Escalating Threat Levels of Bacterial Infection Can Be Discriminated by Distinct MAPK and NF-κB Signaling Dynamics in Single Host Cells

Graphical Abstract

Highlights

- Live-cell imaging reveals heterogeneity in host signaling during infection
- NF-κB and JNK dynamics vary with bacterial location, pathogenicity, and replication
- Activation of JNK is a signature of an escalation in bacterial threat level

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In Brief

Lane et al. use live-cell imaging to connect macrophage NF-κB and JNK signaling dynamics with distinct bacterial inputs. They identify a role for host signaling dynamics in bacterial threat assessment, and this may enable TLRs, a general bacterial detection system, to generate stimulus-specific responses.

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Escalating Threat Levels of Bacterial Infection Can Be Discriminated by Distinct MAPK and NF-κB Signaling Dynamics in Single Host Cells

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SUMMARY

During an infection, immune cells must identify the specific level of threat posed by a given bacterial input in order to generate an appropriate response. Given that they use a general non-self-recognition system, known as Toll-like receptors (TLRs), to detect bacteria, it remains unclear how they transmit information about a particular threat. To determine whether host cells can use signaling dynamics to transmit contextual information about a bacterial stimulus, we use live-cell imaging to make simultaneous quantitative measurements of host MAPK and NF-κB signaling, two key pathways downstream of TLRs, and bacterial infection and load. This combined, single-cell approach reveals that NF-κB and MAPK signaling dynamics are sufficient to discriminate between (1) pathogen-associated molecular patterns (PAMPs) versus bacteria, (2) extracellular versus intracellular bacteria, (3) pathogenic versus non-pathogenic bacteria, and (4) the presence or absence of features indicating an active intracellular bacterial infection, such as replication and effector secretion.

INTRODUCTION

The innate immune system faces an extraordinary signal discrimination challenge, as the number and type of bacterial inputs it must distinguish are far in excess of the number of Toll-like receptors (TLRs) available for detection. Two features of TLR signaling in particular appear ill suited to the discriminatory capacity of the innate immune network. First, although TLRs discriminate microbe from self via the recognition of pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2002; Medzhitov et al., 1997; O’Neill et al., 2013; Poltorak et al., 1998), these features are not specific to pathogens or even intact bacteria (Medzhitov, 2001; Vance et al., 2009). Second, distinct TLRs recognize different bacterial PAMPs; for instance, TLR2, TLR4, and TLR5 are cell-surface-localized TLRs that recognize bacterial lipoproteins, lipopolysaccharide (LPS), and flagellin respectively, while endosomal TLRs such as TLR9 recognize unmethylated CpGs in bacterial DNA (Gay et al., 2014). Additional cytosolic receptors (NOD1/2) also function to detect intracellular PAMPs, such as peptidoglycan (Medzhitov, 2007). Despite their recognition of distinct ligands, these receptors subsequently converge on many of the same set of signaling pathways, including nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPKs), and interferon regulatory factors (IRFs), to execute a transcriptional response (Kawai and Akira, 2010). Despite these constraints, certain features of a bacterial infection, such as viability, replication, and pathogenicity, have been shown to induce a specific response in immune cells (Blander and Sander, 2012; Ivanov and Roy, 2013; Saliba et al., 2016; Sander et al., 2011; Vance et al., 2009). How a system that responds to general stimuli can nonetheless discriminate an early, active infection remains largely mysterious.

The solution may lie in the dynamic aspects of host and pathogen interaction during infection. Recently, it has been proposed that the context in which PAMPs activate TLRs may be of paramount importance for the execution of tailored downstream responses (Vance et al., 2009). These contextual cues, designated “patterns of pathogenesis,” encompass key features of an intracellular bacterial infection that an immune cell needs to be able to detect—from bacterial internalization, intracellular bacterial replication, and death to the bacteria-mediated perturbation of host processes. This latter feature is specific to pathogens, such as Salmonella typhimurium, and typically involves the secretion of bacterial effector proteins into the host cytosol via a type 3 secretion system (T3SS) (Jennings et al., 2017). The coupling of the initial TLR-sensing of the bacterial input with the detection of these contextual cues could expand the discriminatory capacity of the host cell and may be critical for detection of pathogens, many of which have evolved mechanisms to minimize detection by the host (Andersen-Nissen et al., 2005; Kawasaki et al., 2004; Rebei et al., 2004). However, there remains a significant gap in our knowledge regarding the cellular mechanisms involved in the transmission and processing of patterns of pathogenesis during infection.

The dynamics of key innate immune signaling pathways offer a compelling candidate for such a mechanism. Cellular signaling dynamics can be used by cells to discriminate between different types and concentrations of ligand in the environment (Albeck et al., 2013; Batchelor et al., 2011; Nandagopal et al., 2018; Purvis et al., 2013) and may be critical for assigning a specific infection load (Andersen-Nissen et al., 2005; Kawasaki et al., 2004; Rebei et al., 2004). Most importantly, the dynamics of key signaling pathways allow cells to assign more than one feature of an infection challenge, as the number and type of bacterial inputs it must distinguish are far in excess of the number of Toll-like receptors (TLRs) available for detection.
and Lahav, 2013; Ryu et al., 2015; Tay et al., 2010). Moreover, these temporal patterns enable cells to conduct complex signal processing behaviors (Antebi et al., 2017) and have been linked to cellular decision-making processes, including cell death and differentiation (Purvis et al., 2012; Santos et al., 2007; Suel et al., 2006). Two of the major signaling arms downstream of TLRs, NF-κB and the MAPKs, display heterogeneous dynamics in single cells; these have been characterized in response to innate immune stimuli, such as purified PAMPs and TNF-α (Cheng et al., 2015; Gutschow et al., 2013; Kellogg et al., 2017; Lee et al., 2014; Nelson et al., 2004; Regot et al., 2014; Sung et al., 2014; Tay et al., 2010). Thus, while an immune cell could co-opt signaling pathways beyond the TLRs to transmit contextual information about a given bacterial stimulus, there is increasing evidence that a cell could also use the dynamics of TLR signaling proteins for this purpose. For example, S. typhimurium infection of fibroblasts has been shown to reduce NF-κB dynamics in infected cells via the secretion of effectors (Ramos-Marquéz et al., 2017), while Cryptococcus neoformans infection of macrophages was found to increase NF-κB signaling by modulating the negative feedback response (Hayes et al., 2016).

These studies show that host-bacterial interactions are heterogeneous and non-static; therefore, dynamic single-cell measurements that simultaneously monitor changes in bacteria together with host signaling are essential to decipher the contribution of innate immune signaling dynamics to bacterial threat assessment. Here, we combine quantitative measurements of NF-κB and MAPK dynamics with those of S. typhimurium infection and load, all inside of individual macrophages over 24 h and find that host cells can encode contextual cues about the specific bacterial threat in MAPK and NF-κB signaling dynamics.

RESULTS

Analyzing Bacterial Threat Assessment by Imaging Host-Pathogen Dynamics in Single Cells

To accurately determine the threat posed by a given TLR stimulus, a host cell needs to be able to discriminate between a number of distinct situations all of which can activate MAPK and NF-κB signaling. To probe the discriminatory capacity of the host cell we examined four common decisions a host cell needs to make about a bacterial input; these include discriminating between (1) free-floating PAMPs versus an intact, living bacterium, (2) extracellular versus intracellular bacteria, (3) pathogenic versus non-pathogenic bacteria, and (4) the presence or absence of features indicating an active intracellular bacterial infection, such as replication and effector secretion (Figure 1A). To investigate these decision points and ask whether a cell can use TLR signaling to both sense and process the stimulus it encounters, both host signaling dynamics and bacterial infection should be measured in individual cells at the same time. We selected a macrophage-like cell line, RAW264.7, for these multiplexed, dynamic measurements of infection since macrophages are key mediators of the innate immune response to bacterial infection as well as a known reservoir for intracellular pathogens, such as S. typhimurium. To conduct live-cell microscopy, we established cell lines expressing live-cell reporters for both NF-κB and MAPKs (Figure 1B; STAR Methods). To quantitatively measure NF-κB signaling, we used a C-terminal fusion between the NF-κB subunit p65 and the fluorescent protein mRuby2; in this instance, the location of the reporter acts as a surrogate for NF-κB activity as upon TLR4 ligation and subsequent IkBα degradation, the fusion protein will translocate from the cytoplasm into the nucleus (Lee et al., 2009). To monitor MAPK signaling in single cells we took advantage of the recently developed kinase translocation reporters (KTRs) for c-Jun N-terminal kinase (JNK) and p38 (Regot et al., 2014). KTR function by translating a phosphorylation event into a localization change; for instance, the JNK-KTR shuttles from the nucleus to the cytoplasm as a function of activation status. Because of the recent work that identified aberrant regulation of ERK signaling in RAW264.7 cells we chose to exclude this MAPK from our study (Gottschalk et al., 2016). In addition, an H2B-iRFP670 fusion protein was used as a nuclear marker to facilitate cell tracking and downstream image analysis (Figure 1B; STAR Methods). To image bacterial infection we generated strains of either pathogenic S. typhimurium or a non-pathogenic strain of Escherichia coli (MG1655) that express the fluorescent protein mCerulean3. We used bacterial fluorescence to identify infected cells and the total intensity of mCerulean3 for each infected macrophage was used to track changes in bacterial levels over time (Figures 1C and 1D; STAR Methods). Together, this combination of reporters allowed us to image and track individual cells during infection and extract quantitative data on both MAPK and NF-κB signaling dynamics and bacterial infection from single cells (Figures 1C and 1D).

