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Deletion of Endogenous Neuregulin-4 Limits Adaptive Immunity During Interleukin-10 Receptor–Neutralizing Colitis

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Background: Growth factors are essential for maintenance of intestinal health. We previously showed that exogenous neuregulin-4 (NRG4) promotes colonocyte survival during cytokine challenge and is protective against acute models of intestinal inflammation. However, the function(s) of endogenous NRG4 are not well understood. Using NRG4$^{-/-}$ mice, we tested the role of endogenous NRG4 in models of colitis skewed toward either adaptive (interleukin-10 receptor [IL-10R] neutralization) or innate (dextran sulfate sodium [DSS]) immune responses.

Methods: NRG4$^{-/-}$ and wild-type cage mate mice were subjected to chronic IL-10R neutralization colitis and acute DSS colitis. Disease was assessed by histological examination, inflammatory cytokine levels, fecal lipocalin-2 levels, and single cell mass cytometry immune cell profiling. Homeostatic gene alterations were evaluated by RNA sequencing analysis from colonic homogenates, with real-time quantitative polymerase chain reaction confirmation in both tissue and isolated epithelium.

Results: During IL-10R neutralization colitis, NRG4$^{-/-}$ mice had reduced colonic inflammatory cytokine expression, histological damage, and colonic CD8^{+} T cell numbers vs wild-type cage mates. Conversely, in DSS colitis, NRG4$^{-/-}$ mice had elevated cytokine expression, fecal lipocalin-2 levels, and impaired weight recovery. RNA sequencing showed a loss of *St3gal4*, a sialyltransferase involved in immune cell trafficking, in NRG4-null colons, which was verified in both tissue and isolated epithelium. The regulation of *St3gal4* by NRG4 was confirmed with ex vivo epithelial colon organoid cultures from NRG4$^{-/-}$ mice and by induction of *St3gal4* in vivo following NRG4 treatment.

Conclusions: NRG4 regulates colonic epithelial ST3GAL4 and thus may allow for robust recruitment of CD8^{+} T cells during adaptive immune responses in colitis. On the other hand, NRG4 loss exacerbates injury driven by innate immune responses.

Lay Summary

Neuregulin-4 (NRG4) is a growth factor that protects the epithelial cells lining the colon from injury and restrains innate (non-specific) immune responses. Here we show that NRG4's role in inflammation is context-specific, and mice that lack NRG4 have impaired adaptive immunity in a model of chronic immune-mediated colitis.

Key Words: ErbB receptor tyrosine kinases, neuregulin growth factors, adaptive immunity, experimental colitis

Introduction

Neuregulin-4 (NRG4) is a member of the neuregulin subfamily of epidermal growth factor–like growth factors that activate ErbB receptor tyrosine kinases. In particular, NRG4 is a selective ligand for ErbB4 with negligible reported binding to other ErbB family receptors.[1] A variety of tissue responses have been described for this ligand, including context-dependent regulation of cellular apoptosis,[2,3] control of cytokine expression,[4,5] tuning of energy metabolism and insulin susceptibility,[6] and others. We previously showed that NRG4 levels in the colon are reduced in mouse models of colitis and in inflammatory bowel disease (IBD)[2] and that the inflammatory cytokine tumor necrosis factor (TNF) downregulates its expression.

Treatment with exogenous NRG4 protects against intestinal inflammation in several experimental models.[2,3,7] Furthermore, deletion of the NRG4 receptor ErbB4 in either macrophages[3] or the intestinal epithelium[8] worsens chemically induced experimental colitis models initiated by innate immune responses. However, the role of endogenously expressed NRG4 in regulating colonic epithelial function, gene expression, and response to insult is unknown, as is the role of NRG4 signaling in adaptive immune response–driven colitis.

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Key Messages

- While neuregulin-4 (NRG4) is protective in acute models of intestinal inflammation, deletion of endogenous NRG4 limits adaptive immunity in the interleukin-10 receptor neutralization model of colitis.
- NRG4 promotes epithelial *St3gal4* expression and $CD8^+$ T cell accumulation in the colon.
- NRG4-ErbB4/ST3GAL4 signaling provides a potential therapeutic target to limit T cell influx in the colon during chronic inflammation.

IBD is a multifaceted disease that broadly involves genetic susceptibility, aberrant immune and epithelial responses, and dysregulation of the intestinal microbiome. One specific example of the complexity of the pathogenesis of IBD is with the alteration of interleukin (IL)-10 signaling. IL-10 is essential to dampen both innate and adaptive immune responses, and genetic mutations in IL-10 signaling are associated with early-onset IBD in children.[9-11] In mice, experimental disruption of IL-10 signaling drives colitis and can promote a dysregulated, colitogenic microbiota.[12-15]

ST3GAL4 is a β-galactoside α2,3-sialyltransferase that regulates production of α2,3-sialyllactose (3SL) by mediating the addition of the sialic acid N-acetylneuaminic acid to lactose.[16] 3SL can in turn stimulate T helper 1/T helper 17 proinflammatory responses,[17] linking this pathway to immunity and inflammation. ST3GAL4 is also involved in the formation of selectin ligands[18] necessary for immune cell function and thus may regulate the development of localized immune responses. Both E-selectin and Sialyl Lewis X, one known selectin binding partner, are expressed during active IBD.[19,20] Furthermore, ST3GAL4 deficient mice show reduced disease in acute dextran sulfate sodium (DSS) and IL-$10^{-/-}$ colitis mouse models.[16,17]

Here, we tested role of endogenous NRG4 in colitis by subjecting whole-body NRG4-null ($NRG4^{-/-}$) mice to an immune-mediated (IL-10 receptor [IL-10R] neutralization) model of colitis.[21] In contrast to the protective role of NRG4 and ErbB4 signaling in innate immunity and injury-driven models of colitis,[2,7,8] whole-body deletion of NRG4 limited T cell influx and adaptive immunity in the IL-10R neutralization model. $NRG4^{-/-}$ mice exhibited reduced colonic levels of *St3gal4*, T cell infiltration to the colon, and inflammatory cytokine levels, and a shift in intestinal microbial species that may favor an anti-inflammatory profile. Overall, our results suggest that while NRG4 signaling reduces innate immunity-driven inflammation[3,5] and protects the epithelium from injury,[2,7] it also participates in T cell recruitment during inflammation driven by disruption of IL-10 signaling. Thus, the role of NRG4 signaling is tissue, cell type, and context dependent. Understanding how to balance these specific responses will be key for future therapeutic targeting of this axis in IBD and other inflammatory disorders.

Methods

Animal Study Approval

The use of all animals and animal experiments were approved and monitored by the Children's Hospital Los Angeles Animal Care and Use Committee (Animal Welfare Assurance #A3276-01). All experiments complied with appropriate ethical animal testing and research regulations.

Animal Experiments

$NRG4^{-/-}$ mice were purchased from the Mutant Mouse Regional Resource Center at University of California, Davis, and then bred and maintained at Children's Hospital Los Angeles. Males and females from either $NRG4^{+/-}$ and/or $NRG4^{-/-}$ breeding cages were generated from mice backcrossed to a C57BL/6 background for at least 8 generations. Male and female cage mates were housed together for at least 2 weeks prior to experiments.

Five-week-old $NRG4^{-/-}$ and wild-type (WT) control animals were used for colonic baseline flow cytometry analysis, while 6- to 12-week-old mice were used for all other baseline experiments. For NRG4 rescue experiments, 6-week-old $NRG4^{-/-}$ mice were intraperitonally injected with either 100 µg/kg NRG4 (Reprokine; Q8WWG1) or phosphate-buffered saline (PBS) for 24 hours. Full-thickness distal colon was collected and flash frozen for later messenger RNA (mRNA) isolation and analysis.

For 16S analysis, stool was collected at both 5 and 9 weeks from 6 WT and 6 $NRG4^{-/-}$ cage mates, along with WT and $NRG4^{-/-}$ dam stool that was collected at weaning. Colonic scrapings were collected on week 9 at euthanasia. All stool and scrapings were stored at -20 °C until 16S processing and analysis.

For IL-10R neutralization, 5 week-old $NRG4^{-/-}$ and WT controls were intraperitonally injected with 1 mg/kg *InVivo*MAb anti-mouse IL-10R (CD210) antibody (Bio X Cell; BE0050) weekly for 5 weeks.[21] All mice were euthanized on week 5 (1 week after the last injection). Postmortem plasma from blood collected by cardiac puncture was used to analyze myeloperoxidase. For DSS colitis, mice were given ad libitum access to water containing 3% DSS for 6 days, followed by 6 days with water containing no DSS. Stool was collected weekly for lipocalin-2 analysis in the IL-10R model and on the last day in the DSS colitis model. Full-thickness distal colon was collected and flash frozen for RNA analysis. Swiss rolls of each colon were formaldehyde fixed and paraffin embedded for histology scoring.[22] Histology scores from the amount and depth of inflammation, the percentage of inflamed crypts, crypt damage, and the percentage of crypts involved in crypt damage (as previously done)[2,5] were determined by an experimentally blinded pathologist. Total score is the sum of each category.

