GH18 family glycoside hydrolase Chitinase A of *Salmonella* facilitates bacterial invasion and survival by modulating host immune responses

Kasturi Chandra¹, Dipshikha Chakravorty^{1,2,#}

¹Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India.

²Centre for BioSystems Science and Engineering, Indian Institute of Science, Bangalore, India.

Corresponding author

Dipshikha Chakravorty

Email: dipa@iisc.ac.in

Telephone: +91 80 22932842

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Abstract

Salmonella is a facultative intracellular pathogen that has co-evolved with its host and has also developed various strategies to evade the host immune responses. *Salmonella* recruits an array of virulence factors to escape from host defense mechanisms. Previously *chitinase A (chiA)* was found to be upregulated in intracellular *Salmonella*. Although studies show that chitinases and chitin binding proteins (CBP) of many human pathogens have a profound role in various aspects of pathogenesis, like adhesion, virulence and immune evasion, the role of chitinase in strict intravacuolar pathogen *Salmonella* has not yet been elucidated. In this study, we deciphered the role of chitinase of *Salmonella* in the pathogenesis of the serovars, Typhimurium and Typhi. Our data propose that ChiA mediated modification of the glycosylation on the epithelial cell surface facilitates the invasion of the pathogen into the epithelial cells. Further we found that ChiA aids in reactive nitrogen species (RNS) and reactive oxygen species (ROS) production in phagocytes, leading to MHCII downregulation followed by suppression of antigen presentation and antibacterial responses. In continuation of the study in animal model *C. elegans*, *Salmonella* Typhi ChiA was found to facilitate attachment to the intestinal epithelium, gut colonization and persistence by downregulating antimicrobial peptides.

Introduction

Salmonella is one of the major foodborne pathogens that causes enteric disturbances in humans and other mammals. Although *Salmonella*-mediated enteric illnesses can be treated,

the high occurrences of drug-resistant strains challenge the pathogen eradication. Human gastrointestinal tract is covered with two distinct types of glycan layers- mucin and complex oligosaccharides (glycocalyx) that protects the enterocytes from the environment [1]. To gain access to the enterocytes, an enteric pathogen like *Salmonella* should be able to cleave the mucinous layer. In various human pathogens, glycoside hydrolases such as sialidases, muraminidases, glucosaminidases, pullulanases, GalNAcases etc. are known to facilitate the bacterial attachment to the host cells [2]. GH18 family protein chitinases and chitin binding proteins were also found to be involved in pathogenesis of several human enteric (*Vibrio cholerae*, *Listeria monocytogenes*, *Serratia marcescens*) [3-7] and non-enteric pathogens (*Pseudomonas aeruginosa*, *Legionella pneumophila*) [8-10]. In all these pathogens, commonality of the presence of mucin-rich environment hinted towards a potentially significant role of chitinases and chitin binding proteins in breaching mucosal barrier. *Salmonella* causes infection in the gut mucosal region which also has a protective mucinous layer. A BLAST search revealed that *Salmonella* Typhimurium exochitinase ChiA (encoded by STM14_0022) showed 20-40% identity with the abovementioned pathogenic proteins. Further *Salmonella* Typhi chitinase (ChiA; STY0018) is 98% similar to the *S. Typhimurium* SL1344 *chiA* (STM0022) that was reported to be upregulated ~12-20 fold in the infected macrophages and ~4-5 fold in the epithelial cells [11, 12].

We infected epithelial cells and phagocytes with the mutant strain and interestingly we found that the mutant was invasion defective in epithelial cells. *Salmonella* is known to remodel the host cell surface glycans to facilitate invasion in the epithelial cells [13-15], we checked the host cell surface glycan modification by lectin-binding assay. Our data suggest that chitinase aids in glycan remodeling by cleaving the terminal sialic acid (Neu5Ac), and Gal- β 1,4-GalNAc, thus making the mannose residues accessible to the bacteria for binding. Further we found that the phagocytes infected with the mutant bacteria produced less antibacterial

molecules. Interestingly, the mutants were significantly less virulent, less persistent, and were unable to dampen host antibacterial and immune responses in the *in vivo* infection models. Moreover, in this study we demonstrated a novel role of ChiA in facilitating extra-intestinal colonization of *Salmonella* Typhi in *C. elegans*. Together our data suggest that chitinase A plays a multifaceted role in *Salmonella* pathogenesis ranging from aiding bacterial invasion in epithelial cells, enhancing antibacterial NO production *ex vivo*, to increasing bacterial persistence in the nematodes and regulating cellular and humoral immune responses *in vivo*.

Results

Chitinase deletion impairs bacterial invasion in human epithelial cells

Since previous reports suggest that chitinases and chitin-binding proteins (CBPs) were involved in adhesion, invasion and *in vivo* pathogenesis of several human pathogens [3-10] and STM ChiA and STY ChiA are 19-24% identical to these pathogenic proteins (**Fig. S1A**), we made isogenic mutants of *chiA* using lambda red recombinase method [16]. The mutants as well as the trans-complemented strain (STY Δ *chiA*:*chiA*) did not show any growth difference *in vitro* (**Fig. S1B, S1C**), suggesting that Chitinase A is non-essential for extracellular life of *Salmonella* sp. Upon entering the host and surviving through the acidic stomach environment, *Salmonella* reaches gut epithelium, where SPI1-T3SS effectors induce membrane ruffling in the enterocytes. This facilitates bacterial entry in the epithelial cells and marks the beginning of *Salmonella* infection [17]. Since earlier report suggested that *chiA* of *S. Typhimurium* SL1344 strain (STM0022) was highly upregulated in intracellular bacteria from epithelial cells, we checked bacterial invasion and intracellular proliferation in Caco2 cells. We found that the *chiA* deletion rendered the bacteria less invasive and hyperproliferative in epithelial cells (**Fig. 1A-D, S1D, S1E**). We next checked the expression of SPI1 and SPI2 effector genes in intracellular bacteria as SPI1 effectors facilitate bacterial

invasion in epithelial cells and SPI2 effectors are required for intracellular survival and proliferation. Surprisingly we found that SPI1 effectors *invF* and *hilA* were significantly upregulated during the early phase of infection in the $\Delta chiA$ mutant bacteria, whereas no significant difference was observed in the expression of SPI2 effector *ssaV* after 16 hours of infection (**Fig. 1E-F**), suggesting that the reduced bacterial invasion in the epithelial cells by $\Delta chiA$ mutant is independent of SPI1 gene expression.

Chitinase A facilitates bacterial entry in epithelial cells by cell surface glycan

modification The intestinal epithelium is covered with mucus and the cells are layered with oligosachharide molecules that forms the glycocalyx. α 2-6, α 2-3, α 1-3, β 1-3 or β 1-6 linked Glycosylation on the host epithelial cells follow a particular array, such as the outermost glycosylation is N- acetylneuraminic acid (Neu5Ac or sialic acid), followed by galactose, N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc) and the innermost moieties mannose and fucose (**Fig. 2A**) [14]. To initiate infection, an enteric pathogen must breach the protective glycocalyx layer. Particularly in the pathogenesis of *S. Typhi*, host cell surface glycoproteins are known to play an important role in typhoid toxin mediated inflammation [18]. Therefore, we checked the abundance of various glycosyl molecules present on Caco2 cells after *Salmonella* infection. Interestingly we observed that after 120 min of infection with the WT bacteria, the host cell surface showed a significant decrease in the abundance of sialylation on the surface glycome, which was not observed for the cells infected with $\Delta chiA$ mutants, suggesting chitinase is involved in removal of the terminal sialic acids (**Fig. 2B; top panel**). This was further validated by the shift of the Neu5Ac-bound SNA-FITC lectins towards lower abundance in flow cytometry analysis (**Fig. 2C; first column**). Consequently, we observed a significant increase in the abundance of Gal- β 1,3-GalNAc on the cells infected with WT bacteria as compared to the cells infected with $\Delta chiA$ strains (**Fig. 2B; middle panel**), which was further corroborated by a significant shift of Gal-bound PNA-FITC lectins

towards higher abundance in flow cytometry analysis (**Fig. 2C; second column**). Finally, we observed a substantial increase in the abundance of mannose-bound concanavalin A-FITC fluorescence on the cell surface of WT bacteria infected cells as compared to the $\Delta chiA$ mutant infected cells (**Fig. 2B; bottom panel**) that was supported by a significant shift of mannose-bound ConA-FITC lectins towards higher abundance in flow cytometry analysis (**Fig. 2C; third column**). The cell surface glycan bound lectin fluorescence was further quantified which further validated these observations (**Fig. S1F**) Together these data suggested that *Salmonella* chitinase helps in the host cell surface remodeling.

