with *S. Typhimurium* cells (37). To better understand the extent to which macrophages respond to *S. Typhimurium* infection, we investigated the time course responses of the macrophages to *S. Typhimurium* infection at the global proteomic level. Our results demonstrate a global macrophage response to *Salmonella* infection.

MATERIALS AND METHODS

Reagents and standard procedures. All of the cell culture reagents and antibodies used and the chemicals used for tryptic digestion were purchased from Invitrogen (Carlsbad, CA), Santa Cruz Biotechnology (Santa Cruz, CA), and Sigma (St. Louis, MO), respectively. Protein concentrations were measured with a bicinchoninic acid (BCA) protein assay kit from Pierce (Rockford, IL). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blot analyses were conducted according to the instructions provided by Invitrogen.

Cell culture and infection with *S. Typhimurium* cells. The RAW 264.7 macrophage-like cell line, which lacks a functional natural-resistance-associated macrophage protein 1 (Nrp1, also called Slc11a1), and *S. Typhimurium* strain 14028 were obtained from the American Type Culture Collection (Manassas, VA). The detailed procedures for maintaining RAW 264.7 macrophages and infecting the macrophages with *S. Typhimurium* strain 14028 were described previously (37). Briefly, *S. Typhimurium* cultures were prepared from frozen stocks in LB medium and grown for 18 h at 37°C with agitation (150 rpm). *S. Typhimurium* cells were harvested, washed once with the same volume of Dulbecco's phosphate-buffered saline without Mg²⁺ or Ca²⁺, and resuspended in 1 ml of Dulbecco's phosphate-buffered saline without Mg²⁺ or Ca²⁺. After their concentrations were determined, *S. Typhimurium* cells were diluted in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and incubated on ice for 30 min. Macrophage cells were washed twice with 2 ml/well of Hanks' buffered saline solution and then infected with *S. Typhimurium* cells at a multiplicity of infection (MOI) of 100. To increase the uptake of *S. Typhimurium* cells, plates were centrifuged at 1,000 × *g* for 10 min. Uptake of *S. Typhimurium* was allowed to occur for 30 min at 37°C in 5% CO₂. This time point was defined as 0 hpi. After being washed with DMEM three times, the cells were incubated in DMEM that contained gentamicin to kill any *S. Typhimurium* cells that remained outside of the macrophages. At different predetermined time points, the cells were washed twice with 2 ml/well of Hanks' buffered saline solution and lysed for 30 min on ice with 0.25 ml/well of cell lysis buffer consisting of 100 mM NH₄HCO₃ (pH 7.8), 0.1% (wt/vol) SDS, and 1% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). After the cell lysates from two plates were pooled, they were precipitated with trichloroacetic acid to remove excess detergents (50). The precipitates were resuspended with 100 mM NH₄HCO₃ (pH 7.8) and then subjected to tryptic digestion.

Tryptic digestion. After protein concentrations were determined by a BCA assay, urea, thiourea, and dithiothreitol were added to the total cell lysates at final concentrations of 7 M, 2 M, and 5 mM, respectively, and the mixture was

incubated at 60°C for 30 min. The samples were diluted 10-fold with 100 mM NH₄HCO₃ (pH 7.8) in the presence of 1 mM CaCl₂ and then subjected to tryptic digestion (Promega, Madison, WI) at a 1:50 (wt/wt) trypsin-to-protein ratio for 3 h at 37°C. The resulting digested peptides were desalted with strong cation-exchange solid-phase extraction columns (Supelco, Bellefonte, PA) (2, 4, 28, 37). The eluted peptides were concentrated with a SpeedVac to a final volume of ~100 µl. A BCA protein assay was performed to determine peptide concentrations prior to liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analyses.

LC-MS/MS analyses. The desalted peptides were separated with an automated reverse-phase capillary LC system designed in house (26). Eluate from the LC was directly electrosprayed into an LTQ-Orbitrap mass spectrometer (Thermo Fisher, San Jose, CA) by using an electrospray ionization interface manufactured in house. The heated capillary temperature and spray voltage were 200°C and 2.2 kV, respectively. Data were acquired for 100 min, beginning at 65 min after sample injection (i.e., 15 min into the gradient). Orbitrap spectra (automatic gain control, 1 × 10⁶) were collected from 400 to 2,000 *m/z* at a resolution of 100,000, followed by data-dependent ion trap MS/MS spectra (automatic gain control, 1 × 10⁴) of the three most abundant ions at a collision energy of 35%. Each sample underwent LC-MS/MS analysis at least four times.

