**Mycobacterium tuberculosis** Responds to Chloride and pH as Synergistic Cues to the Immune Status of its Host Cell

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**Abstract**

The ability of Mycobacterium tuberculosis (Mtb) to thrive in its phagosomal niche is critical for its establishment of a chronic infection. This requires that Mtb senses and responds to intraphagosomal signals such as pH. We hypothesized that Mtb would respond to additional intraphagosomal factors that correlate with maturation. Here, we demonstrate that [Cl⁻] and pH correlate inversely with phagosome maturation, and identify Cl⁻ as a novel environmental cue for Mtb. Mtb responds to Cl⁻ and pH synergistically, in part through the activity of the two-component regulator phoPR. Following identification of promoters responsive to Cl⁻ and pH, we generated a reporter Mtb strain that detected immune-mediated changes in the phagosomal environment during infection in a mouse model. Our study establishes Cl⁻ and pH as linked environmental cues for Mtb, and illustrates the utility of reporter bacterial strains for the study of Mtb-host interactions in vivo.

**Introduction**

*Mycobacterium tuberculosis* (Mtb) causes a chronic infection in approximately one third of the human population and remains an important public health problem [1]. The macrophage (MØ) is the major host cell for much of Mtb’s life cycle, and a defining feature of Mtb’s pathogenesis is its ability to arrest full maturation of the phagosome in which it resides [2,3]. Indeed, Mtb mutants that fail to arrest phagosomal maturation have reduced survival during MO infection [4]. However, Mtb remains subject to multiple stresses within the phagosome, which may act as important environmental cues for Mtb [5]. Proper sensing of such signals informs Mtb of its surroundings, allowing the bacterium to respond appropriately to ensure its survival and replication. Elucidating the cues that Mtb recognizes during infection, and the possible interplay between such signals, is critical for a complete understanding of the impact of the microenvironment on Mtb pathogenesis and persistence, and Mtb’s interaction with fundamental host cell processes.

One environmental cue that has received particular attention is pH; the Mtb phagosome acidifies to an intermediate pH of 6.4 [3,4,6], and even in medium, the bacterium exhibits a profound transcriptional response to acidic pH [5,7,8]. The abolition of phagosome acidification during bacterial uptake by MØs, through treatment with concanamycin A, eliminates a majority of Mtb’s transcriptional response, indicating the importance of pH as a signal for the bacterium in sensing and responding to its environment [5]. The process of acidification does not, however, proceed in isolation. Specifically, acidification (increase in [H⁺]) must be counterbalanced by efflux of other cations from the phagosome, and/or by the uptake of a counter anion. We hypothesized that Mtb might also take advantage of this counterbalancing factor as an environmental cue, expanding the sensitivity and dynamic range of its ability to define its immediate environment. Cell biological studies have established Cl⁻ as a major counter anion during acidification of the endosome [9–11]. Several Cl⁻ channels are known to be present on the endosomal membrane [12,13], although it remains controversial which of these channels are involved in the counter-balancing of increased [H⁺] during endosomal maturation [14,15]. More recent studies have also proposed efflux of cations, such as K⁺, as a counter mechanism to increased [H⁺] in the lysosome [16]. The existence of such mechanisms have not, however, been formally shown for phagosomes. In this context, it is of particular note that the Mtb phagosome has been reported to possess a high [Cl⁻] [17]. The impact of common ions and changes in their concentration on Mtb during infection is a concept that is just beginning to be appreciated [18]; however, much remains to be determined regarding their physiological significance.

In this study, we show that [Cl⁻] increases during phagosome maturation, mirroring a decrease in pH within the compartment. Mtb modulates its transcriptional profile in response to [Cl⁻], and reacts to the environmental cues of pH and [Cl⁻] in a synergistic manner, with the two-component regulatory system *phoPR* playing...
**Author Summary**

*Mycobacterium tuberculosis* (MtB) is the causative agent of tuberculosis, a disease that remains a major global health problem. To ensure its long-term survival in the host, MtB must be able to sense and respond to changes in its immediate environment, such as the pH differences that occur in the phagosome in which it lives. Knowledge of the external signals that MtB recognizes during infection is critical for understanding the impact of the microenvironment on MtB pathogenesis and persistence, and how MtB interacts with its host cell. We show here that [Cl\(^-\)] correlates inversely with pH as the phagosome matures, and identify [Cl\(^-\)] as a novel cue that MtB responds to, in synergy with pH. By constructing a MtB strain that fluorescently reports on changes in [Cl\(^-\)] and pH, we find using a mouse model of infection that environmental alterations in MtB’s phagosomal home are mediated at the local level by activities of the host immune system. Our study demonstrates how a pathogen can exploit linked environmental cues during infection, and shows the value of reporter bacterial strains for MtB-host whole animal studies.

