Heterogeneity of *Salmonella*-host interactions in infected host tissues

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Abstract

Infected host tissues have complex anatomy, diverse cell types, and dynamic inflammation. Traditional infection biology approaches largely ignore this complex host environment and its impact on pathogens, but recent single-cell technologies unravel extensively heterogeneous host-pathogen interactions in vivo. *Salmonella* are major model pathogens in this field due to the availability of excellent mouse disease models and facile molecular biology. The results show how *Salmonella* stochastically vary their virulence, exploit differential nutrient availability, experience and respond to widely varying stresses, and have disparate fates ranging from vigorous proliferation to eradication within the same host tissue. Specific *Salmonella* subsets drive disease progression, while others persist during antimicrobial chemotherapy. Further elucidation of the underlying mechanisms could provide a basis for improved infection control.

Introduction

During infection, pathogens often colonize host tissues with complex anatomy, diverse cell types and microenvironments with different physico-chemical parameters and divergent molecular composition. Pathogens can adopt a large variety of physiological states and stress defense programs in these highly heterogeneous environments. This externally triggered pathogen heterogeneity will add to the inevitable internal variation due to stochastic molecular fluctuations in the pathogen. The resulting rich diversity of pathogen behavior has been largely ignored until recently, in part because available methodology provided only bulk average readouts that could not resolve variation between pathogen subpopulations. However, in the past few years, single-cell approaches have been starting to reveal fascinating diversity of host-pathogen interactions in infected tissues [1-3].

These data provide the basis for a paradigm shift to single-cell pathogen infection biology for better understanding fundamental mechanisms that determine course of disease and treatment outcome. Individual pathogen-host encounters involve divergent cellular and molecular mechanisms that lead to disparate outcomes within the same tissue that range from local pathogen eradication to vigorous proliferation in adjacent infection foci [1-3]. Disease progression hence does not reflect a general inability of host immunity to control the pathogen. Instead, the host seems often have powerful effector mechanisms that efficiently kill pathogen, but fails to employ these mechanisms against all dispersed pathogens, resulting in local lack of control. Likewise, antimicrobial chemotherapy might rapidly kill a large fraction of pathogens, but some pathogen subsets might hide in microenvironments that are poorly reachable for drugs [4], or adopt physiological states that make them tolerant against antibiotics [5-7]. Such surviving pathogens will require extended treatments to minimize the risk of relapses. We need to understand better the pathogen subsets that escape efficient immune control and antimicrobial chemotherapy to enable more efficient infection control strategies.

Salmonella infections in mice provide unique opportunities for developing concepts and approaches that might be broadly applicable to other infection models. In particular, well-characterized mouse infection models, facile *Salmonella* genetics and suitability for numerous experimental approaches, as well as extensive literature make *Salmonella* one of the best-studied

pathogens. The mouse is a natural host of various *Salmonella enterica* serovars. Low doses of *Salmonella enterica* serovar Typhimurium can cause systemic infections in genetically susceptible mice that reproduce some aspects of human typhoid fever (which is caused by human-adapted serovars Typhi and Paratyphi) [8], and the recent re-establishment of experimental human models of typhoid/paratyphoid fever [9,10] offers exciting possibilities to compare at murine and human infections under well-controlled infection conditions. Infection of genetically resistant mice can lead to chronic infections with low but stable *Salmonella* tissue loads in spite of a strong immune response [11]. Mice do not normally develop diarrhea, but disruption of the normal gut microbiota by a single dose of streptomycin overcomes *Salmonella* colonization resistance, and provides a versatile and widely used enteritis model [12].

Suitable animal and cell-culture infection models, together with facile molecular biology have made *Salmonella* a prime pathogen for developing numerous innovative approaches. This includes in vivo expression technology (IVET) [13], signature-tagged mutagenesis (STM) [14], differential fluorescence induction (DFI) [15], ex vivo proteomics [16], population dynamics with wild-type isogenic tagged strains (WITS) [17], fluorescence dilution (FD) [18], TIMER growth rate reporter [19], ex vivo isolation of pathogen subpopulations [20], dual RNA-seq [21], single-cell RNA-seq of infected cells [22,23], etc. As part of this general history of *Salmonella* as a suitable model pathogen for developing novel methodology, single-cell techniques such as confocal microscopy, flow cytometry coupled with informative fluorescent reporter constructs, and single cell RNAseq are starting to yield unique insights into *Salmonella* in vivo heterogeneity in expression, growth rate, stress exposure, antimicrobial tolerance, and single-cell fates. Some of these methods have been recently covered in other reviews [2,24-26]. Here, we will focus on the results that have been obtained and open questions in this new field.

