Enterobacter sakazakii Enhances Epithelial Cell Injury by Inducing Apoptosis in a Rat Model of Necrotizing Enterocolitis

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Necrotizing enterocolitis (NEC) is an inflammatory intestinal disorder that affects 2%–5% of all premature infants. Enterobacter sakazakii, a common contaminant of milk-based powdered infant formula, has been implicated as a causative agent of sepsis, meningitis, and NEC in newborn infants, with high mortality rates. However, the role played by E. sakazakii in the pathogenesis of NEC is, to date, not known. Here, we demonstrate for the first time that E. sakazakii can induce clinical and histological NEC in newborn rats. E. sakazakii was found to bind to enterocytes in rat pups at the tips of villi and to intestinal epithelial cells (IEC-6) in culture, with no significant invasion. Exposure to E. sakazakii induced apoptosis and increased the production of interleukin-6 in IEC-6 cells and in the animal model. These data suggest that E. sakazakii could be a potential pathogen that induces NEC and triggers intestinal disease by modulating enterocyte intracellular signaling pathways.

Enterobacter sakazakii is an important emerging neonatal pathogen that is associated with outbreaks of meningitis, sepsis, and necrotizing enterocolitis (NEC) [1]. E. sakazakii is prevalent in certain milk-based powdered infant formulas, cereals, chocolate, potato flour, and pasta [2–4]. The number of E. sakazakii–related infections has substantially increased during the past 10 years owing to the increased use of infant formula. The US Food and Drug Administration has issued several warnings regarding E. sakazakii infection in newborns.

NEC is an inflammatory intestinal disorder that affects 2%–5% of all premature infants and is associated with high mortality rates. Three principal factors have been implicated in the pathogenesis of the disease: an immature intestinal epithelial barrier, abnormal bacterial colonization, and formula feeding. Although a variety of bacterial species have been implicated in the development of NEC, including Enterobacter, Clostridium, and Staphylococcus species, no single pathogen has yet been shown to cause the disease [5]. Enterobacter species have been associated with NEC and isolated from the blood, skin, cerebrospinal fluid, peritoneal fluid, urine, and respiratory tracts of affected infants [6, 7]. Milk-based infant formulas contaminated with E. sakazakii have been implicated in outbreaks of NEC, suggesting that this pathogen may be important for disease pathogenesis.

The establishment of disease depends on successful pathogen colonization and the ability of a microbe to adhere to host surfaces, such as the intestinal epithelial layer [8]. E. sakazakii adheres to endothelial cells and to neoplastic epithelial cell lines [9]. However, the associations between E. sakazakii and nontransformed intestinal epithelial cell lines remain poorly understood. Pathogen binding may trigger a number of different host responses, ranging from alteration in intracellular signaling pathways, chemokine and cytokine release, and induction of apoptosis. A variety of inflammatory molecules, including tumor necrosis factor (TNF)–α, nitric oxide, and interleukin (IL)–6, have been implicated in...
the pathogenesis of NEC [10]. In particular, elevated levels of IL-6 have been found in blood and stool samples of infants with NEC [11]. Apoptosis is a programmed cell death, which under homeostatic conditions exists in harmony with cellular mitosis. Experimental NEC is associated with increased enterocyte apoptosis [12]. We therefore hypothesized that colonization of the intestine by *E. sakazakii* may contribute to the pathogenesis of NEC by stimulating the release of inflammatory factors and inducing the apoptosis of enterocytes. The present study examined the causative relationship between *E. sakazakii* and NEC in a rat model of the disease.

**METHODS**

**Bacterial strains, cells, and reagents.** *E. sakazakii* (strain 51329), *Escherichia coli* DH5-α, and rat intestinal epithelial cells (IEC-6; passages 20–26) were obtained from the American Type Culture Collection. IEC-6 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, 1 U/mL insulin, 100 U/mL penicillin G, and 100 U/L streptomycin. Rodent formula (Esbilac) was obtained from PetAg.

**Transformation of *E. sakazakii* with green fluorescent protein (GFP) plasmid.** *E. sakazakii* were grown for 8 h in Luria broth at 37°C, centrifuged to pellet down bacteria, washed, and treated with 0.1 mol/L calcium chloride for 30 min on ice. Then, *E. sakazakii* were transformed with a GFP plasmid, as described elsewhere [13]. Colonies were assessed for GFP expression by viewing them under ultraviolet light, and further cultures were grown from a single colony.