**Results/Discussion**

[Cl\(^-\)] increases during phagosomal maturation

We first sought to establish the dynamics of [Cl\(^-\)] during maturation of the phagosome with model particles. The fluorescent [Cl\(^-\)]-sensitive, pH-insensitive compound 10,10’-Bis[3-carboxy-ethylpropyl]-9,9’-biacridinium (BAC) [9] was synthesized as a trifluoracetate salt, and coupled to IgG beads. As previously reported, BAC fluorescence is quenched by Cl\(^-\) in a concentration-dependent manner, and is unaffected by pH changes (Figure S1 in Text S1) [9]. To track [Cl\(^-\)] changes during phagosomal maturation we attached Alexa Fluor 594 (AF594) as a calibration fluorophore to the BAC-IgG beads. These dual-color Cl\(^-\) sensor beads were added to murine bone marrow-derived MOs and fluorescence measured in a microplate reader. We observed an increase in AF594/BAC fluorescence ratios over time, indicating an increase in [Cl\(^-\)] as the phagosome matured (Figure 1A). This increase in [Cl\(^-\)] was also observed with phagosome maturation in MOs derived from human monocytes (Figure 1B). To calibrate AF594/BAC ratios to actual [Cl\(^-\)], we treated MOs that had phagocytosed Cl\(^-\) sensor beads with bafilomycin A1 and the ionophores nigericin and monensin in buffers of known [Cl\(^-\)]. By fitting a polynomial regression to the standard curve (Figure S2 in Text S1), we calculate that phagosomal [Cl\(^-\)] reached a maximal concentration of \(~70-95 \text{ mM}\). As this is a population-based measurement, we note that this value range underestimates the [Cl\(^-\)] that can be reached in individual phagosomes (see below).

We further examined the dynamics of [Cl\(^-\)] increase during phagosome maturation by tracking individual beads during phagocytosis by live-cell time-lapse microscopy. These experiments showed that the switch from low [Cl\(^-\)] to high [Cl\(^-\)] occurred for most beads, although a subset remained in phagosomes with low [Cl\(^-\)] (Figure 1C and Video S1). Imaging of populations of Cl\(^-\) sensor bead-containing cells at given time points illustrated the heterogeneity in [Cl\(^-\)] attained in individual phagosomes, with measurements indicating that a [Cl\(^-\)] greater than 120 mM was reached in some phagosomes (Figure 1D). Similar results were obtained in MOs derived from human monocytes (data not shown). Cl\(^-\) sensor beads present in media alone and imaged in parallel did not show significant changes in fluorescence, demonstrating that the decrease in BAC fluorescence observed in the phagocytosed beads has a biological basis and is not due to bleaching of the fluorescent signal during imaging (Video S2).

In examining these results, we noted that the increase in [Cl\(^-\)] mirrored the kinetics of the decrease in phagosomal pH previously reported [23]. In order to quantify this correlation directly within a single experiment, we coupled BAC to IgG beads in combination with the red fluorescent pH sensor pHirodo, which exhibits an increase in fluorescence as pH decreases. Measurement of the fluorescence profile of the beads during phagocytosis by MOs showed the previously observed quenching of BAC signal indicative of increased [Cl\(^-\)] as the phagosome matured (Figure 1E). pHrodo fluorescence on the same particles exhibited an inverse profile, increasing in intensity over time, signaling a decrease in pH (Figure 1E). Analysis of the phagocytosis of the BAC/pHirodo indicator beads by live-cell time-lapse microscopy further verified these results at the individual phagosomal level (Figure 1F and Video S3). Similar profiles were observed in MOs derived from human monocytes (data not shown). We also verified that BAC/pHirodo beads imaged in media alone did not show such changes in fluorescence profile (Video S4).

Further support for the relation between [Cl\(^-\)] and pH during phagosomal maturation was demonstrated by the failure of phagosomal [Cl\(^-\)] to increase when MOs were treated with bafilomycin A1 (Figure S3A in Text S1). Similarly, addition of bafilomycin A1 to the MOs after phagosomes containing the Cl\(^-\) sensor beads had initially been allowed to mature resulted in increased BAC fluorescence, indicating a reversal of the Cl\(^-\) accumulation upon dissipation of the pH gradient (Figure S3B in Text S1). Together, these results demonstrate that [Cl\(^-\)] increases during phagosomal maturation, and supports the proposed functional relationship between acidification of the endosomes and [Cl\(^-\)] increase [9–11].

MtB regulates gene expression in response to Cl\(^-\), with a subset corresponding to pH responsive genes

MtB shows a marked transcriptional response upon exposure to acidic pH, and we have previously shown that almost half of the MtB genes upregulated during an early stage of MO infection are induced in a pH-dependent manner [5]. Given our results indicating [Cl\(^-\)] increase during phagosomal maturation and the link between [Cl\(^-\)] and acidification, we compared the transcriptional profiles of MtB grown in regular 7H9 media to those grown in 7H9 media supplemented with 250 mM NaCl for 4 hours. The number of genes (32) upregulated on exposure to high [Cl\(^-\)] was noticeably fewer than the hundreds previously reported to be
induced under acidic pH (Table 1) [5,7]. Strikingly however, a significant number of genes that were upregulated in the presence of high [Cl$^-\$] (18/32) were genes that also showed increased expression during exposure to acidic pH (Table 1).

The upregulated gene expression detected by microarrays was validated by semi-quantitative real time PCR (qRT-PCR) for several genes. These experiments were also carried out on samples exposed to acidic pH (pH 5.7), and showed data consistent with the microarray analysis (Figure S4 in Text S1). While our microarray platform allows for the global analyses of gene expression changes, it does have a flattened dynamic range [5,24], and the qRT-PCR data indicate that the actual level of induction is considerably greater. These experiments indicate that Mtb responds transcriptionally to Cl$^-$, and further reinforce the idea that pH and Cl$^-$ may function as interconnected environmental cues for Mtb during the course of infection.