***Salmonella* ChiA is required for stabilization of the SCVs in epithelial cells**

In the epithelial cells, *Salmonella* resides in a double membrane compartment known as *Salmonella*-containing vacuoles (SCVs). *Salmonella* replicates in the epithelial cells by inhibiting the fusion of SCVs with lysosomes. SCVs are specialized endosomes that are marked with the LAMPs, Rab7, Rab11 and vATPases. Since several reports suggested that disruption of SCV leads to bacterial hyperproliferation in the cytoplasm [19], we checked whether the enhanced proliferation of the mutant bacteria after the loss of chitinase is caused by defect in SCV maintenance. We found that after 16 hours of infection, $\Delta chiA$ mutant bacteria did not co-localize with the late-endosomal marker LAMP1 (**Fig. 3A, S1G, S1H**), suggesting disruption of SCVs in the mutant bacteria infected cells. Upon counting the number of SCV-bound and cytoplasmic bacterial population, we found that 81.6% of STM $\Delta chiA$ and 87.2% of STY $\Delta chiA$ quit the vacuole, while very less WT bacteria (STM WT 12.2%, STY WT 8.2%) did not co-localize with LAMP1 (**Fig. 3B-C**). We further quantified the cytosolic bacterial population by chloroquine resistance assay and found significantly higher number of cytosolic mutant bacteria after 16 hours of infection (**Fig. 3D-E**) suggesting that chitinase deletion leads to SCV destabilization in epithelial cells and hyper-proliferation of the cytoplasmic bacteria.

Chitinase aids in bacterial survival in phagocytes by suppressing antimicrobial responses

After successfully invading the epithelial cells, *Salmonella* reaches lamina propria, where it is taken up by macrophages, DCs and neutrophils which marks the beginning of systemic spread of the pathogen. To understand the role of chitinase in phagocytic cell infection, we infected U937 monocytes and BMDCs. We found that $\Delta chiA$ mutants were invasion defective in U937 monocytes, while the mutants showed enhanced survival in the monocytes as compared to the WT strains (**Fig. 4A-D, S2A, S2B**). While STM WT and STM $\Delta chiA$ showed similar invasiveness, surprisingly STY $\Delta chiA$ showed increased invasion and better survival in BMDCs (**Fig. 4E-H, S2C, S2D**). Higher invasiveness of STY $\Delta chiA$ in naturally phagocytic cells hints towards that this strain might be highly immunogenic. Phagocytic cells are known to inhibit intracellular bacterial growth by production of reactive nitrogen species and reactive oxygen species [20]. Estimation of nitric oxide produced by the infected BMDCs suggested that $\Delta chiA$ mutant infected cells produced significantly less nitric oxide (**Fig. 4I**). We further checked the survival of the WT and $\Delta chiA$ in the absence of NO using NOS2^{-/-} BMDCs and observed that WT bacteria survived similar to the $\Delta chiA$ mutants (**Fig. 4J-K**), suggesting that chitinase might be involved in the induction of NO in DCs. Furthermore, $\Delta chiA$ infected peritoneal macrophages (PM) showed significantly less ROS level as compared to WT infected cells (**Fig. 4L**), indicating that chitinase might be regulating RNI and ROS level in the infected cells. NO is an important cell signaling molecule that is produced to kill many human pathogens, such as *Salmonella*, *Mycobacterium* and *Listeria* etc [20]. Previous studies suggested that low level of NO enhances T cell survival [21], while very high NO is capable of inhibiting T cell proliferation [22]. To check whether lesser production of NO in response to $\Delta chiA$ mutant infection has any effect on antigen presentation and T cell expansion, we looked into the proliferation of CD8⁺ T cell population

using OT1 transgenic mouse (C57BL/6-Tg(TcraTcrb) 1100Mjb/J). The TCR of this transgenic mouse recognizes OVA₂₅₇₋₂₆₄ when presented by MHC-I molecules. This TCR recognition of MHC-I bound cognate peptide results in CD8⁺ T cell proliferation, which can be measured by incorporation of ³H thymidine in the DNA of the proliferating population. We found that with $\Delta chiA$ mutant infection, the expansion of CD8⁺ T cell population was significantly higher in response to the antigen stimulation (**Fig. 4N**). Since APCs such as macrophages and DCs possess MHC-I and MHC-II molecules on the cell surface in order to induce both CD8⁺ T cells and CD4⁺ T cells population, respectively, we detected the surface MHC-II molecules on activated PMs. We found that upon $\Delta chiA$ infection, the surface MHC-II level was similar to uninfected cells, while WT infection significantly reduced the surface MHC-II level (**Fig. 4O, S2E**). Further immunofluorescence analysis verified that $\Delta chiA$ infection did not change the surface MHC-II molecules on macrophages (**Fig. 4M, S2F**). Together these data suggests that *Salmonella* ChiA dampens host antimicrobial responses leading to enhanced pathogen survival.

Chitinase facilitates *in vivo* invasion, survival and pathogenesis of *Salmonella* Typhimurium

To further delineate the role of chitinase in *Salmonella* infection *in vivo*, we orally infected 6 weeks old C57BL/6J mice with lethal dose of the bacterial strains (10^8 CFU/animal) and observed for survival. *Salmonella enterica* serovar Typhimurium mimics the systemic typhoidal disease in murine model. STM $\Delta chiA$ infected cohort showed enhanced animal survival as compared to the STM WT infected cohort (**Fig. 5A**), suggesting a role of chitinase A during infection *in vivo*. We also found that STM $\Delta chiA$ mutant bacteria were shed prior to the STM WT and the $\Delta chiA$ mutant was defective in PP colonization after 2 hours of oral gavage (**Fig. 5B-C**). Further, we orally infected C57BL/6J mice with sublethal dose of *Salmonella* strains (10^7 CFU/animal) and bacterial CFU from liver, spleen, mesenteric lymph