Enzyme-Linked Immunosorbent Assay

Fecal lipocalin-2 was analyzed from homogenized fecal contents using Mouse Lipocalin-2 DuoSet ELISA (R&D Systems; DY1857), per manufacturer instructions. Myeloperoxidase levels were analyzed by using 10% of plasma, in PBS, from postmortem cardiac puncture by Mouse Myeloperoxidase DuoSet ELISA (R&D systems; DY3667), per manufacturer's instructions.

Real-Time Quantitative Polymerase Chain Reaction

mRNA from full-thickness tissue or colonic organoids was extracted using an on-column total RNA isolation kit (OMEGA Biotek; R6834-02), and complementary DNA was generated with a high-capacity complementary DNA reverse transcriptase kit (Applied Biosystems; 4368814).

Quantitative gene expression analysis using TaqMan assays (Thermo Fisher Scientific) (Table 1) was performed using an Applied Biosystems StepOne thermocycler. The average fold change, calculated by the $2^{-\Delta\Delta Ct}$ method, represents the gene expression relative to WT or PBS control groups as appropriate, with *Hprt* as the reference gene.

Microbial 16S Ribosomal RNA Community Profiling

Cage mate mice of WT or NRG4$^{-/-}$ genotypes were weaned and co-housed. Fresh fecal pellets were obtained from mice at 5 and 9 weeks of age. Mice were euthanized and the colonic mucosa scraped with a razor blade to obtain a representation of the mucosal adherent microbiome at 9 weeks old. Fecal and tissue samples were flash frozen and stored at -80 °C prior to profiling.

Sequencing of the V4 16S ribosomal RNA bacterial variable region using polymerase chain reaction (PCR) primers targeted to 515/806 was performed by MR DNA. After 30 cycles of PCR amplification, samples were multiplexed and pooled for generation of an Illumina DNA library. Sequencing was performed on the MiSeq platform following the manufacturer's (Illumina) guidelines. Sequence data were processed by MR DNA. Sequences that were <150 bp or containing ambiguous bases were filtered out; the remaining sequences were quality filtered using a maximum expected error threshold of 1.0, dereplicated, denoised, and filtered to remove chimeras and point errors. The resulting zOTUs were taxonomically classified using BLASTn against a closed-reference, curated database derived from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) to obtain species-level nearest-neighbor annotations, when possible.

Raw and percentage-normalized abundance matrices were loaded into R software Version 3.3 (R Foundation for Statistical Computing) for analysis and principal component visualization. Overall taxonomic diversity was quantified by generating rarefaction curves using the vegan 2.5-7 package. The PERMANOVA routine was used to perform statistical evaluations of genotype-based and other metadata-based associations of microbial community structure. The randomforest 4.7-1 package was used to identify individual taxa that facilitate discrimination of WT and NRG4$^{-/-}$ stool microbiomes.

Table 1. Quantitative polymerase chain reaction assays.

Gene	Life Technologies TaqMan Gene Expression Assay
Hprt	Mm03024075_m1
St3gal4	Mm00501503_m1
Tnf	Mm00443258_m1
Il1b	Mm00434228_m1
Il6	Mm00446190_m1
Ifng	Mm01168134_m1
Muc2	Mm01276696_m1
Itgb7	Mm00442916_m1
Ccr9	Mm02528165_s1
Itgal	Mm00801807_m1
Il10ra	Mm00434151_m1
Il10rb	Mm00434157_m1
Ptprc	Mm01293577_m1
Mki67	Mm01278617_m1
Ccnd1	Mm00432359_m1
Nrg4	Mm00446254_m1

RNAScope In Situ Hybridization

Sections from paraffin-embedded, colonic Swiss rolls were probed using an RNAScope Mm-ST3GAL4 probe (Advanced Cell Diagnostics; 543061), and processed using the RNAScope 2.5 HD Detection (Advanced Cell Diagnostics; 322310), per manufacturer's protocol.

Mucus Thickness Quantification

Distal colonic tubes containing fecal pellets were collected and fixed in Carnoy's fixative. Sections were stained with Alcian blue to visualize the mucus layer; thickness of the layer was measured in ImageJ Version 1.5.3 (National Institutes of Health).

T Cell Isolation

CD8+ T cells were isolated from the spleens of NRG4$^{-/-}$ and WT mice using the EasySep Mouse CD8+ T Cell Isolation Kit (STEMCELL Technologies; 19853) according to manufacturer provided instructions. Cells were stimulated with anti-CD28 (5 µg/mL) and/or TNF (PeproTech; 100 ng/mL) depending on experimental conditions and collected for RNA expression analysis.

Colonic Organoids

Colonic organoids (colonoids) were generated and passaged from NRG4$^{-/-}$ and WT mouse colons, based on previously published protocols.[23-25] Briefly, colonic crypts were extracted by incubation in chelation buffer (2 mM EDTA and 43.4 mM sucrose in D-PBS), followed by trituration in a shaking buffer (43.8 mM sucrose and 54.9 mM sorbitol in PBS). Isolated crypts were then embedded in Matrigel (BD Biosciences) and overlaid with Intesticult (STEMCELL Technologies). For experiments, cultures were starved in Dulbecco's Modified Eagle Medium (DMEM) for 24 hours to limit the effect of exogenous growth factors contained in Intesticult that may affect NRG4-mediated signaling pathways.

RNA Sequencing and Analysis

RNA extracted from NRG4$^{-/-}$ and WT distal colon homogenates were barcoded using Illumina index primers and sequenced on a NextSeq machine to obtain single-ended reads of 75 bp length to a depth of ~10 M reads/sample by SeqMatic LLC). Kallisto[26] was used to pseudoalign the FASTQ files to the mouse transcriptome, for estimated transcript per million abundances. For a pathway enrichment comparison, transcript abundances were summed to obtain a per-gene expression value, then input into enrichr.[27-29] Immune cell population estimates were determined by CIBERSORT (http://cibersort.stanford.edu), using the LM22 signature per 100 permutations.[30] These results were normalized as the relative abundance to the total predicted cells. The data are available online as Gene Expression Omnibus dataset GSE206965.

Single Cell Mass Cytometry (CyTOF) Processing and Analysis

For CyTOF preparation, colonic mucosal peelings from 4 WT and 5 NRG4$^{-/-}$ cage mates were extracted under a

dissection microscope on week 5 of IL-10R neutralization colitis. Separated peelings were segmented and incubated in a digestion solution (0.2 Wunsch units/mL Liberase TM + 200 Kuntz units/mL DNase I in DMEM/12 + 15mM HEPES) at 37 °C for 30 minutes, with continuous shaking at 175 rpm. A neutralizing solution (DMEM/F12/HEPES + 10% fetal bovine serum) was added to the digested tissue, and the tissue was triturated and passed through a 70-µm-pore cell strainer. The strainer was then washed with neutralization solution and isolated cells/solution were centrifuged at 300 *g* for 8 minutes. After washing the pellet in a fluorescence-activated cell sorting buffer (HEPES-buffered saline + 1% bovine serum albumin) and centrifuged, a red blood cell lysis solution (BioLegend; 420301) was used to resuspend the cells for 5 minutes at room temperature. This was followed by 2 more fluorescence-activated cell sorting buffer washes and centrifugation steps. The pellet was then passed through a 40-µm-pore cell strainer, and dead cells were removed using EasySep Dead Cell Removal Kit (STEMCELL Technologies), per manufacturer's instructions. Live cells were resuspended in PROT1 fixative (Smart Tube Inc) prior to processing by the Children's Hospital Los Angeles Single Cell, Sequencing, and CyTOF Core using the Fluidigm Maxpar staining protocols of a mouse immune cell panel (Table 2). Stained cells were run on a Fluidigm Helios operating system.

Flow Cytometry

Colons and spleens from 6 NRG$^{-/-}$ and 6 WT cage mates were extracted and processed similar to previously reported methods.[31] For colons, dissected tissues were segmented into 2-mm pieces and placed in digestion solution (0.2 Wunsch units/mL Liberase TM + 200 Kuntz units/mL DNase I in DMEM/12 + 15mM HEPES) for 30 minutes at 37 °C with continuous 180 *g* agitation. Tissue was then triturated, passed through a 70-µm-pore cell strainer, washed with DMEM:F12 + 10% fetal bovine serum, and with HEPES-buffered saline[32] with 0.5% bovine serum albumin. Cells were isolated from spleens using a method similar to STEMCELL Technologies T cell isolation extraction method: extracted spleens were washed in EasySep Buffer (STEMCELL Technologies; 20144), minced on a 70-µm-pore cell strainer, and washed with EasySep Buffer. Cells were centrifuged at 300 *g* at 4 °C for 10 minutes. This was followed by a 5 to 10 minutes red blood cell lysis and wash with PBS. After centrifugation, cells from the spleens and colons were processed at the same time.

Cells were blocked for 15 minutes at 4 °C with 5% mouse/rat serum + Fc block (anti-CD16/32 antibody) (BioLegend; "truStain fx"), and probed with a mixture of preconjugated antibodies for 30 minutes at 4 °C. Antibodies (working dilutions) were targeted to CD45 (BioLegend; 103107; 1:250), CD8 (BioLegend; 100751; 1:100), CD4 (BioLegend; 100451; 1:200), and CD3 (BD Biosciences; 563565; 1:200), followed by washes. Propidium iodide (Life Technologies; R37108) was added to cells before analysis on a BD LSR II. Compensation was adjusted using references obtained by the analysis of single antibodies to unstained cells. Flow cytometry analysis was performed by FlowJo Version 9.