Phagocytosis of a Pathogen Induces NF-κB and MAPK Responses that Are Distinct from Those Induced by Other Types of Bacterial Inputs

When a population of cells is exposed to bacteria, the stimuli experienced by any individual cell can be quite heterogeneous; for instance, certain cells in the population become infected by the bacteria they encounter and retain these bacteria in intracellular vacuoles, while other host cells remain as uninfected bystanders (Gog et al., 2012). These uninfected bystanders either directly encounter extracellular bacteria or are stimulated only by the PAMPs shed from the bacteria into the environment. Despite this heterogeneity, the signaling response to infection has chiefly been addressed at the population level (Procyk et al., 1999; Rosenberger et al., 2000), leaving open the question of whether cells can distinguish and process these different inputs to produce a unique signaling response. To better characterize the signaling response associated with infection we exposed RAW264.7 cells expressing both the JNK-KTR and the NF-κB reporter to S. typhimurium grown to late-stationary phase, and imaged these host cells for 24 h. We used the fluorescent signal from S. typhimurium to separate cells in each well into two subpopulations, infected and uninfected bystanders. In agreement with a previous study (Gog et al., 2012), we found that the majority of the macrophage population remained uninfected after exposure to S. typhimurium (Figure S1A). The median response for each subpopulation over the first 6 h revealed that at low multiplicity of infections (MOIs), 1 and 2.5, the infected cell population displayed increased JNK and NF-κB signaling compared to uninfected bystanders, which showed a minimal response (Figures 2A and 2B). Under infection conditions at a higher MOI, 10, differences in the median response of the two subpopulations were abrogated for both
reporters, with a more rapid activation of JNK in infected cells the most obvious difference between the two (Figures 2A and 2B). A comparison of the relative differences in the median signaling responses of the two subpopulations as a function of MOI confirmed that the subpopulations were more distinct at lower MOIs and that JNK signaling was a better discriminator of infection than NF-κB (Figures 2A, 2B, S1B, and S1C).

Although we observed differences in the average response of the two subpopulations to S. typhimurium, it is known that averages mask many features of the individual cell response (Lahav et al., 2004; Nelson et al., 2004). Therefore, we next asked what features of the JNK and NF-κB single-cell responses could distinguish infected from uninfected bystander cells (Figure 2C).

This analysis revealed that at the single-cell level JNK and NF-κB are differentially used to encode S. typhimurium infection and exposure. In comparison to uninfected bystanders, S. typhimurium-infected cells have an overall increase in JNK activity, illustrated by the time to the first peak, maximum amplitude, number of peaks, and area under the curve (AUC). On the other hand, the time to the first peak and maximum amplitude of the NF-κB response encode the presence of the bacteria, both inside and outside of the cell, while only the number of peaks and the AUC of NF-κB encode information about whether the cell is infected. The ability of the cell to encode infection in JNK and NF-κB single-cell dynamics improves as the MOI is decreased. Overall, this feature-based analysis suggests that NF-κB dynamics may encode the general response to bacterial exposure while those of JNK may be more specifically connected to infection.

Next, to determine whether the act of phagocytosis could account for the differences between our two subpopulations, we then exposed cells to latex beads but found that particle uptake alone failed to induce a JNK response and only induced a transient period of NF-κB activity (Figure S2A). We observed similar trends for p38 responses as for JNK (Figures S3A–S3C); however, due to the higher signal-to-noise ratio of the

Figure 1. A Multiplexed Imaging Approach to Study Host-Pathogen Dynamics during Infection

(A) During infection, a host cell needs to both detect bacteria and assess the scale of the threat posed to the host. We examined four types of decisions a host cell needs to make in order to initiate an appropriate response to the bacterial input. Schematic illustrates the four decisions and the conditions we used to assess them.

(B) Dual host-pathogen imaging setup. RAW264.7 cells with MAPK-KTR-Clover (JNK, p38) and NF-κB-mRuby2 reporters were arrayed in 96-well imaging plates, exposed to bacteria or PAMPs, and imaged. Bacterial strains express mCerulean3. MAPK and NF-κB reporters translocate between the nucleus and cytosol as a function of activity. The host signaling dynamics and bacterial levels were tracked in individual cells over 24 h.

(C) Selected images from a single host cell infected with S. typhimurium followed over 24 h show JNK-KTR-Clover, NF-κB-mRuby2, and S. typhimurium-mCerulean3. Scale bar, 5 μm.

(D) Signaling dynamics for both JNK (green) and NF-κB (purple) reporters together with changes in S. typhimurium levels over time (blue) for the cell shown in (C). C/N ratio refers to cytoplasmic over nuclear intensity. N/C ratio refers to nuclear over cytoplasmic intensity. Salmonella intensity refers to the total bacterial fluorescence intensity in the macrophage.
Figure 2. JNK and NF-κB Signaling Dynamics Distinguish the Response of Cells to S. Typhimurium Infection from Other Types of Bacterial Inputs

(A and B) (i) Differences in host signaling between infected and uninfected bystander cells. Host cells were exposed to S. typhimurium at an MOI of 1, 2.5, or 10, imaged, and cells were grouped according to infection status. Median signaling dynamics of (A) JNK and (B) NF-κB for infected (blue) and uninfected bystander (black) cells are shown. Shaded areas represent a 68% confidence interval calculated by bootstrapping. Data shown are for the first 6 h. C/N ratio refers to cytoplasmic over nuclear intensity. N/C ratio refers to nuclear over cytoplasmic intensity. Yellow bars indicate the time points at which differences between the two subpopulations were significant using the Kolmogorov-Smirnov test with a confidence level of p = 0.01. (ii) Comparison of the scale of the signaling differences between the subpopulations across MOIs. Shown is a scatter plot of the median infected cell response against the median uninfected bystander response for all time points shown in (i). Data points are colored according to MOI. The black line represents x = y. See Figure S1 for plots of individual MOIs.

(C) Features of the single-cell traces of JNK and NF-κB distinguish S. typhimurium infected cells from uninfected cells. For each cell, the following features were extracted from the first 6 h of the time course for both JNK and NF-κB—number of peaks, time to the first peak, maximum signal amplitude in the first 2 h, and area under the curve (AUC) for 0–6 h. Mean and standard deviation are shown and significance for each set of infected and uninfected bystander measurements was calculated using a two-sided independent t test. *p < 0.01, **p < 0.001.

(D) Response to S. typhimurium infection is distinct from stimulation with heat-killed bacteria. Comparison of the median signaling dynamics of JNK and NF-κB in cells infected with S. typhimurium (blue) and the response of cells exposed to heat-killed S. typhimurium (green). Shaded areas represent a 68% confidence interval calculated by bootstrapping. MOI 2.5 was used for both samples. Data shown is for the first 6 h. Yellow bars indicate the time points at which differences between the two subpopulations were significant using the Kolmogorov-Smirnov test with a confidence level of p = 0.01.
Figure 3. *S. typhimurium* Infection Is Defined by Both the Activity and Duration of JNK and NF-κB Signaling

(A) Schematic of signaling classification strategy. The signaling state of individual cells was classified as being either dual JNK and NF-κB responder, NF-κB only responder, JNK only responder, or non-responder according to whether the signaling response for each reporter was above a threshold (see STAR Methods) for at least 30 consecutive min during the first 6 h of the time course. Thresholds are indicated by the dotted green (JNK) and purple (NF-κB) lines. Representative signaling traces for individual cells belonging to each of these groups are shown. Note that high-level JNK activation is rarely seen in the absence of an accompanying activation of NF-κB and this is evident in the low-level JNK response of the JNK only responder cell trace.

(B) The types of signaling activation states differ between *S. typhimurium* infected and uninfected bystander cells. For each subpopulation, infected and uninfected bystanders, the JNK and NF-κB signaling responses of individual cells were classified over the first 6 h according to the criteria in (A) and shown is the fraction of cells in each activation state across MOIs.

(C) JNK and NF-κB signaling dynamics of dual responder cells only were compared across each of the following conditions: *S. typhimurium*-infected cells, *S. typhimurium*-uninfected bystanders, or LPS-treated cells. Schematic of a sample signaling trace is shown to highlight two features of the signaling dynamics that were used to generate the plots shown in Figures 3D and 3E. Dotted line indicates activity threshold. Traces are grayed out where the activity is above the threshold and this is used in (D) to calculate the total time (hours) in which a given cell is active. Time of final JNK or NF-κB activity is the latest point in the time course at which the signal was above the threshold (dotted line) and is indicated by the orange arrow and is used in (E).

(D) The extent of JNK and NF-κB signaling in dual responder cells depends on the stimulus. For each condition—LPS 100 ng/mL (gray), *S. typhimurium* uninfected bystanders (black), and *S. typhimurium* infected cells (blue)—dual responders were first identified based on the criteria in Figure 3A. For each condition, we compared the fraction of dual responder cells that had at least "x" h of reporter activity. Hours of activity is calculated as indicated in Figure 3C. LPS n = 141.
JNK reporter, we chose to focus on it together with the NF-κB reporter for the remainder of our study. Overall, our data suggest that host cells can discriminate between intracellular and extracellular bacteria and that MAPK and NF-κB responses are sufficient to make this distinction, but at higher inocula of bacteria, the discrimination capacity of the host cell is decreased, potentially due to the higher concentration of environmental PAMPs. Based on these results, all subsequently described experiments used lower MOIs of bacteria, unless otherwise noted.

