

Dysregulation of Noncanonical NF- κ B Signaling in Gastrointestinal Diseases

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biomedical and Veterinary Sciences

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August 11th, 2023
Blacksburg, VA

Keywords: NIK, inflammation, differentiation, cancer, immunology

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ABSTRACT

Regulation of host health is intricately coordinated by a diverse interplay of immune cells detecting assaults from pathogens via recognition of pathogen associated molecular patterns (PAMPs) to mount an immune response, as well as detecting damage associated molecular patterns (DAMP) to indicate an area of damage and signal tissue repair. The gastrointestinal tract is a major signaling hub for such immune responses, as intestinal epithelial cells (IECs) compose the epithelial barrier, immune cells surveillance breached barriers to regulate the gut microbiome, and intestinal stem cells (ISCs) proliferate to replenish the IEC pool. One such method for regulating these cellular functions downstream of PAMPs/DAMPs within the gastrointestinal tract is via NF- κ B signaling. This cellular signaling pathway is activated by one of two pathways: the well-defined canonical NF- κ B pathway and the understudied noncanonical NF- κ B pathway. The noncanonical NF- κ B pathway is unique as it requires NIK, the NF- κ B-inducing Kinase, to further elicit signal transduction of this pathway. Noncanonical NF- κ B activation is critical to maintaining gut health, as signaling is regulated at a precise level to ensure a balance of pro-/anti-inflammatory signals to elicit a proper damage response. Any perturbations to NIK-activated signaling significantly predisposes the gastrointestinal niche towards chronic inflammatory conditions of the gastrointestinal tract.

In this work, we explore the potential involvement of dysregulated noncanonical NF- κ B signaling in inducing chronic inflammatory diseases of the gut, including Eosinophilic Esophagitis (upper GI tract), Celiac Disease/Non-Celiac Gluten Sensitivities (small intestine), Inflammatory Bowel Disease (entire intestine/large intestine), and an inflammatory subtype of colorectal cancer being Colitis-Associated Colorectal Cancer (large intestine). We study this pathway via the use of murine models bearing genetic deletions, cellular models, and the generation of miniature organs (i.e. “organoids”) in petri dishes. Further, we assess varying levels of NF- κ B signaling through the genetic deletions of NIK and RelA to inhibit noncanonical and canonical NF- κ B pathways, respectively. Reciprocally, we also examine overactivated signaling via loss of the negative regulatory NLRs, which are proteins that function to impede NF- κ B signaling. Clinical relevancy of this work is evaluated using biopsy samples collected from human patients with active disease states. Culminating our work, we find that noncanonical NF- κ B signaling levels is both tissue- and cell-type specific in driving disease formation. Finally, we conclude our findings by suggesting the promise of NIK as a potential candidate for disease biomarkers and a target for future drug development.

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GENERAL AUDIENCE ABSTRACT

Redness, swelling, heat, pain, and loss of function – these are the five signs of inflammation. Under normal physiological conditions, inflammation is the body's conserved evolutionary response by serving as the first line of defense against infections propagated by foreign invaders like pathogens (i.e. bacteria, viruses, fungi), while also signaling to the immune system to resolve tissue damage. Therefore, properly maintained pro-inflammatory signaling is critical to ensuring a healthy state. However, an imbalance in pro- and anti-inflammatory signaling elicits a long-term, low-grade form of inflammation termed “chronic inflammation”. Unresolved chronic inflammation can persist for several months or even years and further predisposes patients to various chronic inflammatory conditions and even inflammation-induced cancer. The NF- κ B cellular signaling mechanism is a central regulator of inflammation and can be activated upon either the canonical NF- κ B or noncanonical NF- κ B pathways. In comparison to its canonical counterpart, the noncanonical NF- κ B is vastly understudied, especially in regards to gastrointestinal health. A unique feature of the noncanonical NF- κ B pathway is the required stabilization of the NF- κ B-inducing Kinase (NIK) protein, which is required for further propagation of this signaling network.

As evidenced by our culmination of works, we reveal that Noncanonical NF- κ B signaling is critical to gut health, as it maintains a precise cellular signaling mechanism within the gut tract by properly maintaining pro- and anti-inflammatory signaling. Additional, downstream implications include regulation of cell division and activation of cell death to elicit a proper damage response. Within this dissertation, we evaluate the understudied noncanonical NF- κ B pathway in various chronic inflammatory diseases of the gut including Eosinophilic Esophagitis (upper GI tract), Celiac Disease/Non-Celiac Gluten Sensitivities (small intestine), Inflammatory Bowel Disease (entire intestine/large intestine), and an inflammatory subtype of colorectal cancer Colitis-Associated Colorectal Cancer (large intestine). Through the use of murine models bearing deletions of genes related to noncanonical NF- κ B signaling (esp. NIK), cell models, and the generation of “mini-organs” organoids from isolated intestinal stem cells, we are able to model the involvement of NIK and noncanonical NF- κ B signaling in maintaining gastrointestinal health. Clinical relevancy of these findings was further evaluated by quantifying noncanonical NF- κ B signaling levels in human biopsies. Culminating our work, we find noncanonical NF- κ B signaling to be context-specific in driving disease formation. Finally, we conclude this work by suggesting the promise of NIK as a potential candidate for disease biomarkers and target for future drug development.

To all the people who stood in my corner and supported me during my PhD, through all the insecurities, celebrations, and in finally owning my confidence - THANK YOU! It truly takes a village, and I treasure all of you for helping me realize my full potential and never letting me for a moment doubt myself or give up on my dreams.

This work is dedicated to all my biggest advocates, including my loving family, caring “lab family”, friends, and dedicated mentors.

ACKNOWLEDGMENTS

Prior to beginning this PhD, I never realized the true magnitude of earning a PhD. As a first-generation college student, a minority, and a STEMInist, you underestimate the enormity of undertaking this task and pursuing a doctoral degree. Although it takes immense individual progress to learn and grow, a PhD is undoubtedly a team effort. This work would not have been made possible without my extensive support network.

First and foremost, I would like to extend a great appreciation to my advisor Dr. Irving Coy Allen for opening his laboratory to me and offering me a position as a PhD student. Under Dr. Allen's mentorship, I was able to work on a medley of intriguing projects, collaborate with several other researchers/engineers/clinicians/veterinarians, find my passion for stem cell biology, and develop as a scientist. Thank you for giving me the space to cultivate my skills/techniques, challenging me, and giving me opportunities to grow. This experience allowed me to become a better, stronger version of myself and an accomplished biomedical researcher. Further, I extend this appreciation to my committee members Drs. Clayton C. Caswell, Nick Dervisis, Xin M. Luo, and Kenneth J. Oestreich who ensured my continued progress over the years and provided me with insightful advice/discussions at our meetings.

To the Allen Lab, you have been the best group of people I could have possibly asked for to earn PhDs alongside. Imran Khan, Jus Tupik, Hannah Ivester, Tamalika Paul, Manali Powar, Maddie Mott, Dr. Brie Trusiano, Dr. Margaret Nagai-Singer, Dr. Rebecca Brock, Dr. Alissa Hendricks-Wenger, and Dr. Kristin Eden, we undoubtedly grew together and supported each other through all our endeavors. I am thankful for the fact that I can proudly say: "we are just as much friends inside the lab, as we are outside the lab". Because of this, you have endearingly become my "Lab Family". Additionally, I have been fortunate to have such great undergraduates as mentees, including Morgan Stephens, Christina Mounzer, Audrey Rowe, Timothy Le, and Chloe Tenenbaum. Your enthusiasm for learning and dedication to assisting me in my projects helped fuel my passion for not only research, but also mentoring. Thank you to our medical students Kacie Hoyt and Benjamin Tintera for their research contributions and providing our lab with a clinician's perspective for the diseases we study. Bettina Heid, our lab manager, thank you for being a wealth of information regarding good laboratory practice, as well as troubleshooting protocols.

At VMCVM, I am thankful for the endless support I have received from the Office of Research and Graduate Studies, including Andrea Green, Jessica Crawford, and Monica Taylor, as well as my colleagues in the BMVS department. Also, I had the pleasure of working closely with Dr. S. Ansar Ahmed as his teaching assistant. I appreciate the commitment he has shown me as Associate Dean in ensuring my success. I welcome Dr. Margie Lee to her position as Associate Dean and thank her for the support she has already shown me. Drs. R. Scott Pleasant, Laura Hungerford, and S. Ansar Ahmed were instrumental in introducing me to this community, when I first explored opportunities for pursuing graduate school and PhD programs. Likewise, I recognize and express gratitude for the hardworking individuals at the Animal Resource and Care Division (ARCD) and Teaching and Research Animal Care Support Service (TRACSS).

This work would not have been made possible without the mice used in this study and the individuals the Caco-2 cells, THP-1 cells, and HEK293T cells have been derived from. Just as HeLa cells collected from Henrietta Lacks is revolutionary to the field of medicine, these individuals also contributed to this field of research.

Of course, I express the utmost appreciation and gratitude to my family for supporting me during this journey. My parents Travis Morrison and Maira Morrison are the very reason I seek to push myself, set high goals for myself, and instilled in me self-discipline. They dedicated so much to me growing up to ensure that I would never give up on my dreams, myself, or my passion for biomedical research. They deserve this PhD as much as I do for listening to me perform practice runs of my talks, reading through most of my drafts/cold emails/cover letters, and just being there for me (I love you to the moon and back!). Likewise, my grandparents “Pop-pop” Richard Morrison, “Lola” Emilia Basalan Cuizon, “Nana” Carol Poppe, “Grandma” Kathi Lebenns, and “Nana” Linda Morrison have been instrumental in providing me general life advice and helping me see the bright side of things. Further, this work would not have been made possible without the endless support and time dedicated to me by my Pop-pop Richard and Nana Carol. I especially thank my grandmother Emilia for showing me what a strong, independent woman looks like. Decades ago, she had the very strength, courage, and perseverance to take a chance in providing for her family by emigrating them from their hard-working, poverty-stricken roots of the Philippines to a brighter future in Iceland. By earning this degree, I hope to show her that her sacrifices were worth it. Of course, this work has been made much easier with the gentle comfort of my loving golden retrievers Lily and Buddy. Truly, my interests in the sciences began early on as my great-grandmother Jean Brennan inspired my curiosity for the environment, its wildlife, and the natural sciences.

To my Uncle & Godfather Dr. Scott Morrison (DVM Class of '99), you have served as one of my greatest inspirations. As early as I can remember, I aspired to follow in your footsteps by attending the Virginia-Maryland College of Medicine (at the time in the hopes of becoming a veterinarian like you). I will always be thankful that my first biomedical research project was in collaboration with you at Rood & Riddle Equine Hospital, as we sought to characterize the microbiomes of equine pressure ulcers. This project made me realize my life's purpose - conducting research in the hopes of better understanding the mechanisms driving disease pathogenesis and how can these findings be applied to better improve human/animal medicine. When the going gets tough, I fondly reflect upon this research experience to remind me of my commitment to this field.

To each and every one of you, as well as those that are not mentioned, thank you!

ATTRIBUTIONS

Chapter Two: HAM and KJH analyzed data and wrote manuscript. HAM and KJH contributed equally to this work and share first authorship. HAM, KJH, CM, HMI performed research, wrote manuscript, and analyzed data. BHB and BS collected samples and reviewed manuscript. ECM and ICA were responsible for conceptualization, investigation, methodology, formal analysis, supervision, writing, data management, and funding.

Chapter Three: HAM was responsible for investigation and writing/editing this manuscript. Design and execution of the experiments were conducted by HAM, KE, YL, and MN-S. KE and HAM were responsible for mouse breeding, genotyping, and general husbandry. Experimental data interpretation was conducted by HAM, YL, PAW, and ICA. HAM, KE, and YL contributed equally to this work and share first authorship. PAW and ICA contributed equally to this work and share senior authorship. All authors contributed to the writing and revising of the manuscript.

Chapter Four: HAM outlined, edited, reviewed manuscript. HAM, BT, AJR and ICA prepared figures and wrote the manuscript.

Chapter Five: VN, KE, and HAM were responsible for investigation and writing this manuscript. VN, KE, and HAM contributed equally to this work and share first authorship. VN, HAM, KE, KK, SS, RB, and DG were responsible for investigation, DS and ICA were responsible for conceptualization, investigation, methodology, formal analysis, supervision, and writing (initial draft, review, and editing).

Chapter Six: HAM and KE analyzed data and wrote manuscript. HAM and KE contributed equally to this work and share first authorship. HAM, KE, and ICA lead and designed studies, performed experiments, analyzed/interpreted data, prepared figures, and wrote the manuscript. AJR, CM, MCS performed experiments. DER, YQ, PAW, SLB, KMH, and EKH were responsible for designing/executing specific studies and providing key reagents and/or expertise. KE is a board-certified pathologist and was responsible for pathology characterization. EKH collected and provided human specimens.

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CHAPTER ONE

Introduction

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Chronic inflammation is inextricably linked to detrimental health conditions, including chronic inflammatory diseases, autoimmune disorders, and cancer. Unmitigated, long-term exposure to inflammation culminates in an onslaught of proinflammatory cytokines, overly robust wound healing, tissue remodeling, and fibrosis (1). Indeed, the World Health Organization (WHO) has described chronic inflammatory diseases as a systemic worldwide issue, with approximately 3 out of 5 people estimated to die from morbidities/co-morbidities associated with underlying chronic inflammation (2). These lasting effects of chronic inflammation are deleterious as Rudolf Virchow first delineated inflammation as a causative agent for cancer in 1863. His discovery of “lymphoreticular infiltrate”, describing the inflammatory infiltration of leukocytes in neoplastic tissue, was the first to correlate inflammation to cancer development (3). As such, inflammation sustained for periods longer than several months, termed “chronic inflammation”, promotes proliferative and survival signaling precursing carcinogenesis. Nearly a century later, Douglas Hanahan and Robert A. Weinberg undertook the formidable task of simplifying cancer into six “Hallmarks of Cancer” (4). This otherwise highly heterogeneous disease is a culmination of diverse neoplasia/hyperplasia of various tissue-types throughout the body, with intricate disease mechanisms and complex cellular signaling cascades that are ever-evolving, making cancer not only difficult to treat but as the oncologist Siddhartha Mukherjee described, “the emperor of all maladies” (5). Hanahan and Weinberg highlight six conserved features that may be pinpointed to better describe cellular/molecular mechanisms driving disease progression, as well as targetable characteristics for future drug development. Although not constituting one of the six original hallmarks of cancer, inflammation was later described as an enabling characteristic that cultivates a microenvironment conducive to promoting tumorigenesis and its subsequent progression (6).

Rather than merely being the bearer of bad news, inflammation is a conserved evolutionary adaptation to protect the host from various bacterial/viral/fungal infections

and to indicate tissue damage following cellular stress/trauma (7). However, this form of acute inflammation is both temporally restricted as it only spans minutes/hours and is restricted in severity. Following recognition of conserved molecular patterns including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs), further downstream signal transduction activates inflammation (3, 7). The NF- κ B pathway is one such pathway downstream of PRR activation that is nearly synonymous with inflammation. Activation of the NF- κ B transcription factor occurs through either the canonical or noncanonical NF- κ B signaling pathways to initiate two very different signaling cascades with diverse biological implications.

The canonical (aka “classical”) NF- κ B pathway is activated by a diverse myriad of pro-inflammatory signaling molecules, including PAMPs, DAMPs, and TNF. Following TNF receptor superfamily receptors/T-cell receptors/B-cell receptor stimulation, the canonical pathway is rapidly activated within minutes as IKK phosphorylates I κ B α at two N-terminal serines resulting in its degradation (8). Under inactive states, I κ B α maintains the NF- κ B transcription factor, constituting of the heterodimer RelA/p50, in the cytoplasm through the I κ B kinase complex consisting of the regulatory subunit I κ B γ and the catalytic subunits IKK α and IKK β . Proteosomal degradation of I κ B α therefore relinquishes RelA/p50 into the cytoplasm permitting its translocation into the nucleus, which activates the transcription of further pro-inflammatory genes including *COX2*, *TNF*, *IL-1 β* , and *IL-6* (9). Activation of the canonical NF- κ B promotes gene transcription related to immunity, cell migration, cell adhesion, cell death, and inflammation (10).

