Review

Experimental In Vivo Models of Bacterial Shiga Toxin-Associated Hemolytic Uremic Syndrome

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Shiga toxins (Stxs) are the main virulence factors expressed by the pathogenic Stx-producing bacteria, namely, *Shigella dysenteriae* serotype 1 and certain *Escherichia coli* strains. These bacteria cause widespread outbreaks of bloody diarrhea (hemorrhagic colitis) that in severe cases can progress to life-threatening systemic complications, including hemolytic uremic syndrome (HUS) characterized by the acute onset of microangiopathic hemolytic anemia and kidney dysfunction. Shiga toxicosis has a distinct pathogenesis and animal models of Stx-associated HUS have allowed us to investigate this. Since these models will also be useful for developing effective countermeasures to Stx-associated HUS, it is important to have clinically relevant animal models of this disease. Multiple studies over the last few decades have shown that mice injected with purified Stxs develop some of the pathophysiological features seen in HUS patients infected with the Stx-producing bacteria. These features are also efficiently recapitulated in a non-human primate model (baboons). In addition, rats, calves, chicks, piglets, and rabbits have been used as models to study symptoms of HUS that are characteristic of each animal. These models have been very useful for testing hypotheses about how Stx induces HUS and its neurological sequelae. In this review, we describe in detail the current knowledge about the most well-studied in vivo models of Stx-induced HUS; namely, those in mice, piglets, non-human primates, and rabbits. The aim of this review is to show how each human clinical outcome-mimicking animal model can serve as an experimental tool to promote our understanding of Stx-induced pathogenesis.

Keywords: Shiga toxin, HUS, animal models, STEC

Shiga Toxins and Hemolytic Uremic Syndrome

Shiga toxins (Stxs) are ribosome-inactivating proteins expressed by several species of pathogenic bacteria that colonize the gastrointestinal tract. They are responsible for a condition known as hemorrhagic colitis or bloody diarrhea. Stxs bind specifically to the neutral membrane glycolipid globotriaosylceramide (Gb3) receptor expressed on the cell surface of various host cells [1, 2]. After binding to Gb3, Stxs are internalized via endocytosis and trafficked to the endoplasmic reticulum (ER) via retrograde transport through the *trans*-Golgi network and Golgi apparatus [3].

Upon entering the host cell cytosol, multi-functional Stxs inhibit protein synthesis and induce pro-inflammatory responses, autophagy, and apoptosis via ER stress triggered by the accumulation of unfolded proteins within the ER [4–9]. Stxs increased the activation of three ER-localized transmembrane stress sensor proteins including inositol-requiring enzyme 1 α (IRE1 α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) [10–14]. Sequentially, the Stx-activated ER stress sensors lead to increased mRNA and protein expression levels of C/EBP homologous protein (CHOP) and death receptor 5 (DR5) to induce apoptosis in

the toxin-sensitive cells [13, 14]. X-ray crystal structures of holotoxins reveal that Stxs have an AB₅ molecular configuration: a monomeric A subunit and a pentameric B subunit. The A subunit possesses enzymatic N-glycosidase activity, and the B subunit clusters bind to the Gb3 receptor on the cell surface prior to internalization [15, 16]. The prototypical Shiga toxin, termed Stx, is produced by *Shigella dysenteriae* serotype 1, which is a Gram-negative, invasive, and facultative intracellular pathogen that causes the most severe form of the epidemic bacillary dysentery that is associated with contaminated water. Both the genus *Shigella* and the toxin are named after the bacteriologist Kiyoshi Shiga, who showed in 1897 in Japan that the microorganism is a causative agent of inflammatory dysentery or large-volume watery diarrhea [17]. While 15 serotypes of *Shigella dysenteriae* and three other *Shigella* species all cause shigellosis (bacillary dysentery), only *Shigella dysenteriae* serotype 1 expresses Stx [18, 19]. In developing countries, shigellosis caused by *Shigella* species continues to be a major threat to public health, particularly in areas where overcrowding, malnutrition, and poor waste water management intersect. Shigellosis caused by Stx from *Shigella dysenteriae* serotype 1 is of particular concern because patients can develop life-threatening, extra-intestinal complications such as hemolytic uremic syndrome (HUS), which is characterized by acute renal failure, central nervous system (CNS) abnormalities, seizures, paralysis, and death [20–26].

