Recurrent Infection Progressively Disables Host Protection Against Intestinal Inflammation

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Abstract

Intestinal inflammation is the central pathological feature of colitis and the inflammatory bowel diseases. These syndromes arise from unidentified environmental factors. We found that recurrent non-lethal gastric infections of Gram-negative *Salmonella enterica* Typhimurium (*ST*), a major source of human food poisoning, caused inflammation of murine intestinal tissue, predominantly the colon, which persisted following pathogen clearance and irreversibly escalated in severity with repeated infections. *ST* progressively disabled a host mechanism of protection by inducing endogenous neuraminidase activity thereby accelerating the molecular aging and clearance of intestinal alkaline phosphatase (IAP). Disease was linked to a Tlr4-dependent mechanism of IAP desialylation and deficiency with resulting accumulation of lipopolysaccharide-phosphate (LPS-P). Oral administration of IAP or the marketed anti-viral neuraminidase inhibitor Zanamivir were therapeutic by maintaining IAP dephosphorylation of LPS-P.

Introduction

Inflammation of the intestinal tract is the defining feature of colitis and the human inflammatory bowel diseases (IBDs) including Crohn's disease and ulcerative colitis (UC). In these syndromes, chronic inflammation disrupts intestinal homeostasis and provokes

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Supplementary Materials

Materials and Methods

Fig. S1–S10

immune-mediated tissue damage (1–3). Disease origins remain mysterious and involve one or more environmental factors as genome sequence variation may play a secondary and sometimes minor role (4). Among multiple human monozygotic twin comparisons, the genetic contribution to the origin of UC is approximately 20%, while in Crohn's disease genetics can play a larger role (5). Among possible environmental origins of disease, pathogenic infection has been studied as a factor in precipitating intestinal inflammation (6). Interestingly, bacterial infections have been linked to seasonal increases in hospital admissions involving intestinal inflammation and IBD (7).

Small inoculum bacterial infections that are brief and self–limited are likely to be the most common of infections, and they may frequently go unreported, potentially leading to an underappreciation of the numbers of infections among individuals. We considered that there may be cumulative effects of repeated small inoculum and sub-clinical infections, which if correct might be detected in a model of human food poisoning. We designed such a study using recurrent low-titer non-lethal gastrointestinal infection by the bacterium *Salmonella enterica* Typhimurium (*ST*), a common human pathogen. Non-typhoidal *Salmonella* (NTS) causes a greater human disease burden than any other foodborne bacterial pathogen in the U.S. causing more than a million illnesses annually (8, 9). Globally, NTS causes 93.8 million cases and 155,000 deaths each year (10), and is responsible for up to 50% of bacteremias among young children in developing countries (11, 12).

Results

ST infection elicits intestinal inflammation by diminishing host IAP levels

Beginning at 8 weeks of age, wild-type C57BL/6J mice were infected by gastrointubation with 2×10^3 *ST* colony–forming units (cfu) every 4 weeks for 6 consecutive months. Following infection, *ST* was detected transiently and predominantly in the small intestine and some lymphoid tissues. The pathogen was cleared by the host to undetectable levels by 21 days post-infection monitoring that further noted the absence of overt symptoms or mortality among the animals (Fig. S1, A and B). The onset of disease required more than a single infection with this low titer while multiple disease signs were evident among the majority of animals prior to the fourth infection. Phenotypes consistent with the onset of intestinal dysfunction included weight loss, reduced colon length, altered stool consistency (diarrhea), and the presence of fecal blood (Fig. 1, A–D and Fig. S1C). By 20 weeks of age and prior to the fourth infection, disease signs were present among the majority of animals and further included an epithelial barrier defect (Fig. 1E). At 32–48 weeks, rectal prolapse was observed among some animals undergoing recurrent infections (Fig. 1F). The frequency of disease signs escalated with successive recurrent infections and persisted for at least 5 months following the cessation of infections.

Reductions of alkaline phosphase (AP) activity and intestinal alkaline phosphatase (IAP) abundance were detected in both the small intestine and intestinal contents, whereas levels of duodenal tissue mRNA that encode IAP were unchanged (Fig. 1, G–I and Fig. S2A). Mammalian IAP is produced exclusively by enterocytes of the duodenum of the small intestine and is released from the cell surface into the lumen where it can dephosphorylate and thereby detoxify the lipopolysaccharide (LPS) endotoxin of Gram-negative bacteria

(13–16). We found that oral supplementation with calf intestinal alkaline phosphatase (cIAP) maintained normal AP activity levels in the intestinal tract among animals receiving recurrent *ST* infections (Fig. 1J). Analysis of LPS isolated from the intestinal tract at 20 weeks of age prior to the fourth infection and following *ST* clearance revealed a four-fold increase in endogenous LPS-phosphate levels in the context of a 50% increase in total LPS, both of which were maintained close to normal levels in mice receiving cIAP treatment (Fig. 1K). Augmentation of AP activity by cIAP treatment also protected against the development of disease signs encompassing weight loss, colon length, diarrhea, fecal blood, and epithelial barrier function (Fig. 1L).

Inflammatory cytokines associated with intestinal tissue inflammation include chemokine ligand 5 (Ccl5), interleukin-1β (Il1b), tumor necrosis factor-α (Tnfa), and interferon-γ (Ifng). Recurrent *ST* infection progressively increased inflammatory cytokine mRNA expression levels predominantly in the colon, unless cIAP treatment was provided (Fig. 1M). Histopathological changes were also predominantly seen in the colon including the infiltration of leukocytes into the lamina propria that included neutrophils, monocytes, and T cells, as well as erosion of the epithelial barrier, and reduced goblet cell numbers, whereas a much lesser effect was observed in the small intestine and in addition only in the ileum (Fig. 1N, Fig. S2b, and Fig. S3). Thus, recurrent non-lethal gastrointestinal infection of *ST* diminished the expression of host IAP activity that normally confers host protection against intestinal inflammation and tissue damage predominantly in the colon. Studies were further undertaken to identify the mechanisms that regulate IAP function.

IAP deficiency is linked to an accelerated rate of de-sialylation and endocytic localization

IAP is synthesized as a glycosylphosphatidylinositol (GPI)-linked glycoprotein residing on the enterocyte cell surface of the duodenum until it is released into the intestinal lumen by phospholipase activity (13, 17). IAP production was investigated by pulse–chase experiments of ex vivo primary enterocyte cultures derived from tissue of the small intestine. Normal rates of IAP synthesis and appearance at the cell surface were observed among all enterocyte cultures regardless of previously cleared infections (Fig. 2A). In contrast, a significant decrease in IAP cell surface half-life with reduced IAP abundance in the culture media were measured among enterocyte samples from mice that had cleared multiple infections (Fig. 2, B and C). Co-localization studies revealed increased co-localization of IAP with markers of early endosomes and lysosomes coincident with reduced cell surface IAP abundance (Fig. 2, D and E).