In contrast, the noncanonical (aka “alternative”) NF- κ B pathway is more tightly regulated and activated by a limited array of signaling molecules (i.e. BAFF, CD40L, RANKL, TNF, TWEAK) to its respective TNF receptor superfamily receptors (i.e. BAFFR, CD40, RANK, TNFR2, FN14) (11). The noncanonical NF- κ B pathway is unique from its canonical counterpart in that it requires stabilization of the NF- κ B-inducing Kinase (NIK) to process the NF- κ B heterodimer p100/RelB into its active form (9). Following stimulation of the TNF receptor superfamily receptor, TRAF3 of the

TRAF3/TRAF2/cIAP1/cIAP2 complex is degraded, allowing cytosolic levels of NIK to stabilize (9). NIK therefore phosphorylates IKK α , which results in the phosphorylation of p100 into its active form of p52 and revealing an NLS for nuclear localization (9). Following nuclear translocation of p52/RelB, gene transcription is relatively limited to the chemokines *CXCL12*, *CXCL13*, *CCL19*, and *CCL21* (9, 11-14). Rather than being predominantly associated with regulating inflammation like the canonical pathway, activation of the noncanonical pathway is attributed to development of lymphoid structures (esp. Peyer's patches within the gut), T cell activation/differentiation, IgA class switching, and B cell maturation (15).

Given that the noncanonical NF- κ B pathway was once considered an auxiliary signaling mechanism should canonical signaling be impaired (8, 16), this pathway is relatively understudied in the field of NF- κ B research, especially regarding the gastrointestinal tract. To date, dysregulated noncanonical NF- κ B signaling has been characterized in several inflammatory disorders and cancers, with both overzealous and inhibition of noncanonical NF- κ B signaling contributing to several disease etiologies, thus emphasizing the delicate balance that must be maintained for homeostasis. Under normal physiologic conditions, proper noncanonical NF- κ B signaling has been shown to regulate lymphoid organogenesis, B cell maturation, osteoclast differentiation, and immune cell trafficking (15). A revived interest in noncanonical NF- κ B research has begun to unveil further biological processes regulated by noncanonical NF- κ B, as well as downstream implications given its dysregulation. Aberrant noncanonical NF- κ B signaling has been well-characterized in related diseases including leukemia (17), myeloma (18), lymphomas (19-22), periodontal inflammation/bone loss (23), and inflammatory osteoclastogenesis (24). Dysregulation of noncanonical NF- κ B signaling has also been observed in various cancers, including but not limited to ovarian cancer (25), breast cancer (26), prostate cancer (27), lung cancer (28), glioblastoma/de-differentiation of astrocytes (29-31), and gastric cancers (32). However, the role of noncanonical NF- κ B signaling in the gastrointestinal tract has been somewhat limited. Recent seminal work characterizes unrestricted noncanonical NF- κ B signaling following loss of its negative regulator NLRP12 in contributing to colitis and colitis-induced

tumorigenesis (12, 33-35). Therefore, overactivation of the noncanonical NF- κ B signaling pathway contributes to chronic inflammation within the large intestine. However, loss of NIK in the esophagus culminates in the presentation of a hypereosinophilic syndrome within the esophagus of the upper gastrointestinal tract (36, 37). Interestingly, noncanonical NF- κ B signaling must be maintained in a precise balance, as both overabundant and insufficient noncanonical NF- κ B signaling contributes to hyperinflammatory conditions of the gastrointestinal tract.

Within this dissertation, we examine the potential role of dysregulated noncanonical NF- κ B signaling, an understudied pathway that activates the transcription factor NF- κ B, in the disease pathogenesis of various chronic inflammatory disorders of the gastrointestinal tract. In **Chapter 2**, we interrogate the whole transcriptome of human Eosinophilic Esophagitis (EoE) patients and healthy control patients to directly delineate the plausible role of noncanonical NF- κ B signaling in this disease. Next, we compare the effects of both overzealous canonical and noncanonical NF- κ B signaling given loss of their respective negative regulators NLRX1 and NLRP12 in altering the gut microbiome and contributing to Celiac Disease (CeD) and Non-Celiac Gluten Sensitivities (NCGS) in **Chapter 3** (38). In **Chapter 4**, we highlight recent works that have found dysregulated NF- κ B signaling to drive Inflammatory Bowel Disease (IBD) pathogenesis (11). We contribute to this field of IBD research by finding that upregulation of noncanonical NF- κ B signaling is associated with loss of responsiveness to anti-TNF therapeutics in human IBD patients in **Chapter 5** (39). In **Chapter 6**, we reveal the cellular/molecular mechanisms underlying the malignant transformation of intestinal epithelial cells following inflammation-induced tumorigenesis in Colitis-Associated Colorectal Cancer (CAC). Finally, we conclude this dissertation in **Chapter 7** and discuss the potential applications of this work, highlighting the promise of NIK and noncanonical NF- κ B signaling as potential targets for drug development and biomarkers for disease.

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CHAPTER TWO

Expression Profiling Identifies Key Genes and Biological Functions Associated with Eosinophilic Esophagitis in Human Patients

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Abstract

Eosinophilic Esophagitis (EoE) is a chronic allergic disease characterized by progressive inflammation of the esophageal mucosa. This chronic inflammatory disorder affects up to 50 per 100,000 individuals in the United States and Europe yet is limited in treatment options. While the transcriptome of EoE has been reported, few studies have examined the genetics among a cohort including both adult and pediatric EoE populations. To identify potentially overlooked biomarkers in EoE esophageal biopsies that may be promising targets for diagnostic and therapeutic development. We used microarray analysis to interrogate gene expression using esophageal biopsies from EoE and Control subjects with a wide age distribution. Analysis of differential gene expression (DEGs) and prediction of impaired pathways was compared using conventional transcriptome analysis (TAC) and artificial intelligence-based (ADVAITA) programs. Principal Components Analysis revealed samples cluster by disease status (EoE and Control) irrespective of clinical features like sex, age, and disease severity. Global transcriptomic analysis revealed differential expression of several genes previously reported in EoE (*CCL26*, *CPA3*, *POSTN*, *CTSC*, *ANO1*, *CRISP3*, *SPINK7*). In addition, we identified differential expression of several genes from the *MUC* and *SPRR* families, which have been limited in previous reports. Our findings suggest that there is epithelial dysregulation demonstrated by DEGs that may contribute to impaired barrier integrity and loss of epidermal cell differentiation in EoE patients. These findings present two new gene families, *SPRR* and *MUC*, that are differentially expressed in both adult and pediatric EoE patients, which presents an opportunity for a future therapeutic target that would be useful in a large demographic of patients.

INTRODUCTION

Eosinophilic Esophagitis (EoE) is a chronic allergic disease that is characterized by progressive inflammation of the esophageal mucosa, which in severe cases may lead to food impaction and esophageal stricture (1, 2). This eosinophil-associated gastrointestinal disorder (EGID) affects up to 50 per 100,000 individuals in the United States and Europe and can lead to significant impairment in quality of life if untreated, demonstrating the need for effective and targeted treatments (3). Epidemiologic data

suggests that the incidence of EoE is exponentially increasing, with some studies reporting up to a 100-fold increase in recent years (4). Risk factors ranging from geographical location and heritable traits to early antibiotic exposure have been implicated in the pathogenesis of EoE(5). EoE affects both adult and pediatric populations, although several differences between the character of the disease in these groups have been reported (6, 7). Children tend to display more acute manifestations of inflammation-like exudates and furrows, whereas adults tend to have more fibrostenotic signs of disease, such as strictures (8). Identifying biologic factors that are shared in both the pediatric and adult EoE populations would pave the way for novel diagnostic and treatment targets for the wide spectrum of this disease.

EoE is diagnosed by presence of symptoms of esophageal dysfunction (dysphagia, food impaction, heartburn, chest pain), histologic confirmation by H&E stain of ≥ 15 eosinophils per high power field (eos/hpf), and the exclusion of other causes of esophageal eosinophilia (4). The treatment goal is symptomatic management and prevention of esophageal remodeling. First-line treatment for EoE is pharmacologic intervention proton pump inhibitors/corticosteroids or empiric elimination diets (9). Recently, several clinical trials repurposing biologics used for other indications in the treatment of EoE have been evaluated with variable success (10). Dupilumab (IL-4R α antagonist) completed its Phase III trial in May 2022 and is currently FDA approved(11). Although originally promising, Mepolizumab (IL-5 antibody) did not meet its primary endpoint in a Phase III study for EoE (12). Monoclonal antibodies targeting IL-13, anti-TNF, and anti-TSLP are still ongoing (10). Limited success therefore offers an opportunity for identifying new EoE-specific markers for potential targeted therapies specific to a disease process.

Seminal work by Rothenberg described the “EoE transcriptome,” including 574 transcripts associated with pathogenesis, including overexpression of the eosinophil-specific chemoattractant eotaxin-3 (*CCL26*) (13). Subsequent work delineated the molecular pattern of epithelial barrier dysfunction in EoE with downregulated filaggrin (*FLG*) and desmoglein (*DSG1*) (14, 15). The *SPINK* family is also dysregulated in EoE patients, likely contributing to the permeability of the epithelium (16). Disruption of the

epithelial barrier promotes the T_H2 response in the esophagus by directly releasing TSLP, IL-33, and IL-25, and enabling relevant antigens to penetrate the esophageal mucosa.

Consistently described as an allergic disease, the presence of T_H2 cells and cytokines have been identified in EoE patients. T_H2 associated cytokines IL-4, IL-5, and IL-13, were induced in patients with active EoE by milk, supporting the allergic etiology of EoE (17). This is corroborated by genetic associations that describe *TSLP* as a promoter of the T_H2 response in EoE subjects (14). This overactive T_H2 response, particularly IL-13 production, further induces an epithelial response and diminishes barrier function as EoE patients have overall decreased *DSG1* and decreased expression of genes related to epithelial structural genes, including *FLG*, *IVL*, and the *SPRR* gene family (18). These genes also comprise the epidermal differentiation complex (EDC) and include the expression of the cytokeratins *KRT4* and *KRT13* in the suprabasal zone, which contain more differentiated cells. Likewise, E-cadherin is expressed in both the basal and suprabasal layers by epithelial cells (18). Therefore, the transcriptome of EoE patients is characterized by a unique esophageal gene expression signature that is indicative of altered esophageal tissue differentiation, impaired barrier function, and overzealous allergic responses.

Regulators of local epithelial barrier dysfunction and the T_H2 response have been well described by molecular studies. In this manuscript, we confirm commonly identified transcriptomic patterns in the pathogenesis of EoE. Further, we extend these results by highlighting the *MUC* and *SPRR* families, which were highly represented among the most differentially expressed gene families in our dataset. These findings may underscore pathways that have yet to be investigated in the development of EoE and represent a potential target for future therapeutic studies.

MATERIALS & METHODS

Subject Recruitment

A total of 24 (12 EoE, 12 Control) subjects were enrolled into this study with informed consent from all participants and/or guardians. All studies were approved by the Institutional Review Board. EoE subjects were individuals with suspected EoE scheduled for esophagogastroduodenoscopy (EGD) for diagnostic confirmation. Control subjects were individuals undergoing EGD for investigative purposes or other non-inflammatory

etiologies. All patients selected were undergoing an initial EGD and not yet prescribed therapeutics for EoE. All relevant data, including health information, laboratory results, pathology results, endoscopy scores, treatment, and questionnaire responses were recorded.

Sample Collection

Tissue samples were obtained during clinically indicated EGD procedures with clinical assessment of Eosinophilic Esophagitis Endoscopic Reference Score (EREFS). Biopsies collected from endoscopically visible areas of active inflammation and healthy mucosa in subjects and controls, respectively. Definitive diagnosis of EoE was made using clinical, endoscopic, and histologic metrics per current guidelines (4), including symptoms of esophageal dysfunction, presence of ≥ 15 eos/hpf, and exclusion of other potential causes of esophageal eosinophilia. Controls were defined as patients who underwent an EGD for symptoms suggestive of EoE but not confirmed with endoscopic or histologic assessment. Samples were then stored in RNeasy (Qiagen) until further processing.

Variable Definitions

Active disease was defined as active inflammation based on endoscopy. Disease duration was categorized by years since diagnosis in 3 groups: <1, 1-5, >5 years. Eosinophilic Esophagitis Reference Score (EREFS) disease activity summary score was recorded at the time of biopsy (19). Eosinophilia grading was developed using eos/hpf histologic data: mild=0-6 eos/hpf, moderate=7-15 eos/hpf, severe=15-30 eos/hpf, and profound= 31+ eos/hpf.

Tissue Processing and Microarray Analysis

Biopsy samples were homogenized in RLT buffer (Qiagen). DNA, RNA, and protein were extracted using the AllPrep kit per manufacturer's protocol (Qiagen). RNA was assessed for quality using NanoDrop™ and stored at -80°C. 10µL RNA (50ng/µL) were plated on a barcoded 96-well plate provided by Thermo Fisher Scientific. Microarray analysis using Clariom™ S Assay for human samples was performed by Thermo Fisher Scientific. Plate normalization, hybridization, and processing are performed as part of the throughput at Thermo Fisher Scientific. Finalized data was distributed in the format of CEL files.

Data Analysis

Population descriptors were expressed as frequency (number and percentage) and median $\pm$ IQR, as appropriate. Microarray analyses were performed using the Thermo Fisher Transcriptome Analysis Console (TAC). CEL files were imported to the TAC software and initial quality control evaluation was conducted. Samples passed quality control for three parameters, including labeling controls, hybridization controls, and positive vs negative AUC thresholds. Principal Components Analysis (PCA) were used to determine if samples clustered based on disease status or other clinical metrics. Differential gene expression (DEG) was used to determine gene families and clusters that were differentially up-/down-regulated between EoE and Control groups, with significance defined as ≥ 2 , ≤ -2 -fold change.

Analysis using ADVAITA Bioinformatics iPathwayGuide, which is an AI-based platform for predictive pathway analysis (ADVAITA Bioinformatics, Ann Arbor, MI, USA), was conducted in parallel. All significant DEGs were used as input to determine the significantly impacted pathways with pathway annotations being derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Significant pathways (false discovery rate adjusted p values < 0.1) were presented as $-\log(p\text{-value})$. The top significantly enriched Gene Ontology (GO) biological processes and cellular components are reported as $-\log_{10}(p\text{-value})$. Significant DEGs for the SPRR and MUC family were input into g:Profiler (<https://biit.cs.ut.ee/gprofiler/gost>) for functional profiling with statistically significant related Reactome pathways reported as $-\log_{10}(p\text{-value})$.

RESULTS

Patient Characteristics

This study utilized tissue samples from 12 EoE and 12 Control subjects. The EoE group was 33.3% female compared to 83.3% female in the Control group (**Table 1**). The age distribution was comparable with a median age of 18 years in both EoE (range: 5-31 years) and Control (range: 5-49) groups. The study population overall was predominantly white (83.3%), non-Hispanic Latino (83.3%). None of the Control samples had evidence of disease activity on biopsy, whereas all the EoE subjects had evidence of active disease at the time of sampling. Half of the EoE subjects had a disease duration of greater than 5 years, 33.3% 1-5 years, and 16.7% less than 1 year. The median disease severity based on

endoscopic reference score assessing EREFS in the EoE group was 3.5 (range: 1-6). Histological grading of the EoE biopsy samples revealed a median eosinophilia count of 31 eos/hpf (range: 15-92). The transcriptomic data was analyzed for the impact of these clinical features on the clustering of samples using principal component analysis (PCA). The samples strongly clustered by disease category, moderately clustered by disease duration, moderately clustered based on sex, and no defined clustering based on age category (**Supplementary Figure 1; 2**). Therefore, disease status better defines the clustering observed, with their being stratification given disease duration. Clustering based by sex may likely be explained by EoE being nearly 4 times more prevalent in males than females (20). We anticipate that clustering effects would become stronger given a larger sample population. Based on the appearance of these clusters, we further explored sample transcriptomics to better understand which gene families are driving the clusters.

Global Transcriptomic Analysis Shows Sample Clustering by Disease State

Microarray analysis returned data on 21,448 genes, 1,059 of which were DEGs between EoE and Control samples. Scatterplot analysis of the DEGs revealed 806 (76.1%) were up-regulated and 253 (23.9%) were down-regulated (**Figure 1A**). The top 20 up-regulated (**Figure 1B**) and down-regulated genes (**Figure 1C**) represented a diverse repertoire of functions. The most differentially expressed up-regulated genes were *JCHAIN* (151.4-fold), *CPA3* (92.8-fold), *ANO1* (57.9-fold), and *CDH26* (55.7-fold) which all had a greater than 50-fold change increase compared to Controls. Overall, the down-regulated genes were more differentially expressed with 3 genes having more than a 100-fold decrease compared to Controls: *CRISP3* (-1411.9-fold), *SPRR2E* (-127.7-fold), and *SPINK7* (-123.9-fold). Hierarchical clustering of up-regulated genes displayed close relationships between all but one of the Control samples, suggesting a similar transcriptomic profile. Similarly, one EoE sample clustered more tightly with the Control group than the EoE group (**Figure 1D**). Interestingly, hierarchical clustering of the down-regulated genes displayed 2 distinct subgroups of EoE patients, while the Control samples remained tightly clustered (**Figure 1E**). The Control sample that clustered with the EoE group in the up-regulated gene heatmap remained clustered with EoE samples in the down-regulated heatmap.