Peptides were identified with the SEQUEST program (14) and filtered with a combination of scores provided in the output files. The minimal threshold filters used were those proposed by Washburn et al. (45). Additional filter thresholds included a minimal Peptide Prophet probability (21) of 0.85 to reduce the false-positive identifications. The peptide false-positive identification rate for the entire data set was calculated by using the forward and reverse sequence identifications (30). To estimate the relative abundance of each identified protein in the sample, the number of peptides observed for each protein in a sample was divided by the total number of peptides (i.e., both macrophage and *S. Typhimurium* peptides) found in the same sample (37). Similar approaches have been reported previously (3, 16, 18, 19, 25, 31, 43). Only proteins identified by at least three unique peptide observations that met both SEQUEST and Peptide Prophet criteria were reported. Student's *t* test was used to compare results between groups. Heat maps were generated with the software tool MeVv4.0 (The Institute for Genomic Research, Rockville, MD) (34). Raw and processed proteomic results can be found at proteomicsresource.org and omics.pnl.gov.

Generation of a *sopB* deletion mutant. *sopB* was deleted by using a PCR-based method described by Datsenko and Wanner (12). A kanamycin resistance cassette was PCR amplified from pKD13 with primers *sopB*-RF1 (5'-TGTCCCACTCCCTATTACAGGAATATTAAACAGCTATGATTCCGGGGATCCGTCGACC-3') and *sopB*-RR1 (5'-ATAGTTACCTCAAGACTCAAGATGTGATTAATGAAGAAATGTGTAGGCTGGAGCTGCTCC-3') and then used to replace *sopB* via λ Red recombination (12). Following the removal of the kanamycin resistance cassette, the deletion was validated by PCR (48). The resulting *sopB* deletion mutant (Δ *sopB*) and the wild type were used to determine the impact of *sopB* deletion on the abundance of SNX6. The infection conditions and Western blot analysis were described above.

RESULTS

LC-MS/MS analysis overview. To investigate the impact of *S. Typhimurium* infection on the macrophage proteome, we used an LC-MS/MS-based proteomic approach to analyze the lysate of *S. Typhimurium*-infected RAW 264.7 macrophages at 0, 2, 4, and 24 hpi, as well as that of the RAW 264.7 macrophages that served as a noninfected control. This differed from our previous work, in which we comparatively analyzed *S. Typhimurium* cells isolated from RAW 264.7 macrophages that lack a functional Nrp1 protein and RAW 264.7 macrophages complemented with Nrp1 in *trans* (37). Consequently, more macrophage proteins (1,006) and fewer *S. Typhimurium* proteins (115) were identified in this study than in the previous one, which included 378 macrophage and 315 *S. Typhimurium* proteins (Fig. 1; see Table S1 in the supplemental material) (37). The peptide false-positive identification rate for the entire data set used in this study was 1.1%.

***S. Typhimurium* proteins.** Among the identified *S. Typhimurium* proteins, 113 were also observed in previous studies of

FIG. 1. Peptide abundances of all identified macrophage and *S. Typhimurium* (STM) proteins. A total of 1,121 proteins are shown, which include 1,006 macrophage and 115 *S. Typhimurium* proteins. Peptide abundances for all identified macrophage and *S. Typhimurium* proteins in the noninfected (NI) control and at different time points after infection are indicated by colors that range from black (peptide abundance, 0%) to red (peptide abundance, $\geq 0.2\%$). Peptide abundance (percent) was calculated by dividing the number of peptides observed for each protein in a sample by the total number of peptides, which included both macrophage and *S. Typhimurium* peptides, determined from the same sample and then multiplying by 100 as described in Materials and Methods.

S. Typhimurium cells isolated from macrophages and/or mouse spleen (6, 37), including DnaK (STM0012), GamC (STM3118), and PhoN (STM4319), whose expression in the *S. Typhimurium* cells isolated from RAW 264.7 macrophages was confirmed previously by Western blot analyses (37). Nearly 30% of the total identified *S. Typhimurium* proteins were the ribosomal proteins that were part of the core proteome for free-living bacteria (9). The two *S. Typhimurium* proteins newly identified in this study were a putative ABC transporter (STM0770) and SopB (STM1091) (see Table S2 in the supplemental material). SopB is a virulence factor that is required for *S. Typhimurium* to survive inside macrophages (29). Whether STM0770 has any role in *S. Typhimurium* virulence needs to be determined. Most of the *S. Typhimurium* proteins were found in the late stages of the infection time course (i.e., 4 and 24 hpi) (Fig. 1), which is consistent with the fact that *S. Typhimurium* cells proliferate in RAW 264.7 macrophages that lack a functional Nrp1 protein (37).

