Macrophages Inhibit Salmonella Typhimurium Replication through MEK/ERK Kinase and Phagocyte NADPH Oxidase Activities*

Host responses during the later stages of Salmonella-macrophage interactions are critical to controlling infection but have not been well characterized. After 24 h of infection, nearly half of interferon-γ-primed murine RAW 264.7 macrophage-like cells infected by Salmonella enterica serovar Typhimurium contained filamentous bacteria. Bacterial filamentation indicates a defect in completing replication and has been previously observed in bacteria responding to a variety of stresses. To understand whether macrophage gene expression was responsible for this effect on Salmonella Typhimurium replication, we used gene arrays to profile interferon-γ-primed RAW 264.7 cell gene expression following infection. We observed an increase in MEK1 kinase mRNA at 8 h, an increase in MEK protein at 24 h, and measured phosphorylation of MEK's downstream target kinase, ERK1/2, throughout the 24-h infection period. Treatment of cells with MEK kinase inhibitors significantly reduced numbers of filamentous bacteria observed within macrophages after 24 h and increased the number of intracellular colony-forming units. Phagocyte NADPH oxidase inhibitors and antioxidants also significantly reduced bacterial filamentation. Either MEK kinase or phagocyte oxidase inhibitors could be added 4–8 h after infection and still significantly decrease bacterial filamentation. Oxidase activity appears to mediate bacterial filamentation in parallel to MEK kinase signaling, while inducible nitric-oxide synthase inhibitors had no significant effect on bacterial morphology. In summary, Salmonella Typhimurium infection of interferon-γ-primed macrophages triggers a MEK kinase cascade at later infection times, and both MEK kinase and phagocyte NADPH oxidase activity impair bacterial replication. These two signaling pathways mediate a host bacteriostatic pathway and may play an important role in innate host defense against intracellular pathogens.

Macrophages serve a central role in host defense against pathogenic microbes by nature of their ability to rapidly recognize bacterial components, phagocytose pathogens, and activate an arsenal of antimicrobial effectors to contain and eliminate the microbe. A macrophage's repertoire of antimicrobial effectors includes the phagocyte NADPH oxidase (phox),1 inducible nitric-oxide synthase (iNOS), cationic antimicrobial peptides, and an endosomal system designed to restrict nutrients and traffic phagocytosed microbes to degradative lysosomes. Phox is a multisubunit complex that can be assembled on intracellular membranes, such as the phagosomal membrane and the plasma membrane. Phox activity produces superoxide that can lead to the generation of other toxic reactive oxygen intermediates (ROI), such as hydrogen peroxide, and combine with nitric oxide to generate peroxynitrite, all of which can directly cause oxidative damage to bacteria (1). Macrophages activate many signaling pathways following recognition of bacterial components, although the relative contribution of each pathway to the induction of antibacterial effectors is not fully understood. For example, macrophages activate MEK/ERK kinase signaling in response to bacterial infection (2). MEK is a mitogen-activated protein kinase kinase that is activated by phosphorylation following Salmonella enterica serovar Typhimurium infection of macrophages in a Raf-dependent or -independent manner (3). Upon activation, MEK phosphorylates the downstream kinase ERK (extracellular signal-regulated kinase), which then dimerizes and translocates to the nucleus where it activates transcription factors such as Elk-1 to modify gene expression (4). MEK/ERK signaling is involved in the activation of oxidative and nitrosative bursts, endosomal trafficking, and increased macrophage differentiation and therefore is a strong candidate for being involved in the augmentation of macrophage defenses against intracellular pathogens (5).

In the murine model of human typhoid fever, Salmonella Typhimurium resides intracellularly within macrophages (6) in a specialized vacuole, and macrophages appear to be a preferred site for bacterial replication (7). As this intramacrophage niche helps to shield Salmonella from killing by components of the innate and humoral immune defenses, the responses of infected macrophages are thought to serve a central role in determining disease outcome (7). The interplay between host resistance factors and bacterial virulence factors are critical to determining the outcome of infection. On the host side, macro-

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1 The abbreviations used are: phox, phagocyte NADPH oxidase; cfu, colony-forming unit; N,N,N’-n-monomethyl arginine; DPI, diphenyleneiodonium; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; IFN-γ, interferon-γ; iNOS, inducible nitric-oxide synthase; L-NMMA, L-N-g-monomethyl arginine; DPI, diphenyleneiodonium; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; IFN-γ, interferon-γ; L-NMMA, L-N-g-monomethyl arginine; DPI, diphenyleneiodonium; ERK, extracellular signal-regulated kinase; L-NMMA, L-N-g-monomethyl arginine; DPI, diphenyleneiodonium; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco's modified Eagle's medium; PBS, fetal bovine serum; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Cytokines secreted during infection, including interferon, may affect bacterial replication by modifying its intracellular environment. Macrophages are required for effective host defense against *Salmonella Typhimurium* in the murine typhoid model (9–11). Cytokines secreted during infection, including interferon (IFN)-γ (12), are essential for host defense against *Salmonella* infection. IFN-γ-primed macrophages may be important in mediating bacterial clearance in immune mice (7), and IFN-γ stimulation up-regulates the expression of many of these antimicrobial effectors and impairs replication of *Salmonella Typhimurium* within macrophages (12). On the bacterial side, while *Salmonella Typhimurium* initiate a pro-inflammatory response by macrophages, some bacteria are able to secure an intracellular niche within a distinct endosomal compartment where replication occurs 4–8 h after infection. Bacterial virulence protein mutants that cannot replicate within macrophages are strongly attenuated for systemic disease within the murine typhoid model, reinforcing the importance of *Salmonella*-macrophage interactions (13, 14).

