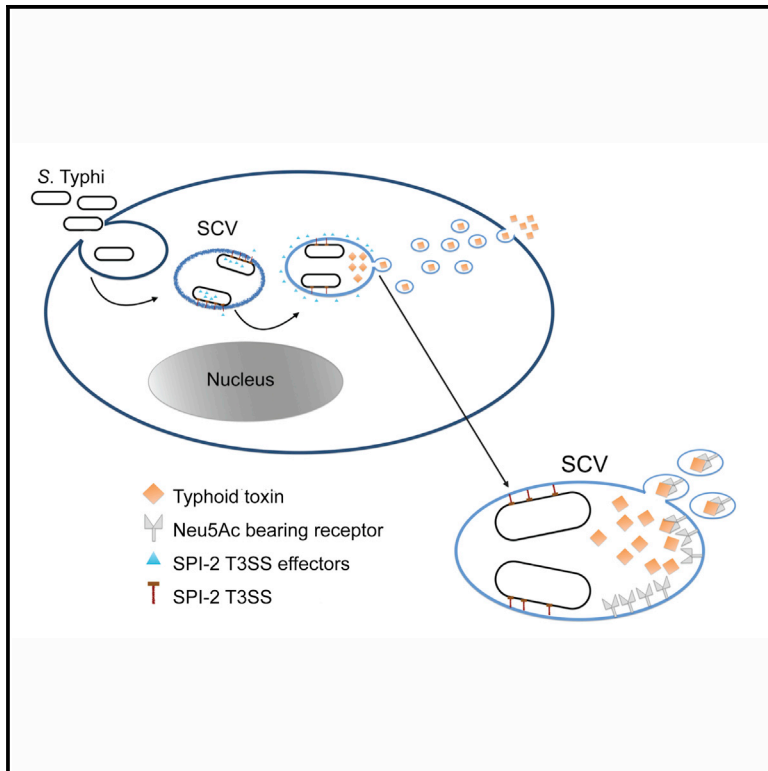# Cell Host & Microbe

Receptor-Mediated Sorting of Typhoid Toxin during Its Export from *Salmonella* Typhi-Infected Cells

Graphical Abstract

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In Brief

Typhoid toxin is produced by intracellular *S.* Typhi and subsequently packaged into transport carriers, which take it to the extracellular space. Chang et al. show that typhoid toxin sorting into the transport intermediates requires a host glycan receptor and engagement by the toxin B subunit, PltB.

Highlights

- A glycan receptor sorts typhoid toxin from the SCV to its transport carrier

- The toxin B subunit, PltB, is required for export

- *N*-glycosylation-deficient cells do not export typhoid toxin

- An *S.* Typhi mutant with altered intracellular trafficking does not export typhoid toxin

Short Article

Receptor-Mediated Sorting of Typhoid Toxin during Its Export from *Salmonella* Typhi-Infected Cells

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SUMMARY

Typhoid toxin is an essential virulence factor of *Salmonella* Typhi, the cause of typhoid fever. Typhoid toxin is secreted into the lumen of *Salmonella*-containing vacuole (SCV), after which it is packaged into vesicle carrier intermediates and released extracellularly through incompletely understood mechanisms. Following export, the toxin targets cells by interacting with human-specific Neu5Ac-terminated glycan receptors. We show that typhoid toxin is sorted from the SCV into vesicle carrier intermediates via interactions of its B subunit, PltB, with specific lumenal sialylated glycan packaging receptors. Cells deficient in *N*-glycosylation or the synthesis of specific gangliosides or displaying Neu5Gc-terminated, as opposed to Neu5Ac-terminated, glycans do not support typhoid toxin export. Additionally, typhoid toxin packaging requires the specific SCV environment, as toxin produced by an *S.* Typhi mutant with impaired trafficking is not properly sorted into vesicles. These results reveal how the exotoxin of an intracellular pathogen engages host pathways for packaging and release.

INTRODUCTION

Salmonella enterica serovars Typhi (*S.* Typhi) is an exclusive human pathogen and the cause of typhoid fever, a major global public health concern (Crump and Mintz, 2010; Dougan and Baker, 2014; Parry et al., 2002; Raffatellu et al., 2008; Wain et al., 2015). A related illness caused by *S. enterica* serovar Paratyphi A (*S.* Paratyphi) is becoming increasingly prevalent in some parts of the world (Baker et al., 2014; Fangtham and Wilde, 2008). Unlike illnesses caused by other *S. enterica* serovars, such as *S.* Typhimurium, which are usually associated with limited gastroenteritis (Grassl and Finlay, 2008; Ohl and Miller, 2001), typhoid fever is a systemic disease (House et al., 2001; Parry et al., 2002; Raffatellu et al., 2008; Wain et al., 2015) that results in ~200,000 annual deaths (Buckle et al., 2012; Crump

