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Research Paper

Salmonella Typhi Colonization Provokes Extensive Transcriptional Changes Aimed at Evading Host Mucosal Immune Defense During Early Infection of Human Intestinal Tissue

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ABSTRACT

Commensal microorganisms influence a variety of host functions in the gut, including immune response, glucose homeostasis, metabolic pathways and oxidative stress, among others. This study describes how *Salmonella* Typhi, the pathogen responsible for typhoid fever, uses similar strategies to escape immune defense responses and survive within its human host. To elucidate the early mechanisms of typhoid fever, we performed studies using healthy human intestinal tissue samples and "mini-guts," organoids grown from intestinal tissue taken from biopsy specimens. We analyzed gene expression changes in human intestinal specimens and bacterial cells both separately and after colonization. Our results showed mechanistic strategies that *S.* Typhi uses to rearrange the cellular machinery of the host cytoskeleton to successfully invade the intestinal epithelium, promote polarized cytokine release and evade immune system activation by downregulating genes involved in antigen sampling and presentation during infection. This work adds novel information regarding *S.* Typhi infection pathogenesis in humans, by replicating work shown in traditional cell models, and providing new data that can be applied to future vaccine development strategies.

1. Introduction

Bacterial pathogens represent a significant global burden to human health resulting in chronic infection, significant mortality, certain cancers, and diminished quality of life [23,38]. *Salmonella enterica* serovar Typhi (STY) is a human-restricted, gastrointestinal pathogen whose successful infection results in Typhoid fever or chronic infection [30]. Typhoid fever is frequently fatal when untreated in pediatric or immunocompromised populations [9,31,51,79]. It affects an estimated 11.9 to 26.9 million people annually [6,49,50], with estimated financial burdens equaling roughly a third of the gross national income for patients in undeveloped areas of Southeast Asia [35]. Current treatments depend on antibiotics; however, STY is rapidly developing antibiotic resistance, thereby increasing both the risk and severity of infection.

STY is a Gram-negative, enteric pathogen of the genus *Salmonellae*, species enterica, subspecies enterica. The subspecies enterica represents

Abbreviations: STY, Salmonella Typhi; STM, *Salmonella* Typhimurium; M cells, Microfold cells; CFU, colony forming units; LB, Luria Burtoni broth; DMEM, Dulbecco's Modified Eagle Medium; PBS, phosphate buffered saline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ISC, intestinal stem cell; DAPT, N-[2S-(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl-1,1-dimethylethyl ester-glycine; qPCR, quantitative reverse transcriptase polymerase chain reaction; FITC, fluorescein isothiocyanate; TEER, trans-epithelial electrical resistance; RNA, ribonucleic acid; LDH, lactate dehydrogenase; H&E, hematoxylin and eosin; PAS, periodic acid Schiff; TEM, transmission electron microscopy; IL, interleukin.

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two serovars: Typhimurium and Typhi. Genetically related, but phenotypically divergent, *S.* Typhimurium (STM) can cause localized inflammation of the small intestine, diarrhea and cramping. Conversely, STY infection has an incubation period of up to two weeks and results in systemic bacteremia with limited or no gastrointestinal symptoms. Comparative genomics between the two serovars reveals 480 STM-unique genes and 600-STY-unique genes [66]; notable STY genetic acquisitions include a capsule-encoding specific pathogenicity island (SPI)[78], two human-like serine-threonine kinases [73] and a typhoid toxin [18,21]. Numerous STY genes contain mutations relative to the STM homologs, with 0.6% of the STM genome encoding pseudogenes compared to 5% in the STY genome [66].

Current STY vaccination strategies include products that are expensive to produce and store. The attenuated oral vaccine Ty21a, which requires several immunizations to create a sustained immune response, confers protection in 62–96% of vaccinated individuals [41]. Alternative vaccine candidates currently in phase I and phase II studies have been generated by targeting virulence genes related to acid resistance, stress response, osmolarity and invasion. These gene targets are combined with gene deletions resulting in limited intracellular replication [24]. In most STY vaccine candidates, deleted genes attenuate infection. A notable exception is the constitutive expression of the STY-specific [82] immune-capsule suppressing regulator [78] *tviA* gene [24]. Despite the development of several vaccine strains against STY, no strategy confers long-term protection in a cost-effective manner.

Vaccine development against typhoid fever is hindered by significant assumptions about how *S.* Typhi causes infection in its human host. Specifically, no data are available regarding STY interaction with the small intestinal mucosa as the first step in the cascade of events ultimately leading to infection. Moreover, there have been no studies evaluating bacterial gene expression and consequences on host gene expression that occur during these critical early moments of infection. Despite fundamental differences in the pathology of infected humans relative to infected mice, and significant genomic differences between the two serovars, our current understanding of STY infection is mainly derived from the STM mouse model. Moreover, the two-week incubation period between STY exposure and onset of disease symptoms demonstrates a critical window in which STY infection is active prior to the onset of clinical disease. In recent years, human-derived organoid systems [19,64,67,80,84] or three-dimensional cell line models [29,60,68,69] have gained traction as a strategy to study host-pathogen interactions. These models have enabled new insight into cellular response during early STM infection to identify the role of bacterial genes n, such as the SPI-1 operon [60], or STM manipulation of signaling cascades [84]; however, few studies combining STY and human-derived organoid monolayers have been published.

