Understanding Human Alpha-Defensin 6-Mediated Inhibition of Flagellar Motility

By

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Abstract

Within the mammalian intestine, various microbes utilize flagellar motility to traverse the mucus layer and reach the epithelium, challenging the spatial segregation established by the host. For many of these microbes, contacting the epithelium to adhere or invade can provide them an advantage over other members of the highly competitive resident microbiota. These actions threaten homeostasis and can result in pathological consequences, which presents a direct risk to the health of the host. In response, the host has a collection of innate and adaptive immune countermeasures that target and inhibit flagellar motility, thereby preserving the spatial segregation of microbes from epithelium. These methods include pattern recognition receptors capable of responding to bacterial flagella, secreted innate effector molecules that inhibit microbial swimming through a variety of mechanisms, and flagella-specific mucosal antibody that can regulate the movement and behavior of flagellated microbes. Together, these host factors possess a redundancy in function that provides the intestinal epithelium with an overlapping defense against motile microbes.

This work elucidates that human α-defensin 6 (HD6), an antimicrobial peptide secreted by Paneth cells in the small intestine, is capable of inhibiting bacterial flagellar motility. HD6 was previously described to protect the host from infection with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) by limiting its invasion and dissemination into peripheral tissues through non-lethal means. It was found that HD6 could bind to bacterial flagellin and self-assemble into large polymers, termed nanonets, which resulted in bacterial agglutination and inhibition of cellular invasion. However, it remained unknown if HD6 could also have an effect on individual bacteria through agglutination-independent mechanisms. Due to the ability of

HD6 to bind flagellin, the major component of the flagellar filament, we hypothesized that HD6 could inhibit the motility of individual flagellated bacteria through a process of binding and self-assembly. Initially, we employed a semi-solid agar bacterial motility assay to demonstrate that the motility of *S.* Typhimurium was hindered after treatment with HD6. The degree of this inhibition was comparable to treatment with α-flagellin IgG, a known inhibitor of flagellar motility. Next, we explored how HD6 affected the motility of individual bacteria by developing a live-cell fluorescence microscopy assay, capable of resolving the movement of single motile bacteria. Through this method, we showed that the population of actively motile *S.* Typhimurium was significantly reduced after treatment with HD6 at bacterial densities where agglutination does not occur and α-flagellin IgG can no longer inhibit motility. We also found that the loss of bacterial motility caused by HD6 coincided with the appearance of immobilized bacteria, which were distinct from actively motile and diffusing bacteria due to an absence of perceptible movement. Using an advanced segmentation and masking strategy, we found the degree of bacterial immobilization was dependent on HD6 concentration. Finally, using a single amino acid variant of HD6, $HD6^{F2A}$, which retains the ability to bind flagellin but cannot self-assemble, we showed that the bacterial immobilization was dependent on HD6's ability to self-assemble. Together, these results suggest a specialized role of HD6 in targeting and inhibiting bacterial motility.

To interrogate the effect of flagellar arrangement and post-translational glycosylation HD6-mediated motility inhibition, future studies can utilize other motile bacteria, such as Pseudomonas aeruginosa. Additionally, outlined within are different mouse models that aim to explore role of HD6 in modulating the commensal microbiota and the adaptive immune system.

In summary, this work expands our understanding of the effects of HD6 on flagellated bacteria, and more broadly provides evidence supporting a specific role of the host immune system in targeting bacterial motility.

Chapter 1

Flagella at the Host-Microbe Interface: Key Functions Intersect With Redundant Responses

Douglas T. Akahoshi and Charles L. Bevins

Abstract

Many bacteria and other microbes achieve locomotion via flagella, which are organelles that function as a swimming motor. Depending on the environment, flagellar motility can serve a variety of beneficial functions and confer a fitness advantage. For example, within a mammalian host, flagellar motility can provide bacteria the ability to resist clearance by flow, facilitate access to host epithelial cells, and enable travel to nutrient niches. From the host's perspective, the mobility that flagella impart to bacteria can be associated with harmful activities that can disrupt homeostasis, such as invasion of epithelial cells, translocation across epithelial barriers, and biofilm formation, which ultimately can decrease a host's reproductive fitness from a perspective of natural selection. Thus, over an evolutionary timescale, the host developed a repertoire of innate and adaptive immune countermeasures that target and mitigate this microbial threat. These countermeasures are wide-ranging and include structural components of the mucosa that maintain spatial segregation of bacteria from the epithelium, mechanisms of molecular recognition and inducible responses to flagellin, and secreted effector molecules of the innate and adaptive immune systems that directly inhibit flagellar motility. While much of our understanding of the dynamics of host-microbe interaction

regarding flagella is derived from studies of enteric bacterial pathogens where flagella are a recognized virulence factor, newer studies have delved into host interaction with flagellated members of the commensal microbiota during homeostasis. Even though many aspects of flagellar motility may seem innocuous, the host's redundant efforts to stop bacteria in their tracks highlights the importance of this host-microbe interaction.

1. Introduction

Microbes inhabit nearly every environment on Earth—ubiquity made possible by their ability to evolve and adapt to widely diverse conditions. One striking adaptation is the ability to move within a given environment, whether that be a pond, the surface of a damp leaf, or inside the human gut [1,2]. Locomotion provides the microbe advantages including the ability to seek nutrient-rich niches and acquire symbionts, as well as the ability to avoid noxious environments [1,3-7]. Many microbes achieve locomotion via flagella, which are "tail-like" organelles that function as a swimming motor [8,9]. The structure and biomechanics of microbial flagella have been extensively studied and reviewed [8,10-18].

Among microbial flagella, the bacterial flagella have been the most extensively studied. Despite taxonomic diversity within the domain Bacteria, the structure and sequence of bacterial flagella are highly conserved [10]. The prototypical flagellum of bacteria is comprised of three protein-based components: basal body, the hook, and filament [8,19]. The basal body is imbedded in the membrane, serving as an anchor for the hook and filament. The hook is

attached to the basal body and together they generate the torque necessary to rotate the filament [8,20]. The main body of a flagellum is the filament, a multimeric polymer composed of between 100-20,000 protein monomers, termed flagellin [21]. Working together, the flagellum apparatus provides bacteria the ability to move in liquid and semi-solid environments in a directional manner, reaching recorded velocities of 30μm/second [8]. Commonly, this flagella-driven movement of bacteria is directed toward beneficial chemical gradients or away from toxic chemical gradients in a process termed chemotaxis [22,23]. Although the synthesis and use of flagella incurs a resource cost for bacteria, this energy debt is outweighed by the ability to gain access to throughout the environment and outcompete non-motile microbes, especially when resources are scarce [23-26].

The gastrointestinal (GI) tract of vertebrates is home to a phenomenally diverse microbial community. In this environment, some microbes incorporate flagella into their lifestyles in order to gain an advantage over their competitors. Indeed, it is well established that flagellar motility is an essential virulence factor for numerous enteric pathogens [27-31]. However, flagellated microbes in the GI tract face a challenge absent in many other environments—a host that is actively sensing and restricting flagellar motility. From the host's perspective, the mobility that flagella impart to microbes carries potential risk. For example, flagella-driven motility can facilitate cellular invasion and translocation across the epithelium. Thus, in response to flagellar activity, the host has developed a repertoire of immune countermeasures to mitigate this threat. This review will provide a perspective on various ways that animal-associated microbes utilize flagellar motility, and highlight the redundant strategies co-evolved by the host to recognize, adapt, and respond to this molecular process. Based on

the prominent use of bacterial models in current literature on the topic, the focus will be on the bacterial flagella.

2. Flagellar motility as a colonization factor

For microbes associated with the GI tract, much of our understanding of flagella is derived from the study of pathogens, since flagellar motility is often key in their successful colonization of the host. Although many different pathogens utilize flagellar motility within the host, it often provides varying degrees of fitness advantage and is not always necessary for host colonization. For example, the use of flagellar motility is integral to the pathogenesis strategy of *Helicobacter pylori* and *Campylobacter jejuni*, as evidenced by its absence hindering their ability to effectively colonize the host [1,27,32-35]. By comparison, *Salmonella enterica* serovar Typhimurium (STM), *Escherichia coli,* and *Pseudomonas aeruginosa* selectively express their flagella at specific times and sites within the host to gain advantage over other members of the microbiota; however, these bacteria ultimately do not require flagellar motility for pathogenesis, and when expressed at the "wrong" time can actually impair colonization of the host [1,27,36-42]. For *Listeria monocytogenes*, flagellar motility appears to play an important role outside of the host where it might increase infectious potential [43]. However, upon transitioning from the cooler external environment to the warmer environment inside the host, Listeria actively downregulates flagella expression and utilizes alternative motility mechanisms [44]. In addition to their fundamental role in motility, flagella can have additional functions for some

bacteria, including secretion, adhesion, and biofilm formation [23,29,45-50]. For example, a recent investigation demonstrated that STM can methylate lysine residues on its flagellin to increase the hydrophobicity of the flagella and thereby facilitate adhesion to the hydrophobic surface of host cells [48].

Altogether, these examples illustrate how flagella expression within the host can have either positive or negative impacts on bacterial fitness. To elaborate on how flagellar motility can be important to microbes within the host, three partially overlapping functions will be discussed: the power to resist flow, the ability to reach the epithelium, and the capacity to travel to nutrient niches within the lumen (Figure 1).

2.1 Flagellar motility, a mechanism to resist flow

Given that microbes often optimize their metabolism to specific nutrient niches, it is important for these microbes to localize to the favorable regions of the host gastrointestinal tract [51]. However, the proximal-distal peristaltic flow of intestinal contents, as well as the rapid turnover and shedding of mucus and epithelial cells, impose a perpetual existential threat to microbes attempting to maintain colonization [52-54]. To overcome these challenges, some commensal and non-commensal bacteria use flagellar motility to stably colonize specific regions of the GI tract [52,55].

A recent study using intravital microscopy of *Danio rerio* (zebrafish) revealed a role of flagellar motility in resisting the peristaltic flow of luminal contents in the GI tract [54]. Zebrafish are an attractive model for viewing microbial motility *in situ* because during their embryonic

stage the fish are transparent, allowing for live imaging of their GI tract [56-58]. Using this approach, a motile strain of *Vibrio cholerae* was shown to stably colonize the zebrafish GI tract and localize to the most proximal regions of the foregut [54]. However, when flagellar function was impeded, the *V. cholerae* was more susceptible to expulsion, thereby diminishing colonization – those remaining were observed in more distal regions of the GI tract [54]. If chemotaxis rather than flagellar motility were inhibited, a similar scenario emerged but with nuanced variation. When chemotaxis was inhibited, *V. cholera* colonized efficiently but localized more distally than their WT counterparts, with a smaller fraction remaining in the proximal region of the foregut [54]. Generally, inhibition of chemotaxis does not impair motility per se, but rather leaves bacteria unable to change directions in response to chemotactic signals within the environment. Without the ability to bias movement toward specific locations, bacteria tend to more uniformly disperse. In the zebrafish gut, this resulted in the dispersal throughout the lumen of chemotaxis-deficient *V. cholera*, while WT *V. cholera* concentrate closer to the epithelium. The localization closer to the epithelium likely provided protection from the flow of intestinal contents, and thus afforded WT *V. cholera* the ability to maintain stable colonization of the proximal foregut [53]. Together, this study elegantly highlights how flagellar motility provides *V. cholerae* the means to resist the peristaltic flow in the intestinal tract (Figure 1A).

While these studies were done in zebrafish, it is likely bacteria utilize flagellar motility to similar effect within the mammalian gut. Indeed, studies on IgA agglutination of bacteria in the mouse intestine showed that agglutinated bacteria were expelled from the host [59]. The proposed mechanism was that agglutination restricted motility of these bacteria, rendering

them unable to resist the proximal-distal flow of intestinal contents [59]. Thus, flagellar motility appears to be a viable strategy for bacteria to maintain their regional localization within the gastrointestinal tract.

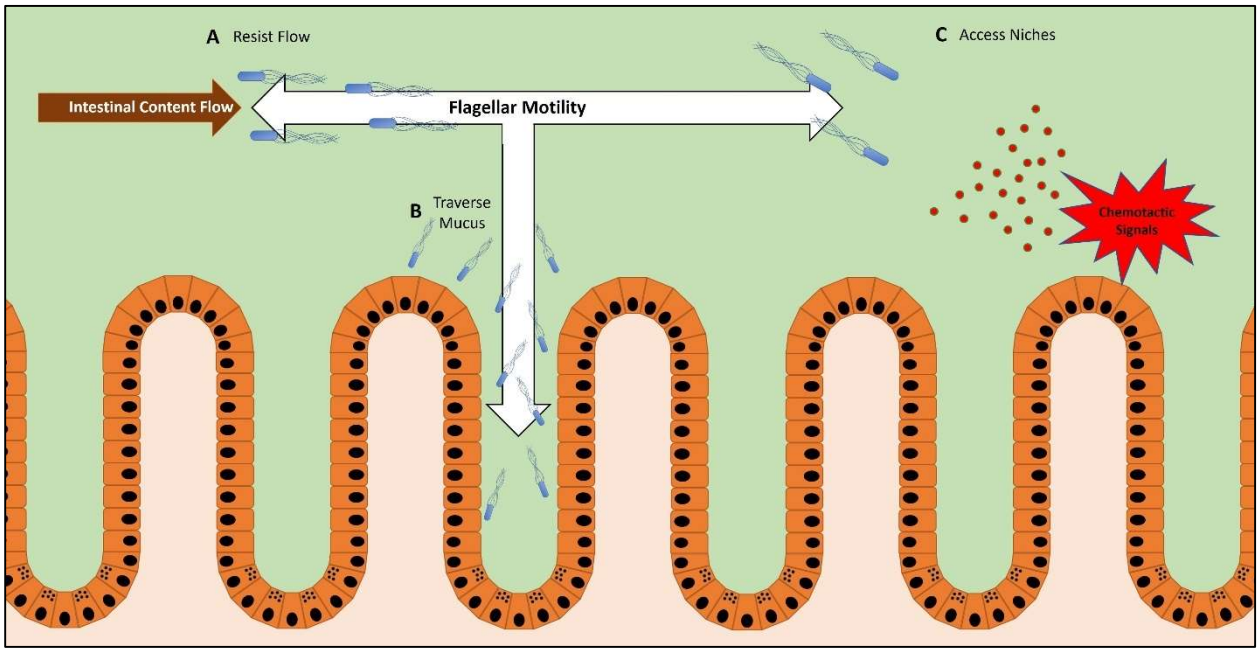

Figure 1| Bacteria utilize flagellar motility for multiple functions within the host. (A) Bacteria use flagellar motility to resist the flow of intestinal contents, maintaining their location within the host GI tract. In the case of *Vibrio cholera*, this allows them to maintain stable colonization of the proximal end of the *Danio rerio* (zebrafish) gut [53,54]. **(B)** Bacteria use flagellar motility to swim through mucus to reach the epithelium. This is a likely method for microbes that need to reach the epithelium in order to facilitate activities such as adhesion, invasion, and translocation [37,60]. **(C)** Bacteria use flagellar motility to swim toward chemotactic signals [36,37,61,62]. These signals can be products of inflammation or epithelial damage, representing vulnerable areas of the mucosal barrier.

2.2 Flagellar motility, a mechanism to acquire proximity

During homeostasis, the GI epithelium establishes a mucus barrier that effectively segregates the luminal microbes away from the epithelial layer [63-65]. In simplest terms, mucus is a macromolecular complex that forms a semi-permeable barrier adjacent to the epithelium [63]. For many enteric pathogens, an aspect of their lifestyle necessitates contact with the epithelium to facilitate activities such as adhesion, invasion, and/or translocation. Thus, these microbes require a mechanism to efficiently overcome the mucus barrier.

Some enteric pathogens employ flagellar motility to traverse the mucus barrier (Figure 1B). *In vivo* evidence using STM models at early timepoints of infection demonstrate that flagellated bacteria are more closely associated with the epithelium than their aflagellate counterparts [37]. While these data suggest the importance of flagella to access the epithelium, a caveat worth noting is that the phenotype might not be solely attributable to the loss of flagellar motility per se, since flagella can perform other functions. More direct data comes from recent work utilizing live-fluorescence microscopy of intestinal explants, which allowed the real-time viewing of individual swimming bacteria interfacing with the mucus barrier [60]. While a majority of STM were observed segregated to the outer mucus layer, a significant fraction of flagellated STM successfully swam through the inner mucus layer and associated more closely to the epithelium [60]. By contrast, aflagellated STM were almost completely confined to the surface of the outer mucus layer [60]. These observations provide visual confirmation that STM can use flagella to swim through mucus towards the epithelium, an important property for this multi-functional organelle.

The real-time visualization of live bacteria in *ex vivo* systems also enables analysis of more subtle swimming behaviors. For example, STM swimming close to the epithelium engage in a phenomenon termed "near-surface swimming" - when STM reach an impassable surface, such as the surface of the epithelium or dense mucus layer, they initiate a circular swimming pattern [60,66]. Although a detailed understanding of this behavior is not clear, an attractive hypothesis is that near-surface swimming is a strategy to find vulnerabilities along a dense mucus layer, or identify potential attachment and invasion sites on an epithelial monolayer [66].

Surprisingly, STM lacking flagella are able to effectively colonize the host and disseminate to peripheral tissues, despite their lack of motility. This raises the question of how these bacteria reach the epithelium, despite an apparent inability to passively diffuse through mucus. A likely explanation stems from experimental observations of bacteria, irrespective of flagellar motility, coming in direct contact with epithelial cells at vulnerable sites, such as the surface-exposed Peyer's patches [60]. In addition, surface migration that does not involve flagella has been described [67]. Such contact and migration could reconcile how aflagellated STM are able to infect the host, albeit less efficiently than their motile counterparts [60]. The findings with aflagellated STM provide precedence for possible mechanisms for how other bacteria without a functional flagellum, such as *Citrobacter rodentium*, are capable of attaching and effacing to epithelial cells in the GI tract [68].

2.3 Flagellar motility, a mechanism to access nutrient niches

The distinct environments of various regions within the GI tract create niches that accommodate microbes with an array of lifestyles [51]. Moreover, within each individual niche there are microniches, owing to factors such as the topography and inflammatory state of the epithelium [51,69,70]. Thus, microbes possessing the necessary metabolic machinery to exploit these microniches can gain a significant advantage over their competitors — as long as they have the ability to detect and physically access these sites [25,26]. For some bacteria, this ability comes in the form of chemotaxis-directed flagellar motility (Figure 1C).