Statistics

Statistical plots and analyses were created using GraphPad Prism Version 8 (GraphPad Software). Each dot represents one biological replicate and is portrayed as the mean ± SEM in dot and bar graphs. Statistical testing includes Student's 2-sided *t* test, 1-way analysis of variance, and 2-way analysis of variance, with Tukey's post hoc test or Kruskal-Wallis test, as appropriate.

Table 2. Cell cytometry (CyTOF) mouse immune cell panel.

Reactivity	Label	Vendor	Catalog #
CD45	89Y	Fluidigm	3089005B
CD11b	115/113In	BioLegend	101249
GD2	139La	BioLegend	357302
CD25	141Pr	BioLegend	102002
Ly6G	142Nd	BioLegend	127602
CD8a	143Nd	BioLegend	100702
CD19	144Nd	BioLegend	115502
CD69	145Nd	BioLegend	104502
T-bet	147Sm	BioLegend	644802
CD140a (PDGFα)	148Nd	BioLegend	135902
CD4	149Sm	BioLegend	100506
CD366 (TIM-3)	150Nd	BioLegend	119702
CD206	151Eu	BioLegend	141702
CD279	152Sm	BioLegend	135202
Ly6C	153Eu	BioLegend	128002
CD62L	154Sm	BioLegend	104402
FSP1	155Gd	Abcam	ab220213
CD223 (LAG-3)	156Gd	BioLegend	125204
CD31	158Gd	BioLegend	102402
CD274 (PD-L1)	159Tb	BioLegend	124302
CD317	160Gd	BioLegend	127002
FAP	161Dy	Sigma-Aldrich	MABC1145
CD170 (SiglecF)	163Dy	R&D Systems	750620
Eomes	164Dy	R&D Systems	MAB8889
FoxP3	165Ho	BioLegend	320002
CD326 (EpCAM)	166Er	BioLegend	118202
TGFβ (LAP)	167Er	BioLegend	141402
CD161 (NK1.1)	168Er	BioLegend	108743
CD102	169Tm	BioLegend	105602
CD140b (PDGFβ)	170Er	BioLegend	136002
Phox2b	171Yb	Santa Cruz Biotechnology	sc-376997
CD3ε	172Yb	BioLegend	100302
F4/80	173Yb	BioLegend	123102
CD44	174Yb	BioLegend	103002
Granzyme B	175Lu	BioLegend	372202
I-A/I-E (MHC)	176Yb	BioLegend	107602
CD11c	209Bi	Fluidigm	3209005B

Results

Whole-Body NRG4 Deletion Protects Against IL-10R Neutralization–Mediated Chronic Colitis

Colonic NRG4 expression is reduced in IBD and in experimental inflammation models (eg, DSS colitis, TNF exposure). We previously showed that administration of exogenous NRG4 reduces injury and inflammation in DSS colitis.[2]

However, the role of endogenously expressed NRG4 in the response to colonic injury and inflammation, and NRG4's role in a T cell–dependent disease, are unknown. To test the role of endogenous NRG4 in a T cell–driven chronic colitis model, we injected NRG4$^{-/-}$ mice and WT cage mates with IL-10R–neutralizing antibody[21] weekly for 4 weeks. After 5 weeks, NRG4$^{-/-}$ mice showed lower tissue pathology scores (measuring epithelial damage and immune cell influx) (Figure 1A, 1B) than WT mice. While both genotypes developed colitis within 1 week as shown by fecal lipocalin-2 levels (Figure 1C), the response was significantly attenuated in NRG4$^{-/-}$ animals compared with WT animals during the early stages of disease development. NRG4$^{-/-}$ mice also showed reduced markers of ongoing inflammation, including plasma levels of the neutrophil-produced factor myeloperoxidase ($P = .0005$), spleen weight ($P = .001$), and increased weight gain compared with WT cage mates (Figure 1D-1F). Colon lengths between genotypes were not different (Figure 1G), but in previous studies this has not been a consistent readout of colitis in this model.[33,34] Expression levels of IL-10 *(Il10)* and IL-10R (*Il10ra* and *Il10rb*) in colonic homogenates were also not different as shown by bulk RNA sequencing analysis (Figure 1H), suggesting that changes in colonic expression of these molecules was not responsible for the attenuated response on the NRG4$^{-/-}$ background.

Colonic Inflammatory Cytokines and CD8+T Cell Numbers are Reduced in Chronic Colitis in NRG4$^{-/-}$ Mice

The chronic colitis mediated by IL-10R neutralization involves substantial recruitment of immune cells that produce inflammatory cytokines including TNF, interferon (IFN)-γ, IL-1β, and IL-6.[21] To test whether the reduced severity of colitis is associated with altered immune cell responses, we assessed levels of these cytokines in colonic homogenates at baseline and after anti-IL-10R antibody treatments. Baseline relative mRNA analysis indicated no difference in cytokine production, but after IL-10R neutralization, IL-1β ($P = .033$) and IFN-γ ($P = .0068$) levels were significantly lower in NRG4$^{-/-}$ vs WT colons (Figure 2A). As both IFN-γ and IL-1β are T cell–produced cytokines, we next asked whether this change in cytokine level represented an altered immune cell profile. Using CyTOF analysis of whole colon homogenates after 5 weeks of IL-10R neutralization, we found that NRG4$^{-/-}$ colons showed an impaired adaptive immune response with significantly reduced CD8$^+$ and CD4$^+$ T cell populations in the total colonic cellularity, and a trend toward reduced numbers of B cells (Figure 2B). Innate immune cell representation (macrophages and natural killer cells) was unaltered. Furthermore, colons from NRG4$^{-/-}$ mice showed reduced expression of T cell–homing genes *Itgb7* and *Itgal* (Figure 2C), suggesting a potential defect in T cell recruitment.

NRG4 Deletion Limits Splenic CD8+T Cells but Does not Alter Intestinal T Cell Populations at Homeostasis

To test if baseline immune population shifts might explain the altered T cell immune profile in colitic NRG4$^{-/-}$ mice, we performed flow cytometric analysis on colon and spleen of 5-week-old animals. In the colon, we found no difference in basal intestinal T cell numbers (Figure 3A) in the total cellularity of NRG4$^{-/-}$ mice vs WT cage mates. This correlated with colonic immune cell profiles predicted by CIBERSORT (Figure 3B), a computational analysis of bulk RNA sequencing data to detect immune cell signatures; this analysis found no significant homeostatic differences in any immune populations. In contrast to the findings in colon, however, NRG4$^{-/-}$ mice showed a moderate reduction in splenic CD8$^+$ T cells ($P = .02$) (Figure 3C). To understand if this represents T cell development or maturation changes that could influence responses in the IL-10R model, we measured IL-10R expression in thymus, spleen, and isolated T cells from NRG4$^{-/-}$ and WT mice. Interestingly, while NRG4$^{-/-}$ thymus showed elevated *Il10ra*, no other changes were seen for IL-10R expression or overall immune cell levels (CD45 [*Ptprc*]) in thymus and spleen (Figure 3D). Isolated T cells from WT vs NRG4$^{-/-}$ mice (Figure 3E) showed no significant differences in IL-10R expression in naïve, anti-CD28-stimulated, TNF-stimulated, or anti-CD28/TNF-stimulated conditions (Figure 3F). To test if T cells may have impaired proliferation, we measured T cell expression levels of the cell cycle regulation and proliferation markers Cyclin D1 and Ki67. While TNF stimulation increased Cyclin D1 levels in TNF treated naïve T cells, there was no difference between WT and NRG4$^{-/-}$ (Figure 3G). Together, these results suggests while there may be a moderate influence of NRG4 on lymphoid tissues, at baseline there are no significant changes to T cell proliferation or colonic T cell levels.

NRG4$^{-/-}$ Mice have Impaired Recovery from DSS Colitis

We next tested if deletion of endogenous NRG4 altered inflammation severity and recovery in DSS colitis—a model not explicitly dependent on adaptive immune responses.[35] We gave mice DSS in drinking water for 6 days to induce injury and inflammation followed by 6 days of recovery; in this model, colitis is driven largely by innate immune cells (neutrophils and macrophages). Relative to WT cage mates, NRG4$^{-/-}$ mice showed increased inflammatory markers including weight loss and fecal lipocalin-2 (Figure 4A, B). Colonic lengths and overall injury scores were not significantly different (Figure C, D), but colonic levels of TNF were elevated in NRG4$^{-/-}$ mice (Figure 4E). Taken together, these data suggest that epithelial repair processes may not be substantially altered but resolution of inflammation is delayed. Thus, while whole-body deletion of NRG4 leads to reduced inflammation in a colitis model driven by adaptive immunity (Figure 1), we actually see increased inflammation in a model more skewed toward innate immunity (Figure 4).