and Mintz, 2010; House et al., 2001; Parry et al., 2002; Raffatellu et al., 2008; Wain et al., 2015). Both *S.* Typhi and *S.* Paratyphi encode typhoid toxin, an unusual AB family exotoxin, which is largely absent from non-typhoidal *Salmonella enterica* serovars (Haghjoo and Galán, 2004; Spanò et al., 2008). Previous studies have shown that administration of purified typhoid toxin to experimental animals can reproduce most of the pathognomonic symptoms of typhoid fever, thus placing this toxin at the center of the pathogenesis of this disease (Song et al., 2013). Unlike all known AB toxin family members (Beddoe et al., 2010; Merritt and Hol, 1995), typhoid toxin exhibits a unique A_2B_5 organization with two enzymatically active "A" subunits, PltA and CdtB, linked to a homopentameric "B" subunit made up of PltB (Song et al., 2013). Typhoid toxin is targeted to cells by its PltB B subunit, which interacts with specific glycans on the surface, glycoproteins podocalyxin 1 (on epithelial cells) or CD45 (on myelocytic cells) (Song et al., 2013). Recent studies have shown that typhoid toxin has unique binding specificity for human glycans (Deng et al., 2014), which is consistent with the observation that typhoid fever occurs only in humans. In contrast to most mammals, whose sialylated glycans are terminated in *N*-glycolylneuraminic acid (Neu5Gc), human sialoglycans are primarily terminated in *N*-acetylneuraminic acid (Neu5Ac) because of the absence of CMP-*N*-acetylneuraminic acid hydroxylase (CMAH), whose coding gene has been deleted by an Alu-mediated event (Varki et al., 2011). Cells or animals expressing Neu5Gc-terminated glycans are resistant to typhoid toxin (Deng et al., 2014).

Typhoid toxin is expressed only by intracellular *S.* Typhi (Haghjoo and Galán, 2004; Spanò et al., 2008), and once synthesized, the toxin is secreted into the *S.* Typhi-containing vacuole (SCV) by a unique transport mechanism (Hodak and Galán, 2013). The secreted toxin is then packaged into vesicle carriers that transport to the extracellular space from where it reaches its target cells (Spanò et al., 2008). Notably, intoxication can only occur via autocrine or paracrine pathways. Toxin transport within infected cells is therefore central to the biology of typhoid toxin. Although some Rab GTPases required for toxin transport have been identified (Spanò et al., 2011), the mechanism by which the toxin is packaged into vesicle carriers and transported to the extracellular space remains unknown. We show here that typhoid toxin sorting into vesicle carriers requires its B subunit and that the interaction of PltB with sialylated glycans on the
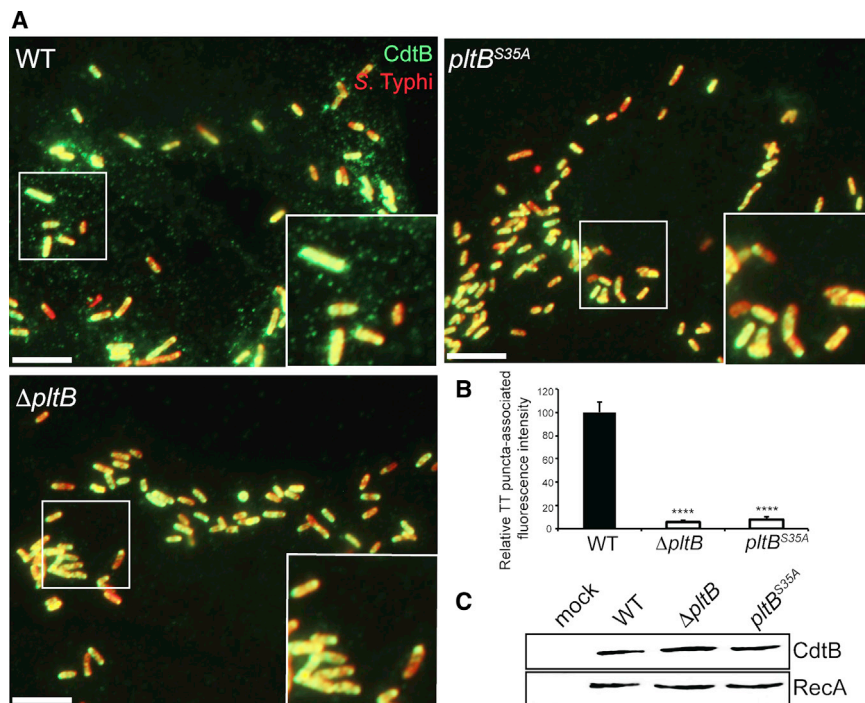
A

B

C

Figure 1. PltB Is Required for the Packaging of Typhoid Toxin into Vesicle Carrier Intermediates

(A) Immunostaining of typhoid toxin in infected cells. Infected cells were stained with antibodies against the FLAG epitope (green) and *S.* Typhi LPS (red). Scale bar, 5 μm.

(B) Quantification of the intensity of typhoid toxin-associated fluorescent puncta, a measure of typhoid toxin carrier intermediates, in infected cells. Values represent relative fluorescence intensity and are the mean ± SEM of three independent experiments in which at least 70 images were analyzed. ****$p < 0.0001$.

(C) Western immunoblot analysis of CdtB-3xFLAG expression in *Salmonella*-infected cell lysates. The levels of the *S.* Typhi protein RecA were used as loading control.

See also Figure S1.

PltB on typhoid toxin sorting, we examined the formation of toxin carrier intermediates in cells infected with an *S.* Typhi mutant strain expressing PltB^S35A. We found no detectable CdtB puncta in cells infected with the *S.* Typhi *pltB*^S35A mutant strain even though the levels of expression of CdtB in this strain were indistinguishable from wild-type (Figures 1A–1C). These results demonstrate that PltB orchestrates the packaging of typhoid toxin into vesicle carrier intermediates and suggest that a glycan receptor on the lumen of the SCV is required for typhoid toxin sorting into vesicle carrier intermediates.