Transcriptomic Data Aligns with Commonly Reported EoE Genes and Pathways

Our dataset confirmed several classic genetic markers of EoE. Among the top 20 up-regulated genes, 8 have been previously associated with EoE. Notably eotaxin-3 (*CCL26*), an eosinophil chemotactic chemokine, which has been extensively reported as a strong driver of EoE (13, 21) was represented in this group (**Figure 2A**). These up-regulated genes have also been reported to play a breadth of roles in EoE pathophysiology including: mast cells (*CPA3*), tissue remodeling (*POSTN*; *CTSC*), and ion channel function/inflammation (*ANO1*; *ALOX15*) (22, 23) (**Figure 2A**). The most highly down-regulated gene (*CRISP3*) has been described as impacting epithelial function in EoE patients (16). *SPINK7*, down-regulated by 132-fold in our dataset, has been implicated in regulating esophageal barrier maintenance and down-regulation may be associated with EoE pathogenesis (16). The downregulated genes have also been associated with cell adhesion and inflammation in EoE, such as *DSG1* (14, 15) (**Figure 2B**). Several of the top pathways based on gene count were associated with barrier cell function and integrity (VEGFA-VEGFR2; Endothelin; EGF/EGFR) (**Figure 2C**). Tight junctions are a critical signaling component of barrier function and were also implicated in pathway analysis performed by ADVAITA iPathwayGuide (**Figure 2D**). The IL-18 signaling pathway, which has been linked to mast cell recruitment in food allergen induced EoE, was also represented in the pathway analysis (24) (**Figure 2C**). Autophagy was one of the top pathways to be implicated by the dysregulated transcriptome (**Figure 2D**). This is consistent with a previous study that found autophagy to be a potential drug target and critical for maintaining metabolic homeostasis within the esophageal epithelia following exposure to pro-inflammatory cytokines (25). Of interest, metabolic pathways was the top pathway predicted with a $-\log_{10}$ p-value of 2.78 (**Figure 2D**). Dysregulation of these predicted pathways is expected to impair functions largely related to intercellular signal transduction, catalytic activity, lipid metabolism, response to stimuli, and keratinocyte differentiation (**Figure 2E**). Pathologic activity is expected to interfere with protein binding, enzyme binding, and cadherin binding (**Figure 2F**). Defects in cadherin binding is another mechanism that may impair barrier function in EoE patients. A study conducted by Doshi *et al.* found that pediatric EoE patients had decreased membrane bound E-cadherin (26). Therefore, loss of E-cadherin is predicted to decrease barrier

integrity by interfering with tight junction interactions. Our findings recapitulate the well-described etiology of EoE as a food allergen directed destruction of the esophageal barrier.

No Significant Involvement of Noncanonical NF- κ B Signaling

Recently, a mouse model deficient in *Map3k14*, which encodes for the central kinase NIK in noncanonical NF- κ B signaling, was characterized as presenting features resembling hypereosinophilic syndrome-like disease (27, 28). As NF- κ B signaling is involved in a diverse range of biological processes and dysregulation of these pathways contributes to inflammatory etiologies, we next assessed the possible involvement of noncanonical NF- κ B in EoE. We hypothesized that genetic deletion of NIK and subsequent inactivation of the noncanonical NF- κ B pathway may be paramount in EoE pathogenesis. Therefore, we also predicted genes encoding for central components within the noncanonical NF- κ B pathway may serve as biomarkers for EoE disease status. We assessed an assortment of nearly 90 genes related to both canonical and noncanonical NF- κ B signaling pathways and found no significant DEGs expressed (**Supplemental Table 1**). These findings reveal that the noncanonical NF- κ B does not participate in EoE pathogenesis at a gene expression level.

Altered Gene Expression in Genes Related to Keratinization

Previous work assessing esophagus-specific transcripts following whole exome sequencing described impaired regulation of epithelial differentiation and keratinization(29). Consistently, keratinocyte differentiation was one of the top GO biological functions predicted from our transcriptome profile in EoE patients (**Figure 2E**). Examination of genes involved in keratinocyte differentiation revealed a distinct enrichment of small proline-rich protein (*SPRR*) genes, as 7 out of 29 genes were significantly downregulated (**Figure 3A**). ADVAITA iPathwayGuide analysis reveals downstream implications of keratinocyte differentiation on epidermal cell differentiation that further affect epithelium development, cell differentiation, and anatomical structure development (**Figure 3B**). We next analyzed our whole-transcriptome dataset specifically for *SPRR* genes and found that all 10 *SPRR* genes were downregulated with 7 genes identified as significant (**Figure 3C**). GO biological analysis of the *SPRR* gene family predicts the involvement of these genes in keratinization,

keratinocyte/epidermal/epithelial cell differentiation, skin/epidermis/epithelium development, and peptide cross-linking (**Figure 3D**). These biological functions are expected to occur within the cornified envelope (**Figure 3E**). Reactome pathway prediction further reinforces the implications genetic dysregulation has on formation of the cornified envelope, keratinization, and developmental biology (**Figure 3F**).

Impaired *MUC* Gene Expression in EoE Biopsies

Mucin expression is a common biomarker for several diseases with interference in production impairing cellular integrity. Within the gastrointestinal tract, the amount of mucins generally increases from esophagus to rectum (30). Under normal physiologic states, the human esophagus produces *MUC1*, *MUC4*, *MUC5B*, and *MUC20* (30). Evaluation of the *MUC* genes indicated that two genes were significantly downregulated: *MUC22* (-36.63-fold) and *MUC21* (-27.49-fold) (**Figure 4A**). *MUC4* was the only gene in this family found to be significantly upregulated (8.08-fold) (**Figure 4A**). To date, these mucins have not been evaluated in the context of eosinophilic esophagitis. However, *MUC4* is associated with normal squamous epithelium; therefore, an increase in this gene expression may be indicative of increased differentiation. However, increased *MUC4* has also been observed other diseased esophagus states, including Barrett's esophagus and high-grade intraepithelial neoplasia (31). A glycoform of *MUC21* with extended carbohydrate chains was found in suprabasal cells and serves as a marker for differentiation in squamous cell carcinoma (32), which suggests another possible marker for esophageal differentiation. Dysregulation of these mucin genes are expected to impact extracellular matrix constituents, particularly involving lubricant activity and structure (**Figure 4B**). GO cellular components largely predicts this dysregulated activity to occur within the Golgi lumen (**Figure 4C**), the site at which mucin dimers move from the ER for assembling of *O*-glycans. As the type and composition of mucins are indicative of tissue, physiologic state, and pathology, mucins contain a spectacle of *O*-linked oligosaccharides that vary between the types of mucins, tissues, and pathologic conditions and is predicted to vary between EoE patients and Controls (**Figure 4D**).

Overall Loss of Epidermal Differentiation Genes in EoE Biopsies

Previous work from the Human Protein Atlas evaluated esophagus-specific transcripts and found that transcripts were predicted to implicate keratinization and differentiation. This unique transcriptome profile was correlated with EoE biopsies lacking adequate tissue differentiation (29). Our work also found common genes related to keratinization and differentiation to be DEGs. Cornulin expression was found to be downregulated in our dataset comparing EoE biopsies to Controls (*CRNN*, -3.69-fold) (**Figure 5A**). Filaggrin (*FLG*, -16.63-fold) and desmoglein 1 (*DSG-1*, -132.09-fold) were also down-regulated in EoE patients (structural integrity of the epithelial barrier) (**Figure 5A**). Further, a member of the SPRR family, *SPINK7* was decreased (-132.09-fold). Keratin-6C (*KRT6C*) was found to be downregulated at -2.35 (**Figure 5A**). This is consistent with our transcriptome profile for EoE patients as functional enrichment GO predicted impaired biological functions related to keratinization, differentiation, and epidermis/epithelium/tissue development (**Figure 3D**). Reactome analysis further predicted formation of the cornified envelope to be impacted (**Figure 3F**). Transcriptome analysis of the EDC genes reveals an overall downregulation of genes related to cornification, proper maturation, and terminal differentiation of squamous epithelial cells.

DISCUSSION

The dataset we present supports previously reported transcriptomic profiles associated with EoE disease states while also offering novel insight by identifying genes previously unreported with EoE. Periostin (*POSTN*) was significantly upregulated and desmoglein 1 (*DSG1*) downregulated compared to Controls in our cohort. These findings support previous models of EoE pathogenesis that describe the interplay of these two genes creating a paradigm induced by IL-13 in which decreased desmoglein function disrupts the epithelial barrier, permitting increased eosinophil adhesion mediated by overactive periostin (14, 15). Another gene, *TSLP*, has also been tied to this inflammatory mechanism by promoting T_H2 differentiation and the pro-inflammatory cycle (33, 34), though this gene was not significantly downregulated in our dataset (fold change -1.07). While this finding is inconsistent with previous reports, several environmental factors have been noted to influence the pathogenesis of EoE. In adults, smoking and other aeroallergens drive T_H2 inflammation(35), whereas in children early antibiotic treatment,

cesarean delivery, and preterm birth were noted as important for pathogenesis (36, 37). The gene perhaps most commonly tied to EoE, eotaxin-3 (*CCL26*), was present among our greatly upregulated genes. Studies have described this gene locus as being under significant epigenetic influence (13, 38). Further investigation of other gene loci may reveal the importance of epigenetics among other markers of EoE. The wide age distribution of our cohort may play a role in the expression levels of classic EoE genes in our analysis given the variable effects of epigenetics and environmental exposures at different ages. It is important to note that Control biopsies collected for this study were collected from patients undergoing exploratory endoscopy for another disease/condition independent of EoE. As a result, a limitation of this study is that Controls were collected from patients that presented with symptoms analogous to EoE but were confirmed non-EoE given histopathological assessment.

Seminal work conducted in 2017 evaluated genes from the Human Protein Atlas to compare esophagus-specific transcripts as they may relate to EoE pathogenesis (29). They found approximately 39% of esophagus-specific transcripts were dysregulated in EoE patients (29). These transcripts were predicted to implicate keratinization/differentiation and correlated with EoE biopsies lacking adequate tissue differentiation (29). This is consistent with our transcriptome profile for EoE patients as functional enrichment GO predicted impaired biological functions related to keratinization, differentiation, and epidermis/epithelium/tissue development (**Figure 3D**). Reactome analysis further predicted formation of the cornified envelope to be impacted (**Figure 3F**). Although there are overlaps in functions between the skin and mucosal surfaces of the gut in forming an effective immune barrier from the external environment, these tissues have distinct anatomical differences. For instance, terminal differentiation of epidermal keratinocytes in the skin results in desquamation and cornified cells at the surface. It is important to note that cornification is atypical within the normal esophageal epithelium, where it is instead composed of nonkeratinized stratified squamous epithelium (39). Beyond the evaluation of the SPRR gene family in EDC, little more is known about its involvement in the normal esophagus or EoE. Here, we document several differentially expressed SPRR genes in human EoE (**Figure 3C**). Work conducted in 2021 analyzed the interplay of SPRR genes and late cornified envelope

(LCE) genes and found co-localization with *SPRR2* and a member of the *LCE3D* in psoriasis (40). *SPRR2A*, *SPRR2B*, *SPRR2D*, *SPRR2E*, and *SPRR2F* were all found to be decreased in EoE biopsies (**Figure 3C**). An emerging school of thought suggests a gut-skin axis linking skin and gut disorders, with the common denominator being dysregulated microbiomes driven by environmental factors, suggesting that more visually apparent skin disorders may give insight to systemic issues occurring simultaneously within the gut (41). We previously showed in a spontaneous murine model for hypereosinophilic-like syndrome that mice lacking noncanonical NF- κ B signaling had systemic eosinophilia, including atopic dermatitis and esophagitis (28). Concomitant atopic dermatitis has also been reported in 2-19% human EoE patient (42). We suggest that a better understanding of EoE pathogenesis may be derived from reinterpretations of the well-studied involvement of the *SPRR* gene family and its implications on cornification.

Current work has sought to better evaluate proper differentiation of epithelial cells within the basal zone (undifferentiated, proliferating cells) and suprabasal zone (differentiated cells) of the esophagus (39, 43). Transcriptome analysis of EDC genes reveals suprabasal zone cells have increased expression of the keratins *KRT4* and *KRT13* (29, 44-46). This work sought to evaluate specific epithelial differentiation markers given pathophysiologic conditions (29). They found increased undifferentiated markers (*KRT5*, *KRT14*) and decreased terminal differentiation marker (*KRT4*) in active EoE patients (29). Although highly expressed in the normal esophagus, decreased cornulin (*CRNN*) expression is observed in EoE patients (29). This was consistent with our dataset comparing (*CRNN*, -3.69-fold) (**Figure 5A**). Other groups further assessed EDC genes as they relate to drug responsiveness (39) and found filaggrin to be down-regulated 16-fold in EoE patients, which is identical to our results (*FLG*, -16.63-fold) (**Figure 5A**). Following treatment with glucocorticoids and/or dietary changes, fillagrin expression was not significantly changed, indicating EDC genes can be reversed following responsiveness to treatment (39). This suggests improper differentiation within the esophagus originates at a genetic level and may cause EoE patients to be susceptible to irregular anatomical development.

Previous work recorded distinct changes in mucin gene expression and production. Within the gastrointestinal tract, the amount of mucins generally increases from esophagus to rectum (30). Mucins produced by the esophagus under normal physiologic states include MUC1, MUC4, MUC5B, and MUC20 (30). MUC1 and MUC4 are expressed in normal squamous epithelium, while MUC5B is expressed by submucosal glands (47). Discrepancy in mucin production can also be associated with pathologic features of the esophagus. For instance, MUC1 and MUC4 levels correlate to the degree of proper differentiation occurring within the epithelia (47), whereas MUC5AC has been associated with tissue remodeling in the lung and found to be upregulated in human EoE patients (48, 49). In our dataset, we found *MUC4* to be increased 8.08-fold, while *MUC1* and *MUC5AC* were not differentially expressed (**Figure 4A**). Another group found *MUC1* and *MUC5B* expression to be downregulated, while *MUC4* was increased (50). We did not find *MUC5B* to be significantly varied in our dataset (**Figure 4A**). Two MUC genes, *MUC21* (-36.63-fold) and *MUC22* (-27.49-fold), were uniquely altered in our transcriptome profiles (**Figure 4A**). To date, MUC22 has not been evaluated in the context of the esophageal epithelium. However, a glycoform of MUC21 with extended carbohydrate chains was found in suprabasal cells and serves as a marker for differentiation in squamous cell carcinoma (32), indicating a potential role of MUC21 *O*-glycosylation in esophageal differentiation, which would be consistent with Reactome pathway predictions (**Figure 4D**). Altered mucin production further recapitulates that improper differentiation occurring within the epithelium may have deleterious downstream effects on barrier function, tissue remodeling, and overall cell integrity within this tissue to culminate in disease and sensitizing this tissue to food allergens.

Our data contributes additional insight into the transcriptome profile of EoE patients and contributes to our understanding of EoE pathogenesis. We highlight two families of genes (SPRR, MUC) that are understudied in the context of EoE and suggest their involvement in improper epithelial cell differentiation of the diseased esophagus. Information culminated from this study suggests potential targets for therapeutic development focusing on EDC. Current treatment options resolve pro-inflammatory signaling to remediate symptoms without directly addressing specific dysfunctional

biological processes, thus necessitating an urgency to develop improved options for EoE. Our data suggests SPRR and/or MUC as targets to resolve EDC and improper tissue damage responses as a viable therapeutic option.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

HAM, KJH, CM, HMI performed research, wrote manuscript, and analyzed data. BHB and BS collected samples and reviewed manuscript. ECM and ICA were responsible for conceptualization, investigation, methodology, formal analysis, supervision, writing, data management, and funding.

FUNDING

This work was supported by the Virginia Maryland College of Veterinary Medicine (I.C.A.); the Virginia Tech Carilion School of Medicine (I.C.A.); National Institute of Allergy and Infectious Diseases (NIAID): K23AI123596 (E.C.M) and R21AI151497(E.C.M); The National Center for Advancing Translational Sciences (iTHRIVE): UL1TR003015 (E.C.M. & I.C.A.). The content is solely the responsibility of the authors and does not necessarily represent the official views of any funding agency.

ACKNOWLEDGEMENTS

The authors would like to thank Bettina Heid, Khan Mohammad Imran, Benjamin Tintera, Brie Trusiano, and Juselyn Tupik for their technical support. We would also like to thank the medical staff and colleagues at the University of Virginia that contributed clinical assistance to this study. We also appreciate the work of iThrive for establishing and facilitating collaborative projects between the University of Virginia and Virginia Tech. Schematics were generated using Biorender.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI's Gene Expression Omnibus (GEO) at GSE228083.

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE228083>.

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TABLES

Table 1. Demographic and clinical characteristics of subjects.