Figure 1. [Cl$^-\$] increases and pH decreases during phagosome maturation. (A and B) [Cl$^-\$] increases during phagosome maturation. BAC/AF594 beads were added to murine bone marrow-derived MØs (A) or MØs derived from human monocytes (B). In each case, sensor beads were also added to wells containing only media, with no MØs ("Media only"). BAC (Cl$^-$-sensitive) and AF594 (calibration fluorophore) fluorescence were tracked with a microplate reader over time. Data are shown as means ± SD from 4 wells. (C) Single Cl$^-$ sensor bead tracking during phagosome maturation. Cl$^-$ sensor beads were added to murine bone marrow-derived MØs and fluorescence for individual beads tracked over time by live-cell time-lapse microscopy (see Video S1). Each line on the graph represents a single bead. (D) Heterogeneity of [Cl$^-\$] in individual phagosomes. BAC/AF594 beads were added to murine bone marrow-derived MØs and fluorescence measured at indicated time points. Each point on the graph represents a single bead. Bars represent mean values. (E) [Cl$^-\$] and pH are inversely correlated during phagosome maturation. BAC/pHrodo beads were added to murine bone marrow-derived MØs and fluorescence measured at indicated time points. Each point on the graph represents a single bead. Bars represent mean values. (F) Single BAC/pHrodo bead tracking during phagosome maturation. BAC/pHrodo beads were added to murine bone marrow-derived MØs and fluorescence for individual beads tracked over time by live-cell time-lapse microscopy (see Video S3). Each color represents a single bead, with solid lines tracking BAC signal and dashed lines tracking pHrodo signal from the same bead.

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Table 1. Overlap between Mtb genes upregulated on exposure to high [Cl\(^{-}\)] or acidic pH.

| Gene Name  | Ratio | Acidic pH induced | Description                        |
|------------|-------|-------------------|------------------------------------|
| MT0772.5   | 1.59  | +                 | PE-PGRS family protein             |
| MT1178     | 1.36  | −                 | HP                                 |
| MT1746.1   | 1.73  | −                 | HP                                 |
| MT2423.1   | 1.34  | +                 | PE family protein                  |
| MT3106.1   | 1.44  | +                 | PE family protein                  |
| Rv0263c    | 1.42  | +                 | CHP                                |
| Rv0264c    | 1.47  | +                 | CHP                                |
| Rv0516c    | 1.58  | −                 | CHP                                |
| Rv1057     | 1.61  | +                 | CHP                                |
| Rv1115     | 1.51  | −                 | HP                                 |
| Rv1187 (rocA) | 1.37 | +                 | Pyrroline-5-carboxylate dehydrogenase |
| Rv1376     | 1.50  | −                 | CHP                                |
| Rv1403c    | 1.96  | +                 | Methyltransferase                  |
| Rv1405c    | 2.98  | +                 | Methyltransferase                  |
| Rv1497 (lipL) | 1.36 | −                 | esterase                           |
| Rv1577     | 1.30  | +                 | phiRv1 phage protein               |
| Rv1705c (ppe22) | 1.86 | −                 | PPE family protein                 |
| Rv1706c (ppe23) | 1.33 | +                 | PPE family protein                 |
| Rv2389c (rpfD) | 1.31 | +                 | Resuscitation promoting factor     |
| Rv2390c    | 2.00  | +                 | CHP                                |
| Rv2450c (rpfE) | 1.31 | −                 | Resuscitation promoting factor     |
| Rv2549c    | 1.35  | −                 | CHP                                |
| Rv2651c    | 1.60  | −                 | phiRv2 phage protease              |
| Rv3093c    | 1.44  | −                 | Oxidoreductase                     |
| Rv3252c (alk8) | 1.33 | +                 | Transmembrane alkane 1-monoxygenase |
| Rv3429 (ppe59) | 1.33 | −                 | PPE family protein                 |
| Rv3613c    | 1.48  | +                 | HP                                 |
| Rv3614c    | 1.53  | +                 | CHP                                |
| Rv3615c    | 1.51  | +                 | CHP                                |
| Rv3616c    | 1.49  | +                 | CHP                                |
| Rv3746c (pe34) | 1.92 | +                 | PE family protein                  |
| Rv3841 (bfrB) | 1.38 | −                 | bacterioferritin                   |

List of genes upregulated >1.3 fold on exposure to 250 mM NaCl for 4 hrs (p<0.05). Genes induced (+) or unchanged (−) by acidic pH, as determined by comparison to references [5,7]. HP, hypothetical protein. CHP, conserved hypothetical protein.