Distinct antibacterial activities have been observed in macrophages at different times during infection (10). The responses of macrophages to intracellular *Salmonella Typhimurium* at later times post-infection are likely critical in mediating the outcome of infection but have not been well characterized, with most of the work centered upon the first few hours of infection. We demonstrate here that IFN-γ-primed RAW 264.7 macrophage-like cells are capable of restricting the bacterial replication that is permitted by naive RAW 264.7 cells. To identify host factors mediating this control of bacterial replication, we have used gene arrays to examine the transcriptional responses of IFN-γ-primed RAW 264.7 cells to intracellular *Salmonella Typhimurium* at 8 h post-infection. We identified up-regulated MEK1 kinase mRNA levels, which were confirmed at the levels of RNA, protein, and kinase activity. MEK activity correlated with inhibition of bacterial replication and induction of bacterial filamentation, an indicator of bacterial stress. MEK kinase and phox activities can impact each other, and we observed that phox inhibitors mimicked the effect of MEK inhibitors in reducing bacterial filamentation. MEK kinase or oxidase inhibitors added later during infection could significantly decrease bacterial filamentation, suggesting that MEK and phox activities at later times are primarily responsible for mediating bacterial filamentation. While phox activity can positively regulate as well as be regulated itself by MEK kinase activity, our results suggest that MEK and phox activities function in parallel to mediate bacterial filamentation.

**Experimental Procedures**

**Growth Conditions of Bacterial and Macrophage Cells**—The *Salmo nella enterica* serovar Typhimurium strain SL1344 was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and stored at −80 °C. Bacterial growth media were as described previously (15). Briefly, *Salmonella Typhimurium* were grown at 37 °C on LB agar plates. Under these infection conditions, macrophages contained an average of 1 bacterium per cell 2 h after infection, as determined by standard plate counts, which permitted analysis of macrophages at 24 h post-infection.

**Immunofluorescence**—IFN-γ-primed RAW 264.7 cells (1 × 10^5 cells/well) were seeded on 12-mm diameter glass coverslips in 24-well plates. Following infection with *Salmonella Typhimurium* for 24 h, fixation was performed with 2.5% paraformaldehyde for 10 min at 37 °C. Fixed cells were washed three times with PBS and blocked in PBS containing 10% normal goat serum for 2 min. Extracellular bacteria were labeled by sequentially overlaying coverslips with a rabbit polyclonal primary antibody to *Salmonella Typhimurium* lipopolysaccharide (LPS; Difco, Detroit, MI) at 1:200 and an Alexa 568-conjugated mouse anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) at 1:400 in PBS + 10% normal goat serum for 20 min. Coverslips were mounted onto 1-mm glass sides using Mowiol (Aldrich). To quantify cells containing filamentous bacteria, only intracellular *Salmonella Typhimurium* were counted (not labeled by the extracellularly applied LPS-specific antibody). Bacteria were scored as “filamentous” when they were >5× longer than their width. At an approximate 5 × magnification, three populations were scored: the number of infected cells containing predominantly filamentous bacteria, the number of infected cells where >50% of intracellular bacteria were of normal size, and the number of infected cells containing bacterial cells that were of normal size. Significance was determined by calculating p values using an unpaired two-tailed t test. The level of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL)-positive (Roche Molecular Biochemicals, Laval, Quebec, Canada) apoptotic cells was less than 10% for all conditions.

**RNA Isolation and Northern Blotting**—At various times post-infection, IFN-γ-primed RAW 264.7 cells were washed once with PBS and scraped to detach the cells from the dish. RNA was then isolated using Trizol according to the manufacturer’s directions (Invitrogen). RNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform. The RNA was then precipitated with 2.5 volumes of 100% ethanol and 0.10 volume sodium acetate, pH 5.2, resuspended in RNA-free water containing RNase inhibitor (Ambion, Austin, TX), and stored at −70 °C. RNA quality was assessed by gel electrophoresis and staining with ethidium bromide. Northern blots were prepared as described previously, using 5–10 μg of total RNA per lane (16). To prepare templates for probe synthesis, cDNA was prepared from total RNA purified from RAW 264.7 cells using oligo(dT) and SuperScriptII reverse transcriptase (Invitrogen). The following primer pairs were designed to amplify portions of the indicated macrophage genes: MEK1, 5′-GGTTGCTTCCAGCCTCCTCCC-3′, 5′-AGATGGTGGCTGCTTTAGGG-3′; GAPDH, 5′-AGAACATCATATGCCTAGCC-3′, 5′-CTGGAGTGAAGAACCAG-3′; and LPS-primed RAW 264.7 cells were prepared by PCR using 50 μg of the appropriate PCR product template, cDNA, antisense oligonucleotide, and modified nucleotides to facilitate repeated stripping of blots (Strip-EZ PCR, Ambion). These single-stranded PCR products were column-purified (Qiagen, Mississauga, Ontario, Canada) and labeled with biotin using psoralen-biotin (Ambion) and cross-linking with 365 nm ultraviolet light. Overnight hybridization at 42 °C was followed by washing probe in UltraHyb (Ambion). The BrightStar non-isotopic de-
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Infection kit (Ambion) was used for probe detection according to the manufacturer’s protocols. Northern blots were quantified by densitometry using an Alphalmager system (Alpha Innotech Co., Santa Barbara, CA).

cDNA Array Hybridization—Atlas™ Mouse cDNA Expression Arrays I (7741–1; CLONTECH, Palo Alto, CA) consisted of a matched set of positively charged membranes containing duplicate spots of 588 murine partial cDNAs. 32P-Radiolabeled first strand cDNA probes were prepared from 5 μg of total RNA from each cell population using Moloney murine leukemia virus transcriptase and pooled primers specific for the 588 genes. Hybridization conditions and data analysis have been described previously (16).

