BACTERIAL COLONIZATION AND TH17 IMMUNITY ARE SHAPED BY INTESTINAL SIALYLATION IN NEONATAL MICE

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Running Title: ST6GAL1 alters the neonatal microbiome

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Abstract

Background: Interactions between the neonate host and its gut microbiome are central to the development of a healthy immune system. However, the mechanisms by which animals alter early colonization of microbiota for their benefit remain unclear. Host-derived carbohydrates, which can serve as metabolic substrates for the expansion of specific commensal and pathogenic bacteria, are one method by which the host may influence interspecies competition in the microbiome. Here, we investigated the role of early-life expression of the α2,6-sialyltransferase ST6GAL1 in microbiome phylogeny and mucosal immunity.

Methods: Fecal, upper respiratory, and oral microbiomes of pups expressing or lacking *St6gal1* were analyzed by 16S rRNA sequencing. Pooled fecal microbiomes from syngeneic donors were transferred to antibiotic-treated wild-type mice, before analysis of recipient mucosal immune responses by flow cytometry, RT-qPCR, microscopy, and ELISA. Intestinal sialylation was characterized by RT-qPCR, immunoblot, microscopy, and sialyltransferase enzyme assays in genetic mouse models at rest or with glucocorticoid receptor modulators.

Results: At weaning, the fecal microbiome of *St6gal1*-KO mice exhibited reductions in *Clostridiodes, Coprobacillus,* and *Adlercreutzia,* but increased *Helicobacter* and *Bilophila*. Transfer of *St6gal1*-KO microbiome induced a mucosal Th17 response, with expression of T-bet and IL-17, and IL-22-dependent gut lengthening. ST6GAL1 was found to be highly expressed in duodenal enterocytes between birth and weaning, driving temporary sialylation of the associated glycocalyx and secretion of active ST6GAL1 into the intestinal lumen. Expression was mediated by the P1 promoter of the *St6gal1* gene, which was efficiently inhibited by dexamethasone.

Conclusions: Intestinal sialylation by the sialyltransferase ST6GAL1 in the neonatal period is a developmentally regulated host mechanism coordinating bacterial colonization in the early gut microbiome. The inability to produce α2,6-sialyl ligands results in microbiome-dependent Th17 inflammation, highlighting a pathway by which intestinal epithelium regulates mucosal immunity. Considering the prevalence of intestinal fucosylation in adult animals, sialic acid may promote an early stage of ecological succession in the developing gut.

Trial registration: N/A

Introduction

The mammalian gastrointestinal tract is densely colonized by microbes that engage the host in a complex symbiotic relationship. During neonatal life, the gut microbiome transitions from the relative sterility of the womb to a diverse and stable community, reflecting increasing exposure to the environment[1]. Disruption of this process by antibiotics, caesarean delivery, or formula feeding results in long-lasting changes to the microbiome, the consequences of which can manifest both locally and systemically[2-5]. Commensal species are of particular importance, as their colonization directly competes with the growth of disease-causing pathogens and educates host immune cells to mount effect responses against viral, bacterial, and fungal pathogens throughout the body[5-12]. However, dysregulated mucosal immune responses can also fuel the pathogenesis of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease[13-16]. Thus, the determinants of colonization of the neonatal microbiome are integral to understanding health in early life and beyond.

Considerable variation in gut microbiota exists between individuals in different geographic locations, with multiple stable community structures conducive to human health[17-19]. Environmental flora and dietary intake are major contributors to the microbiome and can rapidly alter its structure and function[20-23]. However, twin studies have demonstrated that microbiome phylogeny is also at least partially heritable[24]. The mechanisms of gene-microbe crosstalk by which the host might influence bacterial colonization to safeguard its associated benefits remain poorly understood[25].

In the gastrointestinal tract, host-derived carbohydrates serve both as metabolic substrates and adhesive receptors for bacteria[26,27]. Whereas certain commensals independently catabolize host mucins, the liberation of sugars from host cells can also fuel the outgrowth of pathogenic

Salmonella and *Clostridiodes* species[28]. Conversely, specific monosaccharides such as fucose,

present within the host glycocalyx, can be upregulated by the host to resist colonization by

Salmonella and *Enterococcus*[29,30]. The incorporation of fucose by the FUT2 fucosyltransferase

is integrated into mucosal immune signaling, being directly regulated by IL-22 released by

lamina propria innate lymphoid cells[31]. Aside from fucose, sialic acid, a nine-carbon terminal

monosaccharide on deuterostome glycans, is a viable metabolic substrate for many bacterial

species[32,33]. An extensive body of literature demonstrates the functional role of leukocyte sialic

acid in adhesive interactions necessary for tissue infiltration, outside-in signaling triggered by

cytokines and growth factors, and cellular differentiation, underscoring its broad relevance in the

immune system[34-41]. Sialic acid moieties are present predominantly as terminal α2,6- or α2,3-

linked structures on glycoconjugates. α2,3-linked sialic acid, acquired by neonates as

oligosaccharides in breastmilk, promotes intestinal inflammation and the adhesion of pathogens

such as *Helicobacter pylori*[42,43]. Although less is known about α2,6-linked sialic acids in host-

microbe dynamics, genetic variation in the α2,6-sialyltransferase *ST6GAL1* is associated with

changes in the human gut microbiome[44]. Elsewhere, sialylation by ST6GAL1 is implicated in a

plethora of physiologic functions including inflammation[45], humoral immunity[46], cancer[47-50],

cellular survival[51,52], and chemo-/radio-resistance[53,54]. Emerging evidence continues to link

glycosylation of intestinal epithelium and its associated mucins with the severity of

gastrointestinal diseases, including inflammatory bowel disease and colorectal cancer[55-60].

However, the regulation and functional importance of host gut sialylation in mediating microbial

colonization and mucosal immunity remain unknown.

In this study, we utilized genetically engineered mouse models to determine the relevance of

α2,6-sialic acids synthesized by ST6GAL1 to microbiome colonization in early life. We found

that ST6GAL1 expression profoundly altered microbiome composition at the time of weaning,

enriching for *Clostridiodes, Coprobacillus,* and *Adlercreutzia* species while inhibiting the

colonization of *Helicobacter* and *Bilophila*. Fecal microbiome transfer experiments demonstrated that the disrupted microbiota of *St6gal1*-KO animals triggered local and systemic Th17 immune responses, which were partly dependent on activation of the aryl hydrocarbon receptor, and promoted epithelial hyperplasia downstream of IL-22. Consistent with its role in neonatal microbiome formation, we find ST6GAL1 is expressed within duodenal epithelium between birth and weaning due to glucocorticoid disinhibition of the P1 promoter during the neonatal stress hyporesponsive period (SHRP)[61,62]. Our results demonstrate a link between a developmentally programmed period in neonatal life and microbiome colonization that is mediated by host induction of an intestinal sialyltransferase, and shed light on possible implications of this process in mucosal immunity.

Materials & Methods

Animal Models: All animal models were approved by Roswell Park Institutional Animal Care and Use Committee under protocol 1071M. Wild-type mice (C57BL/6J) were purchased from Jackson Laboratory and regularly replenished by backcrossing. St6gal1-KO mice were generated as described previously and backcrossed onto a C57BL/6J background for at least 15 generations[63]. dP1 mice were generated in lab and extensively validated to have undetectable P1 transcripts of ST6GAL1 in the liver and reduced circulatory ST6GAL1 activity[64]. µMT mice were purchased from Jackson Laboratory, and µMT/St6gal1-DKO double knockouts were generated in multiple crossings between single knockouts, followed by genotyping and phenotyping analyses, as previously reported[39]. Standard housing conditions, which do not guarantee exclusion of specific pathogens, were used unless otherwise indicated.