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Mtb carrying an rrv2390c promoter-GFP fusion functions as a Cl\(^{-}\) and pH reporter

To perform analyses of Cl\(^{-}\) and pH as environmental cues for live Mtb, we utilized the microarray and qRT-PCR results to select candidate genes for construction of a reporter Mtb strain that would be responsive to both changes in [Cl\(^{-}\)] and pH. We focused on the rrv2390c-rpfD operon, which appeared particularly promising as both genes in the operon showed robust induction under conditions of high [Cl\(^{-}\)] or acidic pH (Figure S4 in Text S1). The promoter region of rrv2390c was cloned upstream of GFP in a replicating plasmid, and transformed into Mtb CDC1551. This CDC1551(rrv2390c::GFP) reporter strain was then grown in media +/− 250 mM NaCl, buffered to pH 7.0 and pH 5.7, without added NaCl. Using FACS analysis, we observed an increase in GFP fluorescence of CDC1551(rrv2390c::GFP) in conditions of high [Cl\(^{-}\)] or acidic pH over time, with peak inductions of 7–9 fold over control in each instance (Figure 2A). To verify the Cl\(^{-}\)-specificity of the response, we tested several other compounds for their ability to induce GFP fluorescence in CDC1551(rrv2390c::GFP), including KCl, arginine-HCl, Na\(_2\)SO\(_4\), and sucrose, in media buffered at pH 7.0. Induction was observed with compounds containing Cl\(^{-}\), but not with Na\(_2\)SO\(_4\) and sucrose, indicating that Cl\(^{-}\) was the agent responsible for the increase in GFP signal, and suggesting that neither Na\(^{+}\) nor osmolarity were contributory factors (Figure S5 in Text S1). Induction of rrv2390c::GFP expression was also reversible, with GFP fluorescence returning to baseline levels within 5 days of removal of the high [Cl\(^{-}\)] stimulus in log-phase bacteria (Figure S6 in Text S1). These data, along with the lack of induction observed with other stressors such as NO and hypoxia (Figure S7 in Text S1), argue for the usefulness of CDC1551(rrv2390c::GFP) as a specific reporter Mtb strain for the intraphagosomal cues of pH and Cl\(^{-}\).
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To determine if Mtb's response to Cl\(^-\) occurs in a concentration-dependent manner, we repeated the time-course induction assays with media containing different [Cl\(^-\)] at pH 7.0. GFP fluorescence of CDC1551(\textit{rv}2390c::GFP) increased as [Cl\(^-\)] rose, showing Mtb's ability to modulate its response to [Cl\(^-\)] in a manner comparable to a rheostat (Figure 2B). In agreement with a previous study reporting Mtb's dynamic response to diminishing pH [24], we also observed increasing GFP signal with decreasing pH for CDC1551(\textit{rv}2390c::GFP) (Figure 2C). These results further demonstrate the usefulness of CDC1551(\textit{rv}2390c::GFP) as a reporter Mtb strain for Cl\(^-\) and pH, and indicate that Mtb's response to these two environmental cues is fine-tuned by its environment.

Cl\(^-\) and pH act synergistically as environmental cues for Mtb

To test whether Cl\(^-\) and pH might act synergistically as intraphagosomal cues, we incubated CDC1551(\textit{rv}2390c::GFP) in media buffered at pH 5.7, with 250 mM NaCl. These conditions resulted in induction of GFP fluorescence to a level (>50 fold) much greater than merely the sum of the GFP signal obtained when the bacteria were grown in conditions with only one cue (high [Cl\(^-\)] or acidic pH) (Figure 3A). qRT-PCR tests on several genes in wild type Mtb (WT) exposed to the different conditions confirmed the synergistic activity (Figure 3B).

This synergy implied cross-talk between regulatory circuits. In particular, we examined the role of the two-component regulator \textit{phoPR}, a system previously shown to be required for expression of the acid and phagosome-regulated locus \textit{aprABC} [24], and whose regulon significantly overlaps the list of genes regulated in a pH-dependent manner during MO infection [5,8]. We found that unlike WT, a \textit{phoP}::Tn mutant carrying the \textit{rv}2390c::GFP reporter failed to induce GFP fluorescence during growth at acidic pH, supporting the critical role of \textit{phoP} in regulating Mtb's response to pH (Figure 3C). Our experiments further indicated that \textit{phoP} also played a role in regulating Mtb's response to Cl\(^-\), as induction of the GFP reporter signal during growth in high [Cl\(^-\)] was reduced in the \textit{phoP}::Tn mutant as compared to WT (1.5–2 fold vs. 7–9 fold) (Figure 3C). Intriguingly, GFP induction with the reporter \textit{phoP}::Tn mutant in conditions of high [Cl\(^-\)] at acidic pH (4 fold) was still greater than that observed with high [Cl\(^-\)] alone, despite the lack of induction with acidic pH as a single signal (Figure 3C). qRT-PCR analyses on a \textit{ΔphoPR} Mtb mutant, as well as a complemented \textit{ΔphoPR} strain (\textit{phoPR}*) confirmed these data. There was decreased induction of target transcript in conditions of high [Cl\(^-\)] alone or high [Cl\(^-\)] at acidic pH in the \textit{ΔphoPR} mutant as compared to WT (3 vs. 5 fold and 12 vs. >50 fold respectively), and no increase in transcript at acidic pH for the mutant (Figure 3D). Genetic complementation restored transcript induction in the mutant to WT levels (Figure 3D).

These results implicate \textit{phoPR} as a regulator that modulates Mtb's response to Cl\(^-\), while also indicating that it is merely one part of a regulatory circuit that impacts this response.