Macrophage proteins. The peptide abundances of most identified macrophage proteins were relatively constant over the time course of infection, which included housekeeping proteins such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cytoplasmic superoxide dismutase 1 (SOD1). Compared to those of the noninfected control, the peptide abundances of 244 macrophage proteins (i.e., 24% of the total identified macrophage proteins) changed significantly (using a fivefold or greater difference between the abundances any time

FIG. 2. Peptide abundances of *S. Typhimurium* infection-affected macrophage proteins. (A) Macrophage proteins whose peptide abundances are significantly affected by *S. Typhimurium* infection. Compared to those of the noninfected (NI) control, the peptide abundances of 244 macrophage proteins changed significantly (using a fivefold or greater difference between any time point during infection and the noninfected control) after *S. Typhimurium* infection. These include 38 proteins that increased in their peptide abundances, 10 proteins that both increased and decreased in their peptide abundances at different time points after infection, and 196 proteins that decreased in their peptide abundances. Peptide abundances for infection-affected macrophage proteins found in the noninfected control and at different time points after infection are indicated by colors that range from black (peptide abundance, 0%) to red (peptide abundance, $\geq 0.07\%$). (B) A selected group of macrophage proteins whose peptide abundances increased following *S. Typhimurium* infection. The proteins shown include iNOS, COX-2, SQSTM1, and PON3. (C) A selected group of macrophage proteins whose peptide abundances decreased following *S. Typhimurium* infection. The proteins shown include SNX6, Pak2, Cdc2a, and Cdc37. Peptide abundance (percent) was calculated as described in the legend to Fig. 1.

point during infection and those of the noninfected control) after *S. Typhimurium* infection. Of these infection-affected macrophage proteins, 38 increased in their peptide abundances, 10 both increased and decreased in their peptide abundances at different time points after infection, and 196 decreased in their peptide abundances (Fig. 2A; see Table S3 in the supplemental material).

iNOS and prostaglandin (PG)-endoperoxide synthase 2 (also known as cyclooxygenase 2 or COX-2) were among the macrophage proteins whose peptide abundances increased after infection. These findings are consistent with previous observations that *S. Typhimurium* infection increased the mRNA and/or protein abundances of iNOS and COX-2 in macrophages (7, 13, 33, 38). Other macrophage proteins whose peptide abundances increased after infection included those with known functions, such as oxidative-stress-induced sequestosome 1 (SQSTM1), paraoxonase 3 (PON3), facilitated glucose transporter (Slc2a1), glucosidase 1 (Mogs), heme oxygenase 1 (Hmox1), DNA methyltransferase (Dnmt1), 2-oxoglutarate 4-dioxygenase (P4ha1), 2-oxoglutarate

FIG. 3. Validation of LC-MS/MS results by Western blot analysis. Peptide abundances (A) and/or Western blot analysis (B) of GAPDH, SOD1, SOD2, iNOS, and COX-2 in the noninfected (NI) control and at different time points after infection. Peptide abundances are indicated by colors that range from black (peptide abundance, 0%) to red (peptide abundance, $\geq 0.01\%$). Peptide abundance (percent) was calculated as described in the legend to Fig. 1.

5-dioxygenase 3 (Plod3), 3'-5' RNA exonuclease (Pnpt1), mitochondrial glycerol phosphate dehydrogenase 1 (Gpd2), asparagine synthetase (Asns), isoleucine-tRNA synthetase (AI327140), and ninein (Nin), as well as those with unknown functions (Fig. 2B; see Table S3 in the supplemental material).

Macrophage proteins whose peptide abundances fluctuated during the time course of infection included hemoglobin X (Hba-x), high-mobility group protein I (Hmga1), methenyltetrahydrofolate cyclohydrolase (Mthfd2), immunoresponsive gene 1 (Irg1), complement receptor type 3 (Itgam), cation-dependent mannose-6-phosphate receptor (M6PR), and four proteins with unknown functions (see Table S3 in the supplemental material).