SCV is essential for the formation of toxin carrier intermediates. We also show that toxin packaging requires the specific environment of the *Salmonella*-containing vacuole since the toxin produced by an *S.* Typhi mutant that does not traffic properly is not sorted into the vesicle carrier intermediates. These results demonstrate a remarkable adaptation of an exotoxin to the biology of an intracellular pathogen.

RESULTS

PltB Is Required for the Packaging of Typhoid Toxin into Vesicle Carrier Intermediates

We hypothesized that the sorting of typhoid toxin into vesicle export carriers must involve a packaging receptor that may interact with a component(s) of the holotoxin and that PltB may be such a component. To investigate this hypothesis, we examined the formation of toxin carrier intermediates in cells infected with an *S.* Typhi *ΔpltB* mutant strain expressing FLAG-epitope-tagged CdtB. We have previously shown that these vesicle carriers can be visualized by immunofluorescence microscopy as discrete puncta irradiating from the SCV that can be quantified by image analysis (Spanò et al., 2008, 2011). We found that cells infected with the *ΔpltB* mutant strain lacked detectable CdtB puncta although the expression level of CdtB in the mutant strain was indistinguishable from wild-type (Figures 1A–1C). As expected from previous results (Spanò et al., 2008), addition of a typhoid toxin neutralizing antibody to the infection media blocked intoxication but did not alter the number and the distribution of the puncta, consistent with the notion that the observed puncta represent toxin export carrier intermediates (Figure S1). A critical residue on PltB (Ser35) is strictly required for its interaction with the typhoid-toxin glycan receptors (Song et al., 2013). To explore the potential role of the glycan-binding ability of

Disruption of Protein *N*-glycosylation Impairs Typhoid Toxin Export from *S.* Typhi-Infected Cells

To further investigate the potential requirement of a glycan receptor for the sorting of typhoid toxin from the SCV into vesicle carrier intermediates, we used CRISPR/Cas9 genome editing to generate a cell line defective in alpha-1,3-mannosyl-glycoprotein 2-β-*N*-acetylglucosaminyltransferase (MGAT1) (Figure S2). This enzyme is required for the initiation of complex and hybrid *N*-glycan synthesis, and therefore it is essential for protein *N*-glycosylation (Schachter, 2010) (Figure 2A). We verified the phenotype of the MGAT1-deficient cell line by examining the mobility in SDS-PAGE of the heavily glycosylated protein LAMP1. In the parent HEK293T cells, and consistent with its heavy glycosylation, LAMP1 migrated as a broadly diffuse species with a lower mobility than that of its predicted molecular weight. In contrast, in MGAT1-deficient cells, LAMP1 migrated as a tight band of the predicted molecular weight of its unmodified form (Figure 2B). Furthermore, binding of exogenously applied, fluorescently labeled typhoid toxin to MGAT1-deficient cells was significantly reduced as predicted by the role of glycosylated receptor proteins on typhoid toxin binding (Song et al., 2013) (Figure S3). To assess the integrity of the endosomal system in the mutant cell line, we examined its ability to take up fluorescently labeled dextran and secrete alkaline phosphatase. We found that both these activities in the MGAT1-deficient cell line were indistinguishable from the parent cell line (Figure S3),
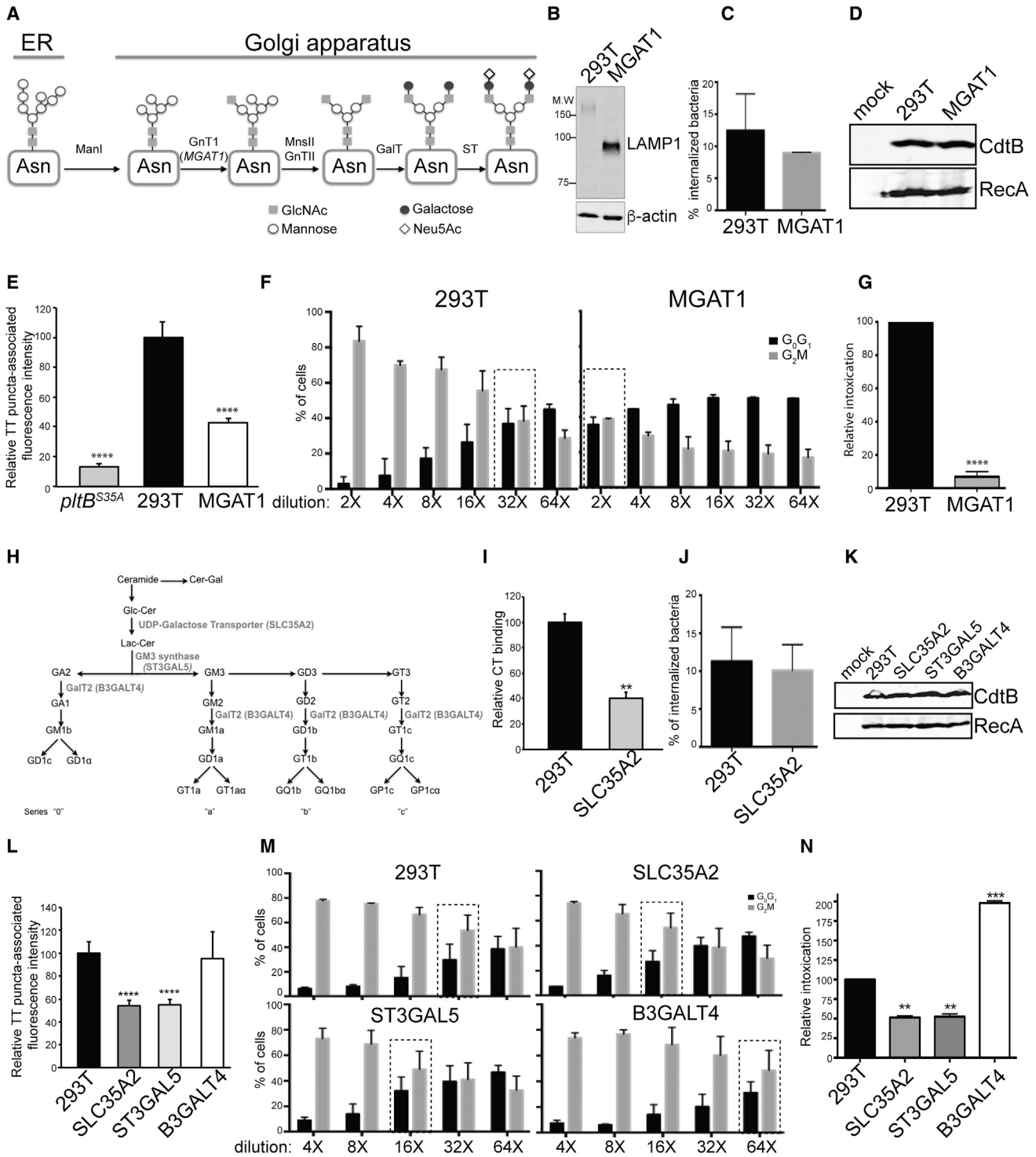
Figure 2. Contribution of *N*-glycosylation and Gangliosides to Typhoid Toxin Sorting into Export Carriers