IAP is also glycosylated during its synthesis in the secretory pathway. We next analyzed the glycan linkages attached to enterocyte IAP using analytical lectins including the α2–3 sialic acid-specific lectin *Maackia amurensis* lectin II (MAL-II) and the galactose-specific lectins *Erythrina cristagalli* agglutinin (ECA) and *Ricinus communis* agglutinin (RCA). These lectins have been validated biochemically and genetically although their binding does not provide full structure resolution of the various types of glycan structures that can present these linkages. A significant reduction of terminal sialic acid linkages coincident with exposure of underlying galactose linkages were measured in the context of recurrent infection (Fig. 2F). No comparative changes to other specific glycan linkages were detected

including the sialylation state of Core 1 O-glycans, or changes in abundance of α2–6-linked sialic acids, using Peanut Agglutinin (PNA) and *Sambucus nigra* (SNA) lectins, respectively. In the absence of infection, we observed a progressive de-sialylation of the glycans attached to nascent IAP on the enterocyte cell surface, indicative of a feature of its molecular aging. This basal rate of IAP de-sialylation was significantly increased by recurrent *ST* infection and was concurrent with increased IAP internalization and degradation in enterocytes (Fig. 2G). Although IAP deficiency appeared to be the predominant factor in disease onset as indicated by the effects of cIAP treatment, multiple enterocyte cell surface glycoproteins were observed to be de-sialylated and internalized from the cells surface of endocytes including sucrase-isomaltase, dipeptidyl peptidase 4, and lactase (Fig. S4). These results suggest that the presence of one or more sialyltransferases establishes the normal half-lives (and consequent abundance) of enterocyte cell surface glycoproteins including IAP.

ST3Gal6 maintains IAP sialylation in protecting against intestinal inflammation

The ST3Gal6 sialyltransferase generates α2–3 sialic acid linkages on glycoproteins and is highly expressed in the intestinal tract (18, 19). In mice lacking a functional *St3gal6* gene, we detected a significant reduction in AP activity and IAP abundance, whereas mRNA encoding IAP was unchanged (Fig. 3A, 3B and Fig. S5A). Although LPS levels in the intestinal tract were similar at 8 weeks of age, a three-fold increase of LPS-phosphate abundance was measured consistent with reduced AP activity (Fig. 3C). Absent ST3Gal6 function, IAP sialic acid linkages were also deficient with increased galactose exposure (Fig. 3D) similar to findings in wild-type mice experiencing recurrent *ST* infection. Glycan alterations were further detected histologically among small intestinal epithelial cells (Fig. S5B). Diminished IAP sialylation in ST3Gal6 deficiency was linked to reduced IAP cell surface residency and increased IAP co-localization with markers of endosomes and lysosomes (Fig. 3, E–I). Thus, IAP sialylation by ST3Gal6 functions to maintain IAP half-life and abundance in the intestinal lumen. The impact of IAP deficiency caused by the absence of ST3Gal6 was further investigated.

Mice aging in the absence of ST3Gal6 were compared with wild-type littermates in the presence and absence of recurrent *ST* infection and cIAP therapy. Spontaneous phenotypes detected in uninfected mice lacking ST3Gal6 included reduced body weight, reduced colon length, diarrhea, the presence of fecal blood, and epithelial barrier dysfunction, all of which were exacerbated by recurrent *ST* infection. The addition of cIAP to drinking water normalized AP activity levels during adult life and reduced or eliminated disease signs (Fig. 4, A–C). Similarly, increased LPS-phosphate levels were closely associated with reduced IAP and the onset of disease signs, whereas total LPS increased only modestly in the intestinal contents of infected mice (Fig. 4D). LPS is generated predominantly in the colon by commensal microbes that may serve as markers or effectors of intestinal inflammatory disease (20).

A survey of microbiota using 16S rDNA probes revealed a 50% increase of intestinal bacterial load attributed primarily to the Gram-negative *Enterobacteriaceae*, consistent with the magnitude of increase of total LPS. These findings were present among both uninfected *St3gal6*-null mice and wild-type littermates subjected to recurrent *ST* infection infection and

ST3Gal6 deficiency (Fig. 4E). The temporal acquisition of these microbiota changes observed at 32 weeks were found to emerge progressively among wild-type mice receiving periodic *ST* infections, and were coincident with the emergence and increase in the severity of disease signs Fig. S6). These findings are also consistent with reports of altered commensal microbiota populations that can contribute intestinal inflammation and which often include elevated levels of *Enterobacteriaceae* (3, 21).

ST3Gal6 deficiency spontaneously increased inflammatory cytokines in the intestinal tissues of uninfected animals, and this was further exacerbated by recurrent *ST* infection (Fig. 4F). Similarly, histopathological findings in the absence of ST3Gal6 correlated with inflammatory marker expression and included leukocyte infiltration, epithelial layer discontinuity, and reduced goblet cell numbers, which were also increased in severity by recurrent *ST* infection (Fig. 4G). These results demonstrated that the ST3Gal6 sialyltransferase is essential to support normal IAP expression and host protection against spontaneous intestinal inflammation. The cause of IAP de-sialylation following *ST* infection was unaccounted for but implicated as a significant trigger of pathogenesis.

Disease onset with neuraminidase induction is Tlr4-dependent and recapitulated by LPS

Neuraminidase (Neu) enzymes, also known as sialidases, hydrolyze sialic acids attached to glycan polymers and are encoded in the genomes of diverse organisms including bacteria, mice, and humans. However, the genome of the *ST* isolate used in our studies does not encode a neuraminidase (22), indicating a host source of the induced Neu activity. Four neuraminidase genes have been identified in mammalian genomes (*Neu1–4*), with Neu1 and Neu3 enzymes expressed in multiple compartments including the cell surface and in blood circulation (23, 24). An increase of neuraminidase (Neu) activity occurred in the small intestine due to *ST* infection. Using Toll-like receptor 4 (Tlr4)-null mice, this induction of host Neu activity was dependent upon Tlr4 function (Fig. 5A). Among mammalian Neu isozymes, only Neu3 abundance and *Neu3* RNA expression correlated with Tlr4-dependent induction of Neu activity (Fig. 5, B and C). Absence of *Neu3* induction due to Tlr4 deficiency resulted in normal IAP expression at the enterocyte cell surface (Fig. 5D). This was coincident with normal levels of sialic acid linkages on IAP and among apical glycoproteins of the small intestinal epithelium (Fig. S5, C and D). AP activity and IAP abundance also remained normal in Tlr4 deficiency and LPS-phosphate levels did not increase significantly following *ST* infections (Fig. 5, E–G). The induction of inflammatory cytokines caused by recurrent *ST* infection was also blocked by Tlr4 deficiency, and disease signs were reduced or eliminated with the maintenance of epithelial barrier function (Fig. 5, H and I).

Tlr4 is activated by binding to its LPS ligand (25, 26). This bacterial sensor of the LPS endotoxin is expressed on all segments of the intestinal tract and is especially prominent in the small intestine (27, 28), where it functions in pro-inflammatory signaling to engage immune cells in the onset and development of disease (29, 30). We investigated whether LPS was itself responsible for Neu induction, IAP deficiency, and concurrent elevations of inflammatory cytokines. Dose-response analyses using commercially obtained LPS were undertaken to determine minimal dosage and timing for further study (Fig. S7, A and B). We

found that gastrointubation of LPS induced Neu activity, Neu3 abundance, and *Neu3* RNA levels with reductions in AP activity and IAP abundance, all of which were dependent upon Tlr4 function (Fig. 6, A–F). The induction of Neu activity by LPS resulted in Tlr4-dependent reductions of sialic acid linkages resulting in galactose exposure on isolated IAP and among apical cell surface glycoproteins of the small intestinal epithelium (Fig. S7, C and D). LPS increased the internalization and co-localization of IAP with endocytic markers coincident with reduced IAP expression at the cell surface (Fig. S7E). Normal IAP levels were retained following LPS administration in Tlr4 deficiency with relatively low abundance of LPS-phosphate (Fig. 6G). LPS–induced inflammatory cytokine induction was also blocked by Tlr4 deficiency with the retention of epithelial barrier function (Fig. 6, H and I). Thus, LPS/Tlr4 signaling resulting from recurrent low-titer *ST* infection is linked to the induction of host Neu3 and increased Neu activity, resulting in IAP de-sialylation and its subsequent pathogenic deficiency. Thus, Neu inhibition may be therapeutic in the context of recurrent *ST* infection.