Inspection of the macrophage proteins whose peptide abundances decreased after *S. Typhimurium* infection revealed that some of the proteins have similar cellular functions, such as regulation of intracellular traffic, cell signaling, the cell cycle, and protein synthesis or degradation. The proteins involved in intracellular traffic included sorting nexin 5 (SNX5), SNX6, SNX9, subunits $\beta 1$ and $\gamma 2$ of the coatamer protein complex (Copb1 and Copg2), vesicle docking protein (Vdp), vesicle-associated membrane protein 8 (Vamp8), tubulin cofactor A (Tbca), and actin-related protein 2/3 complex subunit 1b (Arpc1b) (8, 11, 35). The cell signaling proteins were serine/threonine kinases 2 and 25 (Stk2 and Stk25), similar to serine/threonine kinase (Pak2), MAPK kinase 3 (Map2k3), v-abl Abelson murine leukemia virus oncogene 2 (Abl2), cell division cycle control protein 2a (Cdc2a), protein phosphatase 1G (Ppm1g), catalytic subunits of protein phosphatase 2a and 5 (Ppp2cb and Ppp5c), protein tyrosine phosphatase (Ptpns1), GTP binding protein 4 (Gtpbp4), developmentally regulated GTP-binding protein 1 (Drg1), RAN GTPase activating protein 1 (Rangap1), and Rho GDP dissociation inhibitor alpha (Arhgdia). The proteins involved in cell cycle control included Cdc2a, Cdc37, apoptosis-antagonizing transcription factor (Aatf), and Ppm1g. The peptide abundances for four tRNA synthetases (i.e., synthetase for histidyl-, alanyl-, lysyl-, or valyl-tRNA or Hars, Aars, Kars, and Vars) were downregulated, which was in contrast to that for isoleucine-tRNA synthetase,

FIG. 4. Effects of *sopB* deletion on the abundance of SNX6. Western blot analyses of RAW 264.7 macrophages that served as a noninfected (NI) control or were infected by the wild type or the Δ *sopB* mutant at different time points. The proteins were separated by SDS-polyacrylamide gel electrophoresis and then probed with specific antibodies as described in Materials and Methods.

whose peptide abundance increased after infection. The *S. Typhimurium* infection diminished the peptide abundances of several proteasome components, including regulatory subunit S9 and core subunits $\alpha 1$, $\alpha 4$, $\beta 2$, and $\beta 7$ (LOC228604, Psme1, Psma4, Psmb2, and Psmb7) (Fig. 2C; see Table S3 in the supplemental material).

Western blot validation. To validate our LC-MS/MS results, we directly analyzed the expression levels of a selected set of proteins (i.e., GAPDH, SOD1, SOD2, iNOS, and COX-2) by Western blot analysis. Although it was not detected by LC-MS/MS, SOD2 was included because the antibody against SOD2 was available in our laboratory. The results of Western blot analysis were in general agreement with those of LC-MS/MS. GAPDH and SOD1 were detected in all of the samples tested. While SOD1 abundance remained relatively constant over the time course of infection, GAPDH abundance decreased slightly at 24 hpi, which might be attributed to its degradation. iNOS and SOD2 were also detected in all of the samples tested, but their abundances increased significantly at 24 hpi. Detection of SOD2 by Western blotting, but not by LC-MS/MS, suggests that the abundance of SOD2 is most likely below the detection limit of the LC-MS/MS method used in this study. COX-2 was undetectable in the noninfected control but was detected at 0 hpi. Compared to that at 0 hpi, the abundance of COX-2 increased moderately at 2 and 4 hpi and significantly at 24 hpi (Fig. 3).

Effects of *sopB* deletion on the abundance of SNX6. As an effector of SPI1-T3SS, SopB is a phosphoinositide phosphatase whose enzymatic activity is involved in regulating intracellular traffic. It increases phosphatidylinositol-3-monophosphate formation on SCV membrane by recruiting Rab5 and Vps34 (27). Functioning as a subunit of the retromer complex, SNX6 contains a phox homolog (PX) domain that binds phosphatidylinositol-3-monophosphate-enriched and highly curved membranes of endosomal vesicles or tubules, where it regulates the traffic of endosomal compartments (11). LC-MS/MS detected SopB in samples infected by *S. Typhimurium* (see Table S2 in the supplemental material) and the decrease in SNX6 peptide abundance at 24 hpi (Fig. 2C). To determine whether SopB had any role in regulating the abundance of SNX6, we generated a Δ *sopB* mutant and used it and the wild type to infect macrophages. Western blot analyses showed that infection by the wild type decreased the abundance of SNX6 at 24 hpi, which was in agreement with the LC-MS/MS results. In contrast, infection by the Δ *sopB* mutant had no negative impact on the abundance of SNX6, which was similar to that of the noninfected controls (Fig. 4). These results suggest that SopB has a functional role in regulating the abundance of SNX6.

