Shiga toxins — from cell biology to biomedical applications

Ludger Johannes and Winfried Römer

Abstract | Shiga toxin-producing *Escherichia coli* is an emergent pathogen that can induce haemolytic uraemic syndrome. The toxin has received considerable attention not only from microbiologists but also in the field of cell biology, where it has become a powerful tool to study intracellular trafficking. In this Review, we summarize the Shiga toxin family members and their structures, receptors, trafficking pathways and cellular targets. We discuss how Shiga toxin affects cells not only by inhibiting protein biosynthesis but also through the induction of signalling cascades that lead to apoptosis. Finally, we discuss how Shiga toxins might be exploited in cancer therapy and immunotherapy.

Enterohaemorrhagic *Escherichia coli*
(EHEC). *Escherichia coli* strains that cause haemorrhagic colitis and haemolytic uraemic syndrome. EHEC constitutes a subset of serotypes called Shiga toxin-producing *E. coli* (STEC), in which these toxins are virulence factors.

Haemolytic uraemic syndrome
(HUS). A life-threatening complication characterized by microangiopathic haemolytic anaemia, thrombocytopenia and acute renal failure.

Institut Curie — Centre de Recherche and CNRS UMR144, Traffic, Signalling and Delivery Laboratory, 26 rue d'Ulm, 75248 Paris Cedex 05, France.
Correspondence to L.J.
e-mail: ludger.johannes@curie.fr
doi:10.1038/nrmicro2279
Published online
21 December 2009

The Shiga toxin family, a group of structurally and functionally related exotoxins, includes Shiga toxin from *Shigella dysenteriae* serotype 1 and the Shiga toxins that are produced by enterohaemorrhagic *Escherichia coli* (EHEC) strains. The existence of different, interchangeable terms to describe very similar toxins has historical reasons. The Japanese microbiologist Kiyoshi Shiga was the first to characterize the bacterial origin of dysentery caused by *S. dysenteriae*, in 1897. In 1977, Konowalchuk discovered a group of *E. coli* isolates that produced a factor that was able to kill Vero cells in culture[1]. The factor was termed verotoxin, and the bacteria were termed verotoxin-producing *E. coli* (VTEC). O'Brien and colleagues recognized in the early 1980s that some *E. coli* isolates produced a toxin that was related to Shiga toxin and named these organisms Shiga-like toxin-producing *E. coli* (STEC)[2]. In 1983, it was recognized that STEC strains are associated with haemolytic uraemic syndrome (HUS)[3]. Researchers eventually realized that they were studying identical or highly related toxins.

Shiga toxin is the prototype of the Shiga toxin family and nearly identical to the *E. coli*-produced Shiga toxin 1 (Stx1), differing by a single amino acid in the catalytic A subunit of the toxin. STEC can produce Stx1 variants (Stx1 and Stx1c), Stx2 variants (Stx2, Stx2c, Stx2d, Stx2e, Stx2f) or variants of both in a range of combinations (TABLE 1). However, severe disease has been epidemiologically linked to the presence of Stx2 (REF. 4). Although Stx1 and Stx2 share a common receptor and possess the same intracellular mechanism of action, they are immunologically distinct and only 56% identical at the amino acid sequence level[5]. Stx2 variants are 84–99% homologous to Stx2.

The Shiga toxins in *S. dysenteriae* and STEC are encoded by diverse lambdoid bacteriophages[6]. These phages are highly mobile genetic elements that play an important part in horizontal gene transfer and, hence, in genome diversification[7]. Located in the late gene region downstream of the late promoters and upstream of the lysis cassette, *stx* genes are highly expressed when the lytic cycle of the phage is activated. Phages regulate the production of Shiga toxins through the activity of phage gene promoters, through the amplification of gene copy number and through toxin release[8,9]. Conditions unique to the periplasm of Gram-negative bacteria are required for toxin subunits to efficiently fold and assemble. Once assembled, toxin secretion seems to be achieved by phage-mediated bacterial lysis[10].

Little is known about the influence of intestinal factors on the production of Shiga toxins. Human neutrophil activation products such as hydrogen peroxide induce toxin release by EHEC[11]. The *stx1* genes are also regulated by iron, with toxin synthesis being repressed at high iron concentrations[12], thus limiting the site of toxin production to the distal small intestine and colon.

Human infection by *S. dysenteriae* or EHEC strains, which results initially in diarrhoea, has been associated with the ingestion of contaminated minced beef, vegetables, milk, juice or water. Frequently, the disease progresses into dysentery and haemorrhagic colitis, which may further develop into life-threatening systemic extra-intestinal complications, such as acute renal failure and central nervous system complications that sometimes result in death[13]. Haemorrhagic colitis is primarily a disease of young children and

Table 1 | **The family of Shiga toxins**

Organism	Toxin	Sequence similarity to Shiga toxin		Characteristics	Synonyms	Cellular receptors
		A subunit	B subunit*			
Shigella dysenteriae	Shiga toxin	N/A	N/A	N/A	N/A	Gb3
STEC	Stx1	97%	98%	N/A	SLTI and VT1	Gb3
	Stx1c	97%	98%	N/A	SLTIc and VT1c	Gb3
	Stx2	53%	64%	Associated with severe disease in humans	SLTII and VT2	Gb3
	Stx2c	53%	61%	N/A	SLTIIc and VT2c	Gb3
	Stx2d	54%	61%	N/A	SLTIId and VT2d	Gb3
	Stx2e	53%	61%	Associated with the piglet edema disease	SLTIIe and VT2e	Gb3 and Gb4
	Stx2f	54%	60%	N/A	SLTIIf and VT2f	Gb3

Gb3, globotriaosylceramide; N/A, not applicable; SLT, Shiga-like toxin; STEC, Shigella toxin-producing *Escherichia coli*; Stx1, Shiga toxin 1; VT: Verotoxin. *This is the sequence similarity for mature B fragments, without signal sequences.

elderly people[14]. The most common strain of *E. coli* that causes haemorrhagic colitis is *E. coli* serotype O157:H7. This toxigenic bacterium naturally inhabits the gastrointestinal tract of domestic animals without any ill effect for the host. Up to 15% of patients with haemolytic colitis caused by *E. coli* serotype O157:H7 go on to develop HUS[15]. STEC strains that produce Stx2 are more likely to cause HUS than those that only produce Stx1 (REF. 16). Moreover, strains producing Stx2 alone were found to cause more severe neurological symptoms in gnotobiotic piglets than strains producing only Stx1 or both Stx1 and Stx2, whereas Stx1-producing strains induce only diarrhoea and no systemic complications[17]. Affected organs in humans and experimental animals show signs of profound vascular damage. The most severely affected patients require blood transfusions and dialysis therapy. Haemodialysis and peritoneal dialysis have been used in the past, depending on the age and weight of patients, and peritoneal dialysis is the treatment of choice for chronic renal failure[18]. Several intervention strategies are currently being developed to prevent organ damage by Shiga toxins, the most prominent of which are summarized in BOX 1.

Toxin structure and cellular receptors

Shiga toxin family members have an AB_5 molecular configuration, as revealed by X-ray crystallography[19,20]: An enzymatically active monomeric A subunit, StxA (which has a molecular mass of 32 kDa) is non-covalently associated with a pentamer of identical B fragments (each B fragment has a molecular mass of 7.7 kDa) that form the B subunit, StxB, which is responsible for binding to cell surface receptors (FIG. 1a). StxB forms a doughnut-shaped structure with a central pore into which the carboxyl terminus of StxA inserts[19]. StxA and the StxB fragments are secreted into the bacterial periplasm[21], where they assemble non-covalently into the holotoxin, as was initially described for heat-labile enterotoxins from *E. coli*[22].

StxA possesses a highly specific RNA *N*-glycosidase activity that cleaves an adenine base at position 4,324 on the α-sarcin loop located on domain VI of 28S ribosomal RNA (rRNA) of eukaryotic ribosomes[23,24], thereby inhibiting elongation factor-dependent aminoacyl tRNA binding and subsequent chain elongation[25]. Bacterial ribosomes are also a substrate for StxA, and exposure to Stx1 results in decreased proliferation of susceptible bacteria[26]. Although Shiga toxins are extremely potent ribosome-modifying enzymes, it should be noted that their action is not limited to the inhibition of protein synthesis. As discussed below, they have several cellular effects, including the induction of cytokine expression by macrophages, which in turn may increase the susceptibility of certain cells to toxins.

StxB binds to the neutral glycosphingolipid globotriaosylceramide (Gb3; also known as CD77 or the P^k blood group antigen), which is present on the surface of cells[27–29], leading to subsequent internalization of the toxin. Stx2e uses globotetraosylceramide (Gb4), which contains an additional terminal β[1–3]-linked *N*-acetylgalactosamine residue. In the absence of StxA, StxB still adopts a pentameric structure that is functionally equivalent to the holotoxin in receptor binding[30].

The first structures of Shiga holotoxin[19] and of the B subunit of Stx1 alone[20] were determined by X-ray crystallography in the absence of receptor molecules. Molecular modelling studies[31,32] and fluorescence energy transfer measurements[33] indicated the presence of two receptor-binding sites per B fragment monomer. In 1998, the crystal structure was determined for the Stx1 B subunit in complex with a trisaccharide receptor analogue of Gb3. This study revealed the existence of three trisaccharide-binding sites per B fragment monomer[34] (FIG. 1b,d). All 15 of the Gb3-binding sites in the B subunit homopentamer are facing in the same direction, distal to the A subunit binding site, thereby identifying the membrane interaction surface. The Gb3-binding sites do not interact. At least one of the binding sites

Box 1 | **Intervention strategies**

Clinical observations indicate that conventional anti-microbial therapies for the treatment of infections by Shiga toxin-producing bacteria have inherent limitations and may even be counterproductive[146–148], as killing the bacteria may accelerate toxin release. Hence, a need exists for the development of new therapeutic modalities. Possible therapeutic options include the use of agents that block Shiga toxin binding at the cell surface or its intracellular transport, thereby protecting people from toxin-mediated pathology and development of haemolytic uraemic syndrome (HUS).

Synthetic Shiga toxin binders

To mimic the cell surface binding of Shiga toxin B subunit (StxB) to several globotriaosylceramide (Gb3) molecules at a time, oligovalent water-soluble globotriose ligands, termed STARFISH, were designed on the basis of the crystal structure of StxB in complex with a Gb3 analogue and were found to have a subnanomolar inhibitory activity[149]. However, the protective activity of STARFISH against Shiga toxins was suboptimal *in vivo*[150]. Recent improvements based on supramolecular templating to create high-avidity inhibitors for multimeric receptors are now being tested[151]. In another study, the trisaccharide moiety of Gb3 has been covalently coupled to an inert silicon-based matrix, Chromosorb-P. The resulting compound was termed Synsorb-Pk[152]. Co-incubation of human renal adenocarcinoma cells with Shiga toxin 1 (Stx1) and Stx2 from *Escherichia coli* and Synsorb-Pk for 1 hour resulted in 50% protection of cells, but clinical trials did not show the expected protective effect in children with diarrhoea-associated HUS[153]. SUPER TWIG, a carbosilane dendrimer carrying variously oriented Gb3 trisaccharides[154], and linear polymers of acrylamide with clustered Gb3 trisaccharides (called Gb3 polymers)[155] are promising therapeutic agents for use against Shiga toxin-producing *E. coli* infection in humans. These Stx neutralizers function in the circulation and in the gut, respectively, and exhibit Stx-binding capacities that are 100,000 times as high as those of Synsorb-Pk. Cytotoxic activities of Stx1 and Stx2 were markedly inhibited, and mice were protected from a challenge with a fatal dose of *E. coli* O157:H7, even when the neutralizers were administered after infection had been established.