NRG4 Promotes ST3GAL4 Expression in the Colonic Epithelium

To identify candidate mediators of the differential susceptibility to IL-10R neutralization colitis in NRG4$^{-/-}$ and WT cage mates, we investigated baseline transcriptomic changes in an unbiased manner by bulk RNA sequencing of colonic tissue. Highly decreased transcripts in NRG4$^{-/-}$ mice included *St3gal4*, *Nxpe4,* and *Hsp8*, with a significant increase in *Bco2* (Figure 5A, 5B). Several of these genes are altered during inflammation (*St3gal4*; *Bco2*)[36,37] or have been previously identified to change expression level in IBD (*Nxpe4*; *Hspa8*)[38,39]. The most significant alteration was the loss of *St3gal4* ($P = .0008$). Furthermore, a BioPlanet[40] 2019 pathways catalogue, using an EnrichR algorithm, of

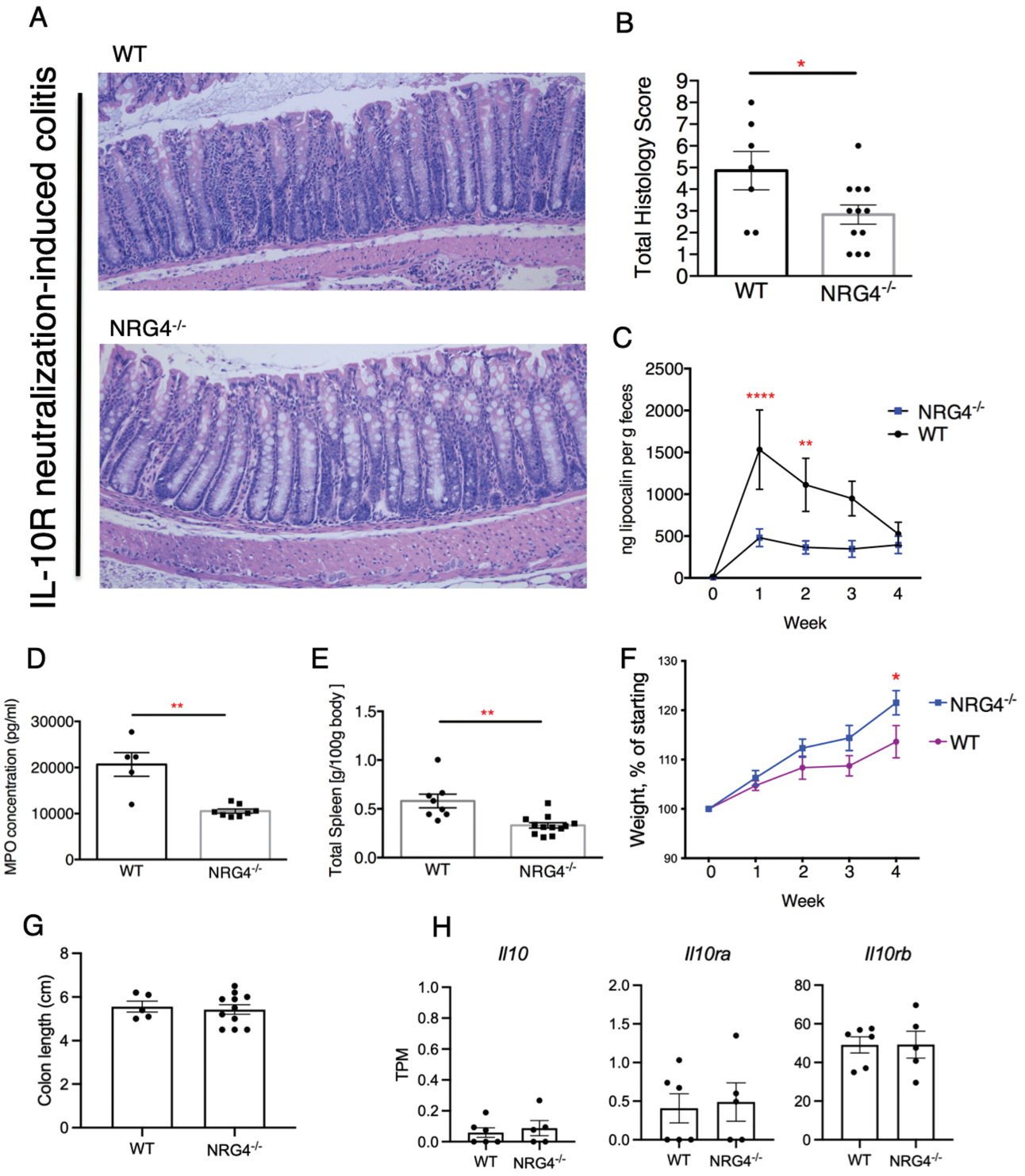

Figure 1. Whole body neuregulin-4 (NRG4) deletion leads to reduced interleukin-10 receptor (IL-10R) neutralization–induced inflammation in the colon. Five-week-old NRG$^{-/-}$ and wild-type (WT) cage mate mice were injected with IL-10R–neutralizing antibody (1 mg/kg) for 4 weeks. **A,** Hematoxylin and eosin–stained sections from paraffin-embedded colonic Swiss rolls collected on experimental week 5. **B,** Total colonic damage score; n = 7 WT and n = 12 NRG4$^{-/-}$. Analyzed by Kruskal-Wallis test (*P < .05). **C,** Fecal lipocalin-2 enzyme-linked immunosorbent assay (****P < .0001, ** P < .001); n = 10 WT and n = 19 NRG4$^{-/-}$ analyzed by 2-way analysis of variance. **D,** Plasma myeloperoxidase (MPO) concentration levels by enzyme-linked immunosorbent assay (**P = .0005); n = 5 WT and n = 8 NRG4$^{-/-}$. **E,** Total spleen weight (**P = .001). **F,** Weekly weights of mice (*P < .05). **G,** Colon length. **H,** Bulk RNA sequencing analysis of colonic homogenates. Data are presented as the mean ± SEM. Statistical testing by Student's 2-sided t tests. TPM, transcripts per million.

