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Single-Cell Analysis: The Differences That Kill

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Using single-cell RNA sequencing, Avraham et al. investigate how variability in macrophage response to infection is controlled by variability within the pathogen population. They find that heterogeneous expression of the *Salmonella* virulence factor PhoP and subsequent cell-wall modifications lead to the bimodal induction of the interferon-response in infected macrophages.

What exactly happens when pathogens penetrate the outer defenses of tissues and start infecting various cells? Since the dawn of modern biology, the battle between pathogens and immune cells has been a central focus, and thanks to powerful new methods that analyze individual cells, we are taking a fresh look at our understanding of infection and immunity. Unlike what traditional population-averaged analyses show, the outcome of pathogen exposure is vastly more complex at the individual-cell level. For example, some host cells completely avoid infection and survive. Other cells become infected and die, survive with the presence of bacteria inside them, or completely clear the pathogens and function normally afterward. The intricate workings of the molecular pathways determining infection and immunity are largely unclear. In this issue of *Cell*, Hung and colleagues take a new look at this fundamental problem using single-cell analysis and ask whether variability in infection outcomes can be explained by the variability among individual bacteria (Avraham et al., 2015). This is a unique approach as compared to most work in the newly emerging field of single-cell immunology. In explaining heterogeneous infection outcomes, the field tends to focus on the state of the host and environment (Snijder et al., 2009), rather than pre-existing variability in the pathogen.

Hung's team focus on the infection of macrophages—first responders of the innate immune system—with *Salmonella typhimurium*, a pathogen that causes typhoid fever and food poisoning in humans. Despite a century of antibiotic treatment and improved hygiene, basic pathogens such as *Salmonella* remain a major health problem, especially in the developing world. Even the developed world is at risk from these basic infections, as evidenced by thousands of *salmonella* infections every year in the USA alone and the recent *E. coli* outbreak in Germany that killed 50 people over the course of a few weeks.

Salmonella typhimurium has specialized molecular tools to avoid, resist, and even hijack the mammalian immune system. Macrophages recognize these pathogen-associated factors and mount transcriptional programs to change their physiology and clear the pathogen. Individual *Salmonella* cells can vary in the manner they express virulence factors. Can the variability in infection outcomes be explained by the variability within the pathogen population? And if so, what virulence factors control this variability? To answer these questions, Avraham et al. first use fluorescent single-cell microscopy to distinguish various infection outcomes: When mixed with *salmonella*, the macrophages could remain uninfected, or become infected with either live or dead bacteria inside. They isolate these single macrophages and use state-of-the-art RNA sequencing (RNA-seq) to determine their transcriptional state by measuring the expression of 535 immune response genes. These genes cluster into distinct groups; however, one cluster shows much higher expression variability between individual cells. These variable genes were related to innate immune recognition of the bacterial virulence factors, including bacterial cell-wall components like lipopolysaccharide (LPS), hinting that the LPS/TLR4 signaling pathway underlies phenotype variability. In particular, Type 1 interferon (IFN) response exhibit bimodal expression in host macrophages, with roughly one third of cells expressing IFN genes at high levels, and the rest at low levels