Probiotic bacteria

A recombinant bacterium based on a non-pathogenic *E. coli* strain was designed that displays a Stx receptor mimic on its surface. This probiotic bacterium has an apparent affinity for StxB that is around 10,000 times as high as that of Synsorb-Pk, and Shiga toxins were indeed very efficiently adsorbed and neutralized. Oral administration of the bacterium completely protected mice from lethal toxin doses[156,157].

Monoclonal antibodies

The use of monoclonal antibodies specific for both Stx1 and Stx2 may be the most attractive treatment modality at this stage[158,159]. These antibodies prolonged the survival of toxin-challenged mice and have also been shown to prevent toxin-associated disease in a piglet model, even when antibodies were given 6–12 hours after toxin application.

Shiga toxin A subunit inhibitors

Small-molecule inhibitors of the catalytic sites of ricin and Stx were designed, based on guanine-like compounds such as pteroic acid[160].

Transport inhibitors

Screens were designed to identify small molecule compounds that inhibit toxin transport along the retrograde route. Examples of some of the identified molecules are Exo1 (REF. 161), Exo2 (REFS 162,163), compounds 75 and 134 (REF. 164), and Golgicide A[165]. In all these cases, application of the inhibitor affected Golgi morphology, and the specificity of the compounds and their protective effect in the host organism remain to be established.

(site 1) is composed of residues from adjacent monomers and therefore only exists in assembled pentameric StxB. Mutational analysis has shown that site 1 and site 2 mediate high affinity receptor binding and are the most relevant for cell cytotoxicity, whereas site 3 mediates the recognition of additional low-affinity Gb3 epitopes[35,36]. Stable isotope-assisted high-resolution NMR methods were used to determine the solution structure of the complex between the Stx1 B subunit and a ^{13}C-enriched Gb3 analogue[37]. In contrast to the crystal structure, only one of the three possible binding sites per monomer, site 2, was occupied by the trisaccharide in solution. The multiple Gb3 binding sites on the homopentameric B subunit explain how the protein achieves a remarkably high affinity for cells (with binding constants in the order of 10^9 M^{-1})[38], despite the much lower affinity of Gb3 molecules for individual binding sites (with binding constants of around 10^3 M^{-1})[39].

Analysis of the crystal structure of Stx2 predicted the presence of the corresponding trisaccharide binding sites on its B subunit but also demonstrated that the conformation at site 2 differs distinctively from that of the Shiga toxin and Stx1 B subunits[19]. Stx1 and Stx2c indeed prefer Gb3 species that differ in the nature of their fatty acid chains[40], and the tightest toxin binding is observed for defined mixtures of these receptor species[41]. Interestingly, Stx1 has a 10-fold higher affinity for Gb3 in cells than Stx2 (REF. 42), but Stx2 has an approximately 400-fold lower LD_{50} in mice than Stx1 (REF. 43). This apparent contradiction may be explained at least in part by differences in their biodistribution.

Gb3 fatty acid heterogeneity[41], hydroxylation[44], chain length and degree of unsaturation[40] may influence the lateral mobility of the lipid in the plasma membrane and affect the conformation of the trisaccharide head group that is presented on the cell surface. Indeed, all these factors and the membrane environment, including cholesterol levels, were shown to be crucial for the recognition process[40,41,44–48]. Interestingly, patients who developed HUS after STEC infection were found to have a higher hydroxylated fatty acid content in their red blood cell Gb3 than

LD_{50}
The median lethal dose of a toxic substance; this is the dose that is required to kill half of the members of a tested population.

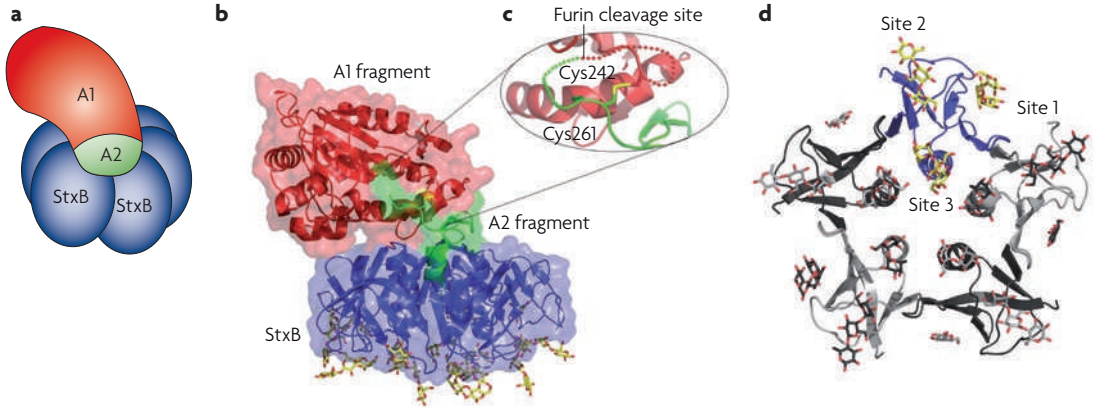

Figure 1 | **Shiga toxin structures. a** | A cartoon of Shiga holotoxin, consisting of one A subunit (StxA), which is cleaved into fragments A1 and A2, and five B fragments that constitute the homopentameric B subunit (StxB). **b** | A ribbon diagram of Shiga toxin, highlighting globotriaosylceramide (Gb3)-binding sites on StxB. Gb3 is shown in a ball-and-stick representation. **c** | An enlargement of StxA at the site of furin cleavage (Arg25-Met252), and showing the disulphide bond (between Cys242 and Cys261) that links the A1 and A2 framents. **d** | A ribbon diagram of an StxB subunit from the membrane-oriented surface, highlighting the three Gb3-binding sites. Gb3 is shown as a ball-and-stick representation. Note the central pore that is lined by α-helices.

patients who did not develop HUS[49], strongly suggesting that Gb3 molecular species differences are also crucial for toxin function in humans.

Shiga toxin trafficking

Extensive studies have been performed on the intracellular trafficking of Shiga toxin and have been reviewed in detailed elsewhere[50–53]. Supplementary information S1 (table) provides an overview of the cellular factors that contribute to the trafficking of Shiga toxins. We limit our discussion below to selected findings concerning endocytosis and retrograde transport that have had a conceptual impact beyond the toxin field. Indeed, StxB has been developed into a cell biology tool[54,55] that allows the quantitative study of trafficking pathways of general importance (reviewed in REF. 52).

Endocytosis. Following the binding to Gb3 receptor molecules at the plasma membrane, Shiga toxin and the other members of the family are internalized into target cells by endocytosis (FIG. 2). Shiga toxin has been found in clathrin-coated pits[56]. When clathrin-dependent endocytosis is inhibited, however, Shiga toxin is still taken up efficiently[57–59], showing that the clathrin pathway is not required for the initial steps of toxin entry into the cell. Indeed, Shiga toxin can induce endocytic plasma membrane invaginations without the help of the cytosolic machinery[60]. In this case, membrane bending results from the toxin-driven clustering of glycosphingolipid receptor molecules (FIG. 2), representing a new mechanism of curvature generation in biological membranes. The toxin-induced invaginations are then processed by cellular machinery involving dynamin, actin and plasma membrane cholesterol.

Retrograde transport. Following its entry into the cell, Shiga toxins localize to early and recycling endosomes. In a pioneering study, Sandvig and van Deurs found that Shiga toxin could eventually be detected in the membranes of the endoplasmic reticulum (ER)[61], strongly suggesting that the toxin was trafficking in a retrograde manner through the secretory pathway (FIG. 2). It was shown subsequently that Shiga toxin can bypass the late endocytic pathway to be transferred directly from early and recycling endosomes to the *trans*-Golgi network (TGN)[62] and, from there, on to the ER. This trafficking seems to be different from the recycling loop that had been identified previously for mannose-6-phosphate receptors, which are cargo-shuttling proteins that were shown to cycle between late endosomes and the TGN[63]. It was then found that the retrograde trafficking route that links the early endocytic pathway to the TGN is also used by several cellular proteins with diverse functions ranging from cell signalling and glucose transport to morphogen trafficking and tissue remodelling (reviewed in REF. 52).

The mechanisms by which Shiga toxins escape from the early endocytic pathway have been intensively studied, and clathrin[57,59] and retromer[64–66] are of crucial importance. Clathrin is a nanodomain organizing protein with established functions in the generation of membrane curvature. Clathrin is recruited to endosomes by a host of proteins, including the phosphatidylinositol-4-phosphate-binding protein Epsin-related protein (EpsinR), for which a role in retrograde transport has been shown[59]. Retromer is composed of a curvature recognition subunit of two sorting nexins (a combination from SNX1, SNX2, SNX5 and SNX6) and a cargo recognition subunit made of three vacuolar protein sorting-associated proteins, VPS26, VPS29 and VPS35 (reviewed in REF. 67). Exactly how clathrin and retromer drive the formation of retrograde transport intermediates on early endosomes remains to be established. Recent evidence suggests that both proteins may function in a sequential manner[65] (FIG. 2). According to this model, clathrin is required for the generation of initial membrane curvature on early endosomes. Cargo uses clathrin adaptors and the cargo

Retromer
A heteropentameric complex that associates with the cytosolic face of endosomes and mediates retrograde transport of cargo molecules from endosomes to the *trans*-Golgi network.

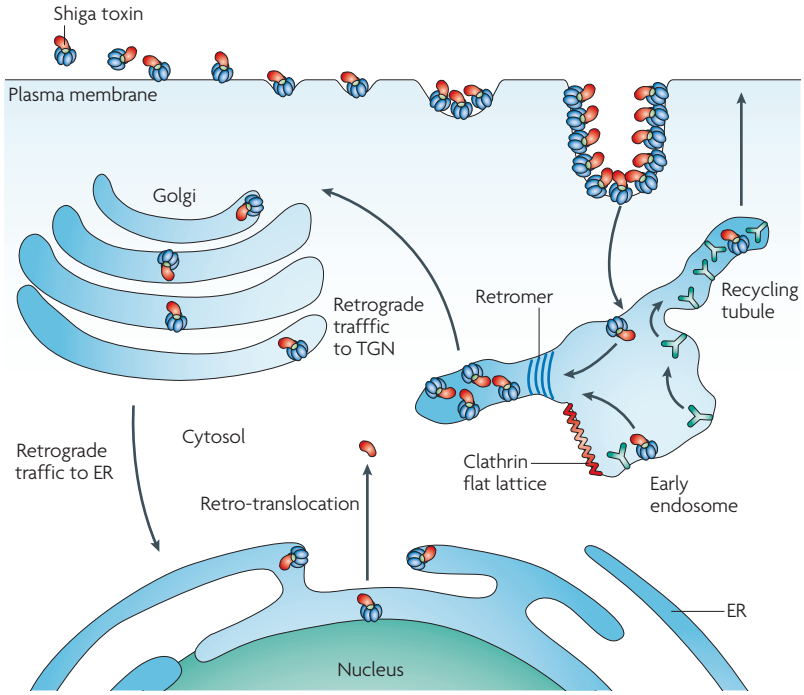

Figure 2 | **Trafficking of Shiga toxins.** An overview of intracellular trafficking of Shiga toxins. Toxin binding to the plasma membrane induces local spontaneous curvature, membrane-mediated clustering and the toxin-driven formation of endocytic invaginations. The toxin then undergoes retrograde sorting in early endosomes, in which retrograde tubules are formed in a clathrin-dependent manner, and Shiga toxins preferentially localize to this tubular environment. Retrograde tubules are processed by scission in a retromer-dependent manner. Shiga toxins bypass the late endocytic pathway and are transferred directly from the early endosome to the *trans*-Golgi network (TGN)[62] and, from there, on to the endoplasmic reticulum (ER). Finally, Shiga toxins use the ER-associated degradation (ERAD) machinery to facilitate retro-translocation into the host cell cytosol. By contrast, the transferrin receptor protein (TfR) (Y-shaped) becomes enriched in recycling tubules in the early endosome and recycles back to the plasma membrane.

recognition subunit of retromer to localize to the sites of transport intermediate formation. Nascent retrograde tubules are then processed through scission into retrograde transport intermediates, probably also involving retromer in a currently ill-defined process. A detailed discussion of this model can be found in REF. 52.