<u>Antibodies:</u> anti-ST6GAL1 (R&D Biosystems), SNA-FITC (Vector Labs), anti-goat-Cy3 (Jackson ImmunoResearch), anti-IL-17-AlexaFluor488 (Invitrogen), anti-CD4-PE (GK1.5, Invitrogen), anti-CD3-PE (17A2, Biolegend), anti-CD4-BV510 (GK1.5, Biolegend), anti-CD8-biotin (53-6.7, eBioscience), streptavidin-PerCP/Cy5.5 (eBioscience), anti-FoxP3-AlexaFluor488 (MF-14, Biolegend), anti-IFN-γ-APC (XMG1.2, Biolegend), anti-IL-17A-PE/Cy7 (TC11-18410.1, Biolegend), anti-IL-22 mAb functional grade (IL22JOP, eBioscience), anti-CD11b-BV711 (M1/70, Biolegend), anti-Ly6G-APC (1A8, Biolegend).

<u>Immunofluorescence Microscopy:</u> Intestinal tissue was separated into small intestine (gastric pylorus to cecum) and colon (cecum to rectum). Mesenteric lymph nodes were isolated by careful dissection of mesenteric fat adjacent to the descending colon. All tissue was snap frozen before sectioning at 10μm thickness onto charged glass microscope slides. Tissue sections were fixed at -20C in acetone, then hydrated in PBS before blocking in 1% BSA for 1 hour. Sections were stained with anti-ST6GAL1 primary antibody and FITC-conjugated SNA lectin overnight, washed thoroughly with PBS, and stained with donkey-anti-goat-Cy3 secondary for 1 hour. For mesenteric lymph nodes, tissues were incubated with anti-IL-17-FITC and anti-CD4-PE overnight. All slides were washed extensively, rinsed with DAPI, air-dried, then mounted with cover slips in 10% glycerol. Images were captured with a Nikon Eclipse E600 microscope with EXFO X-cite 120 light source, Spot RT3 camera, and Spot Software.

<u>Sialyltransferase Assay:</u> Sialyltransferase activity within mouse tissues was determined using an artificial O-benzyl conjugated Gal-β1,4-GlcNAc acceptor, as has been described before[65]. Briefly, serum or lysed cells were incubated at 37C with artificial acceptor and tritium-labeled CMP-sialic acid for 1 hour. The resulting reaction mix was applied to a SepPak column and extensively washed, then eluted with methanol. Radioactive counts in the sample were quantified using a Beckman Coulter LS 6500 scintillation counter. α2,6-sialylated product was

precipitated with SNA-agarose beads, and SNA-reactive fraction once again counted to quantify α2,6-sialyltransferase activity. Remaining α2,3-sialyltransferase activity was inferred.

Glucocorticoid Experiments: To test the effect of exogenous glucocorticoids, mice at postnatal day 10 were given a single intraperitoneal injection of 5μg Dexamethasone (Sigma) in mineral oil, or vehicle only control. 24 hours after treatment, mice were euthanized, and tissues collected for histologic and RNA analysis. In other experiments, glucocorticoid receptor was blocked in adult mice (age 8-12 weeks) with three consecutive days of intraperitoneal injections of 40μg RU-468 (Sigma) in 50μl mineral oil or vehicle only. On day 4, mice were euthanized, and tissues collected for histologic and RNA analysis.

RNA Analysis: Tissue was preserved in Tri Reagent (MRC Inc.) at -80C prior to extraction. RNA extraction was performed in accordance with manufacturer instructions, and RNA concentration and purity quantified immediately. cDNA synthesis was performed with iSCRIPT cDNA synthesis kit (Bio-rad) on normalized amounts (0.5-2μg) of RNA, and RT-qPCR analysis performed with iQ SYBR-Green kit (Bio-rad). Primer sequences, melting temperatures, and references can be found in Figure S7. For liver and intestinal ST6GAL1 expression, cycles of amplification were normalized to β2-microglobulin or β-actin control, and relative expression (2^{dCT}) presented. For lamina propria gene expression, relative expression was normalized to WT microbiome transplant recipients (2^{ddCT}).

16S rRNA Sequencing: Mouse fecal pellets were collected fresh in sterile tubes. Oral microbiome samples represent anterior tongue tissue, collected immediately after sacrifice. Upper respiratory tract microbiome samples were collected immediately post-mortem by anterior dissection to the trachea and caudocephalad sterile saline lavage of the nasopharynx and nasal cavities, with the mouse being held in a supine position. 1ml of sterile saline was

used for lavage, and output collected from the nares. All samples were collected within 2 days of weaning and immediately stored at -80C.

The sequencing libraries were prepared using a two-step PCR method for targeting a ~500bp region of the 16S V3 and V4 rDNA. The first PCR (25-cycle) used 25ng of DNA to amplify the target region, where the PCR primers have overhang adapter sequence necessary for the second PCR step. After purification, the amplicon from the first stem is amplified with 8 cycles of PCR using the Nextera Index Kit (Illumina Inc.), which uses primers that target the overhang adaptor sequence added during the first round of PCR. The second round PCR adds one of 384 different combinations of indexed tags to each sample which allows pooling of libraries and multiplex sequencing. Prior to pooling, each individual sample's amplified DNA is visualized on a Tapestation 4200 D1000 tape (Agilent Technologies) for expected amplicon size, purity and concentration. Validated libraries are pooled equal molar in a final concentration of 4nM in Tris-HCl 10 mM, pH 8.5, before 2 x 300 cycle sequencing on a MiSeq (Illumina, Inc.).

Paired-end fastq reads were demultiplexed, processed and analyzed using QIIME v1.9.1. Operational taxonomic units (OTUs) were assigned using QIIME's uclust-based open-reference OTU-picking pipeline using Greengenes bacterial 16S rRNA reference (v13.8); bacterial sequences were aligned using PyNAST. These alignments were refined by removing chimeric sequences using ChimeraSlayer. OTUs with less than 0.001% assigned sequences were removed from each sample to avoid biased and inflated diversity estimates. Positive and negative control samples were checked against the whole batch and then removed from the data. Relative abundance bar plots were generated at the Class, Family and Genus levels. Results were summarized estimating alpha-diversity scores using inverse Simpson's diversity index. Phylogenetic composition plots at different taxonomic levels, sample-to-sample heatmaps and principal component analysis (PCA) plots were also generated.

Statistical analyses and comparisons were carried out using DESeq2 (v1.20.0) and phyloseq

(v1.26.0) packages from R (v3.5.0). This methodology implements a likelihood ratio test using a

generalized linear model assuming the outcome variable is Negative-Binomial distributed. Bi-

taxa plots were used to examine relevant OTUs, subsetting those having absolute fold-change

values greater than 2 (absFC > 2) and pval < 0.05. Bi-taxa plots are displayed pairing Phylum

(L2) vs Class (L3), Family (L4) and Genus (L5). K vs W and D vs U were compared to find

differentially abundant OTUs, accounting for animal sex, and summarized in a Venn Diagram at

Class, Family and Genus levels. Although initially excluded due to high sample variation,

Clostridiodes genus was significantly abundant in condition W compared to K (pval < 3.4e-8)

and U compared to D (pval < 1.3e-7).

Bacterial Quantification: At 21 days of age, fresh fecal pellets were collected in sterile tubes.

Pellets were digested in proteinase K-containing buffer at 55C overnight, precipitates removed

in 6M NaCl solution, and remaining DNA isolated by ethanol precipitation[66]. DNA yields were

quantified and a normalized 0.5ng of DNA used as template in qPCR analysis (SYBR Green,

Bio-rad). Eubacteria-specific primers were used to quantify total bacterial DNA, which is

presented relative to amplification of an unrelated host gene.