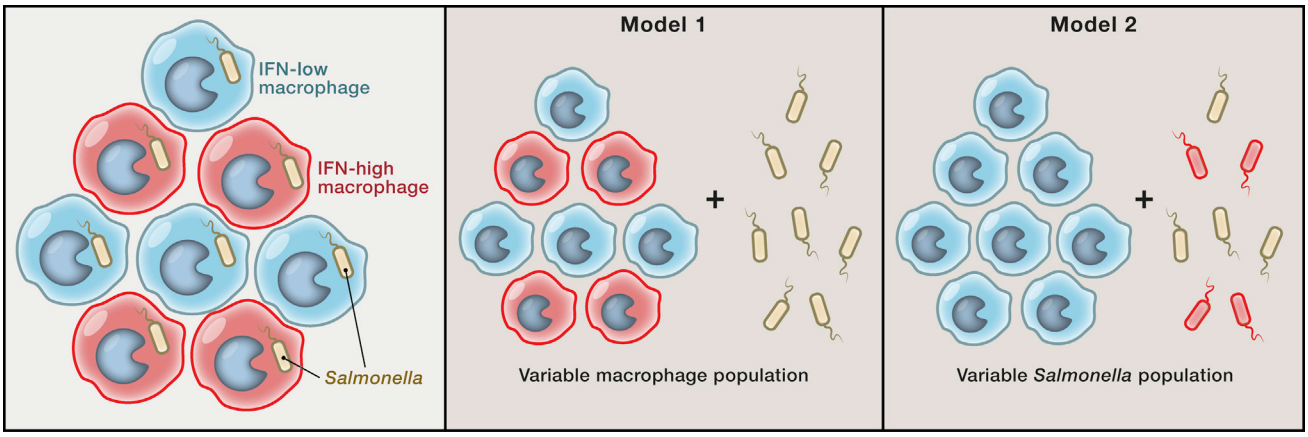

Figure 1. Infection Variability in Macrophages
When infected with single *Salmonella* cells, the macrophage population exhibits heterogeneous infection outcomes, with cells expressing either high (red) or low (blue) levels of *interferon (IFN)* genes. Avraham et al. (2015) show that the macrophage IFN expression variability is not due to variability in the macrophage population as shown in model 1, but is due to the variability in the infecting *Salmonella* cells as described by model 2.

(Figure 1A). The type 1 IFN response leads to secretion of a range of signaling molecules and regulation of cell fate decisions. Bimodality in IFN expression can indicate an underlying stochastic element controlling the IFN pathway (Tay et al., 2010). Intrigued with this bimodal expression pattern, the researchers narrow their focus onto the IFN response genes to uncover the molecular mechanisms driving infection variability.

Using a clever combination of fluorescent reporters, single-cell microscopy and single-cell RNA-seq, the authors ultimately identify the *Salmonella* virulence factor PhoP as underlying the variability in the macrophage infection outcome. Macrophages with high expression of PhoP response genes are also enriched for a Type 1 IFN response. Analysis with mutant strains shows that *Salmonella* expressing PhoP modify the cell-wall component LPS, allowing them to better induce the potentially lethal IFN response in the macrophages they infect. Finally, the researchers perform two conclusive in vivo experiments: They first inject mice with LPS extracted from either $PhoP^{-}$ or $PhoP^{+}$ bacteria, and observe the same correspondence between Type 1 IFN in macrophages from these mice. To further test the functional relevance of their findings, they use a mouse model of septic shock—which produces a cytokine storm leading to infection-related deaths—and again inject mice with modified or unmodified LPS. As expected, the PhoP-modified LPS confers a higher mortality rate in mice, and this effect can be reversed by co-administering a drug (BX795) inhibiting the Type1 IFN response.

What does this all mean? At the fundamental level, we now know that the heterogeneity in pathogen population can control the variability in host immune response (Figure 1). Understanding how pathogenic factors control infection outcomes can lead to better treatment options. Avraham et al. nicely adds to studies on the functional roles of "biological noise," by considering not only the diversity on the host side but also the pathogen side (Snijder et al., 2009; Tay et al., 2010; Snijder and Pelkmans, 2011; Kellogg and Tay, 2015). Of course, the host factors are still involved in the final outcome, and it remains possible that other macrophage-related factors, such as their signaling history, also contribute to the observed phenotypes.

From the perspective of the pathogen, *Salmonella* seems to be using a bet-hedging strategy by diversifying the composition of their cell-wall, as if to wear different battle dress uniforms, to either camouflage themselves from the immune system or to directly attack it. Bet hedging is well studied in systems biology (Veening et al., 2008), and these results constitute a medically relevant example of this interesting phenomenon.

At the molecular level, bimodal expression of *IFN* genes is another example of a digital signaling event (Tay et al., 2010), where a binary switch controlling PhoP expression in *Salmonella* leads to a bimodal gene expression in macrophages. It would be interesting to find out what this switch is and how it could be manipulated with drugs.