**E. sakazakii infection experiments using the rat model of NEC.** Animal experiments were approved by the Institutional Animal Care and Use and Biosafety Committees of Childrens Hospital Los Angeles. NEC was induced in newborn rats by formula feeding and hypoxia [14]. Briefly, timed-pregnant rats were purchased from Harlan, and labor was induced with oxytocin at term. Six experimental groups were used. One group received 0.2 mL of clean Esbilac formula twice daily (the formula feeding [FF] group); a second group received the same feeding protocol as the FF group and thrice-daily hypoxia exposure (~5 min each) with 5% O₂ and 95% N₂ (the FF+H group); a third group received 0.2 mL of clean Esbilac formula once daily and 0.2 mL of formula containing a known quantity of *E. sakazakii* once daily (the FF+ES group); a fourth group received the same feeding protocol as the FF+H group and thrice-daily hypoxia exposure (the FF+H+ES group); and a fifth and a sixth group received the same protocols as the FF+ES and the FF+H+ES group, except that *E. sakazakii* was replaced with *E. coli* (the FF+EC group and the FF+H+EC group, respectively). NEC was graded microscopically by a pathologist blinded to the groups, from grade 0 (normal) to 4 (severe) on the basis of pathological manifestations, including submucosal edema, epithelial sloughing or obliteration, neutrophil infiltration, intestinal perforation, and necrosis [14].

**Binding and invasion assays using IEC-6 cells.** Various doses (1 × 10⁻³–1 × 10⁷ cfu/well) of *E. sakazakii* were added to confluent monolayers of IEC-6 cells separately and incubated for 2–12 h. Medium was changed every 1 h (after 2-h incubation) to limit bacterial multiplication. The cells were washed with Hank’s balanced salt solution. Monolayers were solubilized with 0.1% Triton X-100 for 8 min. The contents were removed, diluted serially, plated onto blood agar, and incubated at 37°C overnight. The number of bacterial colonies was recorded, and the percentage of binding was calculated on the basis of inocu-
cells, the experiments were performed as described for binding, except that the cells were incubated with gentamicin (75 μg/mL) for 60 min after completion of the initial incubation, to kill extracellular bacteria.

Cytokine profiling after exposure to *E. sakazakii*. IEC-6 cells were grown to confluence and incubated with *E. sakazakii* (1 × 10⁷ cfu/well) for 1–6 h. The experimental medium and bacteria were collected and spun twice at 3000 rpm for 10 min to collect the supernatants. Cytokine concentrations in various samples were measured by the Luminex assay [15]. Total RNA was extracted from the infected cells by means of the RNeasy Kit (Qiagen). Reverse-transcription polymerase chain reaction (RT-PCR) for IL-6 was performed using equal amounts of cDNA for 30 cycles, with forward primer 5'-GATGGTTGCTGACAGCCACTGCTTT-3' and reverse primer 5'-TTGGATGATCTTTGGTCCTTAGCCA-3'.

Evaluation of apoptosis in vitro and of intestine in rat pups after *E. sakazakii* exposure. IEC-6 cells, grown to 90% confluence in 4-well chamber slides, were infected for varied periods with *E. sakazakii*, washed, and fixed with 1% paraformaldehyde. The cells were then stained using the ApopTag Red In Situ Apoptosis Detection Kit (Chemicon) along with 4',6-diamidino-2-phenylindole (DAPI) and were visualized using a fluorescent microscope. Additionally, apoptosis of IEC-6 cells infected with *E. sakazakii* was assessed using a carboxyfluorescein caspase-3 detection kit (ApoLogix). The number of apoptotic cells per high-powered field were determined by fluorescent microscopy. Intestinal segments were obtained, placed in 1% paraformaldehyde, and embedded in paraffin. Cryosections were also obtained and stained with DAPI. Immunofluorescent microscopy was used to assess the association of GFP-labeled *E. sakazakii* with the intestinal epithelial layer. Apoptosis was assessed after staining both paraffin sections and cryosections with ApopTag Red stain.