How do Shiga toxins, which bind to the Gb3 glycolipid in the exoplasmic membrane leaflet, communicate with the cytosolic sorting machinery, and why does their trafficking depend on clathrin in the early endosomes but not at the plasma membrane? The first point may be related to the finding that Shiga toxins induce membrane reordering[60] and associates with membrane environments that form detergent-insoluble complexes after cell lysis[68,69]. This fractionation behaviour has been correlated with efficient retrograde sorting in HeLa cells, and the absence of detergent-insoluble domains has been correlated with inefficient retrograde sorting in human monocyte-derived macrophages and dendritic cells that are totally toxin resistant[68]. Toxin-induced changes in lipid repartition in the exoplasmic leaflet may drive similar nanocompartmentalization at the level of the cytoplasmic leaflet and favour the recruitment of sorting machinery.

The requirement of clathrin on endosomes but not at the plasma membrane may be two sides of the same coin: although the toxin induces curvature at the plasma membrane through dynamic cluster formation[60], once clustered the toxin may localize preferentially to the curved environment of retrograde tubules of early endosomes, leading to geometry-driven sorting. Shiga toxins are transported from the TGN in a coat protein complex I (COPI)-independent manner through the Golgi apparatus to the ER[70,71]. The exact mechanisms that underlie this trafficking step still remain largely unexplored.

Retro-translocation. Shiga toxins do not induce pore formation. Instead, they rely on host cell machinery to translocate the catalytic A subunit across cellular membranes and to gain access to the cytosol. The mechanisms by which this occurs are starting to be understood. During the early entry process (probably at the stage of early endosomes), a protease-sensitive loop (residues 242–261) located in the C-terminal region of StxA is cleaved by the membrane-associated endoprotease furin at site Arg251-Met252 (FIG. 1), dividing StxA into the catalytic A1 fragment (consisting of residues 1–251; 27.5 kDa) and an StxB-associated A2 fragment (consisting of residues 252–293; 4.5 kDa)[72]. The A1 fragment remains linked with the StxA2–StxB complex by virtue of a disulphide bond between Cys242 in StxA1 and Cys261 in StxA2 (REFS 72–76). This disulphide bond is ultimately reduced in the ER lumen, liberating the enzymatic A1 fragment, which is subsequently translocated to the cytosol.

Shiga toxins have been co-immunoprecipitated with ER chaperones and translocon components, suggesting that the toxins use the cellular ER-associated protein degradation (ERAD) pathway. Indeed, Shiga toxins were found to interact with HEDJ (also known as ERdj3 and DNAJB11), BiP and 94 kDa glucose-regulated protein (GRP94) in pre-associated large multi-chaperone complexes before their transport across the ER membrane[77–80]. Furthermore, immunoprecipitation studies demonstrated that substantial amounts of HEDJ-associated Shiga toxin interact with the integral membrane Sec61 translocon core unit[79]. It was suggested that HEDJ and its interacting partners recruit misfolded proteins to the Sec61 translocon and that Shiga toxins exploit this machinery for their own transport across the ER membrane[79] (FIG. 3).

Membrane translocation seems to be the rate-limiting step for the intoxication of cells by Shiga toxins. Most of the cell-associated StxA is cleaved but remains bound to StxB, as the disulphide bond between the A1 and A2 fragments fails to be reduced. Only a small fraction (4%) of StxA seems to be translocated to the cytosol[81], consisting of cleaved, reduced and StxB-separated A1 fragments. Interaction of the A1 fragment with the ERAD machinery does not seem to involve a specific export sequence but rather relies on a degenerated structural element, such as a hydrophobic sequence in its C-terminal region[78]. It remains to be determined whether such a sequence directly interacts with the lipid bilayer to induce conformational changes that are then recognized by the ERAD machinery, as has been suggested for other protein toxins[82].

Coat protein complex I
A large coat protein complex that initiates the vesicle budding process on the *cis*-Golgi and retrograde transport to the rough endoplasmic reticulum.

Chaperone
A protein that assists in the folding or unfolding of target proteins.

ER-associated degradation
(ERAD). A cellular pathway that targets misfolded proteins in the endoplasmic reticulum for ubiquitylation and subsequent degradation by the proteasome.

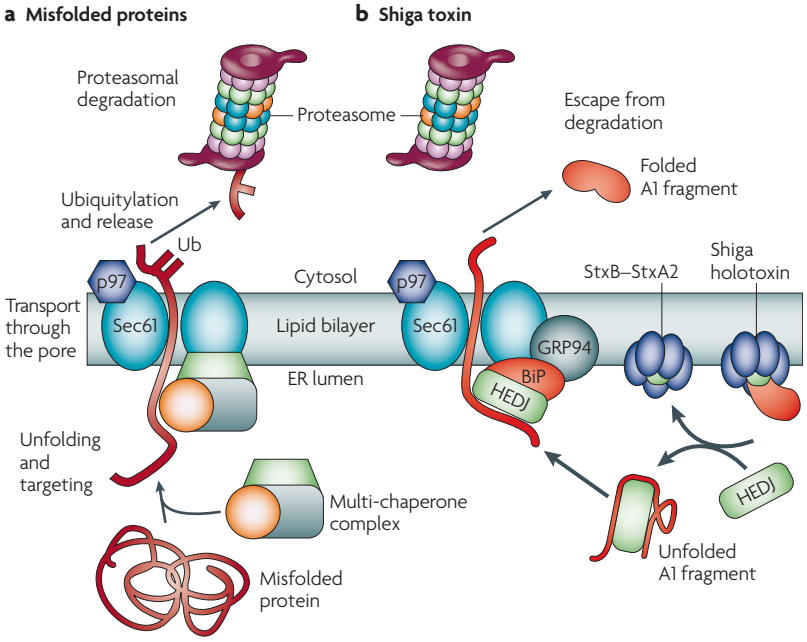

Figure 3 | The **endoplasmic reticulum-associated protein degradation pathway.** **a** | A simplified view of misfolded protein translocation across the endoplasmic reticulum (ER) membrane. Misfolded proteins are recognized by ER-localized luminal chaperone complexes and targeted to a protein-conducting translocation channel termed the translocon, which includes Sec61. Polypeptides are then tagged by ubiquitin (Ub) at the cytosolic face of the ER membrane, and the polyubiquitylated polypeptides are degraded by the proteasome after release from the ER membrane into the cytosol. The cytosolic chaperone p97 (also known as VCP), which is an AAA (ATPase associated with various cellular activities) ATPase, together with its cofactors, may be a driving force of retro-translocation. **b** | Translocation of Shiga toxins. Following cleavage by furin, the Shiga toxin A subunit is released from the B subunit following disulphide bond reduction. The A subunit associates with host ER chaperones (HEDJ (also known as ERdj3 and DNAJB11), BiP and 94 kDa glucose-regulated protein (GRP94)) and is targeted to the translocon channel for retro-translocation into the cytosol.

Unlike misfolded endogenous proteins, the A1 fragment of Shiga toxins must avoid the proteasome until it reaches its substrate in the cytosol. It is very probable that the A1 fragment is protected from degradation in the cytosol by its lack of lysine residues, together with its ability to refold rapidly[83]. Lactacystin, a proteasome inhibitor, increases the amount of cytosolic A1 fragment by 30% and enhances cytotoxicity by 50%, suggesting that a fraction of the translocated A1 fragment can be degraded by the proteasome machinery[81], as described for ricin and cholera toxin[84,85].

Intoxication by Shiga toxins

The kidney and gastrointestinal tract are the most commonly affected organs in patients with HUS, but clinical manifestations can also be seen in some patients at the level of the central nervous system and other organs. The reader is referred to reviews focused on disease-related issues for further information on this topic[13,86]. In short, Shiga toxins can directly inhibit protein biosynthesis. In this way, commensal bacteria in the lumen of the gut can be targeted, thereby reducing their proliferation and providing a competitive advantage for Shiga toxin-producing bacteria. Other targets are host cells. In the case of the intestinal mucosa, initial damage is caused by bacterial products other than toxins, as human gut enterocytes express very low levels of Gb3 (REFS 27,87). However, Shiga toxins damage the microcirculation, causing vasculitis, which exacerbates mucosal damage. Infarction of the mucosa leads to bleeding into the bowel and bloody diarrhoea. This may favour bacterial survival, as essential nutrients, including iron and other growth promoters, are provided. Although it was long thought that the function of Shiga toxins in HUS pathogenesis was limited to their role as protein biosynthesis inhibitors, it is now clear that these toxins also trigger many signalling cascades that influence their own trafficking as well as other cellular functions such as cytokine secretion and the induction of cell death by apoptosis. Notably, the toxin-induced release of pro-inflammatory cytokines from monocytes or macrophages may favour disease progression by upregulating Gb3 expression on endothelial cells. Another effect of Shiga toxins is the cytokine-stimulated activation of polymorphonuclear leukocytes, which release reactive oxygen metabolites that cause endothelial cell injury. Interleukin-8 (IL-8) seems to play a crucial part in this context. The complex modulation of the immune system by Shiga toxins may dampen specific immunity and, through local inflammation, enhance cytotoxicity.

Ribotoxic stress. The most prominent mechanism by which a signalling response is induced by Shiga toxins stems from the modification of ribosomes and is termed the ribotoxic stress response[88]. The 3′ end of 28S ribosomal RNA (rRNA) functions in aminoacyl tRNA binding, peptidyltransferase activity and ribosomal translation. When these activities are disrupted, owing to the rRNA modification induced by Shiga toxins, for example, JUN N-terminal kinase (Jnk) proteins and mitogen-activated protein kinase (MAPK) p38 are activated and extracellular signal-regulated kinase 1 (ERK1; also known as MAPK3) and ERK2 (also known as MAPK1) signalling is altered[89–92]. Ribotoxic stress is specific in that not all protein synthesis inhibitors induce this pathway. How damage in the 3′ end of 28S rRNA triggers the downstream signalling kinases is still not understood. One possibility is that elongation factors or other related ribosome-binding factors interact with the MAPK signalling cascade.

Triggering signalling pathways. On binding to their receptors in the plasma membrane, Shiga toxins induce the recruitment and activation of Gb3-containing, glycolipid-enriched membrane fractions of several tyrosine kinases, such as spleen tyrosine kinase (SYK) in HeLa cells[93], YES in ACHN renal cells[94] and LYN in Burkitt's lymphoma Ramos cells[95]. Once activated, kinases acquire increased detergent solubility[94,95]. Activation of SYK leads to rapid tyrosine phosphorylation of several proteins, including clathrin heavy chains, and has an effect on toxin uptake[93]. Inside cells, the toxins also activate kinases such as protein kinase Cδ (PKCδ)[96] and p38 (REF. 97). In the case of PKCδ, a direct effect on the retrograde transport of the toxin has been observed[96], and the role of p38 in ribotoxic stress is mentioned above.

Interleukin-8
A chemokine of the C-X-C subfamily that is known to recruit polymorphonuclear cells into areas of infection.

Mitogen-activated protein kinase
(MAPK). One of a family of threonine-directed kinases with important roles in the regulation of diverse cellular functions, including cytokine release. MAPKs are categorized into three main pathways and include the extracellular signal-regulated kinases (ERKs), the JUN amino-terminal kinases (Jnks; also called the stress-activated protein (SAP) kinases) and MAP kinase p38.