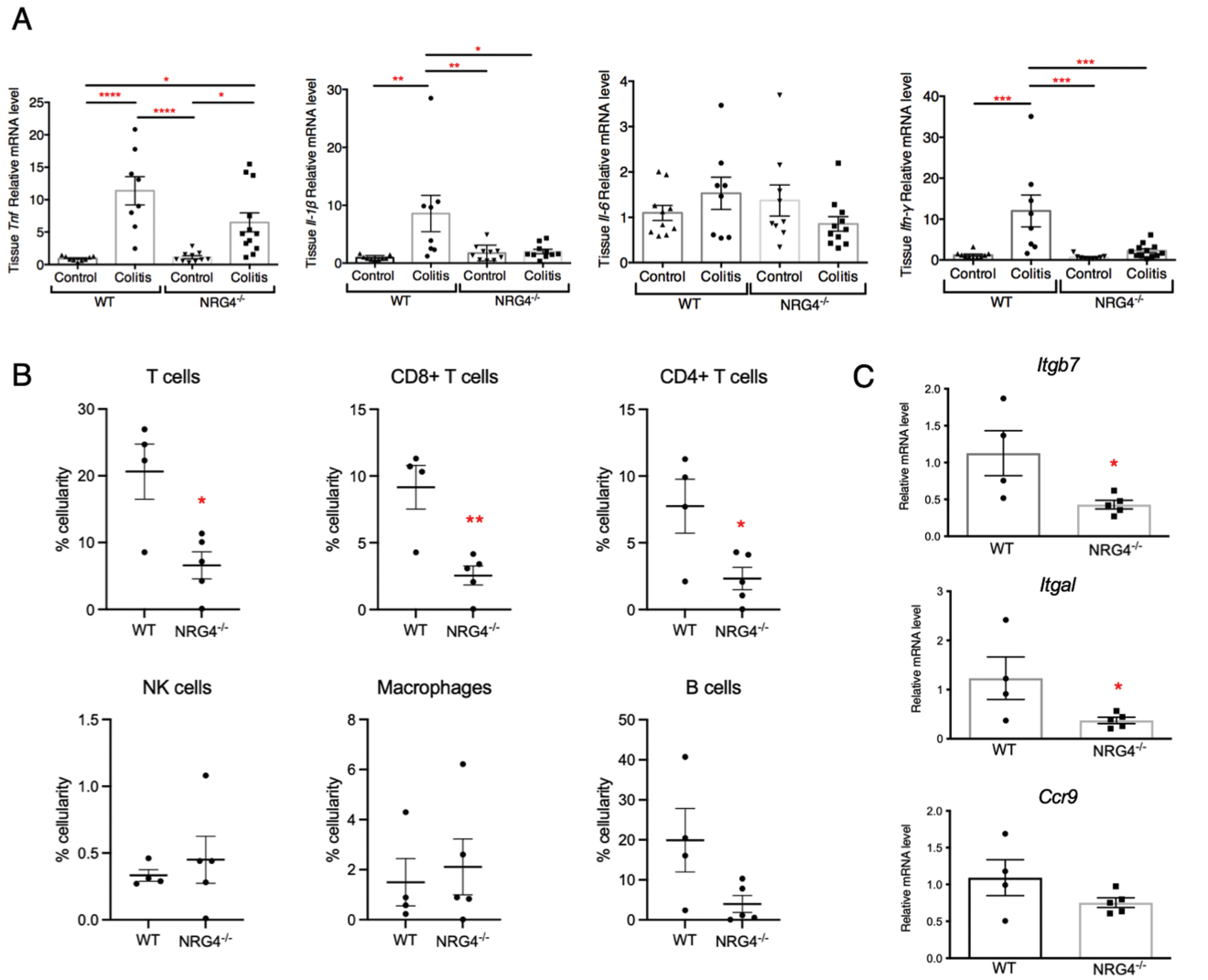

Figure 2. Neuregulin-4 (NRG4) deletion reduces inflammatory cytokines and T cell infiltration in interleukin-10 receptor (IL-10R) neutralization colitis. **A,** Relative messenger RNA (mRNA) levels (quantitative polymerase chain reaction) of *Tnf*, *Ifnγ*, *Il1b*, and *Il6* from full-thickness colonic homogenates after 5 weeks of IL-10R neutralization colitis, compared with baseline colon homogenates; n = 10 baseline WT, n = 10 baseline NRG4$^{-/-}$, n = 8 colitic WT, and n = 12 colitic NRG4$^{-/-}$, analyzed by 1-way analysis of variance. The following outliers were identified and removed by ROUT testing (Q = 1.0%): *Tnf* (1 from the WT control group), *Ifnγ* (1 from the NRG4$^{-/-}$ control group), *Il1b* (1 from the WT control and 2 from the NRG4$^{-/-}$ colitis groups), and *Il6* (1 from the NRG4$^{-/-}$ control and 1 from NRG4$^{-/-}$ colitis groups). **B,** CyTOF analysis of colonic cells isolated after IL-10R–induced injury, expressed as % of total cellularity in the sample. **C,** Expression of homing genes were analyzed by quantitative polymerase chain reaction in colonic homogenates. Data are presented as the mean ± SEM. Statistical testing used Student's 2-sided *t* tests. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

NRG4$^{-/-}$ downregulated genes indicated a significant loss in membrane trafficking, immune system signaling, and O-glycan biosynthesis, known functions of ST3GAL4 and/or other sialyltransferases (Figure 5C).[41,42]

As *St3gal4* was the largest and most significant change in this analysis, we next wanted to determine if a direct causal relationship exists between NRG4 and *St3gal4*. First, we verified and extended our RNA sequencing results. *St3gal4* localization by RNAscope in situ hybridization showed a striking absence of expression in the colonic epithelial crypts of NRG4$^{-/-}$ mice (Figure 6A). This loss of *St3gal4* was also seen by quantitative PCR analysis of colonic epithelial organoids generated from these mice (Figure 6B) and colonic tissue from these mice (Figure 6C). Furthermore, the loss of tissue *St3gal4* persisted after IL-10R neutralization colitis in NRG4$^{-/-}$ mice compared with WT mice ($P < .0001$).

Next, we wanted to determine if exogenous treatment with NRG4 could rescue *St3gal4* loss in NRG4$^{-/-}$ colons, and thus show a direct interaction. We injected NRG4$^{-/-}$ mice intraperitonally with 100 µg/kg NRG4 or PBS. After 24 hours, this treatment resulted in a significant induction of *St3gal4* expression ($P = .0011$) in colonic homogenates (Figure 6D). To determine if *St3gal4* effects are intestinal-specific, we analyzed thymus and spleen for *St3gal4* levels and found no difference between WT and NRG4$^{-/-}$ mice (Figure 6E, 6F). As *St3gal4* has potential roles in mucus stabilization, we also analyzed the thickness of the colonic mucus layer between WT and NRG4$^{-/-}$ littermates, but found no difference (Figure 6G). Together, these results suggest an intestinal epithelial-specific role for NRG4 in regulating *St3gal4*, that may impact adaptive immune responses in colitis.

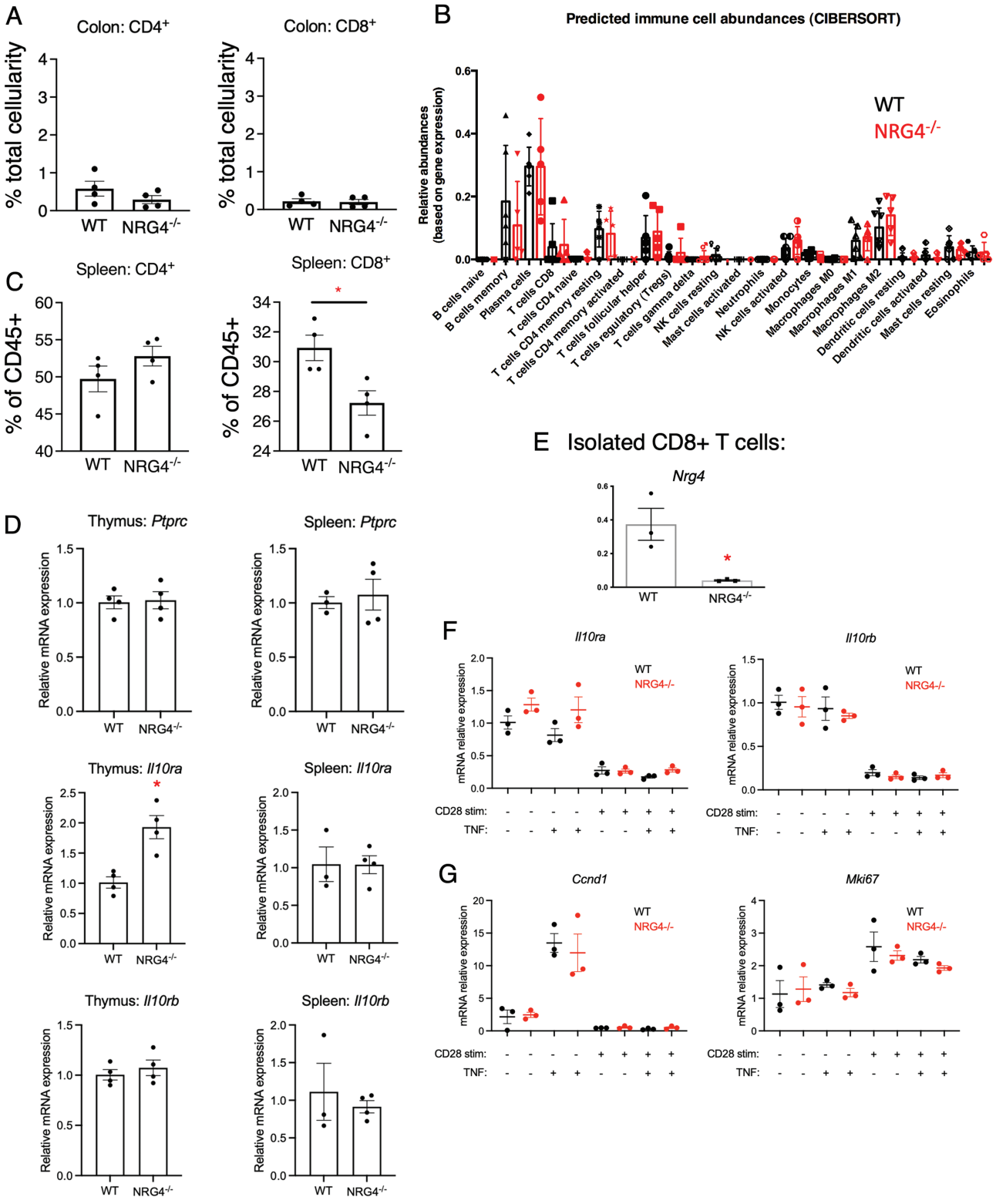

Figure 3. Neuregulin-4 (NRG4) deletion moderately limits splenic CD8+ T cells but does not alter CD8+ T cell proliferation. **A,** Colonic cells were isolated from 5-week-old NRG4$^{-/-}$ mice and wild-type (WT) cage mates and analyzed by flow cytometry; n = 4 WT and n = 4 NRG4$^{-/-}$. **B,** Baseline RNA sequencing from colonic homogenates in adult NRG4$^{-/-}$ and WT cage mates was analyzed using CIBERSORT to predict changes in immune cell abundance between groups; n = 6 WT and n = 5 NRG4$^{-/-}$. **C,** Splenocytes were isolated and analyzed by flow cytometry. **D,** Thymus and spleen collected from unchallenged mice were analyzed by quantitative polymerase chain reaction for expression of CD45 (*Ptprc*) and the interleukin-10 receptor (*Il10ra*, *Il10rb*). **E,** Isolated CD8+ T cells were analyzed for *Nrg4* expression. **F** and **G,** Isolated T cells were stimulated and/or treated with tumor necrosis factor and analyzed for (F) interleukin-10 receptor or (G) Cyclin D1 and Ki67. Data are presented as the mean ± SEM. Analyzed by Student's 2-sided *t* test. **P* < .05. mRNA, messenger RNA.