Therapeutic effect of Zanamivir with maintenance of IAP abundance and function

The marketed antiviral drug Zanamivir inhibits influenza neuraminidase activity (31), and has been used in research on mammalian Neu isozymes (24, 32, 33). At pharmacological dosages, Zanamivir inhibits Neu2, Neu3, and Neu4, but not Neu1 (32, 33). We found that oral treatment with Zanamivir maintained normal Neu activity levels in animals experiencing recurrent *ST* infections (Fig. 7A). Zanamivir did not block the physical induction of Neu3, which persisted for at least 20 weeks following the last *ST* infection (Fig. 7B). However, the inhibition of Neu activity by Zanamivir maintained host AP activity, IAP abundance, and normal lectin-binding patterns among apical glycoproteins of the small intestinal epithelium (Fig. 7C, Fig. 7D, and Fig. S8). The maintenance of IAP expression and activity was linked to the retention of relatively low LPS-phosphate levels that were otherwise elevated by *ST* infection (Fig. 7E). Zanamivir inhibited IAP de-sialylation coincident with normal IAP expression at the enterocyte cell surface (Fig. 7, F and G). Zanamivir also inhibited the induction of inflammatory cytokines and reduced the appearance of disease markers of intestinal inflammation including alterations of commensal microbiota, and barrier dysfunction (Fig. 7, H–J and Fig. S9). In comparisons with the acute and chronic model of chemically induced colitis using dextran sodium sulfate (DSS) (34, 35), Zanamivir had no effect, whereas ST3Gal6 deficiency exacerbated disease signs, consistent with the presence of different mechanisms of inflammation that may be responsive to IAP treatment (Fig. S10). Our findings together indicate a disease mechanism of environmental and pathogen origin that encompasses the different locations and functions of the small intestine and colon (Fig. 8).

Discussion

An increasingly severe colitis developed from recurrent low-titer non-lethal transient gastric infections of the Gram-negative pathogen *ST*. In this mouse model of repeated human food poisoning, the host rapidly cleared the pathogen. Nevertheless, these subsequent recurrent infections progressively disabled a mechanism in the host that normally protects against spontaneous intestinal inflammation. This anti-inflammatory mechanism operates primarily

in the colon but is dependent upon IAP production and release from duodenal enterocytes of the small intestine. *ST* infection targeted this protective mechanism by activating host Tlr4 function in the duodenum and thereby inducing host Neu activity with elevated Neu3 expression at the luminal surface of the enterocyte. Neu induction accelerated the rate of nascent IAP desialylation on the enterocyte cell surface, reducing IAP half-life, causing IAP internalization and degradation, and resulting in a downstream IAP deficiency in the colon. IAP deficiency was linked to deficient dephosphorylation of LPS endotoxin molecules produced by commensal microbiota. This Tlr4-dependent disease manifested primarily in the colon with signs more similar to UC than to Crohn's disease, and was linked to increases of the pro-inflammatory Tlr4 ligand, LPS-phosphate. Similarly, the genetic disruption of ST3Gal6-dependent sialic acid linkage formation during IAP synthesis caused IAP deficiency resulting in a spontaneous colitis that increased in severity with age and which was exacerbated by recurrent *ST* infections. In both cases, diminished glycoprotein sialylation among enterocytes resulted in reduced IAP half-life and deficiency with markedly elevated LPS-phosphate abundance in the colon. Consistent with a Tlr4-dependent mechanism, LPS administration alone recapitulated Neu3 induction and IAP deficiency, bypassing the requirement for IAP deficiency to increase colonic LPS-phosphate levels in provoking inflammation.

Intestinal inflammation failed to resolve following the discontinuation of periodic recurrent infections and persisted for months afterwards as a lasting outcome. The degree of this persistence may be determined in part by initial infection titers and the time between recurrent infections, and may result from multiple mechanisms. One possibility is the generation of epigenetic modifications to inflammatory gene promoters regulated by Tlr4 function that result in the persistence of inflammatory cytokine expression and may explain observations of the slow resolution of inflammatory processes (36, 37). The enterocyte *Neu3* allele is perhaps similarly regulated in this way as its expression remained induced long after periodic *ST* infections were discontinued. It is also possible that the escalating inflammation resulting from increased recruitment and activation of innate and adaptive immune cells reaches a point wherein the degree of immunological activation and damage to the epithelium is not easily reversed or attenuated. Disease persistence was also coincident with microbiota alterations that we found emerged concurrently with disease signs and endured following the discontinuation of recurrent infections. Interestingly, acute enteric infections with *Yersinia* can trigger gut microbiota dysbiosis and chronic inflammation after pathogen clearance in Tlr1-deficient mice (38). The microbiota alterations we observed predominantly involved *Enterobacteriaceae*, which are frequently imbalanced in studies of intestinal inflammatory disease (21,38).

Neuraminidases function in a variety of processes including pathogen virulence, glycan catabolism, and biological signaling (39–41). Neu function may differ among isozymes and origins, such as indicated from caecal sources in contributing to DSS-induced colitis (34). The *Neu3* gene of mammals includes presumptive binding sites for transcriptional factors Stat-3, Rreb-1, MyoD, Ik-2, Pax-2, Aml1a, Hoxa9, and Meis-1, and may employ alternate promoters controlled by Sp1/Sp3 transcription factors (42). Indeed, Stat-3 and Sp1 transcription factors are activated by LPS (43, 44). Transcriptional activation of *Neu3* may further underlie Neu3 induction in cancers of colon, renal, and prostate tissues, and mice

lacking Neu3 exhibited fewer colitis-associated colonic tumors (45–47). Our studies of an *ST* isolate that lacks an annotated and demonstrable neuraminidase gene have further linked the degree of *Neu3* RNA induction to elevation of Neu activity, supporting the view that host Neu3 is involved in the desialylation of the IAP glycoprotein thereby causing IAP internalization and deficiency. However it remains possible that the Neu activity we have measured comes from an unannotated Neu enzyme or the endogenous microbiota, neither of which can be resolved until Neu3-deficient mice are further investigated. Neu3 activity is primarily active towards gangliosides, however studies reported significant but lesser activity towards glycoprotein substrates (48). It is also possible that Neu3 could somehow act indirectly via desialylation of its canonical ganglioside substrates. Nevertheless, Neu3 has been reported to desialylate the epidermal growth factor receptor (EFGR) glycoprotein (49). Moreover, data similar to our findings herein have further implicated Neu3 in the desialylation of circulating glycoproteins in the blood linked to a mechanism determining the various half-lives of plasma proteins (24).

Regulation of enterocyte IAP trafficking by sialylation extends this recently discovered mechanism of secreted protein aging and turnover to include the determination of protein half-lives at the cell surface. Although *ST* infection resulted in the desialylation and internalization of multiple enterocyte glycoproteins expressed at the cell surface, the disease phenotype was largely due to IAP deficiency. This represents an example of a specific glycan linkage that is commonly found on secreted and cell surface proteins as having a biological purpose more restrictively associated with one or few such glycoproteins (50–52). This can be explained in part by the presence of multiple sialyltransferases operating in the intestinal tract that are responsible for the sialylation of different subsets of bioactive glycoproteins and that function in different biological processes, such as findings involving ST3Gal6 deficiency herein compared with other studies of ST3Gal4 deficiency (34). Additional factors that could influence IAP expression and disease onset include mutations of glycan acceptor sites of IAP and transcriptional or mutagenic modifications to relevant glycosyltransferase and glycosidase genes, each of which can contribute to glycoprotein function (53). In the control of IAP half-life, increased internalization and degradation of desialylated IAP infers that a sialic acid-binding lectin analogous to the mammalian siglecs of leukocytes (54) may normally bind nascent sialylated IAP on the enterocyte surface to inhibit premature IAP endocytosis thereby allowing subsequent release into the lumen. Alternatively, the exposure of underlying galactose may unmask cryptic ligands for galactose-binding lectins such as the galectins that provoke glycoprotein endocytosis (50, 55).