In the stomach, *H. pylori* requires chemotaxis to occupy microniches within the gastric gland, which confers a competitive edge over other strains [61,71]. In response to gastric ulcers, some *H. pylori* strains sense chemotactic signals associated with epithelial damage and travel to those sites via flagellum-powered migration [62,72]. *H. pylori* presence at the ulcer sites delay healing, thereby maintaining the microniche and benefiting from the associated nutrients [62].

Similarly, STM employs chemotaxis in the distal intestinal tract to gain a competitive advantage in the inflamed colon [36,37]. Specific methyl-accepting chemotaxis proteins are expressed by STM, allowing them sense and swim towards nitrogen-rich nutrient niches [36] (Figure 1C). These niches are often generated as byproducts of the inflammatory response of the host, likely derived from the reactive oxygen and reactive nitrogen species secreted by the epithelium [36]. According to proposed models, the inflammatory response induced by STM leads to the creation of these nutrient niches, which attracts STM migration via flagellum-driven chemotaxis [36]. Thus, through their use of flagellum-driven chemotaxis, both *H. pylori* and STM

illustrate how the flagellum can be used as a tool to reach specific nutrient microniches within the host and secure a competitive advantage over other microbes. Interestingly, a recent study demonstrated that "minicells", a term coined for chromosome-free nanosized bacterial vesicles, could be engineered to express flagella and bias their movement towards chemical gradients, which resulted in their accumulation at the chemoattractant source [73].

3. A host's perspective to flagella-driven motility

In isolation, microbes swimming in the lumen via flagella-driven motility would not appear to cause harm or pose a threat to the host. However, as noted, some microbes use this flagellar motility as a step towards epithelial adherence and translocation, a process detrimental to host fitness. To mitigate this threat, the host expends considerable resources to maintain an arsenal of often redundant countermeasures to impair flagella-driven motility [74,75]. For example, adaptive immunity, and in particular mucosal production of flagellin-specific IgA, is also of vital importance in keeping motile bacteria in check [76]. The next two sections will delve into the mechanisms that the host uses to hinder flagella-driven motility, including both innate and adaptive immune responses.

3.1 Innate Immune Countermeasures

Innate immunity is marked by providing the host with an ever-ready or immediately inducible collection of molecules that can effectively mitigate microbial threats and restore homeostasis.

With respect to innate immune molecules and responses that may counter the possible deleterious consequences of flagella-driven motility, five mechanisms have been identified and highlighted here.

3.1.1 Mucus barrier

Mucus plays an essential role in maintaining a stable homeostasis between the host and microbes, in part, acting as a physical barrier to fortify the epithelium [63-65,77]. The effective barrier properties of mucus are especially evident in the colon, where the extraordinary density of resident microbes are partitioned away from the epithelial surface. Based on FITC-dextran permeability assays, the dense inner layer of colonic mucus immediately adjacent to the epithelium is selectively permissive to particles smaller than 0.5 μm in size. While the shapes and dimensions of microbes vary, many bacteria fall in the range of 0.5-1.0 μm in length; thus, they would not passively diffuse through the mucus barrier due to Brownian motion. Accordingly, experiments utilizing fluorescence *in situ* hybridization (FISH) to label bacteria show that the vast majority of the microbes reside either in the lumen or penetrate only to the more porous outer mucus layer, suggesting an inability to pass into the inner mucus layer adjacent to the epithelium. Yet, some microbes can be observed swimming through the inner mucus and able to reach the epithelium, most likely utilizing flagellar motility [60].

While flagellar motility allows microbes to penetrate the mucus layer, a percentage of these bacteria become “entrapped” in the mucus and are immobilized [60]. Somewhat counterintuitively, entrapment within the mucus layer appears to be not dependent on the

expression of flagella per se, but rather on the locomotion imparted by flagellar motility [60]. As such, bacteria possessing a functionally disabled flagellum do not become entrapped within the mucus layer. The exact mechanism that enables mucus to target motility of actively swimming microbes for entrapment remains unclear. In addition to this intrinsic ability to hinder motility, mucus also harbors an array of host derived immune factors that reinforce barrier function [78]. These will be addressed in some detail.

Table 1. Innate Immune Effector Molecules

Effector Molecule	**Proposed Mechanism**	**References**
Mucus	Barrier Scaffold for Other Effectors Bulk Flow from Crypts	60,63-65,77-79
HD6	Agglutination Inhibit Flagella Activity (?)	80-82
LYPD8	Inhibit Flagella Activity	83-85
ZG16	Agglutination Redox Switch	86,87

3.1.2 Flagellar-binding proteins and peptides

Various innate immune proteins bolster the efficacy of the mucosal barrier against noxious microbes, and some are embedded in mucus [78]. These effector molecules include peptides and proteins secreted by the epithelium. While many of these molecules are microbicidal, a subset provide protection through alternative mechanisms such as inhibition of motility and spatial segregation. Included in this latter group are human α-defensin 6 (DEFA6, HD6), Ly6/Plaur domain containing 8 (LYPD8), and zymogen granular protein 16 (ZG16), all of which appear to affect the motility and localization of bacteria, although by different, non-bactericidal mechanisms (Figure 2A).

3.1.2.1 HD6

HD6 is produced by Paneth cells in the crypts of the small intestine [80,88,89]. Unlike most processed and folded α-defensins that are potently bactericidal, HD6 lacks antimicrobial activity [80]. Recent studies have reported antimicrobial activity of proteolytic fragments of HD6 [90], and activity against anaerobic commensal bacteria for HD6 when its disulfide bonds are reduced [91]. Instead of microbicidal activity, processed and folded HD6 binds proteins on bacterial surfaces and polymerizes to form macromolecular fibrils and "nets", which can agglutinate bacteria into immobilized aggregates [80-82]. During STM infection, the presence of HD6 in transgenic knock-in mice significantly limited invasion and dissemination of STM to peripheral tissues, without decreasing the bacterial density in the intestinal lumen [80]. Consequently, these mice were more able to survive the infection compared to their WT counterparts [80]. Interestingly, HD6

appears to selectively target non-glycosylated proteins; this is significant since bacterial surface proteins, which include flagellin, are not typically glycosylated [80], although exceptions exist [34,92-94]. Thus, HD6 can provide the host a mechanism to immobilize flagellated bacteria, such as STM. Orthologs of HD6 are reported in Rhesus macaques and some other non-human primates, but not in murids [80].

3.1.2.2 LYPD8

LYPD8 is abundant in the large intestine where it is produced by colonic epithelial cells [83]. Like HD6, LYPD8 lacks discernable bactericidal activity; nevertheless, it also provides protection from pathogen challenge [83,95]. When LYPD8 is absent from the colonic mucus, bacteria localize closer to the epithelium. Additionally, *in vitro* data show that LYPD8 inhibits bacterial motility, suggesting that LYPD8 maintains the spatial segregation of bacteria and epithelium by preventing motile bacteria from swimming through the mucus layer [83-85]. In support of this hypothesis, LYPD8 selectively targets flagellated bacteria; however, LYPD8 does not bind flagellin, implying it may instead bind to another component of the flagellum (Figure 3A) , such as the hook or basal body [83]. Unlike HD6, LYPD8 does not appear to agglutinate bacteria, and the molecular mechanism by which LYPD8 interferes with bacterial motility remains unclear.

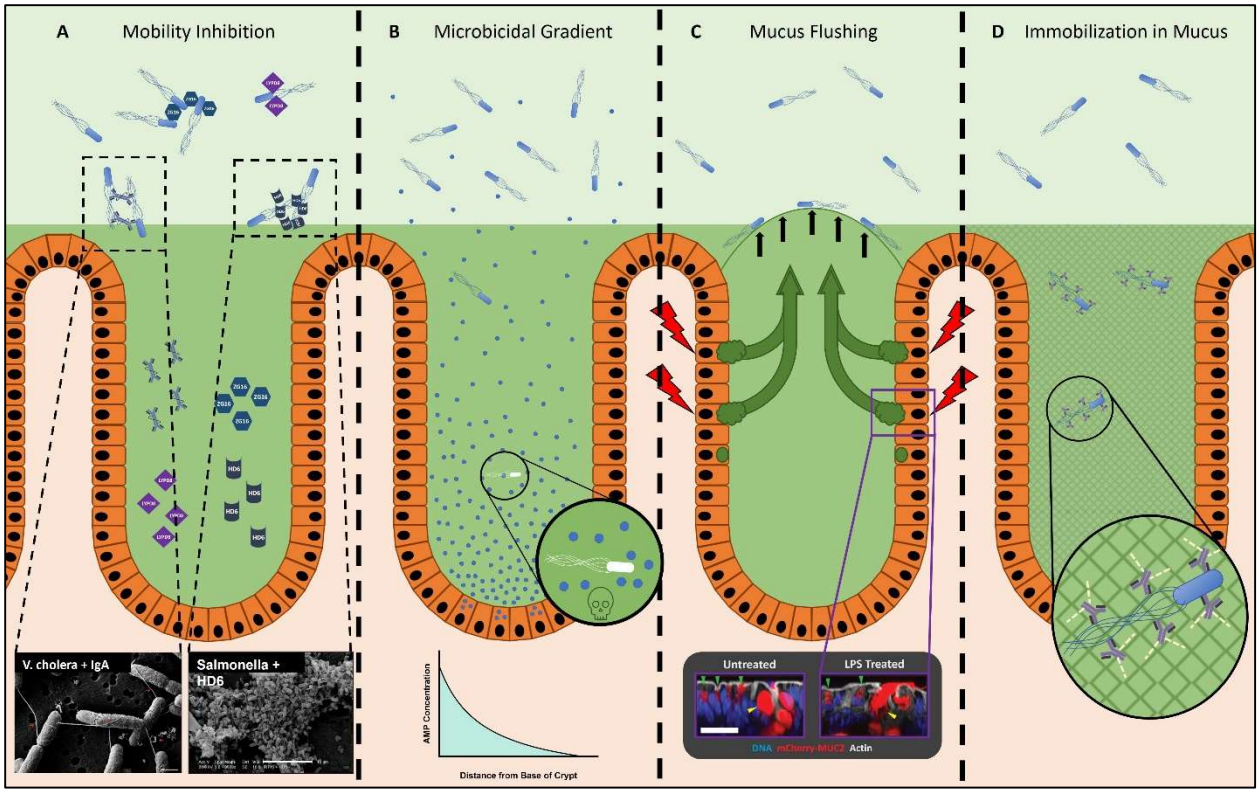

Figure 2| The host prevents microbes from accessing the epithelium. (A) Innate effector molecules and antibody are present in the GI tract lumen and inhibit bacterial motility through various means. ZG16 (blue hexagons) aggregates motile Gram-positive microbes in the outer mucus layer, away from the epithelium [86]. Lypd8 (purple squares) inhibits bacterial motility in the colon through a currently unknown, non-agglutinating mechanism [83]. Dotted boxes depict examples of SEM micrographs of sIgA (Left) and HD6 (Right) agglutinating bacteria, taken from Levinson et al. 2015 [59,96] and Chu et al. 2012 [80], respectively. HD6, human α-defensin 6; LYPD8, Ly6/plaur domain containing 8; ZG16, zymogen granular protein 16. **(B)** Microbicidal effector molecules in the small intestine form a concentration gradient, with the highest concentration located in the crypt [97]. It is likely that microbes able to localize closer to the crypts will be subjected to a more inhospitable or fatal environment, as depicted in the magnified image. AMP, Antimicrobial Peptide. **(C)** Sentinel goblet cells specialized goblet cells located at the entrance of colonic crypts [79]. After stimulation with a bacterial ligand such as flagellin or LPS (red lightning bolt), these sentinel goblet cells will secrete mucus into the crypt. This is likely a method to detect microbes that have traveled through the inner mucus layer (possible by flagellar motility) and initiate the secretion of extra mucus (green arrows), thereby effectively flushing the microbes away from the crypt (black arrows). Dotted box depicts a sentinel goblet cell secreting mucus (right) in response to stimulation by a bacterial ligand, compared to an unstimulated sentinel goblet cell (left), taken from Birchenough et al. 2016 [79]. **(D)** IgG-bound bacteria become immobilized in mucus due to the collective minor and non-covalent interactions (green dashed lines) between IgG and mucus (grey mesh) [98-100].

3.1.2.3 ZG16

ZG16 is also produced by the colonic epithelium and found in the colonic mucus [86]. Similar to the effects of removing LYPD8, the absence of ZG16 also leads to bacteria localizing closer to the epithelium [86]. However, in contrast to both LYPD8 and HD6, which appear to bind to protein ligands, ZG16 *i*) contains a CXXC motif on its flexible carboxy terminus that may serve a redox switch to alter its protein conformation [87], and *ii*) is a lectin that specifically binds to the peptidoglycan of Gram-positive bacteria [86]. After binding, ZG16 aggregates bacteria, thereby limiting their motility [86]. Through this mechanism, ZG16 likely fortifies the barrier to motile Gram-positive bacteria, which ultimately restricts their access to the colonic crypts and epithelium [86]. Consequently, mice lacking ZG16 possess a higher burden of Gram-positive 16sRNA in circulation and peripheral tissues, implying that the closer proximity to the epithelium of Gram-positive bacteria leads to increased rates of translocation [86].

3.1.3 Effector gradients

In addition to the repertoire of effector proteins that function via mechanisms of motility inhibition, bactericidal peptides and proteins produced in the GI tract also contribute to the maintenance of spatial segregation. At the base of small intestinal crypts, secretory Paneth cells constitutively secrete antimicrobial peptides and proteins (AMPs) into the lumen, creating a concentration gradient with highest concentrations at the base adjacent to the intestinal stem cells [97] (Figure 2B). Consequently, luminal bacteria entering the crypt would initially be exposed to mucus harboring a lower concentration of AMPs, and as such, subjected to less bactericidal

activity; however, bacteria able to swim into the crypt would be exposed to elevated concentrations of AMPs, a more inhospitable (and potentially lethal) environment [97,101]. In support of this model, Paneth cell ablation does not result in microbial overgrowth in the lumen as one might initially expect, but instead results in increased rates of bacterial translocation likely due to a more permissive environment near the epithelium and crypt [102]. Therefore, the minefield of AMPs produced by Paneth cells may provide an "incentive" to stay out of the crypt, and instead remain distant from the epithelial surface.

The importance of this Paneth cell function is highlighted in the context of chronic inflammatory bowel disease (IBD), where the morphology and function of Paneth cells are impaired [103-107]. In Crohn's disease of the small intestine, a major type of IBD, the expression of Paneth cell α-defensins is reduced, potentially related to genetic impairments in bacterial recognition and the autophagy response [103,104]. Since Paneth cell ablation is linked to increased rates of translocation, a similar scenario might occur in Crohn's disease wherein the reduction of Paneth cell products permits microbes to swim to the small intestinal epithelium and breach the barrier to initiate inflammation [102]. Thus, the higher levels of flagellin-specific antibody found in Crohn's disease patients could be due to a luminal environment more permissive to flagellar motility and access of flagellated bacteria to the mucosa [108]. Consistent with this model, the major cell type in the small intestinal epithelium expressing TLR5 is the Paneth cell [109]. During homeostasis, this pairing suggests the potential for a nuanced relationship between Paneth cells and motile bacteria. However, with the inflammatory conditions characteristic of Crohn's disease, Paneth cells are likely under stress that results in impaired function, which could be further exacerbated by excessive TLR5 signaling due to elevated number of flagella.

Since Paneth cells play a role in maintaining host-microbe homeostasis, their impaired function may perpetuate the chronic inflammation that impedes a return of homeostasis.

3.1.4 Sentinel Goblet Cells

Recently, a specialized subset of goblet cells, termed sentinel goblet cells, were discovered in the colonic epithelium positioned adjacent to the entrance to the crypt [79]. What defines this subset of goblet cells is their ability to secrete appreciable amounts of mucus in response to pattern recognition receptor (PRR) stimulation. Notably, TLR5 is among the PRRs expressed by sentinel goblet cells, and as such, stimulation with flagellin is sufficient to induce mucus secretion. Birchenough et al. propose a model wherein sentinel goblet cells hold watch at the crypt opening; if microbes bypass the inner mucus layer and come in close proximity to the epithelium, then these cells will secrete a burst of mucus to effectively "flush" the crypt (Figure 2C). Since flagellar motility provides microbes the means to reach the colonic epithelium, and a ligand to stimulate TLR5, the function of sentinel goblet cells may be to protect vulnerable crypts from flagellated microbes.