(A) Simplified schematic representation of the *N*-linked protein glycosylation pathway in mammalian cells. Asn, Asparagine; ManI, Mannosidase I; GnT1, N-acetylglucosaminyl transferase I; MsnII, Mannosidase II; GalT, Galactosyltransferase; ST, Siayltransferase; Neu5Ac, N-Acetylneuraminic acid; GlcNac, *N*-acetylglucosamine.

(B) Analysis of protein glycosylation. Lysates from HEK293T and MGAT1-deficient cells were examined by western immunoblot for the mobility of the glyco-protein LAMP-1 in SDS-PAGE.

(C) *S*. Typhi invasion of HEK293T and MGAT1-deficient cells determined by the gentamicin protection assay. Results represent the percentage of the inoculum that survived the gentamicin treatment and are the mean ± SD of three independent determinations.

(legend continued on next page)

indicating that introduction of the mutation did not grossly alter the cell's endosomal system. We then infected the parent and MGAT1-deficient cells with *S.* Typhi expressing FLAG-epitope-tagged CdtB (to track typhoid toxin) and examined the formation of vesicle carrier intermediates by fluorescence microscopy. We found a markedly reduced number of fluorescent puncta associated with typhoid toxin vesicle carrier intermediates in MGAT1-deficient cells relative to the parent cell line despite indistinguishable levels of *S.* Typhi invasion and typhoid toxin expression in both cell lines (Figures 2C–2E; Figure S4). Since the formation of typhoid toxin carrier intermediates is reduced in MGAT1-deficient cells, we reasoned that the levels of typhoid toxin exported to the extracellular medium should also be reduced. We therefore quantified typhoid toxin export in the parent and MGAT1-deficient cells infected with *S.* Typhi by examining the amount of toxin activity in the infection media. We found that, consistent with the observed deficiency in the formation of export carrier intermediates, the level of typhoid toxin activity in the infection medium of MGAT1-deficient cells was significantly reduced in comparison to the parent cell line (Figures 2F and 2G). Taken together, these results indicate that typhoid toxin requires an *N*-glycosylated protein packaging receptor for its PltB-mediated sorting from the SCV into vesicle export carrier intermediates.

Contribution of Gangliosides to Typhoid Toxin Sorting into Export Carriers

Although the elimination of *N*-glycosylation significantly disrupted typhoid toxin export, the phenotype was less severe than that of a PltB mutant unable to bind glycans, suggesting the existence of an alternative route for typhoid toxin sorting. We have previously shown that typhoid toxin can also bind other moieties containing sialic acid, such as gangliosides (Song et al., 2013). Furthermore, gangliosides have been implicated in the vesicle traffic of viruses and some bacterial toxins (Cho et al., 2012; Ravindran et al., 2013). Therefore, we investigated the potential contribution of gangliosides to the sorting of typhoid toxin into vesicle export carrier. To this end, using CRISPR/Cas9 genome editing, we generated a cell line defective in SLC35A2, an enzyme required for the synthesis of lactosylceramide, a