[Cl\(^-\)] and pH in the Mtb phagosome change during MØ infection

Having established that Mtb's response to Cl\(^-\) and pH is interconnected in vitro, we next pursued these studies in the context of MO infection by Mtb. To make use of the \textit{rv}2390c::GFP reporter for these intracellular studies, we first moved the construct into a replicating plasmid containing mCherry driven by the constitutive promoter \textit{smyc} [24,25], to generate the strain CDC1551(\textit{rv}2390c::GFP, \textit{smyc}::mCherry) (Figure S8 in Text...
S1). This allows visualization of all bacteria regardless of reporter induction levels, and an internal calibration of the GFP signal. Activation of MOs prior to infection with Mtb is known to increase the maturation stage and lower the pH of the bacteria-containing vacuoles [26,27], which should increase induction of GFP expression as a function of both pH and [Cl\(^-\)]. Resting or activated murine bone marrow-derived MOs were infected with the reporter Mtb strain, and samples examined by confocal microscopy. We observed increased GFP fluorescence as the infection progressed, with significantly more induction of GFP signal in the activated MOs (Figures 4A and 4B). This difference in the microenvironment experienced by Mtb during infection of resting or activated MO was even more starkly illustrated by pre-incubating the reporter Mtb in conditions of high [Cl\(^-\)] prior to MO infection. In this case, the inoculating bacteria had an increased level of rv2390c\(^{+}\)-driven GFP expression at the start of infection, and exhibited an enhanced divergence in GFP signal between the resting and activated MOs (Figures 4C and 4D). These experiments indicate that Mtb experiences different Cl\(^-\) and pH during MO infection, dependent on the activation status of the host MO, and points to dynamic regulation of its gene expression in response to these environmental cues.

**Figure 3. Links between Mtb’s response to Cl\(^-\) and pH.** (A) Mtb responds synergistically to Cl\(^-\) and pH. CDC1551(rv2390c::GFP) was grown in vitro in media at pH 7.0 (control, circles), pH 7.0+250 mM NaCl (squares), pH 5.7 (triangles), or pH 5.7+250 mM NaCl (diamonds). Samples were taken over time, fixed, and GFP signal analyzed by FACS. Data are shown as means ± SD from 3 independent experiments. (B) Mtb’s synergistic response to Cl\(^-\) and pH is reflected transcriptionally. qRT-PCR of gene expression in WT grown as in (A) for 4 hrs. Fold induction is as compared to WT grown in media at pH 7.0. Data are shown as means ± SD from 3 technical replicates. (C) phoPR is required for Mtb’s response to pH and plays a role in its Cl\(^-\) response. CDC1551(rv2390c::GFP, phoP::Tn) was grown in vitro in media at pH 7.0 (control, circles), pH 7.0+250 mM NaCl (squares), pH 5.7 (triangles), or pH 5.7+250 mM NaCl (diamonds). Samples were taken over time, fixed, and GFP signal analyzed by FACS. Data are shown as means ± SD from 3 independent experiments. (D) Complementation of ΔphoPR restores Mtb’s response to pH and Cl\(^-\). qRT-PCR of rv2390c expression in WT, ΔphoPR, and the complemented mutant (phoPR\(^{+}\)) grown as in (C) for 4 hrs. Fold induction is as compared to the corresponding strain grown in media at pH 7.0. Data are shown as means ± SD from 3 technical replicates.

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### Reporter Mtb strains directly reveal Mtb’s microenvironment during *in vivo* infection, and demonstrate the impact of host immune pressure on environmental cues

The MO experiments above demonstrate the feasibility of using the CDC1551(rv2390c::GFP, smyc::mCherry) reporter strain to reveal important aspects of Mtb’s microenvironment during infection. We sought to test the utility of this reporter system in a whole animal infection where the infection foci will likely present regional variation in immune responsiveness and heterogeneous levels of MO activation. To probe if we could detect regional variation in immune-mediated modulation of infected MOs, we infected C57BL/6j WT or isogenic interferon-γ\(^{-/-}\) (IFNγ\(^{-/-}\)) mice with Erdman/rv2390c::GFP, smyc::mCherry) via intranasal inoculation. IFNγ\(^{-/-}\) mice fail to properly activate their MOs on infection and are susceptible to Mtb, developing a disseminated infection that is fatal [28,29]. The Erdman strain was used for these experiments, as it establishes robust infection in mice. *In vitro* tests show that the Erdman reporter strain responds similarly to both Cl\(^-\) and pH (Figure S9 in Text S1).

Infected mice were sacrificed at 14 and 28 days post-challenge, and lung tissue examined by confocal microscopy. We observed significantly higher GFP fluorescence in the reporter strain in WT vs. IFNγ\(^{-/-}\) mice at each time point examined (Figures 5A and 5B). In the case of IFNγ\(^{-/-}\) mice, we also noted a disseminated infection, in agreement with previous studies (Figure 5A) [28,29]. These results faithfully reproduce our MO experiments since IFNγ\(^{-/-}\) mice, which are unable to activate their MOs, exhibit reduced expression of the GFP reporter signal.