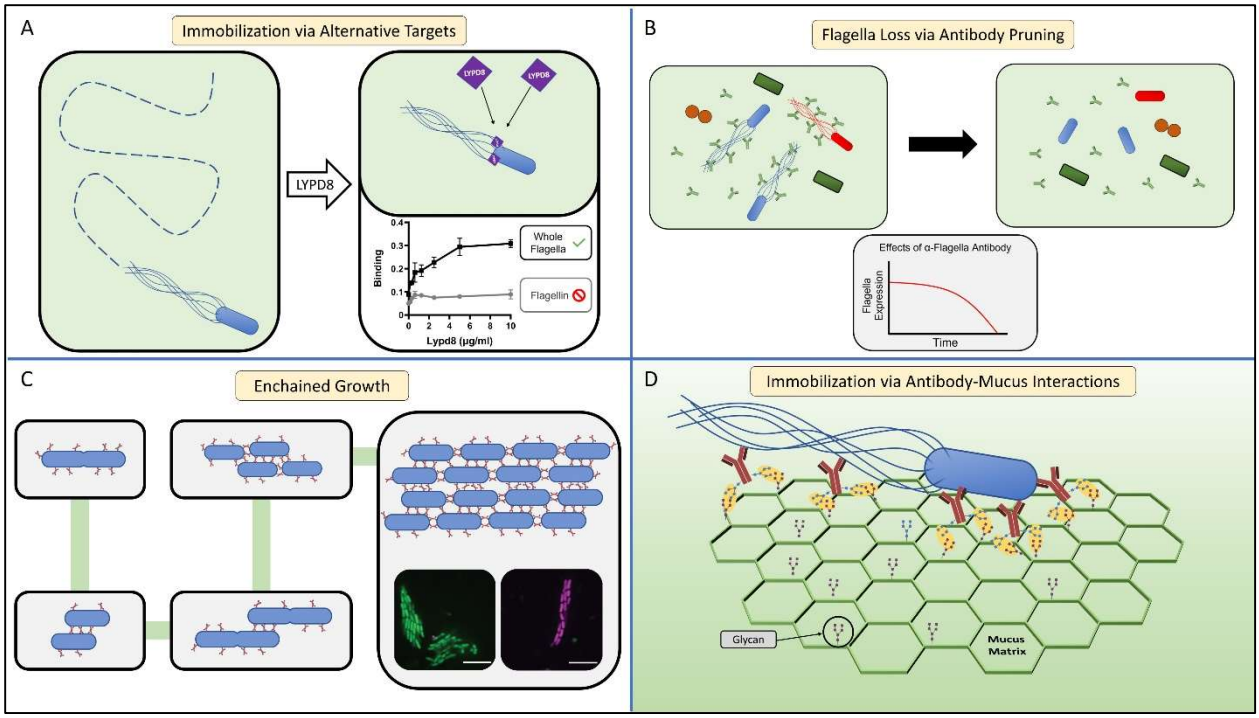

Figure 3| The host immune system can inhibit bacterial motility through multiple mechanisms. (A) Host effector molecules can inhibit flagellar motility through non-agglutination means by binding sites on the flagellum other than flagellin. LYPD8 (purple diamond) inhibits the flagellar motility of STM by binding to the flagellum [83]. Graphs display ELISA experiments showing that LYPD8 binds the flagella (right), but does not specifically target flagellin (left) (adapted from [83]). LYPD8, Ly6/Plaur Domain Containing 8. **(B)** The host can influence the microbiota's expression of flagellar genes through antibody-mediated pruning [74,110]. A sizable fraction of antibody (green bifurcating structures) in the GI tract bind can bind to the flagella of commensal microbes (left panel), resulting a significant decrease of flagellated bacteria within the microbiota (right panel). While this antibody-mediated pruning results in a decrease of flagella expression within the microbiota, the overall composition of the microbiota remains largely unchanged. *In vitro* experiments show that the anti-flagellin antibody causes *E. coli* to down regulate its expression of flagellin over time (graph). **(C)** In the GI tract, antibody-mediated agglutination can occur through a process termed enchained growth [59,96]. By this mechanism, bacteria that are actively dividing within the GI tract are coated in antibody (red line structures). Upon successful fission, the daughter cell is immediately linked to the mother cell from antibody crosslinking. Over multiple cycles of division, an agglutinated aggregate is formed consisting of clonal population of bacteria. Fluorescence microscopy micrographs depict examples of these clonal agglutinated aggregates (inset micrographs). **(D)** Antibody-mucin interactions facilitate the immobilization of motile bacteria within the mucus matrix (green hexagonal structure). In this model, glycans present on the surface of antibody (brown bifurcating structures) and mucin form weak, non-covalent interactions (yellow ovals) [98,99]. When motile bacteria within the mucus are coated by antibody, the numerous antibody-mucus interactions create a cumulatively strong non-covalent interaction that immobilizes the bacteria.

3.2 Adaptive Response

Like the innate immune system, the adaptive immune system can effectively inhibit microbial motility, mainly through luminally secreted antibody. Although neutralization, opsonization, and complement activation are the typical mechanisms ascribed to antibody activity, alternative mechanisms are employed within the GI tract lumen to address flagellar motility during both homeostatic and inflammatory states. These mechanisms include pruning, agglutination, and immobilization.

3.2.1 Antibody Pruning

During homeostasis, plasma cells in the GI tract continually secrete commensal-specific IgA and IgG into the intestinal lumen [111]. While these antibodies target a number of microbe-surface antigenic sites, a sizable fraction bind specifically to bacterial flagellin [74,112,113]. Within the diverse gut microbial community, many bacteria possess the genes necessary to produce functional flagella [74]. However, meta-transcriptomic data indicate that a relatively small proportion of those bacteria are actively expressing flagellar genes in the intestine under homeostatic conditions [74].

Notably, the presence of this flagellin-specific antibody repertoire appears largely dependent on TLR5 expression, consistent with studies indicating that TLR5 selectively enhances flagellin-specific CD4 T cell responses [74]. Using a TLR5 genetic knock-out mouse model, Cullender and colleagues found that TLR5 expression facilitates flagellin-specific antibody production, and in turn is inversely linked with expression of flagellar genes in

commensal microbes [74]. Consequently, in mice lacking TLR5, flagellated microbes are more free to swim within the mucus, which establishes new baseline conditions where flagellar motility is less restricted and the spatial segregation of microbes and epithelium is compromised. The mechanism driving the inverse relationship between flagellin-specific antibody and flagella expression was termed microbial "pruning", wherein microbes alter their expression of surface molecules in response to being bound by antibody (Figure 3B) [74,110]. While the exact molecular mechanism explaining how antibody causes this effect remains unknown, observations of *E. coli* sensing mechanical stimuli through their flagella could provide a possible explanation [114-116]. Thus, the host utilizes antibody as a tool to inhibit expression of flagella by colonizing microbes, thereby creating conditions of homeostasis that include the restricted motility of commensal microbes.

3.2.2 Agglutination

For close to a century the process of antibody-mediated agglutination has been utilized in clinical medicine and basic research to identify the "serotype" of bacteria, such as STM and *E. coli*, based on specific bacterial-surface antigens. Simply mixing isolated bacteria with immune serum (or purified antibodies) yields visible aggregates of agglutinated bacteria. While the practical application of antibody-mediated *in vitro* agglutination is clear, our knowledge of how agglutination occurs *in vivo* is less complete.

In the GI tract, antibody-mediated agglutination was thought to occur through the same mechanism as with *in vitro* agglutination assays, the so-called classical agglutination model.

This model was based on antibody-coated, planktonic bacteria colliding and sticking together, over time forming larger and larger aggregates. In this model the rate of agglutination is heavily dependent on the concentration of bacteria. However, even when *in vivo* bacterial concentrations are orders of magnitude below the predicted minimum concentration for classical agglutination to occur, antibody-mediated agglutination is still observed [59]. Moor and colleagues solved this enigma by showing that when bacterial concentrations are too low for classical agglutination to occur, the active replication of antibody-coated bacteria creates the conditions allowing agglutination to occur—a process termed enchained growth [59,96]. The enchained growth model holds that during conditions where bacteria are replicating in the presence of antibody, the newly replicated daughter cells initially will be linked to their mother cells, which after a few cycles of division will result in the agglutination of an entire lineage (Figure 3C). While the rate of classical agglutination is a function of the initial bacterial concentration, the rate of agglutination through enchained growth is a function of the rate of bacterial division.

Once agglutinated, the bacteria are no longer motile even if possessing functional, and sometimes actively rotating, flagella [59,96]. As a consequence, agglutinated bacteria are eventually expelled from the host, likely due to the peristaltic flow of intestinal contents, in a process previously described for the zebrafish model [53,54,59,96]. An interesting outcome of enchained growth is that the bacteria within an agglutinated aggregate are monoclonal, as opposed to a heterogeneous mixture of bacteria found in classically agglutinated aggregates [59]. Thus, when the agglutinated bacteria are expelled from the host, enchained growth could ultimately lead to the extinction of particular bacterial clones from the metagenome, thereby

reducing the genetic diversity of a specific bacterial strain or species [59,117]. Although antibody-agglutination does not need to specifically target microbes utilizing flagellar motility, agglutination renders flagellated bacteria non-motile and thereby susceptible to clearance from the host.

3.2.3 Immobilization

While agglutination may be an effective strategy when concentrations of bacteria are high or when they are actively dividing, what strategies might be better suited for single flagellated microbes swimming through mucus towards the epithelium? Schroeder and colleagues found that antibody imbedded in mucus can immobilize microbes, independent of agglutination and neutralization [98,99]. This ability depends on a synergy between antibody and mucus. The Fc domain of IgG has weak interactions with mucins, which are dependent on antibody glycosylation. In isolation, these weak interactions are unable to prevent antibody diffusion within the mucus [98,99]; however, like Velcro, a cumulative force from multiple weak interactions occurring between IgG-coated bacteria and mucus leads to the bacterial immobilization (Figure 3D) [98-100]. Through this mechanism, antibody can restrict mobility irrespective of bacterial concentration or division.

4. Concluding Remarks

From a microbe's perspective, current literature supports that flagella can provide microbes a fitness advantage in the host, because of the ability to localize to beneficial sites. Luminal bacteria can use flagella-driven chemotaxis to locate nutrient micro-niches produced by inflammation or epithelial damage, which they can exploit for a growth advantage over competitors. Alternatively, some bacteria utilize motility to access the epithelium, permitting lifestyles that require adherence, invasion, and/or translocation. From the host's perspective, flagellar motility can represent varying degrees of risk, which over an evolutionary timescale resulted in the development of numerous and often redundant defense mechanisms that collectively work to inhibit flagellar motility and/or eliminate flagellated microbes.

When a host is exposed to a novel flagellated microbe, the innate immune system can combine motility-inhibiting peptides and proteins, along with increased mucus secretion, to limit access to the epithelium and promote the elimination of immobilized microbes. While these initial barriers are not impermeable, the collective effects of innate responses reduce microbe-epithelium interactions and limit potential damage to the mucosa. When required, additional immune resources are recruited to eliminate the threat. An antibody response can provide long term fortification of the mucus through motility inhibition. In this case, upon subsequent re-challenge by the antibody-targeted microbe, its flagellar motility will be met with a combined response of both the innate and adaptive immune system. Interestingly, many of the mechanisms of motility inhibition are non-lethal, which may reflect a strategy aimed to avoid collateral damage to the cohabitating microbiota. Additionally, the host's active "pruning" or attenuation of commensal flagella expression could represent a co-evolved

strategy where microbes benefiting from membership in the commensal microbiota need to follow the "rules" set by the host and not employ flagella.

Although innate effectors and antibody may target different epitopes and with different affinities, there are redundancies in their mechanisms of motility inhibition. For example, agglutination is a method employed by both antibody and innate effector molecules such as HD6. Likewise, the agglutination-independent immobilization of microbes occurs with both antibody and innate effectors such as LYPD8. While antibody accomplishes this through cumulative antibody-mucus interactions, the mechanism employed by LYPD8 remains to be elucidated. Thus, our understanding of this mechanism of antibody-mediated motility inhibition could provide clues on how LYPD8, or other innate molecules are able to inhibit motility.

Technological innovations in imaging and sequencing are advancing our understanding of the roles of microbial motility within the host, as well as the motility-directed countermeasures of the host. While the exact molecular mechanism explaining how antibody causes this effect remains unknown, observations of *E. coli* sensing mechanical stimuli through their flagella could provide a possible explanation [118]. This technique could allow future studies to broaden the scope of our understanding, for example, from one based largely on model enteric pathogens, to include how flagellated members of the commensal microbiota also use motility within the host. Likewise, further investigations of the host mucosal immune system will likely uncover novel effector molecules, synergies, and molecular mechanisms that inhibit microbial motility. The intersection between motile microbe and host remains a complex and nuanced subject, with many of its intricacies still unknown.

7. References

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Chapter 2

Flagella-Driven Motility is a Target of Human Paneth Cell Defensin Activity

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Abstract

In the mammalian intestine, flagellar motility can provide microbes competitive advantage, but also threatens the spatial segregation established by the host at the epithelial surface. Unlike microbicidal defensins, previous studies established that the protective activities of human α-defensin 6 (HD6), a peptide secreted by Paneth cells of the small intestine, resides in its remarkable ability to bind microbial surface proteins and self-assemble into protective fibers and nets. Given its ability to bind flagellin, we proposed that HD6 might be an effective inhibitor of bacterial motility. Here, we utilized advanced automated live cell fluorescence imaging to assess the effects of HD6 on actively swimming *Salmonella enterica* in real time. We found that HD6 was able to effectively restrict flagellar motility of individual bacteria. Flagellin-specific antibody, a classic inhibitor of flagellar motility that utilizes a mechanism of agglutination, lost its activity at low bacterial densities, whereas HD6 activity was not diminished. A single amino acid variant of HD6 that was able to bind flagellin, but not self-assemble, lost ability to inhibit

flagellar motility. Together, these results suggest a specialized role of HD6 self-assembly into polymers in targeting and restricting flagellar motility.

Introduction

A critically important biological boundary for host health and survival exists at the intestinal mucosa, where a complex ecosystem of microbes intimately colonizes an organ system tasked with nutrient uptake [1-3]. Here, colonizing microbes can benefit the host in many physiological functions, including nutrient acquisition [4-10]. Yet, harmful microbes can access this anatomic site to cause disease, often through a strategy of contacting and breaching the intestinal epithelium [11-13]. Effective immunity in the intestine must allow for a controlled colonization with the symbiotic microbiota, while being ever ready to protect the host from pathogens [13-18].

Colonization of the mammalian intestinal tract requires microbes to overcome many challenges, including intense competition with co-colonizers for nutrients, displacement by peristalsis, toxicity inherent to host digestive factors such as bile salts and hydrolytic enzymes, and host-derived immune effector molecules [5,19-21]. In such an environment, locomotion can afford microbes a competitive advantage by providing an ability to access nutrient-rich niches and avoid displacement or noxious microenvironments [22-24]. Many microbes achieve locomotion via flagella, whip-like organelles that can provide effective propulsion [25,26]. In addition to certain commensal microbes utilizing flagella to facilitate their intestinal

colonization, numerous enteric pathogens use flagellar motility as an essential virulence factor [27]. As such, from the host's perspective, flagella-mediated motility carries potential risk, since motility can enable microbes to breach of protective barriers responsible for spatial segregation, and even facilitate their epithelial invasion and translocation into underlying tissue [12,27]. In turn, the mammalian host has developed a repertoire of immune countermeasures to mitigate potential threat from flagellar motility, including mechanisms of flagellar detection and response by both innate and adaptive immune mechanisms [24,28-31].

Paneth cells are specialized secretory epithelial cells of the small intestine that play a crucial role in maintaining mucosal integrity and homeostasis through their secretion of a mixture of proteins and peptides that target intestinal microbes [32,33]. Through delivery of these molecules into the luminal environment, Paneth cells help mediate two interrelated functions: defend the host from enteric pathogens and maintain homeostasis with the colonizing microbiota [32-34]. A prominent secretory component of the Paneth cell are α-defensins, a group of tri-disulfide, cationic peptides with antimicrobial properties [32,35,36]. Whereas Paneth cells of some mammals express multiple α-defensin paralogs, humans express only two—human α-defensin-5 and -6 (HD5 and HD6, respectively) [32,37,38]. Like most other mammalian α-defensins, HD5 possesses direct microbicidal activity through a mechanism that targets the microbial membrane, which provides the host with both significant protection from enteric pathogens, as well an ability to reshape the composition of the microbiota [39-42]. Initially, the activity of HD6 was anticipated to mimic that of HD5. Indeed, transgenic mice engineered to express HD6 in Paneth cells gained significant protection from lethal challenge with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) [43]. However, antimicrobial assays revealed that mature

HD6 lacked the microbicidal activity of HD5, leaving open the question of how expression of this peptide protected the HD6 transgenic mice, and what might be the function of this abundant peptide in the human intestine [43,44]. The conundrum was resolved when studies showed that protection of the transgenic mice from lethal challenge with *S.* Typhimurium resulted from a microbicidal-independent mechanism that prevented bacterial invasion and translocation across the epithelium [43]. Moreover, HD6 could block *S.* Typhimurium invasion of epithelial cells *in vitro*, an activity not observed with its bactericidal counterpart HD5 [43]. The ability of HD6 to block invasion was not limited to *S.* Typhimurium, but was also observed with other diverse bacterial pathogens that employ completely different cellular invasion pathways [43,45,46]. This activity against distinct and diverse bacterial pathogens implies a broad-spectrum molecular mechanism, a characteristic feature of innate immunity.

But how is HD6 able to provide this protection? An inherent activity of HD6 stems from its striking ability to self-assemble into high-order polymers, an activity not found with either HD5, endogenous mouse α-defensins or most other α-defensins [43,45,46]. Scanning- and transmission-electron microscopy images show that the 32-residue HD6 peptide can spontaneously polymerize to form macromolecular fibrils reaching microns in length, which can then form a meshwork termed "nanonets" [43,45,46]. In addition to self-assembly, HD6 can also bind to a variety of different bacterial proteins, including flagellin, fimbriae, and invasin [43]. The proposed protective mechanism thus entails a two-step process, where there is initial (stochastic) binding of HD6 to microbial surface molecules (such as fimbrin or flagellin), which then triggers the self-assembly process that forms HD6 macromolecular fibrils and nets that entangle microbes and prevent invasion of host cells. Indeed, bacteria exposed to HD6 are

observed to be agglutinated in webs of nanonets [43,45,46]. However, during the earliest stages of infection, or close to the epithelium during homeostasis, single bacteria propelled by flagellar motility might better typify encounters with HD6 at the mucosal surface as it is secreted from Paneth cells and beneath a blanket of protective mucus. However, the low bacterial concentration would preclude HD6-induced agglutination [47]. Thus, we considered that an ability of HD6 to halt flagellar motility under these conditions could be vitally important to the host.

Herein, we used live-fluorescence microscopy to assess the effects of HD6 on motile, flagellated *S.* Typhimurium in real time with high resolution. Through this methodology, we determined that HD6 is able to inhibit flagellar-driven movement of individual *S.* Typhimurium, an activity not observed with a monoclonal antibody to flagellin. Through the use of the HD6 variant ($HD6^{F2A}$) that retains the capacity to bind flagellin, but lacks an ability to self-assemble, we found that self-assembly is necessary for HD6 to inhibit bacterial motility. Together, the findings elucidate the molecular underpinnings of an HD6 activity to inhibit flagella-driven motility, suggesting a unique role for this human α-defensin in host-microbe interactions at the intestinal mucosa.