Although our results revealed distinct responses for infected and uninfected bystanders, we considered that it was possible that infected cells simply recognized a phagocytosed bacterium as a concentrated “bag of PAMPs.” To address the question of whether cells can discriminate between phagocytosis of a pathogen and stimulation with high concentrations of PAMPs we first stimulated cells with either heat-killed S. typhimurium or a purified PAMP, LPS. Both stimuli produced remarkably similar responses with rapid activation of both JNK and NF-κB signaling (Figures S4A and S4B). However, when we compared the average response of cells infected with live S. typhimurium to those exposed to heat-killed S. typhimurium, we observed that these stimuli elicit distinct signaling profiles, such as the timing, amplitude, and duration of the response (Figures 2D, S4C, and S4D). Overall, our initial findings indicate that in the absence of confounding PAMP stimulation, internalization of the pathogen S. typhimurium is recognized as a distinct stimulus from either extracellular S. typhimurium or PAMPs, suggesting that whether the stimulus transmitted during TLR activation is intracellular or extracellular can lead to differences in MAPK and NF-κB signaling responses.

**Internalization of S. Typhimurium Is Defined by Both the Activity and the Duration of JNK and NF-κB Signaling**

With differences identified between infected and uninfected bystander subpopulations, we next wanted to use our single-cell data to better understand whether activation of both TLR signaling arms is coordinated in the response of each subpopulation to S. typhimurium. We took advantage of our dual JNK and NF-κB reporter line to ask whether there was differential activation of the signaling arms in cells belonging to each subpopulation. For each cell, we used a threshold for each reporter (STAR Methods) to classify the signaling responses over the first 6 h into one of four groups: a cell either activated both JNK and NF-κB (dual responders), only NF-κB, only JNK, or neither (Figure 3A). For each subpopulation, infected and uninfected bystanders, the fraction of cells belonging to each activation group was calculated across MOIs. This revealed that independent of MOI, the majority of infected cells were classified as dual responders (Figures 3B and S5B). In contrast, uninfected bystanders were heterogeneous in their activation state and the fraction of dual responders depended on the MOI used. Furthermore, using our time-series data, we tracked the transition of these cells from the non-responder to the dual responder state. When we did this, we found that from the outset, infected cells steadily transitioned from the single NF-κB responder state to the dual responder state. In contrast, for uninfected bystanders, we found that it was not simply the case that they were slower to activate but instead only a minority of cells made the transition to become dual responders (Figure SSC). Together, these data suggest that the single and dual responder states represent different stages along a continuum of signaling activation and that the host cell decision to engage both TLR signaling arms is differentially regulated, depending on whether the stimulus is inside or outside the cell.

While dual responders are, thus, a hallmark of infected cells, they are also found in uninfected bystanders, although at a lower frequency, and in cells stimulated with high concentrations of LPS (Figures 3B, S5A, and S5B). Moreover, the transition of cells to the dual responder state was found to occur more rapidly and uniformly in response to high concentrations of LPS than in either S. typhimurium infected or uninfected bystander cells (Figure SSC). The induction of dual responders by these distinct stimuli led us to ask whether a cell can use signaling dynamics to encode the stimulus that triggers dual responders or if instead dual responders across all three of these populations are in fact alike. To examine this possibility, we first identified dual responders in each of the populations: S. typhimurium infected, uninfected bystanders, and LPS-stimulated cells (Figure 3C; STAR Methods) according to the criteria shown in Figure 3A. Next, we determined the total hours of JNK and NF-κB activity above a threshold for cells belonging to each group (Figure 3C). We used this to plot the fraction of cells in each group with a specified number of hours of activity (Figure 3D). When we compared the distributions of signaling duration between the dual responder groups in this way, we observed that the duration of JNK signaling was longest in S. typhimurium-infected cells, while NF-κB signaling duration was longest in LPS-stimulated cells. The increased importance of JNK signaling in infected cells was further confirmed when we compared the time at which both JNK and NF-κB signaling ended between the dual responder populations (Figures 3C and 3E). We found once again that JNK signaling in infected cells outlasted that of the other groups while contrasting results were observed for NF-κB, with signaling persisting longer in LPS-stimulated cells compared to the other dual responder populations (Figure 3E). Based on a previous study that observed an increase in total NF-κB levels in macrophages many hours after stimulation with LPS (Sung et al., 2014), we were curious whether using this measurement rather than the nuclear/cytoplasmic intensity ratio might reveal further differences in NF-κB signaling between the types of stimuli. Indeed, we found that similar to LPS, S. typhimurium exposure results in an increase in total NF-κB levels over time; however, this increase was similar between infected and uninfected bystander cells (Figure S6A), as was both the level and frequency of induction (Figure S6B). Furthermore, when we replotted the data in Figure 3D using this measure of NF-κB activity, it did so.
Figure 4. Extended JNK Signaling Dynamics Are a Signature of Non-pathogenic Bacteria

(A) Hours of JNK (left) and NF-κB (right) activity for *S. typhimurium* (n = 69) or *E. coli* (n = 248) infected cells. Mean is indicated by the black bar and adjacent number (h). Standard deviations for *S. typhimurium* data are (JNK = 4 h, NF-κB = 2.2 h) and for *E. coli* data are (JNK = 5.2 h, NF-κB = 2.8 h). p Values calculated using a two-sided independent t test (*p < 0.05, **p < 0.001).

(B) Sustained periods of JNK signaling in *E. coli*-infected cells. Representative images of the JNK and NF-κB reporters and the fluorescent *E. coli* from a single infected cell. JNK activity is on for extended periods as shown by the predominantly cytoplasmic localization of the reporter. *E. coli* is cleared from the cell shortly after the 13.3 h time point shown. Scale bar, 5 μm.

(C) (i) Shown are sample traces of a cell with long (left) or short (right) pulses of activity. A pulse of activity, marked by a black box, begins when the signal crosses the threshold (dotted line) and ends when it next dips below the threshold. The longest pulse of activity is shown in yellow. Traces are grayed out where the activity is above the threshold. (ii) The maximum pulse length of JNK (left) and NF-κB (right) activity is shown for LPS-stimulated cells, and *S. typhimurium* and *E. coli* infected and uninfected bystander cells. p Value calculated using a two-sided independent t test (**p < 0.001).

(D) Traces of JNK (green), NF-κB (purple), and *E. coli* (blue) for two cells infected with *E. coli*, which show either delayed (top) or rapid (bottom) clearance of the bacteria as indicated by the arrow. Data in the top plot correspond to the cell shown in Figure 4B.

(E) JNK signaling persists after *E. coli* is cleared from the cell. For each *E. coli*-infected cell that cleared the bacteria, we compared the time at which the bacteria were cleared to the time of final (i) JNK or (ii) NF-κB activity. The time of *E. coli* clearance is the time at which the bacterial signal goes to zero and the time of final JNK or NF-κB activity is the latest point in the time course at which the signal was above the activity threshold (see Figure S6D). Hexagons are colored according to cell density. Data shown are for MOI 1 (n = 178 cells). In the case of JNK, 90% of cells were found to have reporter activity after the bacteria had been cleared while for NF-κB only 53% of cells were found to have reporter activity after bacterial clearance.

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not improve the separation between the three groups of cells (Figure S6C). While this induction of NF-κB is known to have important consequences for cellular responses, in the absence of evidence linking it specifically to infection we used our nuclear/cytoplasmic ratio as a measure of NF-κB activity for the remainder of this study. Based on these results, JNK appears to be the signaling factor that differentiates infected cells from either bystander cells or cells exposed to LPS, while NF-κB appears to function as a more general sensor of bacterial threat. In addition, although cells under different bacterial inputs may activate both TLR signaling arms, features of the signaling dynamics are stimulus specific, suggesting that information about the nature of the bacterial threat might be encoded in the signaling dynamics.

**Pathogenic and Non-pathogenic Bacteria Induce Distinct JNK Dynamics**

Although the presence of a bacterium inside a host cell represents an escalation in the threat level, further assessment of the intracellular bacterium is needed before an appropriate response can be induced. For instance, a critical decision an infected host cell has to make is whether the internalized bacterium is pathogenic or not. While pathogenicity has been linked to divergent cytokine responses (Ivanov and Roy, 2013) it is unclear what role, if any, MAPK and NF-κB signaling play in relaying such information. To test whether pathogenicity of the internalized bacterium is associated with distinct MAPK and NF-κB signaling dynamics, we compared the macrophage response to a pathogen, *S. typhimurium*, to that of a related Gram-negative, non-pathogenic *E. coli* (MG1655) (see STAR Methods).

We first compared the response of infected and uninfected bystanders to *E. coli*. The frequency of infected cells after exposure to *E. coli* was higher than that observed using *S. typhimurium* (Figures S1A and S7A). However, similar to our results with *S. typhimurium*, we found that *E. coli* induced a stronger average signaling response in infected rather than uninfected bystanders, which could be more clearly identified at lower MOIs (2.5 and 1) (Figure S7B). Notably, this response was stronger than that seen in *S. typhimurium*-infected cells (Figures 2A, 2B, and S7B), perhaps reflecting strategies evolved by *S. typhimurium* to avoid detection by the innate immune system. Despite this stronger response, infection by *E. coli* was still encoded in features of both JNK and NF-κB dynamics, most notably those associated with increased signaling—number of peaks and AUC (Figure S7C). These results suggest that the initial TLR signaling response may contribute to a cell’s ability to differentiate pathogen from pathogen.