Fecal Microbiome Transplantation: At age 7-10 days, wild-type C57BL/6J mice were

administered a combination of vancomycin (0.5g/L;VWR), ampicillin (1g/L;Sigma), neomycin

(1g/L;VWR), and metronidazole (1g/L;Beantown Chemical) in acidified drinking water for 14

days. On day 15-17, fecal pellets from WT or St6gal1-KO mice of age 20-35 days were

collected (minimum n=7), vortexed for 3 minutes in sterile PBS, and administered to antibiotic-

pretreated mice by 50µl gastric gavage at 0.4mg/ml concentration. After the final transfer, mice

were euthanized between days 18-20 for analysis. Where indicated, mice were also

intraperitoneally administered 200µg AhR inhibitor CH-233191 (Sigma) in 25µl of mineral oil (or

vehicle control) prior to each FMT gavage. In IL-22 neutralization experiments, mice were

intraperitoneally administered 1.25µg of anti-IL22 neutralizing mAb per day (or isotype control)

between the initiation of transplantation and analysis.

Cytokine Quantification. Serum samples were diluted according to manufacturer's instructions to

fall within dynamic range of the specific ELISA assay. In all cases, recombinant cytokine

standards were utilized in duplicate to quantify the analyte within serum. Kits used include IL-1β,

IL-6, IL-10, IL-17 (all Invitrogen) and soluble RAGE (R&D).

Lamina Propria Isolation and Flow Cytometry. Small intestines were flushed of fecal matter,

then carefully stripped of adipose tissue and Peyer's patches. Guts were separated into

segments, inverted, and epithelial layer dissociated at 37C for 15 minutes using EDTA and DTT

containing buffer. Remaining submucosal tissue was further digested in collagenase-containing

solution at 37C for 30 minutes, filtered, washed, and cells enumerated. Cells were seeded at

4x10^6 cells/ml in 100µl RPMI with 10% FBS and stimulated for 5-6 hours with PMA and

ionomycin-containing cell activation cocktail (Biolegend). Cells were then collected and stained

for cell surface markers (CD3, CD4, CD8), and fixed for 20 minutes in 2% formalin. Washed

cells were permeabilized either with saponin-containing buffer (BD Cytoperm, BD Biosciences)

with antibodies for cytokines (anti-IFN-γ, anti-IL-17A) or with 0.1% Triton-X 100, followed by

anti-FoxP3 antibody in staining buffer. Cells were analyzed within 24 hours of animal

euthanasia.

Results

ST6GAL1 modifies the postnatal gut microbiome.

Comprehensive changes in the glycosylation of intestinal epithelium occur during neonatal life[67,68]. In particular, α2,6-sialylation of the small intestinal epithelium reaches a maximum during early postnatal life, followed by a sweeping transition from terminal sialylation to fucosylation of glycans after weaning[69,70]. However, the functional importance of this characteristic expression of sialic acid in the pre-weaned animal remains elusive.

We hypothesized that the presence of α2,6-linked sialic acid in the neonatal gastrointestinal tract, either due to endogenous enterocyte expression or the ingestion of breast milk ST6GAL1 and sialylated oligosaccharides, alters the microbiome at weaning. To test this, we performed 16S rRNA sequencing of fecal pellets collected immediately upon weaning. To control for mouse genomic background and genetic drift, we performed parallel comparisons of *St6gal1+/+* and *St6gal1-/-* mice on wild-type (C57BL/6J) and B cell-deficient *Ighm-/- (*µMT.B6) backgrounds. To control for environmental factors, mice were provided identical cages, food and water. Poisson analysis and principal component analysis demonstrated distinct clustering of mice microbiomes by genotype, suggesting that community heterogeneity could be partially attributed to host genotype at the *Ighm* and *St6gal1* loci (*Fig 1a*). In particular, variation due to ST6GAL1 status was captured in the first principal component, representing 31.9% of total variation (*Fig 1a*). Original taxonomic units (OTU) were assigned to known taxonomic groups based on sequence identity (*SUPP Fig 1*). Statistically significant ($p_{adj}<0.05$) differences with a minimum 2-fold difference in means (FC>2) were identified between WT and *St6gal1-KO*, or µMT and µMT/*St6gal1-DKO* microbiomes at the class, family, and genus taxonomic levels (full comparisons in SUPP Fig 2). Although numerous specific differences in microbial phylogeny were observed, ST6GAL1 expression did not significantly alter global microbiome diversity (*Fig 1b*). Parallel analyses of the upper respiratory tract microbiome (obtained by caudocephalad saline flush of trachea and nasopharynx) and oral microbiome (obtained by tongue tissue) from

mice at the same age revealed only weak and inconsistent clustering of samples by host genotype, highlighting that the influence of ST6GAL1 on the microbiome was restricted to the gastrointestinal system (*SUPP Fig 3,4*).

Previous studies have identified multiple fecal microbiome enterotypes in healthy adult humans, each representing stable community structures characterized by the high abundance of a single bacterial genus adapted to specific host and environmental factors[71]. To understand whether expression of ST6GAL1 was associated with the described microbiome enterotypes, we analyzed the levels of enterotype-defining genera *Bacteroides, Prevotella,* and *Ruminococcus* in WT and *St6gal1-KO* mice. *St6gal1-KO* mice harbored significantly elevated levels of *Ruminococcus* OTUs, with a corresponding trending increase in *Bacteroides* and trending decrease in *Prevotella* OTUs (*Fig 1c*). These results are consistent with a potential role for ST6GAL1 in promoting the type 2 enterotype, characterized by a higher abundance of *Prevotella* species, efficient thiamine biosynthesis, and catabolism of fiber and host-derived mucins[22,72].

To identify reproducible changes in specific OTUs associated with ST6GAL1 expression, we identified statistically significant (FC>2, $p<0.05$) differences between comparisons of WT and *St6gal1-KO* mice on C57BL/6J and B6.μMT backgrounds and limited our analysis to OTUs that exhibited similar changes in both comparisons (SUPP FIG 5). We first noted that ST6GAL1 expression was strongly associated with the presence of two Firmicutes genera, *Coprobacillus* and *Clostridiodes* (*Fig 1d, left*). Mice lacking ST6GAL1 had virtually no detectable reads assigned to either of these closely related OTUs. We also observed that abundance of the equol-producing Actinobacteria genus *Adlercreutzia* was reduced in ST6GAL1 deficient animals (*Fig 1d, right*). In contrast, several Proteobacteria genera were expanded in ST6GAL1-deficient animals, including *Helicobacter* and *Bilophila* OTUs (*Fig 1e*).

ST6GAL1 deficiency triggers a Th17 response via the microbiome.

Commensal and pathogenic gastrointestinal microbes can have profound effects on the education and activation of mucosal immune cells. The neonatal period is hypothesized to be a critical window during which both protective anti-microbial responses and tolerance of gut commensals develop[1,5]. Given the influence of ST6GAL1 on microbiome phylogeny during a period of early bacterial colonization, we hypothesized that expression of ST6GAL1 may alter the mucosal immune response by selective pressure on certain bacterial species. To test this, we reconstituted antibiotic-depleted, genetically identical neonatal wild-type mice with fecal microbiome from either WT or *St6gal1-KO* donors. To deplete endogenous microbiota, neonatal WT mice were administered a broad-spectrum antibiotic mix (vancomycin, neomycin, ampicillin, metronidazole) starting at postnatal day 7-10 for two weeks to deplete intestinal colonization, as has been reported elsewhere[73]. One day after the cessation of antibiotics, mice were given 50μl of homogenized fecal pellets (normalized to 40mg/ml) in sterile PBS by oral gavage, once per day for three consecutive days (*Fig 2a*). In our hands, antibiotic treatment depleted detectable fecal bacterial DNA to ~1% of native levels, and fecal microbiome transplantation restored pre-depletion levels, with no significant difference between mice receiving WT or *St6gal1-KO* feces (*Fig 2b*). qPCR analysis of reconstituted fecal pellets demonstrated that KO to WT fecal transplantation reproduced the elevated levels of *Helicobacter* seen in *St6gal1-KO* mice (*Fig 2c*). Overall mouse weights remained unchanged after fecal transplantation (SUPP Fig 6a). Given the well-described role of *Helicobacter* species as pathogens in both humans and rodents, we hypothesized that transplantation of the fecal microbiome of *St6gal1*-KO mice would induce an anti-bacterial immune response at the mucosa. In order to capture changes in immune cell polarization and cytokine production induced by fecal transplantation, mice were euthanized 1-2 days post-transplantation and small intestinal lamina propria cells isolated for