To further examine the impact of host immune pressure on determining Mtb’s microenvironment, we used host inducible nitric oxide synthase (iNOS) expression as an indicator of immune activation in WT mice at 28 days post-infection. This allowed us to compare Mtb resident in regions with vs. without an active immune response, within a single infected WT host. A first observation was that most Mtb were located in iNOS-positive regions of the mouse lung tissue (Figures 5C and 5D). Significantly however, we found greater reporter GFP fluorescence in the...
bacteria residing in iNOS-positive regions vs. those located in iNOS-negative regions (Figure 5D). This result reinforces the concept that host immune pressure can impact substantially on the cues that Mtb responds to in its microenvironment, and that reporter Mtb strains can be exploited to shed light on the signals the bacteria are exposed to during in vivo infection. In particular in the context of the rv2390c’::GFP reporter, it suggests that Mtb experiences a microenvironment with higher }\text{Cl}^{2-}\text{ and more acidic pH during infection of a host with an activated immune system. While the complex nature of in vivo infection means that it remains possible that there are yet other, unidentified, factors that also contribute to the differential induction of GFP fluorescence observed, the apparent specificity of the rv2390c’::GFP reporter supports the notion of }\text{Cl}^{2-}\text{ and pH being at least two of the major drivers of the phenotype observed. This is also consistent with the increase acidification of Mycobacterium-containing phagosomes in activated MOs reported previously [4,27,30], and supports the contention that the bacteria are delivered live to a compartment that represents a more hostile environment.

In order to further validate the utility of reporter strains for studying Mtb infection, we performed additional experiments to examine the possibility of generating a second, independent reporter Mtb strain that would respond to different environmental cues from the rv2390c’::GFP reporter strain. In particular, we pursued in vivo studies with a hspX promoter-driven reporter strain. hspX is a much-studied Mtb gene often used as a marker of expression of the dos regulon, known to respond to hypoxia and NO [19–22]. As expected, in vitro, GFP induction of an Erdman/hspX’::GFP, smyc’::mCherry) reporter strain varied with O2 tension and NO (Figures 6A and 6B). Confocal microscopy analyses of lung tissue from mice infected with Erdman/hspX’::GFP, smyc’::mCherry showed significantly greater induction of Mtb reporter GFP fluorescence in WT vs. IFNγ−/− mice at both 14 and 28 days post-infection (Figures 6C and 6D). We also observed much greater induction of hspX’-driven GFP...
signal at 28 days vs. 14 days post-infection, in accord with the reported time-frame of iNOS synthesis during Mtb infection in WT mice (Figures 6C and 6D) [31]. Immunofluorescent staining of iNOS further showed significantly higher hspX'-driven GFP fluorescence in Mtb residing in iNOS-positive vs. negative regions in WT mice (Figure 6E). Together with the Erdman rv2390c::GFP, smyc::mCherry results above, these experiments illustrate that both reporter Mtb strains reliably detect and respond to localized regions of immune activation in vivo, and support the usefulness of reporter Mtb strains for studies of Mtb-host interactions.

Concluding remarks

Our finding that Mtb can utilize Cl⁻ as an environmental cue, in synergy with pH, is a first illustration of a pathogen exploiting interlinked host signals during phagosome maturation. Importantly, Mtb responds to these cues not just in vitro but also during in vivo infection, where these signals are modulated by immune activity of the host. Most studies on Mtb and its response to environmental cues have centered on in vitro assays and homogeneous bacterial cultures.

While these constitute an important foundation they provide little insight into how Mtb senses and responds to environmental cues in vivo, where the heterogeneity linked to location and immune activation is critical in determining the productiveness of the diverse subpopulations of Mtb present in an infected host [32]. In the current study we validated the two reporter strains for their ability to respond to stresses relevant to their survival in vivo, and support the usefulness of reporter Mtb strains for studies of Mtb-host interactions.
bacteria such as the ones developed in this current study. We feel that these strains represent a new generation of tools to probe the fitness of Mtb \textit{in vivo}. These strains should enable us to functionally dissect the TB granuloma to identify privileged regions of bacteria growth, or hostile areas of immune containment. We also predict that these strains will be valuable in probing for drug action and tissue penetrance, through enhanced stress, as one tries to improve drug availability \textit{in vivo}.

Extending beyond Mtb, our results also have potential implications for other intracellular organisms that similarly experience compartments with a range of decreased pH, such as the bacteria \textit{Coxiella burnetti} [33] and \textit{Brucella} [34], and the parasite...
Leishmania [35]. Might these microbes also respond to Cl\textsuperscript{−}, and is the ability to use Cl\textsuperscript{−} and pH as synergistic cues a more widespread phenomenon? In bacterial studies, Cl\textsuperscript{−} has largely been examined only within the context of salt tolerance and osmolarity. Few reports have studied Cl\textsuperscript{−} in the context of bacterial-host interactions, although Radhke and colleagues proposed that increased [Cl\textsuperscript{−}] aided Listeria monocytogenes phagosomal escape by increased activation of listeriolysin O [36].