To compare the signaling of macrophages infected with pathogenic versus non-pathogenic bacteria we first compared the duration of the JNK and NF-κB response between *S. typhimurium* and *E. coli*-infected cells. We found that the mean duration of JNK signaling was 3 h longer in *E. coli*-infected cells than in *S. typhimurium*-infected cells (9.2 h versus 6.2 h) (Figure 4A). To a lesser degree, we also observed an increased NF-κB response in *E. coli*-infected cells (3.7 h versus 3 h) (Figure 4A). These results raised the question of whether a specific pattern of signaling dynamics was associated with the observed increase in the duration of signaling. NF-κB dynamics have been shown to display a classic pulsing behavior across multiple cell types and stimuli (Nelson et al., 2004; Sung et al., 2014; Tay et al., 2010), and although less well characterized at the single-cell level, JNK dynamics have typically been found to also be pulsatile (Fosbrink et al., 2010; Regot et al., 2014). Furthermore, for several proteins differences in temporal signaling patterns have shown to have significant effects on cellular phenotypes (Purvis et al., 2012; Santos et al., 2007; Traverse et al., 1992); in the context of innate immune signaling, distinct NF-κB dynamics have recently been linked to differences in gene expression in single cells (Lane et al., 2017). To determine whether infection with a pathogen or non-pathogen induced a distinct form of JNK signaling dynamics, we began by surveying the time-lapse movies for any gross changes in JNK activity. We observed numerous examples of *E. coli*-infected cells with long pulses of JNK activity (Figure 4B). Given this, we wanted to determine whether under distinct stimulation conditions there were differences in whether macrophages had long or short pulses of JNK or NF-κB signaling activity. To do this, we determined the maximum pulse length for both JNK and NF-κB across a range of inputs (Figure 4C). In contrast to the short, pulsing behavior observed in cells stimulated extracellularly with either LPS, *S. typhimurium*, or *E. coli*, we found that cells infected with bacteria had longer periods of JNK activity (Figure 4C). Moreover, the duration of these sustained periods of JNK signaling was increased in cells infected with *E. coli* compared to *S. typhimurium* (Figure 4C). Consistent with previous studies, we observed short bursts of NF-κB activity across all stimuli (Figure 4C). Taken together, these results suggest that bacterial infection induces a shift from short to longer, sustained pulses of JNKs signaling and that the duration of this signaling is significantly different for pathogenic and non-pathogenic bacteria.

We were intrigued by the sustained pulses of JNK signaling observed in *E. coli*-infected cells, specifically because most of these host cells eradicated or “cleared” the bacteria during the imaging period. In contrast, we did not observe bacterial clearance at a high frequency when cells were infected with *S. typhimurium*. We decided to investigate this clearance behavior further and observed that after an initial pulse of activity many *E. coli*-infected cells displayed long periods of sustained JNK signaling, which did not terminate after the bacteria had been cleared (Figure 4D). To determine the relationship between JNK signaling activity and *E. coli* clearance, we compared the time at which the *E. coli* signal disappeared to the time at which JNK or NF-κB signaling ended for every infected cell that subsequently cleared the bacteria (Figures 4E and S7D). Notably, we found JNK signaling persisted after the bacteria had been eliminated in 90% of cells, and in some cases, cells were still signaling 18–20 h after the infection had been eradicated (Figure 4E). NF-κB signaling duration showed no clear association.
with *E. coli* clearance. These results were invariant across MOI, indicating that the persistence of JNK signaling after *E. coli* clearance is independent of external PAMP concentration (Figure S7E).

In several contexts, sustained JNK signaling has been linked to apoptosis (Chen et al., 1996; Davis, 2000; Lin, 2003), and as such, we were curious as to how *E. coli*-infected cells tolerated this signaling behavior. NF-κB has been shown to negatively regulate JNK activity in response to TNF-α, and in this particular context, deletion of NF-κB induced a transition from a transient to a sustained JNK signal (Reuther-Madrid et al., 2002; Tang et al., 2001). Given this, we took advantage of our single-cell data measuring both TLR pathways in the same cell to explore the relationship between NF-κB activity and sustained JNK signaling during *E. coli* infection. To do this, we used the activation thresholds previously used to identify responder status (Figure 3B) to now classify single cells at each time point as having either both pathways active, only JNK, only NF-κB, or neither. Visualizing these data as a heatmap revealed that early during *E. coli* infection, both JNK and NF-κB activity are coupled (Figure 4F). However, later in the time course, these two branches of TLR signaling become decoupled, with long periods of JNK signaling associated with an absence of NF-κB signaling (Figure 4F). Together, these data suggest that persistent JNK signaling after the bacterial stimulus has disappeared is associated with an absence of NF-κB signaling, and such opposing activation status may be critical for macrophages to tolerate sustained JNK signaling.

**Pathogen-Induced Processes Are Associated with Extended JNK Signaling in Infected Cells**

In contrast to soluble PAMPs, bacterial infections pose a more complex processing challenge for the host cell as an internalized bacterium is, in fact, a dynamic signal that can undergo several changes while inside a macrophage. For instance, once inside a macrophage, signals from the intracellular environmental can trigger bacterial replication and/or the secretion of effector proteins into the host cytosol. Measuring both NF-κB and MAPK dynamics in single cells infected with *S. typhimurium* into those containing either replicating or low-level/non-replicating bacteria (Figure 5A; STAR Methods), but since we can follow changes in bacterial levels over time, we can ask how signaling differs in these cells even before signs of replication emerge. To determine if changes in bacterial replication state are associated with specific signaling patterns, we first grouped *S. typhimurium*-infected cells into those with bacterial replication or those with low-level/no replication (Figure 5A). Next, to track how the signaling activity of each group changed over the time course we divided the JNK and NF-κB reporter dynamics for each cell into 2 h windows, and for each window, a cell was marked as either active or inactive (Figure 5B). Finally, we calculated the fraction of cells in each group with activity in each of the time windows. When we considered JNK signaling, we observed that the subpopulations with replicating and low-level/non-replicating bacteria began to separate at around 8–10 h, with extended JNK signaling in cells with replicating bacteria (Figure 5C). In contrast, the NF-κB activation profiles were indistinguishable between these two groups.

An additional change that can take place in intracellular *S. typhimurium* is the translocation of effector proteins across the vacuole into the host cytosol via the T3SS encoded by the Salmonella pathogenicity island-2 (SPI-2) (Cirillo et al., 1998; Hensel et al., 1998). Therefore, we next asked whether effector secretion contributes to the signaling profile of infected cells. To investigate this, we infected cells with either the wild-type *S. typhimurium* or the ssaV mutant (Hensel et al., 1998). In the absence of ssaV, *S. typhimurium* is unable to assemble the SPI-2 encoded T3SS and effector secretion inside of host cells is abolished. We separated the infected macrophages according to both bacterial replication status and genotype. Once more, NF-κB activity did not differ across the groups (Figure S8A); however, the prolonged JNK signaling that we observed in cells with replicating wild-type bacteria was abolished in the ssaV mutant (Figure 5D). This suggests that effector secretion modulates host signaling, either by host detection of the effector or by effector-induced alterations in host signaling. Together, these results indicate that JNK signaling is connected to phenotypic changes in the bacterial population while they are inside the host cell and further establish JNK signaling dynamics as a defining signature of bacterial infection. Overall, we conclude that host cell responses to infection with *S. typhimurium* are dictated not only by contextual cues during the initial infection event but also by subsequent replication levels and effector secretion.

**DISCUSSION**

It is well established that TLRs allow a host cell to detect the presence of bacteria and that in response host cells elicit a response commensurate with the level of threat; however, it remains largely unknown how information gleaned through a general detector system such as the TLRs is translated into a stimulus-specific response. Our work here expands the role of NF-κB and MAPK dynamics beyond PAMP detection (Lane et al., 2017; Regot et al., 2014; Sung et al., 2014) to include the processing of complex bacterial signals both inside and outside of host cells. Measuring both NF-κB and MAPK dynamics in single cells exposes the remarkable capacity of the TLR signaling system to discriminate between diverse bacterial inputs. These results add to the growing body of literature on how individual cells can use such dynamics to encode complex information about their environment (Antebi et al., 2017; Batchelor et al., 2011; Nandagopal et al., 2018; Purvis and Lahav, 2013; Purvis et al., 2012), and we expect that these temporal studies will be a critical complement to the often sequence-based profiling of bacterial infection of single cells at single time points (Avraham et al., 2015; Gierahn et al., 2017; Saliba et al., 2016).

Our results also firmly establish that the NF-κB and MAPK signaling response to soluble PAMPs and bacteria are distinct. Prior to our study, there was conflicting evidence for this, as while several early studies had found that the cellular response to bacterial infection mimicked that due to LPS (Procyk et al., 1999; Rosenberger et al., 2000), others identified differences in the response (Royle et al., 2003). Using single-cell analysis to disentangle the contribution of PAMPs from that of bacterial...
infection, we are able not only to observe different signaling dynamics in infected cells but also to identify one potential source of variation in experimental findings, namely the influence of bacterial dose. At high doses of bacteria, the resulting levels of PAMPs in the environment become saturating. This overrides the capacity of the host cell to distinguish between soluble PAMPs and an internalized bacterium, leading to remarkably similar signaling profiles in the infected and uninfected bystander cell.