Salmonella growth rate

Early studies revealed extensive differences in *Salmonella* growth rate, gene expression, and proteome in gut lumen vs. mucosal tissues and spleen of infected mice [12,16,27-29]. A major switch occurs in *Salmonella* that invade gut epithelial cells and turn on expression of a type three secretion system encoded on *Salmonella* pathogenicity island 2 (SPI-2), which is associated with intracellular growth. A recent study showed that many *Salmonella* originating from the gut lumen and arriving at mesenteric lymph nodes are in an extended lag phase [30], perhaps while they re-program their gene expression as required for the new tissue microenvironment.

Maybe more surprisingly, *Salmonella* shows also extensive heterogeneity even within a single host organ. In the enteritis model, *Salmonella* splits into two intestinal subpopulations with one rapidly proliferating subset with low virulence gene expression and another more slowly growing subset with high levels of the invasion-associated type three secretion system encoded on *Salmonella* pathogenicity island 1 (SPI-1) [31] (Fig. 1). This heterogeneity seems to reflect stochastic variations in *hilD* gene expression [32], but the underlying molecular mechanisms remain unclear. The SPI-1 ON subset invades the mucosa, causes inflammation, and is partially cleared by the host immune system. However, the gut inflammation that is triggered by this SPI-1 ON subset suppresses competing gut microbiota thus enabling the SPI-1 OFF subset to thrive. This is a striking example of "division of labor" or cooperative virulence among pathogen subpopulations. Another advantage of bistable expression of SPI-1 is the maintenance of a well-growing subset. This subset competes effectively against cheater mutants that completely switch off SPI-1, but exploit benefits generated by wild-type subsets with high SPI-1 activity [33].

Salmonella also shows highly heterogeneous growth rates when they reside mostly intracellularly in systemic mouse tissues such mesenteric lymph nodes, spleen, and the gall bladder epithelium [18,19,34,35]. Early after oral infection and tissue invasion, a small *Salmonella* subset remains in an extended lag phase with no detectable cell division [18]. This extensive lag phase is triggered by induction of toxin proteins of toxin/anti-toxin modules such as an aminoacyl-tRNA

101 acetylase TacT [36], possibly in response to low pH and poor nutrient availability in intracellular
102 *Salmonella*-containing vacuoles [34]. However, as disease progresses such growth-arrested subsets
103 become very rare [19,34] due to overgrowth by replicating *Salmonella* and perhaps some wake-up
104 from lag phase and/or clearance by host immunity.

105 Growth-arrested subsets in the mesenteric lymph nodes stay at more constant levels in the
106 mouse enteritis model, in which endogenous gut microbiota are largely eradicated by prior
107 streptomycin treatment [30]. Under these conditions, *Salmonella* maintain very high loads in the gut
108 lumen with bistable SPI-1 expression (see above). Only the SPI-1 ON subset continuously travels to
109 the mesenteric lymph nodes [37]. Many of the new arrivers remain apparently in extended lag
110 phases, especially when the reside in classical dendritic cells (but not in interstitial dendritic cells)
111 [30] (Fig. 1).

112 In presence of a normal gut microbiota, *Salmonella* colonizes the gut lumen only transiently
113 and *Salmonella* colonizes the Peyer's patches and disseminates to systemic tissues such as
114 mesenteric lymph node, spleen, and liver. When disease signs become visible, practically all
115 *Salmonella* cells in these various tissues grow, but their division rates are highly divergent [19]. A
116 minor fast-growing subset drives bacterial tissue loads and disease progression, but much of its
117 offspring slows down to mostly moderate growth rates, while only a minority maintains the high
118 division rates. Subset isolation, proteomics, and metabolic network analysis of fast vs. slow growing
119 subsets suggest that these growth rates reflect at least in part differential supply of nutrients such as
120 nucleosides and amino acids. Surprisingly, diverse host cells such as red pulp resident macrophages
121 and neutrophils support a similar range of intracellular *Salmonella* growth rates, and what
122 determines differential nutrient supply is still unclear. Although we have yet no evidence for
123 additional heterogeneity-promoting factors such as *Salmonella* internal stochastic variations, such
124 contributions might play an important role (as they apparently do in the gut for virulence gene
125 expression, see above).