flow cytometry. Our analysis of several major hematopoietic cell types, including total myeloid cells, neutrophils, total T cells, as well as CD4+ and CD8+ T cell subsets, revealed no significant differences in abundance (SUPP Fig 6b-c). However, we noted a striking enrichment in IL-17 producing CD4+ T cells in mice receiving *St6gal1-KO* microbiota (*Fig 2d*). In contrast, levels of FoxP3+ Tregs or IFN-γ producing Th1 cells were not significantly altered. The induction of a Th17 phenotype is thought to result from production of IL-1β and IL-6, which skew undifferentiated CD4+ T cells towards expression of ROR-γt, resulting in "non-pathogenic" Th17 cells that express IL-17 and IL-10[74]. Antigen-presenting dendritic cells produce pro-inflammatory IL-23, leading to further differentiation of Th17 cells into a "pathogenic" phenotype[75,76]. A major mechanism by which IL-17 production and the Th17 phenotype is stabilized in the gut lamina propria is by dietary and microbial tryptophan metabolites that engage the aryl hydrocarbon receptor[77,78]. When we administered the AhR inhibitor CH-233191 concurrently with fecal microbiome transplantation, the enrichment of Th17 cells within the lamina propria of *St6gal1-KO* FMT recipients was completely abrogated (*Fig 2e*). In order to gain a comprehensive understanding of the immune pathways activated by *St6gal1-KO* microbiota, we analyzed small intestinal lamina propria RNA by qPCR. In our analysis, Treg-associated immunosuppressive genes (*FoxP3, Il10, Tgfb1*) and Th2-associated *Gata3* were unaltered by the *St6gal1-KO* microbiome (*Fig 2f*). Meanwhile, several Th1 and Th17-associated genes (*Tbx21, Il17a, Il22*) were markedly upregulated, with related genes trending but not significantly increased (*Rorc, Il23*) (*Fig 2f*). Downstream epithelial genes associated with Th17 pathways (*Reg3b, Reg3g*) and inflammatory cell recruitment (*Il6, Cxcr2, Nos2*) were not altered in our experiments (*Fig 2f*). Collectively, these data suggest that *St6gal1-KO* microbiota induce a local Th17 immune response, in part by activation of the aryl hydrocarbon receptor.

To understand whether this Th17 response was a localized or systemic change, we analyzed the draining mesenteric lymph nodes, spleen, and blood of recipient animals. Within the

mesenteric lymph nodes, we noted an increase in CD4+ T cells staining positive for IL-17 in mice receiving *St6gal1-KO* microbiota, suggesting a parallel process to the mucosa was occurring in a secondary lymphoid organ (*Fig 2g, yellow cells*). To understand if the IL-17 response had spread systemically, we analyzed the RNA levels of *Il17a* within the spleen. Here as well, mice receiving *St6gal1-KO* microbiota expressed significantly increased levels of IL-17a mRNA (*Fig 2h*). Finally, analysis of serum cytokines indicated that although KO recipients did not exhibit increased systemic inflammation, as evidenced by pro-inflammatory cytokines IL-1β and IL-6, or anti-inflammatory cytokine IL-10, they did have greatly increased blood IL-17A (*Fig 2i*). Other tested serum factors are shown in SUPP Fig 6e-f. Collectively, these results demonstrate that a genetic inability to express the sialyltransferase ST6GAL1 results in gut microbiome changes, which induce a systemic enrichment of IL-17 and IL-22 producing Th17 cells.

In contrast to IL-17, which primarily functions to recruit neutrophils and stimulate the production of IL-6, GM-CSF, G-CSF, and IL-1, IL-22 primarily acts upon epithelial cells and can have a strong pro-survival and pro-proliferative effect[79-81]. In line with this, we also observed that mice receiving *St6gal1-KO* microbiota developed a longer gastrointestinal tract within days of treatment (*Fig 3a*). This difference in length was present both in the small intestine and colon (*Fig 3b*). Consistent with a microbiome-dependent mechanism, administration of the AhR inhibitor CH-233191 equalized intestinal lengths between treatment groups (*Fig 3c*). Finally, to test whether gut lengthening was dependent on Th17-dependent production of IL-22, we administered an anti-IL-22 neutralizing antibody during microbiome transplantation, which was also able to normalize the changes in gut length (*Fig 3d*). These results suggest that the microbiome of ST6GAL1-deficient mice induces a Th17 response dependent on AhR activation, which promotes a lengthening of the gastrointestinal tract by IL-22-dependent epithelial proliferation.

Expression of ST6GAL1 in the neonatal intestine.

Although our findings demonstrate a previously unknown function of ST6GAL1 in altering the colonization of bacteria in the pre-weaning period, the specific cell types responsible for ST6GAL1 expression in this period, as well as its mechanisms of regulation, remained unclear. We analyzed the expression of *St6gal1* mRNA in various segments of intestinal tract in pre-weaned (post-natal day 10, 19) and weaned (post-natal day 44) mice. In suckling mice, *St6gal1* mRNA in the duodenum was significantly higher than in the ileum and colon (*Fig 4a*). In contrast, intestinal *St6gal1* expression shifted distally to the jejunum and ileum in adult mice (*Fig 4a*). At all times examined, there was minimal evidence of *St6gal1* expression in the colon. Using primers specific for the 5'-untranslated regions (5'UTR) of *St6gal1*[82], we found that both expected tissue-specific P1 and P3-dependent transcripts contributed to duodenal *St6gal1* expression (primer sequences in SUPP FIG 7). Notably, P1-dependent *St6gal1* transcripts were limited to the duodenum, where they were upregulated over 20-fold compared to more distal portions of the intestine. P3-dependent transcripts, thought to exist constitutively at low levels in most cells and tissues, were similarly present in the duodenum to a lesser extent (*Fig 4b*)[82]. Cell lysates were collected from duodenum, jejunum, ileum, and colon at similar times and subjected to immunoblot analysis for ST6GAL1 protein. At postnatal day 13 (PND13), ST6GAL1 protein levels were highest in the colon, possibly due to the ingestion of enzyme from maternal-derived milk and its accumulation in the distal GI tract, as ST6GAL1 is abundantly expressed in colostrum from lactating mammary glands[83]. By postnatal day 19, an ST6GAL1 surge was noted in the duodenum, jejunum, and ileum, consistent with endogenous mRNA levels (*Fig 4c*). Interestingly and quite unexpectedly, by adulthood (postnatal day 44), the vast majority of ST6GAL1 protein was found in colon (*Fig 4c*).