DISCUSSION

This study represents the first global proteomic analyses of the time course responses of RAW 264.7 macrophages to *S. Typhimurium* infection. A total of 1,006 macrophage proteins were identified, of which 219 were also found in our previous analysis (37). Compared to those of the noninfected control, the peptide abundances of 244 macrophage proteins (or 24% of the total identified macrophage proteins) changed significantly after *S. Typhimurium* infection. The functions of these *S. Typhimurium*-affected macrophage proteins were diverse and ranged from production of antibacterial NO (i.e., iNOS) or production of PG H₂ (i.e., COX-2) to regulation of intracellular traffic (e.g., SNX5, SNX6, and SNX9). The functional diversity of *S. Typhimurium*-affected macrophage proteins demonstrates the broad impact of *S. Typhimurium* infection on the macrophage proteome, which suggests a global macrophage response to *S. Typhimurium* infection. Western blot analysis confirmed LC-MS/MS results for GAPDH, SOD1, iNOS, COX-2, and SNX6. The validation of these identified proteins demonstrates that the applied proteomic methods can be used to discover relevant protein changes.

The results of this study are in agreement with previous observations. For instance, LC-MS/MS and Western blot analyses consistently showed that the protein abundances of iNOS and COX-2 increased significantly at 24 hpi. While iNOS produces antibacterial NO, COX-2 converts arachidonic acid to PG H₂, which is the precursor of all PGs, prostacyclins, and thromboxans. After synthesis, the PGs are secreted from macrophage cells, where they function as autocrine agents to regulate macrophage functions, including the production of proinflammatory cytokines and NO. Induction of iNOS and COX-2 in macrophages by *S. Typhimurium* and their roles in controlling *S. Typhimurium* infection are well documented (7, 10, 13, 33, 38, 39).

Our results also revealed that while cytoplasmic SOD1 abundances remained relatively constant, mitochondrial SOD2 abundances increased at 24 hpi, which is consistent with the transcriptome analysis results that show an increase in the SOD2 mRNA level after *S. Typhimurium* infection of human macrophages (13). Functioning as an antioxidant enzyme, SOD catalyzes the dismutation of superoxide anion to reduce the oxidative stress that cells encounter (49). In addition to SOD2, the peptide abundance of oxidative-stress-induced SQSTM1 and PON3 increased after *S. Typhimurium* infection. Although whether SQSTM1 has any antioxidant role is unclear, PON3 protects lipids from oxidation (5, 36). Thus, induction of SOD2, as well as oxidative-stress-induced SQSTM1 and PON3, suggests an increased oxidative stress condition in mitochondria and most likely in the cytoplasm after infection. *S. Typhimurium*-mediated macrophage infection results in activation of phagocyte NADPH oxidase and iNOS in the cytoplasm, where they generate antibacterial reactive oxygen and nitrogen species, respectively (10, 40). These reactive oxygen and nitrogen species thus most likely cause the increased oxidative stress condition in the cytoplasm and mitochondria. It should be noted that *S. Typhimurium* infection can damage macrophage mitochondria and result in release of cytochrome *c* from the mitochondria, which leads to macrophage apoptosis (20). It will be very interesting to investigate whether SOD2

has any role in *S. Typhimurium*-induced macrophage apoptosis.