Salmonella tolerance to antimicrobial chemotherapy

Heterogeneous in vivo growth rates can have a dramatic impact on antimicrobial chemotherapy. Non-growing *Salmonella* that reside in in mesenteric lymph nodes seem to be partially resilient against early high-dose treatment with fluoroquinolone antibiotics. However, if such "survivors" retain actual colony-forming capabilities (i.e., full viability) seems to depend on subtle experimental details [19,30,34,38]. It has also been proposed that *Salmonella* detected in mesenteric lymph nodes during therapy might actually represent *Salmonella* dynamically entering from unidentified intestinal sites during therapy, instead of locally persisting *Salmonella* [38]. As SPI-1 expression is required for tissue invasion and reaching the mesenteric lymph nodes, antibiotic treatment actually favors survival of the virulent subset in the apparently privileged mesenteric lymph nodes during antimicrobial therapy, providing a fascinating connection between cooperative virulence and antimicrobial tolerance [37]. Fluoroquinolones retain partial bactericidal activity against non-dividing bacteria in vitro, suggesting that additional in vivo factors might further enhance survival of this *Salmonella* subset. Such factors could include diminished bactericidal activity of fluoroquinolones at high concentrations and/or low oxygen tension [39].

During systemic infection, a non-proliferating *Salmonella* mutant is largely resilient against high-dose fluoroquinolone treatment [40], but it is unclear if this is relevant for normal infections. To assess this issue, we orally infected mice with wild-type *Salmonella* and started treatment after appearance of clinical disease signs (day 5 post oral infection) with only moderate doses [19]. Under these more clinically representative conditions, killing efficacy again strongly correlates with *Salmonella* growth rates. However, instead of non-growing *Salmonella* (which are rare under these conditions), abundant moderately growing *Salmonella* subsets with still substantial antimicrobial tolerance are mostly responsible for slow eradication during treatment. Together, these data show how *Salmonella* in vivo phenotypic heterogeneity can impair antimicrobial chemotherapy, even in

absence of any inheritable resistance. This *Salmonella* in vivo tolerance could reflect internal stochastic fluctuations in *Salmonella* (as widely studied in vitro). On the other hand, host factors such as stresses in the *Salmonella*-containing vacuole that induce high persister frequencies [33], differential nutrient access leading to a wide range of growth rates and antimicrobial tolerance [19] seem to have a major impact.

Salmonella **stress exposure and fates**

In addition to differential nutrient access, individual *Salmonella* cells also experience widely varying stress conditions as part of antimicrobial host attacks [20]. In particular, regional accumulation of inflammatory monocytes expressing high levels of inducible nitric oxide synthase (iNOS) exposes local *Salmonella* to nitric oxide (NO). Isolation and proteome analysis of the affected *Salmonella* subset (using a NO-inducible reporter) showed that they respond by specific upregulating of just three NO detoxification/repair proteins that effectively alleviate NO toxicity. As a result, iNOS has no impact on *Salmonella* fitness during the first week of acute infection. In parallel, *Salmonella* spreading to new host cells experience transient oxidative bursts that expose them to a variety of reactive oxygen species (ROS) [20]. The affected *Salmonella* subset upregulates specific detoxification enzymes such as catalase KatG and peroxidase AhpCF, in addition to a general high baseline level of other detoxification enzymes (superoxide dismutase SodCI, peroxidase TsaA). This *Salmonella* defense is effective against moderate oxidative bursts employed by red pulp resident macrophages, but insufficient to cope with the more powerful oxidative bursts in neutrophils and inflammatory monocytes [19]. These latter cell types use the abundant enzyme myeloperoxidase to convert superoxide and peroxide to highly reactive hypochlorite (bleach) at the *Salmonella* surface [41]. Hypochlorite immediately reacts with any biomolecule confining its damaging action to the *Salmonella* envelope and the immediate surroundings, thereby minimizing collateral host tissue damage.

These data suggest that some *Salmonella* can effectively cope with certain host stresses with little impact on their fitness. Indeed, slow- and fast-growing *Salmonella* experience similar NO and ROS stress based on their proteome profiles [19], suggesting that these stresses have limited impact on division rates. On the other hand, neutrophils and inflammatory monocytes kill a significant number of *Salmonella* cells using oxidative bursts.