A limitation of snapshot measurements like RNA-seq, however, is that they do not account for the dynamical changes in single cells. This can lead to misleading results if care is not taken: grouping expression levels in unsynchronized cells can be skewed by dynamically changing expression levels, especially for genes that are not at the steady state (and often, immune genes are not at steady state when induced with pathogen signals) (Tay et al., 2010; Kellogg and Tay, 2015). On the other hand, there are not many ways to measure gene dynamics except for live-cell microscopy with the few fluorescent reporters currently available to us. Nevertheless, there are known "master regulators," transcription factors like NF-κB, IRF3, and AP-1 that control the expression of genes studied here, and incorporating time-lapse microscopy by tagging these proteins in live cells could reveal more information on pathogen-host interactions (Tay et al., 2010; Kellogg and Tay, 2015; Selimkhanov et al., 2014). This kind of work would also benefit from new microfluidic and optogenetic methods, allowing better control of cell-cell interactions and creating more precise and realistic conditions in vitro (Kellogg et al., 2014; Frank and Tay, 2015; Toettcher et al., 2013). These tools would

be particularly useful in isolating the effects of paracrine and autocrine signals and in more precisely controlling the dosing and timing of pathogen inputs.

This work demonstrates the power of high-content single-cell techniques like RNA-seq in understanding pathogen-host interactions, but there is much left to do, both on the technical and biology sides. The researchers collected a very rich dataset on macrophage transcription and highlight the PhoPQ-IFN link, but there could be more to discover in this treasure chest of functional single-cell data.

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Helping the Help for CD8+ T Cell Responses

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Eickhoff et al. and Hor et al. use time-lapse intravital microscopy to show an unexpected choreography of CD4+ and CD8+ T cells "dancing" between different dendritic cell sub-populations during priming of cytotoxic immune responses to viruses.

The idea that clonal selection is at the basis of adaptive immune responses was first proposed in the Fifties by Nils Jerne and Sir Macfarlane Burnet. It was not, however, until the end of the Nineties that the field accepted that dendritic cells (DCs), first identified in 1973 by Ralph Steinman, are the antigen-presenting cells that support clonal selection and initiate adaptive immune responses in lymph nodes. The first visual in situ dynamic evidence of early interactions between naive T lymphocytes and DCs came in the early 2000s when two-photon intravital imaging methods were developed in immunology (reviewed in Pittet and Mempel, 2008). These early studies revealed a high degree of unexpected complexity in these interactions. First, clonal selection occurs within a complex tissue environment and within specific regions of lymphoid tissue. Second, the encounter of an antigen-presenting cell and a T cell specific for that particular antigen is a rare, non-random event. Dendritic cells accumulate in certain regions of lymph nodes and T cells migrate along preferential tracks, guided by combinations of chemokines. Third, the interactions between T cells and DCs have a specific controlled duration, which is critical for clonal expansion and T cell differentiation into effector and memory cells.

A key critical level in the initiation of immune responses, however, had not yet been addressed: DCs are a heterogeneous cell population that includes multiple cell subtypes with different functions. DCs fall into two main lineages (sometimes referred to as CD103+ and CD11b+ lineages), each including lymphoid tissue resident and migratory cells (Merad et al., 2013). One of the lineages (CD103+, CD8aa+, and XCR1+) specializes in the induction of CD8+ T cell responses and the presentation of internalized antigens on class I MHC molecules (cross-presentation) and will be referred to as XCR1+ DCs. The other subtype is more heterogeneous. CD11b+ DCs present internalized antigens on preferentially class II MHC molecules and induce CD4+ T cell and B cell responses (Merad et al., 2013). The current view of DC biology therefore underlines a repartition of antigen presentation to CD4+ and CD8+ T cells between DC subpopulations.

On the other hand, effective anti-viral cytotoxic immune responses by CD8+ T cells are strictly dependent on CD4+ T cells. In the absence of CD4+ T cells, CD8+ T cell responses are weak and lack long-lasting memory protection (Janssen et al., 2003; Shedlock and Shen, 2003). Different models have been proposed to account for these observations. The first proposed that CD4+ T cell help requires direct interactions between the three cell types (CD4+, CD8+ T cells, and DCs) (Ridge et al., 1998). The likelihood of this three-way cell interaction was questionable, and subsequent studies showed that the three cell types do not need to interact