Preparation of Protein Extracts and Western Blots—IFN-γ-primed RAW 264.7 cells (5 × 10^5/well) were seeded in six-well tissue culture plates and incubated overnight. At various times post-infection, cells were collected into 100 μl of boiling 5× SDS-PAGE loading buffer. Total protein lysates were resolved on a 12% acrylamide SDS-PAGE gel, electrotransferred to nitrocellulose membrane, and blocked with 5% skim milk in Tris-buffered saline-0.1% Tween 20. Antibodies were used at the following concentrations: rabbit anti-MEK1, 1:1000 (New England Biolabs, Beverly, MA); rabbit anti-phosphorylated MEK1, 1:1000 (New England Biolabs; kindly provided by Dr. B. Ellis, University of British Columbia); rabbit anti-ERK1, 1:2000 (New England Biolabs, Beverly, MA); monoclonal phosphospecific anti-p44/p42 (ERK1/2), 1:200; (New England Biolabs); and monoclonal anti-actin (ICN, Montreal, Quebec, Canada). Blots were incubated with primary antibodies overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and detected by enhanced chemiluminescence (Amersham Biosciences, Baie d’Urfé, Quebec, Canada). Western blots were quantified by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Chemical Inhibitors of MEK Kinase, NADPH Oxidase, and iNOS—IFN-γ-primed RAW 264.7 cells were pretreated with inhibitors for 30 min prior to infection at the following concentrations: 50 μM PD98059 (Calbiochem), 50 μM U0126 (Promega, Madison, WI), 4 μM diphenylenedioimid (DPI), Sigma), 250 μM acetovanillone (apocynin; Aldrich), 1 mM ascorbic acid (Sigma), 30 mM N-acetylcyesteine (Sigma), 2 mM N(G)-monomethylarginine (L-NMMA, Molecular Probes), or 2 mM N(G)-monomethylarginine (o-NMMA, Molecular Probes). Fresh inhibitors were added immediately after infection, at 2 h, and 6–8 h post-infection to ensure potency. Control cells were treated with equivalent volumes of DMSO per ml of media. To remove inhibitors from pretreated cells, monolayers were washed three times with PBS at 8 h post-infection and then cultured for 16 h in DMEM containing 10% FBS and 10 μg/ml gentamicin.

Quantification of Intracellular ROIs and Extracellular Nitrite—Intracellular ROIs were quantified by a luminol-enhanced chemiluminescence assay as described previously (17, 18). Briefly, 1 × 10^5 IFN-γ-primed RAW 264.7 cells were seeded per well in six-well tissue culture plates and primed with IFN-γ for 24 h. Cells were pretreated with inhibitors or Me2SO in media and infected as described above. After 8 or 24 h of infection, cells were washed once with PBS, scraped into 200 μl of substrate warmed to 37 °C (PBS containing 10% heat-inactivated FBS, 5 × 10^-5 M luminol (5-aminob-2,3-dihydro-1,4-pthalazinedione, Sigma) as an indicator of ROIs, and 50 units/ml superoxide dismutase (Sigma) and 2000 units/ml catalase (Sigma) to remove extracellular ROIs). Duplicate samples of 100 μl each were transferred to a clear-bottomed white 96-well plate, and chemiluminescence (light) units were quantified for 20 min using a TECAN spectrophotometer/luminometer (Männedorf, Switzerland), and the light units detected per minute over this time period were calculated. Nitrite concentration in extracellular medium of infected cells after 24 h was measured using a Griess reagent kit (Molecular Probes) according to the manufacturer’s instructions.

RESULTS

IFN-γ Priming of RAW 264.7 Cells Restricts Salmonella Typhimurium Growth—IFN-γ is essential for clearance of Salmonella Typhimurium within the murine epithoid model, and we have shown previously that IFN-γ has pleiotropic effects on macrophage transcriptional responses at early times to Salmonella Typhimurium infection (16). To establish a model for investigating macrophage responses that are effective in restricting Salmonella Typhimurium replication, we assessed the effect of IFN-γ on the ability of RAW 264.7 macrophage-like cells to control intracellular numbers of Salmonella Typhimurium. As shown in Fig. 1A, the number of intracellular Salmonella Typhimurium increased 6-fold over a 22.5-h period in RAW 264.7 cells. These cells permit Salmonella Typhimurium replication after 4–8 h, although avoidance of macrophage-mediated killing could partially contribute to the increase. In contrast, intracellular bacterial numbers did not increase in RAW 264.7 cells primed with IFN-γ over this same period (Fig. 1A). We hypothesized that IFN-γ-primed RAW 264.7 cells provide a more relevant model for studying macrophage responses that are effective in limiting Salmonella Typhimurium infection, as host factors should be maximally expressed in IFN-γ-primed RAW 264.7 cells that restrict intracellular bacterial numbers. This choice of model was strengthened by the observation that both primary murine macrophages and IFN-γ-primed RAW 264.7 cells restrict the intracellular load of Salmonella Typhimurium (19). Macrophages Induce Bacterial Filamentation at 24 h Post-infection—IFN-γ priming of macrophages restricts intracellular Salmonella Typhimurium replication, but the precise mech-
anisms for this control are unclear (20). To better understand the interactions between macrophages and Salmonella Typhimurium, IFN-γ-primed RAW 264.7 cells were infected with Salmonella Typhimurium expressing green fluorescent protein (GFP) and examined by fluorescence microscopy. While intracellular bacteria exhibited normal morphology after 8 h of infection, 47 ± 12% of infected cells contained filamentous bacteria that were >3× the length of a typical bacterium after 24 h (Fig. 1B). Filamentous bacteria were observed using another Salmonella Typhimurium strain (14028s), indicating that filamentation is shared by more than one strain of Salmonella Typhimurium (data not shown). Filamentous bacteria had partial or absent septa, suggesting a defect in completion of cell division, an indicator of bacterial stress (21, 22).