Characteristic		EoE (n=12)	Control (n=12)
Sex- No. (%)			
	Female	4 (33.3)	10 (83.3)
	Male	8 (66.7)	2 (16.7)
Age- Median (range)		18 (5-31)	18 (5-49)
Age Category- No. (%)			
	0-10 yr	1 (8.3)	1 (8.3)
	11-20 yr	6 (50.0)	6 (50.0)
	21-30 yr	3 (25.0)	1 (8.3)
	31-40 yr	2 (16.7)	3 (25.0)
	41-50 yr	-	1 (8.3)
Race- No. (%)			
	White	9 (75.0)	11 (91.7)
	Bi/Multi Racial	1 (8.3)	-
	African American	-	1 (8.3)
	American Indian	-	-
	Asian	-	-
	Native Hawaiian	-	-
	Not identified	2 (16.7)	-
Ethnicity- No. (%)			
	Hispanic/Latino	2 (16.7)	1 (8.3)
	Non-Hispanic Latino	9 (75.0)	11 (91.7)
	Unknown	1 (8.3)	-
Active Disease- No. (%)		12 (100)	0 (0)
Disease Duration- No (%)			
	<1 yr	2 (16.7)	7 (58.3)
	1-5 yr	4 (33.3)	2 (16.7)
	>5 yr	6 (50.0)	3 (25.0)
EREFS- median (range)		3.5 (1-6)	0 (0-2)
Eos/hpf- median (range)		31 (15-92)	0 (0-2)

Abbreviations: EoE, Eosinophilic Esophagitis; Eos/hpf, Eosinophils per high power field; EREFS, Endoscopic Reference Score Assessing Edema, Rings, Exudates, Furrows, and Strictures.

FIGURE LEGENDS

Figure 1

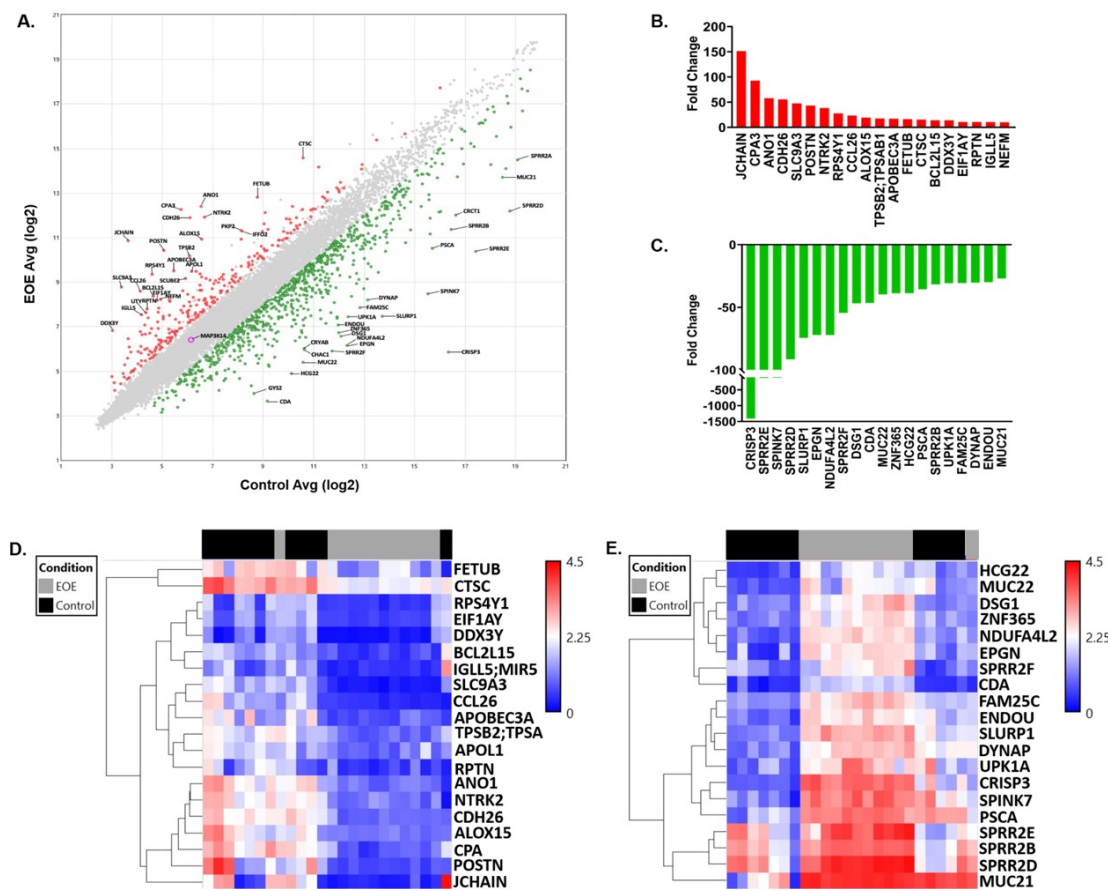


Figure 1. Global transcriptomic analysis of esophageal biopsies. **A)** Scatter plot of differentially expressed genes between EoE and Control subjects. **B)** Top 20 upregulated genes. **C)** Top 20 downregulated genes. **D)** Heatmap depicting hierarchical clustering of

top 20 upregulated genes. **E)** Heatmap depicting hierarchical clustering of top 20 downregulated genes.

Figure 2

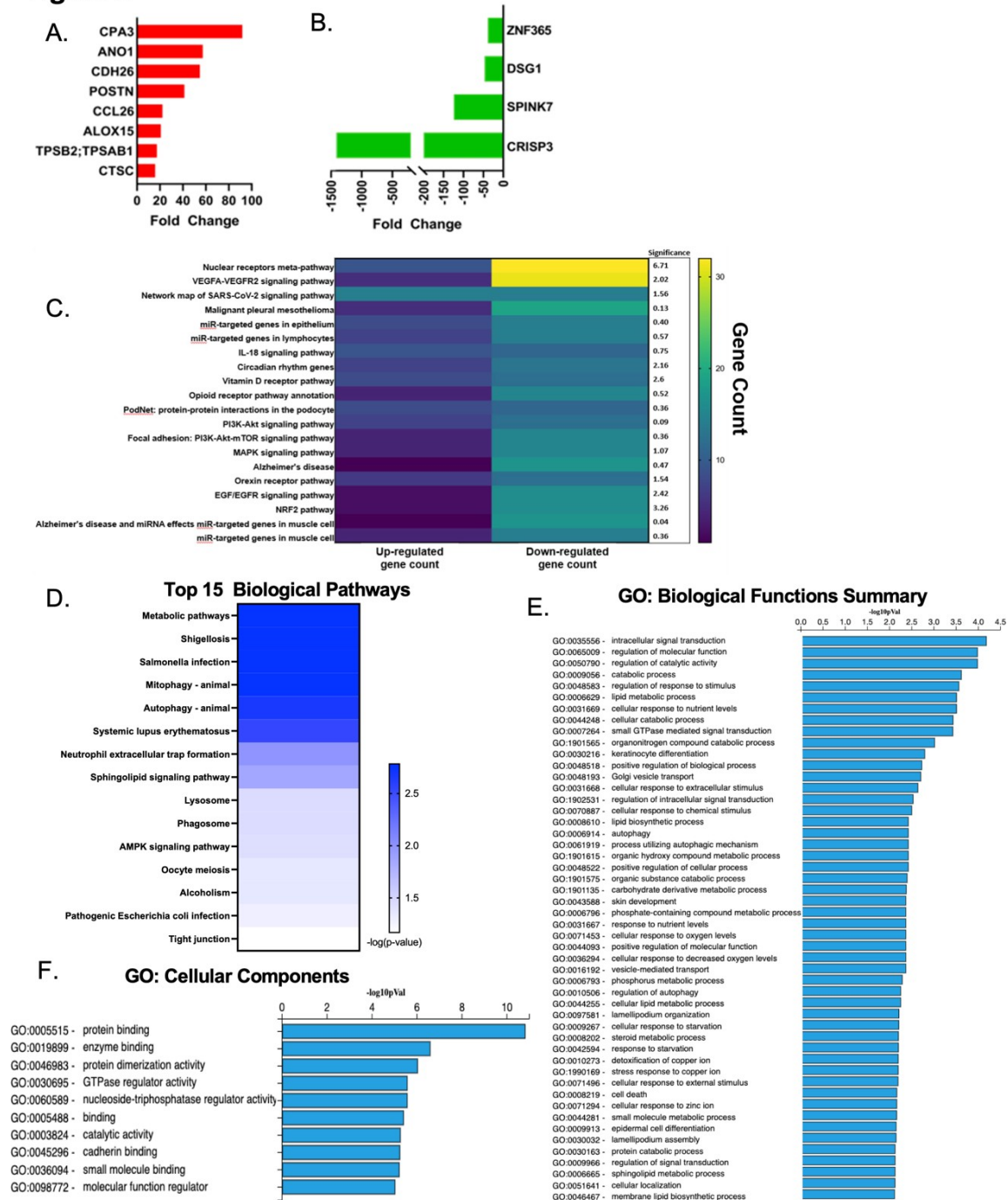


Figure 2. Genes commonly associated with EoE. A) Upregulated genes commonly associated with EoE. **B)** Downregulated genes commonly associated with EoE. **C)** Heatmap of top pathways underlying transcriptomic expression indicated by gene count. **D)** Top 15 Biological Pathways. **E)** GO: Biological Functions Summary. **F)** GO: Cellular Components.

Figure 3

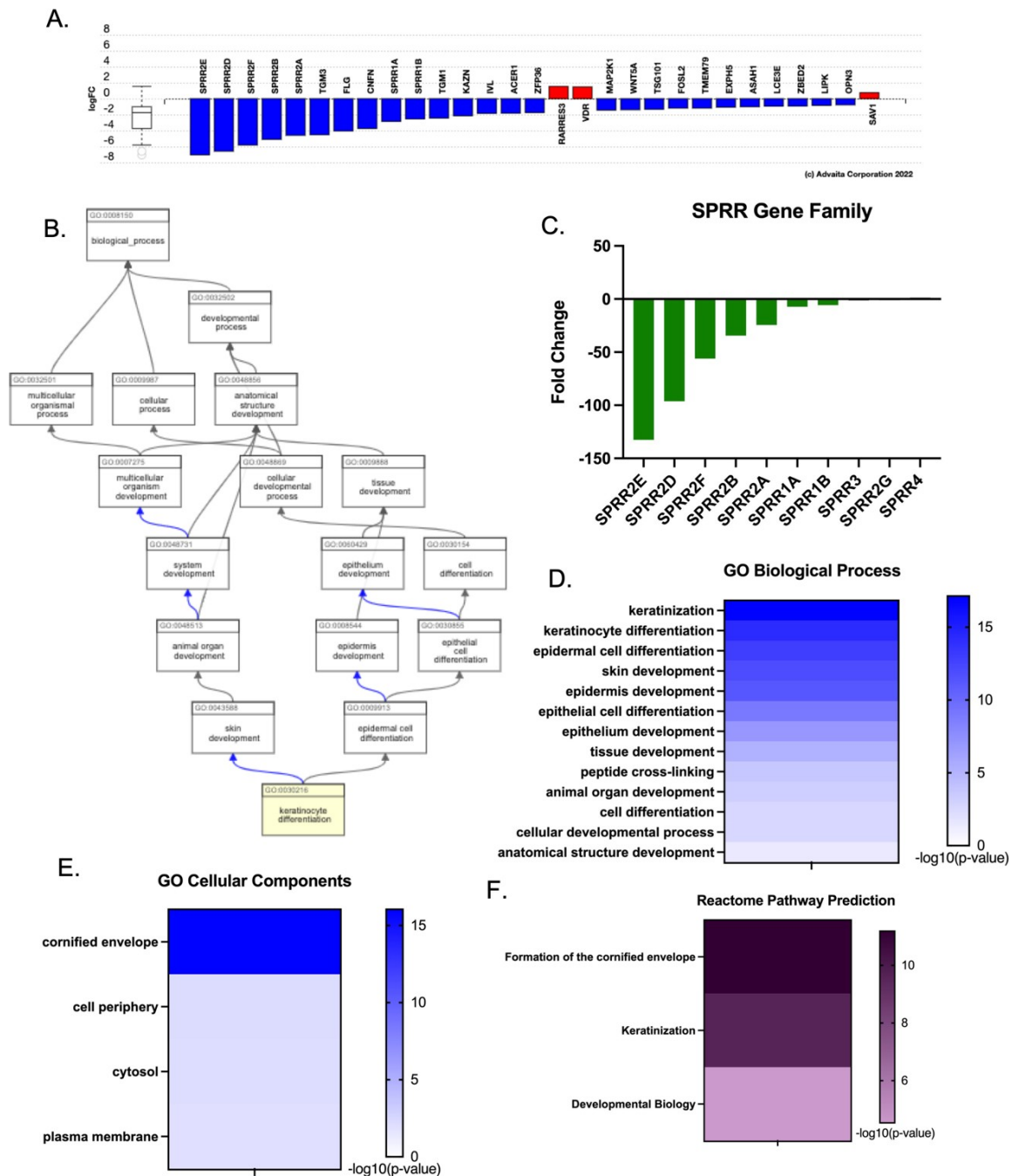


Figure 3. EoE Characterized by Impaired Keratinization Given Downregulated SPRR Genes. **A)** Dysregulation of genes related to keratinization. **B)** Downstream effects of keratinocyte differentiation. **C)** SPRR gene expression, **D)** GO Biological Processes, **E)** GO Cellular Components, **F)** Reactome Pathway Prediction.

Figure 4

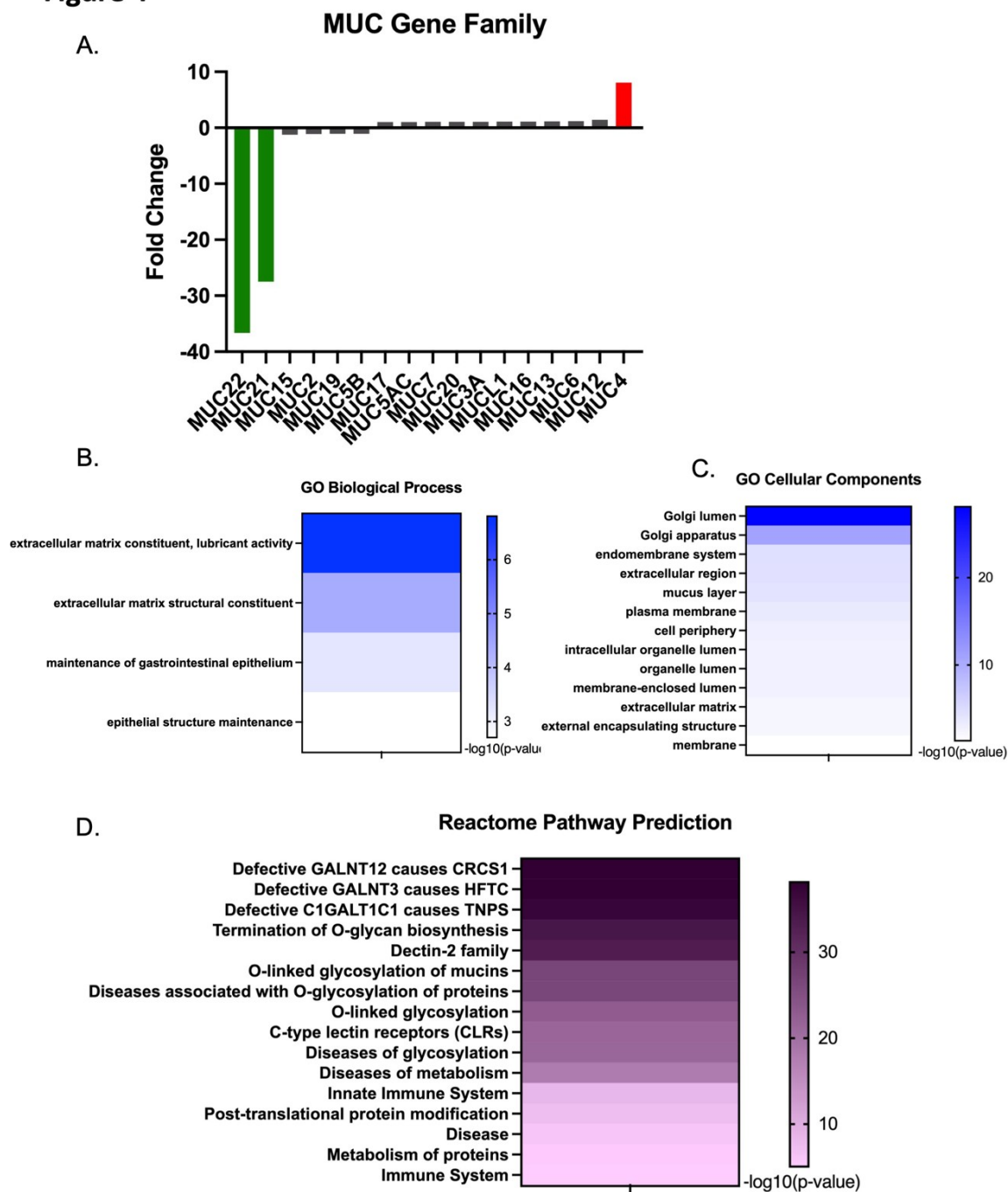


Figure 4. Unique Expression of MUC genes in EoE samples. A) Gene expression of MUC gene family. **B)** GO Biological Processes for MUC gene family. **C)** GO Cellular Components for MUC family. **D)** Reactome Pathway Prediction for MUC gene Family.