Diminished alkaline phosphatase activity has been described in patients with colitis and celiac disease and oral alkaline phosphatase supplementation is under study to treat inflammatory diseases including the IBDs (56–59). Moreover, Neu3 protein abundance and activity is increased in human IBD patients (60). In animal studies, IAP deficiency contributes to colitis and allowed greater bacterial passage from the intestinal lumen to the mesenteric lymph nodes (16, 35, 56, 61). We have found that IAP is highly regulated and that *ST* infection disables IAP function in host protection while progressively eroding microbial barriers by successive rounds of what might otherwise be considered as unproductive infections. Environmental Gram-negative pathogens that access and infect the

small intestine may have a similar strategy towards misappropriating host Tlr4 function to diminish IAP activity and increase colonic LPS-phosphate levels thereby provoking intestinal inflammation. This further emphasizes the dual nature of host Tlr4 function, which may be either advantageous or disadvantageous depending upon the context of exposure and perhaps the severity of infection. The link identified herein between host Tlr4 function and Neu3 induction appears to favor the pathogen as it is currently unclear why Tlr4 signaling is linked to the induction of enterocyte Neu3 expression. This likely reflects contextual features of infection and possible advantageous host responses that remain to be discovered. IAP augmentation and Neu inhibition nevertheless represent candidate therapies for intestinal inflammation resulting from recurrent infections of the *ST* pathogen in a model of repeated human food poisoning that escalates the host production of LPS endotoxin and Tlr4 ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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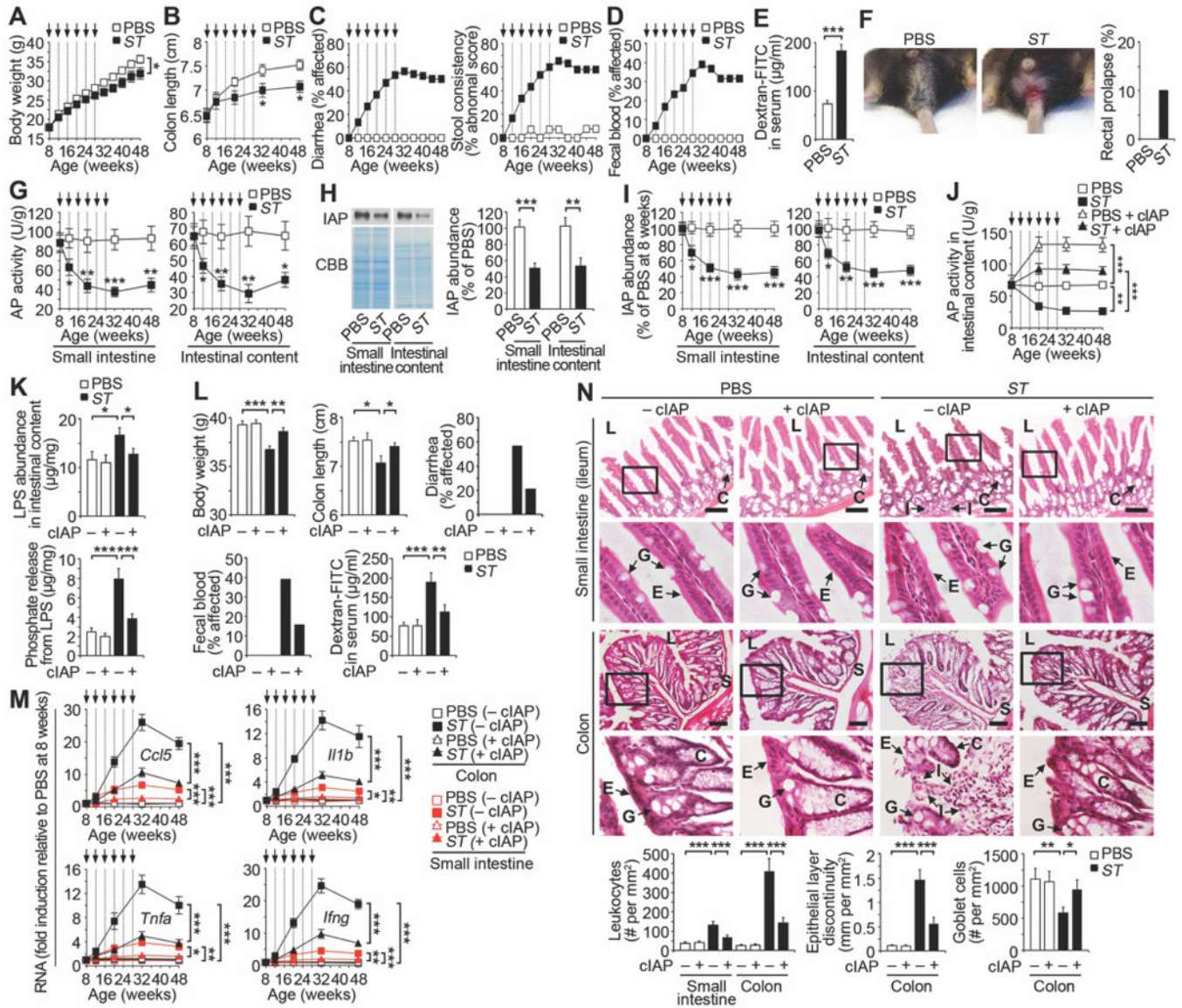
One Sentence Summary

A leading cause of human food poisoning disrupts a mechanism of host protection in causing chronic intestinal inflammation.

Fig. 1. Recurrent *ST* infection diminishes the abundance and protective role of IAP

Wild-type mice were analyzed during a course of recurrent *ST* infection (2×10^3 cfu) or uninfected (PBS) at indicated time points (arrows). (**A**) Body weight (*ST*, n = 20; PBS, n = 19). (**B**) Colon length (n = 40 per condition). (**C**) Diarrhea and stool consistency (*ST*, n = 19; PBS, n = 13). (**D**) Fecal blood (*ST*, n = 19; PBS, n = 13). (**E**) Intestinal epithelial barrier function (n = 8 per condition) at 20 weeks of age prior to the fourth infection. (**F**) Rectal prolapse (*ST*, n = 30; PBS, n = 20) at 32–48 weeks of age, or 4–20 weeks following last *ST* infection (representative image). (**G**) AP activity (n = 40 per condition). (**H**) Immunoblot blot analysis of IAP at 20 weeks of age prior to the fourth infection (n = 8 per condition). (**I**) Relative IAP abundance (n = 40 per condition). (**J**) AP activity +/− calf IAP (cIAP) (n = 40 per condition). (**K**) LPS abundance and phosphate released from LPS (n = 8 per condition) at 20 weeks of age. (**L**) Body weight (n = 10 per condition), colon length (n = 8 per condition), diarrhea (*ST*, n = 23; PBS, n = 14; *ST* + cIAP, n = 19; PBS + cIAP, n = 15), fecal