node (MLN) and PP was enumerated after sacrificing the animals after indicated time intervals. We found that STM $\Delta chiA$ mutant infected animals showed less bacterial burden in each of the organs and higher body weight as compared to the STM WT infected animals (**Fig. 5D-H**). Since our *ex vivo* data suggested that the $\Delta chiA$ mutant was unable to induce NO production which is known to affect T cell survival, we checked whether chitinase mediated NO upregulation has any effect *in vivo*. We also found a significant increase in the spleen length after 20 days of infection with STM $\Delta chiA$ bacteria, as compared to the STM WT infected mice (**Fig. S3A, S3B**). We isolated total splenocytes from these infected spleens and checked the activated CD4⁺ T cell population and T cell proliferation by flow cytometry. We found that STM $\Delta chiA$ infection leads to a significant increase in CD4⁺ T cell population, as well as an increase in the activated CD25⁺ T cells (**Fig. 5K**). Analysis of T cell mediated cytokine response revealed that there was a significant increase in the pro-inflammatory cytokines IL2 and IFN γ , in the serum isolated from $\Delta chiA$ infected animals (**Fig. 5I-J**), whereas there was no difference in the anti-inflammatory cytokine levels (**Fig. S3C, S3D**). Lesser IL2 in STM WT infected mice serum as compared to that in $\Delta chiA$ mutant infected serum further strengthens the previous finding that ChiA aids in dampening of T cell activation, as IL2 is a key marker of CD8⁺ T cell proliferation [23]. Previous reports suggested that high level of IFN γ can induce B cell proliferation and enhance IgG2a and IgG3 production [24]. Therefore, we looked into the role of chitinase in anti-*Salmonella* immune response by detecting the anti-*Salmonella* IgG titer from infected mice serum. Interestingly, we found a significant increase in the anti-*Salmonella* antibody titer in the serum obtained from STM $\Delta chiA$ mutant infected cohort (**Fig. 5L**). We further used the polyclonal convalescent sera isolated from STM $\Delta chiA$ infected mice to probe against STM WT-mCherry whole cell lysate to test the polyclonality of the sera. Multiple dense bands against various *Salmonella* proteins were obtained after incubating the membrane with sera

collected from STM $\Delta chiA$ mutant infected cohort (**Fig. S3E**). Together these data suggest that *Salmonella* chitinase A is essential for restricting innate and humoral immune responses against *Salmonella*.

Chitinase helps in *Salmonella* Typhi pathogenesis in *C. elegans*

Salmonella Typhi is a human obligatory pathogen that does not cause a significant infection in mice because of the presence of TLR11 [25]. Long before TLR11^{-/-} mice model came into existence, Labrousse *et al.* suggested *Caenorhabditis elegans* can be used as an alternative host to study *S. Typhi* pathogenesis [26]. Given that *C. elegans* pharyngeal lumen is rich in chitin, it served as a suitable host to study the role of chitinase in bacterial pathogenesis [27]. We began with checking the bacterial CFU in the infected worms after 24 hours and 48 hours of continuous feeding. We found that the STY $\Delta chiA$ and STY $\Delta chiA:pQE60$ strains showed a higher bacterial burden after 24 hours of continuous feeding (**Fig. S3F**), but the fold change of bacteria were lesser than that of the STY WT and STY $\Delta chiA:chiA$ strains (**Fig. 6A**). We further checked animal survival after infecting the worms with different bacterial strains and found that all the *Salmonella* Typhi strains are pathogenic to the animals as compared to *E. coli* OP50, while STY $\Delta chiA$ showed slower death (TD₅₀ 330±8hrs) in the worms as compared to the STY WT (TD₅₀ 190±10hrs) and STY $\Delta chiA:chiA$ (TD₅₀ 270±12hrs) strains (**Fig. 6B**). Together these data suggest that chitinase deletion renders *Salmonella* Typhi less virulent in *C. elegans* infection. We further checked bacterial colonization in the worm's gut using the transgenic worm FT63 strain that expresses GFP in the epithelial cells. We visualized the bacterial colonization in the worms gut after 24 hours and 48 hours of continuous feeding. We used STM $\Delta invC$ mutant as a control which is known to be invasion defective in nonphagocytic cells [28]. We found that *S. Typhi* $\Delta chiA$ showed less colonization than STY WT after 24 hours continuous feeding, while the colonization was significantly reduced after 48 hours feeding (**Fig. 6C**), suggesting chitinase is required for

245 successful gut colonization in *C. elegans*. Percent colonization was measured as the ratio of
 246 the diameter of the lumen occupied by the bacteria to the total diameter of the gut (**Fig. S3G**).
 247 Interestingly STM $\Delta invC$ did not show any defect in colonization in the *C. elegans* gut,
 248 suggesting SPI1 effector InvC is not essential for colonization in the worms gut. We next
 249 checked if *S. Typhi* utilizes chitinase to colonize the chitin-rich pharyngeal lumen by
 250 infecting N2 worms with different strains of *Salmonella* and stained the chitin-rich parts of
 251 the worms using eosin Y. We found that after 24 hours of continuous feeding, luminal STY
 252 WT and STM $\Delta invC$ bacteria colocalized with the chitin-rich regions of the pharyngeal wall
 253 and terminal bulb (grinder), whereas STY $\Delta chiA$ bacteria did not show any colocalization
 254 with the pharyngeal wall (**Fig. 6D**), suggesting *Salmonella* Typhi utilizes chitinase to
 255 colonize the chitin-rich pharynx and terminal bulb. Additionally, we looked into the role of
 256 chitinase in bacterial persistence in the worms gut by infecting the worms with different STY
 257 strains, followed by feeding onto *E. coli* OP50. We hypothesized that if a bacterial strain can
 258 adhere to the gut lumen effectively, it will remain adhered to the gut lumen and proliferate
 259 even when the worms are fed with *E. coli* OP50. We observed that after 24 hours of feeding
 260 on STY $\Delta chiA$ followed by 24 hours feeding on *E. coli* OP50, the STY $\Delta chiA$ was unable to
 261 persist in the gut, whereas STY WT showed significantly higher colonization in the
 262 pharyngeal lumen (**Fig. S3H**). When we further extended the infection for 48 hours, followed
 263 by 24 hours of *E. coli* OP50 feeding, we observed that STY WT showed profound
 264 colonization of the gut lumen, while STY $\Delta chiA$ colonization was diminished (**Fig. 6E**),
 265 suggesting *Salmonella* utilizes chitinase to attach to the lumen wall for enhanced persistence
 266 in the worms. Interestingly, after 24 hours of continuous feeding STY WT attached to the
 267 luminal wall, but not STY $\Delta chiA$ strain. (**Fig. S4A**). After 48 hours of continuous feeding, we
 268 detected STY WT and STM $\Delta invC$ bacteria in the extra-intestinal tissues of the worms, while
 269 STY $\Delta chiA$ did not show extra-intestinal colonization (**Fig. 7A, S4A, S4B**), suggesting that

chitinase might be required to invade extra-intestinal tissues of the worms. To the best of our knowledge, this study is the first report suggesting an extra-intestinal invasion/colonization by *Salmonella* Typhi in *C. elegans*.

Salmonella* chitinase is important for alteration of metabolism and antibacterial defense in *C. elegans

Grinder, a part of the terminal bulb, is the complex structure that helps in uptake and grinding of bacteria before it passes to the intestine where the nutrients get absorbed. In a healthy and well-fed state, worms feed at the average rate of 200 pumps/min. Since we observed that *Salmonella* uses chitinase to colonize chitin-rich organs (**Fig. 6D**), we next looked into the nutritional state of the worms by counting the number of pharyngeal pumps per min. We found a significant reduction in the number of pharyngeal pumps/min after 72 hours of STY WT and STY $\Delta chiA:chiA$ infection (**Fig. 7B**). Further, *in vivo* oxidative stress was quantified using CL2166 worms, that possess oxidative stress inducible GFP. STY WT and STY $\Delta chiA:chiA$ infected worms showed significantly higher oxidative stress and ‘bag of worms’ phenotype (**Fig. S4C, S4D**). We next checked the nutritional fitness of the worms by measuring the lipid content using oil red O staining. Oil red O (ORO) is a fat-soluble dye that stains neutral lipids [29]. Interestingly we observed significant fat-loss in the worms fed with STY WT and STY $\Delta chiA:chiA$ for 24 hours and 48 hours as compared to STY $\Delta chiA$ infected wormed (**Fig. 7C, S4E**). We further checked whether chitinase deletion has any effect on the immune response of the worms. Although our data suggest both STY WT and STY $\Delta chiA$ downregulated p38 MAP kinase pathway genes *pmk1* and *mek1* equally (**Fig. S4F**), p38 MAP kinase pathway regulated antimicrobial peptides were differentially expressed. While *clec85*, *lys7*, *ilys2* expressions were severely downregulated in both STY WT and STY $\Delta chiA$ infection, all of them showed significant rescue in the worms infected with STY $\Delta chiA$ bacteria (**Fig. 7D**). Interestingly, we observed that STY WT infection

induced downregulation of antimicrobial peptide *sppI* was completely rescued upon STY $\Delta chiA$ infection, while *abf2* was significantly upregulated upon infection with STY $\Delta chiA$ (Fig. 7E), indicating an important contribution of chitinase in dampening the antimicrobial responses of the host.