Figure 5

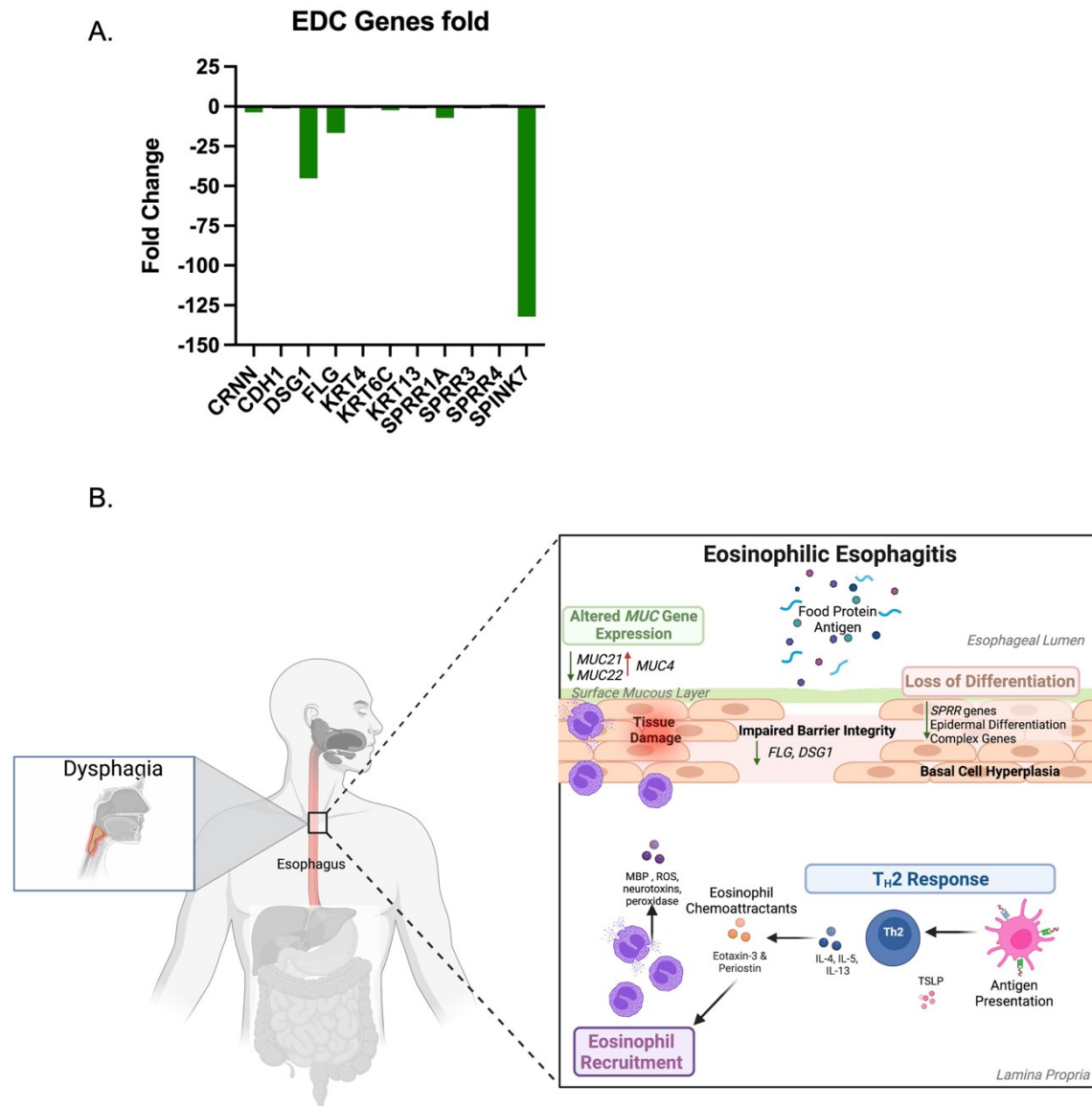
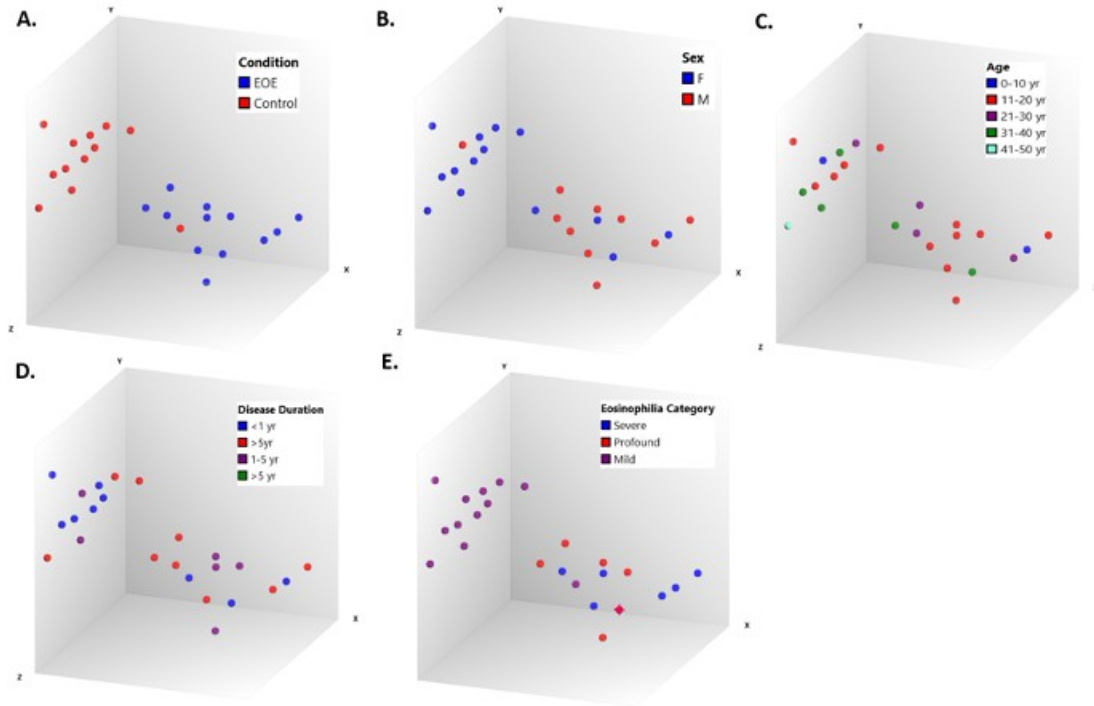


Figure 5. Proposed Model for EoE. A) Gene expression related to epidermal differentiation complex (EDC). **B)** EoE biopsies are characterized by dysregulation of SPRR genes and MUC21, which are predicted to impair differentiation of squamous epithelial cells and further impair their capacity to cope with prolonged inflammatory conditions.

SUPPLEMENTARY MATERIAL

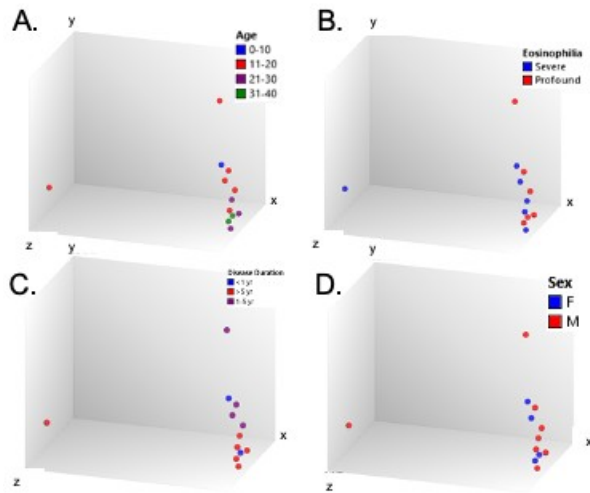
Supplementary Figure 1



Supplementary Figure 1. Disease state, not clinical features drives clustering of samples by Principal Components Analysis (PCA). A) PCA of samples by condition, B) sex, C) age, D) disease duration and E) eosinophilia category: mild=0-6 eos/hpf,

moderate=7-15 eos/hpf, severe=15-30 eos/hpf, and profound= 31+ eos/hpf. (No EoE samples collected for the moderate eosinophilia range.)

Supplementary Figure 2



Supplementary Figure 2. PCA analysis of EoE samples shows samples lack further apparent clustering by other clinical features. A) PCA of samples by age. **B)** PCA of samples by eosinophils category: severe=15-30 eos/hpf, profound= 31+ eos/hpf **C)** PCA of samples by disease duration **D)** PCA of samples by sex.

Supplemental Table 1: Assorted genes associated with canonical and noncanonical NF- κ B genes. No significance observed for any genes encoding for proteins within NF- κ B signaling pathways. Differential gene expression reported as fold change comparing EoE and control groups. Significance defined as ≥ 2 , ≤ -2 -fold change.

Symbol	Fold Change	Symbol	Fold Change	Symbol	Fold Change
AKT1	1.27	LTBR	-1.52	TNFRSF8	1.24
BCL10	-1.51	MALT1	-1.93	TNFSF10	1.32
BCL2	1.18	MAP3K1	-1.98	TNFSF13B	-1.09
BCL2L1	-2.14	MAP3K14	1.22	TRADD	1.07
BIRC2	1.15	MYD88	-1.24	TRAF2	1.26
BIRC3	-1.07	NFKB1	1.24	TRAF3	1.69
CASP1	1.4	NFKB2	1.52	TRAF6	-1.26
CASP4	1.22	NFKBIA	-1.16	TNFSF12	1.01
CASP5	1.24	NFKBIE	1.32	XIAP	-1.4
CCL19	1.04	NLRC3	1.02	ZFP91	1.32
CCL20	-1.58	NLRC5	-1.03		
CCL21	-1.01	NLRP1	-1.1		
CCL22	-1.71	NLRP12	-1.16		
CCL5	1.19	NLRP3	1.06		
CCR7	1.14	NLRP6	1.25		
CD27	1.04	NLRX1	-1.46		
CD40	1.42	NOD1	-1		
CD40LG	-1.03	NOD2	1.2		
CD70	1.09	PTGS2	1.12		
CHUK	1.14	REL	-1.07		
CXCL12	1.06	RELA	-1.6		
IRAK3	1.3	RELB	1.51		
CXCL13	-1.11	RIPK1	1.1		
IRAK4	-1.23	STAT3	-1.12		
CXCL2	-1.02	STUB1	-1.23		
CXCL3	1.27	SUMO1	1.13		
CXCR4	1.4	TLR2	-1.13		
CXCR5	-1.07	TLR3	1.73		
CYLD	1.31	TLR4	1.2		
EGFR	-1.07	TNF	1.03		
FBXW7	-1.65	TNFAIP2	-1.33		
HSPA8	1.14	TNFAIP3	1.5		
IKBKB	-1.07	TNFRSF10A	1.77		
IKBKG	-1.03	TNFRSF10B	1.72		
IL6	1.06	TNFRSF11A	-1.96		
IRAK1	1.1	TNFRSF12A	-1.11		
IRAK2	-1.75	TNFRSF13C	-1.1		
LTA	1.05	TNFRSF1A	-1.07		
LTB	1.16	TNFRSF1B	1.38		

CHAPTER THREE

NLRX1 Deficiency Alters the Gut Microbiome and Is Further Exacerbated by Adherence to a Gluten-Free Diet

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ABSTRACT

Patients with gluten sensitivities present with dysbiosis of the gut microbiome that is further exacerbated by a strict adherence to a gluten-free diet (GFD). A subtype of patients genetically susceptible to gluten sensitivities are Celiac Disease (CeD) patients, who are carriers of the HLA DR3/DQ2 or HLA DR4/DQ8 haplotypes. Although 85-95% of all CeD patients carry HLA DQ2, up to 25-50% of the world population carry this haplotype with only a minority developing CeD. This suggests that CeD and other gluten sensitivities are mediated by factors beyond genetics. The contribution of innate immune system signaling has been generally understudied in the context of gluten sensitivities. Thus, here we examined the role of NOD-like receptors (NLRs), a subtype of pattern recognition receptors, in maintaining the composition of the gut microbiome in animals maintained on a GFD. Human transcriptomics data revealed significant increases in the gene expression of multiple NLR family members, across functional groups, in patients with active CeD compared to control specimens. However, *NLRX1* was uniquely down-regulated during active disease. NLRX1 is a negative regulatory NLR that functions to suppress inflammatory signaling and has been postulated as preventing inflammation-induced dysbiosis. Using *Nlrp1*^{-/-} mice maintained on either a normal or gluten-free diet, we show that loss of NLRX1 alters the microbiome composition and a distinctive shift further ensues following adherence to a GFD, including a reciprocal loss of beneficial microbes and increase in opportunistic bacterial populations. Finally, we evaluated the functional impact of an altered gut microbiome by assessing short- and medium-chain fatty acid production. These studies revealed significant differences in a selection of metabolic markers that when paired with 16S rRNA sequencing data could reflect an overall imbalance and loss of immune system homeostasis in the gastrointestinal system.

INTRODUCTION

Adherence to a gluten-free diet (GFD) is currently the only treatment available to patients with gluten sensitivities like Celiac Disease (CeD) and non-celiac gluten sensitivity (NCGS). Although being an extensive dietary change, gluten exclusion has yet to be meaningfully evaluated in host health. Also of significant interest, current “fad

diets” promote the dietary exclusion of gluten in healthy individuals without any prior diagnoses of gastrointestinal disorders with the generally unsubstantiated claims that this lifestyle change promotes gut health and anti-inflammatory properties. However, wheat-derived oligosaccharides are likely prebiotic in nature (1). Therefore, entire elimination of gluten-containing foods, namely wheat-based sources, is anticipated to have dysbiotic effects by impairing bacterial metabolic activity and subsequently metabolite production, such that gut barrier function and overall host health are compromised.

Upon exposure, typically from dietary intake, gluten-sourced gliadin and glutenins are incompletely digested and remain active in the gastrointestinal tract in patients with gluten sensitivities (2-6). These partially degraded gluten fragments and peptides transverse the epithelial barrier to elicit a strong adaptive T-cell immune response (7-9). Translocating to the lamina propria of the small intestine, type 2 transglutaminase (TG2), a CeD autoantigen, deamidates partially-digested gluten peptides, causing TG2 to autocatalyze itself and increasing the affinity of gluten peptides to DQ2/8 to antigen presenting cells, thus inducing T-cell driven auto-destruction of small intestinal epithelia (10). This induces the production of pro-inflammatory cytokines including IFN- γ , TNF, IL-15, and IL-8 and decreases anti-inflammatory cytokines like IL-10 (11, 12). Adaptive immune system dysfunction has been widely studied in CeD. However, it is also clear that elements of the innate immune system also significantly contribute to disease pathogenesis. For example, particular gliadin peptides have enhanced resistance to degradation, particularly gliadin P31-43 (13). These peptides robustly activate innate immune responses via activation of IFN- α and TLR7/MyD88 signaling. Mechanistically, gliadin fragments mimic a viral response by activating TLR7 signaling impairing endocytic trafficking and subsequently activating NF- κ B signaling (13). This indicates a major contribution of dysregulated innate immune responses in gluten sensitivities and likely involvement of nucleotide-binding domain and leucine-rich repeat-containing proteins (NLRs) which negatively regulate NF- κ B signaling.

Tightly regulated NLR signaling is critical for maintaining the host defense against infection and dysbiosis, while activating the host immune response against damage that may accrue as a result of GFD-induced dysbiosis. This form of pattern recognition receptor (PRR) signaling functions to maintain symbiosis of the gut

microbiome by surveilling the gut mucosa for potentially breached mucosal barriers and infiltration of pathogenic, opportunistic bacteria by recognizing highly conserved pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). NLRs can be divided into multiple subgroups, including inflammasome forming NLRs (14-19) and regulatory NLRs (20-25). NLRX1 and NLRP12 are negative regulators of inflammation that impact a variety of inflammatory pathways and have been implicated in several diseases (26-40) and may be further implicated in patients with gluten sensitivities like CeD and NCGS. As CeD patients present with dysbiosis that is further exacerbated by adhering to GFD (41, 42), tightly regulated NLR signaling is likely critical for ensuring symbiosis of the gut microbiota both prior to and following adherence to GFD and protecting against gastrointestinal pathologies, specifically related to gluten sensitivity.