blood (*ST*, n = 23; PBS, n = 14; *ST* + cIAP, n = 19; PBS + cIAP, n = 15) at 48 weeks of age (20 weeks following last *ST* infection), and intestinal epithelial barrier function (n = 8 per condition) at 20 weeks of age prior to the fourth infection. (**M**) Cytokine mRNA expression (n = 30 per condition). (**N**) H & E-stained intestinal tissues at 48 weeks of age (20 weeks following last *ST* infection). L, intestinal lumen; E, epithelial layer; C, crypt; G, goblet cell; S, submucosa; I, infiltration of leukocytes. Graphs are representative of 16 fields of view (4 mice per condition). All scale bars: 100 μm. Error bars, means ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; Student's *t* test (A), (B), (E), (G), (H), and (I) or one-way ANOVA with Tukey's multiple comparisons test (J) to (N).
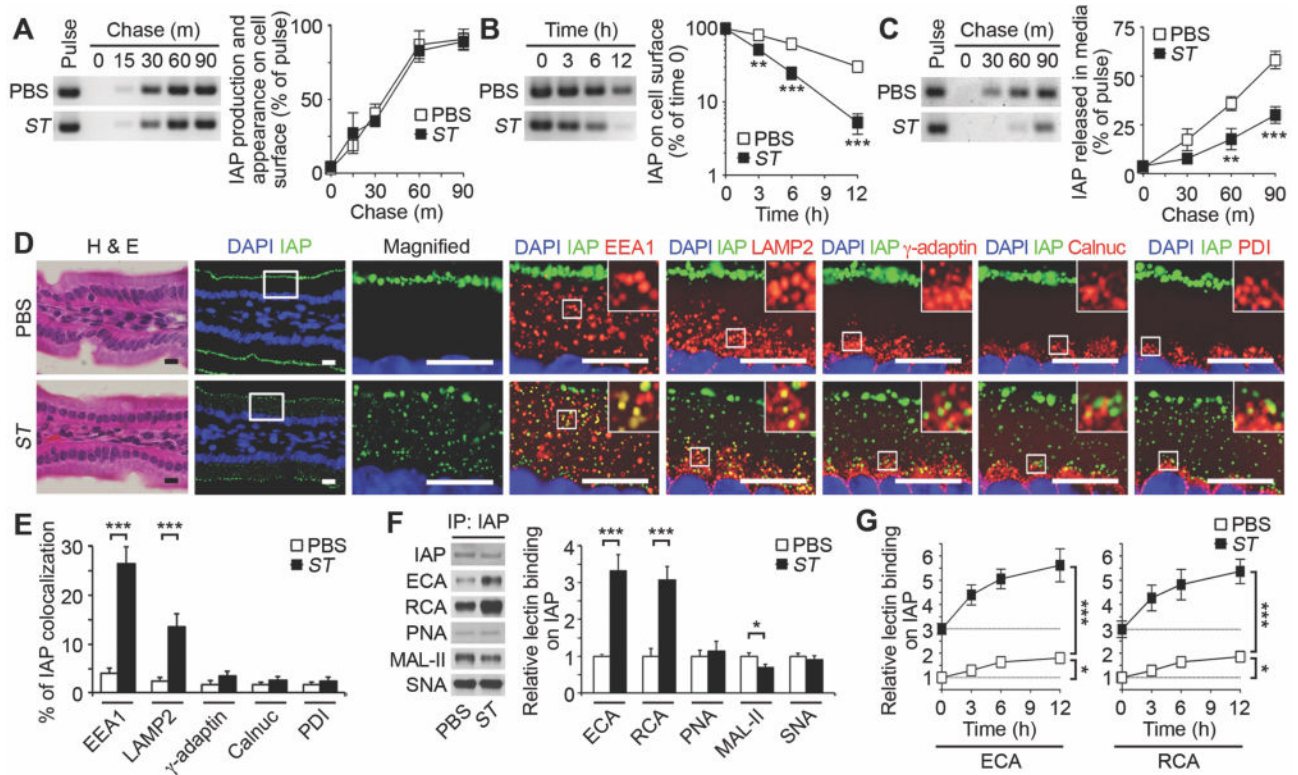
Fig. 2. Mechanism of IAP regulation during recurrent *ST* infection

(**A**–**C**) Pulse–chase analyses of IAP synthesis, trafficking and cell-surface half-life among cultured primary enterocytes isolated from wild-type mice at 20 weeks of age (prior to fourth *ST* infection). (**D** and **E**) In situ localization and intracellular co-localization of IAP in duodenum sections stained with H & E or with fluorescent antibodies to IAP (green) and intracellular compartment proteins (red) including early endosomes (EEA1), lysosomes (LAMP2), trans-Golgi (γ-adaptin), cis-Golgi (Calnuc), or the endoplasmic reticulum (PDI), depicting the percentage of IAP co-localization (yellow). Graphs are representative of ten fields of view (four mice per condition). Scale bars: 10 μm. (**F**) Lectin blot of IAP from small intestine. (**G**) Lectin binding of IAP on cultured enterocytes following cell-surface biotinylation. (**A**–**G**) Wild-type mice at 20 weeks of age prior to fourth infection. (**A**–**C** and **G**) n = 6 per condition. (**F**) n = 8 per condition. Error bars, means ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; Student's *t* test (A), (B), (C), (E), and (F) or one-way ANOVA with Tukey's multiple comparisons test (G).
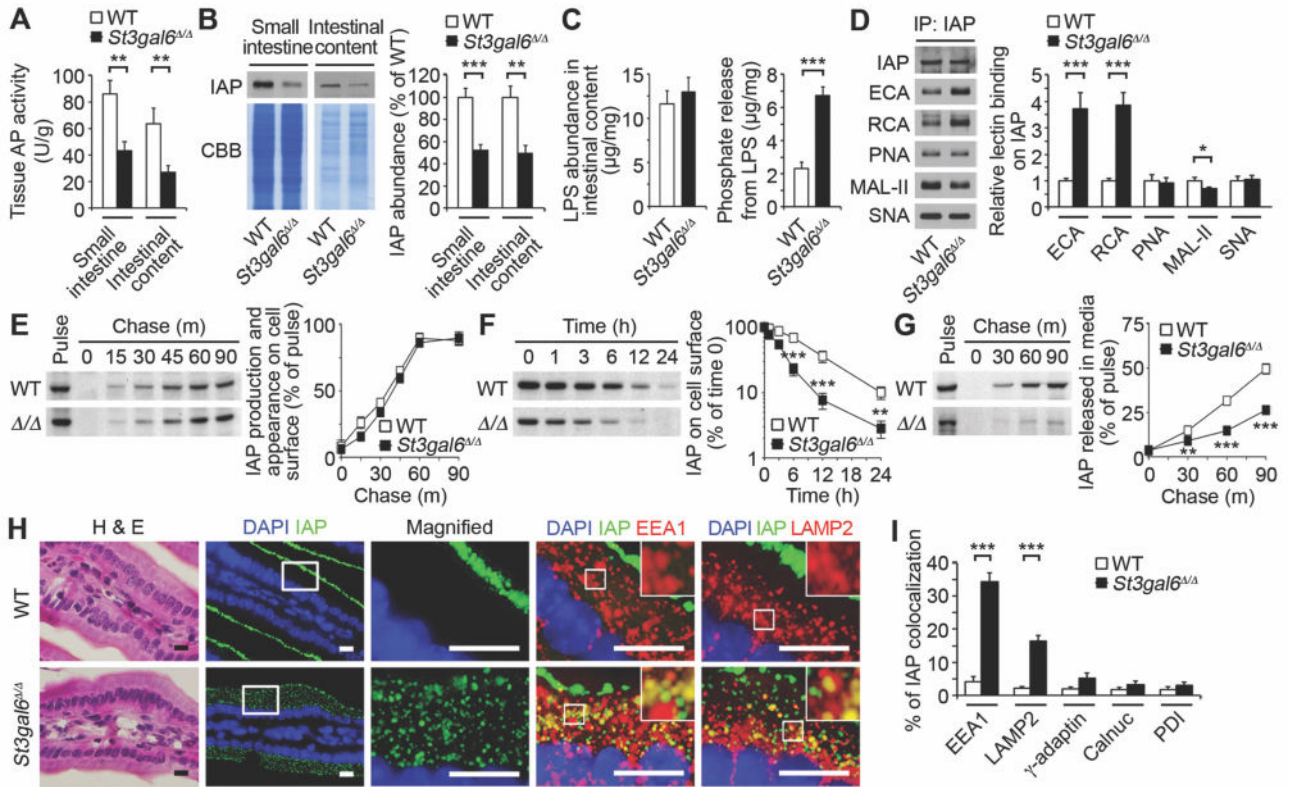
Fig. 3. Mechanism of IAP regulation by ST3Gal6 sialylation