Expression of ST6GAL1 occurs in diverse cell types, including those of the epithelial, endothelial, mesenchymal and hematopoietic lineages, all of which are present within the small intestine[84-86]. To understand which cell types were responsible for the neonatal spike of *St6gal1* transcripts, we employed a histology approach comparing the small intestines of wild-type, global *St6gal1-KO*, and *dP1* pups (which lack the promoter to generate the P1-transcripts for *St6gal1*). To analyze the morphologic distribution of sialic acid, we used SNA, a lectin from *Sambucus nigra* that specifically recognizes the α2,6-sialyl product of ST6GAL1. Consistent with our previous observations, we noted very high SNA reactivity within the submucosa, muscularis layers, and adventitia, which was partially attenuated in *dP1* mice and virtually absent in global *St6gal1-KO* mice (*Fig 4d*). This SNA reactivity increased between postnatal day 1 and 12 but remained constant in intensity thereafter. In contrast, the small intestinal epithelium exhibited maximal ST6GAL1 staining at postnatal day 12, with corresponding SNA reactivity, which was not detected after weaning on postnatal day 24 (*Fig 4d*). Epithelial ST6GAL1 was not evident in *St6gal1-KO* mice and was reduced in mice lacking the P1 promoter (*Fig 4d*). However, epithelial SNA reactivity in *dP1* pups was similar to full knockouts, confirming that P1-dependent ST6GAL1 expression is a major determinant of epithelial sialylation (*Fig 4d*).

α2,6-sialyltransferase activity towards Gal-β1,4-GlcNAc acceptors, indicative of ST6GAL1 and ST6GAL2, was quantified in total intestinal cell lysates. To control for potential ST6GAL2 activity, St6gal1-KO tissues were used as a negative control for all samples (not shown). In contrast to the RNA and protein expression data, we were unable to detect any spike in sialyltransferase activity in the neonatal duodenum of suckling pups, with detectable but minimal activity downstream in the jejunum and ileum. However, the colon exhibited strikingly high α2,6-sialyltransferase activity, increasing nearly five-fold between postnatal day 13 and 44 (*Fig 4e*, top). Furthermore, α2,6-sialyltransferase activity was detectable in the homogenized fecal pellets of suckling WT, but not age-matched *St6gal1*-KO or dP1 mice, nor adult WT mice (*Fig*

4e, bottom). Together, these observations further suggest that ST6GAL1, though expressed proximally in the duodenum, accumulates in the colon of suckling mice.

Rodents undergo a period of stress hyporesponsiveness between birth and weaning, during which blood adreno-corticotropic hormone (ACTH) and glucocorticoid levels remain low and unresponsive to most stressors. Upon weaning, the stress of maternal separation triggers a surge of glucocorticoid production that drives the maturation of both pulmonary and gastrointestinal tissues[62,87]. Given the significant downregulation of ST6GAL1 expression in the proximal small intestine upon weaning, we hypothesized that glucocorticoid sensitivity was involved in its expression. First, we attempted to inhibit neonatal ST6GAL1 expression by providing an early bolus of systemic glucocorticoids to neonatal mice. Postnatal day 10 pups were given a single dose of intraperitoneal dexamethasone and analyzed after one day for changes in expression of *St6gal1* at the RNA level. We observed that dexamethasone reduced duodenal transcripts ~4-fold, attributable to a reduction of transcripts expressed under the P1, but not P3 promoter (*Fig 4f*, left). Downregulation of ST6GAL1 in the proximal small intestine was also observed by immunofluorescence microscopy (*Fig 4f, right*). To understand if endogenous glucocorticoid-mediated repression was involved in the low levels of intestinal ST6GAL1 expression in adults, we treated 6-8 week old adult mice with the steroid receptor inhibitor RU-486 for 3 successive days, then analyzed ST6GAL1 expression within intestinal tissues. Total *St6gal1* transcripts were elevated within the duodenum and jejunum, largely attributable to an increase in P1-dependent transcripts (*Fig 4g*). Although the inherent variability in this system precluded statistical significance, our results indicate that ST6GAL1 expression in the neonatal period is likely secondary to glucocorticoid disinhibition during the stress hyporesponsive period.

Discussion

The neonatal period represents a time of rapid evolution for the microbiome. Central to these changes is the balance between symbionts and pathogens, mediated by complex host and environmental factors that are poorly understood. Our results implicate ST6GAL1 as a previously unrecognized genetic factor facilitating the colonization of specific microbes of the Firmicutes phylum. The scavenging of host sialic acid by pathogenic and commensal bacteria for catabolism relies on the expression of three genes of the Nan cluster (NanA, NanK, and NanE), as well as the deacytelase NagA and deaminase NagB[33,88]. Presence of the Nan cluster within bacterial genomes is limited to species of the Gamma-Proteobacteria and Fusobacteria phyla, as well as Bacillales, Clostridioides, and Lactobacillales of the Firmicutes phylum[89,90]. In our data, colonization by bacteria of the genus *Clostridiodes* was completely dependent on host expression of ST6GAL1, consistent with the expansion of pathogenic *Clostridioides* species in response to sialic acid liberated by other environmental microbes observed by others[28]. Spore-forming bacteria such as Clostridia dramatically expanded in abundance in the human gut after the first year of life, consistent with their ability to tolerate the increasingly anoxic conditions of the developing colon[91]. In contrast to adults, wherein certain *Clostridiodes* species may cause infection, the accumulation of *Clostridioides* in the early postnatal period directly competes with adhering/effacing pathogens, protecting mice from life-threatening infection[92]. Our findings also indicate that α2,6-sialyl ligands support the key isoflavone-producing species *Adlercreutzia*, the presence of which is associated with improved blood lipids and reduced diet-induced obesity[93,94]. To the best of our knowledge, there have not been any published studies of the ability of the genus *Adlercreutzia* to metabolize sialic acid[95-97]. We also show an association between ST6GAL1 expression and reduced colonization of *Helicobacter* and *Bilophila* species, which are collectively implicated in the pathogenesis of infection, cancer, and metabolic dysfunction[98-100]. Interestingly, free sialic acid has been shown to have anti-*Helicobacter*

properties both *in vitro* and *in vivo*[101-106]. Bile-metabolizing bacteria of the *Bilophila* genus, which expand in response to dietary fat intake, were recently identified as pathobionts, capable of promoting colitis[99,100]. Overall, our data support a model that both agrees with and expands upon the existing literature on milk-derived glycans, highlighting a health-promoting role for ST6GAL1 in altering the balance of commensals and pathogens colonizing the neonatal gut[107]. Our microbiome data do not distinguish between the effects of α2,6-sialyl glycans in milk oligosaccharides and gut glycocalyx, but future experiments using a combination of cross-fostering and tissue-specific knockouts may shed light on the relative importance of these two pathways. Neither do we here investigate whether the described microbiome changes persist into adult life. However, our previous findings of neutrophilia and inflammatory tissue damage in *St6gal1*-deficient mice allow for the possibility that microbiome-related processes may still contribute to a tendency towards inflammation in these animals[36,64,108-112].

As a fundamental metabolic substrate, carbohydrates shape microbial fitness within the gastrointestinal ecosystem[113,114]. Host-derived glycans exist both on the plasma membrane glycocalyx and mucins secreted into the extracellular space[114]. The glycosylation of major mucins such as Muc2, along with host expression of associated glycosyltransferases, varies along the intestinal tract in a regiospecific and microbiome-dependent manner[115]. Complete normalization of intestinal mucus layers requires at least 6 weeks of microbial colonization, with stereotyped shifts in major bacterial populations that may mimic those seen during initial colonization of the neonatal intestine[116]. At weaning, a surge in microbial exposure activates IL-22 production, which promotes expression of glycocalyx-associated MUC17, along with a global transition from sialylated to fucosylated glycans in the intestinal mucosa[67,117]. Fucosylation of enterocytes prevents the expansion of disease-causing pathobionts, as part of a dynamic paracrine circuit regulated by type 3 innate lymphoid cells[29,30]. As the immune system matures, infiltrating adaptive CD4+ Treg and Th17 cells gradually dominate the symbiotic relationship

with commensal species, counteracting ILC3s to normalize lipid metabolism in enterocytes[118].