Genomic analyses show that *Shigella* spp. are very closely related to *Escherichia coli*. Moreover, several strains of *E. coli* express one or more toxins that are genetically, structurally, and functionally related to Stx [27]. These bacteria are collectively termed Shiga toxin-producing *E. coli* (STEC),

and the Shiga-like toxins they produce are termed Shiga toxin type 1 (Stx1) and Shiga toxin type 2 (Stx2). The serotype is classified into five types (A to E; Enterotoxigenic, Enteropathogenic, Enterohemorrhagic, Enteroinvasive and Enteraggagative) because STEC is related to both severity and frequency basing on the reported outbreaks (Table 1) [27]. Stx1 and Stx2 are 98% and 60% homologous, respectively, to Stx [28, 29]. In addition, each Stx has variants that are classified according to disease progression (Table 2). In particular, Stx2e induces edema in piglets and binds to Gb4 [30]. In developed countries, STEC remains a public health concern because of the potential for contaminated foods to be distributed on a countrywide basis. This potential was highlighted by recent multi-state outbreaks in the United States of America, which involved contaminated beef products or vegetables [31, 32]. As with infections caused by *Shigella dysenteriae* serotype 1, patients infected with STEC develop hemorrhagic colitis, which may progress to life-threatening systemic sequelae such as HUS and CNS impairment [25, 33, 34].

Bacteremia is rare in patients with EHEC infection, but the bacteria-secreted Stxs are widely known as the principal contributors of organ damage. Recently, a 10-month-old pediatric HUS patient with stool positive confirmation of the Shiga toxin-producing O104 *Escherichia coli* developed severe chronic renal failure, retinal and choroidal hemorrhages as well as neurologically minor physical disabilities with blindness [35]. In the clinical cases reviewed at the University Children's Hospital Zurich between 1995 and 2007, three of 69 examined HUS patients presented ocular involvement with visual impairments [36]. Notably, previous study showed that manifest renal

Table 1. Serotypes of Shiga toxin-producing *Escherichia coli*.

Sero – pathotype ¹	Serotype	Frequency of association with disease	Involvement in outbreaks	Association with HUS and HC ²
A	O55:H7, O157:H7, O157:NM,	High	Common	+
B	O26:H11, O103:H2, O111:H8, O111:NM, O121:H19, O145:NM,	Moderate	Uncommon	+
C	O5:NM, O91:H21, O104:H21, O113:H21, O121:NM, O165:H25 and others	Low	Rare	+
D	O7:H4, O69:H11, O80:NM, O84:H2, O98:NM, O103:H25, O113:H4, O117:H7, 119:H25, O132:NM, O146:H21, O165:NM, O171:H2, O172:NM, O174:H8 and others	Low	Rare	-
E	O6:H34, O8:H19, O39:H49, O46:H38, O76:H7, O84:NM, O88:H25, O98:H25, O113:NM, O136:H12, O136:NM, O143:H31, O153:H31, O156:NM, O163:NM, O177:NM and others	Not implicated	Not implicated	-

¹Adapted from Gyles et al., 2007 [27].

²HUS = hemolytic uremic syndrome; HC = hemorrhagic colitis.

Table 2. Characteristics of the Shiga toxin family.

Organism	Toxin	Amino acid homology* (%)	Disease progression	Receptor
<i>Shigella dysenteriae</i>	Stx	-	D → HC → HUS	Gb3
STEC	Stx1	-	D → HC → HUS	Gb3
	Stx1c	(A) 97; (B) 95	D → HC → HUS	Gb3
	Stx1d	(A) 93; (B) 92	D	Gb3
	Stx2	-	D → HC → HUS	Gb3
	Stx2c	(A) 100; (B) 97	D → HC → HUS	Gb3
	Stx2c2	(A) 100; (B) 97	D	Gb3
	Stx2d	(A) 99; (B) 97	D	Gb3
	Stx2d _{activatable}	(A) 99; (B) 97	D → HC → HUS	Gb3
	Stx2e	(A) 93; (B) 84	D → piglet edema	Gb3 & Gb4
	Stx2f	(A) 63; (B) 57	D	Gb3

¹Adapted from Johannes *et al.*, 2010 [30].