(**A**) AP activity. (**B**) IAP protein abundance. (**C**) LPS abundance and phosphate released from LPS of intestinal content. (**D**) Lectin blot of IAP from small intestine. (**E–G**) Pulse–chase of IAP synthesis and trafficking and IAP cell-surface half-life among cultured primary enterocytes isolated from uninfected ST3Gal6-deficient mice and wild-type littermates at 8–10 weeks of age. (**H** and **I**) In situ localization and intracellular co-localization of IAP in duodenum, depicting the percentage of IAP co-localization (yellow). Graphs are representative of ten fields of view (4 mice per genotype). Scale bars, 10 μm. (**A–I**) ST3Gal6-deficient mice and wild-type littermates at 8–10 weeks of age, uninfected. (**A–D**) n = 8 per condition. (**E–G**) n = 6 per condition. Error bars, means ± SEM. ***P < 0.001; **P < 0.01; *P < 0.05; Student's *t* test (A) to (G) and (I).
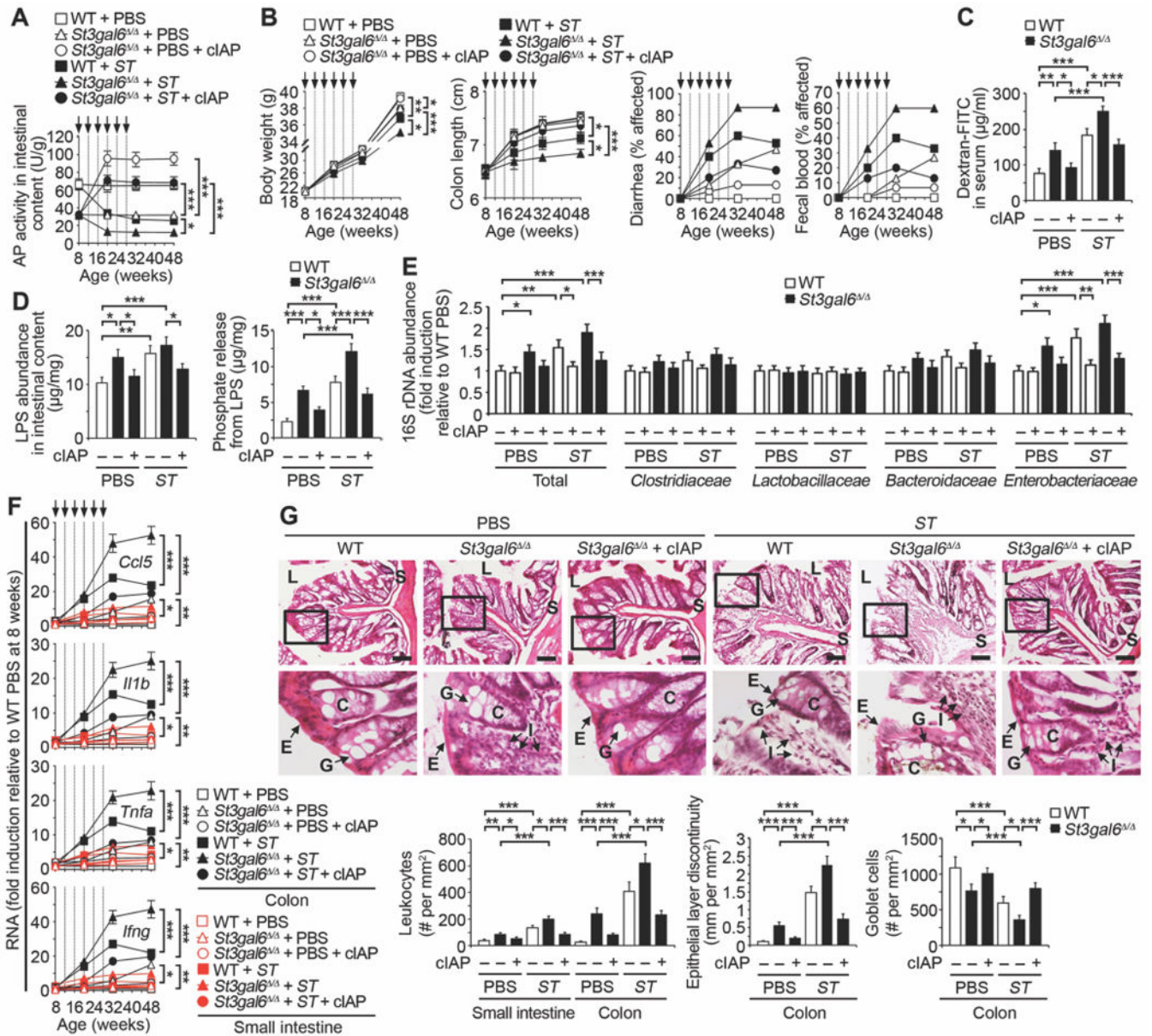
Fig. 4. ST3Gal6 sialylation of IAP in preventing intestinal inflammation