Discussion

Salmonella is a facultative intracellular human pathogen that has co-evolved with its host and has also developed various strategies to evade the host's immune responses. The detailed understanding of the metabolism and the ease of genetic manipulation has made *Salmonella* an excellent the model to study the role of metabolism related proteins in the light of host-pathogen interaction. Although *Salmonella* pathogenesis is governed by classical virulence factors such as adhesins, invasins and toxins, emerging reports suggest that various unique metabolic proteins are important in various aspects of *Salmonella* pathogenesis. Several reports suggest that *Salmonella* can utilize a large pool of chemically diverse host nutrients, such as carbohydrates, lipids, amino acids etc [30]. One such carbon metabolism related protein encoding gene *chiA* (STM0022) was found to be highly upregulated in intracellular *Salmonella* Typhimurium str. SL1344 isolated from infected macrophages and epithelial cells [11, 12]. Bacterial chitinases belong to GH18 and GH19, which are getting recognized as bacterial virulence factors along with several other structurally similar glycosidases such as sialidases, muraminidases, N-acetylgalactosidases etc [2]. Although *Salmonella chiA* was upregulated during infection, the role of this chitinase in *Salmonella* pathogenesis remains elusive. To answer this question, we generated isogenic $\Delta chiA$ mutant by one-step gene inactivation method. We did not find any significant difference in the *in vitro* growth among the two strains. To test whether *chiA* upregulation in infected cells has any significance in the

pathogenesis, we infected epithelial cells and phagocytes with the mutant strain. Interestingly, we found that the mutant was invasion defective in epithelial cells. Previous reports suggested that *Salmonella* remodels the host cell surface glycans to facilitate invasion in the epithelial cells [13-15]. Our observations from the lectin-binding assay suggests that chitinase aids in glycan remodeling by cleaving the terminal glycosyl molecules and making the mannose residues accessible to the bacteria for binding. We further found that absence of ChiA leads to destabilization of the SCV and hyper-proliferation of the mutant bacteria in the cytoplasm of the epithelial cells. The $\Delta chiA$ mutants survived better than WT strains in the phagocytes, suggesting the $\Delta chiA$ mutants were protected from phagocytes mediated bacterial killing. Additionally, we found that the phagocytes infected with the mutant bacteria produced less antimicrobial molecules such as NO and ROS. We also found that ChiA was important for downregulating the MHC-I molecules on the dendritic cells, leading to the inhibition of CD8⁺ T cell proliferation and subsequent antigen presentation. In coherence with the available literatures [22], the enhanced T cell proliferation could be attributed to the absence of NO induction by the $\Delta chiA$ mutant strains. We further showed that absence of *chiA* failed to downregulate the surface MHC-II molecules on the activated macrophages, which is a well-known phenomenon during *Salmonella* infection [31]. *In vivo* infection in C57BL/6 mice suggested that STM $\Delta chiA$ mutant was unable to invade the Peyer's patches, leading to an early fecal shedding and enhanced pathogen clearance. STM $\Delta chiA$ mutant infected cohort presented significantly less bacterial burden in the liver, spleen, MLN and PP, as well as they showed increased survival, suggesting that the STM $\Delta chiA$ mutant is less virulent *in vivo*. Analysis of total splenic lymphocytes by flow cytometry suggested that the $\Delta chiA$ mutant infected cohort had an increased activated T cell population (CD4⁺CD25⁺) in the spleens, suggesting an intensified immune response in these mice. This was corroborated by significant increment in the pro-inflammatory cytokines and anti-STM IgG antibody titer

in the STM $\Delta chiA$ infected mice sera. In the invertebrate *C. elegans* model, chitinase helps in bacterial attachment to the pharyngeal lumen. Additionally, we found that the chitinase helps in *Salmonella* Typhi colonization and persistence in the worms, since deletion of *chiA* leads to bacterial clearance from the worm's gut. In addition, our data suggest that *Salmonella* Typhi chitinase might help in extra-intestinal tissue invasion in the worms. Chitinase was found to be regulating the fat-responsive immune response such as antibacterial peptide synthesis in the worms. We found significantly higher expression of antimicrobial peptides genes *spp1* and *abf2* when the worms were infected with STY $\Delta chiA$ strain, hinting towards a potential role of chitinase in modulating innate immune response in the worms. Together our data suggest that *Salmonella* Chitinase regulates different aspects of pathogenesis, ranging from aiding in invasion in the epithelial cells, impairing the activity of professional antigen presenting cells to as diverse as immune response regulation in various hosts (**Fig. 7F**) and emerges as a novel virulence factor.

Materials and Methods

Bacterial strains

All *Salmonella* Typhimurium strains used in this study are listed below with their genetic description. *Salmonella enterica* serovar Typhimurium strain 14028S was used as the wild type strain, and was also the parental background for all the mutant strains used in this study, i.e. $\Delta chiA$ and $\Delta invC$. All strains were grown and maintained in Lennox broth (LB; 0.5% NaCl, 1% casein enzyme hydrolysate and 0.5% yeast extract) at 37°C under shaking conditions. *Salmonella enterica* serovar Typhi strain CT18 was used as the wild type strain, and was also the parental background for the mutant strain used in this study, i.e. $\Delta chiA$. *S. Typhi chiA* was trans-complemented in pQE60 plasmid in 5' NotI-*chiA*-BamHI 3' direction.

368 This plasmid was transferred to STY $\Delta chiA$ strain to make complement strain.
 369 Complemented strain STY $\Delta chiA:chiA$ and empty vector strain STY $\Delta chiA$;pQE60 strains
 370 were maintained on LBA supplemented with ampicillin (50 μ g/ml). The mCherry expressing
 371 strains were cultured in Lennox broth with 50 μ g/ml Ampicillin at 37°C in shaking condition.
 372 List of strains used in this study.

Strain name	Description	Reference
<i>S. Typhimurium</i> ATCC 14028S (STM WT)	Wild type (WT)	Kind gift from Prof. M. Hensel (Division of Microbiology, University of Osnabrück, Germany)
STM $\Delta chiA$	Isogenic knockout strain for the gene <i>chiA</i> ; Kan ^r	This study
STM $\Delta invC$	Isogenic knockout strain for the gene <i>invC</i> ;	Kind gift from Prof. M. Hensel (Division of Microbiology, University of Osnabrück, Germany)
<i>S. Typhi</i> CT18 (STY WT)	Wild type (WT)	PGIMER, Chandigarh
STY $\Delta chiA$	Isogenic knockout strain for the gene <i>chiA</i> ; Kan ^r	This study
STY $\Delta chiA:chiA$	Isogenic complement strain for $\Delta chiA$ expressing <i>chiA</i> under the T5	This study

	promoter present in the pQE60 plasmid; Kan ^r Amp ^r	
STY Δ <i>chiA</i> :pQE60	Isogenic complement strain with empty pQE60 plasmid; Kan ^r Amp ^r	This study
mCherry tagged strains	Respective strains carrying pFPV-mCherry plasmid, Amp ^r	This study