²D = diarrhea; HUS = hemolytic uremic syndrome; HC = hemorrhagic colitis.

injury in STEC-associated HUS infant patients may be induced with the low concentration of Stxs present in polymorphonuclear leukocytes (PMN) [37]. Ramos *et al.* demonstrated that the Stxs induced the activation of PMN, resulting in the production of reactive oxygen species and increasing CD11b and CD66b expression [38]. PMN in HUS patients contribute to renal dysfunction by causing inflammation and thrombosis of the microvascular system induced by the action of neutrophil extracellular traps (NET) [39]. Multiple studies sought to elucidate the pathogenesis of Stx-induced HUS by developing various in vivo models that involve administration of purified Stxs; these models reproduce many of the pathophysiological changes seen in patients infected with Stx-producing bacteria [40–45]. For example, mouse and baboon models reveal that the toxins induce production of various pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF- α) and interleukin (IL)-1 β , and that this elicits excessive inflammatory responses that may cause renal damage and accelerate the death of the animals [46, 47]. These findings are supported by studies of other infections; such studies show that activation of innate immune responses is critical for both elimination of infectious agents and toxins and for healing and/or regeneration of damaged tissues [48]. Tissue healing may include activation of cell survival and apoptotic signaling cascades [49].

While many studies have been performed in in vitro models to delineate the pathogenesis of Stxs [7, 50], far fewer in vivo studies with animal models showing pathophysiological features of Shiga toxicosis have been conducted. Furthermore, how the innate immune responses

to Stx mediate damage to the colon and the development of potentially fatal complications such as HUS remains to be fully explored. In this review, we will summarize the experimental in vivo models that have been used to study Stx-induced pathogenesis, the knowledge that has been gained from these studies, and the current progress in the development of therapeutic interventions against Stx.

In Vivo Studies on Stx-Induced HUS Pathogenesis

Various animal models have been developed for the in vivo study of STEC pathogenesis. In many models, the animals are infected by an STEC, which travels to the intestine and starts to secrete the Stx. The toxin then passes through the intestinal mucosa and enters the bloodstream, where it mainly binds to neutrophils (Fig. 1). The toxin then travels to the target organs (Fig. 2). Other models take advantage of the fact that the Stxs reach the target organs via the circulation: they are induced by intravenous or intraperitoneal injection of the Stx alone. Since the main target organ of circulating Stxs is the kidney, many animal models that recapitulate the pathogenic outcomes in the kidney or intestine that are seen in humans have been developed. Moreover, since HUS-related CNS associates with serious consequences, animal models of this complication have also been developed. An up-to-date list of Stx studies in animal models of HUS is presented in Table 3.

Rodents

Rodents such as the rat and the mouse are the most commonly used animals for preclinical research because

Fig. 1. Summary of Shiga toxin-induced HUS pathogenesis.

A & E lesions, attaching and effacing lesions; CNS, central nervous system; ER, endoplasmic reticulum; HUS, hemolytic uremic syndrome; Stx, Shiga toxin.

the maintenance costs are relatively low and the animals are easy to handle. They are also very useful for studies on STEC pathogenesis because the clinical outcomes of STEC infection in rodents are similar to those in humans; namely, renal failure, dehydration, diarrhea, and death. The first mouse model of STEC pathogenesis was generated by treating mice with an antibiotic (streptomycin or mitomycin C) that reduced the normal flora in the intestine and then feeding them with an STEC [51–53]. The antibiotic treatment allowed estimation of the ability of STEC to colonize the intestine in the absence of competition from the intestinal flora. Such competition was also eliminated by using germ-

free mice [54, 55]. Notably, when germ-free mice were both injected with TNF- α and inoculated with STEC, the mice developed systemic disease, including neuronal damage and inflammation in the CNS as well as glomerular lesions; these signs were even readily observed when a low STEC dose was used [54]. However, the fact that the antibiotic-treated and germ-free mouse models lack gut flora limits their usefulness for studying the natural disease outcomes of STEC infection. Therefore, several researchers developed mouse models of STEC pathogenesis in the context of a normal bowel flora [56, 57]. These physiologically more relevant models include that of Mohawk *et al.*: they showed