Our study further raises the question of what roles other common ions might have on bacterial-host interactions. Although ions, such as iron, that serve as essential micronutrients and are actively sequestered by the host have long been recognized as important focal points for bacterial-host interactions [37], the possible impact of more common ions, like Cl\textsuperscript{−}, remain largely unstudied. In addition to Cl\textsuperscript{−}, we speculate that other common ions, such as K\textsuperscript{+}, might also act as a signal for infecting bacteria. There are several known bacterial K\textsuperscript{+} transporters [38], and these also impact on important aspects such as pH and membrane

Molecular Devices Gemini EM fluorescence plate reader was used for bottom read signal detection (BAC – Ex. 365 nm/Em. 505 nm, AF594 – Ex. 590 nm/Em. 617 nm, pHrodo – Ex. 560 nm/Em. 585 nm), with 4 replicate wells/condition, and temperature control at 37°C. In experiments to establish a calibration curve, at the end of the assay (2 hrs) described above, the MOs were washed 3x with pre-warmed Cl\textsuperscript{−}-free buffer (1.54 mM KH\textsubscript{2}PO\textsubscript{4}, 2.71 mM Na\textsubscript{2}HPO\textsubscript{4}, 69 mM Na\textsubscript{2}SO\textsubscript{4}, 5 mM dextrose, 1 mM calcium acetate, 1.35 mM K\textsubscript{2}SO\textsubscript{4}, 0.5 mM MgSO\textsubscript{4}), and then placed in buffer supplemented with specific [NaCl], 200 mM baflomycin A1 (Sigma), 10 μM nigericin (Calbiochem), and 10 μM monensin (Enzo Life Sciences). After incubation to allow equilibration, the BAC and AF594 fluorescence signals were read on a plate reader as above.

For live-cell time-lapse microscopy, cells were imaged with a Leica SP5 confocal, equipped with a stage enclosed temperature control system. A 364 nm laser line was used for excitation of BAC fluorescence, a 594 nm laser line for Alexa Fluor 594 fluorescence, and a 543 nm line for pHrodo. Emission detection was set at +/− 15 nm of the peak emission λ in each case. 10 z-slices over a 12 μm range were acquired at each time point, using the Leica Application Suite Advanced Fluorescence program. Velocity software (PerkinElmer) was used for analysis and tracking of individual beads.

Materials and Methods

Ethics statement

All animal procedures were conducted in strict compliance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals”. The animal protocol was reviewed and approved (protocol number 2011-0086) by the Institutional Animal Care and Use Committee, Cornell University, under the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, US Department of Agriculture, and the Public Health Service guidelines for the care and use of animals as attested by the National Institutes of Health. All efforts were made to minimize suffering.

Cell culture

Bone marrow-derived MOs were isolated from C57BL/6j WT mice (Jackson Laboratories), and maintained in DMEM (Corning Cellgro), and for confocal live-cell time-lapse microscopy, 2 mM L-glutamine, 1 mM sodium pyruvate, 90% FBS (Thermo Scientific), 20% L-cell conditioned media, 2 mM L-glutamine, 1 mM sodium pyruvate and antibiotics (penicillin/streptomycin) (Corning cellgro), at 37°C in a 5% CO\textsubscript{2} atmosphere. Monoctyes isolated from peripheral mouse bone marrow were grown in DMEM containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics, and allowed to fully differentiate into MOs before use in assays.

Cl\textsuperscript{−} measurement assays

Generation of Cl\textsuperscript{−} and Cl\textsuperscript{−}/pH sensor beads are described in the Supplementary Materials and Methods. For plate reader assays, 2×10\textsuperscript{5} MOs/well were seeded in a 96-well black plate (Corning Costar), and for confocal live-cell time-lapse microscopy assays, 4×10\textsuperscript{6} MOs/well were seeded in a Lab-Tek II 8-chambered coverglass (Nalge Nunc International). MOs were washed 3x with pre-warmed assay buffer (PBS, pH 7.2, 5% FBS, 5 mM dextrose, 1 mM calcium acetate, 1.35 mM K\textsubscript{2}SO\textsubscript{4}, 0.5 mM MgSO\textsubscript{4}), and sensor beads added at 2–3 beads/MO in assay buffer. Acquisition of data on a plate reader or by confocal imaging was initiated within 2–3 minutes of bead addition. A

Mtb strains and culture

The Mtb strain CDC1551 was the parental strain for all in vitro and MO infection experiments. Strains used in mice infections were in the Erdman strain background. Routine culture of Mtb was as previously described [24]. The phoP::Tn mutant was from BEI (#NR-17476), and has been previously described [24]. Details of the construction of a CDC1551 ΔphoPR mutant and its complemented strain are described in the Supplementary Materials and Methods.

Microarray and qRT-PCR analyses

Log-phase Mtb (OD\textsubscript{600}∼0.6) was used to seed 10 ml cultures at OD\textsubscript{600} = 0.3 in 7H9 media buffered at pH 7.0, +/−250 mM NaCl, in standing vented T-25 flasks. RNA samples were collected after 4 hours of treatment, and five biological replicates were tested. RNA isolation, amplification, labeling and analyses by microarrays were carried out as previously described [5]. This microarray dataset is available in the ArrayExpress database under accession number E-MTAB-1374, and on the TB Database website [42]. qRT-PCR experiments were conducted on cDNA generated from amplified RNA as previously described [24].

Fluorescent reporter Mtb strains and in vitro assays

To generate CDC1551/rv2390c::GFP, a 704 bp region immediately upstream of rv2390c was PCR amplified, placed in front of GFPmut2 [43] in a modified replicating plasmid pSE100 [24], and transformed into CDC1551. The rv2390c::GFP, smyc::mCherry reporter strain was constructed by cloning of rv2390c::GFP into the replicating plasmid pCherry3 [25], and transformation into CDC1551 or Erdman. To construct the Erdman/\texttt{hsp}X::GFP, \texttt{smyc::mCherry} reporter, a 558 bp region upstream of the \texttt{hsp}X start codon was PCR amplified and cloned upstream of GFPmut2 in the pSE100 vector. The \texttt{hsp}X::GFP fusion was then subcloned into the pCherry3 plasmid and transformed into Erdman. Selection in all cases was carried out on 7H10 agar containing 50 μg/ml hygromycin.