Salmonella Induces MEK1 Kinase mRNA and Activity—To examine whether macrophage gene expression was responsible for this effect on bacterial replication at later infection times, we used gene array analysis to profile the transcriptional responses of IFN-γ-primed RAW 264.7 cells to intracellular Salmonella Typhimurium after infection for 4, 8, and 24 h. Hybridization of cDNA arrays indicated that MEK1 kinase mRNA levels were elevated in IFN-γ-primed RAW 264.7 cells at 8 h post-infection but not at 4 h post-infection (data not shown). This observed modest increase in MEK1 mRNA after 8 h of Salmonella Typhimurium infection was confirmed by Northern blot analysis. As seen in Fig. 2A, MEK1 mRNA was transiently up-regulated at 8 h and 18 h post-infection, was not observed prior to 8 h, and reduced to the level in uninfected cells at 24 h (n = 3). The increase in MEK1 mRNA 8 h after infection ranged from 1.2- to 2.5-fold relative to uninfected cells in each of eight experiments (Fig. 2B). We observed similar kinetics in the increase in MEK1 mRNA abundance in cells stimulated with 1 μg/ml Salmonella Typhimurium lipopolysaccharide for 8 or 24 h (LPS; data not shown). This increase in MEK1 mRNA after infection for 8 h was abrogated when cells were treated with the MEK kinase inhibitor U0126 prior to infection, suggesting that transcriptional up-regulation of MEK1 is mediated by prior kinase activity (Fig. 2A and quantification in Fig. 2B).

While activation of MEK/ERK kinase cascades have previously been shown to occur within 1 h of Salmonella Typhimurium infection or LPS stimulation (2), MEK kinase activity at much later times of infection or its transcriptional regulation following infection has not previously been reported. The observed elevation of MEK1 mRNA level in Salmonella Typhimurium-infected IFN-γ-primed RAW 264.7 cells relative to uninfected cells was followed by increased MEK1 protein abundance at 24 h, as determined by Western blot analysis (Fig. 2C). A modest increase in MEK1 protein of 1.5 ± 0.2-fold was detected at 24 h post-infection when normalized to actin protein and relative to uninfected cells (n = 4). This is of a comparable magnitude to the induction of MEK mRNA at 8 h post-infection. At the times when increases in MEK mRNA and protein abundance were measured, MEK protein was phosphorylated, an essential step in activation of MEK kinase. MEK phosphorylation was maximal at 1 h but remained sustained at a modest level in infected cells throughout 24 h, as seen in longer exposures of Western blots (Fig. 2C and data not shown). MEK activity could be detected throughout the infection period, as measured by Western blot analysis of phosphorylation of its downstream targets, the ERK1/2 kinases. As seen in Fig. 3, A–C, phosphorylation of ERK1/2 was maximal within 1 h following stimulation, but phosphorylation remained elevated throughout the 24-h period examined when compared with uninfected cells (quantification of ERK2 phosphorylation in Fig. 3D). MEK1 abundance and activity were similar in IFN-γ-primed RAW 264.7 cells infected by Salmonella Typhimurium or stimulated with 1 μg/ml purified Salmonella Typhimurium LPS over 24 h. Therefore, up-regulated MEK1 mRNA, protein, and activity in IFN-γ-primed RAW 264.7 cells during a 24-h infection by Salmonella Typhimurium can be triggered, at least in part, by bacterial LPS.

Increased MEK1 Activity Correlates with Bacterial Filamentation—Since both MEK kinase activity and bacterial filamentation were observed at 24 h post-infection, and MEK/ERK kinases are strong candidates for augmenting macrophage defenses against intracellular pathogens, we investigated whether there was a connection between induction of MEK kinase signaling and bacterial filamentation. IFN-γ-primed RAW 264.7 cells were pretreated with the MEK inhibitor PD98059 or Me6SO as a control and infected with Salmonella Typhimurium expressing GFP. Remarkably, MEK inhibition by PD98059 caused a 76 ± 10% reduction in the number of cells containing predominantly filamentous bacteria (representative immunofluorescence shown in Fig. 4A and quantification in Fig. 4B). Similar results were obtained using U0126, another