common precursor for the synthesis of all gangliosides (Schnaar, 2016) (Figure 2H; Figure S2). To verify the phenotype of the SLC35A2-deficient cell line, we tested the binding of fluorescently labeled cholera toxin, which is known to recognize GM1 gangliosides as its main receptor at the plasma membrane (Fishman et al., 1993). Consistent with the predicted depletion of gangliosides, the binding of cholera toxin was significantly impaired in the SLC35A2-deficient cell line relative to its binding to the parent cell line (Figure 2I). Furthermore, the binding of exogenously applied, fluorescently labeled typhoid toxin was also reduced (Figure S3). Assessment of the ability of the mutant cell lines to take up dextran or secrete alkaline phosphatase indicated that introduction of the mutation did not grossly alter the endocytic and secretory machineries of the mutant cell lines (Figure S3). We then tested the efficiency of the formation of typhoid toxin export carrier intermediates by examining the CdtB-associated fluorescent puncta and typhoid toxin export to the extracellular milieu in *S.* Typhi-infected mutant cells. We found a reduced number of typhoid toxin export carriers and lower levels of typhoid toxin in the infection media in the SLC35A2-deficient cells, although the relative reduction was less substantial than that observed in *N*-glycosylation-deficient and MGAT1-deficient cells (Figures 2L–2N; Figure S4). Levels of *S.* Typhi invasion and typhoid toxin expression in the SLC35A2-deficient cells were indistinguishable from those observed in the parent cell line (Figures 2J and 2K). Similar results were obtained in cells rendered deficient in the GM3 synthase encoded by the ST3GAL5 gene (Figures 2L–2N; Figure S4). This enzyme encodes the synthesis of GM3, which is the precursor for the synthesis of all glycosylated gangliosides (Schnaar, 2016) (Figure 2H). Interestingly, disruption of B3GALT4, which encodes a β-1,3-galactosyltransferase 4 involved in the synthesis of complex, highly glycosylated gangliosides (Schnaar, 2016) (Figure 2H), resulted in increased typhoid toxin export (Figures 2L–2N; Figure S4). We hypothesize that the impairment of the synthesis of these gangliosides may result in the increase of other less complex gangliosides that may be more efficient at typhoid toxin sorting. Taken together, these results indicate that gangliosides can also contribute to typhoid toxin sorting into export carriers intermediates.

(D) Western immunoblot analysis of CdtB-3xFLAG expression in the indicated uninfected (mock) or *Salmonella*-infected cell lysates.

(E) Quantification of the intensity of typhoid toxin-associated fluorescent puncta, a measure of typhoid toxin carrier intermediates, in infected cells. Values represent relative fluorescence intensity and are the mean ± SEM of three independent experiments in which at least 70 images were analyzed. ****p < 0.0001.

(F and G) Quantification of typhoid toxin export into the infection medium. Infection media obtained from the indicated *S.* Typhi-infected cells were diluted as indicated and applied to HEK293T cells. 48 hr after treatment, the cell cycle profile of the different cells was analyzed by flow cytometry, and the percentage of cells in G_2M (a measure of typhoid toxin toxicity) was determined. Values are mean ± SD of three independent determinations (F). A measure of the relative toxicity (G) was obtained by comparing the dilutions of the infection media preparations (marked with dashed rectangles) that resulted in a ratio between the number of cells in G_0G_1 and G_2M that, when compared across samples, showed statistically insignificant differences. ****p < 0.0001.

(H) Simplified schematic representation of the major ganglioside synthesis pathway (Svennerholm, 1964). Cer, ceramide; Gal, galactose; Glu, glucose.

(I) Binding of fluorescently labeled cholera toxin B subunit to the indicated cells was measured by flow cytometry. Values represent relative fluorescence and are the mean ± SEM of three independent determinations. **p < 0.01.

(J) *S.* Typhi invasion of HEK293T and SLC35A2-deficient cells determined by the gentamicin protection assay. Results represent the percentage of the inoculum that survived the gentamicin treatment and are the mean ± SD of three independent determinations.

(K) Western immunoblot analysis of CdtB-3xFLAG expression in the indicated uninfected (mock) or *Salmonella*-infected cell lysates.

(L) Quantification of the intensity of typhoid toxin-associated fluorescent puncta in the indicated infected cells. Values represent relative fluorescence intensity and are the mean ± SEM of three independent experiments in which at least 90 images were analyzed. ****p < 0.0001.