Assessment of intestinal damage by electron microscopy. Segments of small intestine were obtained from rat pups subjected to FF+H or FF+H+ES and washed 3 times in 0.1 mol/L cacodylate buffer for 15 min each. The intestinal lumen was exposed, postfixed with 2% osmium tetroxide in 0.1 mol/L cacodylate buffer for 15 min each. The images were captured using an FEI Quanta 200 ESEM microscope under low vacuum conditions.

**RESULTS**

Effect of *E. sakazakii* on NEC in a rat model. Previous experiments at our laboratory established a reproducible rat model of NEC [10]. In this model, the combination of formula feeding and hypoxia leads to macroscopic and microscopic intestinal inflammation, similar to that seen in human NEC. Findings include segmental small-bowel necrosis, edema, neutrophil infil-
tration, epithelial sloughing, and intestinal perforation or sepsis [16]. Because *E. sakazakii* has been implicated as a potential pathogen in human NEC, we investigated whether it was capable of increasing disease severity in the rat model. A laboratory *E. coli* strain, DH5-α, was used as a control. The appropriate dose of *E. sakazakii* was determined by measuring mortality rates in pups fed formula containing $1 \times 10^3$, $1 \times 10^4$, and $1 \times 10^7$ cfu/dose. Formula containing $1 \times 10^3$ cfu/dose together with hypoxia did not increase disease severity significantly compared with that in controls, whereas $1 \times 10^7$ cfu/dose conferred >95% mortality in rat pups by experimental day 4 (data not shown). However, $1 \times 10^5$ cfu/dose added to formula produced clinical symptoms consistent with grade 3 NEC in the rat pup model by day 4 (figure 1A). Rat pups subjected to FF+H+ES had marked abdominal distention and abdominal wall discoloration, compared with those subjected to FF+H (figure 1A–1D). Abdominal discoloration and distention are commonly found in severe human disease. The animals in the FF+H group had a mortality rate of 40%, compared with 70% for those in the FF+H+ES group. Rat pups in the control groups (those subjected to FF+EC or FF+H+EC) did not have a higher mortality rate than those in the FF and FF+H groups (findings similar to those shown in figure 1D). Furthermore, all pups treated with *E. sakazakii* had bacteremia at the completion of the experiment, suggesting systemic disease and loss of intestinal barrier integrity, whereas no bacteremia was detected in the groups not fed *E. sakazakii* or *E. coli* (data not shown).

Histological examination of intestine from day 4 in control pups (the FF group) revealed intact villus architecture (figure 2A), whereas villus tip sloughing and blunting was observed in both the FF+H and FF+ES groups (figure 2B and 2C). In contrast, histological grading of pups in the FF+H+ES group revealed more intestinal injury and inflammation than in the FF+H group ($P = .02$) (figure 2D). Pups subjected to FF+H+EC or FF+EC showed no significant intestinal damage compared with controls (figure 2E and 2F). Although supplementation with *E. sakazakii* increased intestinal injury, no significant difference was observed with *E. sakazakii* feeding alone ($P = .078$ for the comparison with FF+H) (figure 2G). Rat pups fed *E. coli* had no significant increase in pathological score relative to baseline. *E. sakazakii*-treated groups had more cellular infiltrates, demonstrated by more neutrophils within the body of the villus and epithelial sloughing.

**SEM of intestine in *E. sakazakii*–treated rat pups.** SEM was performed to observe more closely the morphological changes in villus architecture after *E. sakazakii* infection. As shown in figure 3A and 3B, intestines of rats in the FF+H group showed intact villi, without a significant number of attached bacteria. In contrast, feeding *E. sakazakii* to rat pups greatly increased intestinal injury and villus disruption (figure 3D). A large number of bacteria were attached to the infected villi, with enterocyte blebbing and formation of gaps in the epithelium (figure 3C and 3E). Higher-magnification photographs showed that bacteria were present at the site of bleb rupture (figure 3F), suggesting that *E. sakazakii* directly damages the epithelial cells of villus tips.