Figure 5. Increased JNK Signaling Is Associated with Changes in Intracellular S. Typhimurium

(A) (i) Infected cells were classified according to the levels of intracellular S. typhimurium: pink (cells with low-level/non-replicating bacteria) or green (cells with replicating bacteria). Images of bacteria, host nucleus (H2B-iRFP670), merged images, and the bacterial objects for a representative cell from each subgroup are shown. Scale bar, 5 μm. (ii) Time series data of S. typhimurium fluorescence for each of the cells shown in (i). The dotted line corresponds to the images in (i). (iii) Host cells belonging to the replication subgroups can be separated based on the peak total bacterial fluorescence detected within a macrophage. For every infected cell, at each time point, the bacteria within the host cell are identified as a single object and the peak total signal of that object over the time course was calculated. Shown is a boxplot of the peak total bacterial fluorescent intensity for cells belonging to each of the groups. p value calculated using a two-sided independent t test (***,p < 0.001).

(B) Schematic of how plots in (C and D) are generated. S. typhimurium infected cells were classified based on bacterial replication. The reporter dynamics of each cell were examined in 2 h windows, shown as boxes, and for each window, the cell was marked as either active (black) if the signal was above the activity threshold (dotted line) for that reporter during the time window or inactive (white) if it was below the threshold. Traces are grayed out where the activity is above the threshold. For the cells belonging to a particular replication subgroup, the fraction of active cells was calculated for each of the 2 h windows.

(C) JNK signaling is associated with S. typhimurium replication. Infected cells were classified according to S. typhimurium replication levels and the fraction of cells with active JNK (left) or active NF-κB (right) across the time course is shown for host cells with replicating (green) or low-level/non-replicating (pink) S. typhimurium. n indicates number of cells in each group. The Pearson correlation coefficient of the datasets is shown in the plot.

(D) JNK signaling is associated with S. typhimurium effector secretion. Cells were infected with either S. typhimurium WT or ssaV mutant. Infected cells were grouped according to bacterial replication and genotype. The fraction of cells with active JNK across the time course is shown for cells containing either replicating (left) or low-level/non-replicating bacteria (right). n indicates number of cells in each group. The Pearson correlation coefficient of the datasets is shown in the plot.
cells. However, at lower doses of bacteria, the distinct signaling response of these two populations became apparent. This is consistent with several studies that found the use of low doses of bacteria to be essential to reveal differences in either the timing of the TLR signaling response to *Shigella* (Kasper et al., 2010) or the transcriptional response to *Legionella* mutants (Losick and Isberg, 2008) and may also suggest that macrophages are more capable of discriminating threat levels at the early phase of an infection than later, when bacterial and PAMP concentrations become high. These findings emphasize the importance of using lower doses of bacteria to reveal how individual cells process bacterial infection events.

The results also highlight the potential of making multiplexed signaling measurements in single cells. First, although the initial signaling response of cells infected with *S. typhimurium* was markedly reduced in comparison to either stimulation with LPS, heat-killed *S. typhimurium*, or infection with *E. coli*, one way that a cell appears to encode the presence of an intracellular bacterium is via dual activation of NF-κB and MAPK. Second, we found that the frequency of dual NF-κB and MAPK activation in uninfected bystanders, as well as in response to LPS, was a function of the concentration of the stimulus. This finding is consistent with a recent study that identified distinct response thresholds for MAPK and NF-κB in macrophages in response to the PAMP lipid A (Gottschalk et al., 2016). However, our results in infected cells indicate that such a danger threshold does not appear to apply to infected cells—once the membrane is breached, the signal received is typically sufficient to activate both NF-κB and MAPK, independent of the level of bacteria in the inoculum. Although pathogens have evolved strategies to minimize detection by the host (Kawasaki et al., 2004; Rebeil et al., 2004), our work suggests that the transmission of contextual cues via dynamics ensures dual activation of NF-κB and MAPK signaling during infection; identification of these cues and bacterial strategies to hinder their transmission should be the focus of future efforts.

A consistent, but perhaps surprising, view that emerged from our study was of the relative importance of MAPK dynamics compared to those of NF-κB during bacterial infection. Expanding upon a recent study of the TLR response to lipid A (Gottschalk et al., 2016), our work confirms that engagement of MAPK signaling is a universal signature of an escalation in the threat to the host cell, be it high doses of PAMPs, infection, or bacterial replication. Identifying the role of MAPKs, and perhaps more importantly, the MAPK-NF-κB combination, in discrimination of the infection threat level, also adds a critical new dimension to the recent finding that NF-κB activation dynamics are altered in *Salmonella*-infected fibroblasts (Ramos-Marqués et al., 2017). Furthermore, JNK signaling was a signature both of cells with replicating *S. typhimurium* and those that can secrete effectors. This period of JNK signaling may be an effort on the part of the host to counter bacterial replication or effector function, or alternatively, *S. typhimurium* may induce JNK signaling to positively regulate its replication. Polarization of macrophages to an M2 state is associated with *S. typhimurium* replication (Eisele et al., 2013; Saliba et al., 2016), and a recently established link between MAPK signaling and M2 polarization (Hao et al., 2017; Jiménez-Garcia et al., 2015) raise the possibility that this later-stage JNK signaling may establish a host-cell environment more permissive to bacterial replication.

Although previous work led us to expect JNK dynamics to be pulsatile (Fosbrink et al., 2010; Regot et al., 2014), we were surprised to find evidence of sustained bouts of JNK signaling lasting many hours in bacterially infected cells. These extended periods of JNK activity were most prevalent in cells infected with non-pathogenic *E. coli*, continued long after the bacteria had been eradicated, and were associated with periods of NF-κB inactivity. Unraveling the trigger for this second wave of JNK signaling and defining the functional consequences of this signaling behavior are key next steps. One potential candidate for the trigger is the release of immune stimulatory molecules from degraded bacteria; degradation of bacteria in phagosomes has previously been linked to increased cytokine production (Herskovits et al., 2007; Wolf et al., 2011). In regard to phenotypic consequences, JNK signaling has previously been linked to macrophage priming in response to the phagocytosis of apoptotic cells (Weavers et al., 2016); thus, macrophages may use this period of sustained JNK signaling as a form of molecular memory of a recent infection event that could prime cells to deal with subsequent infections. Future efforts aimed at connecting distinct JNK dynamics to phenotypes will require the integration of signaling dynamics with other forms of single-cell profiling, such as single-cell transcriptomics or cytokine secretion.

Our results support the idea that the scale of the bacterial threat may be encoded in the dynamic pattern of innate immune signaling proteins. However, the origin of such contextual cues and how this information is transmitted into the host cell remain open and intriguing questions. Contextual cues about the bacterial threat probably emerge from several different sources involving both the bacteria and the host. For instance, differences in any or all of the following could make critical contributions to threat assessment: (1) the type of PAMP presented; for instance, *S. typhimurium* is known to be heterogeneous in its modification of LPS (Avraham et al., 2015) and the acylation state of LPS is known to differ between *S. typhimurium* and *E. coli* (Kawasaki et al., 2004); (2) the subcellular location where bacterial stimuli trigger host receptors, be it on the cell surface, phagosome, or in the cytoplasm (Kagan and Barton, 2014); and (3) the overall activation profile of multiple TLR family members (Aderem, 2003). Future studies aimed at analyzing signaling dynamics in the context of TLR-knockout macrophages in combination with bacterial mutants with modifications in particular PAMPs will be instrumental in ascertaining the origin and transmission of these contextual cues during infection and addressing whether pathogens have evolved strategies to interfere with this threat assessment by the host.

In conclusion, our study has identified extensive heterogeneity in the single-cell signaling response to infection and established NF-κB and MAPK signaling dynamics as a key conduit for the transmission of contextual information about the bacterial threat. While major efforts are underway to understand and develop treatments to combat the growing threat of antimicrobial resistance, such efforts will be stymied by our inability to reprogram or mobilize the response of host cells in a controlled way during infection. Expanding our molecular knowledge of cellular responses during infection to include temporal patterns of signaling will be instrumental in developing a more comprehensive map of cellular decision making and will reveal new opportunities for...
disease control. Signaling dynamics have emerged to expand the signal processing capacity of host cells beyond that originally deemed possible; our work, together with that of many other groups, positions the exploration of dynamics along with their integration with multi-omics datasets as a fruitful avenue for future exploration.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Cell Lines and Cell Culture
- **METHOD DETAILS**
  - Generation of Cell Lines
  - Bacterial Strains
  - Reagents
  - Time-Lapse Microscopy
  - Image Processing
  - Identification of Bacterial Objects and Classification of Infection and Replication Status
- **Selection of a Threshold for Reporter Activation Status**
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
- **DATA AND SOFTWARE AVAILABILITY**

SUPPLEMENTAL INFORMATION

Supplemental Information can be found with this article online at https://doi.org/10.1016/j.cels.2019.02.008.

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AUTHOR CONTRIBUTIONS

K.L. and M.A.-T. did experiments and analysis; T.K. worked on image analysis and bacterial segmentation and tracking; K.L., M.A.-T., D.M.M., and M.W.C. conceived the project; and D.M.M. and M.W.C. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


## STAR METHODS

### KEY RESOURCES TABLE

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## CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Markus Covert (mcovert@stanford.edu). Constructs will be available from Addgene.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Lines and Cell Culture
RAW264.7 (male) cells from ATCC and its derivatives were maintained in DMEM supplemented with 10% FBS (Omega Scientific), 2mM L-Glutamine (Life Technologies), and 1X Penicillin/Streptomycin (Life Technologies) at 37°C, 5% CO2.