This highly divergent impact of stress on different *Salmonella* subsets that coexist in the same infected host tissue resolves apparently contradicting previous bulk average data on the impact of host ROS. The accumulation of neutrophils and monocytes around growing *Salmonella* infection foci and the resulting increasingly potent local control, could also explain the early finding that *Salmonella* tissue loads are mostly driven by spreading and formation of new infection foci, whereas old foci are less productive for *Salmonella* growth [42]. The host clearly has the capability to kill *Salmonella* effectively, and an eventually fatal disease outcome reflects the insufficient ability to employ these mechanisms rapidly enough against all emerging infection, rather than a general superiority of *Salmonella* virulence mechanisms and/or weak host immunity. This is a striking parallel to tuberculosis, where successful host control and vigorous *Mycobacterium tuberculosis* growth occur in close vicinity in the same infected host tissue [3].

Salmonella **heterogeneity as a tool for in-depth analysis of specific mechanisms**

The published data show a large complexity of concomitant individual host-pathogen encounters in vivo with diverse molecular mechanisms and disparate outcomes for dozens of distinct *Salmonella* subsets. Ongoing studies reveal even more *Salmonella* heterogeneity, and it will be challenging to understand and interpret all these overlapping complexities. On the other hand, the comparison of distinct *Salmonella* subsets actually simplifies the analysis of individual host conditions, as it enables to compare affected subsets directly to unaffected *Salmonella* subsets from the same tissue (which can serve as ideal “controls”). As an example, this approach revealed a highly focused *Salmonella*

response to local host NO involving just three proteins, compared to much more complex in vitro responses that can be further convoluted by interference with the iron-dependent transcriptional regulator Fur [43].

Conclusions

Single-cell analysis of *Salmonella* heterogeneity in infected host tissues is a newly emerging field. Results obtained so far already reveal a previously unanticipated rich infection biology with dozens of subsets as a result of superimposed distinct molecular stress mechanisms and differential nutrient access. As we gain deeper insight, we discover more and more heterogeneity and it is likely that almost every *Salmonella* might face and specifically responds to a unique host microenvironment. The data also show a crucial importance of some particular types of host-pathogen encounters for disease progression and ultimate outcome. On the other hand, we have clearly obtained only the first glimpses of highly complex scenarios in infected tissues.

We still largely lack a molecular understanding of the mechanisms that drive the divergent outcomes of individual encounters. Host microenvironments clearly influence local *Salmonella*, but in vitro single-cell studies suggest that intracellular *Salmonella* itself can also differentially modulate activities of its host cell [22,23]. In addition to this complex interplay, stochastic variation in *Salmonella* and host cells could further complicate the interactions. In the context of antimicrobial chemotherapy, differential antagonistic host attacks (such as nitric oxide [44,45]) but also inefficient drug penetration of certain tissue compartments [4] could delay eradication. For all these mechanisms, we have yet limited in vivo evidence.

Maybe the most important questions are how we can leverage the increasing knowledge of key *Salmonella* subsets for improving infection control. Can we help the host to direct its potent *Salmonella*-killing cells more comprehensively to all newly emerging infection foci? Which properties of resilient *Salmonella* subsets with high tolerance to antibiotics might exploitable for specific targeting of these crucial subsets (e.g., [46])? Answering these questions is important since

227 *Salmonella* remains a major threat to human health [47]. In fact, this threat might even become more
228 serious because of rapidly rising antimicrobial resistance and the lack of efficacious vaccines against
229 major *Salmonella* serovars. Finally, methods that have been developed primarily using *Salmonella* as
230 a model pathogen might be applicable to other major human pathogens, and exciting new approaches
231 from other infectious diseases such as PET imaging for visualizing infection focus dynamics in live
232 animals [48] might be informative for salmonellosis.