Fig. 2. Pathways of Shiga toxin infection and its action in target organs. CNS, central nervous system; HUS, hemolytic uremic syndrome.

that when BALB/c mice with an intact commensal flora were orally inoculated with *E. coli* O157:H7, approximately 30% died. The dead mice exhibited intestinal colonization and renal tubular damage on necropsy [36]. Moreover, multiple mouse models of STEC pathogenesis have been developed by simply injecting purified STEC Stxs, which are the most important virulence factor of STECs: these injections cause severe renal damage and ultimately kill the mice, regardless of the status of the host's intestinal flora [35, 58]. In addition, when mice were injected with Stxs together with lipopolysaccharide, which is another essential virulence factor of STEC, almost all of the features of HUS that are seen in humans were observed [42, 56].

Generally, it is accepted that Stxs are a major cause of not only HUS but also CNS damage. HUS occurs after the Stx enters the systemic circulation, resulting in CNS damage in severe cases. Patients with STEC-induced HUS are more likely to progress to CNS dysfunction. These CNS alterations are a major cause of child mortality after acute illness [23, 59–62]. Moreover, the mortality rate associated with HUS with CNS dysfunction is 2–3 times higher than that associated with HUS alone [21, 23, 59, 63–65]. In terms of the pathogenic mechanisms by which Stxs induce CNS disturbances in children, HUS with neurologic involvement can lead to visual system impairment, including blindness [65, 66]; indeed, a recent study shows that Stxs induce apoptosis and ER stress in the retinal pigment epithelium, which plays an important role in maintaining proper visual function [67]. Moreover, experiments in mice with oral

STEC infection-induced encephalopathy [68] show that Stxs weaken the blood-brain barrier (BBB) by damaging blood vessels. Recent reports suggest that injection of Stx2 into murine blood vessels damages the striatum, leading to motor deficits and neurovascular injury [69]. In addition, several studies show that mice injected intraperitoneally with 0.025–2.5 $\mu\text{g/kg}$ Stxs develop nervous system symptoms such as hind limb paralysis, lethargy, shivering, abnormal gait, and seizures [68, 70, 71].

In rat models, intraperitoneal injection of culture supernatant from STEC results in histopathological outcomes in the kidney, including acute glomerular necrosis and microvascular thrombosis, which are also seen in STEC-infected humans [72]. STEC-induced CNS damage was also generated in rats by intracerebroventricular administration of purified-Stx2 (6 ng/mice): confocal microscopy revealed neuronal death and glial cell damage [73, 74]. A clue to how Stx induces CNS damage was initially provided by Rensmeester and Hulsman: they showed that the CNS edema and neurological symptoms of patients after epileptic seizures associate with changes in brain aquaporin (AQP) expression [75]. Two other studies showed that exposure of rats and mice to Stx reduced AQP4 expression around blood vessels in the brain [76, 77]. Moreover, heat shock protein 70 (Hsp70), a chaperone protein, interacts with the stress sensor protein IRE1 α to protect host cells from ER stress [78]. In vitro experiments showed that Stx2 treatment induces apoptosis and decreases proliferation of B92 and primary rat glial cells by

Table 3. Animal models of Stx injection or STEC infection.