**E. sakazakii adherence to IEC-6 cells in vitro and to enterocytes in newborn rats.** The infection of rat pups showed that *E. sakazakii* interacts with and damages intestinal epithelial cells, which could be responsible for the onset of NEC. To understand the mechanisms involved in *E. sakazakii*–induced intestinal epithelial cell damage, we developed an in vitro model of *E. sakazakii* infection using IEC-6 cells in culture. Initially, we determined the effect of inoculum size on *E. sakazakii* binding to IEC-6 cells after 2 h of infection. *E. sakazakii* bound to IEC-6 cells at a frequency of 0.5% at an inoculum of $1 \times 10^4$ cfu/well.
and the frequency increased to 2.5% when 1 × 10^7 cfu/well were added (figure 4A). Subsequent increases in inoculum sizes did not result in greater binding.

Of note, the degree of invasion of *E. sakazakii* into IEC-6 cells was very low (frequency of 0.01%), suggesting that *E. sakazakii* could not efficiently invade intestinal epithelial cells. Because we observed optimal binding and invasion with 1 × 10^7 cfu/well, time course experiments were performed with 1 × 10^7 cfu/well over a period of 6 h. As shown in figure 4B, the binding of *E. sakazakii* to IEC-6 cells increased significantly (4-fold) at 6 h compared with 2 h after infection, reaching a plateau after 6 h. However, the invasion was increased only 2-fold (0.01% at 2 h vs. 0.02% at 6 h). Light microscopic analysis of *E. sakazakii* binding to IEC-6 cells revealed that the bacteria bound diffusively to enterocytes, a pattern similar to that seen with SEM (figure 4C). To examine whether *E. sakazakii* binds to enterocytes in vivo, we examined cryosections of intestine obtained from rat pups that had been fed GFP-labeled *E. sakazakii*. The sections were stained with DAPI to enable viewing of villus structure. GFP-labeled *E. sakazakii* was seen mainly on the villus surface in FF + H + ES-treated rats (figure 4C). Interestingly, the association was seen only in the distal small intestine and not in the proximal areas, suggesting that specific receptors for *E. sakazakii* binding may be expressed in the distal small intestine. Notably, the distal small intestine is the intestinal region most frequently affected in both human and experimental NEC [14].

**Induction of apoptosis by *E. sakazakii* in IEC-6 cells and enterocytes in newborn rats.** SEM experiments demonstrated that administration of *E. sakazakii* causes damage to the tips of villi, suggesting that the bacterium might kill the cells to which it attaches. To examine whether interaction between *E. sakazakii* and IEC-6 cells induces apoptosis, IEC-6 cells were stained with ApopTag stain after incubation of the cells with 1 × 10^7 cfu/well for 0–12 h. This method identifies apoptotic cells by staining fragmented DNA, which appears during the late stage of apoptosis. The number of apoptotic IEC-6 cells increased with increasing time of *E. sakazakii* infection (figure 5A). Caspase-3 activation, an early marker of apoptosis, was also examined. Quantification of apoptotic cells suggested that the increase in apoptosis reached statistical significance by 4 h and appeared to plateau at 6 h after infection, when 70% of IEC-6 cells demonstrated signs of apoptosis (*P* < .001) (figure 5B). Furthermore, staining of GFP-labeled *E. sakazakii*–infected hypoxic intestinal segments with ApopTag stain revealed more apoptosis in enterocytes than in controls (figure 5C). In the control sample, apoptosis was seen mainly at the villus tip, but with *E. sakazakii* infection it was everywhere in the villus epithelium. Apoptotic changes were almost entirely absent in the FF group (data not shown). These results indicate that *E. sakazakii* induces apoptosis in intestinal epithelial cells, both in vitro and in vivo.

**Induction of a proinflammatory cytokine response by *E. sakazakii* in intestinal epithelial cells.** Cytokines activate local and systemic inflammatory responses that may contribute to the pathogenesis of NEC. It is possible that *E. sakazakii* increases NEC by stimulating epithelial inflammatory cytokine expression. To examine the production of cytokines in intestinal epithelial cells, we collected supernatants from IEC-6 cells treated with *E. sakazakii* and assayed for cytokine production. Exposure to *E. sakazakii* leads to a time-dependent increase in IL-6 production after 2 h of infection, compared with that in FF + H-treated controls (*P* = .018) (figure 6A). Of note, IL-1α was also detected after 2 h of infection; however, there was no apparent
correlation with *E. sakazakii* dose (data not shown). Other cytokines, including interferon-γ, TNF-α, IL-2, IL-4, IL-10, and IL-12, were also assayed, but levels were not detectable.