Our findings add to the understanding of the development of IL-17-dependent responses in the

neonate, underscoring a role for ST6GAL1 in preventing Th17 accumulation in the gut prior to

weaning. This is achieved by the careful calibration of bacterial colonizers during the suckling

period, prior to the wholesale changes brought about by an adult diet. Surprisingly, despite high

ST6GAL1 expression in the duodenum, the majority of ST6GAL1 protein and α2,6-

sialyltransferase activity accumulates distally within the colon, consistent with previous studies

showing detectable ST6GAL1 protein in the colon[115]. These observations are important in

explaining how neonatal ST6GAL1 expression in the proximal gastrointestinal tract might alter

microbiome composition in the distal gastrointestinal tract. Furthermore, the disproportionately

high α2,6-sialyltransferase activity in the colon of adult mice (~100-fold higher than upstream

tissues) may indeed be attributable to a previously reported protein co-factor of ST6GAL1 in that

tissue[119].

Glucocorticoids are widely recognized for their importance in lung and gut epithelium

maturation[120,121]. Previous studies have documented the ability of glucocorticoids to upregulate

enzymes involved in dietary carbohydrate metabolism, as well as *Fut2*, effecting an increase in

fucosylation during the post-weaning period[122-124]. In early works, we noted that transcription of

St6gal1 was mediated by a number of distinct promoter/transcription initiation regions[83,125,126].

Others have shown that intestinal ST6GAL1 is upregulated in the presence of microbes and

downregulated during colitis and in response to a high protein diet[115,127]. In our previous studies,

transcription of *St6gal1* in the neonatal intestine was associated with expression from the P1

promoter, previously known only to be utilized by the liver[128]. Here, our data expand on earlier

findings and indicate that glucocorticoids also potently inhibit ST6GAL1 expression via the P1

promoter, placing ST6GAL1 among a suite of other investigated intestinal enzymes whose

expression levels are linked to serum glucocorticoids[129-131]. Ultimately, glucocorticoids likely play

a central role in the transition from sialylated to galactosylated and fucosylated glycans in the intestinal epithelium that occurs with weaning[132]. Although not tested in this study, we hypothesize that this surge of glucocorticoid production and sensitivity is most likely triggered by the maternal separation of weaning, terminating the neonatal period of stress hyporesponsiveness[62].

In humans, *Helicobacter pylori* is regarded as a carcinogen for its role in the pathogenesis of gastric adenocarcinoma and MALT lymphoma[133]. In mice, *Helicobacter hepaticus* is a pathogen that causes hepatitis, colitis, and colorectal cancer[134]. Several lines of evidence point to a central role for bacteria of the *Helicobacter* genus in our data. A greater abundance of *Helicobacter* in ST6GAL1-deficient animals is consistent with a number of previous reports demonstrating anti-Helicobacter activity of soluble sialic acid preparations by at least two independent mechanisms. Firstly, sialic acid has direct antibacterial properties towards *Helicobacter* species both *in vitro* and *in vivo,* an effect that is augmented by co-administration of antioxidant catechins[103-106]. Secondly, sialylated gastrointestinal mucins, particularly those of the stomach, act as decoys for *Helicobacter* strains expressing the sialic acid binding SabA adhesin by competing with sialylated structures in the gastric epithelial surface necessary for initiating infection[135-138]. Physiologically, it remains a matter of speculation whether neonate-derived or maternal-derived free sialic acid is more important in preventing *Helicobacter* infection. Although a physiological role for milk-derived sialic acid in protecting the neonate from *Helicobacter* colonization is plausible, human studies in multiple populations have failed to demonstrate any association between breastfeeding and *Helicobacter* colonization[139-142]. *Helicobacter* infection induces a robust but plastic CD4+ T cell response with both Th1 and Th17 characteristics, characterized by expression of T-bet and ROR-γt, as well as by production of IFN-γ, IL-17, and IL-22[143]. Ensuing inflammation often targets other bacterial species and is associated with increased tissue damage in both humans and mice[144,145]. In our experiments,

fecal transplantation of *St6gal1*-KO microbiome recapitulated an elevated abundance of

Helicobacter DNA and provoked a local and systemic Th17-mediated immune response within

days, consistent with the initial stages of *Helicobacter* infection. Interestingly, we show that the

Th17 response is highly dependent on AhR engagement in our model, paralleling findings that

Ahr-deficient mice develop rectal prolapse associated with uncontrolled *H. hepaticus*

infection[146]. During persistent infection, *Helicobacter pylori* often achieves immune escape by

inducing tolerance after an early failure of Th17-mediated pathogen clearance, a process

dependent on dendritic cells and c-Maf+ regulatory T cells[147,148]. Our findings add to existing

literature by demonstrating the importance of early life expression of ST6GAL1 in reducing the

colonization of *Helicobacter* pathogens. In addition to the possibility of using oral sialic acid as a

treatment for *Helicobacter* infections, our data hint at the importance of the neonatal stress

hyporesponsive period in ensuring appropriate microbial colonization in the gut.

Conclusions: Intestinal sialylation by the sialyltransferase ST6GAL1 in the neonatal period is a

developmentally regulated host mechanism coordinating bacterial colonization in the early gut

microbiome. The inability to produce α2,6-sialyl ligands results in microbiome-dependent Th17

inflammation, highlighting a pathway by which intestinal epithelium regulates mucosal immunity.

Considering the prevalence of intestinal fucosylation in adult animals, sialic acid may

promote an early stage of ecological succession in the developing gut.

Declarations:

Ethics approval: This manuscript does not report studies involving human participations, human data, or human tissues.

Consent for publication: All authors consent to publication of the submitted manuscript and vouch for the integrity of data presented within. There are no individual person's data in any form.

Availability of data and materials: All data generated or analysed during this study are invluded in this published article and its supplementary information files.

Competing interests: The authors declar that they have no competing interests

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Figures
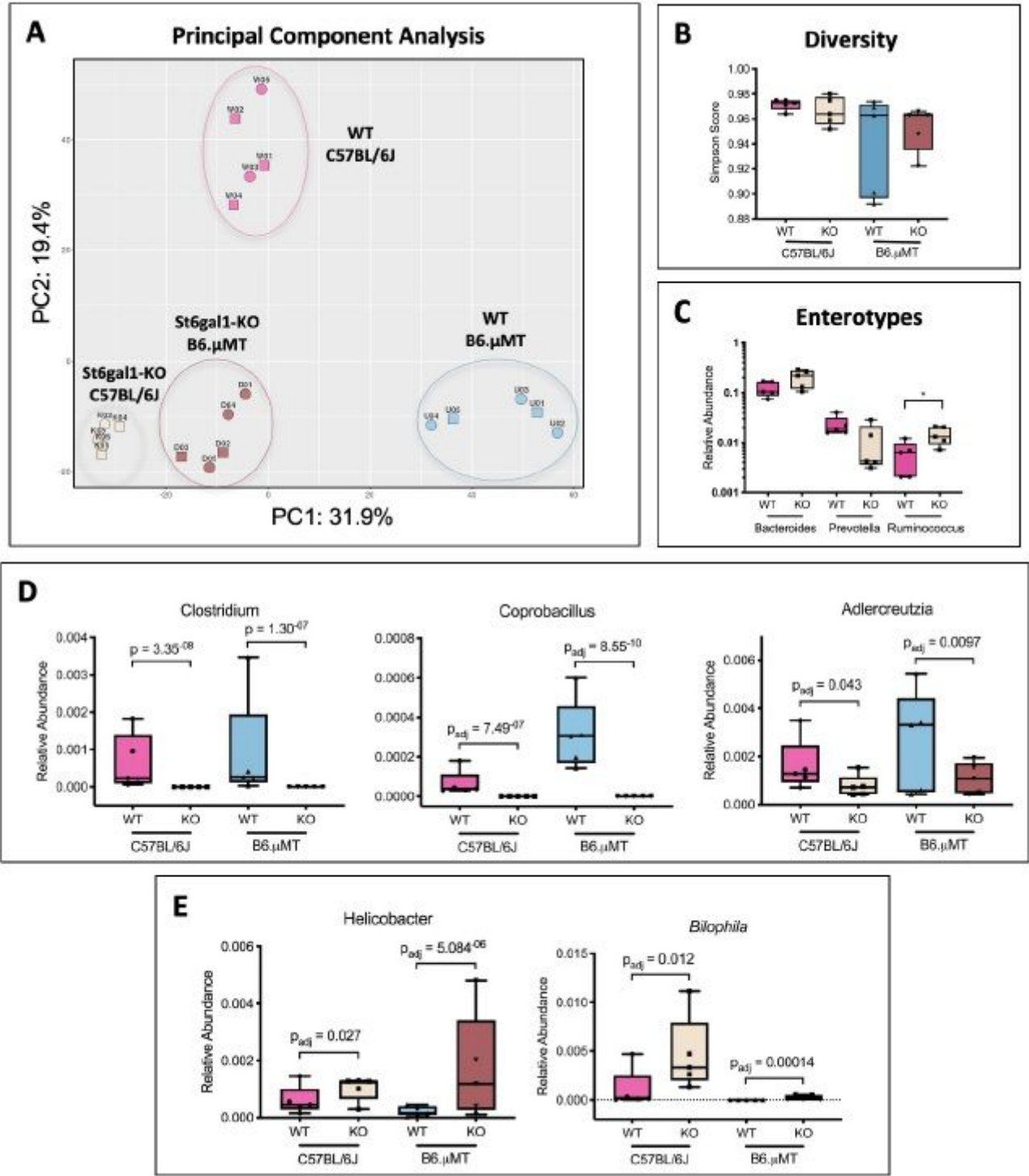
Figure 1

ST6GAL1 Influences Neonatal Fecal Microbiome Composition. Fecal pellets were collected from mice of indicated genotypes upon weaning and extracted DNA subjected to 16S rRNA sequencing. (A) Principal components analysis of 16S sequencing data for ST6GAL1-sufficient and deficient mice on C57BL/6J and B6.µMT backgrounds. (B) Simpson diversity score for microbiomes derived from indicated

genotypes. (C) Relative abundances of Bacteroides, Prevotella, and Ruminococcus genera, indicative of enterotypes observed in the human microbiome. (D) OTUs assigned to Clostridioides, Coprobacillus, and Adlercreutzia genera were enriched in ST6GAL1-expressing animals.(E) OTUs assigned to Helicobacter and Bilophila were depleted in ST6GAL1- expressing animals.
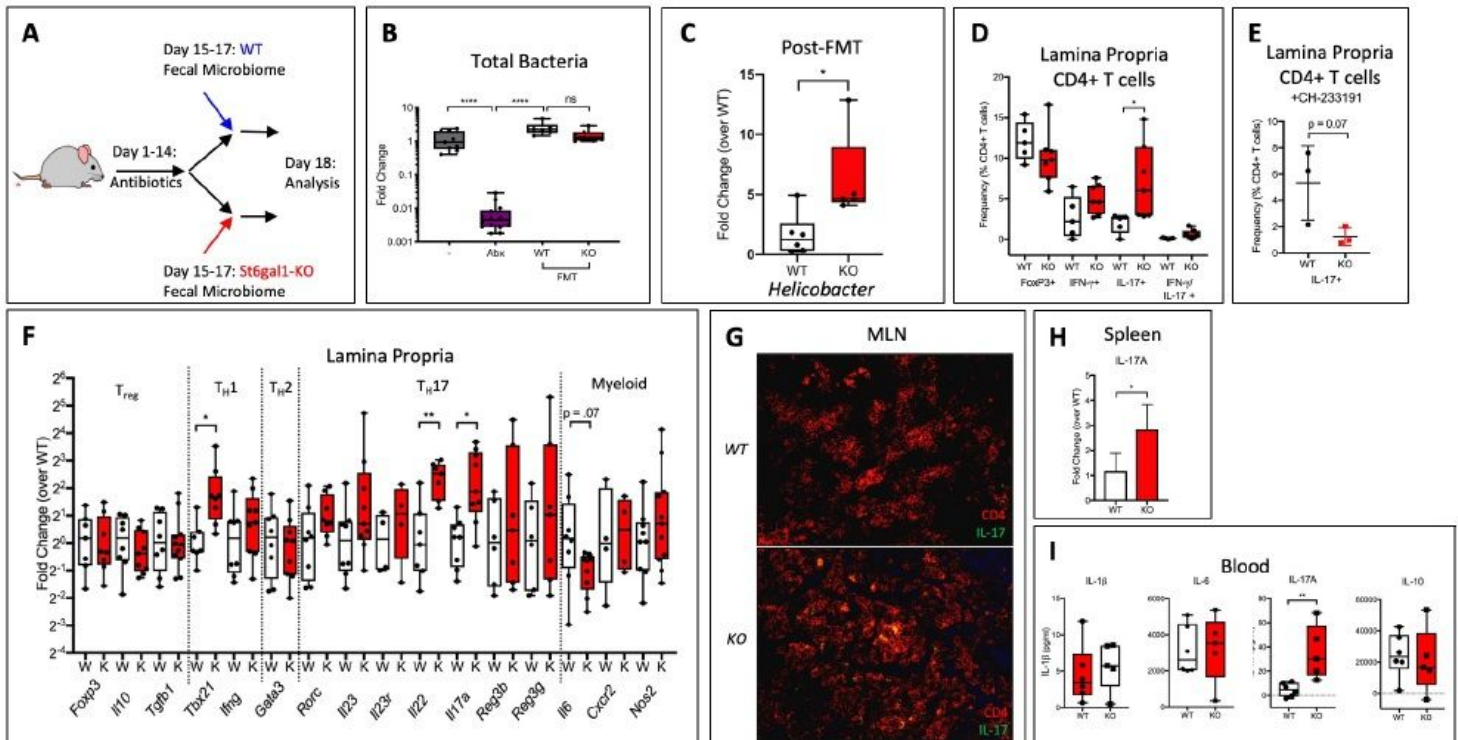
Figure 2

ST6GAL1 Deficiency Promotes Microbiome-dependent Local and Systemic Th17 Responses. (A) Wild-type mice were treated for 7 days with antibiotics (ampicillin, vancomycin, metronidazole, neomycin) in drinking water, then given fecal microbiome transplants (FMT) with either WT or St6gal1-KO feces from postnatal day 20-35 donor mice. After 4-8 days, mice were sacrificed for analysis. (B) Depletion of fecal microbiome by antibiotics and restoration by FMT. (C) Reconstitution of elevated Helicobacter prevalence in mice receiving St6gal1-KO microbiome. (D) Quantitation of frequency of CD4+ T cell subsets with indicated fecal microbiome transfer donor genotype. (E) Treatment with CH-233191 depletes Th17 cell increase induced by St6gal1-KO microbiome transfer. (F) qPCR analysis of total lamina propria cells between mice receiving WT or St6gal1-KO fecal microbiome. (G) Immunofluorescence staining of mesenteric lymph nodes for CD4 (red) and IL-17 (green). (H) qPCR analysis of IL-17A expression within the spleen. (I) Serum analysis of indicated cytokines after FMT.
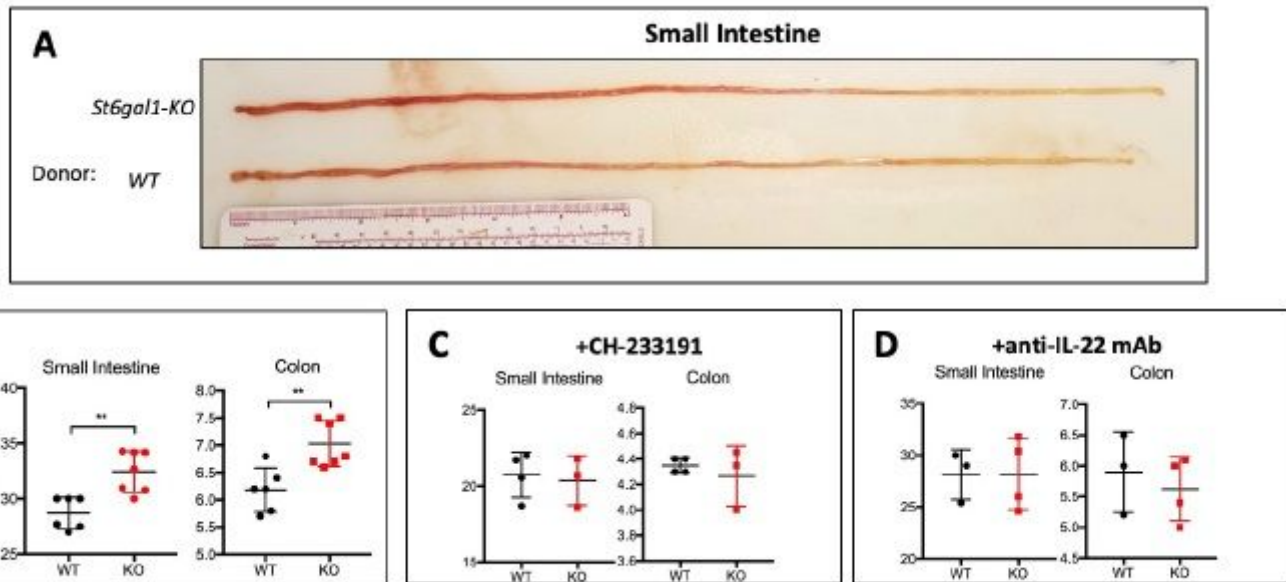
Figure 3

ST6GAL1 Deficient Microbiome Promotes Gut Lengthening via an AhR/IL-22 Pathway. (A) Representative image of small intestine from FMT recipients at time of sacrifice. (B) Length of small intestine and colon between mice receiving WT or KO FMT, with or without concurrent administration of (C) CH-233191 or (D) neutralizing anti-IL-22 mAb.
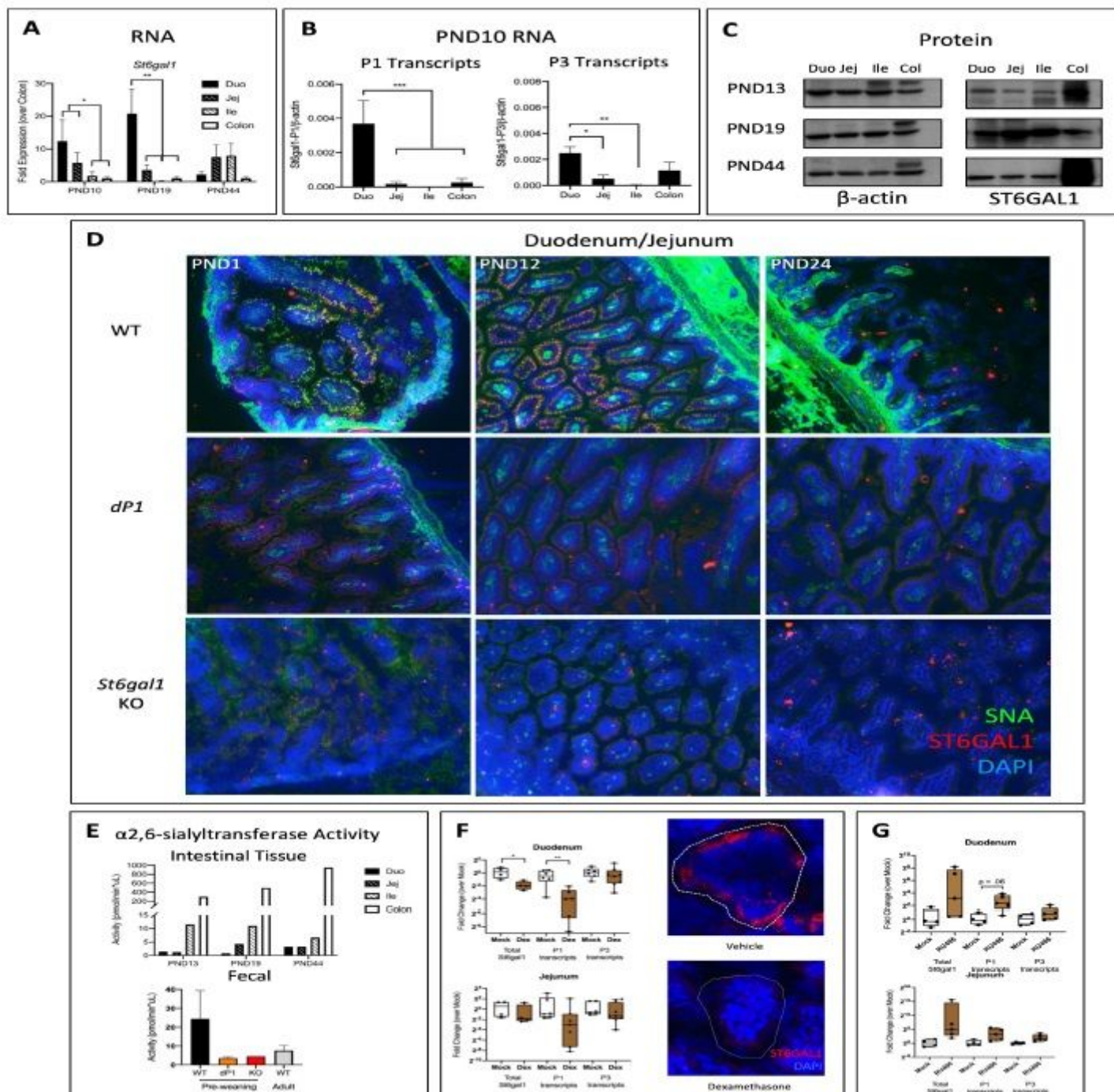
Figure 4

ST6GAL1 Expression in the Neonatal Duodenum is Mediated by the P1 Promoter and Inhibited by Glucocorticoids. (A) Relative expression of St6gal1 transcripts within the duodenum, jejnum, ileum, and colon on postnatal days (PND) 10, 19, and 44. (B) Relative abundance of P1-dependent and P3-dependent St6gal1 transcripts on postnatal day 10. (C) Immunoblot for ST6GAL1 from total tissue of duodenum, jejunum, ileum, and colon on postnatal days 13, 19, and 44. (D) Frozen sections of mouse total small intestine tissue at postnatal days 1, 12, and 24 were stained for ST6GAL1 (red) and with Sambucus nigra lectin (green). Comparison of wild-type (WT), P1 promoter conditional knockout (dP1),

and global St6gal1 KO mice is shown. (E) α2,6-sialyltransferase activity in indicated WT gastrointestinal tissues at indicated ages (above) and in fecal pellets at 10d of age (below). (F) Postnatal day 10 mice were given a single bolus of intraperitoneal dexamethasone, then sacrificed after 24 hours and total RNA levels of total, P1-specific and P3-specific St6gal1 transcripts quantified in duodenum and jejunum (left). Proximal small intestine was stained for ST6GAL1 protein in vehicle and dexamethasone-treated PND15 mice (right). (G) Adult WT mice were treated with intraperitoneal RU-486 or vehicle control for 3 days, then abundance total, P1-specific and P3-specific St6gal1 transcripts quantified in duodenum and jejunum.