Animal	Inoculated material	Concentration	Route	Organ	Outcome	Ref
Mouse	Purified Stx2	0.5 and 50 ng/20 g	Intraperitoneal	Brain	N, D	[71]
Mouse	VTEC/VT2	5×10^{10} CFU/1 to 4 ng	Oral/Intraperitoneal	Brain	N, D	[68]
Mouse	Purified Stx1 and Stx2a	Stx2a at 7 ng/ml and Stx1 at 1,500 ng/ml	Intraperitoneal	Kidney, brain	N, R	[120]
Mouse	STEC	10^{10} CFU	Oral	Intestine	I, C	[51]
Mouse	STEC	10^{10} CFU	Oral	Intestine	R, C	[52]
Mouse	STEC	5×10^3 CFU	Oral	Intestine	I, D, C	[53]
Mouse	STEC	10^{9-10} CFU	Oral	Kidney, intestine	R, N, C	[54]
Mouse	STEC	10^{2-7} CFU	Oral	Kidney, intestine	R, I, C	[55]
Mouse	STEC	10^{5-9} CFU	Oral	Intestine	I, D, C	[57]
Mouse	Purified Stx1 and Stx2	400 ng and 1 ng	Intraperitoneal and intravenous	Kidney	R, D	[58]
Mouse	Purified Stx2	1 to 5 ng/20 g	Intravenous	Kidney	R, D	[35]
Mouse	STEC	10^{7-8} CFU	Oral	Kidney, intestine, brain	R, N, I	[56]
Mouse	Purified Stx2	225 ng/kg	Intraperitoneal	Kidney	R, D	[42]
Mouse	Purified Stx2	50 ng/kg	Intravenous	Kidney	R, D	[70]
Mouse	Purified Stx2	5 to 0.44 ng/mouse	Intravenous	Brain	N, D	[69]
Mouse	Stx	0.075 ng/g	Intravenous	Kidney	R, D	[121]
Mouse	Purified Stx2	0.15 ng/g	Intravenous	.	D	[122]
Mouse	Purified Stx2	100 ng per mouse	Intraperitoneal	Kidney	R	[123]
Rat	Culture supernatant from the recombinant <i>E. coli</i> (sStx2)	Approximately 20 µg/kg	Intraperitoneal	Kidney, intestine	R, I	[72]
Rat	Culture supernatant from the recombinant <i>E. coli</i> (sStx2)	1 ml/100 g (b.w.) Stx2 (400 ng of Stx2/ml)	Intraperitoneal	Brain	N	[76]
Rat	Purified Stx2	6 µl of Stx2 (1 ng/µl)	Intracerebroventricular	Brain	N	[74]
Rat	Purified Stx2	10 pg/g	Intravenous	Brain	N	[124]
Rat	Purified Stx2	6 µl of Stx2 (1 ng/µl)	Intracerebroventricular	Brain	N	[73]
Monkey	STEC	10^{11} CFU	Oral	Intestine	I	[80]
Baboon	Purified Stx1	2.0 µg/kg	Intravenous	Kidney, brain	R, I	[81]
Baboon	Purified Stx1	50 to 200 ng/kg	Intravenous	Kidney, intestine, brain	R, N, I	[47]
Baboon	Purified Stx1 and Stx2	Stx1 at 10, 50, or 100 ng/kg or Stx2 at 10, 50 ng/kg	Intravenous	Kidney	R	[83]
Baboon	Purified Stx1 and Stx2	100 ng/kg	Intravenous	Kidney	R, T	[82]
Baboon	Purified Stx1 and Stx2	Stx1 at 10, 50, or 100 ng/kg or Stx2 at 10, 50 ng/kg	Intravenous	Kidney	R, D	[84]
Calf	STEC	10^{10} CFU	Oral	Intestine	I, C	[85]
Calf	EHEC O104:H4	10^{10} CFU	Oral	Intestine	I, C	[86]
Calf	STEC	3×10^{10} cfu	Intramuscular	Intestine	I	[125]
Chick	STEC	10^5 CFU	Oral	Intestine	I	[126]
Chick	STEC	1.6×10^9	Oral	Intestine	I	[93]
Chick	STEC	10^8 CFU	Oral	Intestine	I	[92]
Piglet	STEC	10^{11} CFU	Oral	.	E	[88]
Piglet	STEC	3×10^9 CFU	Oral	Brain	N, D	[89]
Piglet	Stx2e	5 to 500 ng/kg	Intravenous	.	E	[87]
Piglet	STEC	10^{10} CFU	Oral	Intestine	I	[90]
Canine	Purified Stx1 and Stx2	0.03 to 0.05 µg/kg	Intravenous	.	D, T	[95]
Rabbit	O153/O157:H7	5×10^8 / 9×10^8 CFU	Oral	Kidney, intestine	R, D, D*	[96]
Rabbit	Purified Stx2	0.1 to 4.0 µg/kg	Intravenous	Brain	N	[99]
Rabbit	VT2	5 mg/kg	Intravenous	Brain	N, I	[98]
Rabbit	VT1	4 µg/rabbit	Intravenous	Kidney, intestine	C	[127]
Rabbit	Purified Stx2	1,200 ng/kg	Intravenous	Kidney, intestine	R, I, T	[97]

R, Renal damage; N, Neurological manifestations; I, Intestinal pathology; D, Death; C, Colonization; T, Thrombosis; D*, Diarrhea; E, Edema; b.w., body weight.

reducing expression of Hsp70 [79].