IL-6 production reached maximal levels within 12 h after infection. In addition, expression of IL-6 was also determined at the mRNA level by RT-PCR. *E. sakazakii*-treated IEC-6 cells showed elevated levels of IL-6 mRNA expression after 60 min (figure 6B). RPS-17, which was used as a control, showed no significant differences between various time points. We further assessed whether *E. sakazakii* induces IL-6 production in neonatal rats. The animals were subjected to FF+H+ES, and serum samples were collected to evaluate IL-6 levels. In agreement with the IEC-6 cell data, rat pups that received FF+H+ES had higher levels of IL-6 after 4 days of treatment than did those subjected to FF+H treatment alone (figure 6C). Taken together, these data suggest that IL-6 may be an important inducible cytokine in the pathogenesis of *E. sakazakii*-induced NEC.

Figure 5. Induction of apoptosis in intestinal epithelial cells by *Enterobacter sakazakii*. IEC-6 cells were grown to 90% confluence in 8-well chamber slides and incubated with *E. sakazakii* for varying periods. Cells were washed, fixed, treated with TUNEL red reagent, and viewed under a fluorescence microscope (A). In separate experiments, infected cells were washed, released from plates with medium containing ethylene glycol tetraacetic acid/1% bovine serum albumin, and stained using the TUNEL red kit, and apoptotic cells were counted (B) (*P < .001). In addition, cryosections of intestines from rat pups in the FF+H (formula feeding and hypoxia exposure) group (left) or the FF+H+ES (formula feeding, hypoxia exposure, and *E. sakazakii*) group (right) were stained with 4',6-diamidino-2-phenylindole and ApopTag stain (C).

Figure 6. Expression of interleukin (IL)–6 in IEC-6 cells and in rat pups infected with *Enterobacter sakazakii*. Confluent monolayers of IEC-6 cells were infected with *E. sakazakii* for varying periods, and the cytokines present in the supernatants were determined by Luminex assay (A). Results are representative of 3 independent experiments performed in triplicate (*P < .05). Total RNA was extracted from the cells, and reverse-transcription polymerase chain reaction was performed for 30 cycles on normalized cDNA, using IL-6–specific primers (B). RPS-17 primers were used as internal controls. In addition, serum samples were collected on day 4 from rat pups subjected to various treatments, and IL-6 production was determined by Luminex assay (C). The production of IL-6 in rat pups in the FF+ES+H (formula feeding, *E. sakazakii*, and hypoxia exposure) group was significantly greater than in controls. FF+H, formula feeding and hypoxia exposure. *P < .05 and **P < .001.
DISCUSSION

Although bacterial colonization is broadly regarded as a key step in the pathogenesis of NEC, there is a paucity of information regarding the roles of individual bacterial species. Several reports have suggested that NEC may be associated with *E. sakazakii*, a common contaminant of infant formula [1, 17, 18]. Here, we have demonstrated for the first time that *E. sakazakii* can induce the clinical and morphological changes of NEC in a rat pup model. Infection with *E. sakazakii* was confirmed in all surviving rat pups, as evidenced by the presence of GFP-labeled *E. sakazakii* within the intestine and in cultures of the intestinal contents of pups fed *E. sakazakii* (data not shown). The enhanced pathogenesis caused by *E. sakazakii* in the compromised intestine has particular relevance to newborn infants in whom NEC develops. Premature infants exhibit a relative intestinal immune deficiency, including decreased production of local antibacterial products [19], decreased levels of immunoglobulin (such as IgA) [20], and altered immune cell production [21], all of which may contribute to a compromised intestinal epithelial layer. In addition, a subgroup of newborn infants at increased risk for NEC have underlying congenital defects, such as pulmonary or cardiac anomalies [22]. These defects may result in global hypoxia and thus lead to intestinal hypoxia. Intestinal epithelial hypoxia has been shown elsewhere to diminish epithelial barrier integrity, increase intestinal permeability, and allow for increased bacterial translocation [23]. Therefore, in accordance with our animal findings, the effects of *E. sakazakii* may be especially enhanced in premature or hypoxic newborns.