The mechanisms by which the Shiga toxins stimulate cytosolic kinases remain to be confirmed, although a protein with an apparent molecular mass of 27.2 kDa that co-immunoprecipitates with Gb3-specific monoclonal antibodies is a possible candidate for linking Gb3 with YES in ACHN cells[98]. Alternatively, Shiga toxin-induced Gb3 clustering in the exoplasmic leaflet may induce lipid repartition in the cytosolic leaflet, leading to aggregation-mediated autophosphorylation of SRC family protein tyrosine kinases[94,95].

The binding of Shiga toxins to the plasma membrane induces remodelling of the actin and microtubule cytoskeleton[99,100]. This process is accompanied by the redistribution of a number of proteins, including ezrin, CD44, vimentin, cytokeratins, paxillin and focal adhesion kinases. Both StxB binding and Gb3-specific antibody binding induces phosphorylation of ezrin, a protein that links the actin cytoskeleton to the plasma membrane. Shiga toxin-induced ezrin phosphorylation is inhibited by lowering the levels of plasma membrane cholesterol or by inhibiting SRC, phosphoinositide-3-kinase or Rho-associated protein kinase 1 (ROCK1)[100].

Cytokine synthesis and release. Many endothelial and epithelial cells in the kidney and the central nervous system are exquisitely sensitive to cytotoxicity induced by Shiga toxins, whereas others, such as peripheral blood monocytes, have been reported to be resistant to intoxication, despite their expression of Gb3 (REFS 101,102). Toxin-resistant cells respond to toxin binding and internalization by synthesizing and releasing pro-inflammatory cytokines. In turn, these cytokines stimulate Gb3 biosynthesis and expression on numerous endothelial cells and sensitize target cells to the cytotoxic action of the toxins[103,104] in a process that often involves signalling through the p38 pathway[105,106]. Therefore, the host innate immune response may exacerbate the vascular damage that is initiated by Shiga toxins (see above).

Shiga toxins regulate cytokine expression through multiple mechanisms. On release from the bacterium, Shiga toxins interact with the intestinal epithelium, where it induces the ribotoxic stress response to cause p38-mediated secretion of the pro-inflammatory cytokine IL-8 (REF. 97). In Vero cells as well as in intestinal epithelial HCT-8 cells, the ZAK isoforms ZAKα and ZAKβ were identified as MAP3Ks (MAPK kinase kinases). ZAK transduces the signal from intoxicated ribosomes to the host MAPKs p38 and Jnk proteins, which in turn initiate a cascade of events that ultimately promote upregulation of IL-8 (REF. 91). Shiga toxins have also been shown to induce synthesis of IL-8 in intestinal Caco-2 and T84 cells[107]. After crossing the intestinal epithelium and entering the vasculature, Shiga toxins stimulate circulating monocytes (which do not undergo apoptosis) to secrete cytokines, including GM-CSF and tumour necrosis factor (TNF), in a p38-dependent manner[89,101,108,109]. Patients with HUS frequently have elevated levels of TNF in their urine[89].

The stimulation of differentiated, macrophage-like cells of the human monocytic cell line THP1 by Shiga toxins activates YES, which then upregulates the expression and activity levels of tissue factor, a key element of the coagulation–inflammation–thrombosis circuit, through phosphoinositol-3-kinase-mediated activation of the inhibitor of NF-κB kinase subunit-β–proteasome–nuclear factor-κB–REL and MAPK/ERK kinase (MEK)–ERK2–early growth response protein 1 pathways[110]. These reactions may also contribute to toxin-induced endothelial cell injury.

Apoptosis. Apoptosis is a cell death pathway that is characterized by several nuclear changes, such as chromatin condensation and DNA fragmentation. The sequential activation of a cascade of cysteine-dependent, aspartate-specific proteases, called caspases, is a key component of the programmed cell death machinery. Although Shiga toxins may not induce apoptosis in all cell types, there is ample evidence to suggest that apoptosis is crucial for the development of vascular lesions and tissue damage following translocation of the toxins into the bloodstream. The data published to date suggest that Shiga toxins signal apoptosis through different mechanisms in different cell types (FIG. 4).

Stx1-induced apoptosis in THP1 cells requires retrograde transport through the Golgi apparatus to the ER and the activation of the executioner caspase, caspase 3 (REF. 111). Incubation of THP1 cells with Stx1 activates the ER stress response, a signalling mechanism triggered by the accumulation of unfolded or misfolded proteins in the ER, which in turn triggers the induction of programmed cell death. Stx1 treatment increases activation of the ER membrane-associated stress sensors IRE1, PRKR-like ER kinase (PERK; also known as EIF2αK3) and cyclic AMP-dependent transcription factor 6 (ATF6) and increases the expression of the transcriptional regulator C/EBP-homologous protein (CHOP; also known as DDIT3) and the death receptor 5 (DR5; also known as TNFRSF10B) at mRNA and protein levels (FIG. 4). The level of the survival factor B cell lymphoma 2 (BCL-2) decreases following Stx1 intoxication, whereas secretion of TNF-related apoptosis-inducing ligand (TRAIL; also known as TNFSF10) increases.

ER stress, elicited by Stx1, leads to Ca^{2+} release from ER stores and the activation of the Ca^{2+}-dependent cysteine protease calpain, which contributes to the early activation (through cleavage) of caspase 8 (REF. 112) (FIG. 4). Cleavage of caspase 8 may directly trigger the activation of caspase 3 or may initiate apoptosis through the mitochondrial pathway by cleavage of the inactive, 22 kDa form of BH3-interacting domain (BID) to the active, 15 kDa form called truncated BID (tBID). One study found that programmed cell death required the delivery of the functional holotoxin (not StxB alone or holotoxin molecules comprised of StxA with point mutations that dramatically reduce the toxin's enzymatic activity) to the ER, suggesting that this organelle indeed serves as the initial location for apoptotic signalling in THP1 cells[113].

Protein kinase Cδ
A member of the protein kinase C family, which contains at least 12 isoforms of serine/threonine kinases that are involved in signal transduction, regulation of gene expression and myeloid differentiation.

GM-CSF
(Granulocyte-macrophage colony-stimulating factor). A protein that is often secreted by macrophages, T cells, endothelial cells and fibroblasts.

Tumour necrosis factor
A cytokine involved in systemic inflammation. Its primary role is the regulation of immune cells.

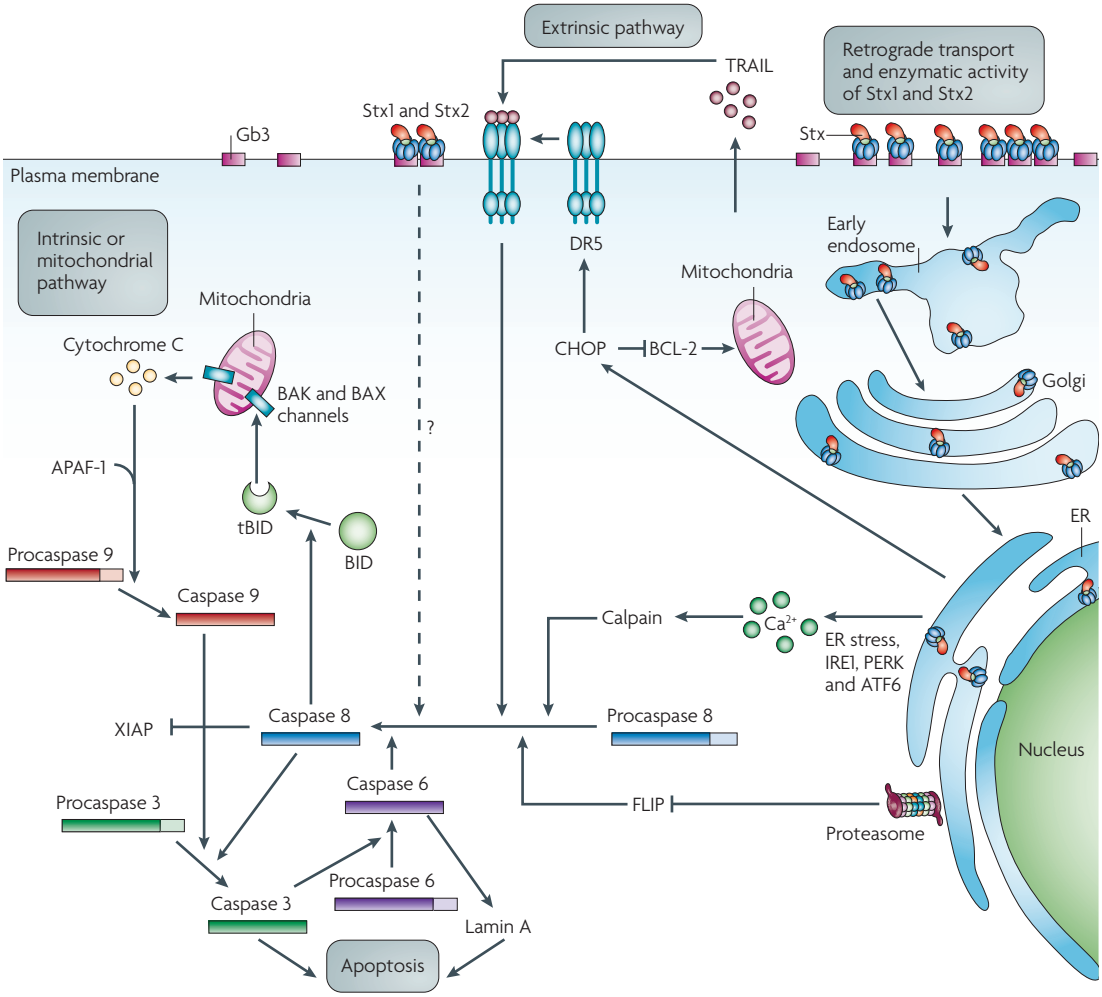

Figure 4 | **Apoptosis pathways that are induced by Shiga toxins.** Shiga toxins induce apoptosis through different mechanisms, resulting in activation of the apoptosis initiator caspase, caspase 8, and the executioner caspase, caspase 3. See main text for details. This figure summarizes findings from different cell types. In most cases, the enzymatic activity of Shiga holotoxins is required for the induction of apoptosis. APAF-1, apoptotic protease-activating factor 1; ATF6, cyclic AMP-dependent transcription factor 6; BAK, BCL-2-homologous agonist/killer; BCL-2, B cell lymphoma 2; BID, BH3-interacting domain; CHOP, C/EBP-homologous protein (also known as DDIT3); DR5, death receptor 5 (also known as TNFRSF10B); ER, endoplasmic reticulum; FLIP, FLICE-like inhibitory protein (also known as CFLAR); Gb3, globotriaosylceramide; PERK, PRKR-like ER kinase (also known as EIF2αK3); tBID, truncated BID; TRAIL, TNF-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis protein.

Similar apoptotic signalling pathways are triggered by Shiga toxins in different cell lines, namely monocytic THP1 cells, HeLa cells and human brain microvascular endothelial cells (HBMEC)[112–115]. In HeLa cells, Stx1-induced apoptosis occurs through a pathway requiring caspase 8, caspase 6 and caspase 3 but not caspase 9 (REF. 114). In these cells, Stx1 was found to increase the expression of the caspase 9 inhibitor X-linked inhibitor of apoptosis protein (XIAP). In HBMEC, Stx2 induces DNA fragmentation, and cleavage activation of caspase 3, caspase 6, caspase 8 and caspase 9 is mediated by CHOP upregulation and the complete degradation of the anti-apoptotic protein FLICE-like inhibitory protein (FLIP; also known as CFLAR), which in turn enhances the activation of caspase 8 (REF. 115).

Apoptosis induced by Stx1 can be associated with enhanced expression of the pro-apoptotic protein BAX[116], and overexpression of BCL-2 protects cells against Stx1-induced cell death[117]. Shiga toxins also inhibit the expression of the anti-apoptotic BCL-2 family member MCL1 (REF. 118).