Non-Human Primates

Despite some ethical and financial problems, primates are still the most suitable animal model for research on infectious diseases because their immune system is very similar to that of humans. Therefore, several studies have assessed the toxic effects of Stxs in primate models. Kang *et al.* intragastrically infected 22 adult *Macaca radiata* with the STEC O157:H7 strain 84-01 and then sacrificed two or three monkeys at various intervals over the next 12 days for histological analysis [80]. Of the 22 monkeys, 17 developed watery (not bloody) diarrhea that lasted at most for 4 days post-infection. Sorbitol-non-fermenting STEC were isolated from the stool samples of these 17 monkeys. The infected monkeys that were sacrificed within 1 day of infection all exhibited mucosal changes in the colon, including vacuolization, tufting, and extrusion of epithelial cells. However, only half of the monkeys had kidney damage, and this was limited to mild tubular vasculopathy.

Another primate model study was conducted by Taylor *et al.* [47]. When they injected baboons intravenously with Stxs, they found that although the outcomes varied depending on the amount of injected toxin, almost all baboons developed acute renal failure and died within 3 days or less after the injection. The renal proximal tubular epithelial cells and the intestinal mucosal epithelial cells, which express receptors for the Gb3 moiety of Stxs, were severely damaged and microthrombosis was detected in renal glomerular tissues. Neurological autopsy revealed mild to moderate cerebral edema and electron microscopy showed perivascular edema in the high-dose group (2.0 µg/kg). A subsequent study by the same group then showed that when six baboons were administered one dose of purified Stx1 (100 ng/kg) intravenously, three had seizures [81]. Moreover, they showed that while intravenous injection with 25 ng/kg Stx2 induced HUS symptoms such as thrombocytopenia, hemolytic anemia, and azotemia, equal amounts of Stx1 did not elicit these effects [82]. It was also found that when baboons are injected intravenously with Stx1 or Stx2, the kidney expresses high levels of cytokine- and chemokine-encoding mRNA and the urine contains pronounced levels of these proteins. These changes are associated with leukocyte infiltration into the renal interstitium [83, 84].

Other Animal Models

It is believed that cattle are the most important sources of the STEC that cause foodborne diseases such as HUS in

humans. Since cattle infected with STEC do not exhibit any serious disease symptoms, researchers have used cattle to study how to reduce STEC colonization in STEC host animals [85, 86]. Dean-Nystrom *et al.* showed that after inoculation of cattle, STEC can be detected within a few days in not only the colon and cecum (which are the main sites of colonization) but also in the intestines and gall bladder [85]. The piglet was developed as a model of porcine edema by injecting animals intravenously with Stx2e, or by oral administration of Stx2e-producing *E. coli* [87, 88]. Furthermore, a STEC-infected piglet model was used to validate vaccines against Stx2, which causes brain injury, dysfunction, and death [89]. Other animals, including chickens, are also considered to be possible reservoirs for STEC, and several studies show that like cattle, the main sites of STEC colonization in chickens are the cecum and colon [90–93].

In the 1990s, a HUS-like disease in dogs with renal failure was reported. This led to the development of canine models of HUS [94, 95]. Raife *et al.* showed that when greyhounds were injected with Stx1 or 2, they develop bloody diarrhea and HUS-like outcomes, including microvascular thrombosis [95]. Rabbits have also been used as animal models for STEC pathogenesis studies because inoculation of rabbits with STEC causes intestine and kidney lesions. This is because these target organs in rabbits bear a homolog of the human Gb3 receptor [96]. Renal injury with diarrhea was observed in Dutch belted (DB) rabbits administered an intravenous injection of Stx2 (1,200 ng/kg); symptom severity was dose-dependent [97]. These rabbit models also show CNS symptoms, including paralysis, recumbency, and ataxia [98, 99]. Takahashi *et al.* showed that intravenously injecting rabbits with 0.1–4.0 µg/kg Stx2 induces vascular endothelial cells in the CNS to secrete TNF-α and IL-1β, and that this induces inflammation of the CNS parenchyma [99].