373

374 Isolation and maintenance of primary cells and cell lines

375 Human colorectal adenocarcinoma cell line Caco2 (ATCC HTB-37) was cultured in
376 complete DMEM media (Lonza), whereas human monocyte cell line U937 (ATCC CRL-
377 1593.2) was maintained in complete RPMI 1640 media (Lonza) with 100 μ M β -
378 mercaptoethanol and differentiated to macrophages using 20 ng/ml PMA for 24 hours prior to
379 infection. Bone-marrow was isolated from either wildtype (NOS2^{+/+}) C57BL/6J mice or
380 NOS2^{-/-} C57BL/6J mice as described previously [31]. Briefly, tibia and femur bones were
381 carefully taken out, caps were removed and the marrow was flushed with RPMI 1640 media
382 using a 26G needle. After making single cell suspension, RBCs were lysed using RBC lysis
383 buffer. Cells were pelleted and grown in complete RPMI 1640 media supplemented with 20
384 ng/ml mGM-CSF (Peprotech), antibiotics and 100 μ M β -mercaptoethanol. After every 2
385 days, the media was replenished. Once approximately 65-70% of the cells were differentiated
386 to dendritic cells (loosely adherent spheres), the cells were collected and used for further
387 experiments. To obtain peritoneal macrophages (PMs), thioglycolate was injected in the
388 peritoneal cavity of C57BL/6J mice. After 5 days these mice were sacrificed and ice cold
389 PBS was injected in the peritoneum to collect the peritoneal exudate. Any residual

erythrocytes were lysed using RBC lysis buffer and the cells were maintained in complete RPMI 1640 media for further experiments.

Generation of deletion mutant

$\Delta chiA$ mutant strains were made using one-step deletion strategy as mentioned by Datsenko and Wanner [16]. Briefly, wild-type *Salmonella* (*S. Typhimurium* 14028S or *S. Typhi* CT18) bacteria transformed with a ‘lambda red recombinase’ expressing plasmid under arabinose inducible promoter (pKD46), was grown in LB with 50 µg/ml ampicillin and was induced with 10 mM L-arabinose at 30 °C to an OD₆₀₀ of 0.35-0.4. Electrocompetent cells were prepared by pelleting the bacterial cells and washing the pellet three times with ice cold, sterile MiliQ water and 10% glycerol, followed by resuspension in 50 µl of 10% glycerol. Kanamycin resistance cassette was amplified from pKD4 plasmid using primers containing upstream and downstream sequences of *S. Typhimurium chiA* gene (STM14_0022) and *S. Typhi chiA* gene (STY0018) fragment. 500 ng of this PCR product was purified and used for electroporation. Transformants were selected on LB agar containing kanamycin plates and were further confirmed with confirmatory primers, *chiA* specific RT primers and kanamycin resistance cassette internal primers.

Infection and gentamicin protection assay

Epithelial Caco-2 cell line was infected with mid-log phase culture of bacteria grown in LB (OD₆₀₀ 0.3), whereas phagocytic U937 derived monocytes and BMDCs were infected with overnight culture (OD₆₀₀ 0.3). The multiplicity of infection (MOI) of 10 was used in each case. Bacterial attachment to host cells was enhanced by centrifuging at 600 rpm for 10 min. After 25 min of infection, cells were treated with gentamicin (100 µg/ml in complete media) for 1 hour to remove extracellular bacteria and then maintained with 25 µg/ml gentamicin for rest of the experiment. 0.1% Triton-X 100 (v/v in 1x PBS) was used to lyse the cells and the

lysate was plated on *Salmonella-Shigella* (SS) agar for *S. Typhimurium* strains and Wilson Blair (WB) agar for *S. Typhi* strains. For invasion assay, cells were lysed after incubation in 100 µg/ml gentamicin treatment (i.e. 1 hour post infection) and percent invasion was calculated with respect to the pre-inoculum used for infection. For intracellular survival assay (ICSA), infected cells were lysed at 2 hours and 18 hours post infection. CFU at 18 hours was divided by CFU at 2 hours to obtain fold replication of the intracellular bacteria. For estimating the cytoplasmic bacterial population, chloroquine resistance assay was performed [32]. Briefly, Caco2 cells were infected by different bacterial strains as mentioned previously. The infected cells were treated with 800 µM chloroquine 1 hour prior to cell lysis and absolute CFU were calculated by plating the cell lysate on selective media.

Quantitative RT-PCR

Bacterial RNA was isolated from infected cells as described previously by Eriksson *et al.*[11]. Briefly, *Salmonella* infected cells were lysed at different time intervals on ice by incubating for 30 minutes with 0.1% SDS, 1% acidic phenol and 19% ethanol in sterile water. Eukaryotic cell debris was removed by centrifuging the cell lysate at 300g for 10 minutes, followed by pelleting bacterial cells at 5000 rpm for 5 minutes. At each time point, bacteria were recovered from a 6-well plate of infected U937-derived monocytes and pooled to isolate RNA. *In vitro* grown bacterial RNA was obtained by growing bacteria statically at 37 °C in RPMI 1640 medium, under 5 % CO₂. The bacterial pellet was resuspended in TRIzol reagent (Takara) and stored at -80 °C. Young adult hermaphrodites were infected with respective bacterial strains for 48 hr. Infected worms were harvested by washing the plates with M9 buffer and pelleting at 1000g for 1 min. The extracellular bacteria were removed by repeatedly washing the pellet for 5-6 times. The worms pellet was resuspended in TRIzol reagent (Takara) and stored at -80 °C. RNA was isolated by phase separation method using chloroform. cDNA was synthesized with reverse transcriptase (GCC Biotech). Quantitative

439 PCR was carried out using SYBR Green Q-PCR kit (Takara). Relative expression with
440 respect to control (*act2* gene) was plotted as fold change. Relative expression with respect to
441 control (16s rRNA gene for bacterial genes and *act2* for *C. elegans* genes) was plotted as fold
442 change.

Gene	Sequence (5'-3')
STM <i>chiA</i> KO FP	TTATGGACCCCGCAGAACGAGCTGCGACAATTTTG AAACGTAAAAGGAAATTTGAAAGTGTAGGCTGGAG CTGCTTC
STM <i>chiA</i> KO RP	GGTAAACCAGGGCTTGAATCATGAAGCCCAATACA TCGGCTTAATACCGTGTACATATGAATATCCTCCTT AG
STM <i>chiA</i> conf FP	GCTGCGACAATTTTGAAAC
STM <i>chiA</i> conf RP	GAAGCCCAATACATCGG
STY <i>chiA</i> KO FP	GGACCCCGCAGAACGAGCTGCGACAATTTTGAAAC GTAAAAGGAAATTTGAAAGTGTAGGCTGGAGCTGC TTC
STY <i>chiA</i> KO RP	CCCCGGTAAACCGGGGCTTGAATCATGAAGCCCAA TACATCGGCTTAATACCGTGTACATATGAATATCCT CCTTAG
STY <i>chiA</i> conf FP	CTGCGACAATTTTGAAACG
STY <i>chiA</i> conf RP	CCAATACATCGGCTTAATACC
STY <i>chiA</i> :pQE60- <i>chiA</i> FP	TACGCCATGGATGGCTACAAGCAAAGTCAAG
STY <i>chiA</i> :pQE60- <i>chiA</i> RP	AGTCGGATCCTTAGTAAGCGCCAAGATCGG
STM/STY <i>chiA</i> RT FP	CGGAAGAGGAAGAAGAGATT