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Salmonella Typhimurium infection increases MEK1 mRNA and protein in IFN-γ-primed RAW 264.7 cells. A, Northern blot analysis of MEK1. RNA was isolated from Salmonella Typhimurium-infected or mock-infected IFN-γ-primed RAW 264.7 cells at 1, 2, 4, 6, 8, 18, or 24 h following infection. The label 8 h + U0126 denotes RNA isolated from cells that were pretreated with the MEK kinase inhibitor U0126 and infected for 8 h. Northern blots were hybridized with a MEK1-specific probe and then stripped and re-probed using a GAPDH-specific probe. A representative experiment is shown (n = 3). B, quantification of Northern blot analysis. Northern blot hybridization signals for MEK1 at 8, 18, and 24 h in uninfected (white bars) or infected cells (gray bars) or at 8 h in infected cells pretreated with U0126 (black bar) were quantified by densitometry and normalized to the hybridization signals for GAPDH and to the level in uninfected cells at each time point. mRNA levels for GAPDH in uninfected cells at 8, 18, and 24 h were equivalent. The mean ± S.D. for the following number of independent experiments is shown: 8 h, n = 8; 8 h + U0126, n = 3; 18 h, n = 3; 24 h, n = 8. * denotes p < 0.01. C, Western blot analysis of MEK1. Protein lysates were prepared from Salmonella Typhimurium-infected (+) or mock-treated (−) IFN-γ-primed RAW 264.7 cells at 1, 2, 4, 6, 8, and 24 h following infection and separated by SDS-PAGE electrophoresis. Western blots were probed with antibodies specific for total MEK1, phosphorylated MEK1, and total ERK1/2, to confirm equal loading of samples. A representative experiment is shown (n = 3).
MEK inhibitor but with a different mode of action (Fig. 4B and data not shown) (23). Both inhibitors were functional in greatly reducing MEK activity under our experimental conditions, as determined by Western blot analysis of ERK1/2 phosphorylation (Fig. 6 and data not shown). This suggests that MEK-dependent control of intracellular bacterial proliferation is mediated through impairment of bacterial cell division, resulting in filamentation.

**MEK1 Activity Controls Intracellular Bacterial Numbers**—To confirm the morphological impairment of bacterial replication revealed by fluorescence microscopy using an independent assay, we counted the number of cfus isolated from infected cells treated with MeSO or MEK inhibitor PD98059 after 24 h. When MEK activity was inhibited in IFN-γ-primed RAW 264.7 cells, we observed a significant (3-fold) increase in the number of intracellular Salmonella Typhimurium that could form colonies on solid media (Fig. 5). Similar results were obtained using the MEK inhibitor U0126 (data not shown), further supporting our observation that control of intracellular bacterial numbers results from increased MEK activity. Neither MEK inhibitor altered internalization of bacteria, as the number of intracellular cfus at 3 h post-infection was comparable between inhibitor-treated and untreated cells (Fig. 5). Both MEK inhibitors were active, reducing ERK1/2 phosphorylation to the basal levels observed by Western blotting in uninfected cells (Fig. 7 and data not shown).

**Phagocyte NADPH Oxidase Also Mediates Bacterial Filamentation**—We proceeded to investigate a mechanism to explain the effect of MEK kinase activity on impairing bacterial replication. The antibacterial effectors phox and iNOS were strong candidates in mediating bacterial stress and inducing filamentation. Both reactive oxygen and nitrogen species can inhibit Salmonella Typhimurium survival in macrophages in vivo (9, 10). ROIs produced by phox can positively regulate MEK kinase activity, and phox and iNOS can be activated by MEK/ERK signaling (24–29). The iNOS inhibitors l-NMMA and Nω-nitro-l-arginine methyl ester hydrochloride (l-NAME) had no significant effect on bacterial morphology when compared with inactive d-NMMA or Me2SO, respectively (Fig. 4 and data not shown). In addition, l-NMMA did not have a synergistic effect when applied with various antioxidants, although l-NMMA was functional in decreasing iNOS activity and the corresponding concentration of extracellular nitrate by the Griess assay (data not shown). By contrast, numerous NADPH oxidase inhibitors and antioxidants had a similar effect to the MEK inhibitors in reducing bacterial filamentation in IFN-γ-primed RAW 264.7 cells (representative immunofluorescence shown in Fig. 4A and quantification in Fig. 4B). The inhibitor DPI, which lowers ROI levels and increases intracellular levels of the antioxidant glutathione, reduced the number of cells containing predominantly filamentous bacteria by 97 ± 5%. Since DPI also inhibits iNOS activity, we used a variety of other chemical inhibitors of oxidative burst to confirm the involvement of oxidative activity in mediating bacterial filamentation. The antioxidant N-acetyl-l-cysteine and the phox flavoprotein inhibitor acacetovanilnone reduced the number of these filamentous bacteria-containing cells by 86 ± 12% and 53 ± 13%, respectively.