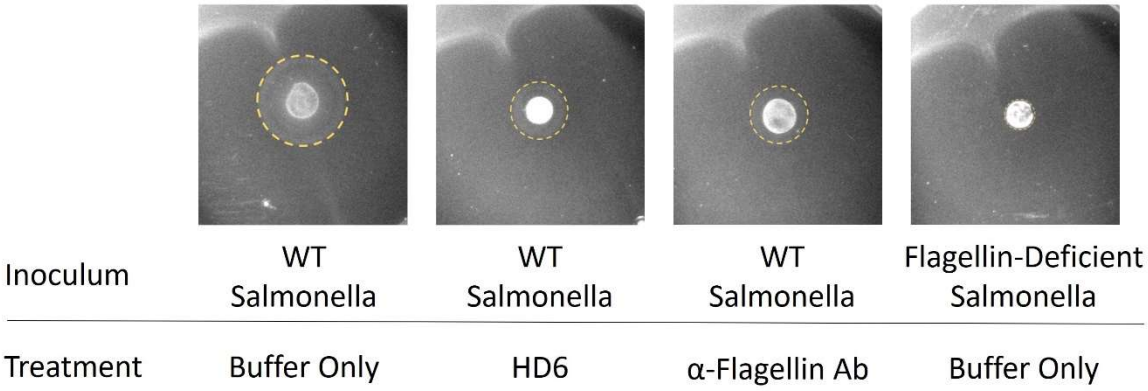
Results

HD6 inhibits bacterial motility

To initially assess the impact of HD6 on flagella-mediated motility, we used a semi-solid agar motility assay with a motile strain of *S.* Typhimurium. Extensive *in vitro* and *in vivo* investigations have characterized the flagellar motility of *S.* Typhimurium, making it a suitable

model for our investigation [25,48]. In these assays, the presence of HD6 significantly inhibited the migration of *S.* Typhimurium, compared to migration with a buffer control (Figure 1A & 1B).

1A



1B

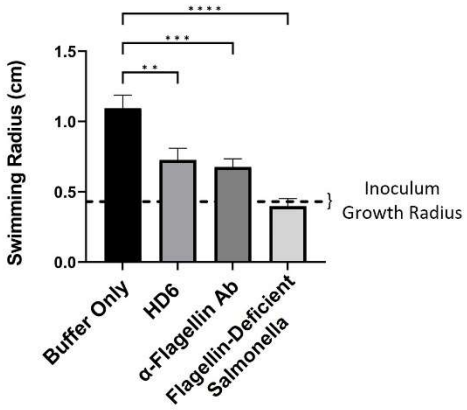


Figure 1| Analysis of HD6 effects on bacterial motility in semi-solid agar. (**A**) Images of semi-solid agar motility plates treated with either HD6 (30 µg), α-FliC IgG antibody (20 µg), or buffer (Tris-Maleate pH 6.4). Plates were then inoculated with either wild type (WT) *S.* Typhimurium or flagellin-deficient mutant, ΔFliCFljB *S.* Typhimurium. After a 6.5-hour incubation at 37°C, photographic images were captured. The outer edge of the bacterial swimming halo is highlighted by the dashed circular yellow line. Images are representative of 2 independent experiments, each with technical replicates. (**B**) Quantification of the bacterial swimming halo radius for each experimental group as in (A). The dashed horizontal line represents the radius of the inner circle, representing the inoculum growth for non-swimming bacteria, averaged for all treatments. Error bars, mean ± SD. Data were pooled from 2 independent experiments, n=6-13 plates, and analyzed using one-way ANOVA comparing experimental groups to buffer control (**P<0.01, ***P<0.001, ****P<0.0001).

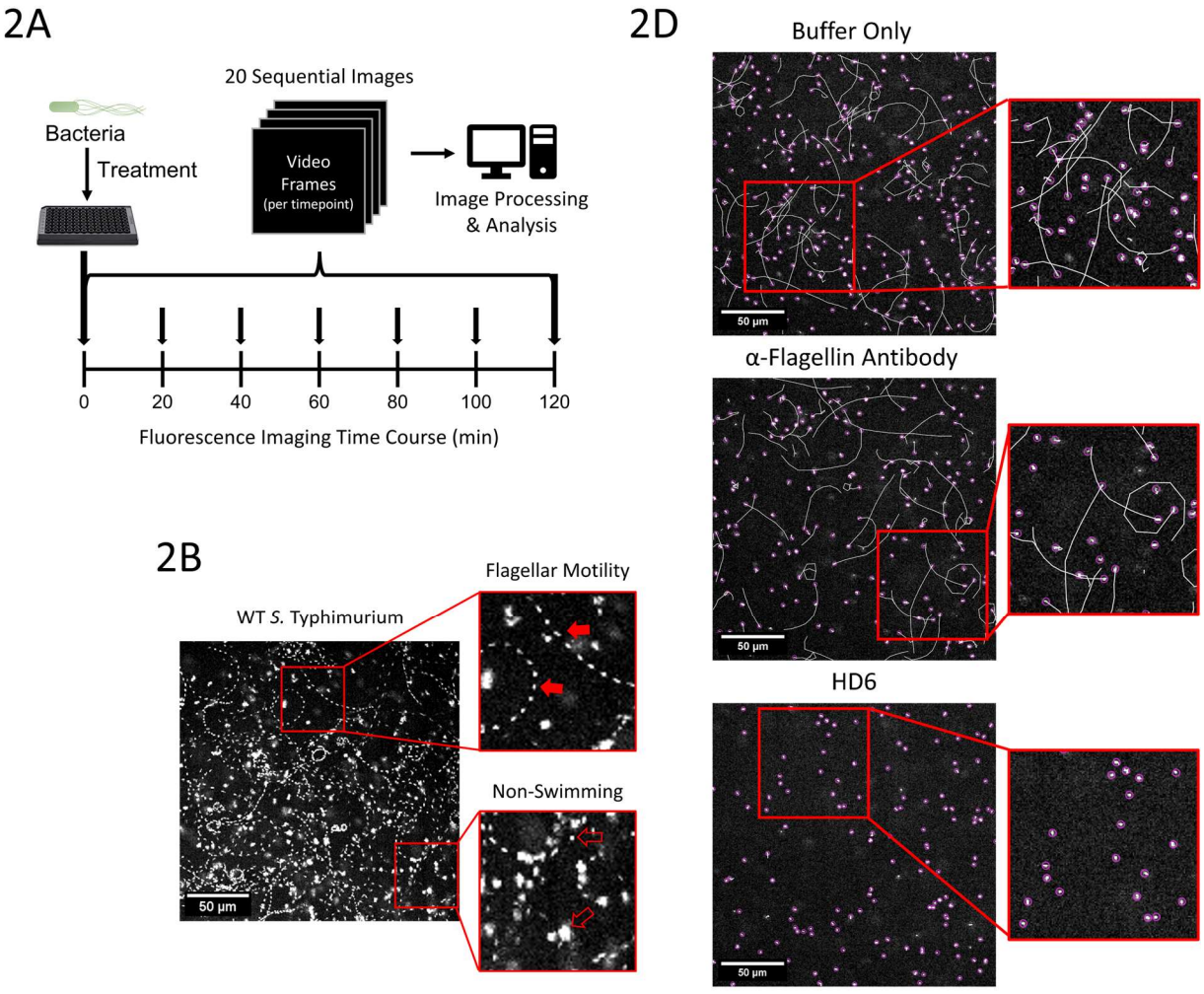
Antibodies that target flagella are effective and classical inhibitors of bacterial motility [49]. A monoclonal IgG specific to *S.* Typhimurium flagellin (α-FliC-IgG) caused a significant degree of motility inhibition to *S.* Typhimurium, comparable to that seen with HD6 treatment (Figure 1A & 1B). In these assays, neither HD6 nor α-FliC-IgG treatment were sufficient to completely inhibit the motility of *S.* Typhimurium, when compared to a nonmotile, flagellin-deficient ΔFliCΔFljB *S.* Typhimurium mutant (Figure 1A & B). The results from these semi-solid agar motility assays establish that HD6 is capable of inhibiting bacterial motility.

A high-resolution live cell fluorescent microscopy assay to visualize bacterial motility

Whereas the agar-plate motility assay yielded a population-level assessment of bacterial motility, it did not provide the resolution necessary to visualize and study the motility of individual bacteria. Therefore, to better delineate the effects of HD6, a high-throughput, automated live cell fluorescence microscopy assay was developed to capture time-course images of the motility of GFP-expressing *S.* Typhimurium under various experimental conditions (Figure 2A). Importantly, our automated and high-throughput strategy allowed experimental parameters to be tested rapidly in parallel, which reduced experimental variability and facilitated replicate assays. Bacterial motility was visualized by capturing twenty frames in rapid succession every twenty minutes for two hours, allowing thorough assessment of movement dynamics. *S.* Typhimurium populations are known to have heterogenous expression pattern of flagellar genes, which results in given populations having both actively swimming as well as non-swimming bacteria, corresponding to flagellated bacteria and those not expressing flagellar

genes, respectively [50-52]. Preliminary experiments established the imaging parameters within which treatment effects could be analyzed (DNS) and demonstrated our ability to record the movement of individual *S.* Typhimurium at a single-cell resolution (Figure 2B, Supplemental Video 1). Consistent with previous investigations on bacterial motility, the movement of individual bacteria could be categorized as either actively motile (swimming) or diffusing (Figure 2B) [52]. Diffusing bacteria (a movement coined by Furter et al. [52]) exhibited a vibration-like movement characterized by short movements in seemingly random directions resulting in minimal displacement (Figure 2B). Actively motile bacteria moved across appreciable distances in either straight, arcing, or circular paths (Figure 2B). Assays of a flagellin-deficient ΔFliCΔFljB *S.* Typhimurium mutant revealed a complete absence of the actively motile population, confirming that the actively motile fraction of WT bacteria locomote via flagella-dependent swimming (Figure 2C, Supplemental Video 2). Thus, this live-fluorescence microscopy assay provided a high-throughput means to effectively observe and characterize the motility of individual bacteria.

Initial experiments using a high bacterial density (1e8 CFU/ml) showed that *S.* Typhimurium were agglutinated into large aggregates by HD6 (Supplemental Figure 1, Supplemental Videos 3-5). Similar results were observed with *S.* Typhimurium treated with α-FliC-IgG (Supplemental Figure 1, Supplemental Video 5). These observations are consistent with previous reports showing that both HD6 and α-FliC-IgG are able to agglutinate *S.* Typhimurium [43,53]. Next, wells were inoculated with a reduced density of *S.* Typhimurium (5e6 CFU/ml). Under these conditions, HD6 retained its ability to inhibit the active motility of *S.* Typhimurium


2A
20 Sequential Images
Bacteria
Treatment
Video Frames (per timepoint)
Image Processing & Analysis
0
20
40
60
80
100
120
Fluorescence Imaging Time Course (min)
2B
Flagellar Motility
WT S. Typhimurium
Non-Swimming
50 µm
2C
Flagellin-Deficient S. Typhimurium
Non-Swimming
50 µm
2D
Buffer Only
α-Flagellin Antibody
HD6
50 µm
2E
****
ns
****
Fraction of Total Population
0.6
0.4
0.2
0.0
Buffer Only
α-Flagellin Ab
HD6

(Supplemental Video 7), but there was no evidence of agglutination (Figure 2D, Supplemental Video 6 & 7). To quantify the impact of HD6 on bacterial movement, individual bacteria were tracked and measured using Trackmate, a particle tracking software plugin for ImageJ that enabled quantitation of bacterial motility based on velocity (Figure 2D) [54,55]. This analysis revealed that HD6 caused a significant reduction in the number of actively motile (swimming) *S.* Typhimurium compared to buffer treatment alone (Figure 2E). Strikingly,

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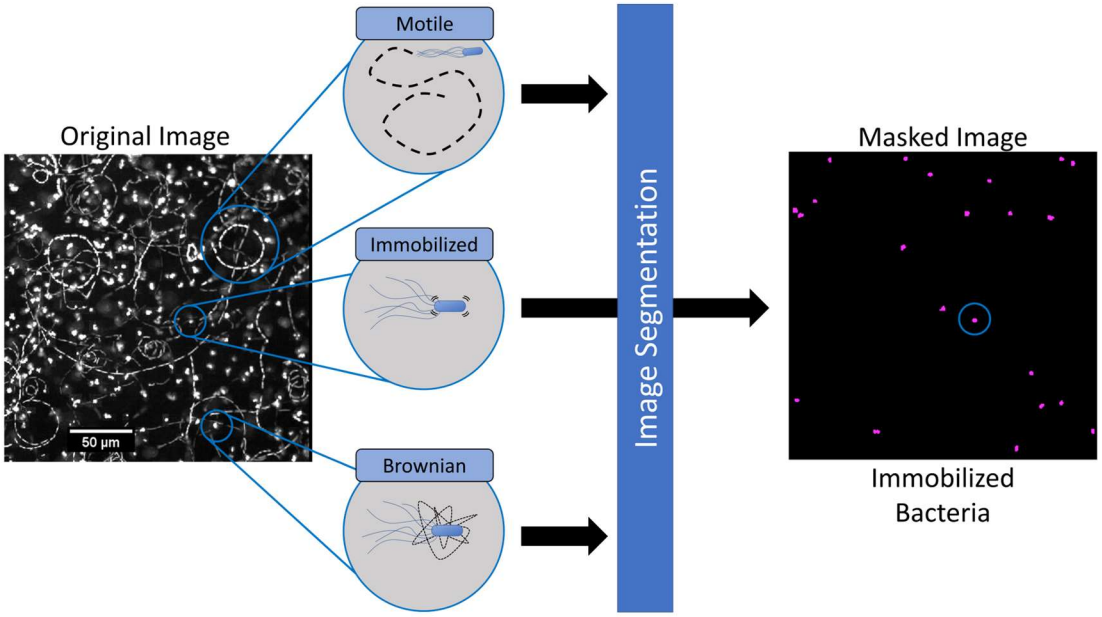
Figure 2| Live-fluorescence microscopy analysis of HD6 and α-FliC IgG effects on *S.* Typhimurium motility. (**A**) Schematic diagram depicting the workflow used for all live-fluorescence microscopy imaging experiments. Top Left, GFP-expressing *S.* Typhimurium are added to wells in a 96-well plate that containing different experimental treatments. Bottom, Images are captured at 20-minute intervals for each in-well position over the course of a 120-minute experiments, for a total capture of 7 timepoints. Top Right, At each timepoint, 20 sequential images are captured and Imaging data are then processed for analysis using Matlab and ImageJ. (**B**) Representative data captured at a single timepoint for GFP-expressing wild type (WT) *S.* Typhimurium in buffer. Data are presented as single superimposed image containing the 20 sequential images captured at a single timepoint, allowing for the detection of bacterial movement over time. Top Inset, Magnified image displaying an example of the characteristic pattern of *S.* Typhimurium moving via flagella driven motility (filled red arrow). Bottom Inset, Magnified image displaying examples of patterns of movement for non-swimming *S.* Typhimurium, with two examples highlighted (open red arrows). (**C**) Representative data captured at a single timepoint as in (B) for GFP-expressing flagellin-deficient mutant ΔFliCFljB *S.* Typhimurium in buffer. Inset, Magnified image displaying examples movement for non-swimming *S.* Typhimurium, with two examples highlighted (open red arrows). No instances of swimming pattern of movement were detected with ΔFliCFljB *S.* Typhimurium (DNS). For (**B**) and (**C**), image data are representative of 8 independent experiments with technical replicates. Scale bar, 50 µm. (**D**) Representative data for GFP-expressing wild type (WT) *S.* Typhimurium treated with either buffer alone (TOP), α-FliC IgG antibody (20 µg/ml, MIDDLE), or HD6 (10 µg/ml, BOTTOM). Images are depicted with a track overlay generated by ImageJ Trackmate. Insets, Magnified images displaying the tracks of both swimming and non-swimming *S.* Typhimurium captured using Trackmate. Track color is randomly assigned based on track ID. Image data are representative of 2 independent experiments with technical replicates. Scale bar, 50 µm. (**E**) Quantification of bacterial swimming expressed as the fraction of the total *S.* Typhimurium population that are moving via flagellar motility, as determined using the velocity track metric from Trackmate analysis. Data were pooled from 2 independent experiments, n=6 wells, and analyzed using one-way ANOVA (Error bars, mean ± SD, ns= P>0.5, **** P<0.0001).

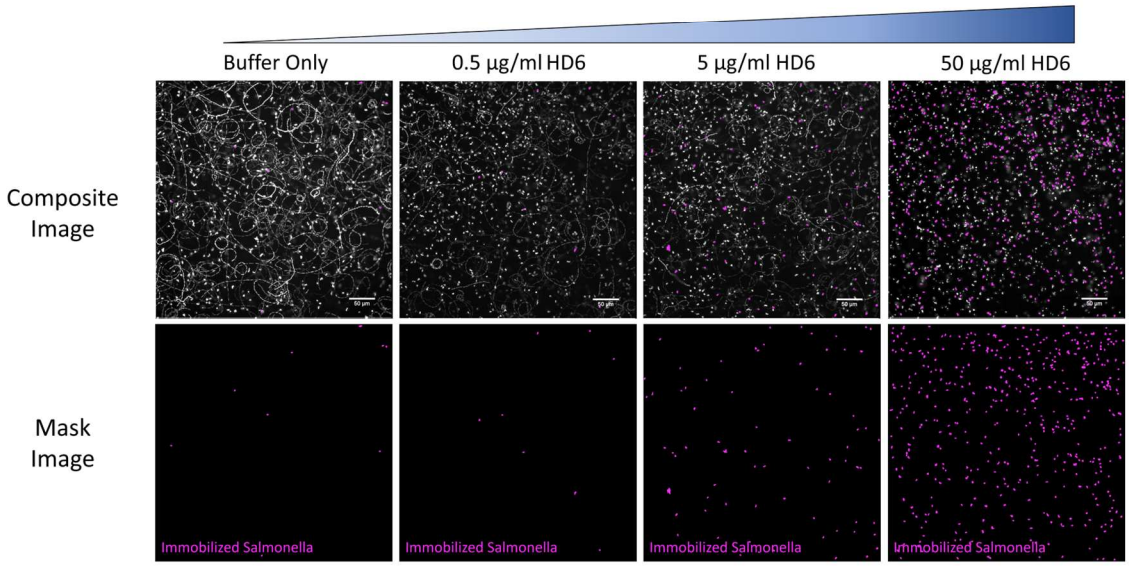
multiple HD6-treated wells had a complete absence of swimming *S.* Typhimurium (Figure 2D & 2E). Colony counting plate assays determined that HD6 showed no microbicidal activity in these assays (DNS), consistent with previous findings [43,45,46]. In contrast to the significant activity on bacterial motility evident with HD6, α-FliC-IgG treatment resulted in no significant change in the population of actively motile *S.* Typhimurium compared to buffer control at this lower bacterial density (Figure 2E). Thus, despite its ability to bind flagellin and agglutinate bacteria at high density, α-FliC-IgG was unable to inhibit the flagellar-driven motility of individual *S.* Typhimurium in these assays. These data suggested that HD6 inhibited bacterial motility by an agglutination-independent mechanisms, that was distinct from inhibition of motility through α-FliC-IgG-mediated bacterial agglutination.