233

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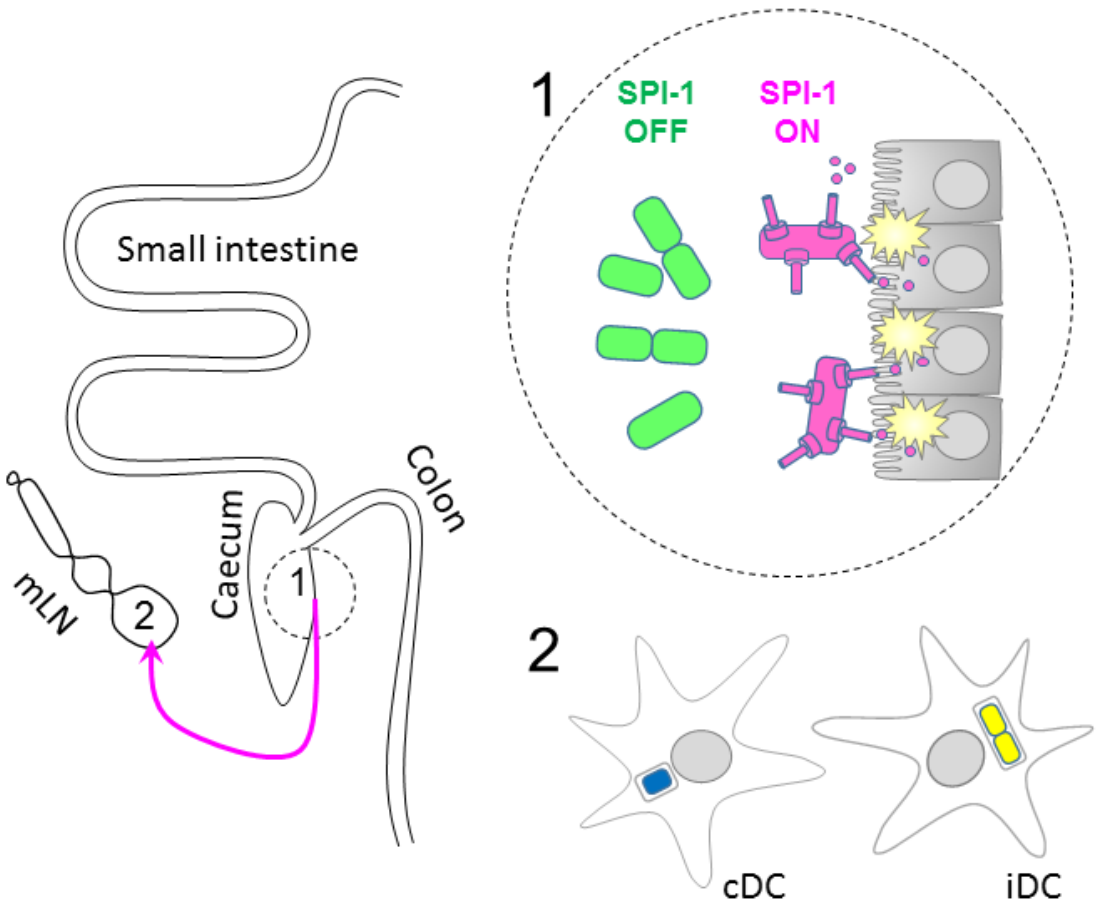
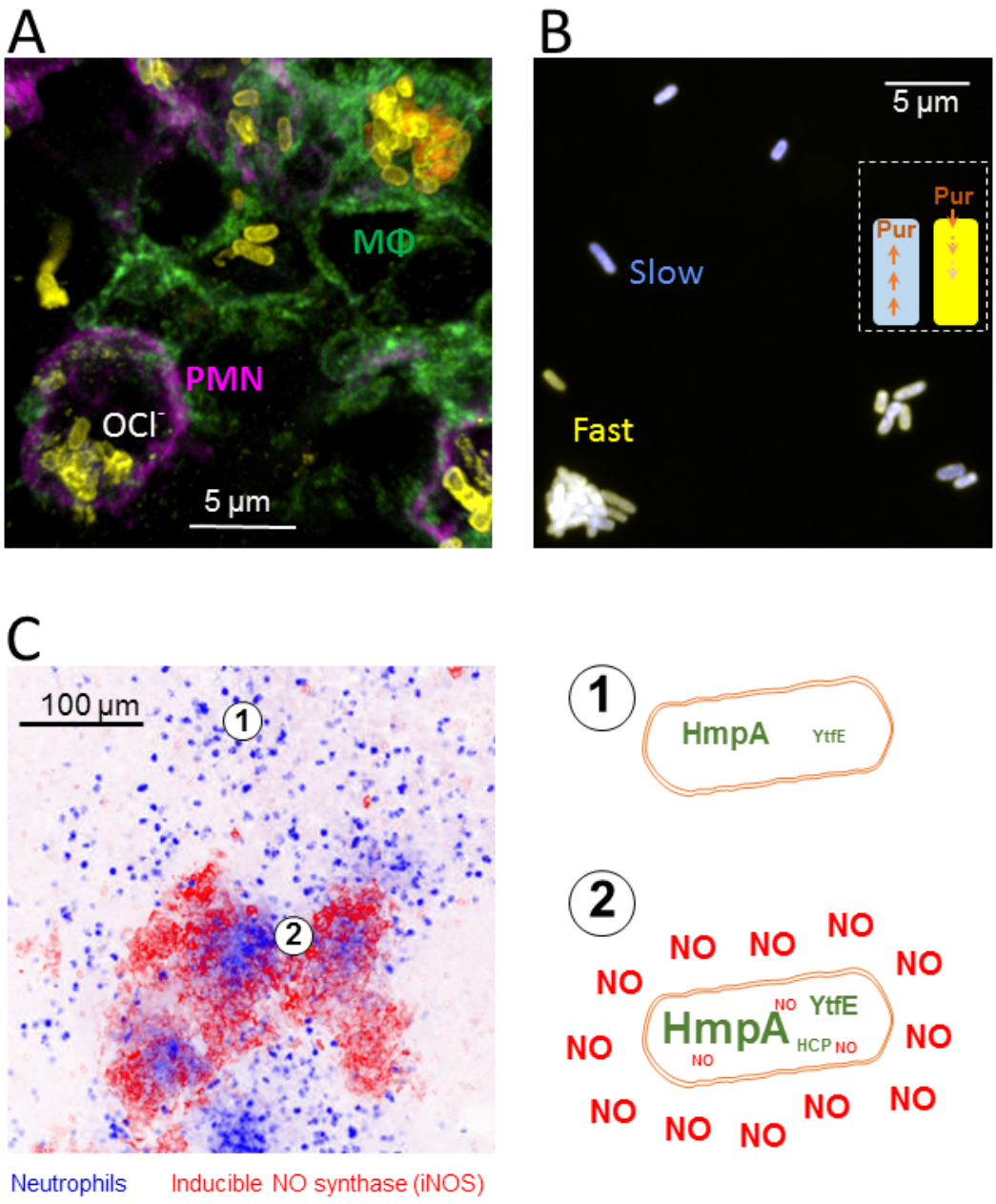