Conclusions and Future Perspectives

The experiments with various *in vivo* models, including mice, piglets, and rabbits, have not only provided important insights into the pathological consequences of Stx, they have also revealed potential pathways that could be targeted by therapies for HUS. These animal models are particularly important because even though non-human primate models probably best reproduce the serious clinical outcomes seen in humans (especially the toxin-induced nephrotoxicity), studies with these models are inevitably limited by the scarcity of monkeys and high costs.

While this review focused on *in vivo* models, it should be mentioned that the *in vivo* model findings have often been initiated, supported, and extended by *in vitro* analyses of the effects of Stx on susceptible cell types, including human monocytes/macrophages, endothelial cells, renal epithelial cells, and neuronal cells [50, 100]. Several interesting *in vitro* models have also been described recently. Thus, Karve *et al.* showed that induced human intestinal organoids (iHIOs) provide important information about the effect of STEC infections on intestinal tissue. iHIOs are differentiated human intestinal tissues that are generated from pluripotent stem cells. When iHIOs are infected with STEC, they produce reactive oxygen species and upregulate their inflammatory responses, including IL-8 secretion [101]. In addition, three-dimensional tissue models have been developed for renal tissue [102] and human cortical renal tubular epithelial cells [103]. Studies using these models showed that exposure to Stx2 increases the production of kidney injury marker 1 and IL-8 and hampers renal tubular cell regeneration [102, 103].

Diarrhea-associated HUS (D+HUS) is a leading cause of pediatric acute renal failure [104, 105]. Despite the many studies with HUS animal models, a therapeutic vaccine that effectively ameliorates D+HUS is not yet available. Instead of vaccine development, toxin-neutralizing therapeutics using several anti-Stxs antibodies were tested in animal models like piglets or rodents, and these antibody treatments effectively rescued the Stx-intoxicated animals from severe mortality [35, 106–108]. In addition, peptide-based neutralizer that directly binds to Stx2 was identified to inhibit Stx binding to Gb3 receptor and successfully protected rodent models from Stx-caused lethality [109–111]. As therapeutic targeting for retrograde trafficking to the Golgi apparatus or the ER of the Stxs, small molecule compounds such as Retro-1, Retro-2 and Exo2 were developed and found to be protective for STEC-infected mice from Stx-induced toxicity [112, 113]. Metal cofactor manganese was found to stimulate degradation of the endosome-to-Golgi transport protein Gpp130 and protect it from Stx1, but not Stx2 in mice [114].

Although many HUS animal model studies show that Stxs upregulate various stress-activated kinase pathways, including p38 MAPK, JNK, ERK, MK2, and ZAK, and that these induce the production of pro-inflammatory cytokines that mediate the tissue damage caused by these toxins [115–117], it has been difficult to find the downstream substrates of these kinase pathways that can be targeted with sufficiently high specificity by a therapy against D+HUS. Further research aiming to identify these targets is warranted.

ZAK-deficient mice were protected from gastrointestinal lysine toxicity by reducing CXCL1 production following depurination of the sarcin-ricin loop [118]. Treatment of rabbit ZAK kinase inhibitor imatinib also reduced the number of neutrophils penetrating STEC-infected colon tissues [119]. Other potential targets for treatments that prevent or ameliorate the Stx-induced acute renal damage in HUS may be the signaling molecules that mediate STEC-induced inflammation, apoptosis, autophagy, and ER stress responses. Further studies in both *in vitro* and *in vivo* models that evaluate inhibitors targeting these signaling molecules are needed. Moreover, studies that further elucidate the pro-inflammatory cytokine-mediated signaling mechanisms that are activated by Stxs will be critical for development of effective therapies against the emerging infectious diseases caused by *Shigella dysenteriae* serotype 1 and STEC.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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