Gb3-specific monoclonal antibody can induce apoptosis-like cell death in Burkitt's lymphoma cells. Strikingly, however, Gb3-specific antibody and Stx1 trigger different apoptotic signalling pathways. Stx1-induced apoptosis involves caspase activation and mitochondrial depolarization, whereas oxidative stress mediates Gb3-specific antibody-induced cell death[119]. In almost all other cells Gb3-specific antibodies do not trigger cell death and StxA is required to induce apoptosis[111,120].

Biomedical applications

The B subunit of Shiga toxins are naturally evolved delivery tools that transport the catalytic A subunit from its site of production in the intestine to peripheral tissue locations. As a delivery tool, the B subunit exhibits molecular characteristics that have been acquired during co-evolution with its hosts, including stability at extreme pH and in the presence of proteases, the capacity to cross tissue barriers and to distribute in the organism, and resistance to extra- and intracellular inactivation (reviewed in REF. 51). The B subunit receptor, Gb3, has a restricted tissue expression pattern and is present on two cell types that are of particular interest in biomedical research into novel strategies for the clinical management of cancer and infectious diseases: cancer cells themselves and dendritic cells, which are antigen-presenting cells with key functions in the induction of primary immune responses.

Tumour targeting. The expression and metabolism of cell surface glycolipids is modified during oncogenic transformation, and altered glycosylation patterns affect tumour invasion and metastasis[121]. Gb3 is overexpressed by various cancer cell lines and human cancers, including colorectal[87,122], breast[123,124] and ovarian[125] carcinomas and Burkitt's lymphomas[126]. These findings suggest that the Gb3-binding specificity of StxB could be exploited to target human tumours.

In pioneering studies, it was found that intratumoral injection of the Stx1 holotoxin inhibits tumour growth in mouse xenograft models[127–130]. Stx1 also eliminates cologenic tumour cells in *ex vivo* purging applications[131]. However, the use of holotoxin as a therapeutic agent in humans may be limited by the fact that the toxic effect of the A subunit is not tumour specific. The B subunit has therefore been coupled to cytotoxic compounds that have preferential effects on cancer cells. Furthermore, contrast agents for non-invasive tumour imaging have also been linked to the B subunit.

Using these linked contrast agents, it was shown that the B subunit targets Gb3-expressing spontaneous adenocarcinomas of the gut following oral uptake or intravenous injection in a transgenic mouse model[132]. The tumours could be detected by non-invasive imaging using fibred confocal fluorescence endoscopy and positron emission tomography. It was also shown that, owing to trafficking through the retrograde route, the B subunit stably associates with tumour cells, in which it can be detected for several days after injection. The concept of using the B subunit as a delivery tool was recently extended to human colorectal carcinoma. Primary cultures of tumoral enterocytes from surgical samples are targeted by the B subunit[87], and it is also efficiently taken up by xenografts of primary human tumours in mice[133]. Interestingly, endothelial cells of tumour neovascularization accumulated the B subunit *in vivo*, which may allow for targeted therapeutic intervention even in cases in which the tumours cells themselves do not express Gb3 (REFS 128,133).

A number of cytotoxic compounds have been coupled to the B subunit, including: the topoisomerase I inhibitor SN38, which is the active metabolite of camptothecin 11 (a compound that is used for the treatment of colorectal carcinomas)[134]; the benzodiazepine RO5-4864, which is a ligand for mitochondrial peripheral benzodiazepine receptors[135]; and photosensitizing agents, such as the porphyrin-based compounds TPP(p-O-β-GluOH)$_3$ (REF. 136) and Chlorin e6 (Ce6)[137]. It was shown that a B subunit–TPP(p-O-β-GluOH)$_3$ conjugate was more efficient at photodynamic cell killing than the porphyrin compound alone, owing to B subunit-induced retrograde delivery into the Golgi apparatus[136]. The efficacy of each of these novel formulations is currently under scrutiny in animal experiments.

Immunotherapy. The unique trafficking pathway of Shiga toxins and the selective expression of Gb3 on human dendritic cells make the B subunit an attractive non-live and non-toxic synthetic vector for the development of therapeutic vaccines to treat human cancers and infectious diseases. When genetically fused or chemically coupled to the B subunit, various exogenous cancer and viral antigens were delivered to the cytosolic compartment of dendritic cells, leading to proteasome-dependent antigen processing, TAP-dependent peptide loading onto neosynthesized major histocompatibility complex class I (MHC class I) molecules in the lumen of the ER and subsequent MHC class I molecule-restricted antigen presentation at the cell surface[138–141]. Dendritic cells are currently considered to be the most potent inducers of CD8$^+$ cytotoxic T lymphocyte (CTL) responses to viral infections and tumours. Vaccination of mice with B subunit-based conjugates indeed induces strong CTL responses with T_H1 polarization. These CTLs are long lasting[142] and also efficient, as they protect mice against challenges by viruses or tumours in prophylactic and therapeutic settings[142,143].

Conclusions

We have discussed the many ways in which Shiga toxins interact with mammalian hosts. Although it is easy to see how damage of the intestinal mucosa would benefit the toxin-producing bacteria by providing improved growth conditions, it is less easy to explain why damage at peripheral sites such as the kidney or central nervous system would be beneficial. It is possible that these organs are not the primary targets. Furthermore, harbouring a bacteriophage that encodes Shiga toxins may protect a host's microbiota from bactivorous predators like the ciliated protozoa *Tetrahymena thermophila*[144,145]. In this respect, humans might be coincidently caught in the crossfire of a microbial predator–prey relationship. However, neither Shiga toxin-binding lipids nor close homologues of the gene that encodes the mammalian Gb3 synthase have been identified in *T. thermophila*[144]. Alternative receptors could be present, or toxins might be captured through the oral apparatus.

Shiga toxins have become powerful tools for the investigation of cellular processes, notably in the context of intracellular trafficking. One of the most important challenges in this context is to come to a functional understanding of how a protein that associates with the exoplasmic membrane leaflet can communicate efficiently with the cytosolic machinery. Addressing this

Topoisomerase I
A group of proteins belonging to the topoisomerase family, which contains enzymes that catalyse ATP-independent breakage of one of the two strands of DNA, passage of the unbroken strand through the break and rejoining of the broken strand. Topoisomerase I enzymes reduce the topological stress in the DNA structure by relaxing the superhelical turns and knotted rings in the DNA helix.

Benzodiazepine
A psychoactive drug, the core chemical structure of which is the fusion of a benzene ring and a diazepine ring. Benzodiazepines have varying sedative, hypnotic, anxiolytic, anticonvulsant, muscle relaxant and amnesic properties, which make them useful in treating anxiety, insomnia, agitation, seizures, muscle spasms and alcohol withdrawal and as a pre-medication for medical or dental procedures.

Major histocompatibility complex class I
(MHC class I). A molecule found on every nucleated cell of the body. It's function is to display fragments of proteins from within the cell to T cells, so that healthy cells will be left alone and cells with foreign proteins will be attacked by the immune system. Because MHC class I molecules present peptides that are derived from cytosolic proteins, the pathway of MHC class I presentation is often called the cytosolic or endogenous pathway.

challenge will require novel technologies for live-time and high-resolution imaging of the dynamics of Gb3 lipids, as well as input from the field of membrane biophysics to formulate concepts that are expected to impact our understanding of membrane mechanical processes beyond the field of Shiga toxin research.

Shiga toxins have started to attract attention as delivery tools, exploiting to this end many of the characteristics that the toxins have naturally acquired through interaction with their hosts. Although in the field of immunotherapy proof of concept could be obtained in the mouse model, the tumour delivery approach is still in an early phase of development. Crucial issues such as the immunogenicity of the vector in long-term, high-dose treatments, its toxicity to peripheral targets such as the kidney and its efficacy as a high-capacity delivery tool need to be explored systematically. This line of research holds the promise of medical benefit from molecules that, as holotoxins, are still a major threat for human health today.