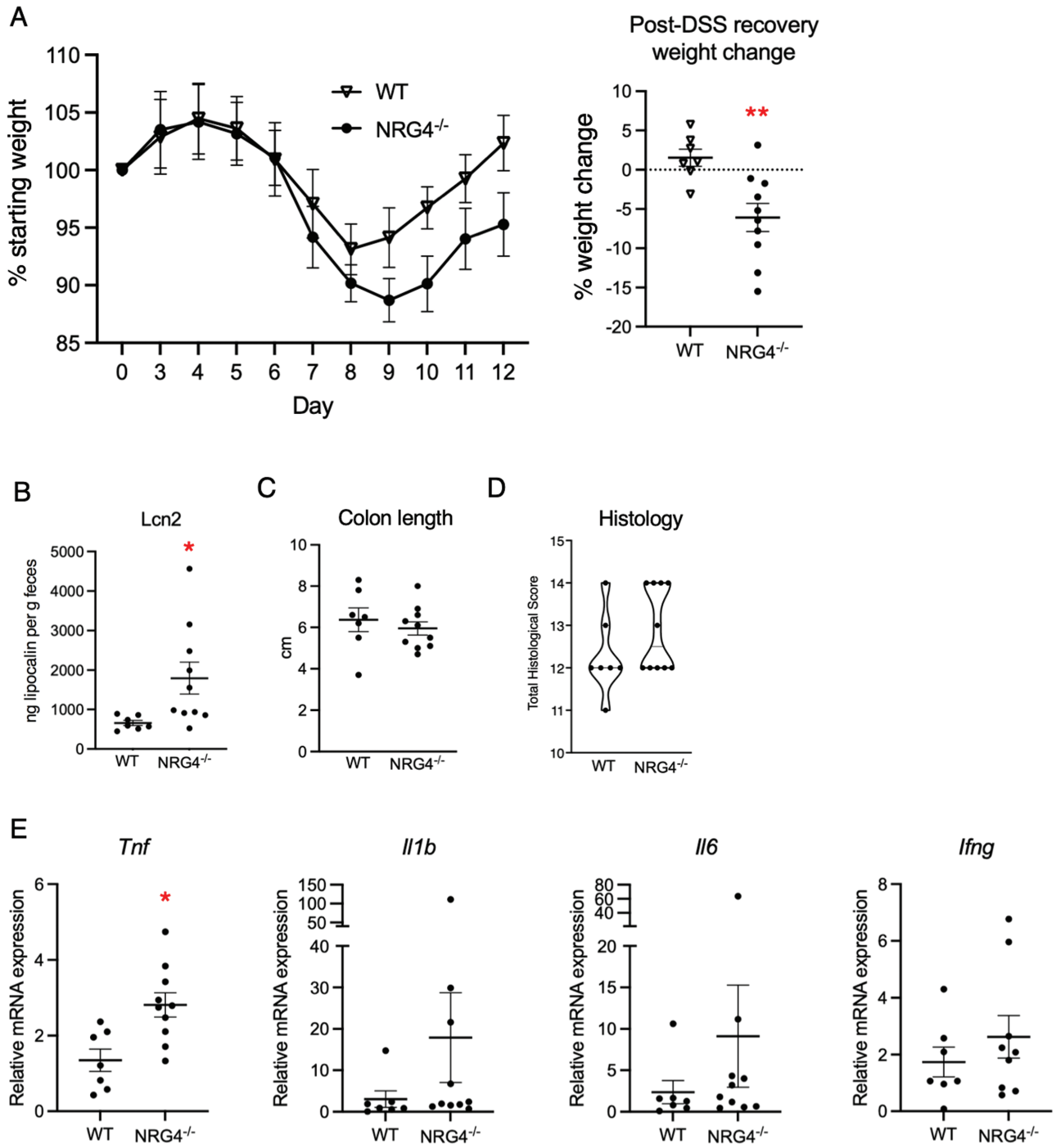

Figure 4. Neuregulin-4–null (NRG4$^{-/-}$) mice have impaired recovery in dextran sulfate sodium (DSS) colitis. NRG4$^{-/-}$ and wild-type (WT) littermates were subjected to a recovery model of DSS colitis (6 days with 3% DSS in drinking water followed by 6 days with no DSS). **A,** Daily weights of mice. **B,** Fecal lipocalin-2 levels. **C,** Colon lengths. **D,** Histological scores. **E,** Cytokine expression in colonic homogenates. Data are presented as the mean ± SEM. Analyzed by Student's 2-sided *t* test. **P* < .05; ***P* < .01. mRNA, messenger RNA.

NRG4 may Influence Microbial Diversity

Dysbiosis of the intestinal microbiota is observed in IBD. ST3GAL4 and 3SL are known to impact early-life bacterial colonization and microbial composition[16,36]; thus, we tested whether microbial taxonomic composition was altered in NRG4$^{-/-}$ mice compared with WT cage mates. We sampled the 16S ribosomal RNA community sequences of the stool microbiome at 5 and 9 weeks of age and of the mucosa-associated microbiome at 9 weeks of age. We found a trend toward increased taxonomic diversity in stool (5 weeks, $P = .09$; 9 weeks, $P = .06$) in NRG4$^{-/-}$ mice compared with WT cage mates (Figure 7A). However, at 5 and 9 weeks, the stool microbiomes appeared globally similar on a principal component plot (Figure 7B). The composition of the mucosa-associated microbiome (intestinal scrapings) at 9 weeks of age also appeared globally similar. Random forest analysis of individual species (Figure 7C-D) showed that NRG4$^{-/-}$ mice had modest but not statistically significant elevations

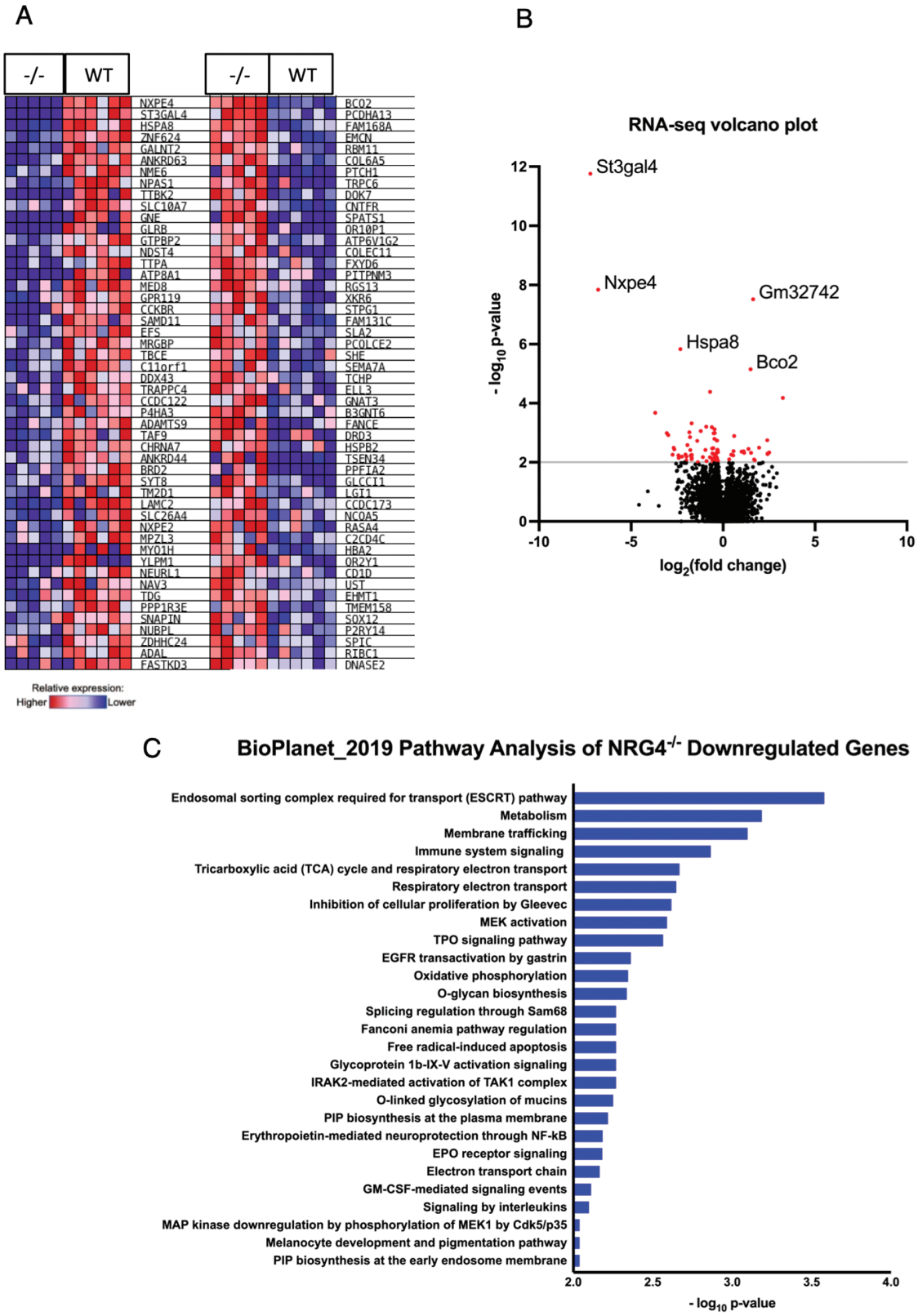

Figure 5. RNA sequencing (RNA-seq) analysis indicates significant loss of *St3gal4* in mice with neuregulin-4 (NRG4) deletion. RNA-seq of full-thickness colonic biopsies from n = 5 NRG4$^{-/-}$ and n = 6 wild-type (WT) cage mates 11 to 12 weeks of age. **A,** Heat map of expression levels of altered genes. Red indicates an increase in relative expression and blue indicates a decrease in relative expression, as shown in the figure legend. **B,** Volcano plot of altered genes identified by RNA-seq; *St3gal4* $P = .0008$. **C,** Pathway analysis of downregulated genes that are above 2 -$\log_{10}$ *P* value by BioPlanet_2019 in NRG4$^{-/-}$ mice compared with WT cage mates.