**MEK Kinase and Phox Activities at Later Times Mediate Bacterial Filamentation**—As seen in Fig. 3, phosphorylation of ERK1/2 is maximal within 1 h of infection but remains elevated for 24 h. To determine whether this sustained activity plays a role in mediating bacterial filamentation, distinct from the early maximal kinase activation, the MEK inhibitor U0126 was added to infected IFN-γ-primed RAW 264.7 cells 1, 2, 4, 6, or 8 h post-infection and compared with cells treated with inhibitors prior to infection. Interestingly, U0126 significantly reduced the number of cells containing predominantly filamentous bacteria relative to MeSO when added up to 8 h after infection rather than pre-infection (Fig. 6A). Similar results were observed when the antioxidant DPI was added post-infection. As shown in Fig. 6B, DPI added up to 4 h post-infection was as potent in decreasing bacterial filamentation as cells pretreated with inhibitors. Inhibition of bacterial filamentation was also observed in cells treated with the MEK inhibitor PD98059 or antioxidant N-acetylcysteine 6 h post-infection (data not shown). Furthermore, cells treated with U0126 or DPI for the first 8 h of the infection and then removed by
Fig. 4. MEK1 and NADPH oxidase activity correlates with bacterial filamentation. A, IFN-γ-primed RAW 264.7 cells were seeded on glass coverslips, pretreated with either chemical inhibitors or MeSO (DMSO) (control), and infected with Salmonella Typhimurium expressing GFP. After 24 h, the monolayers were fixed and the coverslips incubated with anti-Salmonella LPS antibody and a red fluorophore-conjugated secondary antibody to label extracellular bacteria. All bacteria shown were extracellular. The effect of various inhibitors on bacterial filamentation was assessed relative to infected cells mock-treated with MeSO. Decreased bacterial filamentation was observed in cells treated with PD98059 to inhibit MEK kinase activity. Decreased bacterial filamentation was also observed in cells treated with DPI to inhibit ROIs. No significant inhibition of bacterial filamentation was observed in cells treated with L-NMMA to inhibit iNOS. Similar results for each inhibitor were observed in ≥3 independent experiments. B, quantification of decrease in bacterial filamentation by MEK kinase inhibitors. Cells were pretreated with each inhibitor, and infected cells containing intracellular Salmonella Typhimurium that were not labeled by the extracellularly applied LPS-specific antibody were counted. The mean percentage of cells containing predominantly filamentous bacteria ± S.D. is shown relative to the percentage of MeSO-treated cells, which was set to 100% (36 ± 8% of infected MeSO-treated cells contained predominantly filamentous bacteria). Pretreatment with the MEK kinase inhibitors PD98059 or U0126 or the oxidase inhibitors and antioxidants DPI, acetovanillone, or N-acetyl-L-cysteine significantly decreased the number of cells containing predominantly filamentous bacteria relative to MeSO-treated cells (* denotes p < 0.01). No significant inhibition of bacterial filamentation was observed in cells treated with l-NMMA to inhibit iNOS relative to cells treated with MeSO or the inactive enantiomer d-NMMA (p = 0.05 and p = 0.85, respectively). For each experiment, 100–250 infected cells were counted per condition. The number of independent experiments for each condition was as follows: MeSO, n = 10; PD98059, n = 8; U0126, n = 4; DPI, n = 5; N-acetylcysteine, n = 3; acetovanillone, n = 3; l-NMMA, n = 4; d-NMMA, n = 3.

Fig. 5. MEK1 activity controls Salmonella Typhimurium replication. IFN-γ-primed RAW 264.7 cells were pretreated with PD98059 (MEK inhibitor, filled bars) or MeSO (control, white bars) and then infected with Salmonella Typhimurium. Extracellular bacteria were removed after 30 min by washing, and the remaining bacteria were killed using gentamicin. At 3 and 24 h following infection, monolayers were lysed with detergent and intracellular bacteria enumerated by plating dilutions on solid medium. The mean ± S.D. is shown. These data are representative of five independent experiments with three wells plated in triplicate per experiment; * denotes p < 0.001.

washing exhibited a similar degree of bacterial filamentation to cells not treated with either inhibitor (Fig. 6, A and B). Taken together, these data suggest that later MEK kinase and phox activities are primarily responsible for mediating bacterial filamentation and can be dissociated from the rapid MEK kinase and phox activities following Salmonella Typhimurium infection, which are maximal at 1 h.

**MEK Kinase and Phox Activities Appear to Function in Parallel to Mediate Bacterial Filamentation**—Phox activity produces ROIs that can enhance MEK kinase activity (25, 26). To assess whether the oxidase induces bacterial filamentation by increasing MEK kinase activity, we treated IFN-γ-primed RAW 264.7 cells with DPI prior to and during Salmonella Typhimurium infection and measured ERK phosphorylation relative to untreated cells. Antioxidant treatment did not reduce MEK kinase activity, as detected by phosphorylation of the downstream ERK kinases at 8 h (data not shown) or 24 h (Fig. 7 and data not shown). In addition, DPI did not attenuate the increase in total MEK kinase protein after 24 h of infection relative to uninfected cells (data not shown). In contrast, the MEK kinase inhibitors PD98059 and U0126 substantially reduced ERK phosphorylation (Fig. 7 and data not shown). These data suggest that phox activity does not augment MEK kinase activity either prior or subsequent to the induction of bacterial filamentation within RAW 264.7 cells.

To assess whether MEK kinase activity mediates bacterial
filamentation by positively regulating oxidase activity (24, 29), we measured the effect of various inhibitors on intracellular ROIs in infected cells. A luminol-based chemiluminescence assay detected elevated levels of intracellular ROIs within IFN-γ-primed RAW 264.7 cells after 6 h of infection by Salmonella Typhimurium, relative to uninfected cells. DPI, N-acetyl-L-cysteine, and acetovanillone were effective in significantly decreasing the products of phox activity (Fig. 8A and data not shown). However, treatment with the MEK kinase inhibitors U0126 or PD98059 did not significantly reduce intracellular ROIs (Fig. 8A and data not shown). Elevated ROIs were measured within infected cells at 24 h, when bacterial filamentation is observed, were similarly reduced by antioxidants and unchanged by inhibition of MEK kinase activity (Fig. 8B). These data suggest that MEK kinase activity does not alter oxidase activity and intracellular ROIs either prior to or during the presence of filamentous bacteria.