While *S.* Typhimurium treated with HD6 had a significant reduction in actively motile bacteria, there did not appear to be a corresponding increase in the diffusing population. Instead, a different population of immobilized *S.* Typhimurium were present, distinguished from the diffusing population by a complete lack of perceptible movement (Figure 2D, Supplemental Video 8). These bacteria appeared to maintain a fixed position within the field of view, often remaining in the same location across timepoints for the entire duration of the experiment. To characterize the kinetics and magnitude of this immobilization, *S.* Typhimurium were imaged upon treatment with an HD6 concentration of 0.5, 5.0, or 50 μg/ml (Supplemental videos 9-12). The degree of immobilization was quantified using an imaging analysis-mask capable of identifying and isolating immobilized *S.* Typhimurium population from the actively motile and diffusing populations (Figure 3A). Treatment with the 0.5 μg/ml concentration of HD6 did not yield a significant change in the proportion of immobilized bacteria compared to buffer control

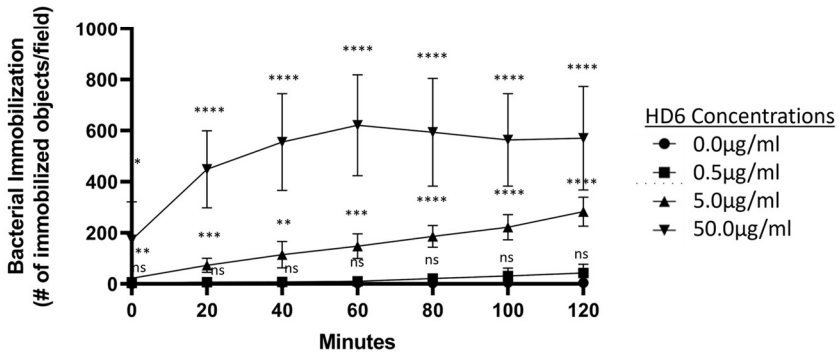
3A


Motile
Original Image
Immobilized
Brownian
50 µm
Image Segmentation
Masked Image
Immobilized
Bacteria

3B


Buffer Only
0.5 µg/ml HD6
5 µg/ml HD6
50 µg/ml HD6
Composite
Image
Mask
Image
Immobilized Salmonella
Immobilized Salmonella
Immobilized Salmonella
Immobilized Salmonella

3C


Bacterial Immobilization
(# of immobilized objects/field)
1000
800
600
400
200
0
0
20
40
60
80
100
120
Minutes
HD6 Concentrations
0.0µg/ml
0.5µg/ml
5.0µg/ml
50.0µg/ml

(Figure 3B & 3C, Supplemental Video 9 & 10). However, at all timepoints the proportion of immobilized *S.* Typhimurium was significantly greater when treated with either 5 µg/ml or 50 µg/ml HD6 as compared to buffer control, and the proportion increased throughout the time course at these concentrations (Figure 3B & 3C, Supplemental Video 11 & 12). Moreover, the proportion of immobilized *S.* Typhimurium at all timepoints was significantly greater after treatment with the 50 µg/ml concentration compared to treatment with HD6 at a concentration of 5 µg/ml (Figure 3B & 3C). These results demonstrate a concentration dependence in the ability of HD6 to immobilize individual bacteria.

Figure 3|. Live-fluorescence microscopy analysis of HD6-mediated *S.* Typhimurium immobilization. (**A**) Diagram depicting the masking strategy used to identify and quantify immobilized GFP-expressing *S.* Typhimurium. Left, An original superimposed image (as in Fig. 2B) displaying examples of the three patterns of movement: motile, immobilized, Brownian motion. Inset circles, schematic depictions of the three patterns of movement. Right, The image mask identifies and then only displays immobilized bacteria, based on invariant spatial location in the 20 sequential images at each experimental timepoint. The resulting masked image displays the immobilized *S.* Typhimurium (red) with the image of a single bacterium highlighted (blue circle). Scale bar, 50 µm. (**B**) Representative unmasked composite data (TOP) and masked image data (BOTTOM), which depict immobilized *S.* Typhimurium observed in the presence of HD6 (0.5, 5.0, 50 µg/ml) or with buffer alone. Composite images consist of an overlay of both the original superimposed image series (white) and the image from the immobilization mask (red). The masked images (BOTTOM) display only immobilized *S.* Typhimurium (red). Data are representative of 3 independent experiments, each with technical replicates. Scale bar, 50 µm. (**C**) Quantification of *S.* Typhimurium immobilization for each experimental group as in (B). The y-axis displays the number of objects detected by in immobilized mask image for each treatment group at each experimental timepoint. The treatment groups were buffer alone (black circle), 0.5 µg/ml HD6 (black square), 5.0 µg/ml HD6 (black triangle), and 50.0 µg/ml HD6 (inverted black triangle). The data for buffer alone and 0.5µg/ml HD6 treatment groups are essentially overlapping, which obscures some of the data points. Data were pooled from 3 independent experiments, n=7 wells. Data for HD6 treatment groups (as compared to buffer alone at each experimental timepoint) were analyzed by multiple-comparison unpaired t-test with Welch correction. Error bars, mean ± SD (ns= P>0.5, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

HD6 self-assembly is necessary for activity

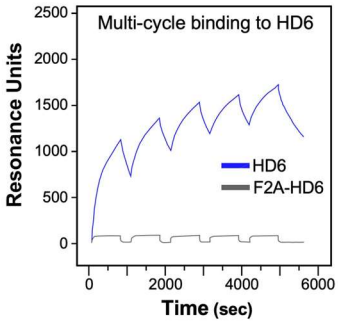
Previous work by our group and others showed that HD6 self-assembles into higher order polymers, which appear to create a meshwork of fibrils termed "nano-nets" that are visible through scanning- and transmission-electron microscopy [43,45,46]. Chairatana et al. found that the F2 residue in HD6 is critical for self-assembly and a HD6^{F2A} variant is unable to form fibrils or agglutinate bacteria (Figure 4A) [45]. Using multicycle-surface plasmon resonance (multicycle-SPR) to analyze the binding kinetics of HD6 and the HD6^{F2A} variant to immobilized HD6, we confirmed that HD6^{F2A} was unable to self-assemble into the higher order polymers seen with native HD6 (Figure 4B). Next, we used multicycle-SPR to analyze binding of HD6 and the HD6^{F2A} variant to immobilized flagellin isolated from *S.* Typhimurium. We found that HD6^{F2A} was able to reversibly bind to flagellin, but subsequently lacked the ability to self-assemble as seen with native HD6 (Figure 4C). Thus, these data indicated that like HD6, the HD6^{F2A} variant can bind to flagellin, but unlike HD6 was unable to self-assemble into higher-order structures.

To test if HD6-mediated immobilization of *S.* Typhimurium is dependent on its ability to self-assemble, we used our live cell microscopy assay to assess bacterial motility in the presence of HD6^{F2A} (Figure 4D, Supplemental Videos 13-16). Quantification of bacterial immobilization revealed that treatment of *S.* Typhimurium with HD6^{F2A} had no significant effect on *S.* Typhimurium motility compared to the buffer control, even at the higher concentration of 50 μg/ml (Figure 4E). When compared to the bacterial immobilization caused by treatment with native HD6, these results indicate that the ability of HD6 to self-assemble is necessary for it to immobilize *S.* Typhimurium.

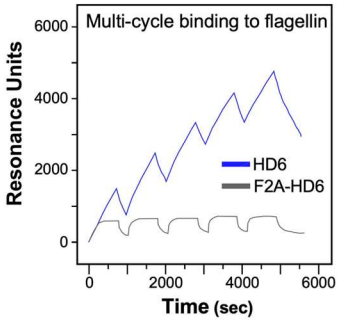
4A


AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL Native HD6
AATCHCRRSCYSTEYSYGTCTVMGINHRFCCL HD6F2A Variant

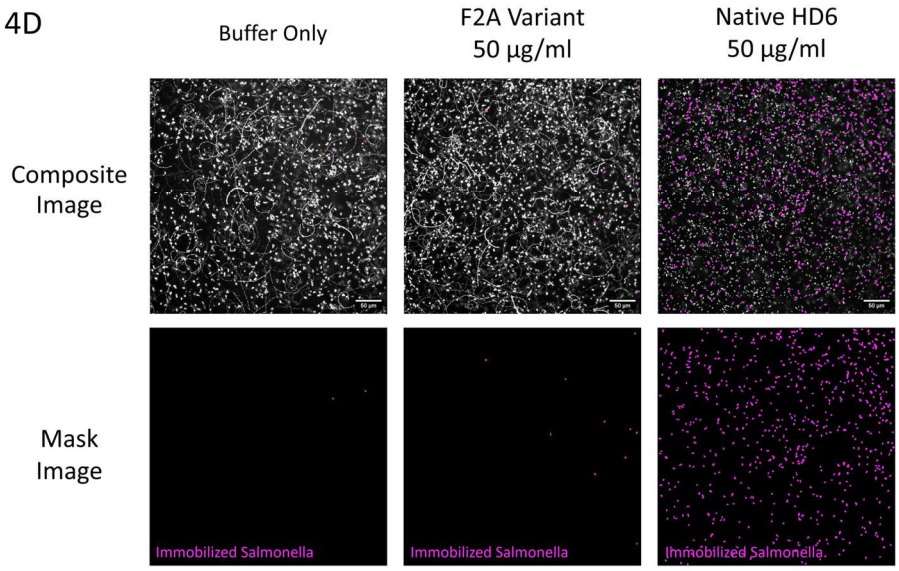
4B


Multi-cycle binding to HD6
Resonance Units
Time (sec)
HD6
F2A-HD6

4C


Multi-cycle binding to flagellin
Resonance Units
Time (sec)
HD6
F2A-HD6

4D


Buffer Only
F2A Variant 50 µg/ml
Native HD6 50 µg/ml
Composite Image
Mask Image
Immobilized Salmonella
Immobilized Salmonella
Immobilized Salmonella

4E

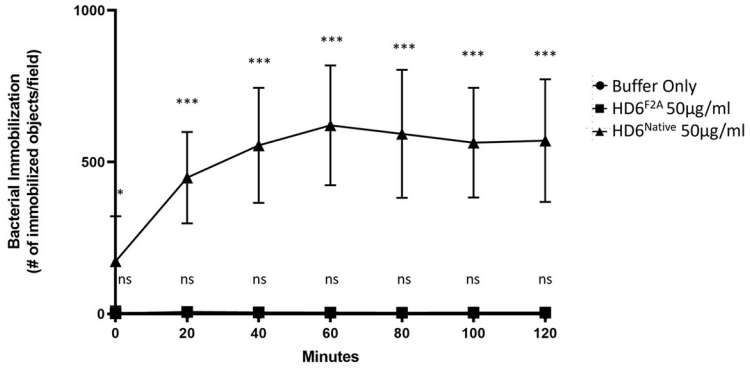

Bacterial Immobilization (# of immobilized objects/field)
Minutes
Buffer Only
HD6F2A 50µg/ml
HD6Native 50µg/ml

Figure 4| Structure-function analysis of HD6 immobilization activity. (**A**) Primary structure of native HD6 and the variant $HD6^{F2A}$ peptides. Position two is highlighted with an arrow, with native F2 (phenylalanine) residue in black, variant A2 (alanine) in red. The connectivity of the three disulfide bonds found in HD6 and typical of α-defensins are depicted (Cys^1-Cys^6, Cys^2-Cys^4, Cys^3-Cys^5). (**B, C**) Surface plasmon resonance (SPR) analysis of binding and self-association. (**B**) Graph showing multi-cycle SPR results of $HD6^{Native}$ or $HD6^{F2A}$ binding to immobilized $HD6^{Native}$. The SPR biosensor presented HD6 and the HD6 (blue) or $HD6^{F2A}$ analytes traversed the biosensors at 20µl/min. Each 15 min cycle included a binding period of 12.5 min, followed by a wash period of approximately 2.5 min. (**C**) Graph showing multi-cycle SPR results of $HD6^{Native}$ or $HD6^{F2A}$ binding to immobilized flagellin. Analysis as in (B) except that the SPR biosensor presented flagellin isolate from *S.* Typhimurium. (**D**) Live-fluorescence microscopy analysis of *S.* Typhimurium immobilization upon treatment with either HD6 or $HD6^{F2A}$. Representative unmasked composite data (TOP) and masked image data (BOTTOM) as in Fig. 4B, which depict immobilized S. Typhimurium observed in the presence of HD6 (50 µg/ml), $HD6^{F2A}$ (50 µg/ml) or with buffer alone. Composite images consist of an overlay of both the original superimposed image series (white) and the image from the immobilization mask (red). The masked images (BOTTOM) display only immobilized *S.* Typhimurium (red). Data are representative of 3 independent experiments with technical replicates. Scale bar, 50 µm. (**E**) Quantification of *S.* Typhimurium immobilization for each experimental group as in (D). The y-axis displays the number of objects detected by in immobilized mask image for each treatment group at each experimental timepoint. The treatment groups were buffer alone (black circle), $HD6^{F2A}$ (50.0 µg/ml, black square), and $HD6^{Native}$ (50.0 µg/ml, black triangle). The data for buffer alone and $HD6^{F2A}$ treatment groups are essentially overlapping, which obscures individual data points. The data for $HD6^{Native}$ are from Fig. 4C, which was performed on the same plate. The data were pooled from 3 independent experiments, n=7 wells. Data for HD6 and $HD6^{F2A}$ treatment groups (as compared to buffer alone at each experimental timepoint) were analyzed by multiple-comparison unpaired t-test with Welch correction. Error bars, mean ± SD (ns= $P>0.5$, *$P<0.05$, ***$P<0.001$).

Discussion

In the mammalian intestinal tract, many microbes use flagella-driven motility to effectively compete for colonization in this challenging environment, including some enteric pathogens that use flagella as an instrument of virulence [12,22,23,27,56]. A noteworthy feature of the immune system of mammals is the multiple overlapping mechanisms for recognition and response to flagellin, the primary protein component of flagella [24,28-30], which highlights a vital importance of targeting flagella to maintain homeostatic balance in host-microbe interactions. In this work, we asked if HD6 can interact with motile bacteria and inhibit flagellar motility. We developed a high-throughput, automated live cell fluorescence microscopy assay that allowed us to visualize and track the movement of individual bacteria under controlled experimental conditions. Using this approach, we demonstrated that HD6 inhibits the swimming motility of *S.* Typhimurium. Analysis of a single residue variant of HD6 revealed that the ability to bind flagellin alone is not sufficient to inhibit motility, but rather that an ability to self-assemble is also necessary. Furthermore, the inhibition of flagellar motility is distinct from flagellin-specific antibody, the classic inhibitor of flagellar motility that utilizes agglutination as a mechanism to halt movement. In assays using low bacterial densities, the flagellin-specific antibody lost its immobilization capability, whereas HD6 activity on individual swimming *S.* Typhimurium was not diminished. Collectively, our data support a model where HD6 can bind to the flagella of individual bacteria and utilize a self-assembly process that ultimately arrests movement and renders the bacterium immobilized.

Spatial segregation of the microbial-colonized lumen from the epithelium is necessary at mucosal sites for the host to maintain a balance between the flourishing microbiota and a

healthy mucosa [5,13,18,57,58]. However, this homeostatic spatial segregation is threatened by flagellated microbes, because of their ability to travel through mucus and gain close proximity to the epithelium [22,52,59]. A site likely critical for the host to limit microbial access is the base of the small intestinal crypts, where stem cells that renew the intestinal epithelium reside [60]. Paneth cells are located immediately adjacent to this vulnerable stem cell niche, and so ideally positioned to secrete molecules to thwart microbial trespassers [32,33,60]. As one of the two α-defensins constitutively expressed by human Paneth cells, HD6 secreted into the crypt could immobilize and restrict the movement of individual flagellar-propelled microbes that had traversed mucus to access the crypt microenvironment.

The distinct mechanism of HD6 activity represents a significant departure from the typical microbicidal activity seen with its counterparts in the α-defensin family [36,43-45,61]. Most microbicidal α-defensin have a high degree of sequence variation from their orthologs, likely due to the biophysical properties responsible for membrane-directed activity being tolerant to amino acid substitutions [61]. In contrast to this majority of α-defensins, HD6 in its mature and folded confirmation, at concentrations 20-fold higher than those used in the current study, lacks demonstrable microbicidal activity *in vitro* [43-45,62]. Also special to HD6 is that it shares near sequence identity with its non-human primate orthologs (Suppl. Fig. 2). Given that HD6 has a unique mechanism whereby it self-assembles into oligomers to prevent microbial invasion of the intestinal epithelium, the high conservation of sequence may reflect requirements for its ability to self-assemble. Chairatana and Nolan demonstrated that HD6 self-assembly is sensitive to mutation, with perturbation hydrophobic residues F2, I22, V25 and F29 preventing self-assembly and attenuating biological activity [45]. They reported that an F2A mutation, in

particular, does not form fibrils nor prevent bacterial invasion of epithelial cells, consistent with the SPR findings and attenuation of biological activity we report here.

Although HD6 is a remarkable outlier within the α-defensin family, we propose that it is part of an integral arm of the innate immune system tasked with maintaining mucosal barrier integrity through recognition and inhibition of flagellar motility. In addition to molecular sensors that detect flagellin, including Toll-like receptor 5, nucleotide-binding domain leucine-rich repeat (NLR)-C4, and NLR family, apoptosis inhibitory protein (NAIP)-5 and -6 [28,63,64], an effector protein in the intestine is LYPD8, a 25 KDa colonocyte-derived protein capable of inhibiting bacterial flagellar motility by binding to a non-flagellin part of the flagellum [65,66]. In addition, akin to the action of flagellin-specific antibody, ZG16 is a lectin produced by goblet cells that can inhibit bacterial motility through a mechanism of agglutination [67]. Together, this evidence of redundancy employed by the host to recognize and inhibit microbial motility suggests these specialized effector peptides and proteins target a critical function not required for microbial replication. As such, this may highlight the value of defense strategy aimed at inhibiting a specific microbial behavior, instead of microbial eradication, which in turn would promote selection for resistance and lead to reciprocal antagonistic adaptation, the so-called "Red Queen effect" [68,69]. A non-sterilizing immune strategy targeting flagellar motility could exert host-beneficial influence not only on the behavior of pathogenic microbes, but also on members of the colonizing microbiota.

