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Phagocyte Roulette in *Salmonella* Killing

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SUMMARY

Salmonella propagate in macrophages to cause life-threatening infections, but the role of neutrophils in combating *Salmonella* has been controversial. In this issue, Burton et al. (2013) use single cell analyses and modeling to explain the ability of *Salmonella* to survive in macrophages, while being killed by neutrophils.

Salmonella serovars cause more than 350,000 deaths per year throughout the world. Although most infections lead to gastroenteritis, the most serious *Salmonella* disease results from extraintestinal infection and bacteremia. The hallmark of these extraintestinal infections is the ability of *Salmonella* to survive within host immune cells, which normally kill bacteria by producing a variety of antimicrobials, including reactive oxygen species (ROS) and reactive nitrogen species (RNS).

It is clear that *Salmonella* survives and replicates primarily in macrophages during systemic infection, and the mechanisms of macrophage survival have been the focus of much research on *Salmonella* pathogenesis (Figueira and Holden, 2012). Upon being engulfed into the macrophage phagosome, *Salmonella* senses and responds to this environment by inducing a variety of virulence factors, including a type III secretion system. Vesicular trafficking and maturation of the phagolysosome are altered, thereby presumably lessening the antimicrobial response and providing a niche for bacterial replication. Most *Salmonella* in macrophages divide only a few times before the bacteria apparently breakout of the host cell, via mechanisms that are not clear, to infect additional macrophages (Mastroeni and Grant, 2013). But viewing systemic disease solely as a function of macrophage survival is too simplistic; not surprisingly, *Salmonella* infection is a dynamic process involving multiple types of immune cells and significant heterogeneity in bacterial cell fate.

The mouse model of *Salmonella* infection provides a powerful tool to study host-pathogen interaction. This animal model, in conjunction with tissue culture systems, has taught us much about *Salmonella* interaction with host cells. But the vast majority of experiments performed with these systems have provided information regarding only the overall population of bacteria. Only recently have investigators used molecular techniques to gain information on the fate of individual cells during infection. In this issue of Cell Host & Microbe, Burton et al.(2013) use single cell analyses and computational modeling to nicely tease apart the differential roles and killing mechanisms in various phagocytic cells. They

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conclude that, whereas *Salmonella* survives in a subset of macrophages, neutrophils and inflammatory monocytes effectively kill the bacteria, primarily via production of lethal concentrations of ROS or hypochlorite (chlorox) in the phagosome.

Orally acquired *Salmonella* invade the intestinal epithelium, replicate in Peyer's patches and subsequently spread to systemic tissues, initially concentrating in the spleen and liver. In agreement with previous studies, Burton et al. (2013) found *Salmonella* almost exclusively in the red pulp of the spleen 4 days after infection. But a significant fraction of the bacteria were located in neutrophils and inflammatory monocytes associated with inflammatory lesions in the tissue. Outside of these lesions, the bacteria were found in macrophages. The authors use danti-LPS antibodies to detect the *Salmonella* cells, but they determined the viability of the bacteria by monitoring release of a cytoplasmic fluorescent protein. The fate of *Salmonella* differed significantly within the cell types, and whereas *Salmonella* survives and propagates in macrophages, neutrophils and inflammatory monocytes efficiently killed the bacteria.

Macrophages and neutrophils normally kill engulfed bacteria by delivering a variety of antimicrobial substances to the phagosome, including proteases, antimicrobial peptides, lactoferrin, and lysozyme. The multi-subunit NADPH-dependent phagocytic oxidase (Phox or NOX2) assembles on the phagolysosome membrane and creates superoxide anion in the phagosome by reducing oxygen. Superoxide is enzymatically or spontaneously reduced to hydrogen peroxide, which is further reduced by free iron in the Fenton reaction to create hydroxyl radical, the nastiest of the ROS (Imlay, 2009). Nitric oxide is produced from arginine and oxygen by the inducible nitric oxide synthase or iNOS(Fang, 2004). ROS and RNS are critical antimicrobial effectors used by both cell types, but their specific roles are controversial, and the absolute mechanisms of bacterial inhibition or killing are unclear (Slauch, 2011). While there are some general similarities in the mechanisms used by macrophages and neutrophils to kill bacteria, the phagosomal environments in the two cell types are strikingly different. The pH of the neutrophil phagosome is basic, whereas the macrophage phagolysosome is acidified. Moreover, neutrophils and inflammatory monocytes, but not macrophages, produce myeloperoxidase (MPO), which produces hypochlorite or other hypohalites from hydrogen peroxide and is a primary consumer of both superoxide and hydrogen peroxide in the phagosome (Winterbourn *et al.*, 2006).