369 **Figure 1**: *Salmonella* heterogeneity in gut-associated tissues in a widely used mouse enteritis model.
370 *Salmonella* colonizes the caecum lumen (which is partially equivalent to the human colon) and splits
371 into two subsets (1). One subset actively proliferates with low virulence gene expression, whereas
372 the other subset has low growth rate and high expression of virulence genes associated with the SPI-
373 1 type III secretion system ("SPI-1 ON"). The SPI-1 ON subset invades the caecum mucosa and
374 triggers inflammation that diminishes the density of competing normal gut microbiota, thereby
375 enabling the SPI-1 OFF *Salmonella* subset to thrive. Although many invading *Salmonella* are killed,
376 some SPI-1 ON *Salmonella* manage to travel inside dendritic cells from the gut to mesenteric lymph
377 nodes (mLN). Many *Salmonella* residing in classical dendritic cells (cDC) do not divide enabling them
378 to tolerate high doses of antimicrobials, whereas *Salmonella* in interstitial dendritic cells (iDC) might
379 proliferate at higher rates and remain sensitive to antibiotics (2).

380



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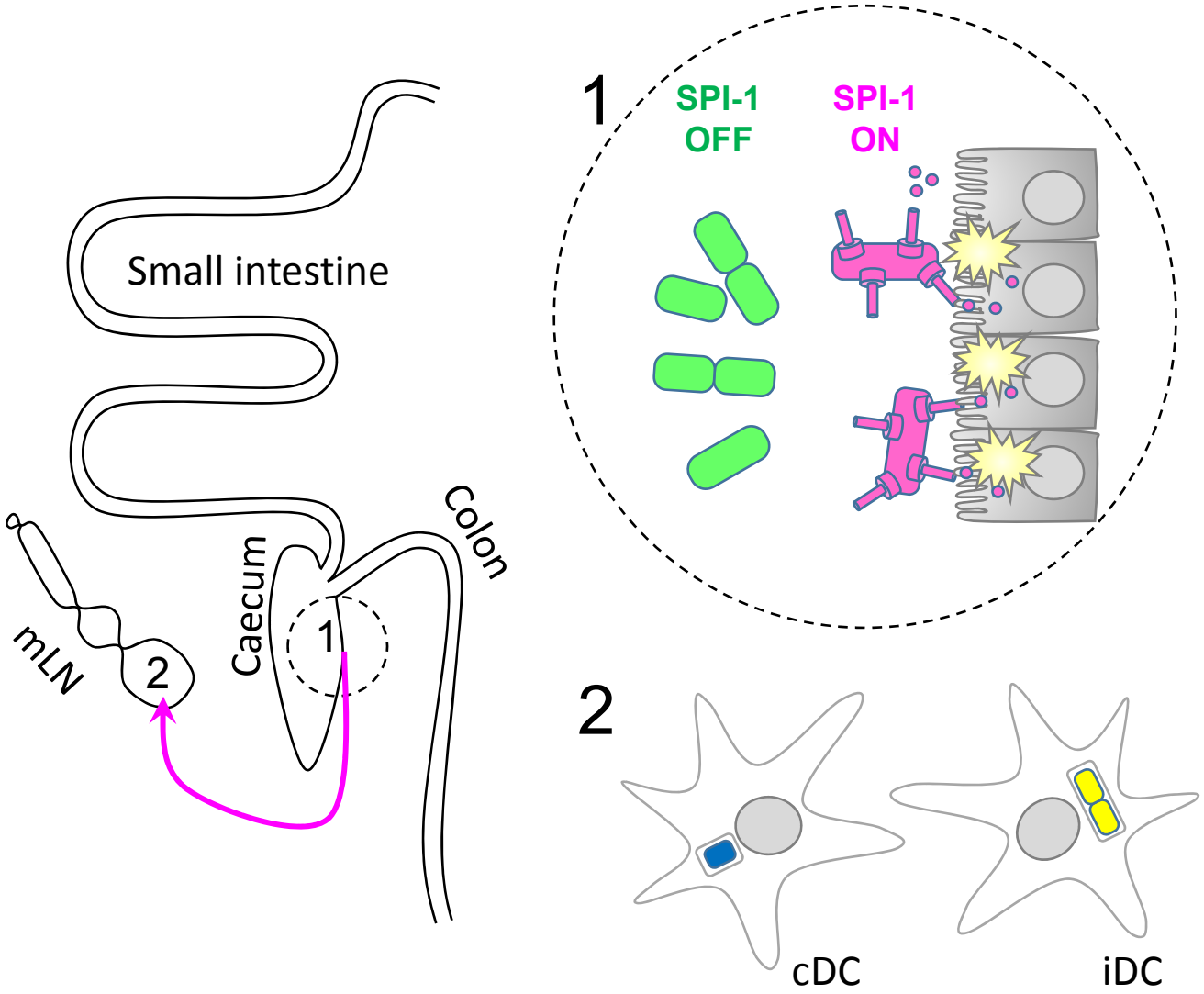
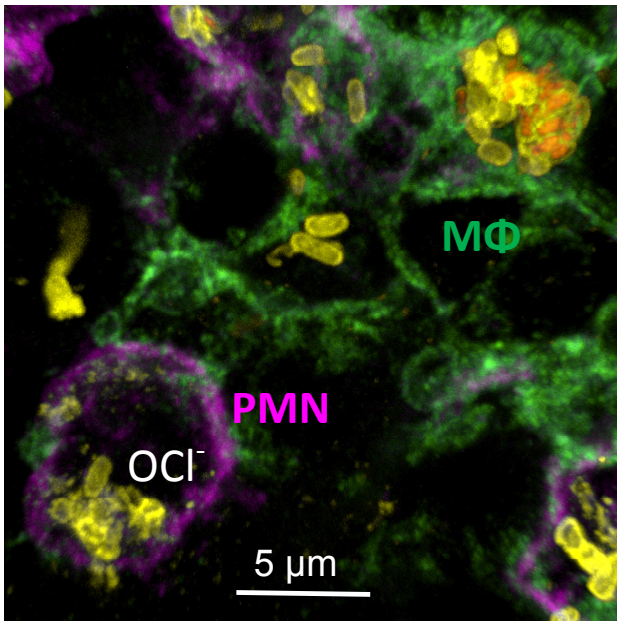
382 **Figure 2**: Divergent fates and properties of *Salmonella* subsets in spleen. **A**) Confocal micrograph of a
383 spleen cryosection. *Salmonella* (stained with an anti-lipopolysaccacharide antibody, yellow) reside in
384 diverse cell types including resident macrophages (MΦ, stained with F4/80, green) and
385 polymorphonuclear leukocytes (neutrophils; PMN, stained with Ly6G, magenta). Some *Salmonella* in
386 macrophages retain internal mCherry fluorescent protein (orange) indicating their viability, whereas
387 most *Salmonella* in neutrophils are killed by OCl^- (bleach) resulting in compromised *Salmonella*