Unlike iNOS and COX-2, whose roles in macrophage-*S. Typhimurium* interaction are well characterized, the roles of most of the infection-affected macrophage proteins identified in this study, including SNX6, in the macrophage-*S. Typhimurium* interaction have yet to be determined. Our results indicate that *S. Typhimurium* virulence factor SopB is involved in regulating the abundance of SNX6. Previous studies showed that *S. Typhimurium* inside SCV was able to alter macrophage intracellular traffic via its T3SS, including SopB, and some host cell signaling proteins, such as PKA and PKB (also known as AKT1), were directly involved in regulating intracellular traffic (1, 22, 23, 27, 29). SopB was detected in *S. Typhimurium*-affected macrophages by LC-MS/MS in this study. The observed downregulation of the peptide abundances of the proteins involved in intracellular traffic (e.g., SNX6) and in cell signaling (e.g., MAPKs) after *S. Typhimurium* infection and the involvements of SopB in regulating the abundance of SNX6 are not only in general agreement with these previous results but also suggest that these *S. Typhimurium*-affected macrophage proteins, especially SNX6, might have important roles in macrophage-*S. Typhimurium* interactions. Future research should focus on what role SNX6 plays in the macrophage-*S. Typhimurium* interaction and the mechanisms by which SopB decreases the abundance of SNX6 in the late stage of the infection time course.

The peptide abundances of several macrophage proteins involved in cell cycle control decreased following *S. Typhimurium* infection. These results are consistent with transcriptome analysis results that also show downregulation of the expression of the genes involved in controlling the cell cycle (33). It is suggested that downregulation of the genes that control the cell cycle may be part of the coordinated efforts by which infected macrophages launch effective antibacterial activity (33).

Differences also exist between our results and those published previously. Our previous results showed that 61% of the macrophage proteins coisolated with *S. Typhimurium* cells were found only at 2, 4, and/or 24 hpi, which is much higher than the percentage (24%) of infection-affected macrophage proteins observed in this study. This difference is attributed to the different samples analyzed in these studies. Because the goal of our previous work was to investigate the *S. Typhimurium* response during macrophage infection, we only analyzed *S. Typhimurium* cells isolated from macrophages. In this study, we measured macrophage cell lysate, as the objective was to determine the macrophage response to *S. Typhimurium* infection. The higher percentage of infection-affected macrophage proteins found in the previous study than in this one suggests that infection increases the interactions between most of the infection-affected macrophage proteins found in the previous study and the isolated *S. Typhimurium* cells but not the abundances of these infection-affected macrophage proteins. Identification of SopB in this study but not in the previous one also indicates that the SopB protein detected is most likely the one secreted into the macrophage cytoplasm.

The percentage (24%) of infection-affected proteins found in this study is much higher than the percentage of affected mRNAs in *S. Typhimurium*-infected human and RAW 264.7

macrophages (i.e., 0.3 to 13%) (13, 33). With the exception of iNOS, COX-2, and SOD2, there is no other overlap between the *S. Typhimurium*-affected proteins identified in this study and the *S. Typhimurium*-affected mRNAs identified in the transcriptome analyses. The reasons for these differences vary and most likely are attributed to a number of different variables that were used in these studies, including different analytical methods, cell lines, *S. Typhimurium* strains, and infection conditions. In transcriptome analyses, the samples were collected at 4 hpi. In the present study, we analyzed the samples collected at 0, 2, 4, and 24 hpi and that of a noninfected control. Analysis of the samples collected at multiple and later (i.e., 24 h) time points following the infection certainly increases the chance of identifying infection-affected macrophage proteins. In previous work, the MOIs used were 4 to 12 and 20, respectively (13, 33). In this study, an MOI of 100 was used. This higher MOI should cause a stronger macrophage response and result in the identification of more infection-affected macrophage proteins. The different macrophages (mouse RAW 264.7 and human U-937), *S. Typhimurium* strains (14028 and SL1344), and media (DMEM and RPMI 1640 medium) used and preinfection treatment of U-937 with phorbol 12-myristate 13-acetate for 46 h are also expected to contribute to the different results obtained from these studies. In addition, it is well known that protein and mRNA abundances are not perfectly correlated (46).

Despite those differences, this is the first report of the time course responses of macrophages to *S. Typhimurium* infection at the global proteomic level, which demonstrates a global macrophage response to *S. Typhimurium* infection. Our results also show for the first time that *S. Typhimurium* infection (i) increases the mitochondrial SOD2 abundance and (ii) decreases the abundance of SNX6, most likely via *S. Typhimurium* virulence factor SopB. Future work should focus on understanding the roles of *S. Typhimurium*-affected macrophage proteins, such as SOD2 and SNX6, in macrophage-*S. Typhimurium* interactions, which we hope will lead to improved host-based therapeutic approaches to intracellular pathogens.

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