Indicated genotypes following *ST* re-infection (arrows) were analyzed in absence or presence of cIAP. (**A**) AP activity (n = 32 per condition). (**B**) Body weight (n = 10 per condition), colon length (n = 32 per condition), diarrhea (n = 30 per condition), and fecal blood (n = 30 per condition). (**C**) Intestinal epithelial barrier function. (**D**) LPS abundance and phosphate released from LPS of intestinal content. (**E**) Commensal microbiome 16S rDNA in intestinal content (n = 10 per condition). (**F**) Inflammatory cytokine RNA in colon and small intestine (n = 24 per condition). (**G**) H & E-stained colon sections. Graphs are representative of ten fields of view (four mice per condition). Scale bars: 100 μm. (**C** and **D**) Data were acquired from mice 20 weeks of age prior to fourth infection. (**E**–**G**) Data were acquired from mice 32 weeks of age and 4 weeks following the last infection. (**C** and **D**) n =
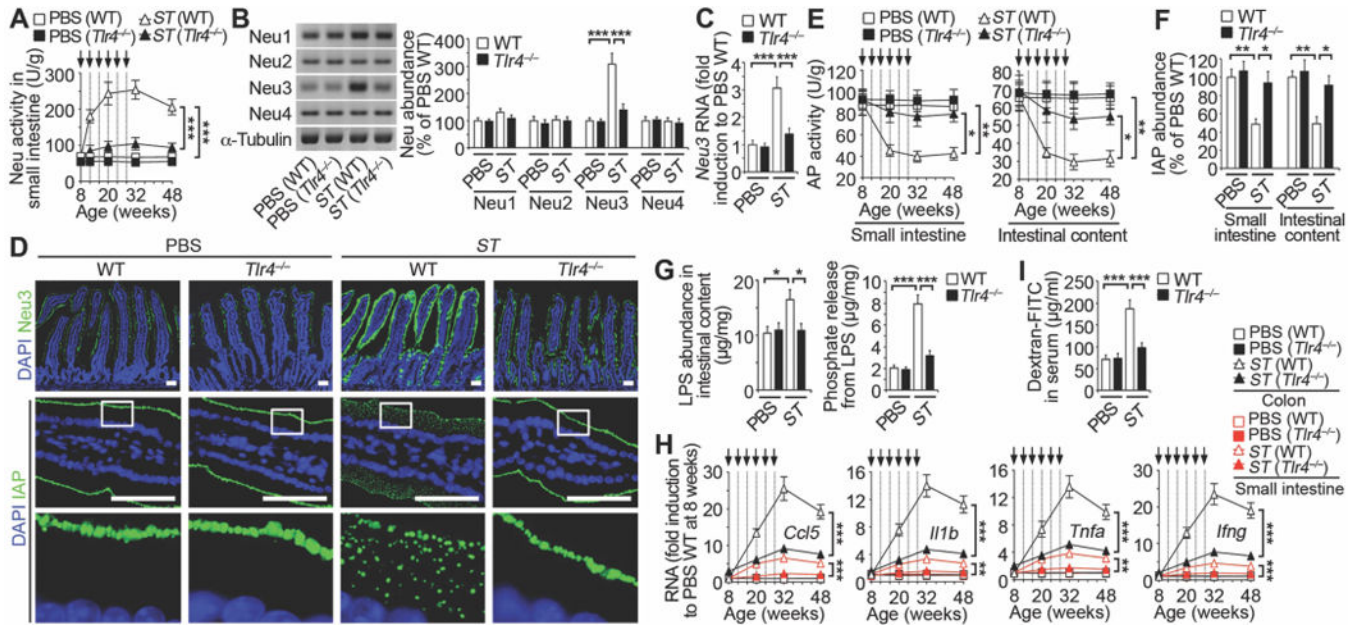
8 per condition. Error bars, means ± SEM. ***P < 0.001; **P < 0.01; *P < 0.05; one-way ANOVA with Tukey's multiple comparisons test (A) to (G).

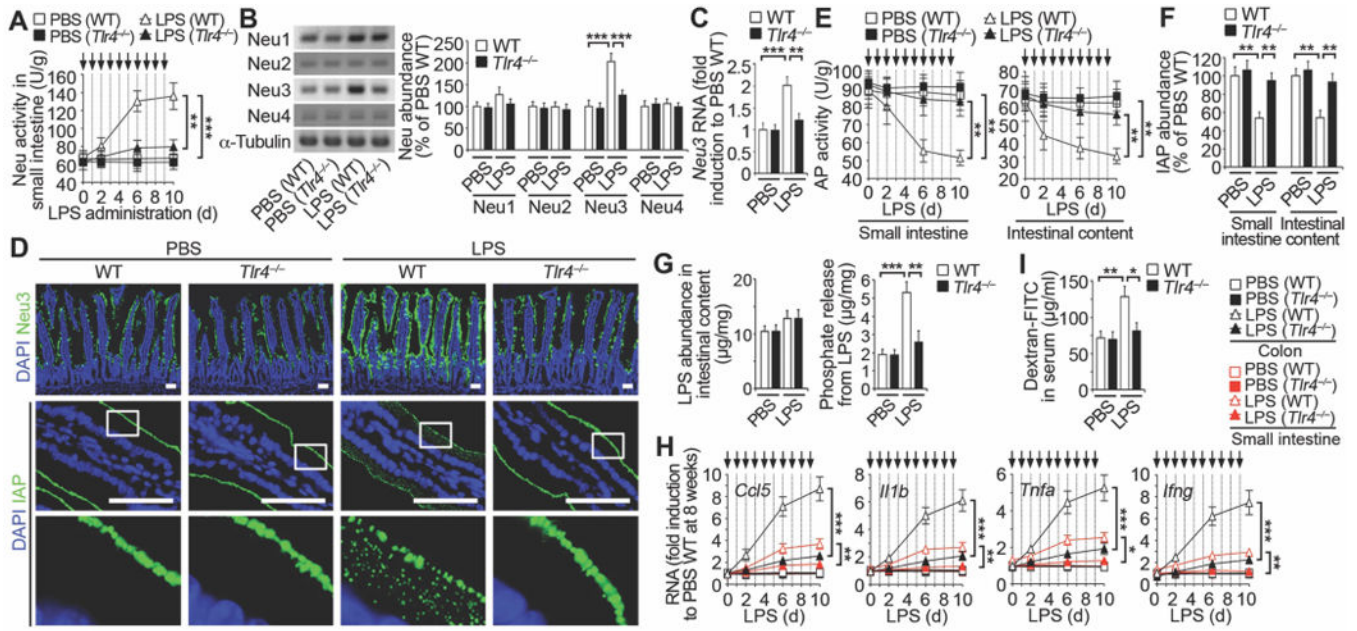**Fig. 5. Host neuraminidase induction by Tlr4 during recurrent *ST* infection**

Wild-type and Tlr4-deficient mice were analyzed following *ST* re-infection or LPS administration (arrows). (**A**) Neuraminidase (Neu) activity (n = 30 per condition). (**B**) Neu1, 2, 3 and 4 protein abundance in small intestine. (**C**) *Neu3* mRNA expression in small intestine. (**D**) In situ localization of Neu3 and IAP in duodenum. Images are representative of ten fields of view (4 mice per condition). Scale bars: 50 μm. (**E**) AP activity (n = 24 per condition). (**F**) IAP protein abundance. (**G**) LPS abundance and phosphate released from LPS of intestinal content. (**H**) Inflammatory cytokine RNA abundance (n = 16 per condition). (**I**) Intestinal epithelial barrier function. (**B, C, F, G, and I**) n = 6 per condition. (**B, C, F, G**, and **I**) Animals were 20 weeks of age and prior to the fourth infection. Error bars, means ± SEM. ***P < 0.001; **P < 0.01; *P < 0.05; one-way ANOVA with Tukey's multiple comparisons test (A) to (I).