NF- κ B signaling is attenuated by NLRX1 and NLRP12 and this signaling pathway has been previously demonstrated as being dysregulated in CeD patients. NLRX1 is a negative regulator of the canonical NF- κ B pathway by associating with TRAF6 and IKK (23, 32, 33, 43). NLRP12 functions to negatively regulate noncanonical NF- κ B signaling by associating with TRAF3 and NIK (34). These NLRs directly attenuate inflammatory signaling in the gut and have been found to be dysregulated in inflammatory bowel disease and colitis associated cancer (23, 34, 44, 45). Their role has been evaluated in maintaining the host immune response to pathogenic microbes and maintaining the colonic microbiota. NLRX1 has been previously demonstrated as a key regulator in maintaining the host-gut microbiota interactions and protecting against gut dysbiosis in colitis (30). Loss of NLRX1 in mice results in impaired gene expression related to tight junction of the intestinal epithelial barrier, which further implicates the expansion of colitogenic bacteria including *Veillonella* and *Clostridiales* upon increased permeability (30). In an oral bacterial infection model, NLRX1 activates the NLRP3 inflammasome following *Fusobacterium nucleatum* infection to elicit a host immune response for bacterial clearance (46). The exact mechanism for microbial responses following NLRX1 activation has been studied extensively in viral work where NLRX1 attenuates Type 1 Interferon production and induces autophagy following viral infection (47). Deletion of NLRP12 in mouse models is associated with dysbiosis of the gut

characterized by decreased commensal bacteria including Lachnospiraceae and increased colonization of enteropathogenic bacteria namely *Erysipelotrichaceae* (34, 36, 37, 48). Current studies have yet to identify the exact molecular underpinnings by which NLRP12 regulates the gut microflora with studies highlighting both the hematopoietic and non-hematopoietic compartments (34, 49). Interestingly, NLRP12's role as a negative regulator in dampening the host immune response has been demonstrated to perpetuate bacterial infection, where decreased production of proinflammatory cytokines and antimicrobial molecules prevents clearance of *Salmonella* in a mouse infection model (50). Due to the varying insights associated with the prior publications, more work is necessary to better define the role of these regulatory NLRs in maintaining gastrointestinal health and disease.

Currently, there is speculation as to whether dysbiosis is a cause or consequence of these etiologies and whether these microbial signatures are genetically inherent or determined by environmental factors like diet. Genetically inherent factors may include depletion of regulatory NLR signaling, such as deficits of NLRX1 or NLRP12, while the primary environmental factor of interest is adherence to GFD. While the gut microbiome of NLRX1- and NLRP12-deficient mice have been evaluated in the context of colitis (26, 30, 34, 37, 49), their potential predisposition towards GFD has yet to be meaningfully demonstrated. Further, innate immune signaling has been overlooked in the context of CeD and NCGS pathology. We therefore hypothesize that the regulatory NLRs, NLRX1 and NLRP12, have a pertinent role in maintaining proper innate immune responses and microbiome homeostasis in the context of GFD, which is of relevance to CeD and NCGS. To evaluate this hypothesis, we assessed the gut microbiome composition and metabolic profile of *Nlrp12*^{-/-} and *Nlrp12*^{-/-} mice maintained on a GFD compared to normal diet to characterize the influence of NLR-deficiency in maintaining the gut microbiome. Our findings imply that loss of NLR signaling can be significantly exacerbated by simple dietary changes, such as switching to GFD, and may contribute to inflammatory diseases like gluten sensitivities.

MATERIALS AND METHODS

Experimental Animals

The generation and characterization of *Nlr1^{-/-}* and *Nlrp12^{-/-}* mice have been previously described (23, 35). Following IACUC approval by Virginia Tech, all experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animal housing conditions include the room temperature being maintained at 22°C with humidity ranging between 30-70%. The light cycle in the room is 14 hours light to 10 hours dark. Wild-type, *Nlr1^{-/-}*, and *Nlrp12^{-/-}* mice were housed in standard specific pathogen-free (SPF) conditions in animal vivariums located at the Virginia-Maryland College of Veterinary Medicine, which is American Association for Laboratory Animal Care (AALAC) accredited. SPF status of the mouse colony is routinely checked using standard best practices. All experiments were conducted with male and female 6-month old mice that derived from a C57Bl/6J background. GFD wild-type, *Nlr1^{-/-}*, and *Nlrp12^{-/-}* mice were bred and fed commercially-available rodent gluten-free diet (*Research Diets #AIN-76A*) for at least three generations. Control mice were maintained on normal diet (*Tekland Global 18% Protein Rodent Diet*) throughout generations (**Supplemental Table 1**).

Fecal Samples

Prior to harvest, necropsy tools were washed and sterilized via autoclave with a designated set of tools used for each genotype. Mice were euthanized in a biological safety cabinet at necropsy. Colonic contents were collected and immediately stored on dry ice. Long-term storage of samples kept at -80°C.

16S rRNA Gene Sequencing and Data Analysis

One fecal pellet from each individual mouse was prepared for DNA extraction and 16S rRNA gene sequencing (n = 6-7 animals per group). Genomic DNA was extracted from fecal pellets using the DNeasy PowerSoil HTP 96 Kit according to the manufacturer's protocol. Genomic DNA was suspended in 100 µl of manufacturer supplied C6 solution.

Extracted DNA was stored at -20°C until further processing. PCR amplification of a region of the mitochondrial 16S rDNA gene was performed on the extracted DNA sample using forward and reverse primers. The primers used include the forward primer GTGYCAGCMGCCGCGGTAA and reverse primer GGACTACNVGGGTWTCTAAT. The forward and reverse primers both contained a 5' adaptor sequence for Illumina sequencing. The 25 µl total volume PCR master mix was prepared for each sample using Promega PCR Master Mix Specifications: 12.5 µl Master Mix, 0.5 µl of each primer, 1.0 µl gDNA, and 10.5 µl DNase/RNase-free H₂O. DNA was amplified under the following cycle conditions: initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 45 seconds at 95 °C, 1 minute at 50 °C, and 90 seconds at 72 °C, and a final elongation at 72 °C for 10 minutes. Sample library pools were subsequently sequenced on an Illumina MiSeq (San Diego, CA) using a v2 500-cycle kit. Proper quality control was conducted prior to sequencing. Sequencing data was analyzed using Mothur V.1.40.4 (51) to group 16S rRNA sequences into OTUs using 97% similarity. Classifications were determined to a group by comparing sequences to the Silva database 132. Relative abundances of bacterial phyla and family were determined per sample through classified OTUs and all taxonomic plots were generated using R (version 4.0.5) Samples were normalized to the same number in the to adjust for variation in sequencing depth. Principal coordinate analysis was used to assess community similarity among all samples. Weighted unifrac distances between communities were displayed in a two-dimensional space (52). Linear discriminant analysis effect size (LEfSe, Galaxy version 1.0; <https://huttenhower.sph.harvard.edu/galaxy/>) was performed to compare the differential bacterial abundance using

default settings (53). This analysis was performed with the default setting (Alpha value for the factorial Kruskal-Wallis test: 0.05 ; Threshold on LDA score: 2).

Short- and Medium-Chain Fatty Acids Identification and Quantification

Fecal pellets were harvested following the above protocol. Approximately 100mg fecal samples were weighed in a biological safety cabinet. 1 mL sterile molecular-grade water (Sigma-Aldrich) was added to each sample. At room temperature, fecal samples were placed on a tilt table for 4 hours and vortexed once every hour to disintegrate larger fecal pieces. Following final vortex, samples were allowed to settle for approximately 30 minutes and 900 μ L of supernatant was collected for analysis. 10 μ L of 85% phosphoric acid was added to the sample to achieve 1% acid concentration. Samples for VFA analysis were passed through a 0.45 μ m nitrocellulose membrane filter and frozen prior to analysis to avoid capillary blockage. Upon analysis, samples were defrosted and centrifuged prior to detection. Volatile fatty acid (VFA) analysis was performed using an Agilent 6890 gas chromatograph (Agilent, Wilmington, DE, USA) equipped with a split injector and a flame-ionization detector. Chemstation software was utilized to perform data analysis. NukolTM fused silica 15 m $\times$ 0.53 mm capillary column with 0.5- μ m film thickness was used to perform VFA separation. The carrier gas helium flow rate was 15 mL/min with a split ratio of 2:1. The oven temperature was held constant at 80°C for 3 min, increased to 140°C at a rate of 6°C per minute, where it was held constant for 1 min. Temperatures were maintained at 200°C for the injector and 250°C for the detector. VFA standards were used to generate a standard curve. VFAs are reported as mg/L. Normalization of readings were performed by dividing by weight of total feces collected in milligrams.

Human Transcriptomic Data Analysis

Expression of genes related to NLR signaling in CeD patients was evaluated using publicly accessible gene expression datasets, as previously described (54-59). Data shown were generated from RNA-sequencing data from duodenal biopsies obtained from 51 CeD patients and 45 healthy controls, as previously published (60). Individual gene expression was based on transformed data, converted to relative gene expression for each

individual target (54-59). Pathway analysis was conducted using Ingenuity Pathway Analysis (IPA) and CompBio (54-59). Data were analyzed using causal analysis approaches in IPA (61). Gene expression and pathway analysis in human subjects were conducted using the following array data series (available through the National Center for Biotechnology Information: <https://www.ncbi.nlm.nih.gov/>): GSE134900.

Statistical Analysis

Data analysis was performed using GraphPad Prism version 9.2.0 (Graph- Pad, San Diego, CA, USA) to assess statistical significance and calculate correlation. Student two-tailed t test was calculated for comparisons between two experimental groups. One-way and two-way ANOVA were calculated for multiple comparisons, with Mann-Whitney or Tukey post-tests for multiple pairwise examinations as appropriate. Changes were identified as statistically significant with $p < 0.05$. Mean values were reported with standard error of the mean. Statistical analyses for α -diversity and β -diversity were compared by nonparametric Mann-Whitney U tests and nonparametric multidimensional ANOVA. Distance-based redundancy analysis determined the contribution of different variables to microbiota profile variations.

RESULTS

Human Transcriptome Analyses Indicate Significant Dysregulation of NLR Signaling in CeD Patients

To evaluate NLR signaling in CeD patients, we conducted both biased and unbiased evaluations of publicly available transcriptomic datasets from human duodenal biopsy samples (GSE134900). This dataset included 51 patients with active CeD and 45 control patients, as previously defined (60). Thirteen NLR family members were represented on the human arrays (**Figure 1**). While this is not a comprehensive list of the full NLR family, representatives from each of the inflammasome and regulatory subsets of NLRs are present on the array, as well as the majority of adaptors, caspases, and modulators of each NLR signaling pathway. In the human CeD patients with active disease, we observed a significant increase in NLR inflammasome signaling (**Figure 1A**;

Supplemental Figure 1B). Specifically, genes associated with the NLRP1, NLRP7, and NLRC5 inflammasomes were significantly up-regulated in the CeD patients (**Figure 1A; Supplemental Figure 1B**). Likewise, we also observed significant up-regulation of the regulatory NLRs associated with pro-inflammatory signaling, including NOD1, NOD2, and CIITA (NLRA) signaling in patients with active CeD (**Figure 1B; Supplemental Figure 1**).

In addition to the pro-inflammatory NLRs, we also observed differences in signaling associated with the regulatory NLRs that attenuate inflammation. We did not see any significant changes in *NLRP2* (**Figure 1C**). However, we did observe significant increases in *NLRC3* and *NLRP12* and significantly downregulated *NLRX1* expression (**Figure 1C**). *NLRX1* was the only NLR represented on the array that was significantly downregulated and demonstrated a unique expression pattern in the CeD patients as illustrated in the heatmap (**Figure 1D**). To better define the mechanisms and pathways impacted by the changes in gene expression, selected targets were analyzed using the CompBio pathway analysis tool, as previously described (57). This program utilizes an artificial intelligence algorithm to identify biological functions most likely impacted by complex changes in gene expression in large datasets. Consistent with the observations discussed above, CompBio predicted increased NF- κ B/TNF signaling, NLR inflammasome signaling, and regulatory NLR signaling pathways (**Figure 2A**). Consistent with CeD, we also observed a significant change in dendritic cell regulation of TH1/TH2 development, GI Inflammation, and epithelial cell tight junction disassembly (**Figure 2A**). Villous atrophy formed a central node in the largest cluster, that also included atopic dermatitis, lactose intolerance, colitis, and IBD (**Figure 2A**). Likely due to the up-regulation of TLR7 and several NLRs associated with host-pathogen interactions, specifically virus recognition, the pathway analysis revealed the general up-regulation of signaling pathways consistent with pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs), which are reflected in the recurrent viral infection node (**Figure 2A**). These findings were confirmed using IPA. The schematic shown in **Figure 2B** is representative of the gene expression data output generated by IPA, showing the predicted biological functions associated with the global changes in gene expression focused on the NLR signaling data (**Figure 1; Supplemental**

Figure 1). Together, these data suggest that incomplete digestion of gluten/gliadin is potentially recognized as a DAMP by pattern recognition receptors in the context of CeD. Likewise, most of the NLRs identified have significant roles in maintaining microbiome homeostasis, with dysregulation often resulting in dysbiosis (30, 37, 62-64). Thus, the gene expression changes are further predicted to significantly impact microbiome homeostasis in CeD patients, as predicted in the pathway analysis (**Figure 2B**).

GFD Distinctively Shifts the Gut Microbiota Composition in Mice with Intact NLR Signaling

Microbiome changes have been explored in CeD patients and can significantly impact NLR signaling (60). The most effective therapy for CeD is a gluten-free diet (GFD). However, the complete removal of gluten is also likely to impact the microbiome in individuals on a GFD. Current studies have yet to investigate the impact GFD has on otherwise healthy individuals void of gluten sensitivities. In an effort to better define microbiome changes associated with GFD, we compared the microbiomes based on diet in wild-type mice with normal, intact NLR signaling (**Figure 3**) and predicted that this dietary change will have minimal impacts on a robust healthy gut microbiome.

We observed that mice maintained on GFD demonstrate significant shifts in the microbial ecology within the GI niche compared to mice maintained on the normal diet (**Figure 3**). These findings demonstrate the effects a GFD has on otherwise healthy individuals. We demonstrate here that bacteria belonging to the Firmicutes phylum dominates the microbiome, while the second most abundant phylum is correlated with diet. The second most abundant phyla for mice fed a normal diet were Bacteroidetes and Actinobacteria for GFD-fed mice (**Figure 3A**). Cladogram and LEfSe differential analysis displaying bacterial abundance showed that mice fed a normal diet exhibited significant variation between wild-type mice fed GFD (**Figure 3B**), whereby mice on a GFD are increasingly characterized by Atopobiaceae, Streptococcaceae, and Erysipelotrichaceae. Investigation at a family level indicates that wild-type mice fed a normal diet had a significantly higher abundance of bacteria belonging to the Lactobacillaceae family and unclassified Lactobacillales which are normally associated with beneficial health benefits and utilized as probiotics (**Figure 3B**).

Further, mice fed a GFD had significantly decreased overall count of observed bacterial species and Shannon diversity (**Figure 4A and B**), highlighting a robust shift in the microbiota following GFD administration, wild-type mice fed a GFD had nearly 2-fold less observed species than those fed a normal diet (**Figure 4A**). Consistently lacking in diversity, wild-type mice administered GFD had significantly increased relative abundance of Actinobacteria that expanded in the community, while Bacteroidetes was reciprocally decreased (**Figure 3A, 4D**). Mice fed GFD also had significantly reduced Shannon Diversity, which demonstrates that both the abundance and evenness of species is reduced in this microbial community (**Figure 4B**). Together, these data indicate that a GFD significantly alters the bacterial composition of the gut microbiome in otherwise healthy mice.

Gut Microbiome of NLRX1-Deficient Mice Has Genetically-Inherent Composition Poised Towards Dysbiosis and is Exaggerated by GFD

Based on the findings that GFD skews the microbiome towards dysbiosis and that CeD patients have significantly altered NLR signaling predisposing individuals towards gastrointestinal disorders (42, 60), we next sought to evaluate the understudied negative regulatory NLRs in modulating the gut microflora *in vivo* using NLR-deficient mouse models following GFD. Our attention first focused on NLRX1, the only NLR found to be significantly downregulated in human CeD patients (**Figure 1C**). We hypothesized that the altered pattern recognition receptor (PRR) signaling and concurrent hyperinflammatory innate immune system signaling associated with loss of NLRX1 (23, 30) would result in significant alterations in the bacterial composition of the microbiome with populations highly composed of pathogenic/opportunistic bacteria. Based on our findings in **Figure 3**, we further postulated that the GFD would amplify this shift in the microbiome.