293FT (female) cells were from Thermo Fisher Scientific and were maintained in DMEM supplemented with 10% FBS (Omega Scientific), 2mM L-Glutamine (Life Technologies), and 1X Penicillin/Streptomycin (Life Technologies) at 37°C, 5% CO2. Cell lines were not authenticated.

METHOD DETAILS

Generation of Cell Lines
Lentivirus was generated using 293FT cells. RAW264.7 cells were infected with lentivirus expressing H2B-iRFP670 and sorted. This parental cell line was serially infected with the relevant lentivirus, selected, and then sorted to generate the following dual signaling reporter lines used in this study:

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The design of the NF-κB reporter construct has previously been described (Lee et al., 2009) and in this study we have used a version in which the mouse p65 cDNA is fused at the C-terminus to the fluorescent protein mRuby2. Expression of the p65 fusion protein is controlled by a 1.5kb promoter fragment upstream of the relA gene (encoding p65).

Bacterial Strains
S. typhimurium strain SL12023 was used throughout this study. mCerulean3 was codon optimized for S. typhimurium to increase the fluorescence signal. SL12023 was transformed with the plasmid pFPV-mCerulean3. The knockout ssaV SL12023 strain was generated using standard red-mediated recombination (Yu et al., 2000). E. coli MG1655 was obtained from the Coli Genetic Stock Center (CGSC#: 7740) and transformed with pFPV-mCerulean3. This strain of E. coli was used for the pathogen/non-pathogen comparison in our study. An alternative choice would be a mutant of S. typhimurium in which the two major pathogenicity islands (SPI-1, SPI-2) that contain virulence proteins are deleted. However, in preliminary experiments with this mutant we achieved much lower numbers of infected cells compared to S. typhimurium WT and thus use of this mutant would have required the use of high MOIs and the associated caveats that come with that.

Reagents
Reagents used were: LPS (Enzo Life Sciences, ALX-581-010-L001), E. coli, Serotype EH100 (Ra) (TLR grade), Fluospheres Carboxylate-Modified microspheres (1.0μm, blue fluorescent).

Time-Lapse Microscopy
The day prior to imaging the relevant RAW264.7 cell strains were plated at 12500 cells/well on a fibronectin-coated (10μg/ml) (Sigma Aldrich, F0895) 96-well glass imaging plate (Fisher Scientific, 164588) and allowed to attach overnight. All bacterial strains were grown overnight in LB low-salt on a rotating wheel. One hour prior to imaging RAW264.7 cells were rinsed three times in imaging media to remove residual Pen/Strep and 150μl of imaging media was added to each well. An AeraSeal film (Sigma Aldrich, A9224) was applied to the plate and a pre-infection image was acquired for each well in the appropriate channels. Time-lapse microscopy was performed with a Nikon Eclipse Ti fluorescence microscope equipped with temperature (37°C) and environmental control (5% CO2) and controlled by Micromanager. The camera used was an Andor Neo 5.5 sCMOS and images were acquired at 10 minute intervals using a 20x/0.75 numerical aperture objective and 3x3 binning. We note that long-term imaging of mCerulean3 can be toxic to mammalian cells; to circumvent this and enable long-term imaging in four channels we made two critical modifications to our standard microscopy protocol. First, we found that we could reduce CFP exposure time if we used mCerulean3 as the fluorescent protein for the high-copy bacterial reporter rather than for any of the mammalian reporters. Second, we found that it was also essential to use an ND-60 setting to enable imaging at 10 minute intervals over 24 hours without compromising host cell viability. Bacterial cultures were re-suspended in PBS and diluted to the appropriate MOI and 5μl of this suspension was then added to each well and the plate was centrifuged for 15 minutes, 200g, 34°C. The imaging plate was then placed back on the microscope and imaged three times during the 30 minute infection period. The plate was then removed from the microscope, cells washed twice with imaging media to remove non-internalized bacteria, and then 200μl of imaging media containing 10μg/ml gentamicin was added to each well. The plate was then placed back on the microscope and imaging recommenced for a total of 24 hours. For stimulation with heat-killed bacteria, overnight cultures were processed as normal and diluted to the appropriate concentration. They were then incubated at 80°C for 30 mins prior to being added to the 96 well-plate of RAW264.7 cells. We note that while the addition of gentamicin is common practice it can have negative consequences for intracellular bacteria (Drevets et al., 1994; Menashe et al., 2008;
VanCleave et al., 2017). To determine whether gentamicin could also influence signaling dynamics, for instance via the release of PAMPs from dead bacteria, we compared JNK and NF-κB signaling responses after exposure to S. typhimurium MOI 2.5 in the presence or absence of gentamicin over a period of 6 hours (Figure S9). We found that while signaling in infected cells was not significantly different between these conditions, there was a difference in the response of uninfected bystanders as determined using the KS test. This difference, though statistically significant, was very small (the mean difference between the conditions was 0.042 for JNK and 0.15 for NF-κB). We also note that the level of JNK signaling in both cases is close to that observed in unstimulated cells, and in the case of NF-κB the signaling is actually lower in the non-gentamicin treated cells. Thus, we feel confident that signaling is largely unaffected by gentamicin.

**Image Processing**

Analysis of the live-cell imaging data was performed using CellTK, custom software developed in Python (https://github.com/CovertLab/CellTK) (Kudo et al., 2018). Briefly, after background subtraction, fluorescent images of nuclei labeled with H2B-iRFP670 were segmented by adaptive thresholding of the Laplacian of Gaussian of an image. Tracking of nuclei from frame-to-frame was performed primarily using a linear assignment problem (LAP) based approach, and the remaining cells that were not captured by this method were subject to watershed separation. After nuclei were tracked, cytoplasmic masks were created by the dilation of nuclei a set number of pixels followed by the exclusion of background pixels. Median values were extracted for nuclear and cytoplasmic masks for each macrophage and used to calculate cytoplasmic/nuclear (C/N) ratio for the JNK-KTR and the p38-KTR and nuclear/cytoplasmic (N/C) ratio for the NF-κB reporter. The output imaging datasets from CellTK were then passed to Covertrace for further data cleaning to remove: 1) cells that were not present at the onset of imaging, 2) cells with suboptimal reporter expression, 3) cells that were not tracked for at least 6 hours, 4) cells with too few cytoplasmic pixels (https://github.com/CovertLab/covertrace).

All data was processed and analyzed using beta versions of both CellTK and Covertrace.

**Identification of Bacterial Objects and Classification of Infection and Replication Status**

Identification of bacteria was carried out as follows (Figure S10A): Extracellular bacteria in the media, resulting either from the initial exposure or released from infected macrophages at later stages of the time course, are typically out of focus compared to intracellular bacteria. Images of fluorescent bacteria were preprocessed to subtract background and minimize detection of out of focus extracellular bacteria. Three preprocessing operations were used: N4 bias field correction algorithm, wavelet-based background subtraction (Galloway et al., 2009), and a noise removal filter using anisotropic diffusion. The high fluorescence signal from the bacteria and the extensive preprocessing steps carried out allowed us to use an intensity of 50 (background signal) as a threshold to detect bacteria. A high-pass filter was also applied and bacterial objects were assigned to the nearest nucleus object found within a set range of 15 pixels. The tendency of intracellular S. typhimurium to cluster around the macrophage nucleus facilitated the connection between bacterial objects and their host cell. The output bacterial datasets then underwent further processing to classify cells as either infected or uninfected bystanders. Our approach aimed to reliably identify infected cells while minimizing misclassification of cells due to sporadic fluorescence signal. Rather than determining infection status on a per time point basis we decided to harness our time series data to assess infection status over an extended time period. First, host cells were classified as infected if they contained a bacterial signal for at least 75% of frames 5-20 (50-200 minutes). We did not require 100% of these frames to have a bacterial object as sometimes the orientation of the bacteria can make the signal weaker causing it to be missed in a frame or two and in the case of E. coli the bacteria can be degraded within this time window. During the later stages of a S. typhimurium infection, cells containing large numbers of bacteria can ‘burst’ and disperse bacteria into both the environment as well as neighboring cells. Cells that were not classified as infected based on the criteria above but which later were found to contain bacterial signal were eliminated from the analysis. All remaining cells were classified as uninfected bystanders.

To track changes in bacterial levels over time we calculated the total bacterial fluorescent intensity in each macrophage as our imaging approach is not sensitive enough to count individual bacteria. This is due to a number of reasons including 1) the use of a 20x objective, 2) the lack of z-stacks which means the total fluorescence from an individual bacterium varies depending on its orientation in the host cell, and 3) the fluorescent protein is expressed from a high-copy plasmid leading to a range of fluorescence in bacterial cells. As a result, to assess replication status we decided to separate cells into two bins: high levels of replication and low/no replication as we don’t have the capacity to reliably distinguish cells with non-replicating bacteria from those that have undergone 1 or 2 division events. To distinguish between replicating and low/non-replicating infections we manually determined the total fluorescence intensity from cells with approximately 2-4 bacteria and based on this we set a total intensity value of 25000 as the threshold that the signal from the bacterial mask had to surpass for at least three continuous frames for a macrophage to be classified as containing replicating bacteria. Note that classification of an infected macrophage as containing replicating or non-replicating bacteria was independent of the time at which the bacterial signal passed the threshold.