For broth assays, Mtb was grown in standing vented T-25 flasks, in 10 ml 7H9 medium buffered at specified pH, with addition of NaCl or other compounds as stated for each experiment. pH 7.0

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medium was buffered with 100 mM MOPS, while pH 5.5–6.5 media were buffered with 100 mM MES. Appropriate antibiotics were added as necessary. NO assays were done in stirred, aerated, cultures and used the NO donor DETA NONOate (Cayman Chemicals) at 100 μM. Hypoxia experiments were conducted in 50 ml culture volumes in 125 ml dual-capped 13mm eyewash flasks (BD Biosciences) with stirring using a magnetic stir bar. Cultures were placed in a hypoxia chamber with adjustable O2 and CO2 controls (BioSpherix), set on a magnetic stirrer within a 37°C incubator. CO2 was set at 7%, while O2 levels were adjusted as required. For all *in vitro* assays, samples were fixed with 4% paraformaldehyde and GFP fluorescence read on a BD FACS LSR II. FACS data were analyzed using FloJo (Tree Star, Inc).

**Macrophage infections**

Infection of murine bone marrow-derived MOs with Mtb were carried out as previously described [24]. Where needed, MOs were activated by treatment with 100 U/ml IFNγ and 10 ng/ml LPS. For infection with CDC1551 (rv2390c::GFP, smyc::mCherry) pre-induced with Cl−, the bacteria were grown in the presence of 250 mM NaCl for 6 days prior to MO infection. Bacteria were at log-phase when MOs were infected. Samples were fixed, imaged and analyzed by confocal microscopy as described below.

**Mouse Mtb infections**

All animal experiments were carried out in accordance with NIH guidelines, and with the approval of the Institutional Animal Care and Use Committee of Cornell University. C57BL/6J WT and their isogenic IFNγ−/− derivatives (Jackson Laboratories) were infected with 10⁵ CFU of Erdman(rv2390c::GFP, smyc::mCherry) or Erdman(hspX::GFP, smyc::mCherry) via an intranasal delivery method. This was accomplished by lightly anesthetizing the mice with isoflurane and administering the bacterial inoculum in a 25 μl volume onto both nares. At sacrifice, the lungs were removed and fixed in 4% paraformaldehyde overnight.

**Confocal immunofluorescence microscopy**

For MO infections, Mtb infected cells on glass coverslips were fixed overnight at indicated time points with 4% paraformaldehyde. Nuclei were visualized with DAPI (Invitrogen). For mouse infections, whole lung lobes were fixed overnight with 4% paraformaldehyde, and stored in PBS prior to processing. Details of sample processing and antibodies used for confocal microscopy imaging are described in the Supplementary Materials and Methods. Samples were imaged with a Leica SP5 confocal microscope, and z-stacks reconstructed into 3D using Velocity software. For quantification of reporter Mtb signal, the fluorescence voxel volume of each bacterium was measured via the mCherry channel, with the corresponding sum of the GFP signal for that bacterium simultaneously measured. Settings for the GFP channel were maintained during imaging of samples within experimental sets to allow comparison of values. At least 100 bacteria were quantified for each condition. Statistical differences between data sets were determined by a non-parametric Mann-Whitney test.

**Supporting Information**

Text S1  Text S1 contains supplemental Materials and Methods, supplemental figures and legends, and the supplemental video legends. (PDF)

**Video S1 Time-lapse of murine bone-marrow derived MO phagocytosis of BAC/AF594 beads.** Time-lapse movie showing phagocytosis of BAC/AF594 beads. BAC (green)/AF594 (red) beads were added to murine bone-marrow derived MOs and imaged every 2 minutes for 60 minutes. 10 z-sections were imaged at each time point, and merged. The movie is compressed into 3 seconds. (MOV)

**Video S2 Time-lapse of BAC/AF594 beads in media alone.** Time-lapse movie of BAC/AF594 beads in media alone. BAC (green)/AF594 (red) beads were placed in assay buffer and subjected to the same number of exposures as the MO phagocytosis experiment in Video S1. The movie is compressed into 3 seconds. (MOV)

**Video S3 Time-lapse of murine bone-marrow derived MO phagocytosis of BAC/pHrodo beads.** Time-lapse movie showing phagocytosis of BAC/pHrodo beads. BAC (green)/pHrodo (red) beads were added to murine bone-marrow derived MOs and imaged every 2 minutes for 60 minutes. 10 z-sections were imaged at each time point, and merged. The movie is compressed into 3 seconds. (MOV)

**Video S4 Time-lapse of BAC/pHrodo beads in media alone.** Time-lapse movie of BAC/pHrodo beads in media alone. BAC (green)/pHrodo (red) beads were placed in assay buffer and subjected to the same number of exposures as the MO phagocytosis experiment in Video S3. The movie is compressed into 3 seconds. (MOV)

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**Author Contributions**

Conceived and designed the experiments: ST NS RBA DGR. Performed the experiments: ST NS. Analyzed the data: ST NS. Contributed reagents/materials/analysis tools: TP RBA. Wrote the paper: ST DGR.

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