(M and N) Quantification of typhoid toxin export into the infection medium (see above) (M). Values are mean ± SD of three independent determinations. A measure of the relative toxicity (N) determined as indicated in (G). **p < 0.01; ***p < 0.001.
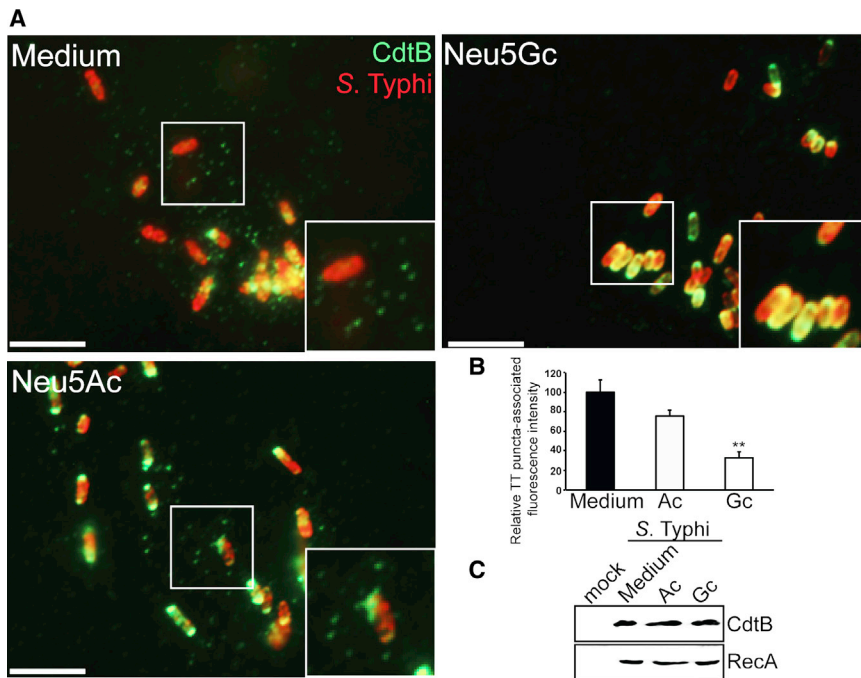
See also Figures S1–S4.

Figure 3. Metabolic Incorporation of Neu5Gc into Human Cells Prevents the Formation of Typhoid Toxin Export Carrier Intermediates

(A) Immunostaining of typhoid toxin in Neu5Ac- or Neu5Gc-treated cells. Cells were infected with *S*. Typhi strains expressing 3xFLAG epitope-tagged CdtB and stained with antibodies against the FLAG epitope (green) and *S*. Typhi LPS (red) and visualized by fluorescence microscopy. Scale bar, 5 μm.

(B) Quantification of the intensity of typhoid toxin-associated fluorescent puncta, a measure of typhoid toxin carrier intermediates, in infected cells treated as indicated. Values represent relative fluorescence intensity and are the mean ± SEM of three independent experiments in which at least 90 images were analyzed. **p < 0.01 versus cells that received Neu5Ac

(C) Western immunoblot analysis of CdtB-3xFLAG expression in the indicated uninfected (mock) or *Salmonella*-infected cell lysates. The levels of the *S*. Typhi protein RecA were used as loading control.

See also Figure S1.

Metabolic Incorporation of Neu5Gc into Human Cells Prevents the Formation of Typhoid Toxin Export Carrier Intermediates

We have previously shown that typhoid toxin exhibits strong binding specificity for Neu5Ac-terminated glycans, which are predominantly displayed in human cells (Deng et al., 2014). To investigate whether Neu5Ac-terminated glycans are also essential for typhoid toxin sorting into its vesicle carriers, we took advantage of the observation that Neu5Gc can be metabolically incorporated into human cell glycans despite the absence of CMAH (Tangvoranuntakul et al., 2003). We therefore compared the formation of typhoid toxin carrier intermediates in human Henle-407 epithelial cells that have been grown in the presence of Neu5Gc or Neu5Ac. We have previously shown that, after growth of these cells in the presence of Neu5Gc, up to 60% of the total sialic acid contained Neu5Gc (Deng et al., 2014). We found that cells that have been fed Neu5Gc showed a significantly reduced number of CdtB-associated puncta in comparison to cells that have been grown in the presence of Neu5Ac or in standard medium (Figures 3A and 3B). Levels of CdtB in the infected cells, on the other hand, were indistinguishable after growth under any condition (Figure 3C). These results indicate that Neu5Ac-terminated glycans are required for typhoid toxin sorting into vesicle transport intermediates and further demonstrate that a glycan receptor in the lumen of the SCV is critical for typhoid toxin packaging into the carrier intermediates.

Typhoid Toxin Packaging into Vesicle Transport Intermediates Requires a Specific Vacuolar Environment

The requirement of a specific glycan packaging receptor for the sorting of typhoid toxin into vesicle carrier intermediates prompted us to investigate whether this sorting event requires *S*. Typhi to reside within a specific intracellular environment.

After internalization mediated by effectors of its pathogenicity island 1 (SPI-1) type III secretion system (T3SS), *Salmonella* resides within a vacuolar compartment known as the *Salmonella*-containing vacuole (Galán, 2001). The composition of the SCV is subsequently modulated by the action of effectors delivered by the pathogenicity island 2 (SPI-2) T3SS (Figueira and Holden, 2012; Ibarra and Steele-Mortimer, 2009). Consequently, the composition of the SCV in cells infected with a *Salmonella* mutant defective in its SPI-2 T3SS is substantially different from the SCV containing wild-type bacteria. Therefore, to investigate the potential requirement of a specific intracellular environment to package typhoid toxin, we examined the formation of typhoid toxin vesicle carrier intermediates in cells infected with an SPI-2 T3SS-defective *S*. Typhi Δ*spiA* mutant. We found that, in HEK293T cells, the *S*. Typhi Δ*spiA* mutant replicated to a similar extent to wild-type (Figure 4A). More importantly, expression of typhoid toxin in wild-type and the Δ*spiA* mutant was indistinguishable (Figure 4B). However, the formation of typhoid toxin export carrier intermediates was significantly reduced in cells infected with the Δ*spiA S*. Typhi mutant strain (Figure 4C; Figure S4). Consistent with this observation, typhoid toxin export from infected cells was also reduced in cells infected with the mutant strain (Figures 4D and 4E). Taken together, these results indicate that the formation of typhoid toxin export carrier, and consequently toxin export from infected cells, requires for *S*. Typhi to reach a specific vacuolar environment.