STM/STY <i>chiA</i> RT RP	CATAGACCACCATTTCACCT
<i>invF</i> FP	AGATCGTAAACGCTGCGAGT
<i>invF</i> RP	CTGCTGCACAAACGACGAAA
<i>hila</i> FP	GCCGGTGACCATTACGAAGA
<i>hila</i> RP	AAGAGAGAAGCGGGTTGGTG
<i>ssaV</i> FP	TATTGATAGGCGCGGACGCTA
<i>ssaV</i> RP	CGCCTTATGGGCCATGTCTTT
<i>phoP</i> FP	GATCTCTCACGCCGGAATT
<i>phoP</i> RP	TGACATCGTGCGGATACTGG
<i>sodA</i> FP	CCTGCCGGTTGAAGAACTGA
<i>sodA</i> RP	GGTTGCTGCTGCTTTTTTCGA
STM 16s rRNA FP	GTGAGGTAACGGCTCACCAA
STM 16s rRNA RP	TAACCGCAACACCTTCCTCC
<i>C. elegans act2</i> FP	ATCGTCCTCGACTCTGGAGAT
<i>C. elegans act2</i> RP	TCACGTCCAGCCAAGTCAAG
<i>C. elegans pmk1</i> FP	CCAAAAATGACTCGCCGTGA
<i>C. elegans pmk1</i> RP	CTTTTGCAGTTGGACGACGA
<i>C. elegans mek1</i> FP	AGCAGCCAATTCCAGAGAGA
<i>C. elegans mek1</i> RP	CGATCAGTCTGCCAGCAATA
<i>C. elegans clec85</i> FP	CCAATGGGATGACGGAACCA
<i>C. elegans clec85</i> RP	CTTCTGTCCAGCCAACGTCT
<i>C. elegans lys7</i> FP	GTACAGCGGTGGAGTCACTG
<i>C. elegans lys7</i> RP	GCCTTGAGCACATTTCAGC
<i>C. elegans ily2</i> FP	TGTTGGATCGCTTTCTTG TG

<i>C. elegans</i> <i>ilys2</i> RP	CATTATGGTTCGGGCCATC
<i>C. elegans</i> <i>spp1</i> FP	TGGACTATGCTGTTGCCGTT
<i>C. elegans</i> <i>spp1</i> RP	ACGCCTTGTCTGGAGAATCC
<i>C. elegans</i> <i>abf2</i> FP	CCGTTCCCTTTTCCTTGCAC
<i>C. elegans</i> <i>abf2</i> RP	GACGACCGCTTCGTTTCTTG

443

444 **Lectin binding assay for cell surface glycan modification**

445 Human colorectal carcinoma cells Caco2 were infected with different bacterial strains as
 446 mentioned before. For confocal imaging, cells were seeded on coverslips prior to infection.
 447 After infection for the specified time, the cells were fixed with 3.5% PFA for 20 min on ice.
 448 For flow cytometry, cells were washed with PBS and treated with 1x Trypsin-EDTA (TE) for
 449 15 min, under 5% CO₂ at 37 °C. After the cells were dislodged from the wells, the TE was
 450 removed and cells were incubated with 1 ml complete media for 20 min under 5% CO₂ at 37
 451 °C for recovery. To avoid non-specific lectin binding the cells were treated with blocking
 452 buffer (PBS+2% FBS) at RT for 15 min. Specific lectins (50µg/ml lectin solution in blocking
 453 buffer for every 10⁶ cells) (Vector Laboratories; #FL-1301, #FL-1071, #FL-1001) were
 454 added to each samples and incubated for 30 min at RT, followed by washing with blocking
 455 buffer. Cells treated with only FITC dye (Merck; #46950) were used as controls.

456 **Flow cytometry and immunofluorescence**

457 Cells were fixed with 3.5% PFA for 20 min on ice. All staining except for the surface
 458 markers (MHC II, CD4 and CD25), were performed in the presence of permeabilizing agent,
 459 0.01% saponin (Sigma) dissolved in 2.5% BSA containing PBS. Flow cytometry analysis
 460 was carried out using BD FACSVerser and BD FACSAria and data were analyzed using BD
 461 FACSDiva software. Immunofluorescence images were obtained using Zeiss LSM 710
 462 and/or Zeiss LSM 880. The images were analyzed using ZEN Black 2012 platform. For

analysis of activated T cell population (CD4⁺ CD25⁺) from infected mice spleen, splenocytes were isolated from mice that survived through 20 days of infection. Total splenocytes were fixed using 3.5% PFA on ice for 20 min, followed by incubation for 1 hour at RT with fluorophore conjugated antibody cocktail in dark. The cells were washed with 1x PBS and analyzed by flow cytometry. Anti-mouse LAMP1 (DSHB; #1D4B) antibody was used for immunofluorescence microscopy. Anti-mouse I-A/I-E (or MHC II) (clone 2G9) FITC (BD Pharmingen; #553623) antibody was used for immunofluorescence microscopy and flow cytometry. Anti-mouse CD4 FITC (Invitrogen; #11-0041-85), Anti mouse CD25 PE (Invitrogen; #12-0251-82) antibodies were used for flow cytometry.

Nitric oxide estimation

Sodium nitrite (Sigma) standards of 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM and 3.13 µM were prepared by diluting 0.1 M stock in deionized distilled water. Conditioned media from infected cells were collected after indicated time intervals for estimation of nitrite by Greiss assay [33]. 1% sulphanilamide solution was made in 5% phosphoric acid. To 50 µl of the standards and the samples (in triplicates), 50 µl acidic sulphanilamide was added and incubated at RT, in dark for 10 min. After incubation, 50 µl of 0.1% NED (N-1-naphthylethylene diamine dihydrochloride) solution was added to it and incubated for 10 min in dark at RT. OD₅₂₀ was measured within 30 min of appearance of purple/magenta colored product using TECAN Infinite Pro 200 microplate reader.

ROS measurement

Intracellular ROS was detected by 2', 7'-dichlorofluorescein diacetate (H₂DCFDA; Sigma) staining. Cells were stained with 10µM DCFDA at 37 °C in dark. After 30 min, cells were washed with ice cold PBS and harvested followed by flow cytometry analysis at 495/530 nm in BD FACSVerse.

T cell proliferation assay

WT BMDCs were infected by incubating the bacteria with DCs for 90min, followed by removal of the bacteria and incubating the infected cells with 25µg/ml gentamicin. Total splenocytes were isolated from the spleen of C57BL/6-Tg (TcraTcrb) 1100Mjb/J mice by mechanical disruption. Erythrocytes were lysed by RBC lysis buffer (Sigma) and cells were maintained in complete RPMI-1640. Finally, non-adherent cells were collected and were used for mixed lymphocyte proliferation assay. The proliferation of the lymphocytes in response to antigen stimuli, was detected by incorporation of the $^3\text{H}_1$ as measured by the scintillation counter.

***In vivo* experiment**

6 weeks old male C57BL/6J mice were used for all the *in vivo* mice experiments. All animal experiments were approved by the Institutional Animal Ethics Committee (CAF/Ethics/670/2019) and the National Animal Care Guidelines were strictly followed. 10^8 CFUs of overnight grown STM WT and STM $\Delta chiA$ mutant bacteria were used for oral infection for animal survival assay. The control group was orally administered with sterile 1x PBS. Animals were observed for 20 days for survival and body weight was documented. For *in vivo* invasion, the animals were euthanized after 2 hours of gavage, and the bacterial CFUs in Peyer's patches (PP) were estimated. To check the bacterial shedding, fecal pellets were collected aseptically from infected cohorts after indicated time. Homogenates were plated on SS agar plates and CFUs were counted. For estimating *in vivo* bacterial burden in different organs, a sublethal dose of 10^7 CFUs of each bacterial strain were used and bacterial CFUs from liver, spleen, MLN and PP were enumerated after indicated time intervals. Spleens were isolated from the animals after 20 days and the length was measured.