1. Konowalchuk, J., Speirs, J. I. & Stavric, S. Vero response to a cytotoxin of *Escherichia coli*. *Infect. Immun.* **18**, 775–779 (1977).
2. O'Brien, A. D. *et al.* Shiga-like toxin-converting phages from *Escherichia coli* strains that cause haemorrhagic colitis or infantile diarrhea. *Science* **226**, 694–696 (1984).
3. Karmali, M. A., Steele, B. T., Petric, M. & Lim, C. Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet* **1**, 619–620 (1983).
4. Boerlin, P. *et al.* Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *J. Clin. Microbiol.* **37**, 497–503 (1999).
5. Jackson, M. P., Newland, J. W., Holmes, R. K. & O'Brien, A. D. Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from *Escherichia coli*. *Microb. Pathog.* **2**, 147–153 (1987).
6. Unkmeir, A. & Schmidt, H. Structural analysis of phage-borne *stx* genes and their flanking sequences in Shiga toxin-producing *Escherichia coli* and *Shigella dysenteriae* type 1 strains. *Infect. Immun.* **68**, 4856–4864 (2000).
7. Herold, S., Karch, H. & Schmidt, H. Shiga toxin-encoding bacteriophages – genomes in motion. *Int. J. Med. Microbiol.* **294**, 115–121 (2004).
8. Neely, M. N. & Friedman, D. I. Functional and genetic analysis of regulatory regions of coliphage H-19B: location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release. *Mol. Microbiol.* **28**, 1255–1267 (1998).
9. Tyler, J. S., Mills, M. J. & Friedman, D. I. The operator and early promoter region of the Shiga toxin type 2-encoding bacteriophage 933W and control of toxin expression. *J. Bacteriol.* **186**, 7670–7679 (2004).
10. Wagner, P. L. *et al.* Bacteriophage control of Shiga toxin 1 production and release by *Escherichia coli*. *Mol. Microbiol.* **44**, 957–970 (2002).
11. Wagner, P. L., Acheson, D. W. & Waldor, M. K. Human neutrophils and their products induce Shiga toxin production by enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **69**, 1934–1937 (2001).
12. Calderwood, S. B. & Mekalanos, J. J. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. *J. Bacteriol.* **169**, 4759–4764 (1987).
13. Proulx, F., Seidman, E. G. & Karpman, D. Pathogenesis of Shiga toxin-associated haemolytic uraemic syndrome. *Pediatr. Res.* **50**, 163–171 (2001).
14. Lingwood, C. A. Role of verotoxin receptors in pathogenesis. *Trends Microbiol.* **4**, 147–153 (1996).
15. Tarr, P. I., Gordon, C. A. & Chandler, W. L. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* **365**, 1073–1086 (2005).
16. Ostroff, S. M. *et al.* Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. *J. Infect. Dis.* **160**, 994–998 (1989).
17. Donohue-Rolfe, A., Kondova, I., Oswald, S., Hutto, D. & Tzipori, S. *Escherichia coli* O157:H7 strains that express Shiga toxin (Stx) 2 alone are more neurotropic for gnotobiotic piglets than are isotypes producing only Stx1 or both Stx1 and Stx2. *J. Infect. Dis.* **181**, 1825–1829 (2000).
18. Offner, G., Brodehl, J., Galaske, R. & Rutt, T. Acute renal failure in children: prognostic features after treatment with acute dialysis. *Eur. J. Pediatr.* **144**, 482–486 (1986).
19. Fraser, M. E., Chernaia, M. M., Kozlov, Y. V. & James, M. N. Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 Å resolution. *Nature Struct. Biol.* **1**, 59–64 (1994).
20. Stein, P. E., Boodhoo, A., Tyrrell, G. J., Brunton, J. L. & Read, R. J. Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*. *Nature* **355**, 748–750 (1992).
21. Donohue-Rolfe, A. & Keusch, G. T. *Shigella dysenteriae* 1 cytotoxin: periplasmic protein releasable by polymyxin B and osmotic shock. *Infect. Immun.* **39**, 270–274 (1983).
22. Hirst, T. R., Sanchez, J., Kaper, J. B., Hardy, S. J. & Holmgren, J. Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harbouring plasmids that encode heat-labile enterotoxins of *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **81**, 7752–7756 (1984).
23. Endo, Y. *et al.* Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA *N*-glycosidase activity of the toxins. *Eur. J. Biochem.* **171**, 45–50 (1988).
24. Saxena, S. K., O'Brien, A. D. & Ackerman, E. J. Shiga toxin, Shiga-like toxin II variant, and ricin are all single-site RNA *N*-glycosidases of 28 S RNA when microinjected into *Xenopus* oocytes. *J. Biol. Chem.* **264**, 596–601 (1989).
25. Hale, T. L. & Formal, S. B. Cytotoxicity of *Shigella dysenteriae* 1 for cultured mammalian cells. *Am. J. Clin. Nutr.* **33**, 2485–2490 (1980).
26. Suh, J. K., Hovde, C. J. & Robertus, J. D. Shiga toxin attacks bacterial ribosomes as effectively as eucaryotic ribosomes. *Biochemistry* **37**, 9394–9398 (1998).
27. Jacewicz, M., Clausen, H., Nudelman, E., Donohue-Rolfe, A. & Keusch, G. T. Pathogenesis of Shigella diarrhea. XI. Isolation of a Shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. *J. Exp. Med.* **163**, 1391–1404 (1986).
28. Lindberg, A. A. *et al.* Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* type 1. *J. Biol. Chem.* **262**, 1779–1785 (1987).
29. Waddell, T., Cohen, A. & Lingwood, C. A. Induction of verotoxin sensitivity in receptor-deficient cell lines using the receptor glycolipid globotriosylceramide. *Proc. Natl Acad. Sci. USA* **87**, 7898–7901 (1990).
30. Donohue-Rolfe, A., Jacewicz, M. & Keusch, G. T. Isolation and characterization of functional Shiga toxin subunits and renatured holotoxin. *Mol. Microbiol.* **3**, 1231–1236 (1989).
31. Nyholm, P. G., Brunton, J. L. & Lingwood, C. A. Modelling of the interaction of verotoxin-1 (VT1) with its glycolipid receptor, globotriaosylceramide (Gb3). *Int. J. Biol. Macromol.* **17**, 199–204 (1995).
32. Nyholm, P. G. *et al.* Two distinct binding sites for globotriaosyl ceramide on verotoxins: identification by molecular modelling and confirmation using deoxy analogues and a new glycolipid receptor for all verotoxins. *Chem. Biol.* **3**, 263–275 (1996).
33. Picking, W. D., McCann, J. A., Nutikka, A. & Lingwood, C. A. Localization of the binding site for modified Gb3 on verotoxin 1 using fluorescence analysis. *Biochemistry* **38**, 7177–7184 (1999).
34. Ling, H. *et al.* Structure of the shiga-like toxin I B-pentamer complexed with an analogue of its receptor Gb3. *Biochemistry* **37**, 1777–1788 (1998).
This paper describes the co-crystal that is formed between the Shiga toxin 1 B subunit and soluble Gb3 receptor analogues. The study shows that there are up to 15 Gb3 binding sites per B subunit pentamer and lays the groundwork for structure–function relationship studies of the B subunit–Gb3 complex.
35. Bast, D. J., Banerjee, L., Clark, C., Read, R. J. & Brunton, J. L. The identification of three biologically relevant globotriaosyl ceramide receptor binding sites on the Verotoxin 1 B subunit. *Mol. Microbiol.* **32**, 953–960 (1999).
36. Soltyk, A. M. *et al.* A mutational analysis of the globotriaosylceramide-binding sites of verotoxin VT1. *J. Biol. Chem.* **277**, 5351–5359 (2002).
37. Shimizu, H., Field, R. A., Homans, S. W. & Donohue-Rolfe, A. Solution structure of the complex between the B-subunit homopentamer of verotoxin VT-1 from *Escherichia coli* and the trisaccharide moiety of globotriaosylceramide. *Biochemistry* **37**, 11078–11082 (1998).
38. Fuchs, G. *et al.* Pathogenesis of *Shigella* diarrhea: rabbit intestinal cell microvillus membrane binding site for Shigella toxin. *Infect. Immun.* **53**, 372–377 (1986).
39. St. Hilaire, P. M., Boyd, M. K. & Toone, E. J. Interaction of the Shiga-like toxin type 1 B-subunit with its carbohydrate receptor. *Biochemistry* **33**, 14452–14463 (1994).
40. Kiarash, A., Boyd, B. & Lingwood, C. A. Glycosphingolipid receptor function is modified by fatty acid content. Verotoxin 1 and verotoxin 2c preferentially recognize different globotriaosyl ceramide fatty acid homologues. *J. Biol. Chem.* **269**, 11138–11146 (1994).
This pioneering work on the importance of Gb3 structure in the recognition by Shiga toxins finds that B subunit binding to different Gb3 molecular species is dependent on fatty acid chain length and saturation degree.
41. Pellizzari, A., Pang, H. & Lingwood, C. A. Binding of verocytotoxin 1 to its receptor is influenced by differences in receptor fatty acid content. *Biochemistry* **31**, 1363–1370 (1992).
42. Head, S. C., Karmali, M. A. & Lingwood, C. A. Preparation of VT1 and VT2 hybrid toxins from their purified dissociated subunits. Evidence for B subunit modulation of a subunit function. *J. Biol. Chem.* **266**, 3617–3621 (1991).
43. Tesh, V. L. *et al.* Comparison of the relative toxicities of Shiga-like toxins type I and type II for mice. *Infect. Immun.* **61**, 3392–3402 (1993).
44. Binnington, B., Lingwood, D., Nutikka, A. & Lingwood, C. A. Effect of globotriaosyl ceramide fatty acid α-hydroxylation on the binding by verotoxin 1 and verotoxin 2. *Neurochem. Res.* **27**, 807–813 (2002).
45. Arab, S. & Lingwood, C. A. Influence of phospholipid chain length on verotoxin/globotriaosyl ceramide binding in model membranes: comparison of a supported bilayer film and liposomes. *Glycoconj. J.* **13**, 159–166 (1996).
46. Boyd, B., Magnusson, G., Zhiuyan, Z. & Lingwood, C. A. Lipid modulation of glycolipid receptor function. Availability of Gal(α1-4)Gal disaccharide for verotoxin binding in natural and synthetic glycolipids. *Eur. J. Biochem.* **223**, 873–878 (1994).
47. Mahfoud, R., Manis, A. & Lingwood, C. A. Fatty acid-dependent globotriaosyl ceramide receptor function in detergent resistant model membranes. *J. Lipid Res.* **50**, 1744–1755 (2008).
48. Nakajima, H. *et al.* Kinetic analysis of binding between Shiga toxin and receptor glycolipid Gb3Cer by surface plasmon resonance. *J. Biol. Chem.* **276**, 42915–42922 (2001).
49. Newburg, D. S. *et al.* Susceptibility to haemolytic-uraemic syndrome relates to erythrocyte glycosphingolipid patterns. *J. Infect. Dis.* **168**, 476–479 (1993).
50. Bonifacino, J. S. & Rojas, R. Retrograde transport from endosomes to the trans-Golgi network. *Nature Rev. Mol. Cell Biol.* **7**, 568–579 (2006).