**Selection of a Threshold for Reporter Activation Status**

We selected a threshold for JNK activation of 0.65 cytoplasmic/nuclear ratio and for NF-κB of 1.5 nuclear/cytoplasmic ratio. Our choice of thresholds stem from a recent paper (Gottschalk et al., 2016) which found that MAPK and NF-κB have different thresholds for activation. Since we saw a similar trend in our movies of RAW264.7 cells stimulated with LPS we decided to use our LPS data to select thresholds that would recapitulate the differential activation thresholds from Gottschalk et al. We then applied these thresholds to our S. typhimurium and E. coli infection data. While our choice of thresholds is rooted in a biological observation it remains an
arbitrary choice. Therefore, to confirm that small changes in our choice of threshold would not lead to significant changes in our conclusions we took the input data for Figures 3B and S5A, varied the thresholds from -50% to +500% and plotted the results (Figures S11A and S11B). We were pleased to see that the trends we observed at our selected thresholds - that JNK and NF-κB have different activation thresholds in response to LPS, that dual responders are a feature of infection and that is invariant across MOI, and that the response of uninfected bystanders is dependent on MOI – held up unless the thresholds were changed by 50% or more. Dual responder activation status for Figure 3 required both reporters to exceed the threshold for at least 3 continuous frames (30 minutes) during the first 6 hours of imaging. 6 hours was selected as the timeframe for this measurement as unlike LPS-stimulated cells which respond rapidly, cells infected with bacteria respond more slowly and we wanted to ensure we captured all of the relevant activation events during this early period of infection. Finally, since the dynamic range of both reporters is different the comparisons throughout our study focus on comparing JNK or NF-κB reporter activity between conditions rather than a direct comparison of differences in the magnitude of the response between JNK and NF-κB in single cells. Sample traces for stimulations showing the activation thresholds are included (Figure S12).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was carried out in Python and a description is available in the main text and associated figure legends.

DATA AND SOFTWARE AVAILABILITY

Image analysis software CellTK is available at (https://github.com/CovertLab/CellTK ) and downstream data processing software at (https://github.com/CovertLab/covertrace). Requests for single-cell trajectory datasets should be directed to the Lead Contact, Markus Covert.
Supplemental Information

Escalating Threat Levels of Bacterial Infection Can Be Discriminated by Distinct MAPK and NF-κB Signaling Dynamics in Single Host Cells

Keara Lane, Marta Andres-Terre, Takamasa Kudo, Denise M. Monack, and Markus W. Covert
Supplementary Figure Legends

Figure S1. Related to Figure 2.
Differences in JNK and NF-κB signaling dynamics between *S. typhimurium* infected and uninfected bystanders increases with decreasing MOI.
A. Percentage of cells infected with *S. typhimurium* using MOI 1, 2.5, and 10. Error bars represent standard deviation. Note the y-axis is scaled from 0-25% due to the low frequency of infected cells under these conditions.
B, C. (i) Comparison of the scale of the signaling differences between the two subpopulations across MOIs. Shown is a scatter plot of the median infected cell response against the median uninfected bystander response for all time points shown in Figures 2A-B for (B) JNK and (C) NF-κB. Data points are colored according to MOI. The black line represents x=y. The light gray lines represent one standard deviation from the diagonal. (ii) Shown is the mean orthogonal distance to the diagonal of data points in (i).

Figure S2. Related to Figure 2.
Phagocytosis of latex beads induces no JNK and minimal NF-κB signaling.
A. RAW264.7 cells were exposed to latex beads and imaged. The median signaling dynamics for JNK and NF-κB are shown for cells that phagocytosed beads ( ). Shaded areas represent a 68% confidence interval calculated by bootstrapping. Data shown is for the first 6 hours.

Figure S3. Related to Figure 2.
p38 signaling dynamics are distinct between *S. typhimurium* infected and uninfected bystander cells.
A. Differences in host p38 signaling between infected and uninfected bystander cells. Host cells were exposed to *S. typhimurium* at MOI 1, 2.5 or 10 and imaged. Cells were grouped according to infection status and the median signaling dynamics of p38 is shown for infected ( ) and uninfected bystander ( ) cells. Shaded areas represent a 68% confidence interval calculated by bootstrapping. Data shown is for the first 6 hours. Yellow bars indicate the time points at which
differences between the two subpopulations were significant using the Kolmogorov-Smirnov test with a confidence level of p=0.01. The difference between the two subpopulations increases as the MOI is decreased.

B. Comparison of the scale of the p38 signaling differences between the two subpopulations across MOIs. Shown is a scatter plot of the median infected cell response against the median uninfected bystander response for all time points in (A). Data for each MOI is first shown individually and the final panel combines data across all MOIs. Data points are colored according to MOI. The black line represents x=y. The light gray lines represent one standard deviation from the diagonal.

C. Shown is the mean orthogonal distance to the diagonal of data points in (B).

**Figure S4. Related to Figure 2.**

Heat-killed bacteria and LPS induce similar signaling responses.

A. (i) Median signaling dynamics for JNK in response to heat-killed *S. typhimurium* (left) at MOI 1, 2.5 or 10 or a range of concentrations of LPS (right). Shaded areas represent a 68% confidence interval calculated by bootstrapping. Data shown is for the first 6 hours. n=number of cells. (ii) Shown is a scatter plot of the median response of cells exposed to heat-killed *S. typhimurium* MOI 10 against the median response to LPS 100ng/ml for all time points in (i). The black line represents x=y. The light gray lines represent one standard deviation from the diagonal.

B. (i) Median signaling dynamics for NF-κB in response to heat-killed *S. typhimurium* (left) at MOI 1, 2.5 or 10 or a range of concentrations of LPS (right). Shaded areas represent a 68% confidence interval calculated by bootstrapping. Data shown is for the first 6 hours. n=number of cells. (ii) Shown is a scatter plot of the median response of cells exposed to heat-killed *S. typhimurium* MOI 10 against the median response to LPS 100ng/ml for all time points in (i). The black line represents x=y. The light gray lines represent one standard deviation from the diagonal.

C, D. Comparison of the scale of the signaling differences between *S. typhimurium* infection and stimulation with heat-killed *S. typhimurium*. Shown is a scatter plot comparing the median response for all time points between the two groups of cells. The black line represents x=y. The
light gray lines represent one standard deviation from the diagonal. MOI 2.5 was used for both samples. Data shown is for the first 6 hours.

Figure S5. Related to Figure 3.
Dual JNK and NF-κB activation depends on the concentration of LPS.
A. RAW264.7 cells were exposed to a range of concentrations of LPS and imaged. The JNK and NF-κB signaling responses for the first 6 hours were used to classify single-cell activation status based on thresholds as detailed in Figure 3A and STAR Methods. The fraction of cells in each activation state across LPS concentrations is shown. Dual responders predominate at high LPS concentrations.
B. The fraction of cells in each of the signaling responder states for cells exposed to (i) S. typhimurium at MOI 1, 2.5, and 10 (Figure 3B) or (ii) a range of concentrations of LPS (Figure S5A).
C. To compare the dynamics of the transition from single to dual responder state, single cells were followed over the course of 6 hours and their responder state was classified at hourly intervals. The fraction of cells in each responder state at hourly intervals up to 6hrs is shown. Data is shown for the following conditions: S. typhimurium MOI 2.5 infected (left), S. typhimurium MOI 2.5 uninfected bystander cells (middle) and cells stimulated with LPS 100ng/ml (right).

Figure S6. Related to Figure 3.
Total NF-κB levels increase in a subset of S. typhimurium infected and uninfected bystanders during the time course.
A. (i) Changes in total nuclear NF-κB levels were determined by normalizing the NF-κB nuclear intensity by the cytoplasmic signal at the start of the time course (N/C\textsubscript{t=0}). (ii) Changes in total cytoplasmic NF-κB levels were determined by normalizing the NF-κB cytoplasmic intensity by the cytoplasmic signal at the start of the time course (C/C\textsubscript{t=0}). The average behavior is shown for untreated cells (left), S. typhimurium infected and uninfected bystander cells (middle), and cells treated with LPS 100ng/ml (right). Shaded areas represent a 68% confidence interval calculated by bootstrapping. Data shown is for the 4-24 hours.
B. To determine whether there was differential NF-κB induction between *S. typhimurium* infected and uninfected bystanders we measured changes in total cytoplasmic NF-κB levels in individual cells as this will identify cells with induction independent of activation state. (i) Maximum fold change in cytoplasmic NF-κB for uninfected bystanders and infected cells is shown. P value calculated using a two-sided independent t-test (NS p > 0.05). (ii) Fraction of cells in both subpopulations with a fold change in cytoplasmic NF-κB above 1.5 are shown.

C. For each condition – LPS 100ng/ml (gray), *S. typhimurium* uninfected bystanders (black), and *S. typhimurium* infected cells (blue) – dual responders were first identified as in Figure 3D but with NF-κB activity determined by normalizing the NF-κB nuclear intensity by the cytoplasmic signal at the start of the time course. For each condition, we compared the fraction of dual responder cells that had at least ‘x’ hours of reporter activity. Hours of activity is calculated as indicated in Figure 3C. Significance calculated by the Kolmogorov-Smirnov test and p value and KS statistic is shown in the inset. (NS p > 0.05).

**Figure S7. Related to Figure 4.**

**The JNK and NF-κB signaling response of host cells to *E. coli***

A. Percentage of cells infected with *E. coli* using MOI 1, 2.5, and 10. Error bars represent standard deviation. Note the y-axis is scaled differently (from 0-100%) to that in Figure S1A due to the higher frequency of infected cells under these conditions.