DISCUSSION

Typhoid toxin is produced by *S*. Typhi within mammalian cells and is then packaged into vesicle transport intermediates, which transport the toxin to cell periphery for its release (Haghjoo and Galán, 2004; Spanò et al., 2008). Thus, the typhoid toxin export pathway is central to its biology, presumably because, during
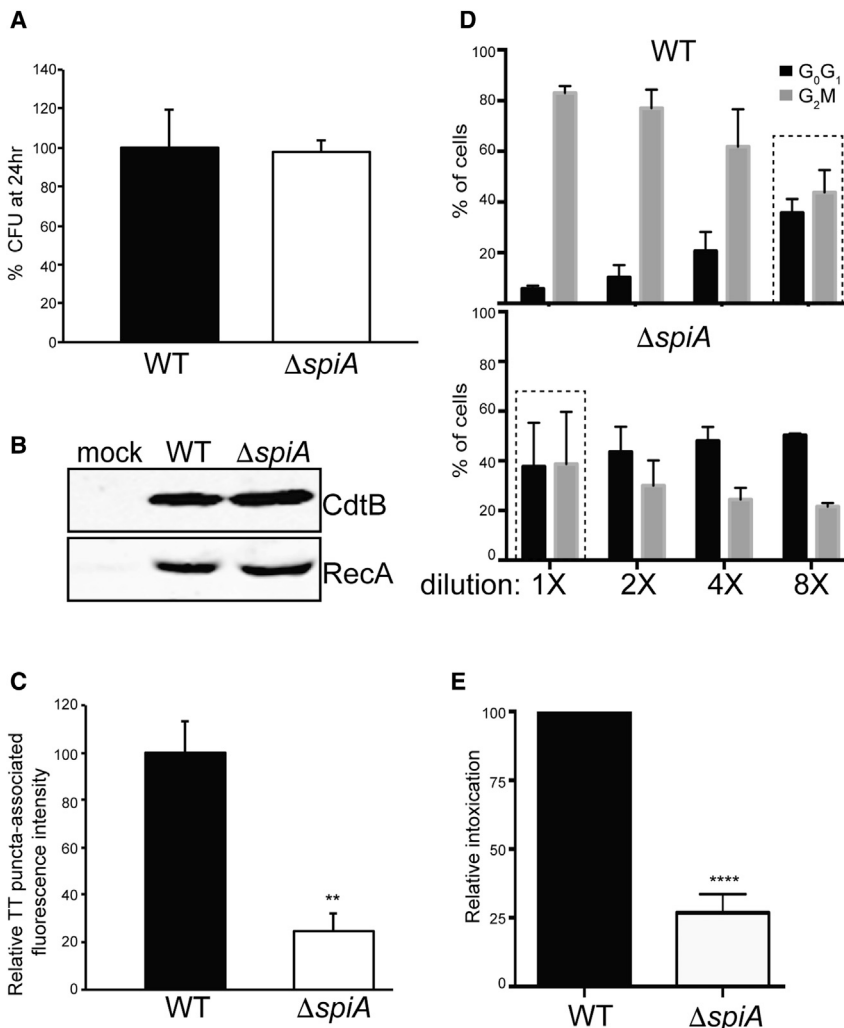
Figure 4. Typhoid Toxin Packaging into Vesicle Transport Intermediates Requires a Specific Vacuolar Environment

(A) Intracellular survival of *S*. Typhi wild-type (WT) and the SPI-2 TTSS-defective *spiA* mutant in HEK293T cells. Values represent percentage of colony-forming units (CFUs) 24 hr post-infection relative to the CFUs 2 hr after infection and have been standardized to the CFUs of wild-type, which was considered to be 100%. Data are mean ± SEM from three independent experiments.

(B) Western immunoblot analysis of CdtB-3xFLAG expression in HEK293T cells infected with the indicated *S*. Typhi strains. The levels of the *S*. Typhi protein RecA were used as loading control.

(C) Quantification of the intensity of typhoid toxin-associated fluorescent puncta, a measure of typhoid toxin carrier intermediates, in HEK293T cells infected with the indicated *S*. Typhi strains. Values represent relative fluorescence intensity and are the mean ± SEM of three independent experiments in which at least 60 images were analyzed. **p < 0.01

(D and E) Quantification of typhoid toxin export into the infection medium. Infection media obtained from HEK293T cells infected with the indicated *S*. Typhi strains were diluted as indicated and applied to uninfected HEK293T cells. 48 hr after treatment, the cell cycle profile of the different cells was analyzed by flow cytometry, and the percentage of cells in G_2M (a measure of typhoid toxin toxicity) was determined. Values are mean ± SD of three independent determinations (D). A measure of the relative toxicity (E) was obtained by comparing the dilutions of the infection media preparations (marked with dashed rectangles) that resulted in a ratio between the number of cells in G_0G_1 and G_2M that when compared across samples showed statistically insignificant differences. ****p < 0.0001.