ELISA for serum cytokines and anti-*Salmonella* IgG

Blood collected from infected animals by cardiac puncture under aseptic conditions, was incubated at RT to facilitate coagulation. Serum was then isolated by centrifugation at 5000 rpm for 10 min at RT and stored at -20 °C for further use. Estimation of serum level of different pro-inflammatory cytokines (IL2 and IFN γ) and anti-inflammatory cytokines (IL10 and IL4) was performed according to the manufacturer's instructions. Anti-*Salmonella* IgG titer was measured by sandwich ELISA as mentioned previously [34]. Briefly, wells were coated with *Salmonella* LPS (200 ng/well; Sigma) at 4 °C overnight. Next day, LPS was removed and the wells were washed with PBST (PBS+0.05% Tween 20), followed by blocking for 1 hour at RT with 5% FBS in PBS to avoid non-specific binding. After blocking, wells were washed with PBST. The serum samples, diluted in blocking buffer, were added to the wells in triplicates and incubated for 2 hours. Subsequently, wells were washed with PBST and anti-mouse IgG (HRP conjugate) was then added to the wells and incubated for 1 hour at RT. Tetramethylbenzidine (TMB; Sigma) was added and the plate was incubated in dark for 20-30 min. The reactions were stopped with 2 N H₂SO₄ and the absorbance was measured at 450 nm.

In vivo* colonization in *Caenorhabditis elegans

C. elegans var. Bristol worms wildtype strain N2, FT63 [xnIs17; dlG-1::GFP + rol-6(su1006)], and CL2166 [dvIs19 III; dvIs (pAF15)gst-4p::GFP::NLS III] strains were maintained on NGM media at 20 °C. L4 or Young adult N2 hermaphrodite worms were used for *in vivo* experiments. 10⁷ CFU of different bacterial strains were seeded on NGM plates and grown for 16 hours. Young adult N2 worms were fed at 20 °C with the different bacterial strains for 24 hours or 48 hours to check bacterial colonization in the worms [35]. Bacterial CFU was enumerated by plating worms' lysate from equal number of infected worms on WB agar plates. Fold change was calculated as the ratio of CFU after 48 hours to CFU after 24

hours. For confocal analysis of the worm gut colonization, FT63 worms were used. mCherry expressing bacterial strains were used to visualize the gut colonization.

Further to check worms survivability, 10^7 CFUs of overnight grown bacterial strains were seeded on 30 mm dishes containing Brain Heart Infusion (BHI) agar media. ~30-40 young adult worms were added at the center of each plate and survival was monitored [36]. Animals were transferred to fresh bacterial plates every day for first 5 days and then after every 5 days. The worms were scored as live or dead at regular intervals throughout the course of the assay. Worms were considered dead when they failed to respond to touch stimulus.

Chitin-rich organs were visualized using Eosin Y stain. After 24 hours of infection, worms were harvested and washed 5 times with M9 buffer, followed by washing the worms pellet with citrate phosphate buffer (0.2 M Na_2HPO_4 , 0.1 M potassium citrate, pH 6.0). The worms were resuspended in 500 μL citrate-phosphate buffer and 15 μL of 5 mg/ml eosin Y (in 70% ethanol) was added to it. Tubes were incubated at RT, in dark for 10 minutes, followed by centrifugation at 1000g for 1 min for washing. The supernatant was discarded and the pellet was washed with citrate phosphate buffer 5 times to remove excess eosin Y.

Effect of bacterial colonization was determined by infecting CL2166 worms for 48 hours with different strains. CL2166 worms possess oxidative stress inducible GFP. Fluorescence of the infected worms was visualized using Zeiss LSM 880 with Multiphoton mode.

Bacterial persistence assay in *C. elegans*

Young adult N2 worms were infected as mentioned previously. After 24 hours or 48 hours of infection, the worms were harvested and washed 5 times with M9 buffer. After indicated time, ~30 worms were mounted for confocal imaging. Rest of the worms were transferred to *E. coli* OP50 plate for further 24 hours. These worms were harvested, washed and imaged as mentioned previously.

Quantification of pharyngeal pumps

The effect of bacterial colonization on the chitin-rich grinder integrity was determined by counting number of pharyngeal pumps per min. Young adult worms were infected as mentioned previously. After indicated infection time, no. of pharyngeal pumps/min was counted for ~25 worms from each infected plate.

Fat estimation by Oil red O

Neutral lipids present in the worms was estimated by Oil Red O (ORO; Sigma) staining [29]. Briefly, solution of Oil Red O was prepared in isopropanol (5 mg/ml) and diluted to 60% in water before use. Synchronized L4 animals were allowed to feed on *E. coli* and STY strains for 48 hr. Worms were harvested in M9 buffer, followed by fixing and permeabilizing using MRWB buffer (160 mM KCl, 40 mM NaCl, 14 mM Na₂-EGTA, 1 mM spermidine-HCl, 0.4 mM spermine, 30 mM Na-PIPES [Na-piperazine N, N'-bis(2-ethanesulfonic acid); pH 7.4], 0.2% β-mercaptoethanol, 0.2% paraformaldehyde) for 1 hour at RT. The animals were stained with 60% ORO at RT. Excess stain was removed by washing twice with 1x PBST (PBS+0.01% Tween 20). Stained animals were mounted on agar pads.

Statistical analysis

Data were plotted using GraphPad Prism 6 software. Statistical analysis was performed using Student's t-test or ANOVA as indicated. The results are expressed as mean ± SEM. p values <0.05 was considered to be significant (p values: ****<0.0001, ***<0.001, **<0.01, *<0.05).

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Author Contribution

KC and DC conceived the study and designed experiments. KC performed experiments, analyzed the data, prepared the figures and wrote the manuscript. DC supervised the work. All the authors read and approved the manuscript.

Competing Interests

The authors declare no competing interests.

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Figure Legends

Fig 1. Chitinase deletion impairs bacterial invasion in human epithelial cells. (A) % invasion and (B) Intracellular proliferation of STM WT and STM $\Delta chiA$ strains in Caco2 cells by gentamicin protection assay. Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3, n=3). Unpaired Student's t test was used to analyze the data. (C) % invasion and (D) Intracellular proliferation of STY WT, STY $\Delta chiA$, STY $\Delta chiA:chiA$ and STY $\Delta chiA:pQE60$ strains in Caco2 cells by gentamicin protection assay. Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3, n=3). One-way ANOVA

was used to analyze the data. **(D-E)** RNA expression level of SPI1 and SPI2 genes in from STM WT and STM $\Delta chiA$ during **(D)** early phase and **(E)** late phase of infection in Caco2 cells. Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3, n=3). Two-way ANOVA was used to analyze the data.

Fig 2. Chitinase helps in glycan remodeling in host epithelial cells. **(A)** Cell surface glycan assembly. **(B)** Representative confocal images of Caco2 cells stained with SNA-FITC (top panel), PNA-FITC (middle panel) and ConA-FITC (bottom panel) lectin after indicated time intervals of STM WT, STM $\Delta chiA$, STY WT and STY $\Delta chiA$ infection (UI- Uninfected). **(C)** Representative flow cytometry histogram showing the cell surface Neu5Ac-bound SNA-FITC (first column), Gal bound PNA-FITC (second column) and mannose bound ConA-FITC (third column) lectin (UI- Uninfected). Solid lines represent MFI after 30 min and dashed lines represent MFI after 120 min. Data are represented as mean $\pm$ SEM of 2 independent experiments (N=2).

Fig 3. Chitinase deletion destabilizes the *Salmonella*-containing vacuoles in the epithelial cells leading to hyperproliferation of the cytosolic bacteria. **(A)** Representative confocal image of Caco2 cells infected with STM WT, STM $\Delta chiA$, STY WT and STY $\Delta chiA$ strains to visualize the intracellular niche of the bacteria. The SCVs were stained for LAMP1 in presence of 0.01% saponin and 2.5% BSA (UI- Uninfected). **(B)** % of STM WT and STM $\Delta chiA$, **(C)** % of STY WT and STY $\Delta chiA$ bacteria inside and outside the LAMP1-stained SCVs after 16 hours of infection was calculated. Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3). **(D-E)** Absolute CFU/ml values of **(D)** STM WT and STM $\Delta chiA$, **(E)** STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ in Caco2 cells in chloroquine resistance assay after 16 hours of infection. Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3, n=3). One-way ANOVA was used to analyze the data.