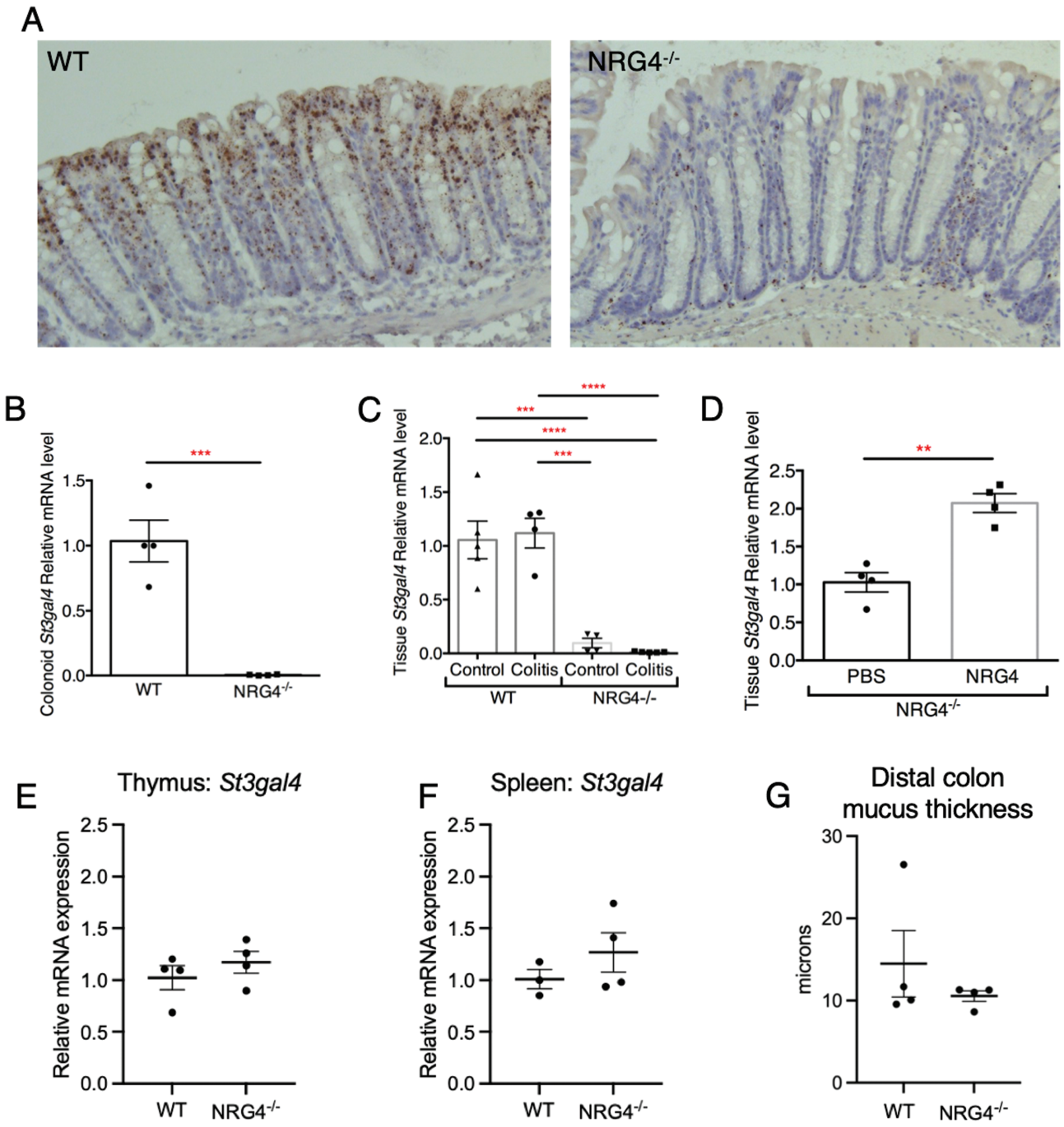

Figure 6. Neuregulin-4 (NRG4) deletion leads to the loss of colonic epithelial ST3GAL4. **A,** *St3gal4* in situ hybridization from NRG4$^{-/-}$ and wild-type (WT) colonic Swiss roll sections at homeostasis. **B,** Relative *St3gal4* messenger RNA (mRNA) expression from NRG4$^{-/-}$ and WT colonic organoids after starvation (***P = .0007), analyzed by Student's 2-sided t test. **C,** Relative *St3gal4* mRNA expression from full-thickness NRG4$^{-/-}$ and WT colonic homogenates at baseline and after interleukin-10 receptor neutralization colitis. Statistical analysis by 1-way analysis of variance, with 1 outlier from the NRG4$^{-/-}$ control group was identified and removed by ROUT testing (Q = 1.0%). **D,** NRG4$^{-/-}$ mice were intraperitonally injected with NRG4 (100 µg/kg) or phosphate-buffered saline for 24 hours. Relative *St3gal4* mRNA expression from n = 4 NRG4$^{-/-}$ and n = 4 WT full-thickness colonic biopsies (**P = .0011). **E,** Thymus *St3gal4* expression. **F,** Spleen *St3gal4* expression. **G,** Mucosal thickness assessed by Alcian blue staining on distal colonic sections. Statistical testing used Student's 2-sided t tests.

in some bacteria that are known to be protective in colitis, such as *Lactobacillus* spp.,[43] *Butyriciccus pullicaecorum*,[44] and *Barnesiella* spp.[45] Taken together, these data suggest that NRG4 loss is associated with subtle changes in the stool microbiome consistent with an anti-inflammatory profile (ie, bacterial diversity and bacterial symbiont abundance).

Discussion

Growth factors play a multitude of roles in maintaining intestinal homeostasis and promoting appropriate responses to injury and inflammation. NRG4 is widely expressed in various tissues of the body and multiple cell types in the intestine. We have previously shown that overall intestinal NRG4 levels

are reduced during colitis, though expression in specific cell types such as inflammatory macrophages actually increases.[2,5] Furthermore, administration of exogenous NRG4 limits disease[2,7] by preventing epithelial apoptosis[2] and suppressing inflammatory macrophage activity.[3,5] Here, we used both unbiased and targeted approaches to understand the role of endogenously expressed NRG4 in colonic homeostasis. Furthermore, we describe how whole-body deletion of NRG4 affects chronic, adaptive immunity–driven colitis.

Our data demonstrate that NRG4 loss reduces colonic T cell influx in IL-10R neutralization colitis, a model of colitis driven largely by adaptive immune mechanisms. NRG4$^{-/-}$ mice also showed significantly lower expression of inflammatory cytokines IL-1β and IFNγ (produced by T cells) during disease. These parameters were not different in unchallenged NRG4$^{-/-}$ vs WT cage mate mice, suggesting that NRG4 does not directly alter homeostatic colonic immune populations. Interestingly, in a model of colitis driven largely by innate immune mechanisms that does not explicitly require T cells (DSS colitis), we did not see protection in NRG4$^{-/-}$ mice. In fact, NRG4$^{-/-}$ mice had exacerbated DSS-induced inflammation (Figure 4). These divergent results suggest that NRG4's role depends on the character of colitis, with its epithelial protective effects and suppression of inflammatory macrophage activity[2,5] likely dominant in disease driven by hyperactive innate immunity and epithelial injury, while chronic adaptive immune responses may in contrast depend to some extent on NRG4.

Given previous work from our lab and others showing that both exogenous NRG4 and its receptor ErbB4 are protective in experimental models of intestinal inflammation including acute DSS colitis,[2,3,5,8] formula feeding/hypoxia neonatal colitis and dithizone/*Klebsiella* neonatal colitis,[7] and cytokine injection,[2] it was somewhat surprising to see protection against IL-10R neutralization colitis with deletion of the endogenous *Nrg4*. However, most growth factor and cytokine signaling pathways have pleiotropic effects that can be context, concentration, or localization dependent. It is possible that the level or source (endogenous vs pharmacological) of signal, specific immune context (primarily innate immunity and ulceration vs adaptive immune–driven inflammation), or timing (acute vs sustained/chronic), or some combination of these, is critical for defining outcomes. Our DSS studies suggest that endogenous NRG4 is important for promoting recovery from acute colitis (Figure 4), whereas our IL-10R neutralization studies suggest effects on adaptive immunity may alter levels of inflammation in chronic T cell–mediated colitis. Additionally, given our findings of moderately reduced CD8+ splenic T cells at baseline (Figure 3), it is possible that NRG4 plays additional unidentified roles in immune cell development that warrant future study. Future studies to address these possibilities will require additional tools such as tissue-specific and inducible deletion or overexpression models.

Multiple T cell subtypes (eg, CD4+, cytotoxic CD8+, memory CD8+ T cells) participate in the colonic immune response.[46] Although our results clearly show systemic NRG4's effect on T cells in chronic adaptive immunity–mediated colitis (Figures 2 and 3), future work will be necessary to understand the precise mechanism of action. Cytotoxic CD8+ T cells participate in IBD pathogenesis,[47-49] whereas helper CD4+ and memory CD8+ T cells may either be pro- or anti-inflammatory.[46,50] Future work to clarify the effect of NRG4 on these subtypes, and whether this response is directly occurring in peripheral and lymphoid tissues, will be important.