Fig. 6. Host neuraminidase induction by Tlr4 and LPS

(**A**) Neu activity in mice at 8 weeks of age prior to repeated LPS administrations (arrows). (**B** and **C**) Neu protein abundance and Neu3 RNA expression in small intestine. (**D**) In situ localization of Neu3 and IAP in duodenum sections, representative of ten fields of view (four mice per condition). Scale bars: 50 μm. (**E**) AP activity prior to repeated LPS administrations (arrows). (**F**) IAP protein abundance. (**G–I**) Phosphate released from LPS of intestinal content, inflammatory cytokine RNA abundance and intestinal epithelial barrier function. (**A** and **E**) n = 24 per condition. (**B**, **C**, **F**, **G**, and **I**) n = 6 per condition. (**H**) n = 16 per condition. (**B**, **C**, **F**, **G**, and **I**) Mice on day 6 following LPS administration. Error bars, means ± SEM. ***P < 0.001; **P < 0.01; *P < 0.05; one-way ANOVA with Tukey's multiple comparisons test (**A**) to (**I**).
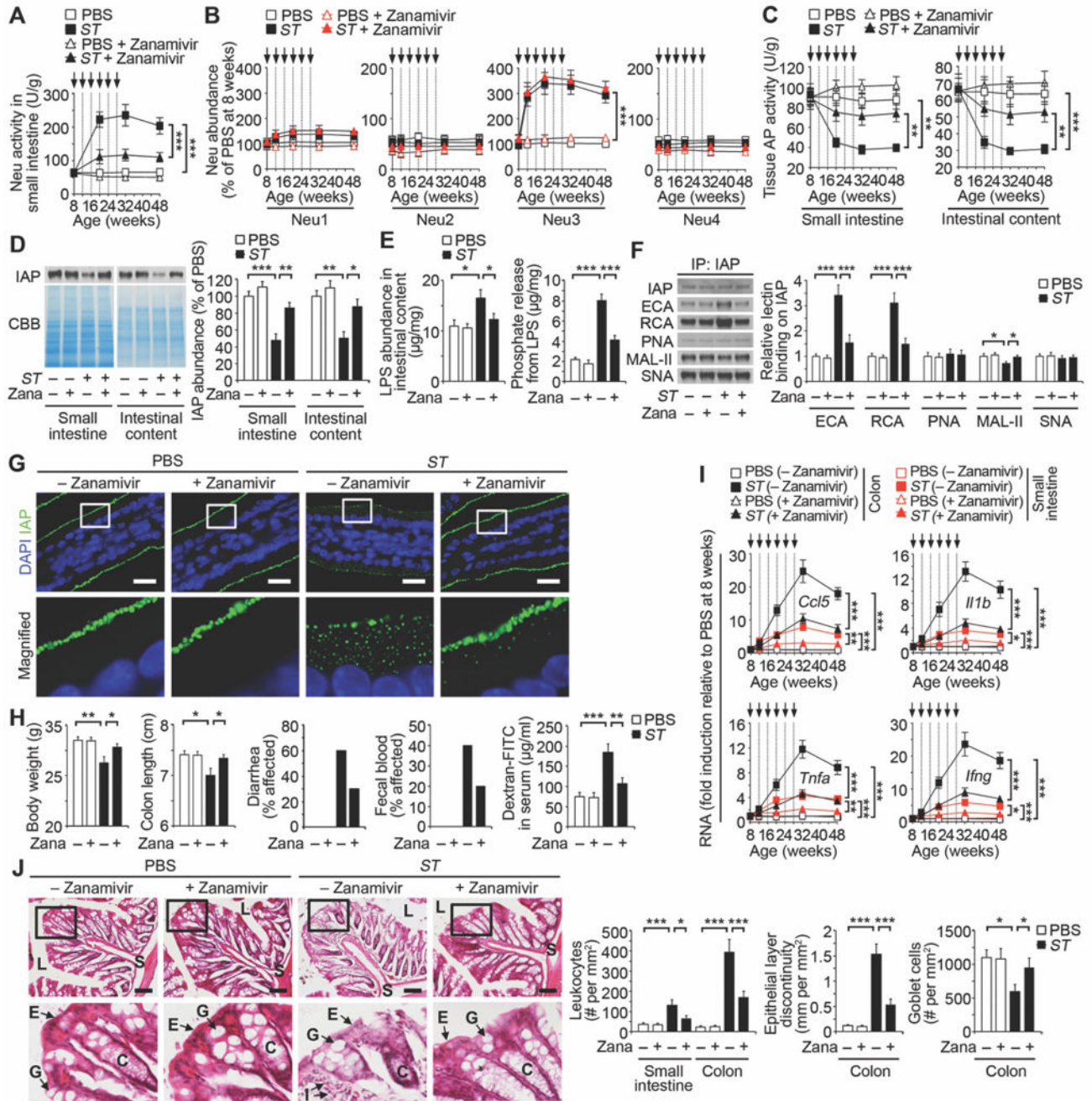
Fig. 7. Effects of neuraminidase inhibitor Zanamivir on intestinal inflammation

Wild-type mice were analyzed at indicated ages prior to *ST* re-infection (arrows) in absence or presence of Zanamivir (Zana) (0.5 mg/ml) provided in drinking water immediately after first infection. (**A**) Neu activity. (**B**) Neu1, 2, 3 and 4 protein abundance in small intestine. (**C**) AP activity. (**D**) IAP protein abundance. (**E**) LPS abundance and phosphate released from LPS of intestinal content. (**F**) Lectin blotting of IAP protein from small intestine. (**G**) In situ localization of IAP in duodenum, representative of ten fields of view (four mice per condition). Scale bars, 20 μm. (**H**) Body weight (n = 10 per condition), colon length (n = 8

per condition), diarrhea (n = 10 per condition) and fecal blood (n = 10 per condition) at 32 weeks of age and intestinal epithelial barrier function (n = 8 per condition) at 20 weeks of age. (**I**) Inflammatory cytokine RNA abundance. (**J**) H & E-stained colon sections at 32 weeks of age. Graphs are representative of ten fields of view (four mice per condition). Scale bars: 100 μm. (**D–G**) Mice at 20 weeks of age. (**A** and **C**) n = 32 per condition. (**B** and **I**) n = 30 per condition. (**D–F**) n = 6 per condition. Error bars, means ± SEM. ***P < 0.001; **P < 0.01; *P < 0.05; one-way ANOVA with Tukey's multiple comparisons test (A) to (J).
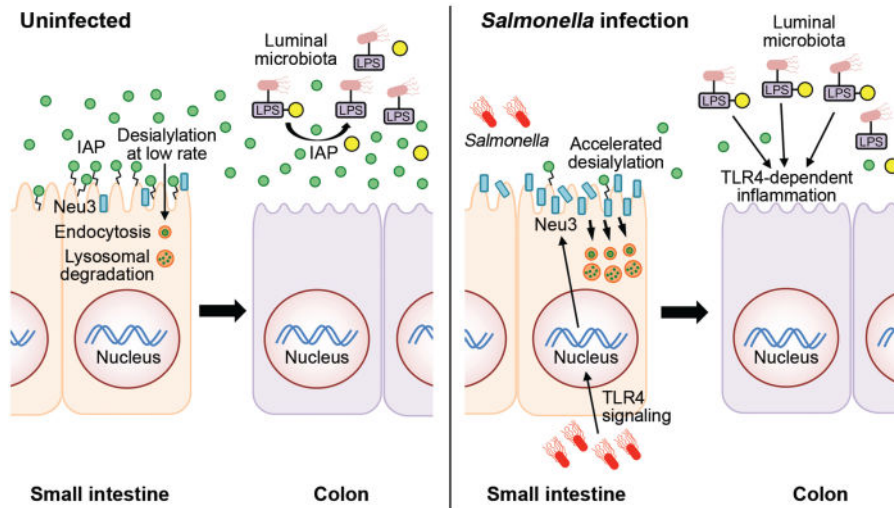
Fig. 8. Model of intestinal inflammation due to recurrent Gram-negative *ST* infection
In the absence of infection of the small intestine, the anti-inflammatory GPI–linked IAP glycoprotein (green circles) is highly expressed on the enterocyte cell surface. IAP is eventually released into the lumen and travels through the intestinal tract to the colon where it detoxifies LPS-phosphate produced by Gram-negative and commensal bacteria via dephosphorylation (yellow circles). Nascent IAP at the enterocyte cell surface undergoes a low rate of desialylation linked to the rate of internalization and degradation involving a normal mechanism of IAP aging and turnover. Enterocytes of the small intestine respond to LPS-phosphate and *ST* infection by activating Tlr4 function, which induces host Neu3 neuraminidase (blue bars) on the enterocyte surface. Increased neuraminidase activity accelerates the rate of IAP de-sialylation and internalization (orange circles), reducing IAP abundance and resulting in increased levels of LPS-phosphate in the colon where Tlr4 activation elicits inflammation and disease.