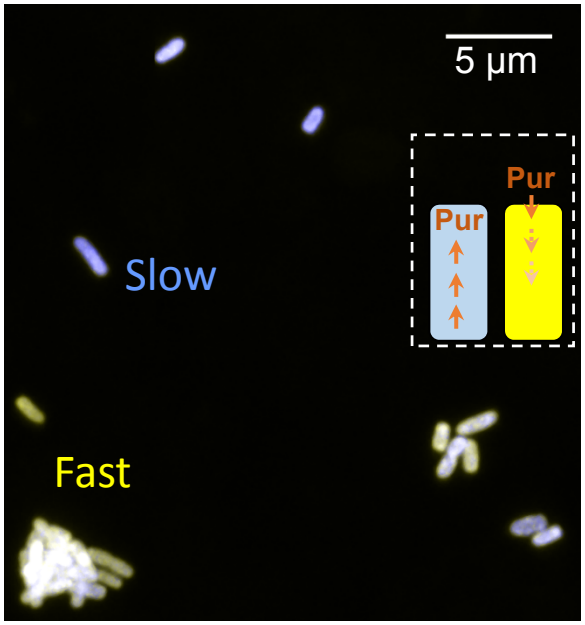
envelope and loss of mCherry. **B**) Divergent growth rates of *Salmonella* in mouse spleen as indicated by the TIMER fluorescent protein. TIMER shows different ratios of a rapidly maturing GFP-like fluorophore (yellow) and a slowly maturing DsRed fluorophore (blue) depending on protein dilution due to *Salmonella* cell division. Fluorescence is shown in false color for better visibility for people with limited red-green discrimination. The inset shows differential access to purine nutrients for slow (poor access, depends on endogenous biosynthesis) and fast (surplus purine availability, can use purine as nutrient) *Salmonella* subsets. **C**) Heterogeneous *Salmonella* exposure and responses to host nitric oxide (NO). The left panel shows a micrograph of an infected spleen section with focal inflammation. Neturophil-rich abscesses are encircled by inflammatory monocytes expressing high levels of inducible nitric oxide synthase (iNOS). *Salmonella* residing in regions with little iNOS expression experience little NO stress and contain baseline levels of defense proteins HmpA (NO dioxygenase) and YtfE (repair of NO-damaged iron-sulfur clusters) (1). *Salmonella* residing in regions with high NO upregulate specifically these defense proteins and the NO reductase Hcp, but no other proteins (2). The enhanced NO detoxification capabilities enable this *Salmonella* subset to diminish NO to non-toxic levels that have no impact on *Salmonella* fitness.


Small intestine
Colon
Caecum
mLN
1
2
1
SPI-1
OFF
SPI-1
ON
2
cDC
iDC

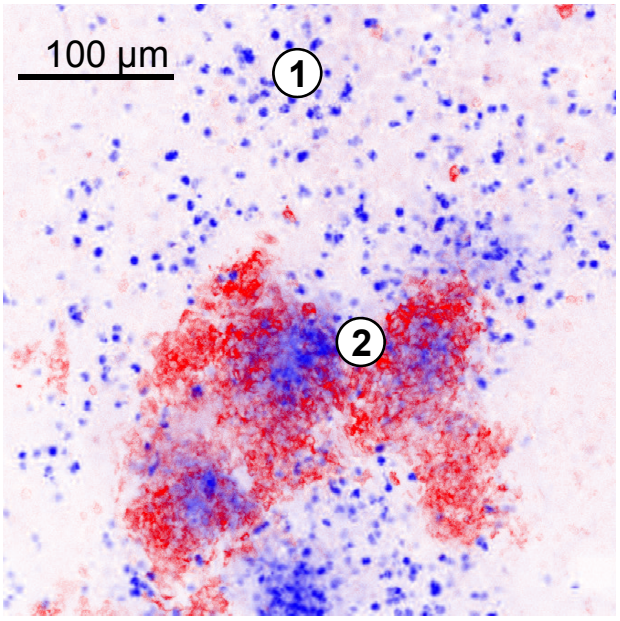
A



B



C



Neutrophils Inducible NO synthase (iNOS)