51. Johannes, L. & Decaudin, D. Protein toxins: intracellular trafficking for targeted therapy. *Gene Ther.* **12**, 1360–1368 (2005).
52. Johannes, L. & Popoff, V. Tracing the retrograde route in protein trafficking. *Cell* **135**, 1175–1187 (2008).
53. Sandvig, K. & van Deurs, B. Delivery into cells: lessons learned from plant and bacterial toxins. *Gene Ther.* **12**, 865–872 (2005).
54. Amessou, M., Popoff, V., Yelamos, B., Saint-Pol., A. & Johannes, L. Measuring retrograde transport to the *trans*-Golgi network. *Curr. Protoc. Cell Biol.* **Chapter 15**, Unit 15.10 (2006).
55. Mallard, F. & Johannes, L. Shiga toxin B-subunit as a tool to study retrograde transport. *Methods Mol. Med.* **73**, 209–220 (2003).
56. Sandvig, K., Olsnes, S., Brown, J. E., Petersen, O. W. & van Deurs, B. Endocytosis from coated pits of Shiga toxin: a glycolipid-binding protein from *Shigella dysenteriae* 1. *J. Cell Biol.* **108**, 1331–1343 (1989).
57. Lauvrak, S. U., Torgersen, M. L. & Sandvig, K. Efficient endosome-to-Golgi transport of Shiga toxin is dependent on dynamin and clathrin. *J. Cell Sci.* **117**, 2321–2331 (2004).
58. Nichols, B. J. *et al.* Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J. Cell Biol.* **153**, 529–541 (2001).
59. Saint-Pol., A. *et al.* Clathrin adaptor epsinR is required for retrograde sorting on early endosomal membranes. *Dev. Cell* **6**, 525–538 (2004).
60. Romer, W. *et al.* Shiga toxin induces tubular membrane invaginations for its uptake into cells. *Nature* **450**, 670–675 (2007).
This work addresses the biological relevance of constructing protein–lipid nanodomains in the cellular uptake of Shiga toxin and proposes a new mechanism for the generation of negative curvature.
61. Sandvig, K. *et al.* Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature* **358**, 510–512 (1992).
A pioneering study that demonstrates that the localization of Shiga toxin in the membranes of the endoplasmatic reticulum is the result of retrograde transport through the secretory pathway.
62. Mallard, F. *et al.* Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of Shiga toxin B-fragment transport. *J. Cell Biol.* **143**, 973–990 (1998).
This article describes the discovery of a transport connection between early and recycling endosomes and the TGN that is different to the recycling pathway of the mannose-6-phosphate receptor between late endosomes and the TGN. The early and recycling endosomes–TGN interface is also used by the cellular protein TGN38.
63. Lombardi, D. *et al.* Rab9 functions in transport between late endosomes and the trans Golgi network. *EMBO J.* **12**, 677–682 (1993).
64. Bujny, M. V., Popoff, V., Johannes, L. & Cullen, P. J. The retromer component sorting nexin-1 is required for efficient retrograde transport of Shiga toxin from early endosome to the trans Golgi network. *J. Cell Sci.* **120**, 2010–2021 (2007).
65. Popoff, V. *et al.* The retromer complex and clathrin define an early endosomal retrograde exit site. *J. Cell Sci.* **120**, 2022–2031 (2007).
66. Utskarpen, A., Slagsvold, H. H., Dyve, A. B., Skanland, S. S. & Sandvig, K. SNX1 and SNX2 mediate retrograde transport of Shiga toxin. *Biochem. Biophys. Res. Commun.* **358**, 566–570 (2007).
67. Bonifacino, J. S. & Hurley, J. H. Retromer. *Curr. Opin. Cell Biol.* **20**, 427–436 (2008).
68. Falguieres, T. *et al.* Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent-resistant membranes. *Mol. Biol. Cell* **12**, 2453–2468 (2001).
69. Kovbasnjuk, O., Edidin, M. & Donowitz, M. Role of lipid rafts in Shiga toxin 1 interaction with the apical surface of Caco-2 cells. *J. Cell Sci.* **114**, 4025–4031 (2001).
70. Girod, A. *et al.* Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum. *Nature Cell Biol.* **1**, 423–430 (1999).
71. McKenzie, J., Johannes, L., Taguchi, T. & Sheff, D. Passage through the Golgi is necessary for Shiga toxin B subunit to reach the endoplasmic reticulum. *FEBS J.* **276**, 1581–1595 (2009).
72. Garred, O., van Deurs, B. & Sandvig, K. Furin-induced cleavage and activation of Shiga toxin. *J. Biol. Chem.* **270**, 10817–10821 (1995).
73. Kurmanova, A. *et al.* Structural requirements for furin-induced cleavage and activation of Shiga toxin. *Biochem. Biophys. Res. Commun.* **357**, 144–149 (2007).
74. Lea, N., Lord, J. M. & Roberts, L. M. Proteolytic cleavage of the A subunit is essential for maximal cytotoxicity of *Escherichia coli* O157:H7 Shiga-like toxin-1. *Microbiology* **145**, 999–1004 (1999).
75. O'Brien, A. D. & Holmes, R. K. Shiga and Shiga-like toxins. *Microbiol. Rev.* **51**, 206–220 (1987).
76. Olsnes, S., Reisbig, R. & Eiklid, K. Subunit structure of *Shigella* cytotoxin. *J. Biol. Chem.* **256**, 8732–8738 (1981).
77. Falguieres, T. & Johannes, L. Shiga toxin B-subunit binds to the chaperone BiP and the nucleolar protein B23. *Biol. Cell* **98**, 125–134 (2006).
78. LaPointe, P., Wei, X. & Gariepy, J. A role for the protease-sensitive loop region of Shiga-like toxin 1 in the retrotranslocation of its A1 domain from the endoplasmic reticulum lumen. *J. Biol. Chem.* **280**, 23310–23318 (2005).
79. Yu, M. & Haslam, D. B. Shiga toxin is transported from the endoplasmic reticulum following interaction with the luminal chaperone HEDJ/ERdj3. *Infect. Immun.* **73**, 2524–2532 (2005).
This investigation finds evidence for a function of HEDJ, other chaperones and the translocon Sec61 in the retro-translocation of Shiga toxin from the ER lumen to the cytosol.
80. Meunier, L., Usherwood, Y. K., Chung, K. T. & Hendershot, L. M. A subset of chaperones and folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent proteins. *Mol. Biol. Cell* **13**, 4456–4469 (2002).
81. Tam, P. J. & Lingwood, C. A. Membrane cytosolic translocation of verotoxin A1 subunit in target cells. *Microbiology* **153**, 2700–2710 (2007).
82. Mayerhofer, P. U. *et al.* Ricin A chain insertion into endoplasmic reticulum membranes is triggered by a temperature increase to 37 °C. *J. Biol. Chem.* **284**, 10232–10242 (2009).
83. Hazes, B. & Read, R. J. Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. *Biochemistry* **36**, 11051–11054 (1997).
84. Deeks, E. D. *et al.* The low lysine content of ricin A chain reduces the risk of proteolytic degradation after translocation from the endoplasmic reticulum to the cytosol. *Biochemistry* **41**, 3405–3413 (2002).
85. Wesche, J., Rapak, A. & Olsnes, S. Dependence of ricin toxicity on translocation of the toxin A-chain from the endoplasmic reticulum to the cytosol. *J. Biol. Chem.* **274**, 34443–34449 (1999).
86. Ray, P. E. & Liu, X. H. Pathogenesis of Shiga toxin-induced haemolytic uraemic syndrome. *Pediatr. Nephrol.* **16**, 823–839 (2001).
87. Falguieres, T. *et al.* Human colorectal tumours and metastases express Gb3 and can be targeted by an intestinal pathogen-based delivery tool. *Mol. Cancer Ther.* **7**, 2498–2508 (2008).
88. Iordanov, M. S. *et al.* Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the α-sarcin/ricin loop in the 28S rRNA. *Mol. Cell Biol.* **17**, 3373–3381 (1997).
89. Foster, G. H. & Tesh, V. L. Shiga toxin 1-induced activation of c-Jun NH_2-terminal kinase and p38 in the human monocytic cell line THP-1: possible involvement in the production of TNF-α. *J. Leukoc. Biol.* **71**, 107–114 (2002).
90. Ikeda, M., Gunji, Y., Yamasaki, S. & Takeda, Y. Shiga toxin activates p38 MAP kinase through cellular Ca^{2+} increase in Vero cells. *FEBS Lett.* **485**, 94–98 (2000).
91. Jandhyala, D. M., Ahluwalia, A., Obrig, T. & Thorpe, C. M. ZAK: a MAP3Kinase that transduces Shiga toxin- and ricin-induced proinflammatory cytokine expression. *Cell. Microbiol.* **10**, 1468–1477 (2008).
92. Smith, W. E. *et al.* Shiga toxin 1 triggers a ribotoxic stress response leading to p38 and JNK activation and induction of apoptosis in intestinal epithelial cells. *Infect. Immun.* **71**, 1497–1504 (2003).
93. Lauvrak, S. U. *et al.* Shiga toxin regulates its entry in a Syk-dependent manner. *Mol. Biol. Cell* **17**, 1096–1109 (2006).
94. Katagiri, Y. U. *et al.* Activation of Src family kinase Yes induced by Shiga toxin binding to globotriaosyl ceramide (Gb3/CD77) in low density, detergent-insoluble microdomains. *J. Biol. Chem.* **274**, 35278–35282 (1999).
95. Mori, T. *et al.* Globotriaosyl ceramide (CD77/Gb3) in the glycolipid-enriched membrane domain participates in B-cell receptor-mediated apoptosis by regulating lyn kinase activity in human B cells. *Exp. Hematol.* **28**, 1260–1268 (2000).
96. Torgersen, M. L., Walchli, S., Grimmer, S., Skanland, S. S. & Sandvig, K. Protein kinase Cδ is activated by Shiga toxin and regulates its transport. *J. Biol. Chem.* **282**, 16317–16328 (2007).
97. Thorpe, C. M. *et al.* Shiga toxins stimulate secretion of interleukin-8 from intestinal epithelial cells. *Infect. Immun.* **67**, 5985–5993 (1999).
98. Shimizu, T. *et al.* An association of 27- and 40-kDa molecules with glycolipids that bind A-B bacterial enterotoxins to cultured cells. *Biochim. Biophys. Acta* **1612**, 186–194 (2003).
99. Hehnly, H., Sheff, D. & Stamnes, M. Shiga toxin facilitates its retrograde transport by modifying microtubule dynamics. *Mol. Biol. Cell* **17**, 4379–4389 (2006).
100. Takenouchi, H. *et al.* Shiga toxin binding to globotriaosyl ceramide induces intracellular signals that mediate cytoskeleton remodeling in human renal carcinoma-derived cells. *J. Cell Sci.* **117**, 3911–3922 (2004).
101. Harrison, L. M., van Haaften, W. C. & Tesh, V. L. Regulation of proinflammatory cytokine expression by Shiga toxin 1 and/or lipopolysaccharides in the human monocytic cell line THP-1. *Infect. Immun.* **72**, 2618–2627 (2004).
102. Ramegowda, B. & Tesh, V. L. Differentiation-associated toxin receptor modulation, cytokine production, and sensitivity to Shiga-like toxins in human monocytes and monocytic cell lines. *Infect. Immun.* **64**, 1173–1180 (1996).
103. Eisenhauer, P. B. *et al.* Tumour necrosis factor alpha increases human cerebral endothelial cell Gb_3 and sensitivity to Shiga toxin. *Infect. Immun.* **69**, 1889–1894 (2001).
104. Louise, C. B. & Obrig, T. G. Shiga toxin-associated haemolytic-uraemic syndrome: combined cytotoxic effects of Shiga toxin, interleukin-1β, and tumour necrosis factor alpha on human vascular endothelial cells *in vitro*. *Infect. Immun.* **59**, 4173–4179 (1991).
105. Stone, M. K., Kolling, G. L., Lindner, M. H. & Obrig, T. G. p38 mitogen-activated protein kinase mediates lipopolysaccharide and tumour necrosis factor alpha induction of Shiga toxin 2 sensitivity in human umbilical vein endothelial cells. *Infect. Immun.* **76**, 1115–1121 (2008).
106. Stricklett, P. K., Hughes, A. K. & Kohan, D. E. Inhibition of p38 mitogen-activated protein kinase ameliorates cytokine upregulated shigatoxin-1 toxicity in human brain microvascular endothelial cells. *J. Infect. Dis.* **191**, 461–471 (2005).
107. Yamasaki, C. *et al.* Induction of cytokines in a human colon epithelial cell line by Shiga toxin 1 (Stx1) and Stx2 but not by non-toxic mutant Stx1 which lacks *N*-glycosidase activity. *FEBS Lett.* **442**, 231–234 (1999).
108. Cameron, P., Smith, S. J., Giembycz, M. A., Rotondo, D. & Plevin, R. Verotoxin activates mitogen-activated protein kinase in human peripheral blood monocytes: role in apoptosis and proinflammatory cytokine release. *Br. J. Pharmacol.* **140**, 1320–1330 (2003).
109. Cherla, R. P., Lee, S. Y., Mees, P. L. & Tesh, V. L. Shiga toxin 1-induced cytokine production is mediated by MAP kinase pathways and translation initiation factor eIF4E in the macrophage-like THP-1 cell line. *J. Leukoc. Biol.* **79**, 397–407 (2006).
110. Murata, K. *et al.* Verotoxin-1 stimulation of macrophage-like THP-1 cells upregulates tissue factor expression through activation of c-Yes tyrosine kinase: possible signal transduction in tissue factor upregulation. *Biochim. Biophys. Acta* **1762**, 835–843 (2006).
111. Kojio, S. *et al.* Caspase-3 activation and apoptosis induction coupled with the retrograde transport of Shiga toxin: inhibition by brefeldin A. *FEMS Immunol. Med. Microbiol.* **29**, 275–281 (2000).
112. Lee, S. Y., Lee, M. S., Cherla, R. P. & Tesh, V. L. Shiga toxin 1 induces apoptosis through the endoplasmic reticulum stress response in human monocytic cells. *Cell. Microbiol.* **10**, 770–780 (2008).
The study provides an integrated view on a major toxin-induced signalling pathway, showing that Stx1 triggers monocytic cell apoptosis through activation of ER stress sensors, increased expression of DR5 and TRAIL, and activation of caspase 8 through a calpain-dependent mechanism.