**DISCUSSION**

In the murine model of human typhoid fever, Salmonella Typhimurium establish a niche within macrophages where they can replicate and cause a systemic disease. Disease is mediated by a dynamic interplay between host responses to bacterial components and bacterial virulence mechanisms that are triggered upon entry into the host environment. We previously used gene arrays to profile the responses of the RAW 264.7 murine macrophage cell line to Salmonella Typhimurium infected or mock-treated for 24 h and then separated by SDS-PAGE electrophoresis. Western blots were probed with antibodies specific for phosphorylated ERK1/2 as an indicator of MEK1 activity and total ERK1/2 to confirm equal loading of samples. B, quantification of Western blots. The level of phosphorylated ERK2 was quantified by densitometry and normalized to the level of total ERK2. The mean ± S.D. are representative of four independent experiments; * denotes p < 0.01.

**FIG. 7.** NADPH oxidase does not mediate filamentation by modulating ERK phosphorylation. A, Western blot. Protein lysates were prepared from IFN-γ-primed RAW 264.7 cells pretreated with Me2SO (DMSO) or various inhibitors that were either Salmonella Typhimurium-infected or mock-treated for 24 h and then separated by SDS-PAGE electrophoresis. Western blots were probed with antibodies specific for phosphorylated ERK1/2 as an indicator of MEK1 activity and total ERK1/2 to confirm equal loading of samples. B, quantification of Western blots. The level of phosphorylated ERK2 was quantified by densitometry and normalized to the level of total ERK2. The mean ± S.D. are representative of four independent experiments; * denotes p < 0.01.

**FIG. 6.** MEK kinase and phox activities at later times mediate bacterial filamentation. A, the MEK inhibitor U0126 was added to infected IFN-γ-primed RAW 264.7 cells 1, 2, 4, 6, or 8 h post-infection and compared with cells treated with inhibitors prior to infection (0 h) or treated with Me2SO (DMSO). Some cells were treated with U0126 for the first 8 h of infection and then washed to remove the inhibitor for the remaining 16 h of infection (8 h chase). B, cells were treated as in A, except that the antioxidant DPI was used. For both A and B, the percentage of cells containing predominantly filamentous bacteria after infection for 24 h was counted. The mean ± S.D. are representative of three independent experiments, with >100 cells counted per condition per experiment; * denotes p < 0.01.
kinase activity observed throughout the 24-h infection period by phosphorylation of ERK1/2 may result from continued shedding of LPS by intracellular bacteria (31). Alternatively, a feedback mechanism of enhancement of MEK activity observed throughout the 24-h infection period.}

**Fig. 8.** MEK kinase does not mediate filamentation by increasing intracellular ROIs. A, intracellular ROIs 6 h post-infection. IFN-γ-primed RAW 264.7 cells (1 × 10^6) were pretreated with inhibitors or Me2SO (DMSO) and harvested 6 h post-infection. Intracellular ROIs were detected using a luminol chemiluminescence assay. Chemiluminescence (light) units were quantified every 20 min and the mean light units detected per minute over this time period ± S.D. is shown. Infection caused a significant increase in intracellular ROIs compared with uninfected cells, which was not significantly altered by treatment with the MEK inhibitor U0126. The oxidative inhibitor DPI significantly reduced intracellular ROIs within infected cells when compared with Me2SO-treated infected cells. The mean ± S.D. is shown and represents duplicate samples from at least three independent experiments; * denotes p < 0.01. B, intracellular ROIs 24 h post-infection. Cells were harvested 24 h after infection and ROIs detected as described in A.

The importance of MEK kinase signaling in effective host response to bacteria is highlighted by the evolution of a MEK-specific phosphatase that is required by *Yersinia* to colonize host cells. In macrophages, signals transmitted through MEK/ERK kinases are known to induce cell proliferation, differentiation, and the expression of proinflammatory genes such as TNF-α (5). MEK can exert its pleiotropic effects on cells by two general mechanisms. First, MEK/ERK signaling has a large number of downstream targets, including multiple transcription factors, phox, and iNOS, while a functional proteomics study recently identified an additional 20 candidate unrecognized MEK/ERK effectors (32). Second, the kinetics of MEK activity impacts the functional effect on the cell. For example, M-CSF causes maximal induction of ERK activity in macrophages after 5 min, leading to cell proliferation. In contrast, LPS stimulation induces maximal ERK activity after 15 min, arresting proliferation and promoting macrophage activation (33, 34). In addition to the previously reported early activation of MEK/ERK cascades, in this study we have shown that *Salmonella* also induce MEK kinase activation at later time points of infection. We have demonstrated that MEK inhibitors can be added 8 h after infection and still inhibit bacterial filamentation. In addition, chemical inhibition of MEK kinase signaling for only the first 8 h of infection results in the same extent of bacterial filamentation after 24 h as cells with functional MEK kinase activity throughout the entire infection. Together, this suggests that the sustained MEK activity at later time points plays a more important role in stressing intracellular bacterial replication than the early induction of MEK activity that peaks at 1 h. Interestingly, inhibition of MEK signaling has the opposite effect in epithelial cells infected by influenza virus (35). In this infection model, intact MEK signaling is necessary for viral replication as it controls nuclear export of viral ribonucleoproteins and the MEK inhibitor U0126 impaired viral production. MEK inhibitors added at 4 h after infection still substantially inhibited replication, providing another example of the dissociation of early ERK activation from later signaling events. MEK signaling appears to be playing a distinct role in macrophages infected by *Salmonella* Typhimurium. Our results emphasize the importance of studying host responses to *Salmonella* Typhimurium infection at times following the initial interactions between bacteria and macrophage, since distinct host responses can occur at different times during the infection.