Our work overcomes these shortfalls by placing STY bacteria directly onto human small intestinal tissue. In this study, we use an ex vivo, human intestinal tissue infection model and a human organoid-derived monolayer model. Infected biopsies were analyzed for transcriptional changes, cytokine profiling and electron microscopy, with specific mechanisms explored using the organoid monolayer model. This work sought to detail critical early events in Typhoid fever development to understand pathogenic mechanisms in human-derived tissue with the goal of identifying novel targets for vaccine development.

2. Materials and Methods

2.1. Bacterial Strains, Growth Conditions and Biopsy Infection

For all experiments, *Salmonella enterica* serovar Typhi strain Ty2 (STY, ATCC® Number: 700931) or serovar Typhimurium strain SL1344 (STM) (kind gift of Bobby Cherayil, Massachusetts General Hospital, Boston MA) were re-streaked bi-monthly on LB-agar plates. For experiments conducted under traditional laboratory conditions, an overnight culture from a single colony of STY or STM was prepared in Miller formula LB-broth (Sigma, St. Louis, MO) at 37 °C with shaking at 225 rpm. The next day, overnight cultures were diluted 1:50 into secondary subcultures and grown to log phase. To prepare the bacteria grown under pro-invasion conditions, a day culture in Miller formula LB broth from a single colony of STY or STM was started at 37 °C without shaking. After 4-8 h growth, a secondary culture diluted 1:50 in Miller LB was prepared and grown overnight at 37 °C without shaking. Bacteria were normalized to an OD600 of 0.5 prior to experimentation, pelleted by centrifugation and resuspended in warm Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) for use in infection. All biopsies were infected with 1×10^8 bacteria in 250 μL DMEM for a maximum of 2 h. Biopsies were mounted using a snapwell system (Corning, Corning NY) with orientation confirmed by a dissecting microscope. Mounted biopsies acclimated for 30 m prior to removal of media and replacement with control DMEM or DMEM containing STY or SL1344. After 2 h of infection, apical and basolateral medium and biopsies were collected. For gentamicin protection assays, after 2 h infection the apical surface was washed 3× in phosphate buffered saline (PBS), and DMEM containing 5μg/mL gentamicin (Gibco) was added for 30 m at 37 °C. Afterward, biopsies were washed in PBS (Gibco) and subsequently homogenized in 0.5% Triton-X (Sigma) in PBS for serial dilution plating to determine CFU/mL recovery.

2.2. Isolation and Generation of Human Organoids; Preparation of Organoid-derived Epithelial Monolayers

The isolation and generation of human organoids was adapted from the protocol published by VanDussen et al. [76]. Briefly, human terminal ileum biopsies were processed in dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA) in PBS with penicillin/streptomycin (P/S, Gibco) to isolate the crypt fractions. This process was repeated 4–5 times; the fractions with the greatest number of crypts were pooled and resuspended in Matrigel (Corning, NY). Spheres were maintained in culture in 1:1 LWRN-conditioned medium and Intestinal Stem Cell (ISC) medium supplemented with Y-27632 (Sigma or Calbiochem, La Jolla, CA) and A-8301 (Sigma or Tocris, Minneapolis, MN). Spheres were fed every 2–3 days and split prior to differentiation. Undifferentiated cells were seeded onto transwell inserts and maintained in culture until monolayers formed. 48 h prior to experiment, monolayers were treated with apical N-[2S-(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl-1,1-dimethylethyl ester-glycine (DAPT) (Calbiochem) to induce differentiation. Monolayers were assessed by quantitative reverse transcriptase polymerase chain reaction (qPCR) and confocal microscopy to identify markers for differentiated epithelial cells, as well as, Trans epithelial electrical resistance (TEER) and fluorescein isothiocyanate (FITC) dextran to determine barrier integrity.

2.3. Human Donors

All protocols for recruitment of human subjects and use of human terminal ileum biopsies were approved by Massachusetts General Hospital / Partners Healthcare IRB (Protocol 2014P002001). Prospective donors without chronic medical conditions who were scheduled to undergo a diagnostic colonoscopy were screened for good general health. Exclusion criteria included pregnancy, a known diagnosis of an autoimmune disorder or any chronic medical condition that would increase the risk from a gastrointestinal biopsy, and an inability or unwillingness to provide written informed consent. Donors signing informed consent contributed four to eight biopsies that were transported to the laboratory and used immediately to maximize tissue viability.

2.4. RNA Isolation and Transcriptomic Analysis

Following treatment (mock or infection) biopsies were immediately snap frozen on dry ice and stored at −80 until use. The control biopsy

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