We also found that whole-body NRG4 deletion leads to substantial changes in the colonic gene expression profile. NRG4$^{-/-}$ colons showed a dramatic loss of epithelial *St3gal4*, which was partially rescued by exogenous NRG4 treatment (Figure 6). This was accompanied by reduced T cell influx, as discussed above. Impaired leukocyte infiltration and reduced colitis was previously reported in murine models after deletion of *St3gal4*. In this setting, colitis sensitivity was rescued by treatment with the ST3GAL4 activity product 3SL.[16,17] Kurakevich et al[17] also determined that 3SL upregulated transforming growth factor β (a known T cell regulator and an inhibitor of P-selectin)[51] and activated dendritic cells isolated from the mesenteric lymph nodes. Furthermore, functional human endothelial ST3GAL4 studies showed that ST3GAL4 is the primary sialyltransferase that regulates the synthesis of E-, P-, and L-selectin ligands.[52] Taken together, these results suggest that the loss of epithelial ST3GAL4 after NRG4 deletion could modulate immune cell influx during IL-10R neutralization mediated injury through multiple possible mechanisms. As T cell recruitment could be pathogenic or protective depending on context, future studies will assess the effects of endogenous NRG4 in additional models. The interaction between NRG4 and ST3GAL4 in the intestine has not previously been identified. Both NRG4 and ST3GAL4 are produced and regulated by many cells and tissues.[53,54] We previously showed that NRG4 is an important factor that is produced in breast milk that promotes early life intestinal health.[7] Similarly, ST3GAL4 and 3SL are critical factors in breast milk, components of milk oligosaccharide signaling mechanisms, that produce a plethora of systemic effects including the regulation of the immune response.[54] Our results suggest that the interaction between NRG4 and ST3GAL4 is an intestinal specific effect (Figure 6).

As ST3GAL4 plays a role in mucin glycosylation[55] and therefore may alter the function of mucus and its effects on the microbiota, we performed analysis of fecal and adherent microbiota in NRG4$^{-/-}$ mice. Previous work by Fuhrer and colleagues demonstrated a significant decrease in *Ruminococcaceae* at baseline in ST3GAL4$^{-/-}$ mice, as determined both by the cross-fostering of WT and ST3GAL4$^{-/-}$ litters and the microbial reconstitution of germ-free mice. *Ruminococcaceae* are abundantly found in IBD patients[56-58] compared with normal control subjects. Though NRG4$^{-/-}$ mice did not display major shifts in bacterial species, a few previously identified protective species showed a trend toward elevation with NRG4 deletion, as determined by random forest plot (Figure 7). NRG4-null mice also demonstrated a trend toward increase in fecal microbial diversity. Furthermore, we demonstrated that epithelial ST3GAL4 is expressed in proximity to both mucus and bacteria, and is lost in NRG4$^{-/-}$ mice (Figure 6). Together, these data suggest that NRG4 could promote selective bacterial colonization and diversity possibly through epithelial ST3GAL4.

In this study, we have shown that NRG4 regulates intestinal epithelial ST3GAL4 expression and may play a role in T cell influx to the intestine during inflammation. In the setting of an adaptive immunity–driven colitis (IL-10R neutralization), NRG4-null mice show a reduction in overall inflammation, but not in innate immunity–driven colitis (DSS). These findings provide important insight into the tissue and context specific effects of NRG4. Together with our previous work showing that NRG4 promotes survival of intestinal epithelial

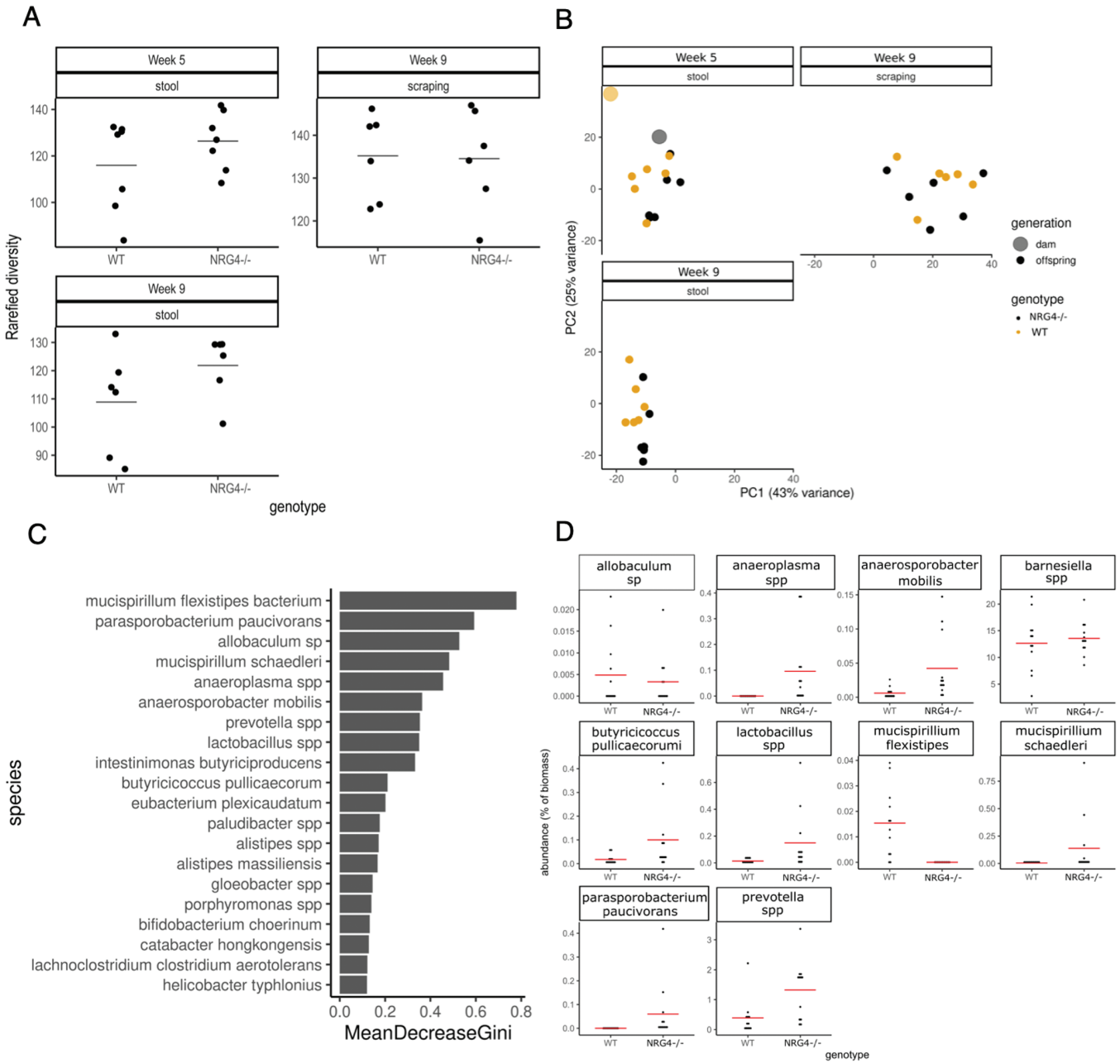

Figure 7. The loss of neuregulin-4 (NRG4) may shift microbial composition, promoting an increase in known protective bacterial species. Six wild-type (WT) mice (WT breeding cage) and 6 NRG4$^{-/-}$ mice (NRG4$^{-/-}$ breeding cage) were weaned together at approximately 3 weeks of age, in 3 different cages. Stool was collected at 5 and 9 weeks of age. Stool from WT and NRG4$^{-/-}$ dams were collected at litter weaning. Colonic scrapings were collected at 9 weeks. **A,** 16S analysis of microbial diversity for each age and genotype visualized from rarefaction. Statistical analysis using Student's 2-sided *t* test; stool at 5 weeks ($P = .09$) and at 9 weeks ($P = .06$). **B,** Principal component (PC) analysis of microbial abundances for each age and genotype, as compared with each dam. **C,** Random forest techniques identify discriminating taxa as those whose elimination reduces the accuracy of the classifier for the tested NRG4$^{-/-}$ and WT genotypes. **D,** The abundance, as determined by the percentage of biomass, from discriminating species predicted by the random forest methods in WT and NRG4$^{-/-}$ cage mates. Statistical testing by Student's 2-sided *t*-test.

cells and death of inflammatory macrophages in the colon, these current results add an additional layer of complexity to how this growth factor can regulate intestinal inflammation.

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Author Contribution

J.K.B. conceived and designed experiments, performed experiments, performed analysis/interpretation of data, and wrote and revised the manuscript. E.B.B. performed experiments and analysis of data and revised the manuscript. C.Y.L. performed analysis/interpretation of data and revised the manuscript. K.K. performed experiments and revised the manuscript. M.K.W. performed histological analysis of tissue, analyzed data, and revised the manuscript. M.A.S. performed

experiments and analysis/interpretation of data and revised the manuscript. M.R.F. performed study supervision, obtained funding, conceived and designed experiments, performed analysis/interpretation of data, and revised the manuscript.

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Conflicts of Interest

M.R.F. and M.A.S. are inventors on a patent held by Children's Hospital Los Angeles on the possible therapeutic use of neuregulin-4 in intestinal inflammation. The other authors have no conflicts.

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