Fig 4. Chitinase induces NOS and ROS generation in phagocytic cells and inhibits of antigen presentation. (A) % invasion and (B) fold proliferation of STM WT and STM $\Delta chiA$ strains in U937 derived monocytes by gentamicin protection assay. Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3, n=3). Unpaired Student's t test was used to analyze the data. (C) % invasion and (D) fold proliferation of STY WT, STY $\Delta chiA$, STY $\Delta chiA:chiA$ and STY $\Delta chiA:pQE60$ strains in U937 derived monocytes by gentamicin protection assay. Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3, n=3). One-way ANOVA was used to analyze the data. (E) % invasion and (F) fold proliferation of STM WT and STM $\Delta chiA$ strains in U937 derived monocytes by gentamicin protection assay. Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3, n=3). Unpaired Student's t test was used to analyze the data. (G) % invasion and (H) fold proliferation of STY WT, STY $\Delta chiA$, STY $\Delta chiA:chiA$ and STY $\Delta chiA:pQE60$ strains in U937 derived monocytes by gentamicin protection assay. Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3, n=3). One-way ANOVA was used to analyze the data. (I) Extracellular NO was estimated by Greiss assay from conditioned media obtained from STM WT, STM $\Delta chiA$, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ infected DCs. (UI- Uninfected). Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3, n=3). Two-way ANOVA was used to analyze the data. (J-K) Intracellular survival of (J) STM WT and STM $\Delta chiA$ and (K) STY WT and STY $\Delta chiA$ strains were calculated in WT and NOS2^{-/-} BMDCs by gentamicin protection assay. Data are represented as mean $\pm$ SEM of 3 independent experiments (N=3, n=3). Unpaired Student's t test was used to analyze the data. (L) Representative flow cytometry plot for ROS estimation by DCFDA assay from STM WT, STM $\Delta chiA$, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ infected and LPS treated peritoneal macrophages. (UI US- Unstained uninfected, UI- Uninfected. LPS- Lipopolysaccharide). (M) Quantification of the MHC-II density per unit

length of the cell membrane of STM WT, STM $\Delta chiA$, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ infected PMs after indicated time (UI- Uninfected). Data are represented as mean $\pm$ SEM of 2 independent experiments. One-way ANOVA was used to analyze the data. (UI- Unstained uninfected, UI- Uninfected). (N) 3H thymidine incorporation assay to assess CD8⁺ T cell proliferation after 20 hours of infection with STM WT, STM $\Delta chiA$, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ (UI- Uninfected, OVA- Ovalbumin, ConA- Concanavalin A). Data are represented as mean $\pm$ SEM of 3 independent experiments. One-way ANOVA was used to analyze the data. (O) Representative flow cytometry plot showing the level of surface MHC-II molecules on PMs infected with STM WT and STM $\Delta chiA$ for 20 hours (UI- Unstained uninfected, UI- Uninfected).

Fig 5. Chitinase facilitates *in vivo* invasion, survival and pathogenesis of *Salmonella Typhimurium*. (A) Survival of the mice infected with lethal dose of STM WT and STM $\Delta chiA$ (PBS= Phosphate Buffered Saline). Data are presented from one independent experiment, representative of 3 independent experiments (N=3). (B) Bacterial shedding in the feces of the animals infected with STM WT and STM $\Delta chiA$. Data are presented as mean $\pm$ SD of one independent experiment, representative of 3 independent experiments (N=3). (C) *In vivo* invasion in Peyer's patches 2 hour post oral gavage by either STM WT or STM $\Delta chiA$. Data are presented as mean $\pm$ SEM of 3 independent experiments. Unpaired Student's t test was used to analyse the data. (D-G) Bacterial burden in (D) liver, (E) spleen, (F) MLN and (G) PP of the infected mice on different days post infection with sublethal dose of STM WT and STM $\Delta chiA$. Data are presented as mean $\pm$ SEM of 3 independent experiments. Unpaired Student's t test was used to analyze the data. (H) Body weight of the infected mice was measured 5 days post infection with sublethal dose of STM WT and STM $\Delta chiA$. Data are presented from 3 independent experiments. (PBS- Phosphate Buffered Saline). (I-J) Pro-inflammatory cytokines (I) IL2 and (J) IFN γ level in serum from STM WT and STM $\Delta chiA$

infected mice after indicated time. Data are presented as mean $\pm$ SEM of 3 independent experiments (N=3). One-way ANOVA was used to analyze the data. **(K)** Flow cytometry analysis of total splenocytes isolated from the spleens isolated from STM WT and STM $\Delta chiA$ infected mice after 20 days. Splenocytes were stained for CD4 and CD25 markers (US-PBS- Unstained splenocytes from PBS infected mouse). Data are presented from one independent experiment, representative of 3 independent experiments (N=3). **(L)** Serum anti-*Salmonella* antibody level was checked by sandwich ELISA after indicated time. Data are presented as mean $\pm$ SEM of 3 independent experiments (N=3). Two-way ANOVA was used to analyze the data.

Fig 6. Chitinase enhances *Salmonella* Typhi pathogenesis in *C. elegans*. **(A)** Bacterial proliferation measured by the ratio of bacterial CFU obtained from infected *C. elegans* after 24 hours and 48 hours continuous feeding on STY WT, STY $\Delta chiA$, STY $\Delta chiA:chiA$ and STY $\Delta chiA:pQE60$ strains. Data are represented as mean $\pm$ SEM of 4 independent experiments. One way ANOVA was used to analyze the data. **(B)** Survival of the worms fed on *E. coli* OP50, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$. Data are presented from one independent experiment, representative of 4 independent experiments. **(C)** Representative confocal images of bacterial colonization in worms gut as observed by infecting transgenic FT63 worms by bacteria expressing mCherry for indicated time. Yellow arrows show presence of intact bacteria in the terminal bulb of the worm. **(D)** Representative confocal images of bacterial colonization on the chitin-rich organs of the worms as detected by eosin Y staining. Yellow arrows colocalization of the bacteria (red) and the eosin-stained chitin containing regions (green). White arrow shows absence of colocalization of the bacteria and chitin-rich organs. **(E)** Representative confocal images showing bacterial colonization in the worms gut after 48 hours of STY WT and STY $\Delta chiA$ feeding followed by feeding on *E. coli* OP50 for subsequent 24 hours.

Fig 7. *Salmonella* chitinase is important for alteration of metabolism and antibacterial defense in *C. elegans*. (A) Representative images of bacterial colonization of the worms gut at higher magnification. Yellow arrows show presence of the STY WT and STM $\Delta invC$ bacteria outside the gut lumen. (B) Quantification of no. of pharyngeal pumps/min in *E. coli* OP50, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ fed worms. Data are represented as mean $\pm$ SD of 3 independent experiments. Two-way ANOVA was used to analyze the data. (C) Representative images of Oil Red O stained worms fed with *E. coli* OP50, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ for 48 hours. (D-E) Quantitative RTPCR analysis of the p38 MAPK dependent antimicrobial peptide genes (D) *clec85*, *lys7* and *lys2* (E) *spp1* and *abf2* in worms fed with *E. coli* OP50, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ for 48 hours. Fold change was normalized over *act2*. Data represents mean $\pm$ SEM of 4 independent experiments. One-way ANOVA was used to analyze the data. (F) Model depicting the role of Chitinase A in bacterial invasion and regulation of host immune response during *Salmonella* pathogenesis in mouse and *C. elegans* host (Created with Biorender.com).

Fig 1

Fig 2**A** **B** **C**

Fig 3

Fig 4

Fig 5

Fig 6

Fig 7

Fig S1

Fig S2

Fig S3

Fig S4

A

C

B

D

E

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