Methods

Bacterial Cultures

Cultures of *S.* Typhimurium (Table S1), strain IR715, were incubated in 5ml of Miller's Luria Broth (BD 248510) under aerobic conditions at 37^{o}C with shaking. *S.* Typhimurium containing the GFP plasmid (pDwb JK1128) were grown with carbenicillin (50 µg/ml). Cultures of ΔFliCΔFljB *S.* Typhimurium (SPN313) were additionally supplemented with kanamycin (30 µg/ml). Subcultures used in microscopy and semi-solid swimming experiments were created by inoculating Miller's Luria broth (5 ml) with the overnight culture (50 µl) and the incubating at 37^{o}C with shaking until the broth had an OD_{600} of 0.2-0.4 (~2 hours).

To create working dilutions of bacteria, the subcultures of *S.* Typhimurium were spun at 4,000 RPM for 5 minutes in 1.5ml microcentrifuge tubes. After the centrifugation, the media supernatant was decanted, and the pellet was resuspended in sterile Tris-Maleate buffer (50mM, pH 6.4), with the volume adjusted to yield the desired bacterial concentration. In microscopy experiments requiring multiple different bacterial concentrations, serial dilutions in Tris-Maleate buffer (50mM, pH 6.4) were prepared. During the process resuspension and dilution of *S.* Typhimurium, mixing utilized gentle pipetting with care to avoid mechanical sheering of flagella from the bacteria.

Peptides and Antibody

$HD6^{Native}$ and $HD6^{F2A}$ were synthesized, folded, verified, and lyophilized as described previously [70,71]. Stock solutions of HD6 peptides were made by dissolving lyophilized peptides in HPLC-grade H_2O to a concentration of 1 mg/ml. For long term storage, HD6 peptide stock solutions were aliquoted into microcentrifuge tubes, snap frozen, and stored at -80°C.

The antibody used for the agglutination microscopy was a monoclonal mouse IgG1 specific for the FliC of *S.* Typhimurium, clone X5A12 (InvivoGen, San Diego, CA, Cat# mabg-flic). Stock solutions were created by dissolving lyophilized antibody in HPLC grade H_2O. Antibody stocks were aliquoted into microcentrifuge tubes, snap frozen, and stored at -80°C.

Semi-solid Agar Motility Assay

Cultured *S.* Typhimurium was suspended in Tris-Maleate buffer (50 mM, pH 6.4) at 1e7 CFU/ml. The semi-solid agar plates were composed of Tris-Maleate buffer (50 mM, pH 6.4), 0.2% w/v casamino acids (BD bacto-tryptone 211705), and 0.3% w/v agar (BD bacto-agar 244520), and prepared the day prior to the experiment. To the center each plate, an aliquot (20µl) was added of either HD6, mouse IgG1 antibody, or Tris-Maleate buffer (50 mM, pH 6.4, as vehicle control). Next, the center of each plate was inoculated with an aliquot (3 µl) of the *S.* Typhimurium suspension. Plates were then incubated at 37°C for 6.5 hours. After incubation, the plates were photographed using a gel imager (UVP, Upland, CA, BioDoc-It Imaging system). The bacterial motility/swimming parameters were assessed for each plate using ImageJ. Thus, for each image, a circular mask of swimming bacteria was generated using the “Threshold” and

"Find Edges" tools. From this mask, the diameter of the circle of swimming bacteria was then measured.

Live-Fluorescence Microscopy

Timelapse fluorescence microscopy of live bacteria was performed using a Nikon Ti-E inverted fluorescence microscope at 37°C. Microscopy experiments were performed using a MATLAB interface for Micromanager to enable custom, fully automated imaging. All images were acquired via epifluorescence illumination using a 20x Nikon apochromat 0.75 NA objective, the X-Cite Xylis LED illumination system, and an Andor Zyla 4.2 sCMOS camera. Experimental assays utilized Cellvis 96-well glass bottom plates (Cellvis, Mountain View, CA). Prior to imaging, buffer and peptide solutions were added to each well and allowed to equilibrate to 37°C in the microscopy chamber for ~30 minutes. After equilibration, each well was inoculated with the working bacterial suspension containing GFP-expressing *S.* Typhimurium immediately prior to the start of the experiment. Time course experiments spanned 120 minutes, and entailed 20 images acquired in rapid succession at 20-minute intervals for each experimental well (T=0, 20, 40, 60, 80, 100, and 120 minutes). Exposure times were either 100 milliseconds or 300 milliseconds, for short and long exposure experiments, respectively.

Agglutination Assay

Prior to imaging, bacterial suspensions containing 5e8 CFU/ml of GFP-expressing *S.* Typhimurium were incubated with either HD6 (50 μg/ml), α-FliC antibody (100 μg/ml), or Tris-

Maleate buffer (50mM, pH 6.8) at 37°C in microcentrifuge tubes (0.5 µl) to allow agglutination. After 30 minutes, 20 µl of this mixture was added to the microscopy plate wells containing to 80 µl of Tris-Maleate buffer (50mM, pH 6.8) equilibrated to 37°C. The final concentrations at the time of imaging were 1e8 CFU/ml GFP-expressing *S.* Typhimurium and either 10 µg/ml of HD6 or 20 µg/ml of antibody.

Bacterial Tracking Assay

To observe the effects on motility of either HD6 or α-FliC antibody at a lower *S.* Typhimurium density, wells containing 5e6 CFU/ml of GFP-expressing *S.* Typhimurium and either HD6 (10 µg/ml) or of monoclonal antibody (20 µg/ml) were analyzed. An equal volume of Tris-Maleate buffer (50mM, pH 6.8) was used as a control. Experimental wells containing solely 5e6 CFU/ml GFP-expressing *S.* Typhimurium did not undergo the 30-minute preincubation step. Imaging was conducted over the course of 120 minutes in the same manner as described above.

Immobilization Assay

To observe concentration dependence of HD6-mediated bacterial immobilization, wells containing 1e7CFU/ml GFP-expressing *S.* Typhimurium were treated with 0.5, 5.0, and 50.0 µg/ml of native HD6 or $HD6^{F2A}$ diluted in Tris-Maleate buffer (50 mM, pH 6.4) buffer. An equal

volume of Tris-Maleate buffer (50mM, pH 6.8) served as a control. Imaging was conducted over the course of 120 minutes as described above.

Image Segmentation

Image processing and segmentation to identify cells for immobilization quantification were performed in MATLAB. First, background subtraction was performed on each frame to remove out-of-focus cells using the MATLAB “imopen” function with a disk-shaped structuring element and radius of 20 pixels (~13.2 microns). Next, sharpening was applied to each frame using "unsharp masking" to enhance cell edges. A single pixel intensity threshold was then determined both for each well and timepoint using the first frame of each timepoint as a reference. Accordingly, following background subtraction and sharpening of the reference frame, a crude background mask was generated by masking pixels in the 98^{th} percentile of intensity (empirically determined to generally include only bright cell pixels), applying a slight dilation using a disk-shaped structuring element and radius of 1 pixels (~0.66 microns), and then taking the complement of the mask using the MATLAB “imcomplement” function. The crude background mask was applied to the reference frame, and a final intensity threshold was determined based on the 99^{th} percentile of the remaining background pixels. For each frame of the well at the given timepoint, and following background subtraction and sharpening, cell masks were generated by applying the calculated intensity threshold, and followed by removal of small non-cell objects ($< 3^2$ microns in area) using the MATLAB "bwareaopen” function.

Immobilization Analysis

A custom strategy was used to identify immobilized objects at each timepoint. For each twenty-frame time-course, the cell masks at frames 1, 10, and 20 were added together to create a composite image with a maximum value of "3" at locations where cells were present in all three frames. Objects in the composite image containing at least 50% of pixels with values of 3 were considered immobilized and were retained in the immobilization mask for that timepoint. All other objects were removed.

After an immobilization mask was created at each timepoint, immobilized objects were quantified using ImageJ. The number of immobilized objects was measured using the ImageJ "analyze particles" function. The total area of immobilized bacteria was calculated using the ImageJ "measure" function, which determines the number of pixels containing a positive signal.

Motility Analysis

The ImageJ plugin Trackmate was used to track the movement of bacteria within the well and obtain data on their movement [54,55]. Movement tracking was performed on wells containing a 5e6 CFU/ml density of *S.* Typhimurium at the 60-minute timepoint.

Raw videos were analyzed with Trackmate. The DoG detector was used to identify individual bacteria within the well. For all samples in a given experiment, the DoG detector parameters were as follows: estimated object diameter of 5, quality threshold of 3, and both pre-processing with median filter and sub-pixel localization turned on. Once bacteria were

detected, the Trackmate "Kalman Tracker" was used to link the positions of the same bacteria across different frames of the video, thus producing a track corresponding to its movement. The Kalman Tracker parameters used for all samples within a given experiment were as follows: initial search radius of 25, search radius of 25, and max frame gap of 2. When necessary, incorrect spot detection and spot linking were manually corrected.

Once correct tracking of the bacteria was achieved, measurements of individual tracks were collected. These metrics included: total track length, track mean and median velocity, track duration, and total track displacement. We observed that incorrect tracking often produced a track with a low track duration. Thus, tracks with a track duration of less than or equal to 2 were omitted from the dataset.

Analysis of the track data was performed using Graphpad Prism. In order to differentiate between swimming and non-swimming bacteria, the median velocity metric was chosen to analyze the tracks. The velocity was calculated by taking the total distance travelled for a track, and then dividing by the total duration of the track, thus normalizing for variations in the duration of tracks. To create a threshold to identify bacteria that are not swimming, tracking was performed on wells containing 5e6 CFU/ml ΔFliCΔFljB *S.* Typhimurium. These bacteria lack flagella, so any movement observed is due to Brownian motion. The median velocities of these tracks were pooled, and the maximum value was chosen as a threshold for the non-swimming bacteria. This threshold was applied to experimental groups to generate a ratio of swimming to non-swimming bacteria.

Surface Plasmon Resonance

Surface plasmon resonance studies were performed as described previously [43]. Amine coupling was used to ligate either HD6 or flagellin to individual flow cells on a CM5 sensor chip. The running buffer (pH 7.4) contained: 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and surfactant P20 (0.005 %). The multi-cycle experiments were performed with an analyte flow rate of 20 µl/min for 12.5 min in each cycle. This was followed by a 2.5 min interval during which the flow cells were perfused by analyte-free buffer until the sample loop had acquired the next 250 µl aliquot of analyte solution. The final analyte injection was in "kinetic mode", with a 10 min dissociation period.

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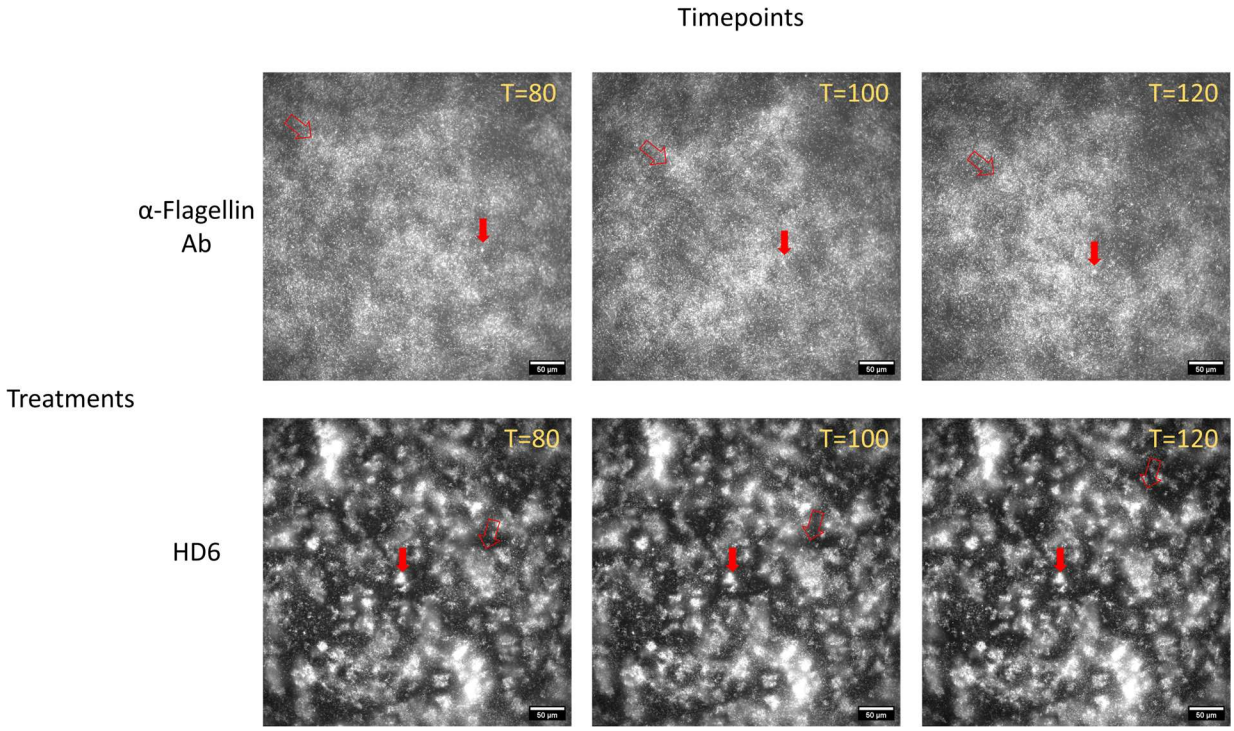
recognition to intestinal inflammation during Salmonella enterica serotype typhimurium infection. Infect Immun *77*, 1904-1916. 10.1128/iai.01341-08.

Supplemental Figures

Supplemental Figure 1



Supplemental Figure 1| Live-fluorescence microscopy analysis of α-Flagellin Ab- and HD6-mediated *S.* Typhimurium agglutination. (**A)** Representative unmasked composite data depicting agglutinated *S.* Typhimurium at high densities (1e8 CFU/ml) in the presence of either 20 µg/ml α-Flagellin Ab (top row) or 10 µg/ml HD6. Areas of transiently higher bacterial density (open red arrow) and high-density bacterial aggregates (closed red arrow) are highlighted as examples of agglutination occurring. Data are representative of 2 independent experiments, each with technical replicates. Scale bar, 50 µm.

Supplemental Figure 2

HD6 Orthologs

```
                    F  C  C    C           C  V   I    FCC
Human               AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL    ---
Chimpanzee          AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL    100%
Gorilla             AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL    100%
Orangutan           SFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL     97%
Silvery Gibbon      SFTCHCRRSCFSTEYSHGTCTVAGVNHRFCCL     85%
NWC Gibbon          SFTCHCRRSCFSTEYFHGTCTVAGVNHRFCCL     81%

Green Monkey        SFTCNCRRSCYSTENSYGTCTIMGVTNRFCCL     82%
Gelada              SFTCNCRRSCYSTEYSYGTCTVMGVSNRFCCL     84%
Rhesus Macaque      SFTCNCRRSCYSTEYSYGTCTVMGVSNRFCCL     84%
Drill               SFTCNCRRSCYSTEYSYGTCTVMGVSNRFCCL     84%
Sooty Mangabey      SFTCNCRRSCYSTEYSYGTCTVMGVSNRFCCL     84%
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HD5 Orthologs

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                      C  CR  G  C        G  CE         LCC
Human                 ATCYCRTGRCATRESLSGVCEISGRLYRLCCR    ---
Chimpanzee            ATCYCRIGHCTILESLSGVCEISGRLYRLCCR     85%
Gorilla               ATCYCRTGPCTNRESLSGVCEISGRLYRFCCR     88%
Orangutan             STCYCRIGLCAAIESYSGRCYINGRRYRLCCR     69%
Silvery Gibbon        STCYCRTGRCAAIESYSGRCYINTRRYRLCCR     72%
NWC Gibbon            STCYCRSGLCAAIESYSGRCYISGRRYRLCCR     72%

Green Monkey          -TCYCRTGRCSRRETYSGSCNINGRLSNLCCR     69%
Gelada                STCYCRTGKCFWRESSAGWCNINGRIYRLCCR     69%
Rhesus Macaque        -TCYCRTGRCYTPEFHSGKCVFNGRTYKLCCR     66%
Drill                 -TCYCRTGRCYTPEFHSGKCLFKGRTYKLCCR     59%
Sooty Mangabey        -TCRCRFGRCSRRESFSGSCNINGRIFSLCCR     63%
```

Supplementary Figure 2| Primary amino acid comparison of HD5 and HD6 orthologs. Highlighted with YELLOW are sequence variant residues for the HD5 and HD6 orthologs, respectively. Shown in RED are the cysteine residues that participate in three intramolecular disulfide bonds (Cys^1-Cys^6, Cys^2-Cys^4, Cys^3-Cys^5). Highlighted with GRAY in the top consensus row are the residues identified as key for structure-function of HD6 and HD5, respectively (Chairatana and Nolan 2014; Rajabi et al. 2012). The column at the left notes the percent of residue identity for HD6 and HD5 orthologs, respectively. Within each group of orthologs, the top cluster includes great apes and the bottom Old World monkeys. Accession numbers for HD6 ortholog sequences are: Human (*Homo sapiens*), AAC50382; Chimpanzee (*Pan troglodytes*), NP001029079; Gorilla (*Gorilla gorilla*), XP004046638; Orangutan (*Pongo abelii*), XP002818825; Silvery Gibbon (*Hylobates moloch*), XP032015057; Northern White Cheek (NWC) Gibbon (*Nomascus leucogenys*), XP003271463; Green Monkey (*Chlorocebus sabaeus*), XP007959810; Gelada (*Theropithecus gelada*), XP025250382; Rhesus Macaque (*Macaca*

mulatta), XP001098733; Drill (*Mandrillus leucophaeus*), XP011851075; Sooty Mangabey (*Cercocebus atys*), XP011933395. Accession numbers for HD5 ortholog sequences are: Human, NP066290; Chimpanzee, NP001012657; Gorilla, XP004046645; Orangutan, XP002818829; Silvery Gibbon, XP032015053; NWC Gibbon, XP003271465; Green Monkey, XP008017319; Gelada, XP025230170; Rhesus Macaque, AY859406; Drill, XP011851059; Sooty Mangabey, XP011890938.