Bacteria respond to stresses such as oxidative stress (8), nutrient stress (36), DNA damage (22), and some antibiotics (21, 37) by arresting DNA replication and/or cell division. Filamentous uropathogenic *Escherichia coli* have been observed during infection of bladder epithelial cells (38). Filamentation or arrest of bacterial septation has been observed in *Salmonella* Typhimurium infection of other cell lines. Mouse and rat fibroblast cell lines, which do not permit replication of *Salmonella* Typhimurium, interfere with bacterial cell division and result in filamentation (39). Recently, Martinez-Lorenzo et al. (40) observed non-septate *Salmonella* Typhimurium in a variety of human cancerous skin-related cell lines and primary melanocytes that also do not permit bacterial replication. These bacteria were exocytosed, non-invasive, and it was suggested that filamentation may facilitate antigen presentation, but neither the host nor bacterial cell signaling responsible for this effect on the bacteria was described (40). We also observed in macrophages that filamentous bacteria were non-invasive (data not shown). Furthermore, we observed that a reduction in the number of filamentous bacteria following treatment with MEK kinase inhibitors correlated with an increased intracellular load of viable bacteria. Therefore, MEK kinase and phox activity contribute to the macrophage’s ability to contain intracellular bacterial numbers by interrupting Salmonella Typhimurium replication, which impairs the infectious cycle. This is one of the first examples of a candidate gene identified by array hybridization for which a functional consequence for host response to infection has been characterized. Bacteria within the *Salmonella*-containing vacuole are subject to numerous stresses, including oxidative and nitrosative chemistry and limitation of necessary cations and nutrients (8). The relative contribution of other host stressors found within the Salmonella-containing vacuole on bacterial replication remains to be determined.

In our model, infection of IFN-γ-primed macrophages by *Salmonella* Typhimurium triggers both MEK kinase signaling and NADPH oxidase activity that can each limit bacterial replication. This effect is manifested at later time points and is distinct from the rapid MEK activation and oxidative burst.
MEK Kinase and NADPH Oxidase Inhibit Salmonella Replication

18761

Salmonella

MEK

P

ERK

ROI

phox

FIGURE 9. Working model for signaling in RAW 264.7 cells mediating filamentation of Salmonella Typhimurium. RAW 264.7 cells infected by Salmonella Typhimurium or stimulated by purified LPS activate both MEK kinase activity, resulting in phosphorylation of the ERK1/2 kinases, and phox activity, resulting in increased intracellular ROIs. MEK kinase and phox appear to function in parallel to inhibit bacterial replication and induce bacterial filamentation, an indicator of bacterial stress.

triggered in macrophages by LPS and phagocytosis. Assembly of the NADPH oxidase enzyme complex can be regulated by MEK kinase activity, since the inhibitor PD98059 attenuates superoxide production within neutrophils (18). Conversely, ROIs can induce MEK kinase activity (25). Our results suggest that MEK kinase and NADPH oxidase likely function in parallel to mediate bacterial filamentation, as inhibition of neither MEK or oxidative activity attenuated activity of the other (Fig. 9). To further test for an interaction between MEK and phox in mediating bacterial filamentation, we measured filamentation in cells treated with suboptimal concentrations of both U0126 and DPI to determine whether there was synergy between MEK and phox activities. In preliminary experiments, two different suboptimal concentrations of U0126 and DPI resulted in a higher percentage of IFN-γ-primed RAW 264.7 cells containing predominantly filamentous bacteria than when cells were treated with both inhibitors simultaneously. Further investigation is required to characterize how MEK-dependent signaling and phox-dependent ROIs impair bacterial replication and induce filamentous morphology. In addition to their direct antibacterial properties, ROIs can also act as signaling molecules and are potent stimulators of many signal transduction cascades in macrophages (41, 42). Phox is required for effective host resistance against Salmonella Typhimurium in the murine typhoid model (10). Early in infection, virulent Salmonella Typhimurium actively secretes one or more virulence proteins that block assembly of a functional multi-subunit phox enzyme on the membrane of the Salmonella-containing vacuole to avoid direct oxidative damage (43). However, we show that at later infection times, phox activity correlates with bacterial filamentation, an indicator of cell stress. Therefore, while Salmonella Typhimurium alters its gene expression upon entering a macrophage to protect itself from direct oxidant damage, the macrophage continues to produce ROIs that may exert antibacterial effects later in infection by an indirect mechanism, perhaps by altering the cellular redox state and subsequent cell signaling. A recent study by Ehrt et al. (44) provides evidence to support this concept. Using gene array expression profiling of macrophages from normal and phox-deficient mice, they observed that 484 differentially expressed genes following Mycobacterium tuberculosis infection and/or IFN-γ stimulation were phox-dependent. This suggests that ROIs may play a dual role during innate immune responses by exerting direct antimicrobial effects on intracellular bacteria as well as impacting the fate of intracellular bacteria by altering macrophage cell signaling.

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