B. Differences in host signaling between *E. coli* infected and uninfected bystander cells. Host cells were exposed to *E. coli* at an MOI of 1, 2.5 or 10, imaged, and cells grouped according to infection status. Median signaling dynamics of (i) JNK and (ii) NF-κB for infected (●) and uninfected bystander (■) cells are shown. Shaded areas represent a 68% confidence interval calculated by bootstrapping. Data shown is for the first 6 hours. Yellow bars indicate the time points at which differences between the two subpopulations were significant using the Kolmogorov-Smirnov test with a confidence level of p=0.01. The difference between the two subpopulations increases as the MOI is decreased.

C. Features of the single-cell traces of JNK and NF-κB distinguish *E. coli* infected cells from uninfected cells. For each cell the following features were extracted from the first 6 hours of the
time course for both JNK and NF-κB – number of peaks, time to the first peak, maximum amplitude in the first 2 hours, area under the curve (AUC) for 0-6 hours. Mean and standard deviation are shown and significance was calculated using a two-sided independent t-test (* p< 0.01, ** p < 0.001).

D. Schematic of features extracted from bacterial and reporter traces that were used to generate Figure S6E and Figure 4D. For each infected cell the time of E. coli clearance is the time at which the bacterial signal goes to zero. Time of final JNK/NF-κB activity is the latest point in the time course at which the signal was above the activity threshold (dotted line).

E. Independent of MOI JNK signaling persists after E. coli is cleared from the cell, while NF-κB shows no association with bacterial clearance. For each E. coli infected cell that cleared the bacteria we compared the time at which the bacteria were cleared to the time of final JNK or NF-κB activity. Hexagons are colored according to cell density. Data shown is for (i) MOI 10 (n=209 cells) and (ii) MOI 2.5 (n=317 cells). In the case of JNK, 89% of cells at MOI of 10 and 85% of cells at MOI of 2.5 were found to have reporter activity after the bacteria had been cleared. For NF-κB, only 57% of cells at MOI of 10 and 55% of cells at MOI of 2.5 were found to have reporter activity after bacterial clearance.

Figure S8. Related to Figure 5.

**NF-κB signaling in S. typhimurium infected cells does not depend on effector secretion**

A. Cells were infected with either S. typhimurium WT or ssaV mutant. Infected cells for each bacterial genotype were grouped according to bacterial replication and the fraction of cells with active NF-κB across the time course is shown for cells containing either replicating (left) or low-level/non-replicating bacteria (right). Both S. typhimurium genotypes show similar NF-κB responses. n indicates number of cells in each group. The Pearson correlation coefficient of the datasets is shown in the plot.

Figure S9. Related to STAR Methods.

**Gentamicin treatment does not alter subpopulation behavior.**

A, B. Differences in host signaling in the presence or absence of gentamicin. Host cells were exposed to S. typhimurium at an MOI of 2.5 in the presence or absence of gentamicin, imaged,
and cells were then grouped according to infection status and gentamicin exposure. Median signaling dynamics of (A) JNK and (B) NF-κB for uninfected bystanders -/+ gentamicin (left) and S. typhimurium infected cells -/+ gentamicin (right) are shown. Shaded areas represent a 68% confidence interval calculated by bootstrapping. Data shown is for the first 6 hours. Yellow bars indicate the time points at which differences between the two subpopulations were significant using the Kolmogorov-Smirnov test with a confidence level of p=0.01. A scatter plot comparing the median cell response of both populations is shown. The black line represents x=y. The light gray lines represent one standard deviation from the diagonal.

**Figure S10. Related to STAR Methods.**
Identification and tracking of bacteria in single cells over time.
A. Schematic of image processing steps to identify fluorescent bacteria and connect them to their corresponding host nucleus.

**Figure S11. Related to Figure 3.**
Results for reporter activation status are relatively insensitive to small changes in the activation threshold used.
A, B. To determine how sensitive our results are to the activation threshold used we changed the JNK and NF-κB thresholds by -50% to +500% and replotted the data on the frequency of different types of responder cells for (A) LPS and (B) S. typhimurium. In both cases the original data from Figure S5A (LPS) and Figure 3B (S. typhimurium) is highlighted with a box. For both stimulus conditions the trends observed did not change appreciably until the activation thresholds were changed by at least 50%.

**Figure S12. Related to STAR Methods.**
Sample single-cell traces of JNK and NF-κB for all conditions.
Sample traces for JNK, NF-κB, and where applicable bacteria, are shown for the following experimental conditions: A) S. typhimurium infected MOI 2.5, B) S. typhimurium uninfected bystanders MOI 2.5, C) E. coli infected MOI 2.5, D) E. coli uninfected bystanders MOI 2.5, E) LPS
100ng/ml, and F) untreated. Note that the scale of the y-axis for both JNK and NF-κB are different for *S. typhimurium* than for the other conditions as it induces a weaker response. The horizontal dashed line represents the activation thresholds used throughout the manuscript.
Figure S1

A

S. typhimurium

% of cells infected

MOI 1  MOI 2.5  MOI 10

B

(i) JNK

(ii)

MOI 1  MOI 2.5  MOI 10

mean distance

C

(i) NF-κB

(ii)
Figure S2

A

JNK

NF-κB

cells with beads, n=230

cells with beads, n=230

C/N ratio

N/C ratio

time (hours)
time (hours)
Figure S3

A  

**p38**

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B  

**p38**

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C  

**p38**

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Figure S4

A. JNK
(i) Heat-killed
- MOI 10, n=111
- MOI 2.5, n=98
- MOI 1, n=102

(ii) LPS
- 100ng/ml, n=151
- 10ng/ml, n=166
- 1ng/ml, n=174
- 0.1ng/ml, n=180
- 0.01ng/ml, n=161
- 0.001ng/ml, n=176

B. NF-κB
(i) Heat-killed
- MOI 10, n=111
- MOI 2.5, n=98
- MOI 1, n=102

(ii) LPS
- 100ng/ml, n=151
- 10ng/ml, n=166
- 1ng/ml, n=174
- 0.1ng/ml, n=180
- 0.01ng/ml, n=161
- 0.001ng/ml, n=176

C. JNK
- SL MOI 2.5
- HK SL MOI 2.5

D. NF-κB
- SL MOI 2.5
- HK SL MOI 2.5
Figure S5

A

- Dual responder
- NF-κB only
- JNK only
- Non-responders

LPS

B

(i)

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C

- S. typhimurium infected, MOI 2.5
- uninfected bystanders, MOI 2.5
- LPS 100ng/ml

fraction of cells per group

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fraction of cells per group

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Figure S6

A

(i) NF-κB

untreated

S. typhimurium MOI 2.5

LPS 100ng/ml

N/C(t=0)

C/C_{t=0}

hours

(ii) NF-κB

N/C(t=0)

C/C_{t=0}

hours

B

(i)

Fold change in cytoplasmic NF-κB

NS

uninfected

infected

(ii)

Fraction of dual responder cells with at least x hours of activity

C

NF-κB

p-val KS

LS 100ng/ml

S. typhimurium uninfected

S. typhimurium infected
Figure S7

(A) E. coli

MOI 1 | MOI 2.5 | MOI 10

% of cells infected

(B) E. coli infected | uninfected bystander

(i) JNK

MOI 1

MOI 2.5

MOI 10

N/C ratio

(ii) NF-κB

MOI 1

MOI 2.5

MOI 10

N/C ratio

(C) E. coli infected | uninfected bystanders

number of peaks | time to first peak (minutes) | max amplitude (0-2hrs) | AUC (0-6hrs)

JNK

MOI 1 | MOI 2.5 | MOI 10

MOI 2.5 | MOI 2.5 | MOI 10

MOI 10 | MOI 2.5 | MOI 10

NF-κB

MOI 1 | MOI 2.5 | MOI 10

MOI 2.5 | MOI 2.5 | MOI 10

MOI 10 | MOI 2.5 | MOI 10

(D) E. coli

signal

Time of E. coli clearance

(E) E. coli

MOI 10

(i) JNK

Time of E. coli clearance

(ii) NF-κB

Time of E. coli clearance

Max amplitude (0-2hrs)
Figure S8

A

Replicating bacteria
- WT (n=30)
- ssaV (n=39)

r = 0.97

Low-level/non-replicating bacteria
- WT (n=39)
- ssaV (n=19)

r = 0.98
Figure S9

A

JNK

uninfected bystanders

S. typhimurium infected

B

NF-κB

uninfected bystanders

S. typhimurium infected

p < 0.01

p < 0.01

p < 0.01

p < 0.01
A

1. Raw images of bacteria expressing a fluorescent protein from a high-copy plasmid

2. Images undergo preprocessing (CellTK) to reduce background and remove out of focus extracellular bacteria
   - N4 bias field correction algorithm
   - wavelet-based background subtraction
   - noise removal filter using anisotropic diffusion

3. Bacterial objects are identified as follows:
   - a threshold of 50 is used to detect signal above background
   - a high pass filter to facilitate object assignment to the correct nucleus
   - this method does not separate individual bacteria within each connected object

4. At each frame all bacterial objects are connected to the nearest nuclear object and share the same object ID number

5. Bacterial traces undergo further ‘cleaning’ to remove cases where the bacteria have been assigned to the incorrect nucleus
Figure S12

A S. typhimurium infected

B S. typhimurium uninfected bystanders

C E. coli infected

D E. coli uninfected bystanders

E LPS 100ng/ml

F untreated

JNK NF-κB Bacteria

S. typhimurium infected

S. typhimurium uninfected bystanders

E. coli infected

E. coli uninfected bystanders

LPS 100ng/ml

untreated