Using an in silico model in combination with *in vivo* expression data for ROS defense enzymes in *Salmonella*, Burton et al.(2013) dissect the contribution of different reactive oxygen species in bacterial killing in both neutrophils and macrophages. MPO is expected to produce significant hypochlorite from hydrogen peroxide (Winterbourn *et al.*, 2006)and the enzyme co-localized with dead *Salmonella*. However, there was surprisingly little increase in bacterial survival in neutrophils from $MPO^{-/-}$ mice, consistent with the limited clinical consequences of genetic loss of MPO in humans. Although the predicted levels of hypochlorite produced are evidently lethal, in the absence of MPO the concentrations of superoxide and hydrogen peroxide are expected to increase significantly. The model suggests that neutrophils produce substantial quantities of superoxide via the NADPH oxidase which, given the high pH of the neutrophil phagosome, remains in the deprotonated form. The superoxide rapidly dismutes to hydrogen peroxide. Burton et al. propose that, in the absence of MPO, hydrogen peroxide, which can readily diffuse across bacterial membranes, overwhelms the bacterial defenses with a cytoplasmic concentration predicted to reach upto15 micromolar, well above previously determined lethal levels (Imlay, 2009). Although they did not consider bacterial scavenging of ROS, a previous model by Winterbourn et al. (2006) predicted that loss of MPO would also lead to superoxide concentrations in the range of 100 micromolar, which could in theory be lethal (Craig and Slauch, 2009). One subtle point is that the Burton model assumes that deprotonated

superoxide does not readily cross the bacterial outer membrane, which, given the presence of porins, might not be valid. Nevertheless, a primary conclusion consistent with both models is that either hypochlorite or ROS (in the absence of MPO) can efficiently kill *Salmonella* at the concentrations produced in neutrophils.

Macrophages generate lower levels of superoxide, but in an acidified phagosome, favoring protonated superoxide that, according to the Burton model, more readily crosses the outer membrane of the bacterium. Consistent with previously published data (Slauch, 2011), the model predicts that in macrophages *Salmonella* is equipped with sufficient defense mechanisms to combat the levels of superoxide and hydrogen peroxide generated. Interestingly, of all ROS defense enzymes, only SodCI, a periplasmic superoxide dismutase, was identified as being essential to protect against lethal oxidative stress in the macrophage phagosome. In the absence of SodCI, the concentration of periplasmic superoxide is predicted to increase more than 4 orders of magnitude to reach a presumably inhibitory concentration. It has previously been suggested that this superoxide damages an unknown extra cytoplasmic target (Craig and Slauch, 2009).

Burton et al. (2013) also perform an elegant experiment to address the relative roles of RNS and ROS by simultaneously monitoring expression of genes responsive to either stress. The iNOS enzyme was predominantly expressed in inflammatory monocytes. The data suggest that *Salmonella* induced effective protective mechanisms to counteract these RNS. Consistent with previous data (Mastroeni et al., 2000; Craig and Slauch, 2009; Slauch, 2011), ROS and RNS seem to function largely independently, with only 10 percent of *Salmonella* (albeit the live cells) demonstrating a significant response to both.

From these and previous studies, we can conclude that neutrophils and inflammatory monocytes are fully capable of killing *Salmonella*, using primarily hypochlorite and/or ROS. The ability of macrophages to kill *Salmonella* is more nuanced; some macrophages are successful, whereas sometimes *Salmonella* gains the upper hand and replicates in a modified phagolysosome. Although reactive oxygen species are important weapons used by macrophages, the defense mechanisms of *Salmonella* are more than capable of normally handling this assault (Slauch, 2011). Importantly, *Salmonella* residing in macrophages are protected against the more lethal neutrophils or inflammatory monocytes. Who wins the battle in an individual macrophage could be dependent on the relative speed with which the bacterium or host cell respond to each other, but it is also possible that there are subsets of macrophages that are permissive for *Salmonella* replication. Indeed, Detweiller and colleagues have shown that *Salmonella* is primarily found in hemophagocytic macrophages during persistent infection (Nix et al., 2007). We have much to learn in these areas, but Burton et al. (2013) have provided significant clarity and models to facilitate future studies.

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