To test this hypothesis, we utilized *Nlrp1*^{+/+} and *Nlrp1*^{-/-} littermates (23), maintained on either normal diet or the GFD as described in the methods. We observed a significant decrease in the number of species in the gut microbiome in the GFD *Nlrp1*^{-/-} mice compared to counterparts fed normal diet (**Figure 4A**). We also observed a significant difference between the *Nlrp1*^{+/+} GFD mice and *Nlrp1*^{-/-} GFD mice, where the

Nlr1^{+/+} animals on the GFD had the lowest levels of species diversity (**Figure 4A**). A significant difference was noted between *Nlr1^{+/+}* and *Nlr1^{-/-}* mice on the normal diet in number of observed species (**Figure 4A**). The results were further confirmed in the Shannon diversity data; however, no difference was observed between the *Nlr1^{+/+}* GFD mice and *Nlr1^{-/-}* GFD mice (**Figure 4B**). Our data further revealed the relative abundance of bacteria belonging to the phyla Bacteroidetes is significantly decreased and Firmicutes is significantly increased in the *Nlr1^{-/-}* mice on the GFD compared to the *Nlr1^{-/-}* mice on the normal diet (**Figure 4C-D**). Principle Coordinate analysis (PCoA) further confirmed that the microbiomes of the *Nlr1^{+/+}* and *Nlr1^{-/-}* mice are inherently unique and clustered separately upon GFD, with clustering not as strongly apparent for mice on the normal diet (**Figure 4E, Supplemental Table 2**). This suggests that diet type and genotype status contribute to microbial community composition in the gut (**Figure 4E**).

We next performed LEfSe analysis to determine the differentially abundant taxa comparing *Nlr1^{+/+}* and *Nlr1^{-/-}* mice fed normal and GFD (**Figure 5**). At the phylum level, the microbiome of *Nlr1^{-/-}* mice fed a normal diet are characterized by Lactobacillaceae, Bacteroidales, Bacteroidia, Bacteroidetes, Muribaculaceae, Burkholderiaceae, Eggerthellaceae, Coriobacteriales, and Coriobacteria (**Figure 5A**). The microbiome of *Nlr1^{-/-}* mice on the GFD are characterized by bacteria belonging to the Bacillales, Staphylococcaceae, Peptostreptococcaceae, Streptococcaceae, and Enterococcaceae (**Figure 5A**). Compounding the differences between the GFD and normal diet in the *Nlr1^{-/-}* mice, we also observed that key bacterial populations were found to be differentially abundant between *Nlr1^{-/-}* mice and *Nlr1^{+/+}* mice on the GFD in the LEfSe analysis, indicating the genetically inherent microbiome of NLRX1-deficient mice is not restored by a GFD. We observed a significant increase in the presence of Enterococcaceae in the *Nlr1^{-/-}* mice compared to the *Nlr1^{+/+}* animals (**Figure 5B**). Increases in Enterococcaceae are known risk factors for increased susceptibility to pathogenic bacteria infection, such as *C. difficile* (65) and enhanced gastrointestinal inflammation (66). At the OTU level, the microbiota of GFD *Nlr1^{-/-}* mice had significantly increased levels of the Enterococcaceae unclassified Otu004 and Muribaculaceae_ge Otu013, (**Figure 5C**). The GFD *Nlr1^{+/+}* had increased

characterization of Erysipelotrichaceae Dubosiella Otu 008(**Figure 5C**). While most of these bacteria are commensal, the overrepresentation of *Enterococcus* spp. in the *Nlrp12*^{-/-} mice fed GFD indicates a unique microbial fingerprint for these NLR-deficient mice.

Microbiota Composition of NLRP12-Deficient Mice Fed GFD Resembles Community Structure of Mice with Intact NLR Signaling

NLRP12 functions as a negative regulator of noncanonical NF-κB signaling, making *Nlrp12*^{-/-} mice more susceptible to gastrointestinal inflammation in the context of inflammatory bowel disease, colitis-associated cancer, and obesity as shown in previous studies (34, 36, 37, 49, 55). Compared to NLRX1-deficient mice, the absence of NLRP12 had minimal effects on the gut microflora. There was no significant variation in *Nlrp12*^{-/-} and *Nlrp12*^{+/+} littermate control mice fed a normal diet or following GFD in observed species, Shannon diversity, or relative abundance of any major phyla (**Figure 6A-B, D**). This suggests that genetic NLRP12-deficiency does not inherently alter the gut microbiome composition. Similar trends were observed in both comparing *Nlrp12*^{-/-} normal diet to *Nlrp12*^{-/-} GFD and comparing *Nlrp12*^{+/+} normal diet to *Nlrp12*^{+/+} GFD, in which number of observed species and Shannon diversity were significantly decreased in GFD mice regardless of genetic deficiency (**Figure 6A-B**). Likewise, there was no significant variation between *Nlrp12*^{-/-} and *Nlrp12*^{+/+} fed a GFD (**Figure 6A-B, D**). Upon further investigation of the microbial composition, the relative abundance of bacteria belonging to the Actinobacteria phyla was increased in GFD mice, while Bacteroidetes was decreased regardless of genetic status (**Figure 6C-D**). The relative abundance of Firmicutes and Proteobacteria were also not found to be significant for any of the comparisons (**Figure 6C-D**).

Further evaluation of the distinctiveness of these microbiome compositions show two overlapping clusters in PCoA is driven by dietary intervention (**Figure 6E, Supplemental Table 3**). These community structures strongly clustered based on diet status. Following dietary intervention in *Nlrp12*^{-/-} mice, LefSE analyses indicate that these two microbiomes indeed have unique identifiers (**Figure 7**). Mice fed a GFD were characterized by Bifidobacteriales and Bifidobacteriaceae (**Fig 7**), with some strains commonly administered as probiotics. Mice fed a normal diet have increased colonization

of Lactobacillaceae, Lactobacillales, unclassified Lactobacillales, and Bacilli, which generally include symbiotic bacteria commonly used for probiotics (**Figure 7**). This trend mirrors what we have observed in wild-type mice (**Figure 3**) in that wild-type mice administered long-term GFD has apparent loss of beneficial gut microbes. It is important to indicate that the microbiomes of *Nlrp12*^{-/-} mice fed a GFD were also represented by bacteria belonging to Bifidobacteriales and Bifidobacteriaceae, which also include probiotic bacteria. Our findings demonstrate that NLRP12-deficiency does not inherently alter the gut microbiome (**Figure 7**). Rather, the microbiome of NLRP12-deficient mice resembles that of wild-type mice and any alterations observed in the bacterial composition is driven by diet.

GFD Alters Metabolite Production in Mice Fed GFD

We found that short-chain fatty acid (SCFA) and medium-chain fatty acid (MCFA) levels significantly vary based on diet type (**Figure 8**). Diet associated alterations in metabolite production were most prominent for acetic and propionic acid (**Figure 8A,G**). Regardless of genotype, exposure to a GFD significantly decreases the production of acetate and propionate (**Figure 8A,G**). Acetate levels were significantly decreased between wild-type ($p = 0.0155$), *Nlr1*^{-/-} ($p = 0.0046$), and *Nlrp12*^{-/-} ($p = 0.0471$) mice on a normal diet compared to respective strains on GFD (**Figure 8A**). Propionate levels were significantly decreased between wild-type ($p = 0.0088$), *Nlr1*^{-/-} ($p = 0.0088$), and *Nlrp12*^{-/-} ($p = 0.0253$) mice on a normal diet compared to GFD (**Figure 8G**). We observed decreased levels of butyrate in *Nlr1*^{-/-} GFD compared to *Nlr1*^{-/-} normal (**Figure 8B**). Minimal deviations in isobutyric, isovaleric, and hexanoic acids are observed, which may not necessarily be genotype independent (**Figure 8D-F**). These aberrant fluctuations in SCFA/MCFA levels gives insight to the shifts in the microbiota may have following GFD.

DISCUSSION

A delicate balance of innate immune signaling mechanisms is needed to maintain gut health and any perturbations, particularly in NLR signaling as demonstrated here, have the potential to disturb the symbiotic relationship between host and microbiota by

promoting hyperinflammatory signaling. Only a few studies have evaluated NLRs in association to CeD. The best described NLR thus far is NOD1, which has been shown to be downregulated in CeD patients (67); whereas, NOD2 and CIITA expression has been reported to be unchanged (68). Studies have yet to evaluate the gut microbiome following NLRX1-deficiency and CeD pathogenesis; however, this negative regulatory NLR, NLRX1, has been evaluated in other gastrointestinal disorders. One study found *Nlrp1*^{-/-} mice to have unique microbiomes that are characterized by colitogenic microbes, particularly belonging to the bacterial taxa *Veillonella* and Clostridiales (30). These microbiomes characteristic of NLRX1-deficiency are in part due to impaired intestinal epithelial cell function and metabolite production, therefore increasing gut barrier permeability and leading to greater colonization of opportunistic pathogens (30). Our data also demonstrate that *Nlrp1*^{-/-} mice have an inherently unique gut microbiome that is further shifted following a GFD (**Figure 4-5, Supp. Fig. 2, Supp. Table 2**). With this distinctive shift in the microbial community, we observed that *Nlrp1*^{-/-} mice had a slight increase in alpha diversity compared to mice with intact NLR signaling (**Figure 4A,B**). Although enhanced bacterial diversity would suggest enhanced ecological fitness, further examination of the bacterial composition of these genetically impaired mice shows increased relative abundance of opportunistic bacteria that are associated with intestinal inflammation, disorders, and infections. Such opportunistic bacteria include unclassified Enterococcaceae (**Figure 5C**). Enterococcaceae are often found in the gut as commensals, yet are opportunistic and elicit infections upon penetration of the gut epithelial barrier (69, 70). Given the NLR-deficiency of these mice, deletion of the negative regulatory NLRX1 is anticipated to further amplify pro-inflammatory signaling in the gastrointestinal tract. Our 16S rRNA sequencing data characterizing the microbiome suggests that loss of NLRX1 alters the gut microbiota in response to administration of a GFD. We further speculate that loss of NLRX1 is conducive towards hyperinflammation as this negative regulator of NF- κ B signaling is rendered inactive allowing for overzealous inflammatory signaling, which further alters the microbiome and likely exacerbates GFD-mediated dysbiosis.

Unlike NLRX1, we did not observe significant changes in the NLRP12-deficient microbiome. The microbiome of NLRP12-deficient mice has been previously

characterized as having reduced bacterial diversity and decreased abundance of the protective commensal Lachnospiraceae contributing to colonic inflammation, as in human Ulcerative Colitis patients (37, 71). The microbiota of NLRP12-deficient mice spontaneously promotes excessive weight gain as a model for obesity and also elicits pro-inflammatory signaling in the colon (36). Further, these mice had diminished bacterial diversity, which is commonly associated with dysbiosis and intestinal inflammation (36). These phenotypes were resolved in mice either treated with antibiotics or housed in germ-free conditions (36). Our data, however, showed little to no variation in *Nlrp12*^{-/-} normal compared to *Nlrp12*^{+/+} mice or *Nlrp12*^{-/-} GFD in comparison to *Nlrp12*^{+/+} GFD at a normal baseline or following GFD (**Figure 6-7, Supp. Table 3**). Our findings indicate that NLRP12-deficiency has minimal effects on regulating the gut microbiota in healthy mice or in GFD-fed mice. Differences in our findings and previous literature may be attributed to our work being conducted at a different facility with different vivarium conditions, use of littermate control animals, or differences in experimental design, including but not limited to primer choice, sequencing methods, and computational analysis. Murine microbiome studies are carefully designed to take into consideration and control for several different factors such as littermate controls, genetic background, maternal inheritance, and vivarium differences in order to ensure reproducibility and draw conclusions that accurately reflect the true nature of these biological processes, all of which were controlled for in our study to limit unaccounted for variation under our specific experimental conditions.

Beyond evaluating the role of NLR signaling in regulating the microbiome, we evaluated the potential effects of GFD in a healthy population that is void of gluten-sensitivities. Of interest, there is an ever-increasing popularity in “elimination diets”. Two of these most popular trends include GFD and cereal-free Paleo diets that promote the exclusion of gluten or cereal grains, respectively, which claim to minimize inflammation and the onset of inflammatory diseases in otherwise healthy individuals. However, claims of such long-term health benefits have yet to be properly and convincingly studied in people overtime. Instead, unnecessary adherence to GFD restricts food sources rich in prebiotics that are necessary for proper bacterial fermentation and metabolic activity. Additionally, healthy individuals, who have neither gluten-

sensitivities nor CeD, are sacrificing well-balanced diets for strict adherence to dietary regimens that increase their risk of malnutrition and mineral/vitamin deficiencies. As a potential model for this exclusion diet in healthy individuals, wild-type mice fed GFD encompassed a significant shift in their gut microflora (**Figure 3**). The gut flora of wild-type mice was not robust enough to withstand the effects of a GFD diet, as depicted by distinctive microbiome composition and metabolite levels (**Figure 3, 8**). This rapid shift in the gut microbiome following GFD may serve as precedent to dysbiosis. In wild-type mice fed GFD for instance there was increased Erysipelotrichaceae family *Faecalibaculum* genus observed. Of interest, bacteria belonging to *Faecalibaculum* genus and Erysipelotrichaceae family have ties to IBD and colitogenic properties (37, 62, 72) ever-highlighting the potential association between IBD and CeD enteropathies, especially given the NLR-deficient mouse models. Our data highlights emerging concerns that may be associated with strict adherence to exclusion diets for individuals void of gluten sensitivities, where loss of beneficial gut microbes and bacterial diversity primes the gut microenvironment for pro-inflammatory signaling attributed to several gastrointestinal pathologies.

Although adherence to a gluten-free diet is the most effective treatment regimen for CeD patients, it is not without faults. Infants carrying the HLA-DQ2 haplotype are genetically predisposed to CeD with their developing gut microbiomes being associated with increased proportions of bacteria belonging to the *Clostridium* species (Firmicutes) and Enterobacteriaceae family (Proteobacteria), and decreased proportions of beneficial *Bifidobacterium* species (Actinobacteria) than infants considered low risk (73). Notably, *Bifidobacterium* is commonly administered as a probiotic to decrease levels of pro-inflammatory TNF, IFN-gamma, and IL-2 (74). We found the *Bifidobacterium* family (Actinobacteria) to be increased in GFD fed NLRP12-deficient mice and mice with intact NLR signaling. However, of interest, NLRX1-deficient mice had the highest levels of *Bifidobacterium* at homeostatic conditions when fed a normal diet, but was greatly reduced by GFD (**Figure 7**). Co-administration of *Bifidobacterium* as a probiotic in CeD pediatric patients currently adhering to a GFD between 6 months to 15 years was found to restore the gut microbiota and decrease the previously heightened levels of acetate and propionate SCFAs (74). In order to maximize the effectiveness of GFD in alleviating

gastrointestinal issues, co-administration of GFD with probiotics should be considered to potentially restore the loss of commensal and beneficial microbes. Alternatively, food technology is currently developing “pseudocereal” formulations of amaranth (75), quinoa (75), buckwheat (75), spirulina (76) and red rice flour (77) that can replace gluten and exhibit prebiotic effects on probiotic bacteria.

Metabolite production is another field of interest where it is predicted that a SCFA fingerprint exists as a biomarker for such gastrointestinal diseases; however, a single SCFA fingerprint has yet to be characterized in CeD patients. Previous literature has reported increased levels of acetic acid (acetate), propionic acid (propionate), and total SCFAs in CeD patients (74, 78-81). However, it is unclear if these changes are associated with CeD or the GFD adopted by these patients. Consistently, we observed decreased acetate and propionate levels following GFD regardless of genetic background (**Figure 8**). Healthy adults on a GFD had decreased levels of SCFAs compared to those on a normal diet (80), which is consistent with our observations comparing GFD and normal diet wild-type mice (**Figure 8**). Comparison of SCFAs in CeD pediatric patients treated with GFD for at least one year, GFD less than one year, and healthy children found that CeD patients that adhered to a GFD for greater than a year closely resembled SCFA levels of healthy children, while CeD patients treated for less than one year had significantly increased SCFA levels (79). We observed a similar trend in our murine study, where GFD mice regardless of NLRX1 or NLRP12 genotype had decreased metabolite levels (**Figure 8**). Acetate levels were significantly decreased between each genotype (wild-type, *Nlr1^{-/-}*, and *Nlrp12^{-/-}*) on a GFD compared to normal diet (**Figure 8A**). Acetate levels are the most abundant SCFA at >50% and are critically involved in the ability of Bifidobacterium to inhibit enteropathogens, therefore ensuring symbiosis of the gut microbiome (80). In *Nlr1^{-/-}* mice, we observed decreased levels of butyrate in mice fed GFD compared to normal diet (**Figure 8B**). Steady levels of butyrate are beneficial in maintaining the gut mucosal barrier and reducing the risk of several gastrointestinal disorders (82-84). Along with butyrate, propionate is critical for the production of gut hormones that regulate appetite and is also involved in the activation of kinase-mediated pathways (85, 86). The complete omission of gluten results in a collateral loss of prebiotics and a deficit of micronutrients that are utilized in the

metabolic activity of microorganisms. Due to this loss, the probiotic effect of beneficial microbes is significantly impaired resulting in expansion of pathogenic/opportunistic microbes.