Table S1. Bacterial Strains.

Strain ID	Relevant Genotype	Reference
IR715	ATCC 14028, Nal^R derivative	[72]
SPN313	IR715 Δ*fliC*(-25 to +1494) *fljB*5001::Mu*d*Cm	[73]
JK1128	IR715 pDwbJK1128, GFP-expression plasmid	This work.
DA1	SPN313 pDwbJK1128, Δ*fliC fljB* with GFP expression	This work.

Supplemental Videos

Video S1 *S.* Typhimurium (5e6 CFU/ml) in buffer alone (tris-maleate pH 6.4), related to figure 2B.

Video S2 ΔFliCΔFljB STM *S.* Typhimurium (5e6 CFU/ml) in buffer alone (tris-maleate pH 6.4), related to figure 2C.

Video S3 High-density *S.* Typhimurium (1e8 CFU/ml) in 20 μg/ml α-flagellin Ab, related to supplemental figure 1.

Video S4 High-density *S.* Typhimurium (1e8 CFU/ml) in 10 μg/ml HD6, related to supplemental figure 1.

Video S5 High-density *S.* Typhimurium (1e8 CFU/ml) in buffer alone (tris-maleate pH 6.4), related to supplemental figure 1.

Video S6 *S.* Typhimurium (5e6 CFU/ml) in buffer alone (tris-maleate pH 6.4), related to figure 2D.

Video S7 *S.* Typhimurium (5e6 CFU/ml) in 10 μg/ml HD6, related to figure 2D.

Video S8 *S.* Typhimurium (5e6 CFU/ml) in in 20 μg/ml α-flagellin Ab, related to figure 2D.

Video S9 *S.* Typhimurium (1e7 CFU/ml) in buffer alone (tris-maleate pH 6.4), related to figure 3B.

Video S10 *S.* Typhimurium (1e7 CFU/ml) in 0.5 μg/ml HD6, related to figure 3B.

Video S11 *S.* Typhimurium (1e7 CFU/ml) in 5 μg/ml HD6, related to figure 3B.

Video S12 *S.* Typhimurium (1e7 CFU/ml) in 50 μg/ml HD6, related to figure 3B.

Video S13 *S.* Typhimurium (1e7 CFU/ml) in buffer alone (tris-maleate pH 6.4), related to figure 4D.

Video S14 *S.* Typhimurium (1e7 CFU/ml) in 0.5 μg/ml $HD6^{F2A}$, related to figure 4D.

Video S15 *S.* Typhimurium (1e7 CFU/ml) in 5 μg/ml $HD6^{F2A}$, related to figure 4D.

Video S16 *S.* Typhimurium (1e7 CFU/ml) in 50 μg/ml $HD6^{F2A}$, related to figure 4D.

Statement of Author Contribution

D.T.A., C.L.B. formulated the original hypothesis and designed the study; D.T.A., D.E.N., W.Y. performed research; D.T.A., D.E.N. designed microscopy protocols; W.L., S.R.C., C.L.B. supervised research and provided funding; all authors analyzed the data; D.T.A. and C.L.B. wrote the paper; all authors commented on the manuscript, data, and conclusions.

Chapter 3

A Prospective Look at Future HD6 Research

Introduction

There are multiple avenues for future research to explore the role the intestinal antimicrobial peptide human α-defensin 6 (HD6) plays in host-microbe interactions and intestinal homeostasis. The collective body of work on HD6 has elucidated many details of HD6 function and activity, yet there are still aspects of HD6 biology that remain unknown. Certain areas have yet to be investigated, including the microbial properties that permit HD6 activity, the effect HD6 exerts on the flagellated members of the commensal microbiota, and the role of HD6 in modulating the adaptive immune system. In this chapter, background research and preliminary data will be presented to outline theoretical approaches to these questions, along with important considerations for experimental design.

Spectrum of HD6 Activity

To date, studies specifically investigating HD6 interactions with flagellated bacteria have predominately used *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) [1]. This is due in part to the structure and regulation of *S.* Typhimurium flagella being well defined [2,3]. A particular advantage of working with S. Typhimurium is their consistent expression of flagella under diverse conditions, making it a particularly convenient bacterium for use in motility experiments [4-6]. Thus, S. Typhimurium has been an instrumental model for the advancement of

our understanding of flagella-dependent agglutination and inhibition of motility by HD6 and other immune factors [1].

The highly conserved structure of the flagellin protein and the flagellar motor within the kingdom Bacteria suggests that HD6 would have similar activity against a wide spectrum of flagellated bacteria [7]. However, the arrangement of flagella on the bacterial surface and the post-translational modifications made to flagellin are key features that could impact HD6 binding and activity against different bacteria. Here, different examples of bacterial flagellar arrangement and post-translational glycosylation will be discussed, along with predictions on how they might influence HD6 function.

Flagellar Arrangement

Many bacteria utilize the flagella motor for locomotion but differ in the way number and organization of flagella across their surface [8]. *S.* Typhimurium, *Escherichia coli* (*E. coli*), and *Listeria monocytogenes* (*L. monocytogenes*) are all examples of peritrichous bacteria, meaning each bacterium has many flagella distributed across the entire surface [2,8]. Synchronous counterclockwise rotation of all the flagella creates a polar flagellar bunder, which facilitates directional locomotion [2]. Compared to other flagellar arrangements, the peritrichous bacteria generally have a higher number of flagella, which can range upwards of 10 or more flagella per cell [9,10].

Monotrichous bacteria represent the opposite end of the spectrum, featuring a single flagellum located at a polar end of the bacterium. While this arrangement provides

monotrichous bacteria with a comparable degree of motility to the peritrichous bacteria, it does so with a significant reduction in the amount of flagellin epitopes present on the bacterial surface. Interestingly, bacterial agglutination experiments using HD6 found that loss of either flagella or fimbriae (both targets of HD6) on the surface of S. Typhimurium led to reduced agglutination activity [1]. From this, one could conclude that the magnitude of HD6 activity is dependent on the density of ligands on the bacterial surface. Consequently, HD6 may have a reduced capacity to bind and affect the motility of monotrichous bacteria because of their lower flagella density. A notable example of a monotrichous bacterium is *Pseudomonas aeruginosa* (*P. aeruginosa*), an opportunistic intestinal pathogen that utilizes its flagellum during its pathogenesis [8,11]. Given the abundance of molecular tools and well-defined motility characteristics for *P. aeruginosa*, it is a good candidate to study the impact of decreased flagellar density on HD6-mediated inhibition of bacterial motility. Similar to monotrichous bacteria, there is a limited presence of flagella on amphitrichous bacteria, which possess only two flagella per bacterium that are arranged with one at either pole. Amphitrichous bacteria present an additional option to explore the relationship between the number of flagellar epitopes on the bacterial surface and the magnitude of HD6 activity. The small intestinal human pathogen *Campylobacter jejuni* (*C. jejuni*) possesses this flagellar arrangement, also making it a potential candidate for study with HD6 [8]. To date, published experiments analyzing HD6 activity against flagellated bacteria have been performed using peritrichous bacteria. Therefore, using bacteria with alternate forms of flagellar arrangement, particularly ones resulting in a lower presence of flagella on the surface, will broaden our understanding and help to define the spectrum of HD6 activity towards flagellated microbes.

Glycosylation

Post-translational glycosylation of flagellin is another factor to consider when determining the spectrum HD6 activity. Chu et al. found that HD6 was unable to bind to gp120, a highly glycosylated HIV protein [1]. However, HD6 was able to bind and self-assemble to enzymatically deglycosylated gp120, suggesting that O- and/or N-linked glycosylation interfere with HD6 binding [1]. Additionally, data demonstrating that HD6 binds to flagellin isolated from S. Typhimurium combined with the observation that the flagellin of S. Typhimurium is not glycosylated provides circumstantial evidence supporting the hypothesis that HD6 targets non-glycosylated proteins [12]. In contrast to *S.* Typhimurium, there are a variety of bacteria that do post-translationally glycosylate their flagella through both O- and N-linkages [8]. Thus, these alternative microbes can be used to directly interrogate if the post-translational glycosylation of flagellin is sufficient to prevent HD6 binding and subsequent activity.

Regarding bacterial post-translational glycosylation, *C. jejuni* has one of most thoroughly studied glycosylation pathways [13-15]. *C. jejuni* is known to heavily glycosylate its flagellin through O-linkages, which accounts for approximately 10% of the total mass of the flagellin protein [16]. Thus, one could predict that HD6 would not be able to bind to *C. jejuni* flagellin, which would prevent any agglutination or motility inhibition. Although non-glycosylated mutants are not available due to the necessity of O-linked glycosylation for C. jejuni to form a stable flagellar filament, observing the effects of HD6 on WT C. jejuni motility would still provide valuable insight into the importance of flagellin glycosylation [8,17].

Alternatively, P. aeruginosa is also know to post-translationally glycosylate flagellin. Like *C. jejuni*, the glycans are attached to *P. aeruginosa* flagellin through O-linkages, although with significantly fewer sites [18]. However, these sites are in the variable region of flagellin, which is exposed to the environment and would be more likely to interact with HD6 [18]. Importantly, *P. aeruginosa* do not require post-translational glycosylation of flagellin to express a stable flagellum, evidenced by the existence of motile mutant strains that lack the enzymes required for glycosylation [19]. Thus, *P. aeruginosa* is an attractive choice to explore whether flagellin glycosylation prevents HD6 binding and activity.

HD6 Effects on Non-Flagella Targets

Flagella are not the only large, multi-subunit protein structure found on bacterial surfaces. Fimbriae, pili, and bacterial secretion systems are also present on many bacteria. Initial characterization of HD6 showed that HD6 is capable of causing the agglutination of flagellin-deficient *S.* Typhimurium due to their expression of fimbriae, multi-functional bacterial surface-protein structures that coat the bacterium [1]. This retention of agglutination activity suggests that HD6 is capable of targeting non-flagellar epitopes. Indeed, live-cell microscopy experiments revealed that HD6 can immobilize flagellin-deficient *S.* Typhimurium (DNS).

Some human pathogens, notably Enteropathogenic *Escherichia coli* (EPEC), utilize fimbriae to adhere to intestinal epithelial cells leading to severe pathology. Interestingly, fimbriae-specific antibodies are capable of blocking bacterial adhesion, likely through a mechanism of agglutination or neutralization [20]. Together, these observations led us to

hypothesize that HD6, like antibody, might similarly protect the host by targeting fimbriae and preventing pathogens from attaching to epithelial cells through agglutination or the neutralization of fimbriae on individual bacteria.

The HD6 transgenic C57Bl/6 mouse is a useful model to study HD6 activity *in vivo*. While mouse models of human EPEC infection do exist, recapitulation of the symptoms and pathogenesis seen in humans typically require alteration of the microbiota to facilitate infection with non-natural mouse pathogens [21]. These models often require the use of antibiotics, which may introduce confounding variables based on microbiota and physiological disruption. Alternatively, *Citrobacter rodentium* (*C. rodentium*) is a natural mouse enteric pathogen that employs a similar adhesion strategy to EPEC and can induce similar symptoms observed during human EPEC infections [22]. Additionally, *C. rodentium* is a naturally non-flagellated bacteria, which allows for observations into HD6 effects specifically on non-flagella targets.

Based on these factors, pilot experiments exploring whether HD6 can provide protection from non-flagellated, fimbriated bacterial pathogens were conducted utilizing *C. rodentium*. The infection model entailed inoculating singly housed HD6 transgenic mice, or wild type control mice, with *C. rodentium* strain DBS100 via water bottle. Mice were singly housed to reduce any effects that may occur from HD6 transfer due to coprophagic behavior. Throughout the course of the infection, body weight changes were recorded, and fecal samples were routinely collected for bacterial enumeration. From the time of inoculation, it takes approximately 3 weeks for the mouse to fully clear the infection, with peak bacterial burden around 5-7 days post infection that corresponds with peak weight loss [22]. Contrary to our initial

hypothesis, no significant differences were observed between HD6 TG and WT bacterial burden throughout the course of infection (Figure 1).

Of course, these results could indicate that HD6 does not affect fimbriae-mediated bacterial adhesion. However, the observations made with non-flagellated *S.* Typhimurium still indicates HD6 can affect fimbriated bacteria. The lack of protection seen during *C.* rodentium infection could be a consequence of *C.* rodentium mainly colonizing the cecum, with virtually no presence in the small intestine. Since HD6 secretion is restricted to Paneth cells in the small intestine, any effect of HD6 in the distal intestinal compartments of the cecum and colon would need to be a result of downstream movement of intestinal contents. It is currently unknown

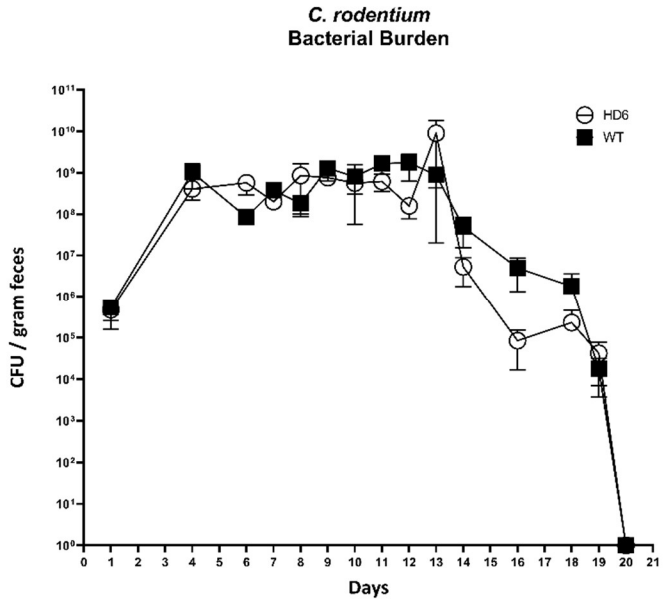

Figure 1| *C. rodentium* infection kinetics of WT vs HD6 TG mice. Graph depicting the colony forming units of *C. rodentium* per gram of feces in WT (closed squares) or HD6 TG (open circles) mice over the course of infection. Limit of detection (LOD) depicted by dashed line. Data from one experiment, N=7 mice, HD6 TG group n=3, WT group n=4 (Error bars, mean ± SD).

whether HD6, and other small intestine-restricted antimicrobials are able exert an effect in distal compartments. Thus, *C.* rodentium infection may not be an optimal model to observe HD6 activity on fimbriated bacterial pathogens.

HD6 Effects on Flagellated Commensal Microbes

While flagellar motility is often associated with pathogens, members of the commensal microbiota also contain the genes necessary to synthesize a functional flagellum [23]. During homeostatic conditions, the host exerts pressure on the commensal microbiota leading to the minimal expression of flagellar genes [23]. Work by Cullender et al. found that in mice, this pressure originates from secreted antibody specific to commensal flagellin [23]. The downregulation of flagellar genes was hypothesized to be a bacterial mechanosensory response to the direct binding of antibody to flagellar structures, functionally resulting in the reduced motility of commensal microbes and stable segregation of microbes to the border of the outer and inner mucus layer [23]. The flagellin-specific antibody response was driven by toll-like receptor 5 (TLR5), a pathogen recognition receptor that recognizes bacterial flagellin [23,24]. TLR5 has been implicated in enhancing both B cell and T cell responses to flagellin, attributed to its role as an endocytic receptor for antigen presenting cells (APC) [23,25,26]. In mice lacking TLR5 expression, there is a significant decrease in commensal flagellin-specific antibody and a subsequent increase in flagellated commensal bacteria [23]. Thus, the TLR5:antibody axis in the intestinal tract plays a key role in suppressing the commensal microbiota's expression of flagella and maintaining spatial segregation [23].

A Modified Mouse Model for HD6 Activity

Since HD6 is highly expressed in the small intestine and is able to bind bacterial flagella, we hypothesized it might regulate flagellated commensal microbes, similar to flagellin-specific antibody, However, the presence of flagellin-specific antibody in the mouse would obfuscate any potential effects HD6 may have on flagellated commensal microbes. As a solution, the HD6 transgene can be transferred onto a $TLR5^{-/-}$ background, which would generate a luminal environment absent of flagellin-specific antibody where the effects of HD6 on flagellated commensal microbes could be observed.

The desired genotype, hemizygous HD6 transgene ($HD6^{Tg/null}$) and homozygous TLR5 knockout allele ($TLR5^{-/-}$), can be achieved through breeding within two generations (Figure 2). The C57BL/6J-Tg(DEFA6)1Hc mouse line would be the HD6 transgene donor, while the C57BL/6J-$TLR5^{Tm1Flv}$ mouse line would be the TLR5 knockout allele donor [1,27]. The first breeding pair would consist of a F_0 $HD6^{Tg/null}$ $TLR5^{+/+}$ male and a F_0 $HD6^{null/null}$ $TLR5^{-/-}$ female (Figure 2, Top Row), which will spawn a F_1 generation that includes either $HD6^{Tg/null}$ $TLR5^{+/-}$ or $HD6^{null/null}$ $TLR5^{+/-}$ genotypes. From this F_1 generation, a $HD6^{Tg/null}$ $TLR5^{+/-}$ male can be crossed with a $HD6^{null/null}$ $TLR5^{-/-}$ female (Figure 2, Middle Row) to produce an F_2 generation that can be used for experiments.

The F_2 generation will be composed of 4 possible genotypes: $HD6^{Tg/null}$ $TLR5^{+/-}$, $HD6^{Tg/null}$ $TLR5^{-/-}$, $HD6^{null/null}$ $TLR5^{+/-}$, $HD6^{null/null}$ $TLR5^{-/-}$ (Figure 2, Bottom Row). The $HD6^{null/null}$ $TLR5^{+/-}$ genotype will likely possess a WT phenotype and can be used for a control group. Similarly, the $HD6^{null/null}$ $TLR5^{-/-}$ genotype will act as a control group to show the effects of the absence of anti-

flagellin antibody without HD6 expression. The HD6$^{Tg/null}$ TLR5$^{+/-}$ genotype will likely present with a HD6$^{Tg/null}$ TLR5$^{+/+}$ phenotype. Finally, the HD6$^{Tg/null}$ TLR5$^{-/-}$ will act as the main experimental group, displaying a phenotype resulting from HD6 transgene expression in the absence of anti-flagellin antibody. It will be necessary to perform a functional validation of the TLR5$^{+/-}$ genotype to confirm that it presents with a wild type (WT) phenotype. Confirmation could be done *in vitro* by stimulating cells isolated from either TLR5$^{+/-}$ or TLR5$^{+/+}$ mice with flagellin and comparing their cytokine production [27].