See also Figures S1 and S4.

infection, its cellular targets may not be the cells that harbor the bacteria but rather other uninfected cells, perhaps those of the immune system. In fact, previous studies in humanized mice are consistent with this hypothesis since they have indicated that typhoid toxin may be important for *S.* Typhi's persistent infection, a process that may require targeting immune cells (Song et al., 2010). Therefore, understanding the mechanisms by which the toxin is exported from infected cells could lead to the development of therapeutic strategies that may help the eradication of *S.* Typhi from chronic carriers.

Cargo selection during vesicle transport is a complex process that requires specific cellular machinery (Dancourt and Barlowe, 2010). Although the components of this machinery vary depending on the cellular compartment or the cargo to be packaged, the packaging mechanisms usually involve transmembrane coat proteins that can select transmembrane protein cargo, additional coat components that deform the membrane and orchestrate the budding process, and specific small GTP-binding proteins that regulate the entire process. In addition, soluble cargo requires specific membrane protein receptors for their recruitment into the budding vesicle. The *Salmonella*-containing vacuole is a highly specialized compartment built through the

action of numerous effectors of its two type III secretions systems (Steele-Mortimer, 2008). Consequently, it is likely that the machinery involved in the packaging of typhoid toxin is different from the packaging machineries that have been described in specific cellular compartments. For example, we have previously identified two Rab-family GTPases, Rab40b and Rab29, that are required for efficient packaging of typhoid toxin (Spanò et al., 2011). Although neither of these GTPases has previously been implicated in endocytic sorting, it is intriguing that Rab29 can be seen decorating the SCV (Spanò et al., 2011), where it may play a regulatory role in cargo selection and/or budding. Here we report another important element for the selection of typhoid toxin for packaging into vesicle carrier intermediates. We found that typhoid toxin packaging is orchestrated by a sialylated glycan receptor on the SCV that specifically interacts with PltB, the B subunit of typhoid toxin. We showed that the glycan receptor must be terminated in Neu5Ac, which is most abundant in human cells but largely absent in glycans from other mammalian cells. In fact, we found that feeding Neu5Gc, which is incorporated into human cell glycans, prevents typhoid toxin packaging into vesicle carrier intermediates. These results are consistent with our previous observations that indicate that typhoid toxin

is exquisitely adapted to humans. Our results also showed that the glycan receptor(s) must be preferentially associated with a glycoprotein, since a cell line unable to perform protein N-glyco-sylation is defective in the formation of typhoid toxin carriers for its export from infected cells. However, we also found that gangliosides also contribute to typhoid toxin sorting, since cell lines defective in the synthesis of gangliosides were defective at packaging and exporting typhoid toxin. Interestingly, ganglio-sides have been previously implicated in the endocytic sorting of various toxins and viruses during their retrograde transport from the plasma membrane to their final destination within the endocytic network (Cho et al., 2012; Ravindran et al., 2013).

Consistent with the requirement of specific machinery to sort and package typhoid toxin into export carriers, we found that the nature of the *Salmonella*-containing vacuole profoundly impacts the packaging process. More specifically, we found that typhoid toxin expressed and secreted by an *S.* Typhi mutant defective in the SPI-2-encoded T3SS is inefficiently packaged into vesicle carrier intermediates. Effectors delivered by this T3SS are essential for the formation of the SCV; therefore, these findings indicate that typhoid toxin has evolved to be packaged specifically within this specific environment.

In summary, our results depict a remarkable adaptation of an exotoxin for the specific biology of an intracellular pathogen. Furthermore, given the importance of typhoid toxin export in its biology, these findings may provide the bases for the development of potential therapeutic strategies that may help with the treatment of typhoid fever.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids
All the *S.* Typhi strains were derived from strain ISP2825 (Galán and Curtiss, 1991) and are listed in Table S1, and all plasmids are listed in Table S2.

Cell Culture, Bacterial Infections, and Quantification of Typhoid Toxin Transport Intermediates and Export
Cell culture, metabolic incorporation of Neu5Ac or Neu5Gc, and bacterial infections were carried out as previously described (Deng et al., 2014). The visualization of typhoid toxin vesicle carrier intermediates was carried out as previously described (Spanò et al., 2008) and quantified using the open source software ImageJ (https://imagej.nih.gov/ij/). *S.* Typhi internalization and replication within cultured cells was carried out using the gentamicin protection assay as previously described (Galán and Curtiss, 1989). The supernatants from infected cells were collected and filtered through 0.2 μm syringe filters at 24 or 48 hr post-infection, diluted as indicated in each experiment, and applied to fresh cells. Toxicity of the different preparations was measured as previously described (Spanò et al., 2008).

Cell Culture and CRISPR/Cas9 Gene Inactivation in Cultured Human Cells
CRISPR/Cas9 genome editing was carried out following standard protocols (Ran et al., 2013) (Figure S2).

Dextran Internalization and Secreted Alkaline Phosphatase Assays
Cells were incubated in the presence of Alexa 488-conjugated dextran for 40 min (molecular weight 10,000; ThermoFisher Scientific) and dextran uptake quantified by fluorescence-activated cell sorting (FACS). To assay, we transfected secreted alkaline phosphatase cells with a plasmid encoding the secreted alkaline phosphatase or co-transfected them as a negative control with a plasmid encoding pArf1-T31N. Next day, the levels of secreted alkaline phosphatase in the culture medium were determined with the fluorescent substrate DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate). The total level of cell-associated alkaline phosphatase was measured after lysis in 0.2% Triton X-100.

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