A primary function of the intestine is to protect the internal environment from the external world, and regulation of enterocyte apoptosis is crucial to healthy maintenance of the gut barrier [24]. In fact, enterocytes are among the most rapidly proliferating cells in the body, and mitotic activity is matched by apoptosis, to maintain normal form and function. Dysregulated enterocyte apoptosis has been associated with several intestinal disorders [25]. Moreover, certain pathogens can increase epithelial apoptosis, thereby contributing to disease pathogenesis [26]. However, an imbalance between normal rates of apoptosis and restitution may reduce barrier integrity, allowing for bacterial translocation and the development of systemic sepsis. In agreement with this concept, our experiments demonstrate that *E. sakazakii* induces significant apoptosis in IEC-6 cells in vitro and in an animal model of NEC, particularly in combination with hypoxia. In contrast, nonpathogenic *E. coli* did not increase disease severity, indicating that some specific traits of *E. sakazakii* are responsible for the pathogenesis of NEC.

Although we did not specifically address the impact of enterocyte apoptosis on intestinal barrier integrity, other investigators have clearly documented a direct relationship between increased enterocyte apoptosis and the loss of barrier integrity [24]. Our SEM experiments revealed intestinal epithelial disarray, blebbing, and holes in the intestinal mucosa in FF+H+ES-treated rat pups, suggesting a necrotic pattern. Therefore, the induction of apoptosis in NEC by bacterial insult or by local inflammatory cytokines may progress to necrosis after prolonged infection, resulting in loss of intestinal barrier integrity. Furthermore, all rat pups subjected to FF+H+ES treatment developed bacteremia after 4 days, suggesting that intestinal translocation of bacteria occurred [27]. Some pathogens are able to enter the systemic circulation through direct invasion of the intestinal epithelium [28]. However, the absence of invasion in our in vitro experiments leads us to speculate that *E. sakazakii* induces intestinal damage by binding to the external surface of enterocytes and inducing apoptosis or necrosis. However, the precise mechanisms of the interaction between *E. sakazakii* and intestinal epithelial cells require further elucidation.

Pathogen-associated molecular pattern molecules, including lipopolysaccharide (LPS), lipoproteins, glycolipids, peptidoglycans, fatty acids, and nucleic acids of bacteria, often interact with various pattern-recognition receptors known as Toll-like receptors (TLRs) [29]. Different TLR family members have been identified and are expressed in a variety of cell types, including macrophages, dendritic cells, and enterocytes [30]. Because LPS is an outer membrane virulence factor of *E. sakazakii*, it is possible that *E. sakazakii* interacts with enterocytes through LPS-mediated binding to TLR4. Previous experiments have demonstrated that LPS facilitates bacterial attachment to Caco-2 cells and increases bacterial translocation [31]. Similarly, we have demonstrated previously that IEC-6 cells express TLR4 [32]. TLR expression may vary with intestinal location and postnatal maturation, thereby providing more opportunity for *E. sakazakii* tobind to certain portions of the intestine and alter the host’s response to commensal or pathogenic microorganisms. Indeed, in mouse models of colitis and NEC, TLR4 knockout mice have reduced inflammatory infiltrates, compared with that in wild-type mice [33]. Studies are in progress to identify the importance of TLRs in intestinal epithelial cell injury caused by *E. sakazakii*.

Alterations in cytokine production, including elevated levels of IL-6, have been associated with human NEC and may be useful clinical markers of disease severity [11, 34, 35]. Our experiments revealed that IL-6 expression and production were elevated in intestinal epithelial cell cultures after exposure to *E. sakazakii*. Increased levels of IL-6 were found in the serum of rat pups after *E. sakazakii* formula feeding; however, the role played by IL-6 in *E. sakazakii*–associated pathogenesis remains to be defined. Thus, the capability of *E. sakazakii* to trigger apoptosis and initiate a proinflammatory response may be fundamental to the pathogenesis of *E. sakazakii*–induced NEC.

In summary, oral administration of *E. sakazakii* increases epithelial injury in experimental NEC. Our findings support the notion that *E. sakazakii* might be a potential pathogen associated with human NEC. Furthermore, *E. sakazakii* likely elicits epithelial damage through enterocyte association without direct inva-
sion. *E. sakazakii* may cause barrier damage by inducing entero-
cyte apoptosis and stimulating expression of the inflammatory
cytokine IL-6. Further understanding of the role played by *E.
sakazakii* in the pathogenesis of NEC may provide clues for de-
veloping new therapeutic strategies against this disease.

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