Special care should be taken when designing a breeding scheme using the C57BL/6J-Tg(DEFA6)1Hc mouse line. Unlike targeted mutation, the insertion of the HD6 transgene into the mouse genome is not site specific, and results in the tandem insertion of multiple transgene copies at a random locus. If the HD6 transgene always maintained as hemizygous, the number of transgene copies will remain stable. However, if the HD6 transgene is homozygous in a mouse, homologous recombination during meiosis has the potential to cause multiple copies of the HD6 transgene to be deleted and alter HD6 transgene expression in its progeny. To preserve the HD6 transgene copy number in the C57BL/6J-Tg(DEFA6)1Hc line, two carriers of the HD6 transgene should never be mated. Although highly unlikely, it may be prudent to sequence the *TLR5* locus of the C57Bl/6J-Tg(DEFA6)1Hc mouse line to confirm that the HD6 transgene was not inserted at that locus, where it could potentially disrupt the gene independent of the TLR5^{tm1Flv} knockout allele.

Due to the phenotypes relating to both antibody and microbiota, care should be taken when planning housing and parental sex. Performing both crosses with a TLR5$^{-/-}$ dam would likely be optimal, since a dam with functional TLR5 would have the potential to vertically transmit flagellin-specific antibody or flagellin-suppressed microbiota to offspring. This transfer could have a confounding effect on the phenotypes by altering the phenotype of the TLR5$^{-/-}$ offspring.

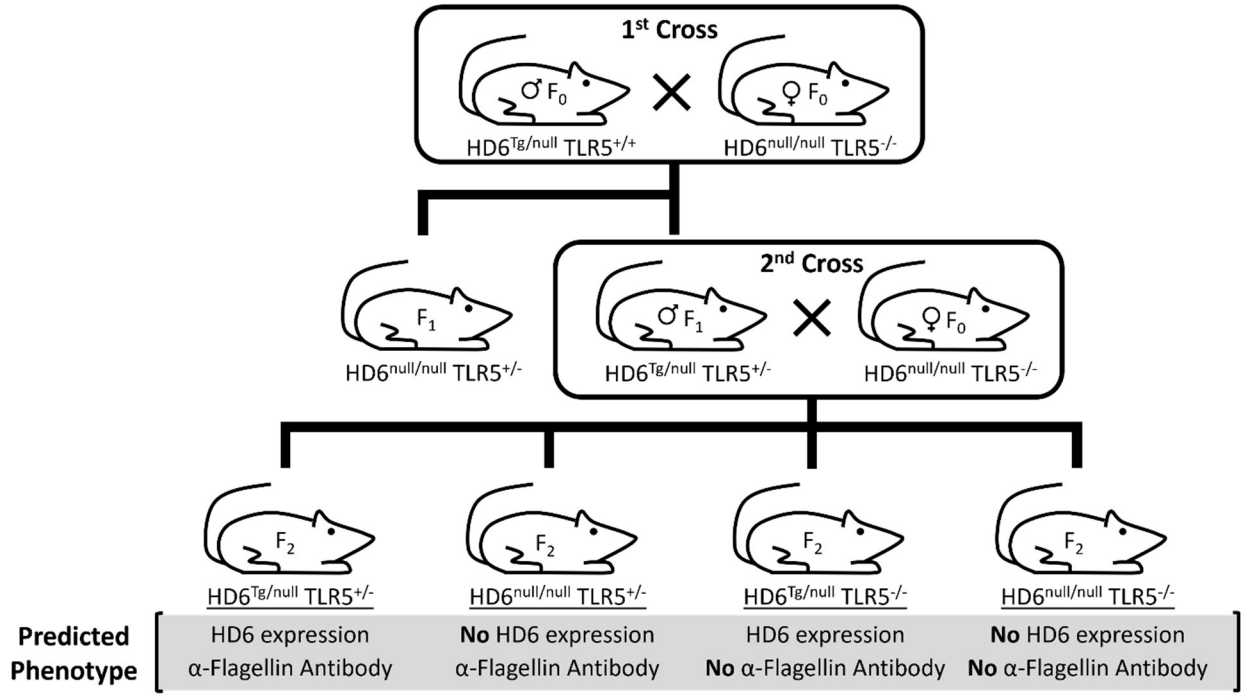

Figure 2| Breeding scheme to transfer the HD6 transgene onto a TLR5$^{-/-}$ background. (**Top Row**) Depiction of F_0 progenitor mating pair, with a male mouse hemizygous for the HD6 transgene (HD6$^{Tg/null}$) and a female mouse homozygous for the TLR5 knockout allele (TLR5$^{-/-}$). (**Middle Row**) Depiction of the F_1 generation and the second breeding pair (box). (**Bottom Row**) Depiction of the F_2 generation which can be used for experiments. The predicted phenotypes are displayed below each genotype.

Measuring Flagella Expression

After generating a HD6$^{Tg/null}$ TLR5$^{-/-}$ mouse, the next hurdle is developing a method to monitor the expression of flagellin by the microbiota. There is a lack of universal primers capable of broadly amplify bacterial flagellin, thus precluding the use of conventional qPCR. Cullender et al. solved this issue through the use of bulk bacterial RNA sequencing to perform a transcriptomic analysis of the entire microbiota [23]. However, this approach has a limited ability to detect changes in flagella expression of individual bacteria.

An alternative approach to attempting to measure the expression of flagellar genes of the entire intestinal microbiota would be to measure the expression of a single bacterial strain. This method would have the advantage of being able generate primers specific to a single flagellin gene, mitigating the potential sampling bias from trying to analyze the diverse array of flagellin sequences. The main consideration with this method would be in selecting a single commensal bacterial strain that can 1) stably colonize without major population fluctuations, 2) express and utilize its flagella during homeostatic conditions, and 3) reach a large enough population density so it can be detected through qPCR methods. A motile mouse-specific commensal *E. coli* strain would be a good candidate for this method. Choosing an *E. coli* strain is beneficial because the microbiota of mice from Jackson Laboratory do not contain culturable densities of bacteria from the family Enterobacteriaceae, which includes *E. coli* [28]. The lack of closely related competitors could be leveraged to easily colonize the mouse with the selected *E. coli* strain [28]. Additionally, choosing *E. coli* would permit the use of MacConkey agar plates to selectively grow and enumerate the density of *E. coli* from fecal pellets, which could then be used to normalize flagellin expression by bacterial density [28].

Measurement of flagellin protein can also be done to complement expression data. This could be done in one of two ways. First, if the mouse has been colonized with a specific, known *E. coli* strain, then a western blot or ELISA could be performed using antibodies specific to that strain's flagellin. The antibody could be either created through immunization with purified flagellin protein or purchased from a commercial source. This method could provide you with an absolute value for the luminal concentration of a specific flagellin. An alternative method, used by Cullender et al., would be to utilize a TLR5 reporter cell line [23]. This cell line contains a GFP expression transgene with a promoter region that can be bound by transcription factors downstream of TLR5. This quantification method involves exposing TLR5-reporter cells to small intestinal protein extracts and measuring the degree of activation[23]. As bacterial flagellin from most bacteria are capable of binding and activating TLR5, the degree of reporter signal provides a measurement of total flagellin present in a given sample. This method can be coupled with a flagellin standard (based on purified *S.* Typhimurium flagellin) to determine an approximate absolute flagellin concentration [23].

HD6 Effects on the Adaptive Immune Response

In the context of the adaptive immune response, flagellin is a unique protein. The ability of flagellin to stimulate TLR5 makes it extremely immunogenic, resulting in the generation of a strong flagellin specific T cell response [29]. Furthermore, TLR5 also acts as an endocytic receptor on dendritic cells, enhancing uptake and presentation to naïve T cells [26]. Together, these factors demonstrate concentrated effort by the host to generate a robust adaptive immune response

to flagellin. This raises the questions of whether the ability of HD6 to interact with bacterial flagellin could also impact this flagellin-specific adaptive immune response.

An Experimental Model to Study HD6 and the Adaptive Response to Flagellin

Agglutinating and concentrating antigen are methods of enhancing the immune response toward a given antigen, through mechanisms of greater receptor activation, enhanced complement deposition and amplification, and more efficient clearance by phagocytes [30,31]. We hypothesize that given its ability to agglutinate bacteria partially based on their expression of flagella, HD6 may play a role in enhancing the flagellin-specific immune response by concentrating flagellin, increasing the efficiency of uptake and antigen presentation by antigen presenting cells (APCs). This mechanism could be prominent during infection with *S.* Typhimurium, where the host generates a strong flagellin-specific T cell response. The kinetics of S. Typhimurium flagellin-specific T cell response to infection can be monitored using SM1 transgenic T cells, which contain a rearranged αβTCR specific to the $FliC_{427-441}$ epitope on *S.* Typhimurium flagellin [32,33]. Interestingly, infection with low doses of S. Typhimurium fail to induce SM1 T cell expansion, suggesting that a certain threshold of antigen availability needs to be met in order to induce an SM1 T cell response [34]. Since the activation of these T cells is dependent on the display of flagellin by APCs, the rate and magnitude of their proliferation can be used to indirectly measure the degree of antigen uptake and presentation by APCs.

To test how HD6 activity may alter the rate of antigen uptake and presentation by APCs, SM1 T cells can be adoptively transferred into HD6 transgenic mice, and the mice subsequently

challenged with oral inoculation of *S.* Typhimurium. The degree of SM1 T cell proliferation and activation in different tissues can be measured by CFSE dye dilution, and CD25 and CD69 expression, respectively. Early time points at day 1, 3, and 5 post infection should be measured to determine if the presence of HD6 enhances the SM1 T cell response. During a typical *S.* Typhimurium oral infection, the activation and expansion of SM1 T cells will first occur in the Peyer's patches, then in the mesenteric lymph node, and finally in the spleen [32]. However, this timeline can be altered dramatically if even a small number of *S.* Typhimurium are able bypass the intestine and enter the bloodstream prematurely, which can occur from minor trauma to the esophagus caused by the initial oral gavage of the inoculum [35]. This relatively minor bacteremia would lead to the early activation of splenic SM1, subsequently obscuring any potential effects of HD6 on antigen uptake and presentation that could be occurring in the small intestine [35]. To mitigate this risk, the *S.* Typhimurium inoculum can instead be administered through a spiked water bottle approach [35]. This method involves spiking the drinking water with a *S.* Typhimurium and allowing the mouse to drink ad libitum overnight [35]. This method produces a similar SM1 T cell activation timeline and *S.* Typhimurium burden to the standard gavage route, while avoiding the potential risk of causing bacteremia [35].

Throughout the course of infection, fecal samples can be collected daily to measure the S. Typhimurium burden, while the spleens, mesenteric lymph node, and Peyer's patches can be collected at specific timepoints to monitor SM1 T cell activation. The congenic CD45.2 isoform bred onto the SM1 transgenic SM1 T cells differentiate them from CD45.1 T cells endogenous to the HD6 transgenic mouse [32]. A pilot experiment using this protocol was performed with tissue collection timepoints at day 1, 3, and 5 post infection. In the Peyer's patch, we observed trends

towards a significant increase in the percent of SM1 T cells of the total CD4 T cells in HD6 TG mice at day 3 and day 5, compared to wild type control mice (Figure 3A). In the MLN, we observed a trend towards a significant increase in the percent of SM1 T cells out of the total CD4 T cells in the HD6 TG mice at day 5, compared to wild type control mice (Figure 3B). No significant differences or trends were observed in the spleen (Figure 3C). Bacterial burden in fecal contents and tissues, as well as percent weight loss, were not significantly different between the HD6 TG mice and WT control mice (DNS). While not significant, repeats of these experiments with a larger n size for each group would provide the power necessary to reveal any significant differences. Additionally, the previously described HD6$^{Tg/null}$ TLR5$^{-/-}$ mouse could be used in this experimental setup to test whether the HD6-mediated agglutination of *S.* Typhimurium could counteract the severely diminished flagellin specific T cell response caused by the loss of TLR5. Together, these initial results justify further pursuit into this line of inquiry.

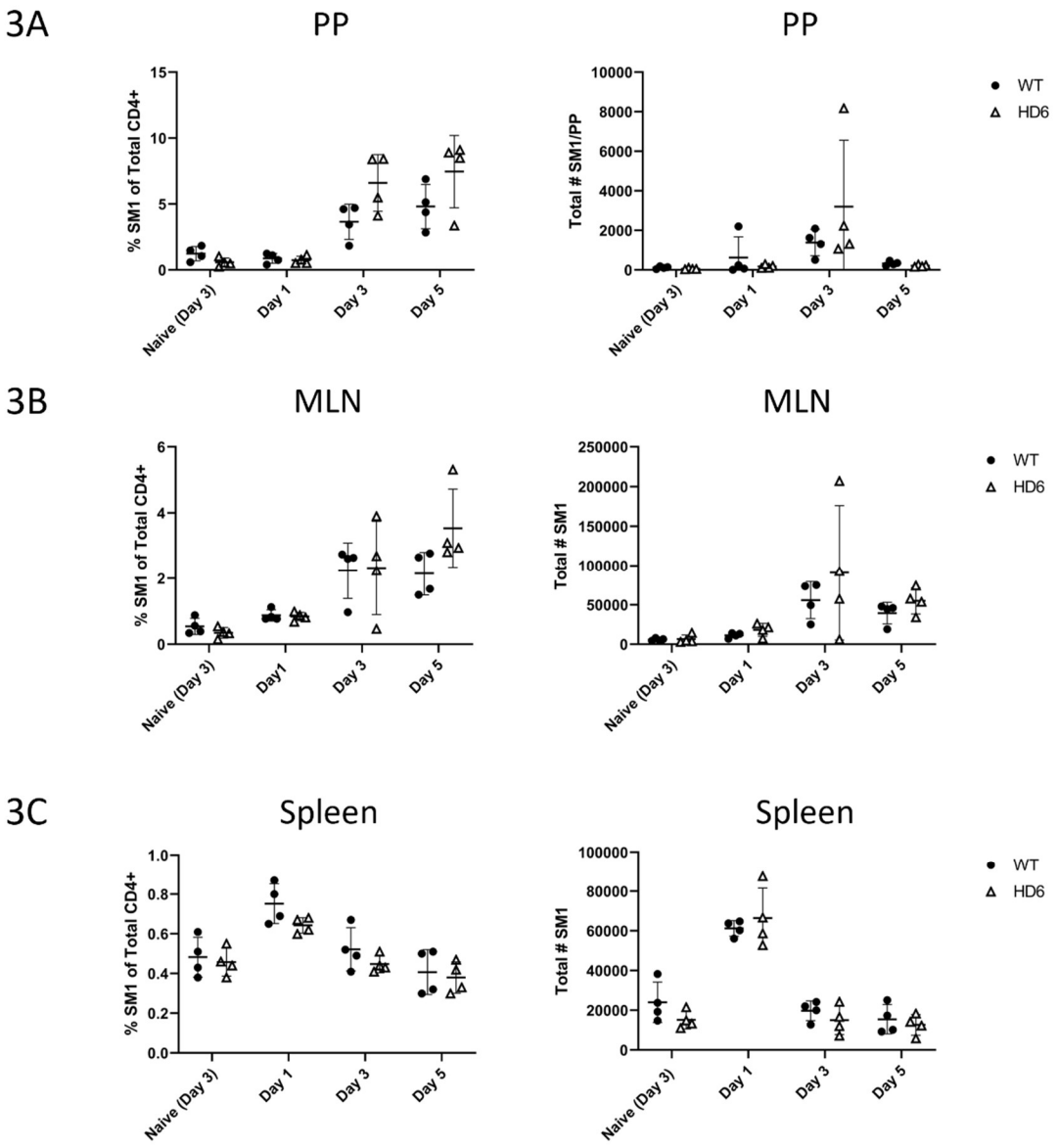

Figure 3| Expansion of adoptively transferred SM1 cells during *S.* Typhimurium infection. Graphs depicting the percent of SM1 T cells out of total CD4+ T cells (left) or total number of SM1 T cells (Right) isolated from (**A**) Peyer's patches (PP), (**B**) mesenteric lymph nodes (MLN), or (**C**) spleens from either WT (closed circle) or HD6 TG (open triangle) recipient mice at specific timepoints post infection with *S.* Typhimurium. For groups labeled Day 1, Day 3, and Day 5, 1 X 10^6 SM1 T cells were adoptively transferred into either WT or HD6 recipient mice prior to infection. Day 1, 3, or 5 refer to the day post infection that the tissues were harvested. For the group Naïve (Day 3), 1 X 10^6 SM1 T cells were adoptively transferred into either WT or HD6 recipient mice but were not infected with *S*. Typhimurium. Tissues from Naïve (Day 3) mice were harvested at the same timepoint as Day 3 mice. Data from one experiment, N=32 mice total, n=4 mice per genotype per timepoint (Error bars, mean ± SD).

Conclusion

HD6 is a unique component of the mucosal immune system, capable of exerting a profound effect on flagellated microbes through non-microbicidal means. Performing its function in the intestinal environment, HD6 is part of a concerted effort made by the host immune system to mitigate the risks posed by flagellar motility and help maintain homeostasis through spatial segregation. Throughout this chapter, future directions of research have been proposed to look at different aspects of HD6 biology, focused on the spectrum of HD6 activity, interactions with the commensal microbiota, and impact on the flagellin-specific adaptive immune response. Future studies will not only provide further characterization of HD6 activity but will also help to expand our understanding of the different, subtle ways the immune system can modulate host-microbe interactions.

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Statement of Author Contribution

Douglas T. Akahoshi wrote and edited the chapter. Douglas T. Akahoshi and Jordan Rixon designed, performed, and analyzed experiments featured in "An Experimental Model to Study HD6 and the Adaptive Response to Flagellin"; Douglas T. Akahoshi designed, performed and analyzed experiments featured in “HD6 Effects on Non-Flagella Targets".