113. Lee, S. Y., Cherla, R. P., Caliskan, I. & Tesh, V. L. Shiga toxin 1 induces apoptosis in the human myelogenous leukemia cell line THP-1 by a caspase-8-dependent, tumour necrosis factor receptor-independent mechanism. *Infect. Immun.* **73**, 5115–5126 (2005).
114. Fujii, J. *et al.* Rapid apoptosis induced by Shiga toxin in HeLa cells. *Infect. Immun.* **71**, 2724–2735 (2003).
115. Fujii, J. *et al.* Shiga toxin 2 causes apoptosis in human brain microvascular endothelial cells via C/EBP homologous protein. *Infect. Immun.* **76**, 3679–3689 (2008).
116. Jones, N. L. *et al. Escherichia coli* Shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family. *Am. J. Physiol. Gastrointest. Liver Physiol.* **278**, G811–G819 (2000).
117. Suzuki, A. *et al.* Bcl-2 antiapoptotic protein mediates verotoxin II-induced cell death: possible association between Bcl-2 and tissue failure by *E. coli* O157:H7. *Genes Dev.* **14**, 1734–1740 (2000).
118. Erwert, R. D. *et al.* Shiga toxin induces decreased expression of the anti-apoptotic protein Mcl-1 concomitant with the onset of endothelial apoptosis. *Microb. Pathog.* **35**, 87–93 (2003).
119. Tetaud, C. *et al.* Two distinct Gb3/CD77 signalling pathways leading to apoptosis are triggered by anti-Gb3/CD77 mAb and verotoxin-1. *J. Biol. Chem.* **278**, 45200–45208 (2003).
120. Williams, J. M. *et al.* Comparison of ribosome-inactivating proteins in the induction of apoptosis. *Toxicol. Lett.* **91**, 121–127 (1997).
121. Hakomori, S. & Zhang, Y. Glycosphingolipid antigens and cancer therapy. *Chem. Biol.* **4**, 97–104 (1997).
122. Kovbasnjuk, O. *et al.* The glycosphingolipid globotriaosylceramide in the metastatic transformation of colon cancer. *Proc. Natl Acad. Sci. USA* **102**, 19087–19092 (2005).
123. Johansson, D. *et al.* Expression of verotoxin-1 receptor Gb3 in breast cancer tissue and verotoxin-1 signal transduction to apoptosis. *BMC Cancer* **9**, 67 (2009).
124. LaCasse, E. C. *et al.* Shiga-like toxin-1 receptor on human breast cancer, lymphoma, and myeloma and absence from $CD34^+$ haematopoietic stem cells: implications for *ex vivo* tumour purging and autologous stem cell transplantation. *Blood* **94**, 2901–2910 (1999).
125. Arab, S., Russel, E., Chapman, W. B., Rosen, B. & Lingwood, C. A. Expression of the verotoxin receptor glycolipid, globotriaosylceramide, in ovarian hyperplasias. *Oncol. Res.* **9**, 553–563 (1997).
126. Wiels, J., Fellous, M. & Tursz, T. Monoclonal antibody against a Burkitt lymphoma-associated antigen. *Proc. Natl Acad. Sci. USA* **78**, 6485–6488 (1981).
127. Farkas-Himsley, H., Hill, R., Rosen, B., Arab, S. & Lingwood, C. A. The bacterial colicin active against tumour cells *in vitro* and *in vivo* is verotoxin 1. *Proc. Natl Acad. Sci. USA* **92**, 6996–7000 (1995).
128. Heath-Engel, H. M. & Lingwood, C. A. Verotoxin sensitivity of ECV304 cells *in vitro* and *in vivo* in a xenograft tumour model: VT1 as a tumour neovascular marker. *Angiogenesis* **6**, 129–141 (2003).
This article documents the expression of Gb3 in tumour neovasculature and describes the antineoplasic effect of Shiga toxin 1 after intratumoral injection in xenografted mice.
129. Ishitoya, S. *et al.* Verotoxin induces rapid elimination of human renal tumour xenografts in SCID mice. *J. Urol.* **171**, 1309–1313 (2004).
130. Salhia, B., Rutka, J. T., Lingwood, C., Nutikka, A. & Van Furth, W. R. The treatment of malignant meningioma with verotoxin. *Neoplasia* **4**, 304–311 (2002).
131. LaCasse, E. C., Saleh, M. T., Patterson, B., Minden, M. D. & Gariepy, J. Shiga-like toxin purges human lymphoma from bone marrow of severe combined immunodeficient mice. *Blood* **88**, 1561–1567 (1996).
132. Janssen, K. P. *et al. In vivo* tumour targeting using a novel intestinal pathogen-based delivery approach. *Cancer Res.* **66**, 7230–7236 (2006).
This study demonstrates that the B subunit targets spontaneous Gb3-expressing adenocarcinomas in a transgenic mouse model and presents different imaging modalities that exploit B subunit-based delivery of contrast agents.
133. Viel, T. *et al. In vivo* tumour targeting by the B-subunit of shiga toxin. *Mol. Imaging* **7**, 239–247 (2008).
134. El Alaoui, A. *et al.* Shiga toxin-mediated retrograde delivery of a topoisomerase I inhibitor prodrug. *Angew. Chem. Int. Edn Engl.* **46**, 6469–6472 (2007).
135. El Alaoui, A. *et al.* Synthesis and properties of a mitochondrial peripheral benzodiazepine receptor conjugate. *ChemMedChem* **3**, 1687–1695 (2008).
136. Amessou, M. *et al.* Retrograde delivery of photosensitizer (TPPp-O-β-$GluOH)_5$ selectively potentiates its photodynamic activity. *Bioconjug. Chem.* **19**, 532–538 (2008).
137. Tarrago-Trani, M. T., Jiang, S., Harich, K. C. & Storrie, B. Shiga-like toxin subunit B (SLTB)-enhanced delivery of chlorin e6 (Ce6) improves cell killing. *Photochem. Photobiol.* **82**, 527–537 (2006).
138. Haicheur, N. *et al.* The B subunit of Shiga toxin coupled to full-size antigenic protein elicits humoral and cell-mediated immune responses associated with a T_h1-dominant polarization. *Int. Immunol.* **15**, 1161–1171 (2003).
139. Haicheur, N. *et al.* The B subunit of Shiga toxin fused to a tumour antigen elicits CTL and targets dendritic cells to allow MHC class I-restricted presentation of peptides derived from exogenous antigens. *J. Immunol.* **165**, 3301–3308 (2000).
140. Lee, R. S. *et al.* Major histocompatibility complex class I presentation of exogenous soluble tumour antigen fused to the B-fragment of Shiga toxin. *Eur. J. Immunol.* **28**, 2726–2737 (1998).
141. Smith, D. C., Lord, J. M., Roberts, L. M., Tartour, E. & Johannes, L. 1st class ticket to class I: protein toxins as pathfinders for antigen presentation. *Traffic* **3**, 697–704 (2002).
142. Vingert, B. *et al.* The Shiga toxin B-subunit targets antigen *in vivo* to dendritic cells and elicits anti-tumour immunity. *Eur. J. Immunol.* **36**, 1124–1135 (2006).
143. Adotevi, O. *et al.* B subunit of Shiga toxin-based vaccines synergize with α-galactosylceramide to break tolerance against self antigen and elicit antiviral immunity. *J. Immunol.* **179**, 3371–3379 (2007).
144. Lainhart, W., Stolfa, G. & Koudelka, G. B. Shiga toxin as a bacterial defense against a eukaryotic predator, *Tetrahymena thermophila*. *J. Bacteriol.* **191**, 5116–5122 (2009).
145. Steinberg, K. M. & Levin, B. R. Grazing protozoa and the evolution of the *Escherichia coli* O157:H7 Shiga toxin-encoding prophage. *Proc. Biol. Sci.* **274**, 1921–1929 (2007).
146. Cimolai, N., Carter, J. E., Morrison, B. J. & Anderson, J. D. Risk factors for the progression of *Escherichia coli* O157:H7 enteritis to haemolytic-uraemic syndrome. *J. Pediatr.* **116**, 589–592 (1990).
147. Proulx, F., Turgeon, J. P., Delage, G., Lafleur, L. & Chicoine, L. Randomized, controlled trial of antibiotic therapy for *Escherichia coli* O157:H7 enteritis. *J. Pediatr.* **121**, 299–303 (1992).
148. Walterspiel, J. N., Ashkenazi, S., Morrow, A. L. & Cleary, T. G. Effect of subinhibitory concentrations of antibiotics on extracellular Shiga-like toxin I. *Infection* **20**, 25–29 (1992).
149. Kitov, P. I. *et al.* Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. *Nature* **403**, 669–672 (2000).
150. Mulvey, G. L. *et al.* Assessment in mice of the therapeutic potential of tailored, multivalent Shiga toxin carbohydrate ligands. *J. Infect. Dis.* **187**, 640–649 (2003).
151. Kitov, P. I. *et al. In vivo* supramolecular templating enhances the activity of multivalent ligands: a potential therapeutic against the *Escherichia coli* O157 AB_5 toxins. *Proc. Natl Acad. Sci. USA* **105**, 16837–16842 (2008).
152. Takeda, T., Yoshino, K., Adachi, E., Sato, Y. & Yamagata, K. *In vitro* assessment of a chemically synthesized Shiga toxin receptor analogue attached to chromosorb P (Synsorb Pk) as a specific absorbing agent of Shiga toxin 1 and 2. *Microbiol. Immunol.* **43**, 331–337 (1999).
153. Trachtman, H. *et al.* Effect of an oral Shiga toxin-binding agent on diarrhea-associated haemolytic uraemic syndrome in children: a randomized controlled trial. *JAMA* **290**, 1337–1344 (2003).
154. Nishikawa, K. *et al.* A therapeutic agent with oriented carbohydrates for treatment of infections by Shiga toxin-producing *Escherichia coli* O157:H7. *Proc. Natl Acad. Sci. USA* **99**, 7669–7674 (2002).
A study describing SUPER TWIG, a compound that neutralizes Shiga toxins in the circulation and protects mice from challenge with a fatal dose of *E. coli* O157:H7. The compound seems highly promising for the development of anti-toxin strategies.
155. Watanabe, M. *et al.* Oral therapeutic agents with highly clustered globotriose for treatment of Shiga toxigenic *Escherichia coli* infections. *J. Infect. Dis.* **189**, 360–368 (2004).
156. Paton, A. W., Morona, R. & Paton, J. C. A new biological agent for treatment of Shiga toxigenic *Escherichia coli* infections and dysentery in humans. *Nature Med.* **6**, 265–270 (2000).
157. Pinyon, R. A., Paton, J. C., Paton, A. W., Botten, J. A. & Morona, R. Refinement of a therapeutic Shiga toxin-binding probiotic for human trials. *J. Infect. Dis.* **189**, 1547–1555 (2004).
158. Mukherjee, J. *et al.* Production and characterization of protective human antibodies against Shiga toxin 1. *Infect. Immun.* **70**, 5896–5899 (2002).
159. Mukherjee, J. *et al.* Human Stx2-specific monoclonal antibodies prevent systemic complications of *Escherichia coli* O157:H7 infection. *Infect. Immun.* **70**, 612–619 (2002).
160. Miller, D. J. *et al.* Structure-based design and characterization of novel platforms for ricin and Shiga toxin inhibition. *J. Med. Chem.* **45**, 90–98 (2002).
161. Feng, Y. *et al.* Exo1: a new chemical inhibitor of the exocytic pathway. *Proc. Natl Acad. Sci. USA* **100**, 6469–6474 (2003).
162. Feng, Y. *et al.* Retrograde transport of cholera toxin from the plasma membrane to the endoplasmic reticulum requires the *trans*-Golgi network but not the Golgi apparatus in Exo2-treated cells. *EMBO Rep.* **5**, 596–601 (2004).
163. Spooner, R. A. *et al.* The secretion inhibitor Exo2 perturbs trafficking of Shiga toxin between endosomes and the *trans*-Golgi network. *Biochem. J.* **414**, 471–484 (2008).
164. Saenz, J. B., Doggett, T. A. & Haslam, D. B. Identification and characterization of small molecules that inhibit intracellular toxin transport. *Infect. Immun.* **75**, 4552–4561 (2007).
165. Saenz, J. B. *et al.* Golgicide A reveals essential roles for GBF1 in Golgi assembly and function. *Nature Chem. Biol.* **5**, 157–165 (2009).

Acknowledgements
We thank L. Roberts, J.M. Lord, J. Wiels and C. Lamaze for helpful discussion, and J. Ménétrey for help with figure 1. Work in the laboratory of the authors was supported by CEFIPRA (The Indo-French Centre for the Promotion of Advanced Research; programme n°3803), the Human Frontier Science Program (grant RGP26/2007), the ImmuCan programme of the Pôle de Compétitivité Médicen Paris Région, the Direction Générale de l'Armement programme of Paracell, the Cancerimmunotherapy project in the European Union's Seventh Framework Programme and the Programme indicatif et coopératif (PIC) on Tumour Delivery at the Institut Curie.

Competing interests statement
The authors declare no competing financial interests.

DATABASES
UniProtKB: http://www.uniprot.org
BAX | BCL-2 | BiP | caspase 6 | caspase 3 | caspase 8 | caspase 9 | CHOP | DR5 | EpsinR | ERK1 | ERK2 | ezrin | FLIP | furin | GRP94 | HEDJ | IL-8 | IRE1 | PERK | SNX1 | SNX2 | SNX5 | SNX6 | tissue factor | TRAIL | VPS29 | VPS35 | XIAP

FURTHER INFORMATION
Ludger Johannes' homepage: http://www.curie.fr/recherche/themes/detail_equipe.cfm/lang/_gb/id_equipe/101.htm

SUPPLEMENTARY INFORMATION
See online article: S1 (table)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF