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HOST RESPONSE AND INFLAMMATION

Enterotoxigenic *Escherichia coli* Enterotoxins Regulate Epithelial to Immune Relay of IL-33 and IL-1Ra Cytokines

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ABSTRACT Enterotoxigenic *Escherichia coli* (ETEC) remain a major cause of diarrheal mortality and morbidity in children in low-resource settings. Few studies have explored the consequences of simultaneous intoxication with heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) despite the increased prevalence of wild ETEC isolates expressing both toxins. We therefore used a combination of tissue culture and murine models to explore the impact of simultaneous ST + LT intoxication on epithelial and myeloid cells. We report that LT induces sustained production of interleukin 33 (IL-33) and interleukin 1 receptor antagonist (IL-1Ra) in T84 intestinal epithelial cells via cAMP production and protein kinase A activation. We demonstrate that combined ST + LT intoxication hastens epithelial transcriptional responses induced more slowly by LT alone. ST- and LT-mediated luminal fluid accumulation *in vivo* correlates with significant increases in IL-33 and IL-1Ra in small intestinal mucosal scrapings. Additionally, IL-33 receptor (IL-33R)-deficient mice are significantly less susceptible to ST-mediated secretion than wildtype mice. In the immune compartment, IL-33 is sensed by myeloid cells, and LT suppresses IL-33-induced tumor necrosis factor α (TNF-α) secretion from macrophages and bone marrow-derived dendritic cells (BMDCs) but amplifies IL-33-mediated induction of IL-6 from BMDCs. In conclusion, our studies suggest that enterotoxin-induced IL-33 and IL-1Ra modulate intestinal inflammation and IL-1 receptor signaling in the intestinal mucosa in response to ETEC enterotoxins.

KEYWORDS ETEC, Escherichia toxins, diarrhea, enteric pathogens, enterotoxins, epithelial cells, innate immunity, myeloid cells

Enterotoxigenic *Escherichia coli* (ETEC) are prominent causes of childhood diarrhea in low-to-middle income countries (LMICs). While mortality rates due to diarrheal disease in LMICs are decreasing, rates of morbidity in the form of physical and cognitive stunting remain high (1, 2). Clinical observations have clearly established that children can be infected with ETEC multiple times before developing protective anti-ETEC antibodies. ETEC are transmitted via the fecal-oral route, bind to the intestinal epithelium using diverse colonization factors, and cause disease through action of the heat-stable enterotoxin (ST) and/or the heat-labile enterotoxin (LT) (3). ST and LT elicit a nonbloody, secretory diarrhea mediated by increased production of cyclic nucleotide second messengers, which leads to luminal ion accumulation followed by osmosis (3).

Although ST and LT are critical virulence factors that cause secretory diarrhea, there is a paucity of data on how these two toxins interact and regulate disease pathogenesis. Previous studies have shown that simultaneous exposure to ST and LT oppose some immunological effects induced following exposure to ST or LT alone (4). Both toxins converge on pathways modulated by intracellular cyclic nucleotide signaling. ST is sensed via the guanylate cyclase C (GC-C) receptor expressed on the apical enterocyte surface and simulates robust cGMP production and protein kinase G (PKG) activation (5, 6). LT is an AB_5 ADP-ribosylating toxin that enters epithelial and immune cells via the B subunit binding to cell-surface gangliosides before undergoing retrograde

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transport to the endoplasmic reticulum, where the A subunit is activated to LTA1 (7, 8). LTA1 then ADP-ribosylates adenylate cyclase, resulting in increased cAMP production and subsequent protein kinase A (PKA) activation (8). Both PKA and PKG phosphorylate the cystic fibrosis transmembrane receptor (CFTR) and inhibit sodium hydrogen exchanger 3 (NHE3) channels to increase the accumulation of luminal ions, causing ETEC-characteristic secretory diarrhea (9). It is hypothesized that simultaneous exposure to ST and LT induces responses that are regulated by cAMP-, cGMP-, and/or dual-specific phosphodiesterases that catabolize cyclic nucleotides. Recent studies have also demonstrated that enterotoxin-induced cyclic nucleotides can be secreted into the extracellular milieu (10, 11) and that cyclic nucleotide catabolism by basolateral cell-surface enzymes function to suppress proinflammatory responses at barrier surfaces via adenosine signaling (12).

We previously demonstrated that ST intoxication of intestinal epithelial cells induces the rapid and transient expression of the epithelial alarmin interleukin 33 (IL-33) and transcripts of the gene encoding the IL-1 receptor antagonist, IL-1Ra (10). IL-33, an IL-1 family member, is a pleiotropic cytokine that has emerged as an activator of type 2 immunity to promote tissue protection, remodeling, and repair in response to helminth infections; however, it is now appreciated that IL-33 is also proinflammatory and regulates hematopoietic and nonhematopoietic cell functions, particularly at mucosal surfaces (13–15). Enterotoxin-mediated induction of IL-33 suggests that it may be a driver of immunomodulation or secretory responses, as recent studies have shown that IL-33 induces degranulation of enterochromaffin cells and serotonin release to hasten intestinal peristalsis (16). Moreover, IL-33 is required for intestinal IgA production (17), consistent with our previous studies showing that ST suppresses the development of fecal anti-ETEC IgA following ETEC immunizations without affecting peripheral anti-ETEC IgG responses (10).

Aside from enterotoxicity, LT increases the immunogenicity of co-delivered antigens, a feature referred to as adjuvanticity (8). Detoxified LT-based adjuvants, including double-mutant LT (dmLT, R192G/L211A), have been engineered to ablate LT-associated enterotoxicity while maintaining LT-associated adjuvanticity (8). dmLT boosts protective humoral immune responses to ETEC antigens in adults, children, and 6-month-old infants (18). Importantly, following parenteral immunization with dmLT-containing formulations, there is significant mucosal homing of antigen-specific T cells via enhanced $\alpha 4\beta 7$ integrin production (19). Recent studies have also shown that increased intracellular cAMP via forskolin or adenosine receptor stimulation can lead to immunosuppressive phenotypes in macrophages (20). Thus, we sought to understand the interplay of ST and LT enterotoxicity on epithelial and immune responses at the intestinal mucosal epithelium.

Both ST and LT induce secretion of proinflammatory IL-8 from human colonic epithelial cells, which may increase the permeability of the intestinal barrier at the site of infection (21). Previous studies have shown that simultaneous exposure to ST + LT results in suppression of LT-induced IL-6 and TNF-α production in murine ceca (4). LT and elevated cAMP enhance IL-6, TNF-α, MIP-1α, MIP-1β, and IL-1β production in THP-1 cells and primary human monocytes (22), and IL-1β increases intestinal permeability in human colonic epithelial cells (23). In the immune compartment, LT intoxication increases expression of the antigen-presentation markers CD80, CD86, and MHCII on dendritic cells (DCs) stimulated with lipopolysaccharide (LPS) (8). Moreover, LT promotes protective Th17 responses via innate IL-1 and IL-23 production, dependent on IL-1R1 (24).

Unlike other highly inflammatory enteric infections caused by *Shigella* species and *Salmonella* species, ETEC infections are generally considered noninflammatory. However, mounting transcriptional and protein-based evidence has demonstrated increased levels of inflammatory mediators following ETEC infection as well (25, 26). Nonetheless, the role of ETEC enterotoxins in inflammation is still unclear, particularly as ETEC infection in young children may not lead to lasting immunity (26, 27). To our knowledge, it is unclear how much ST and/or LT is made during an active ETEC infection or whether both toxins are made and delivered simultaneously. Since many of the most diarrheagenic ETEC strains

produce both ST and LT, we examined how LT and simultaneous ST + LT intoxication affects epithelial IL-33 and IL-1Ra production and subsequent epithelial and immune cell responses to these enterotoxin-induced mucosal cytokines.

RESULTS

Summary. Our work demonstrates that LT induces IL-33 and IL-1Ra production in T84 intestinal epithelial cells, a process that requires catalytic activity and the ability of LT to bind to intestinal epithelial cells. Moreover, we show that IL-1Ra protects intestinal epithelial cells from IL-1β-mediated production of IL-8, suggesting that ETEC enterotoxins alter IL-1 family signaling at the mucosal interface. We also show that simultaneous exposure to ST and LT hastens LT-mediated transcriptional changes in the epithelium. We use the patent mouse model to show that IL-33 and IL-1Ra recovery coincides with intestinal fluid accumulation following ST or LT intoxication. Moreover, pretreatment of mice with the cGMP-elevating phosphodiesterase inhibitor vardenafil before ST gavage results in heightened levels of recoverable IL-33. Additionally, IL-33R knockout mice are less susceptible to ST-mediated secretion compared to wild-type animals. In the immune compartment, we show that IL-33- and LPS-mediated production of TNF-α can be suppressed by LT and other cAMP-inducing agents in RAW 264.7 macrophages and bone marrow-derived dendritic cells (BMDCs) and that LT enhances IL-33-mediated activation of RAW 264.7 macrophages. LT also enhances IL-33- and/or LPS-mediated IL-6 production in BMDCs. In summary, these data suggest that IL-33 can drive immunosuppressive adaptive responses depending on the enterotoxin profile of the infecting ETEC isolate. These data provide a rationale for targeting IL-33 and IL-1 receptor signaling as an ETEC therapeutic.

LT induces IL-33 in epithelial cells. Previous studies have shown that ST is associated with sudden onset of luminal secretion, while LT intoxication is associated with a slower onset of luminal secretion but sustained over a longer time (4). However, the importance of the different toxin kinetics on the development of epithelial and immune responses has not been widely explored. We have previously shown that ST and the Food and Drug Administration (FDA)-approved ST mimic, linaclotide, rapidly induce *IL 33* gene expression and IL-33 protein production in human and murine intestinal epithelial cells in a cGMP-dependent manner (10). Many of the same pathways induced by elevated cGMP levels can also be induced by elevated cAMP levels, and previous studies have shown that dual-specificity phosphodiesterases link cGMP and cAMP metabolism (28). Here, we used quantitative PCR (qPCR) to show that LT and dmLT, despite detoxification, induce epithelial *IL 33* transcript expression following 24-h intoxication of T84 intestinal epithelial cells (Fig. 1A). Unlike ST, neither LT nor dmLT induce *IL 33* transcript expression following 3-h intoxication (10). Our studies support *IL 33* induction via non-Toll-like receptor (TLR) agonists as previously described (29). Next, we confirmed that neither LT nor dmLT induce IL-33 production in T84 cell lysates following 3-h intoxication (Fig. 1B) but that both LT and dmLT induce IL-33 in T84 cell lysates following 24-h intoxication (Fig. 1C).

We previously demonstrated that elevated levels of cGMP drive epithelial IL-33 production in T84 cells following ST intoxication (10). Previous studies have demonstrated that dmLT maintains the ability to induce some intracellular cAMP in target cells, albeit at a reduced level compared to native LT (30), which could explain dmLT-mediated induction of IL-33. To support this prediction, we next tested whether IL-33 production was linked to LT-mediated induction of cAMP. The LT holotoxin is an AB_5 toxin, consisting of an enzymatically active A subunit (LTA) and a binding B pentamer (LTB) (3). We show that neither the LTA nor LTB subunits alone induce significant IL-33 alone, and LT-mediated production of IL-33 is dependent on protein kinase A (PKA) activity, since addition of PKA inhibitor H89 abolishes IL-33 induction (Fig. 1C). Moreover, we show that the cAMP-elevating agent forskolin (FSK), which increases production of cAMP via adenylate cyclase, and calcium ionophore A23187 induce IL-33 expression in T84 cells, but the catalytically inactive LT mutant LT-E112K, which fails to induce cAMP, does not induce IL-33 (31) (Fig. S1). These data show that cAMP and calcium signaling, which can converge on PKA activation (32), can

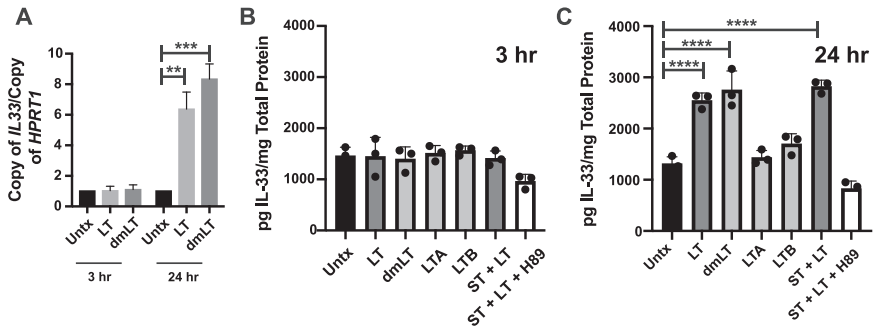

FIG 1 Heat-labile enterotoxin (LT) intoxication induces epithelial interleukin 33 (IL-33). T84 intestinal epithelial cells were treated with 1 μg LT or the indicated LT construct. A total of 1 μg heat-stable enterotoxin (ST) and 50 μM H89 were used where indicated. Following intoxication, RNA was isolated, or lysates were collected for IL-33 quantification via enzyme-linked immunosorbent assay (ELISA). (A) Use of 1 μg LT and 1 μg double-mutant LT (dmLT) induces significant upregulation of *IL 33* following 24-h intoxication relative to untreated (Untx) cells. (B, C) Use of 1 μg LT and 1 μg dmLT does not induce significant protein expression of IL-33 in cell lysates after 3-h intoxication (B) but does induce IL-33 protein expression in cell lysates after 24-h intoxication (C). The plotted data are representative of at least three independent experiments and are presented as means $\pm$ SD. All of the data were analyzed via one-way analysis of variance (ANOVA). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Untx is for untreated controls.

independently mediate the expression of IL-33. In the case of LT-mediated induction of IL-33, GM1 binding, ADP-ribosylating activity, and PKA activation are required.

LT induces IL-1Ra in epithelial cells. Previous studies have demonstrated that LT-mediated induction of IL-1β and IL-1α in DCs is important for promoting Th17 immunity (24). Moreover, inhibition of inflammasome activity via caspase-1 inhibitor or addition of IL-1Ra blocks IL-17 production in dmLT-activated human peripheral blood mononuclear cells (PBMCs) (33). IL-1Ra is capable of sequestering soluble IL-1β and limiting otherwise proinflammatory responses (34). Our previously published RNA-sequencing data (10) shows that ST induces the transcription of *IL1RN* (the gene encoding IL-1Ra) in T84 cells, suggesting that IL-33 and IL-1Ra can be induced following enterotoxin exposure and that enterotoxins may have the potential to reroute IL-1 family cytokine signaling (10). We use qPCR here to show that LT and dmLT induce robust *IL1RN* expression in T84 intestinal epithelial cells following 24-h intoxication (Fig. 2A). Neither LT nor dmLT induce IL-1Ra production in T84 cell lysates following 3-h intoxication (Fig. 2B), but both LT and dmLT induce significant IL-1Ra production following 24-h intoxication (Fig. 2C). Next, we show that neither LTA nor LTB alone induce significant IL-1Ra (Fig. 2C), and IL-1Ra production is dependent on PKA activity since cells treated with the PKA inhibitor H89 do not produce significant levels of IL-1Ra following LT intoxication. Moreover, catalytically inactive LT mutant LT-E112K is unable to induce IL-1Ra, while FSK induces IL-1Ra in T84 cells (Fig. S2), suggesting that cAMP mediates the induction of epithelial IL-1Ra by LT and dmLT. Previous studies have shown that Caco-2 cells respond to IL-1β by producing IL-8, whereas T84 cells do not (23). To demonstrate the functionality of IL-1Ra in an epithelial system, we show that basolaterally applied IL-1Ra suppresses the ability of IL-1β to induce IL-8 secretion from Caco2 cells (Fig. 2D). These data suggest that epithelial IL-1Ra production could reroute IL-1β signaling from the immune compartment following exposure to ETEC.

Combined ST and LT intoxication alters gene expression in T84 cells. ETEC can produce LT alone or in combination with ST, so the outcomes of simultaneous exposure to ST and LT remain an important area of investigation. We have previously shown that ST intoxication of T84 cells modulates expression of epithelial genes in Gene Ontology (GO) pathways related to (i) regulation of intracellular signal transduction, (ii) cytoskeletal organization, (iii) oxidation reduction processes, and (iv) immune effector responses (10). High cGMP concentrations (such as those induced by ST) can cross-activate PKA, making it extremely difficult to delineate ST-specific changes in gene response (28).

Here we isolated RNA from T84 intestinal epithelial cells intoxicated with LT or ST + LT for 3- and 24-h and compared the expression of target genes at both time points. The

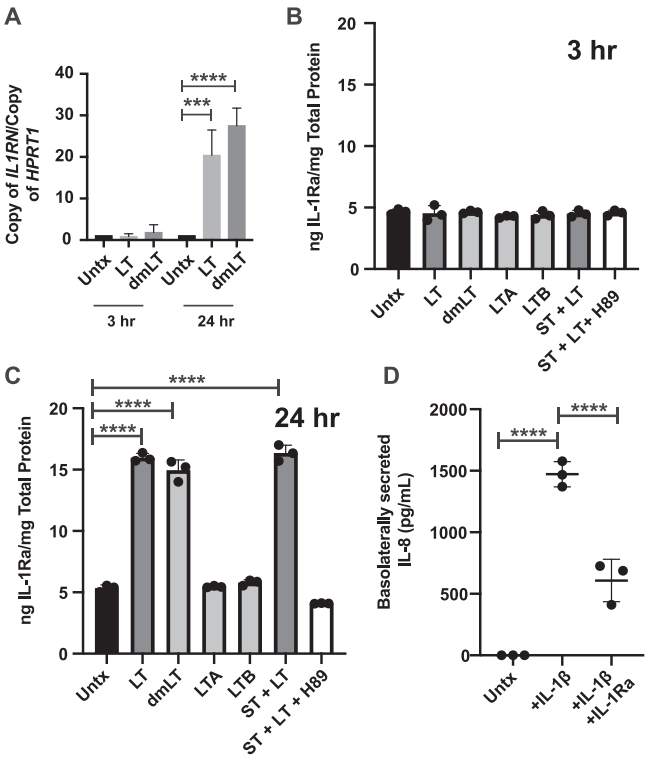

FIG 2 LT intoxication induces epithelial IL-1Ra. T84 intestinal epithelial cells were treated with 1 μg LT or the indicated LT construct. 1 μg ST and 50 μM H89 were used where indicated. Following intoxication, RNA was isolated, or lysates were collected for IL-1Ra quantification via ELISA. (A) Use of 1 μg LT and 1 μg dmLT induces expression of *IL1RN* after 24-h intoxication. (B, C) Use of 1 μg LT and 1 μg dmLT does not induce significant protein expression of IL-1Ra in cell lysates after 3-h intoxication (B) but does induce IL-1Ra protein expression in cell lysates after 24-h intoxication (C). Caco2 cells were plated on 24-well plate Transwell inserts and grown to confluence. IL-1β was added basolaterally following a 30-min preincubation with 200 ng rIL-1Ra where indicated. Supernatants were collected 18 h post-treatment. (D) Preincubation with IL-1Ra reduces the amount of secreted epithelial IL-8 induced by IL-1β. The plotted data are representative of at least three independent experiments and are presented as means $\pm$ SD. All of the data were analyzed via one-way ANOVA. ***, $P < 0.001$; ****, $P < 0.0001$. Untx is for untreated controls.

panel of genes probed includes some of those previously reported to be modulated by ST alone, including laminin A3 (*LAMA3*), serpin E1 (*SERPINE1*), matrix metallopeptidase 1 (*MMP1*), matrix metallopeptidase 10 (*MMP10*), nitric oxide synthase 2 (*NOS2*), interleukin 8 (*CXCL8*), superoxide dismutase 2 (*SOD2*), dehydrogenase/reductase 9 (*DHRS9*), and early growth response 2 (*EGR2*).

Following 3-h intoxication, LT induces the expression of *CXCL8* 50-fold and *EGR2* 32-fold (Fig. 3A). We have previously shown that 3-h ST intoxication induces *CXCL8* 30-fold in T84 cells (35). Interestingly, we show that 3-h simultaneous ST + LT intoxication results in only 1.9-fold induction of *CXCL8*, suggesting simultaneous exposure to both ST and LT suppresses *CXCL8* production. On the other hand, *EGR2*, a gene induced by cAMP, is more robustly induced following 3-h simultaneous ST + LT intoxication (88.7-fold) compared to 3-h LT intoxication (31.8-fold) (Fig. 3A). *EGR2* encodes early growth response 2 protein, which has been shown to be important for immune cell development, and other *EGR* genes are induced following increases in cAMP and GMP (36).

Most genes previously shown to be induced by 3-h ST intoxication, including *SERPINE1*, *MMP10*, *CXCL8*, *MMP1*, *DHRS9*, *CYP3A5*, *LAMA3*, *SLC7A11*, *SOD2*, *NOS2*, and *SLC11A2*, are not affected by 3-h LT intoxication (Fig. 3A). On the other hand, many of the same transcriptional changes seen following 3-h ST intoxication can also be seen following 24-h LT intoxication (Fig. 3B). These data suggest that LT intoxication may promote acute (3-h) and sustained (24-h) transcriptional responses. Typically, 24-h simultaneous ST + LT intoxication (Fig. 3B) results in more robust changes compared to

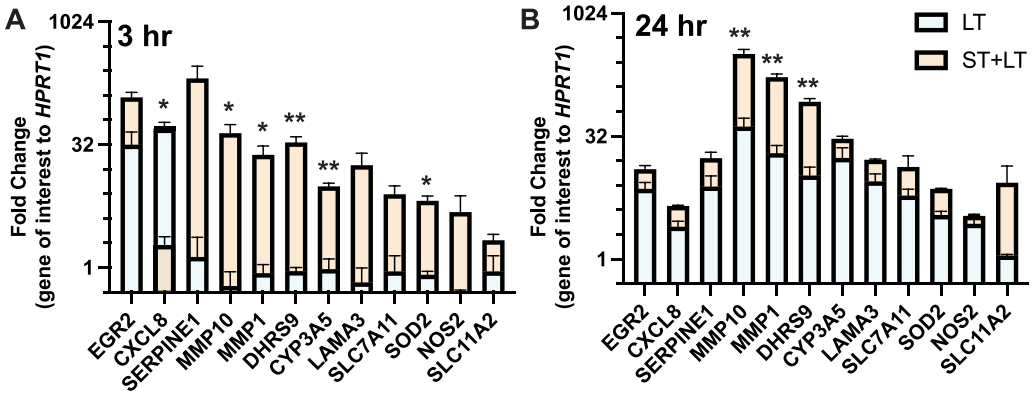

FIG 3 ST hastens LT-induced gene expression in epithelial cells. T84 intestinal epithelial cells were intoxicated with 1 μg LT and/or 1 μg ST for 3 h (A) or 24 h (B). RNA was isolated and reverse-transcribed to cDNA for quantitative PCR (qPCR) analysis. The genes were normalized to the housekeeping gene *HPRT1* and are presented as the means of three replicates ± SEM. *, $P < 0.05$ and **, $P < 0.01$ represent *t* test analysis for gene expression following LT intoxication compared to ST + LT intoxication following 3 or 24 h.

LT alone. Of note, 24-h LT intoxication upregulates *DHRS9* (for dehydrogenase/reductase 9) 8-fold, while 24-h ST + LT intoxication upregulates *DHRS9* 73-fold (Fig. 3B). Additionally, the genes encoding matrix metallopeptidase proteins (MMPs) (*MMP1* and *MMP10*) are more highly induced following 24-h ST + LT intoxication compared to 24-h LT intoxication. LT intoxication upregulates *MMP10* 41-fold, while ST + LT intoxication upregulates *MMP10* 280-fold following 24-h intoxication. Similarly, LT intoxication upregulates *MMP1* 19-fold, while ST + LT intoxication upregulates *MMP1* 148-fold following 24-h intoxication (Fig. 3B). In general, however, most transcriptional changes following 3-h intoxication appear reliant on ST activity and, following 24-h intoxication, depend on LT activity. The 24-h LT-induced transcriptional changes mirror those seen following 3-h ST intoxication. Together, these data suggest that ST hastens the epithelial transcriptional change that would ensue with LT. Moreover, simultaneous 24-h ST + LT intoxication amplifies the transcriptional response induced by LT alone.

***In vivo* enterotoxin-induced secretion correlates with increased mucosal IL-33 and IL-1Ra.** The patent mouse model has been used to provide insights into the secretory mechanisms of enterotoxins in a complex mammalian host. Secretory responses from ST begin rapidly following oral gavage in outbred mice (4), likely attributed to GC-C receptor expression on intestinal epithelial cells and rapid intracellular signal transduction (3, 5). On the other hand, secretory responses from LT peak 3 h following oral gavage (4), likely due to the sequence of events required for LT internalization, processing, and activation (8). Here, we compare ST-, LT-, and ST + LT-mediated secretion with tissue IL-33 and IL-1Ra levels in mice following both 30 and 180 min of intoxication. Following 30-min intoxication, only animals gavaged with ST (with or without LT) demonstrate a significant increase in gut-to-carcass ratios (Fig. 4A, left panel), compared to saline-gavaged animals. Moreover, there is no significant difference in the gut-to-carcass ratio between animals intoxicated with ST and those intoxicated with ST + LT (Fig. 4A, left panel). Following 30 min of intoxication, we found significantly elevated levels of IL-33 in the mucosal scrapings of ST- and ST + LT-treated animals, suggesting that IL-33 recovery following 30 min of intoxication is due to the rapid effects of ST (Fig. 4B, left panel). Following 30 min of intoxication, mice gavaged with ST or ST + LT also had increased levels of IL-1Ra detectable in small intestinal mucosal lysates (Fig. 4C, left panel). We have previously shown that ST intoxication results in significant recovery of IL-33 from mucosal scrapings (10), and we can increase ST-mediated induction of IL-33 in mucosal scrapings by pretreating the animals with an IP injection of the cGMP-elevating phosphodiesterase inhibitor vardenafil before ST gavage (Fig. S3).

Following 3-h intoxication, only animals that were gavaged with LT (with or without ST) demonstrated a significant increase in gut-to-carcass ratios (Fig. 4A, right panel) compared to saline-gavaged animals. Moreover, there is no significant difference in the gut-to-carcass

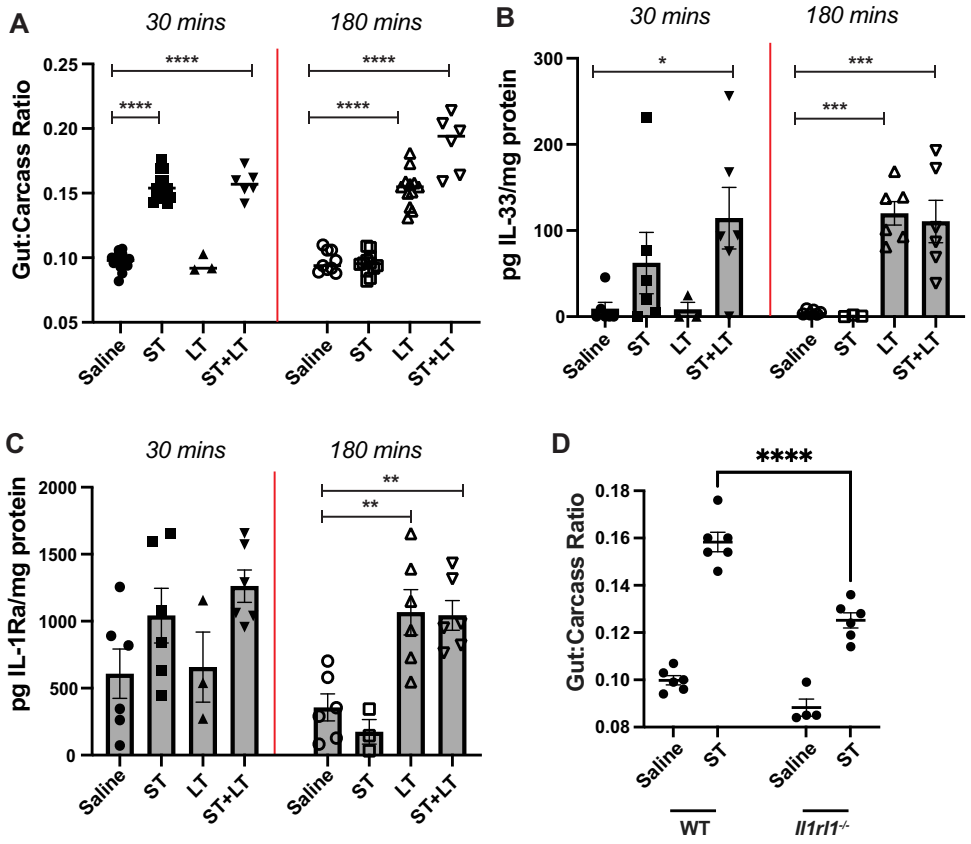

FIG 4 ST- and LT-mediated enterotoxicity coincide with waves of transient mucosal IL-33 and IL-1Ra. BALB/c female mice (6 weeks old) were fasted overnight and orally gavaged with ST (25 μg), LT (125 μg), or both for 30 or 180 min. At the experiment endpoint, the gut-to-carcass ratio was measured for each animal. (A) ST and ST + LT induce significant luminal secretion after 30-min intoxication (left panel). LT and ST + LT induce significant luminal secretion after 180-min intoxication (right panel). (B) IL-33 levels in small intestinal mucosal scrapings were measured via ELISA. ST and ST + LT induce IL-33 in lysates after 30-min intoxication (left panel), while LT and ST + LT induce IL-33 in lysates after 180-min intoxication (right panel). The same mucosal scrapings were used to measure IL-1Ra via ELISA. (C) ST and ST + LT induce IL-1Ra in lysates after 30-min intoxication (left panel), while LT and ST + LT induce IL-1Ra in lysates after 180-min intoxication (right panel). Female Il1rl1 knockout C57BL/6J mice (6 to 8 weeks old) were fasted overnight and orally gavaged with ST (25 μg) for 30 min. At the experiment endpoint, the gut-to-carcass ratio was measured for each animal. Knockout mice show a decreased gut-to-carcass ratio following ST gavage compared to wild-type (WT) BALB/c animals following ST gavage. The data represent pooled results of at least three independent experiments and are presented as means ± SEM. All data in panels A to C were analyzed via one-way ANOVA. (D) The data were analyzed via *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; **** $P < 0.0001$.

ratio between animals intoxicated with LT and those intoxicated with ST + LT (Fig. 4A, right panel). Following 3 h of intoxication, we found significantly elevated levels of IL-33 in the mucosal scrapings of LT- and ST + LT-treated animals, suggesting that IL-33 recovered after 3 h of intoxication is due to the more prolonged action of LT (Fig. 4B, right panel). Following 3 h of intoxication, we also found significantly elevated levels of IL-1Ra in the mucosal scrapings of LT- and ST + LT-treated animals (Fig. 4C, right panel). We did not find increased levels of IL-1Ra in luminal secretions (data not shown), but we found increased levels of IL-1β in mucosal scrapings from LT-gavaged animals following 3-h intoxication (Fig. S5). Compared to saline-gavaged animals, neither ST nor LT induce cell lysis in the patent mouse assays (PMAs), as measured by lactate dehydrogenase (LDH) in mucosal secretions (Fig. S4). Next, we show that IL-33 receptor (IL-33R)-deficient animals are significantly less susceptible to ST-mediated fluid accumulation than wild-type animals in the patent mouse model (Fig. 4D).

ETEC enterotoxins modulate IL-33 signaling to macrophages. Innate immune cells respond to signals from both host and pathogen. Previous studies have shown

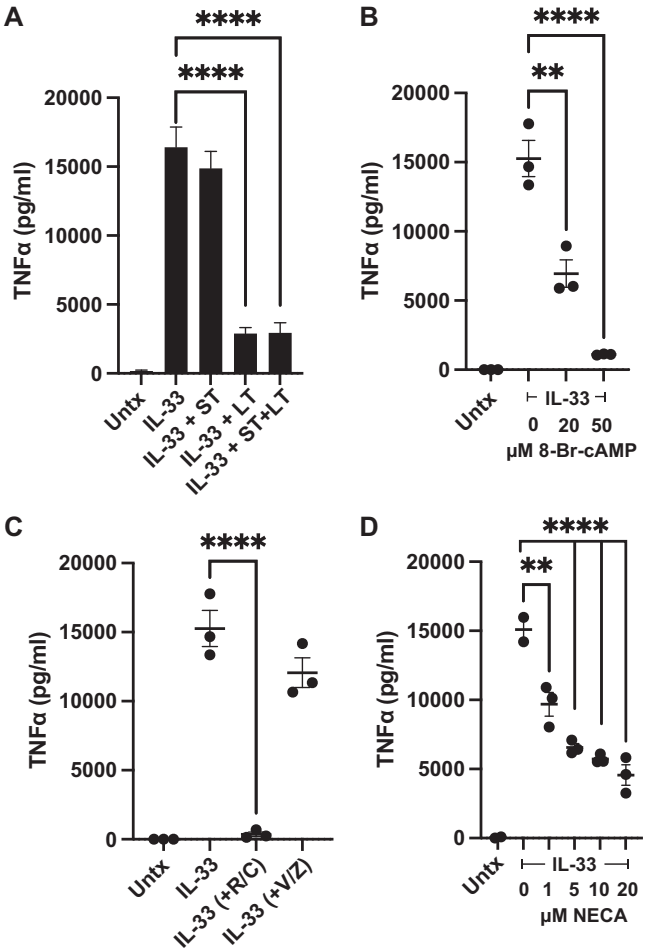

FIG 5 LT suppresses IL-33-induced TNF-α in RAW 264.7 murine macrophages via cAMP signaling. RAW 264.7 macrophages were treated with 50 ng IL-33, 1 μg ST, and/or LT, or the indicated LT-based metabolite for 24 h. Supernatants were assessed for tumor necrosis factor α (TNF-α) via ELISA. (A) IL-33-induced TNF-α secretion is inhibited in the presence of LT. (B) The cell-permeable cAMP analog 8-Br-cAMP suppresses IL-33-induced secreted TNF-α in a concentration-dependent manner. (C) cAMP-elevating PDEis (rolipram and cilostazol [R/C]) but not cGMP-elevating phosphodiesterase inhibitors (PDEis) (vardenafil and zardaverine [V/Z]) suppress IL-33-induced TNF-α secretion. (D) The cell-impermeable adenosine analog 5′-*N*-ethylcarboxyamide adenosine (NECA) suppresses IL-33-induced secreted TNF-α in a concentration-dependent manner. The plotted data are representative of at least three independent experiments and are presented as means $\pm$ SEM. All of the data were analyzed via one-way ANOVA. **, $P < 0.01$; **** $P < 0.0001$. Untx is for untreated controls.

that IL-33 induces TNF-α production from myeloid cells, including macrophages and bone marrow-derived dendritic cells (BMDCs) (37). Published research has also shown that LT polarizes myeloid responses to LPS; LT suppresses LPS-induced TNF-α and exacerbates LPS-induced IL-6 in immune cells (38, 39). We therefore examined the impact of LT and ST on IL-33 signaling to myeloid cells that might be present in the small intestinal lamina propria during active ETEC infection.

We first used RAW 264.7 macrophages to quantify outcomes of simultaneous exposure to IL-33 (derived from the host) and enterotoxin(s) (derived from the pathogen). Our data show that LT, but not ST, suppresses IL-33-mediated TNF-α production in RAW 264.7 macrophages (Fig. 5A), which implicates increased intracellular cAMP levels as causal. As further support, we demonstrate that IL-33-mediated induction of TNF-α can be suppressed via the cell-permeable cAMP analog 8-Br-cAMP (Fig. 5B), as well as the cell-impermeable adenosine analog 5′-*N*-ethylcarboxyamide adenosine (NECA) (Fig. 5D). Moreover, we demonstrate that the cAMP-elevating phosphodiesterase

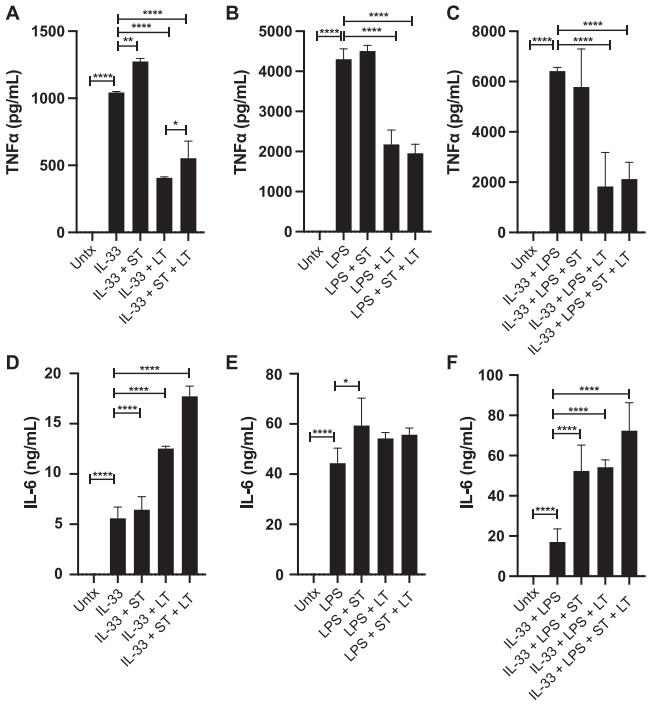

FIG 6 LT suppresses IL-33- and LPS-induced TNF-α and enhances IL-33- and LPS-induced IL-6 in murine bone marrow-derived dendritic cells (BMDCs). Mature BMDCs were treated with IL-33 (20 ng) and/or lipopolysaccharide (LPS) (50 ng) in the presence or absence of ST and/or LT (1 μg each) for 24 h. Supernatants were collected and assayed for TNF-α and IL-6 via ELISA. The presence of LT suppresses IL-33- and LPS-induced TNF-α (A to C) while enhancing IL-33-induced IL-6 (D to F). The plotted data are representative of at least three independent experiments and are presented as means $\pm$ SD. The data were analyzed via one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Untx is for untreated controls.

inhibitor combination of rolipram or cilostazol (R/C) suppresses IL-33-mediated induction of TNF-α, but the cGMP-elevating phosphodiesterase inhibitor combination of zardaverine and vardenafil (V/Z) (10) does not significantly affect IL-33-mediated induction of TNF-α (Fig. 5C). Taken together, these data suggest that the combined activity of LT and IL-33 suppresses TNF-α production from macrophages, similar to the effects of LT on endotoxin-induced TNF-α production. We also show that IL-33 induces expression of CD80, CD40, and MHCII on RAW 264.7 macrophages, which are all enhanced by LT but not by NECA (Fig. S6).

Next, we examined the effects of ST and LT intoxication on IL-33-mediated TNF-α secretion in bone marrow-derived macrophages (BMMs). As expected, BMMs do not induce TNF-α following 24-h stimulation with IL-33 (data not shown) and induce low levels of TNF-α only following 72-h IL-33 stimulation (Fig. S7). The low levels of IL-33-induced TNF-α are consistent with published studies describing low IL-33R expression on BMMs (40). However, more recent studies have demonstrated that BMMs indeed respond to IL-33 by shifting central metabolism to induce immunosuppressive pathways via unique transcriptional programming (41, 42). Our studies show that LT and NECA suppress IL-33-induced TNF-α in BMMs via increased intracellular cAMP, the impact of which remains to be explored in future studies.

ETEC enterotoxins polarize dendritic cell responses to IL-33. Next, we compared the ability of BMDCs to respond to host IL-33 and pathogen LPS in the presence of ETEC enterotoxins. First, we show that BMDCs stimulated with IL-33, LPS, and IL-33 + LPS produce TNF-α following 24-h treatment (Fig. 6A to C).

Moreover, we demonstrate that IL-33 stimulation in the presence of ST significantly increases IL-33-mediated TNF-α production, but IL-33 stimulation in the presence of LT suppresses IL-33-mediated production of TNF-α (Fig. 6A). Simultaneous

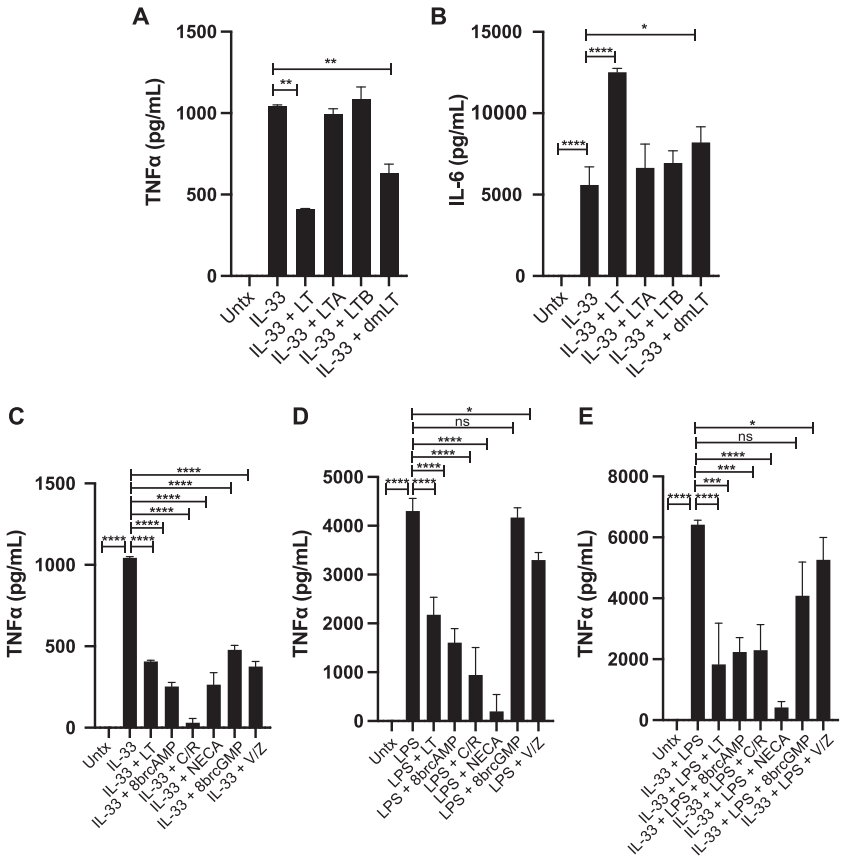

FIG 7 TNF-α suppression by LT requires intact holotoxin and cAMP signaling in BMDCs. Mature BMDCs were treated with IL-33 (20 ng) and/or LPS in the presence of LT, LTA, LTB, or dmLT (1 μg each), 8-Br-cAMP or 8-Br-cGMP (50 μM), rolipram and cilostazol (50 μM each), vardenafil (30 μM) and zardaverine (20 μM), or NECA (20 μM) for 24 h. (A, B) Supernatants following treatment with LT and LT subunits/mutants were assessed for TNF-α (A) and IL-6 (B). (C to E) Supernatants treated with PDEis, NECA, or 8-Br-cAMP/8-Br-cGMP in the presence of IL-33 with and without LPS were assessed for TNF-α. The plotted data are representative of at least three independent experiments and are presented as means $\pm$ SD. The data were analyzed via one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Untx is for untreated controls.

exposure to IL-33 + ST + LT increases TNF-α production compared to IL-33 + LT but remains suppressed compared to IL-33 alone (Fig. 6A). It has been shown that cAMP induced by FSK blocks IL-33-induced TNF-α production in DCs (43), so it is not surprising that we see the same effect with LT. On the other hand, ST does not affect LPS-induced TNF-α production in BMDCs, but LT maintains its ability to suppress LPS-induced TNF-α production. Of note, however, LPS is a more potent inducer of TNF-α than is IL-33 (Fig. 6B). Consistently, when BMDCs are stimulated with both IL-33 and LPS, ST does not affect TNF-α production, but LT suppresses TNF-α production (Fig. 6C).

On the other hand, both ST and LT significantly enhance IL-6 production following IL-33 stimulation, an effect that is further enhanced in the presence of both ST and LT (Fig. 6D). However, ST but not LT enhances LPS-mediated induction of IL-6 (Fig. 6E). When BMDCs are stimulated with both IL-33 + LPS, ST and LT independently enhance IL-6 production, but simultaneous ST + LT exposure induces more IL-6 than either toxin alone. Interestingly, costimulation with IL-33 + LPS reduces IL-6 production in BMDCs induced by LPS alone (Fig. 6F). Taken together, these data indicate that LT intoxication polarizes TNF-α and IL-6 production following IL-33 and LPS stimulation. Importantly, the direct effect of ST on IL-33-mediated induction of TNF-α is surprising since no one has described the presence of guanylate cyclase C (GC-C) on DCs (3). Surprisingly, we did not find any measurable IL-10 following IL-33 stimulation of BMDCs (44).

Last, we show that LT polarization of IL-33-induced TNF-α and IL-6 is dependent on functional AB5 protein assembly. Neither LTA nor LTB suppress IL-33-mediated induction of TNF-α, although dmLT retains the ability to suppress IL-33-mediated TNF-α production (Fig. 7A). Moreover, we show that neither LTA nor LTB can promote IL-33-mediated production of IL-6, although dmLT can significantly increase IL-6 production (Fig. 7B) in BMDCs. We next used small molecule mimics and phosphodiesterase inhibitors (PDEis), which prevent the breakdown of cyclic nucleotides, to determine the impact of altering perceived intracellular cAMP and cGMP levels. Interestingly, we show that IL-33-mediated induction of TNF-α can be suppressed by increasing intracellular cAMP levels via LT, cell-permeable 8-Br-cAMP, the PDEi combination of rolipram and cilostazol (C/R), and NECA (Fig. 7C). IL-33-mediated induction of TNF-α production can also be suppressed by increasing intracellular cGMP levels via cell-permeable 8-Br-cGMP, and the PDEi combination of zardaverine and vardenafil (V/Z) (Fig. 7C). Similarly, LPS-mediated induction of TNF-α production can be suppressed by increasing intracellular cAMP levels via LT, cell-permeable 8-Br-cAMP, and the PDEi combination of C/R. However, LPS-mediated induction of TNF-α production is not effectively suppressed by increasing intracellular cGMP levels via cell-permeable 8-Br-cGMP, or the PDEi combination of V/Z (Fig. 7D). Likewise, increased intracellular cAMP levels, but not increased intracellular cGMP levels, suppress combined IL-33 + LPS-induced TNF-α production (Fig. 7E). Together, these data suggest that cAMP and cGMP pathways may be used to discriminate between host alarmins and pathogen-derived motifs.

DISCUSSION

Despite the adjuvant effect of LT and data demonstrating that vaccine-induced anti-ETEC immunity is possible (18), young children in low-resource settings do not develop long-lasting anti-ETEC immunity following infection and are often subject to reinfection by the same or similar isolates. Our laboratory has shown that ST suppresses anti-ETEC fecal IgA titers when included in ETEC oral immunization formulations (10), supporting the idea that ETEC have evolved mechanisms to subvert host immunity despite carrying LT. Here, we provide further evidence that IL-1 family members IL-33 and IL-1Ra may be central in mediating immune responses to enterotoxins and discuss how signals from the host (IL-33) and pathogen (ST, LT, and LPS) are handled by immune cells.

IL-33 is a pleiotropic cytokine that binds IL-33R and induces type 2 innate lymphoid cell (ILC2) proliferation and immunity via tryptophan hydroxylase 1 in the lamina propria following helminth infection (45). IL-33 overexpression increases secretory cell proliferation and differentiation (46) in response to intestinal pathogens. However, emerging literature describes functions for IL-33 outside Th2 immunity. IL-33R-expressing enterochromaffin cells secrete serotonin in response to IL-33 to hasten gut transit for parasite clearance (16). IL-33 also expands regulatory T-cell (Treg) populations (46) and induces iron recycling in splenic macrophages via immunosuppressive citric acid cycle intermediates such as itaconate (41). Our previous studies demonstrate that ST binds zinc and iron (35). It is possible that ST and LT are sensed via similar mechanisms of iron depletion in surrounding intestinal tissue, which implicates IL-33 in the battle for mucosal metals during enteric infection.

In the present work, we demonstrate that 24-h intoxication with LT and dmLT induce IL-33 and IL-1Ra in T84 intestinal epithelial cells. We also show that IL-33 and IL-1Ra induction depends on cAMP production, as the LTA and LTB subunits or the LT mutant E112K fail to induce IL-33 or IL-1Ra production. Interestingly, IL-1 family cytokines, including IL-1β, IL-1α, and IL-33, have been implicated as drivers of mucosal immunity following enteric infection (16, 47, 48), and IL-1R1-dependent signaling coordinates epithelial regeneration in response to intestinal epithelial damage (49). IL-1Ra is a decoy protein that ablates IL-1 signaling (34) and may silence proinflammatory cytokine responses for pathogen immune subversion. For instance, epithelial-derived IL-1Ra increases rates of uropathogenic *E. coli* colonization of bladder cells (50). In support, we show that recombinant IL-1Ra can block IL-1β-induced production of IL-8 by

epithelial cells, thereby inducing an immunosuppressive phenotype. Immune cell-derived IL-1β is known to be induced by numerous mitogens, including LT (24). For *in vivo* differentiation, 80 to 90% of small intestinal lamina propria IL-17-producing cells require IL-1 signaling, but IL-33 and IL-1β exert opposing functions in the control of functional adaptation of regulatory T cells at mucosal surfaces. IL-1β drives Th17 differentiation, while IL-33 drives Th2 differentiation. IL-33 also maintains the suppressive function of Tregs (46), while the absence of IL-33R promotes acquisition of Th17 phenotypes (51).

ETEC has long been considered noninflammatory, but previous studies show that ETEC induces inflammatory responses in intestinal mucosa (25), and acute cholera infection induces innate defense inflammatory pathways in duodenal mucosa (52). When we examined host gene expression patterns in T84 intestinal epithelial cells based on toxin combination (LT versus ST + LT) and timing (3 h versus 24 h), we discovered that 24-h LT intoxication induces expression of genes important for pathways related to (i) regulation of intracellular signal transduction, (ii) cytoskeletal organization, (iii) oxidation reduction processes, and (iv) immune effector responses. However, very similar signatures arise after just 3 h if cells are intoxicated with ST + LT. Thus, combined exposure to ST and LT may alter the host response to ETEC or create an environment that promotes colonization. Overall, ST hastens transcriptional changes to a genetic program that LT would also induce following more sustained exposure.

T84 tissue culture cell experiments were carried out for 3 or 24 h to understand the effects following enterotoxin exposure on gene expression and functional protein responses, but previous studies show that intestinal secretory onset is much quicker following ST intoxication (30 min) and LT intoxication (3 h) *in vivo* (4). The secretory effects of enterotoxins may involve multiple tissue compartments and/or specialized cell types not reflected in homogenous T84 cell populations. We show that enterotoxin-mediated secretion directly correlates with increased IL-33 and IL-1Ra recovered from small intestinal mucosal scrapings. Since ST specifically activates cells expressing the GC-C receptor, the fast action of ST may forestall LT-mediated cellular reprogramming. Of note, we find that LT but not ST intoxication induces production of IL-1β in intestinal mucosa.

IL-33 induction may be driven by a combination of increased cyclic nucleotide signaling and mechanosensory stimuli, such as luminal swelling following fluid accumulation (46). Thus, we hypothesized that enterotoxin-mediated IL-33 found in murine mucosal scrapings was directly related to physical secretion into the intestinal lumen and suggests that IL-33 and IL-33R are drivers of the secretory response. In support, IL-33R knockout mice are significantly less susceptible to ST compared to wild-type mice. Together, these data suggest that the IL-33/IL-33R axis could be targeted for the development of novel therapeutics to lessen the severity and duration of secretory diarrheal diseases.

Because lamina propria immune cells are in close juxtaposition to the epithelium, we next examined how ETEC enterotoxins and, in our working model, epithelial-derived IL-33 impact inflammatory mediators produced by myeloid cells. Our presented data are in line with published works and show that IL-33 signaling in RAW 264.7 macrophages and BMDCs induces production of TNF-α and IL-6 (43, 53). However, when we stimulate BMDCs and RAW 264.7 macrophages with IL-33 in the presence of LT but not ST, TNF-α induction is suppressed, while IL-6 induction is enhanced. TNF-α upregulates IL-1 cytokines produced by monocytes (IL-1α, IL-1β, and IL-1Ra), so decreased levels support an immunosuppressive phenotype (54). However, IL-6 is a known stimulator of Th17 differentiation and an inhibitor of Treg differentiation (43), and IL-33 has been shown to increase the number of Tregs present in models of inflammation in both the lung and intestine (46, 55), so the consequence of induced IL-6 production remains unknown.

One additional point to discuss is that cAMP catabolism to adenosine has been described in myofibroblasts (56), and adenosine receptors differentially regulate type 2

cytokine production via IL-33-activated bone marrow cells and macrophages (57). Secreted cAMP may therefore be broken down into immunomodulatory adenosine, supporting the effects we see with the adenosine analog NECA. Moreover, IL-33 in the presence of LT appears to polarize macrophages and DCs to an M2d-like phenotype, defined as stimulation through adenosine receptors, via increased intracellular cAMP production. Previously published work shows that M2 polarization downstream of IL-33-induced ILC2 proliferation helps alleviate dextran sulfate sodium-induced colitis (58), but the short- and long-term global impact on myeloid cell programming remains an area of ongoing investigation. Alternatively, secreted cGMP could act as a suicide inhibitor for cell-surface phosphodiesterases, blocking cAMP turnover, and leaves open the possibility that intracellular cAMP is sufficient to modulate epithelial and immune responses.

Our data suggest that ST induces a more rapid inflammatory response than LT, which may benefit the bacterium. We hypothesize that the combined effect of host IL-33 and pathogen-derived toxins is an important factor in ETEC subversion of long-lasting immunity. The balance of IL-1 family signaling in response to ETEC enterotoxins remains an area of high research interest, especially as the extracellular transport of cyclic nucleotides following ST and LT intoxication (10, 11) appear to drive cytokine induction (12). Alternatively, our data suggest that host- and pathogen-specific responses could be relayed by cGMP and cAMP. This is supported by the fact that ST mimics the host endogenous peptides guanylin and uroguanylin, which signal via the GC-C receptor to induce intracellular cGMP for basal salt and water homeostasis. These data provide the framework for future studies to understand how enterotoxins modulate IL-1 family signaling for immune subversion. Perhaps further investigation will help us understand why children cannot mount effective anti-ETEC responses until after age five.

MATERIALS AND METHODS

Animals. Female BALB/c mice (6 to 8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). All of the procedures described herein were approved by the Institutional Care and Use Committee at Tulane University School of Medicine (protocol 768). IL-33R (also called ST2 and *il1rl1*$^{-/-}$) knockout animals were provided to us by Chad Steele (Tulane University, School of Medicine) under agreement from Andrew McKenzie at the Medical Research Council Laboratory of Molecular Biology in the United Kingdom (59).

Enterotoxins, cell lines, and chemicals. ST was purified from supernatants of recombinant strain 9115 as described previously (35). LTh, LTA, LTB, dmLT, and E112K were purified as previously published (30). T84 cells (CCL-248) and Caco2 cells (HTB-37) were purchased from ATCC. T84 cells and Caco2 cells were maintained and cultured on 24-well tissue culture-treated plates or on Transwell inserts as previously described (35). All experiments requiring T84 and Caco2 intestinal epithelial cells were conducted below passage 10 at approximately 75% confluence when grown on 24-well culture plates and when transepithelial resistance (TER) was greater than 1,000 $\Omega \cdot cm^2$ for T84 cells and greater than 500 $\Omega \cdot cm^2$ for Caco2 cells when grown on Transwell inserts. RAW 264.7 macrophages (ATCC TIB-71) were purchased from ATCC and cultured on 12- or 24-well tissue culture plates to 70 to 80% confluence for treatment. Phosphodiesterase inhibitors zardaverine (29685), vardenafil (9001800), cilostazol (15035), and rolipram (31111) were purchased from Cayman Chemicals. 8-Br-cAMP (18141), 8-Br-cGMP (15992), NECA (21420), and H89 (10010556) were purchased from Cayman Chemicals. FSK (F3917) and calcium ionophore A23187 (C7522) were purchased from Sigma. Recombinant IL-33 (3626-ML), IL-1Ra (480-RM), and IL-1β (201-LB) were purchased from R&D Systems. *Salmonella minnesota* R595 LPS was purchased from InvivoGen (tlrl-smlps).

RNA isolation and qPCR. T84 cells were grown to 80% confluence on 24-well tissue culture-treated plates. T84 cells were intoxicated with ST, LT, ST + LT, or dmLT for 3- or 24-h followed by RNA extraction with the Qiagen RNeasy kit. RNA isolated from untreated T84 monolayers at the indicated time point served as the control. RNA was quantified using a NanoDrop C, and 1.0 μg RNA was reverse-transcribed using iScript^{TM} cDNA Synthesis kit with and without reverse transcriptase (Bio-Rad Inc.). qPCR was carried out using PrimeTime qPCR probes from IDT, including HPRT1, Hs.PT.58v.45621572; IL-33, Hs.PT.58.21416460; IL1RN, Hs.PT.58.4381999; EGR2, Hs.PT.58.40402328; CXCL8, Hs.PT.58.39926886; SERPINE1, Hs.PT.58.3938488; MMP10, Hs.PT.58.38586852; MMP1, Hs.PT.58.38692586; DHRS9, Hs.PT.58.14588753; CYP3A5, Hs.PT.58.2827789; LAMA3, Hs.PT.58.836388; SLC7A11, Hs.PT.58.20878688; SOD2, Hs.PT.58.25533008; NOS2, Hs.PT.58.14740388; and SLC11A2, Hs.PT.58.23097204. 5 ng input cDNA was used for each qPCR. The reactions were carried out with PrimeTime gene expression master mix (IDT) on a CFX Connect (Bio-Rad). We did not see genomic DNA contamination of our RNA samples as tested by performing qPCRs on RNA samples without reverse transcriptase. Transcript levels were calibrated to housekeeping gene HPRT1. Changes in mRNA expression were determined using the comparative C_T method.

Patent mouse model. The adult patent mouse assays (PMAs) were carried out as previously described (10). In brief, 6-week-old BALB/c females were fasted overnight, allowed water *ad libitum*, and orally gavaged the following morning with saline, ST (25 μg), LT (125 μg), or both ST and LT. The mice were sacrificed via CO_2 asphyxiation and cervical dislocation 30 min or 3 h postgavage, as previously described (4). The gut-to-carcass ratio was measured, and luminal secretions and mucosal scrapings were collected. Mucosal scrapings were lysed using 1× cell lysis buffer from R&D containing protease inhibitor (Roche, 11836153001).

Isolation and differentiation of bone marrow progenitor cells. Bone marrow progenitor cells were isolated as described previously (60). In brief, both femurs and tibias were disarticulated from euthanized mice and flushed with serum-free RPMI using a 25 5/8-gauge needle through a 70-μm filter. For BMDC differentiation, progenitor cells were spun for 5 min at 4°C and 500 × *g* and resuspended in 35 mL cRPMI (10% FBS [Fisher, FB12999102], 1% HEPES [Thermo Fisher, 15630080], 1% penicillin-streptomycin [Thermo Fisher, 15140122], and 1% GlutaMAX [Thermo Fisher, 35050079]) containing 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Invitrogen, 14-8331-80). BMDCs from one mouse were grown in a single T175 flask and fresh cRPMI containing 20 ng/mL. GM-CSF was added 2 days after seeding and then fully replaced 4 days after seeding. Seven days after seeding, BMDCs were plated in 12- or 24-well plates at a concentration of 1e6 cells/mL with 10 ng/mL GM-CSF. The cells were rested overnight and then treated as specified for experiments. For BMMs, the cells were spun for 5 min at 4°C and 500 × g and resuspended in 35 mL complete Dulbecco's modified Eagle's medium (cDMEM) (10% newborn calf serum [NBCS] [Thermo Fisher, 16010167], 5% horse serum [Fisher Scientific, 26-050-088], 1% HEPES, 1% sodium pyruvate [Thermo Fisher, 11360070], 1% GlutaMAX, 1% penicillin-streptomycin [pen-strep]) containing 20 ng/mL M-CSF (R&D Systems, 416-ML-010). BMMs from one mouse were grown in a single T175 flask, and 35 mL of medium containing 20 ng/mL M-CSF was added to each culture on day 5 and completely replaced on day 6. Seven days after seeding, BMMs were plated in 12- or 24-well plates at a concentration of 1e6 cells/mL in medium containing no M-CSF. The cells were rested overnight and then treated as specified for experiments.

Treatment of immune and epithelial cells. BMDC and BMM media were replaced with cRPMI without GM-CSF and cDMEM without M-CSF, respectively, prior to treatment. BMDCs, BMMs, and RAW 264.7 macrophages were treated for 24 h as specified in the figure legends. Epithelial cell medium was replaced before treatment for the indicated duration. At the experimental endpoint, tissue culture plates were spun for 5 min at 4°C and 500 × *g* to pellet cellular debris. The supernatants were collected and stored at −20°C for use in R&D DuoSet ELISAs. The cells were collected for flow cytometry staining or lysed with radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, protease inhibitor tablet [Roche, 11836153001]) to procure cell lysates where indicated.

Cytokine/chemokine measurements. Cell lysates from T84 cells and PMA mucosal scrapings or secretions from BMDCs, BMMs, RAW 264.7 macrophages, and Caco2 cells were collected following saline, ST, and/or LT (and LT construct) intoxication. The samples were clarified, and protein content was interpolated via Bicinchoninic acid (BCA) assay (Thermo Fisher, 23225) for mucosal scrapings and cell lysates. The samples were applied to IL-33, IL-1Ra, IL-1β, TNF-α, or IL-6 DuoSet ELISAs (R&D Systems) according to the manufacturer's instructions. The experiments were repeated three times. Cytokine quantities are plotted as pg of cytokine per unit mass for cell lysates and total pg or ng/mL for secretions and supernatants.

Flow cytometry staining and analysis. RAW 264.7 macrophages were collected following trypsinization. 500 μl cell sorting buffer (CSB; 1× phosphate-buffered saline [PBS], 2% newborn calf serum [NBCS], 2% NaN_3, 1% 0.5 M EDTA, pH 8.0) was added to collected cells, and the samples were spun at 500 × *g* for 5 min at 4°C to wash. The cells were blocked for 15 min at 4°C using in-house Fc block derived from 2.4G2 hybridoma cell (ATCC, HB-197) supernatant. Stains from BioLegend were added directly to cells following Fc block incubation: fluorescein isothiocyanate (FITC) F4/80 (123108), PerCP/Cy5.5 CD80 (104722), APC CD40 (124612), and PE/Cy7 I-A/I-E (107629). Aqua Fluorescent live/dead stain was added to samples to quantify live cells (Invitrogen, L34966). The cells were stained for 30 min at 4°C protected from light. Following staining, the cells were washed with CSB and then fixed for 20 min at room temperature in 2% PFA protected from light. The cells were washed once more with CSB and resuspended in 500 μl fresh CSB for analysis via flow cytometry on a BD LSR Fortessa analyzer.

LDH cytotoxicity assay. Small intestinal secretions from animals used for PMAs were used to measure LDH using the CyQUANT LDH cytotoxicity assay (Invitrogen, C20300) according to the manufacturer's instructions. LDH measurements from PMA secretory samples are reported as "percent lysis" of mucosal lysate per mg of total protein as determined by BCA analysis.

Statistical analysis. All of the statistical analyses were performed using Prism 9 software (GraphPad, Inc.). Unpaired *t* tests were used to analyze experiments containing two groups. In experiments with more than two groups, statistical analysis was performed using unpaired one-way analysis of variance (ANOVA), followed by Tukey's or Bonferroni's *post hoc* analysis as appropriate. The *P* values are described in the figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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J.P.B. conceived experiments. N.I.M., S.R.S., I.E.H., D.B.M., and C.P.P. conducted the experiments and analyzed the data. N.I.M. and J.P.B. wrote the manuscript. N.I.M., C.P.P., and J.P.B. edited the manuscript.

We declare no conflict of interest.

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