Overall, the culmination of our findings suggests that GFD as a dietary intervention, although the standard of care for CeD and NCGS patients, has limitations. This treatment regimen completely omits food sources containing gluten to prevent any robust autoimmune responses to partially digested gluten fragments and gliadins. While this is a critical avoidance therapy necessary to control these diseases, the significant change from a normal diet disrupts the delicate homeostasis of the microbiome and shifts it towards dysbiosis. Due to this shift in food intake, diet-associated changes to the microflora also interfere with metabolite production. Specifically, GFD decreases SCFA production, which is commonly upregulated in CeD patients, but at the cost of microbiome symbiosis. Our study reveals that adherence to a GFD in otherwise healthy mice results in the loss of beneficial microbes, which suggests that administration of GFD could be paired with probiotics to further promote gut health by reintroducing lost populations of commensals. Using murine models to further explore the role of NLRX1 and NLRP12 deficiencies in microbiota maintenance, we found the gut microbiome of NLRP12-deficient mice did not significantly differ from wild-type mice with intact NLR signaling. However, we found that NLRX1-deficient mice have a genetically inherent gut microbiome that may be primed for dysbiosis following GFD. This, therefore, unveils a potential role of appropriate NLRX1 signaling in regulating the gut microbiome and its potential contribution to CeD and NCGS pathogenesis.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Design and execution of the experiments were conducted by H.A.M., K.E., Y.L., and M.N.-S. K.E. was responsible for mouse breeding, genotyping, and general husbandry. Experimental data interpretation was conducted by H.A.M., Y.L., P.A.W., and I.C.A.

H.A.M., K.E., Y.L. contributed equally to this work and share first authorship. P.A.W. and I.C.A contributed equally to this work and share senior authorship. All authors contributed to the writing and revising of the manuscript.

FUNDING

Grants were awarded from the US National Institutes of Health (I.C.A.; R03 DK105975 and K01 DK092355); the Via College of Osteopathic Medicine (VCOM) One Health Center Seed Funding (I.C.A.); Virginia Maryland College of Veterinary Medicine Internal Research Competition (I.C.A.), and the Virginia Tech Institute for Critical Technology and Applied Sciences (I.C.A.). This work was supported, in part, by the Intramural Research Program of the National Institute of Environmental Health Sciences (ES101965 to P.A.W.).

ACKNOWLEDGMENTS

The authors would like to acknowledge the assistance of the Teaching and Research Animal Care Support Service (TRACSS) at the Virginia Maryland College of Veterinary Medicine, as well as our technician, graduate students, and undergraduate research students, including Veronica Ringel-Scaia, Christina Mounzer, and Bettina Heid. We would also like to especially acknowledge Jody Smiley from the Virginia Tech Sludge Lab for assistance with fecal metabolite analysis. We gratefully acknowledge outstanding technical support in 16s sequencing from Jonah Ventures.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.882521/full#supplementary-material>

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI's Sequence Read Archive (PRJNA808753).

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FIGURE LEGENDS

Figure 1

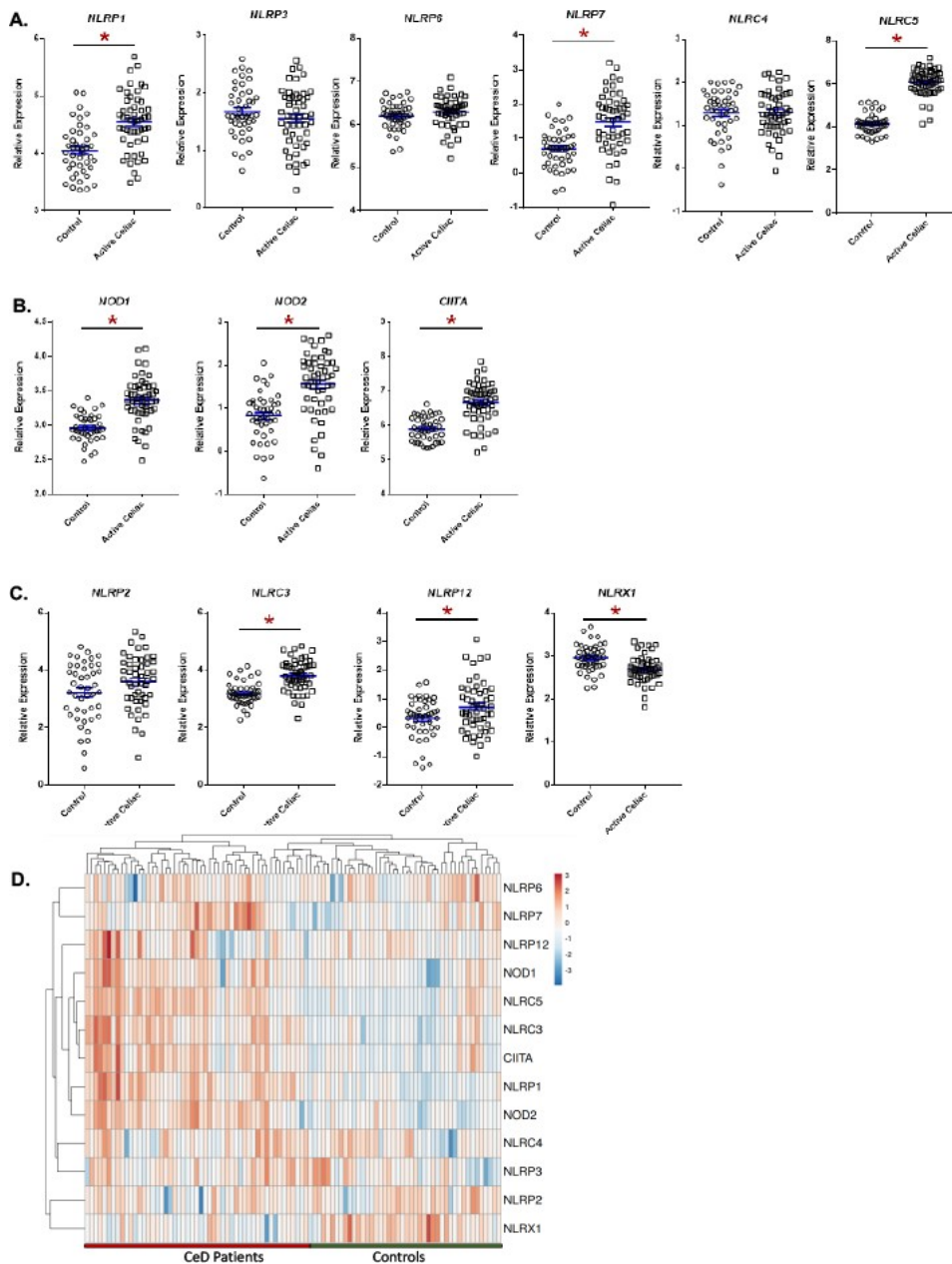


Figure 1. Significant Dysregulation of NLR Signaling in Active CeD Patients. (A) Inflammasome-forming NLR signaling trended higher in active CeD patients than controls, with significant variation observed in *NLRP1*, *NLRP7*, and *NLRC5*. **(B)** Gene expression of the regulatory NLRs *NOD1*, *NOD2*, and *CIITA* were significantly upregulated in active CeD patients. **(C)** Gene expression of the negative regulatory NLRs also trended higher in active CeD patients, with significant upregulation observed in

NLRC3 and NLRP12. NLRX1 is the only negative regulatory NLR that was significantly downregulated. **(D)** Gene expression heatmap of assorted NLRs in CeD patients. * $p < 0.05$.

Figure 2

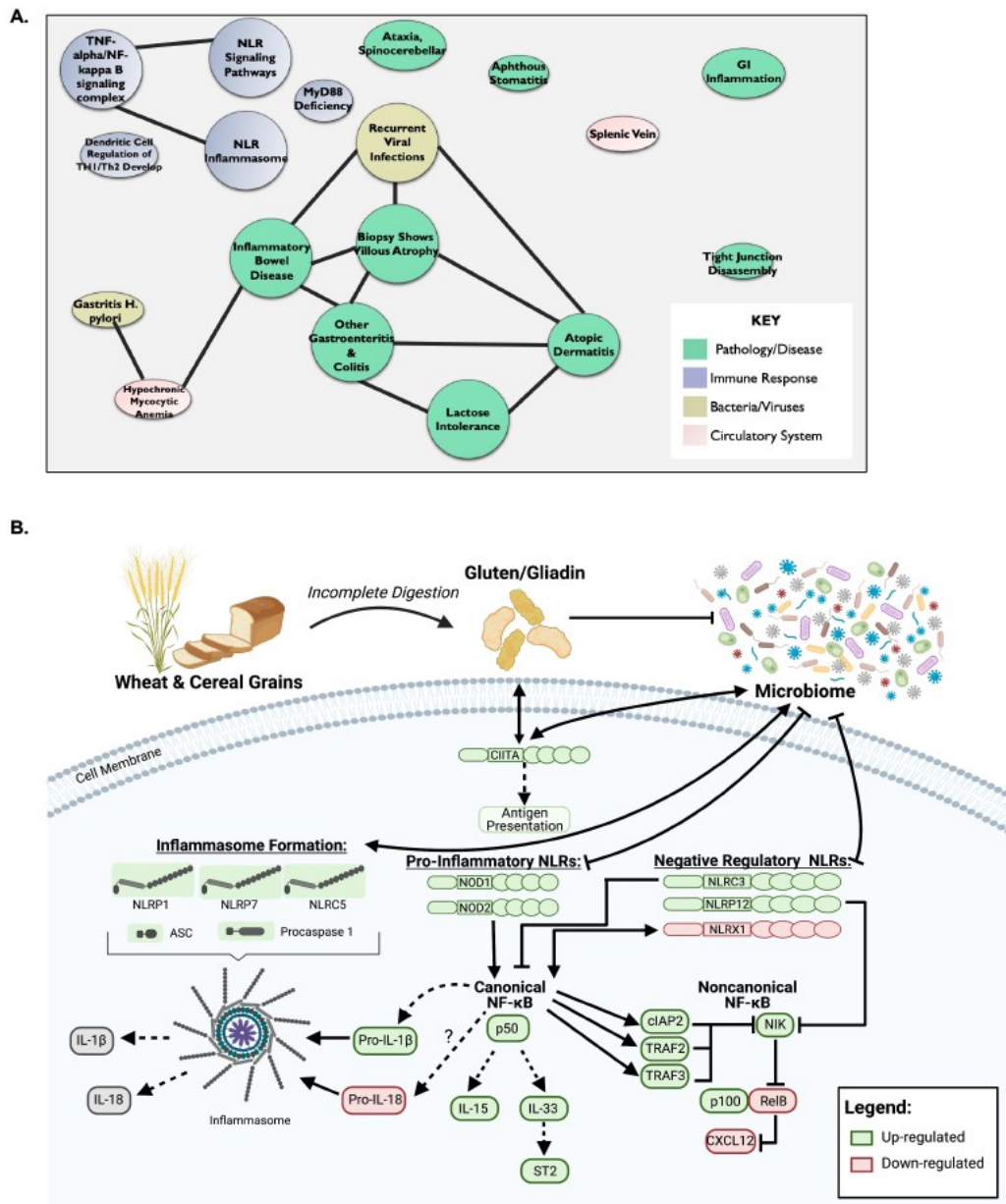


Figure 2. Dysregulation of NF- κ B Pathway Downstream of Impaired NLR Signaling May Potentiate CeD Pathogenesis. (A) CompBio AI algorithm predicted increased NF- κ B/TNF signaling, NLR inflammasome signaling, and regulatory NLR signaling that is consistent with CeD pathology. **(B)** Schematic representative of IPA data output predicts that partial digestion of gluten is recognized as a DAMP by PRRs to signal NLRs in CeD.

This results in downstream activation of canonical NF- κ B and inflammasome formation, while inhibiting noncanonical NF- κ B.

Figure 3



Figure 3. Mice with Intact NLR Signaling Display a Distinctive Microbiome Depending on Diet-type. (A) The microbiomes of mice with intact NLR signaling is dominated by the Firmicutes phyla. The second most abundant phyla is driven by diet type with normal diet being characterized by Bacteroidetes and GFD by Actinobacteria. **(B)** LEfSe differential analysis displaying LDA scores reveals that significant differences

exist between the microbial populations (GFD = red and normal diet = green. The graph was generated using the LEfSe program. GFD (red), $n = 7$; normal diet (green), $n = 7$.

Figure 4

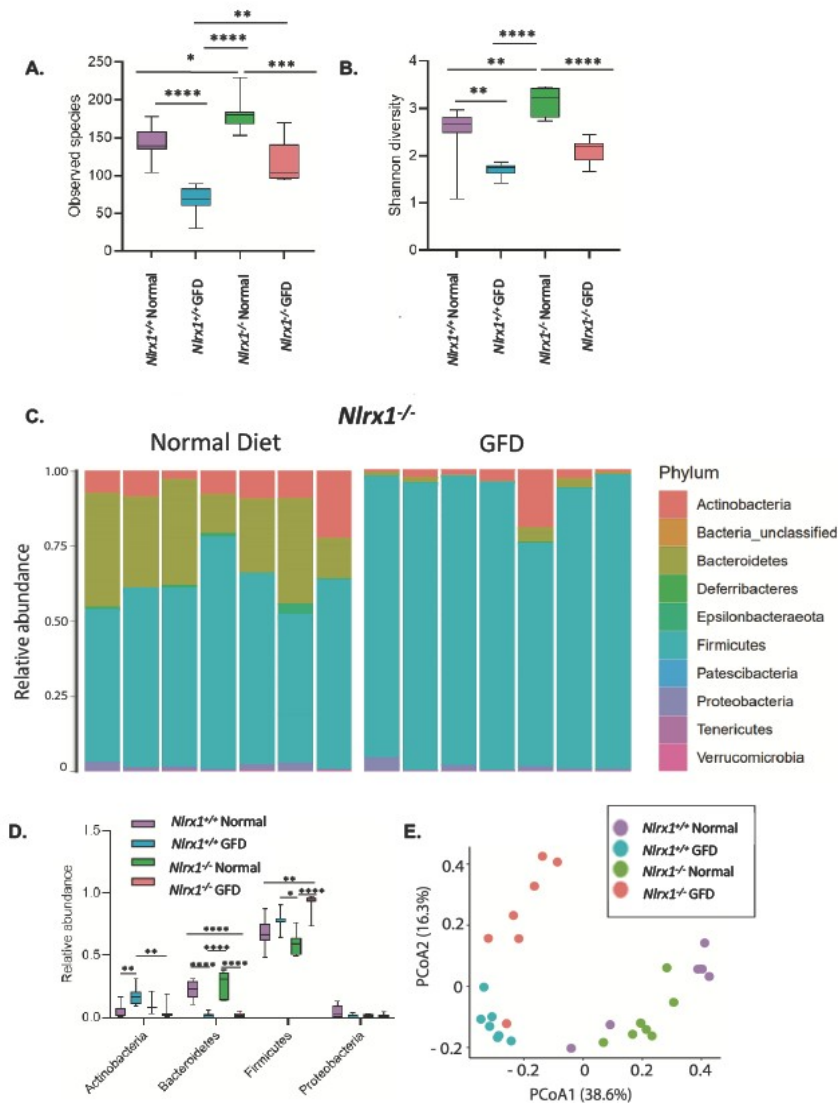


Figure 4. NLRX1-deficient Mice Have Inherently Unique Microflora Diversity. (A) *NLRX1^{-/-}* mice have significantly increased observed species of bacteria when fed a normal diet compared to GFD. (B) Assessment of Shannon Diversity depicts *NLRX1^{-/-}* mice to have significantly decreased diversity on a GFD compared to a normal diet. (C) The most abundant phyla present in the gut microbiota is Firmicutes. Bacteroidetes is the second most abundant phyla. Relative abundance is driven by diet, as these levels were

significantly diminished in GFD mice. **(D)** There is significant variation in relative abundance of phyla present amongst the different treatment groups. **(E)** There is a clear clustering of the groups in PCoA analysis with clusters being apparent for both genetic status and diet type. GFD, $n = 7$; normal diet, $n = 7$. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$

Figure 5

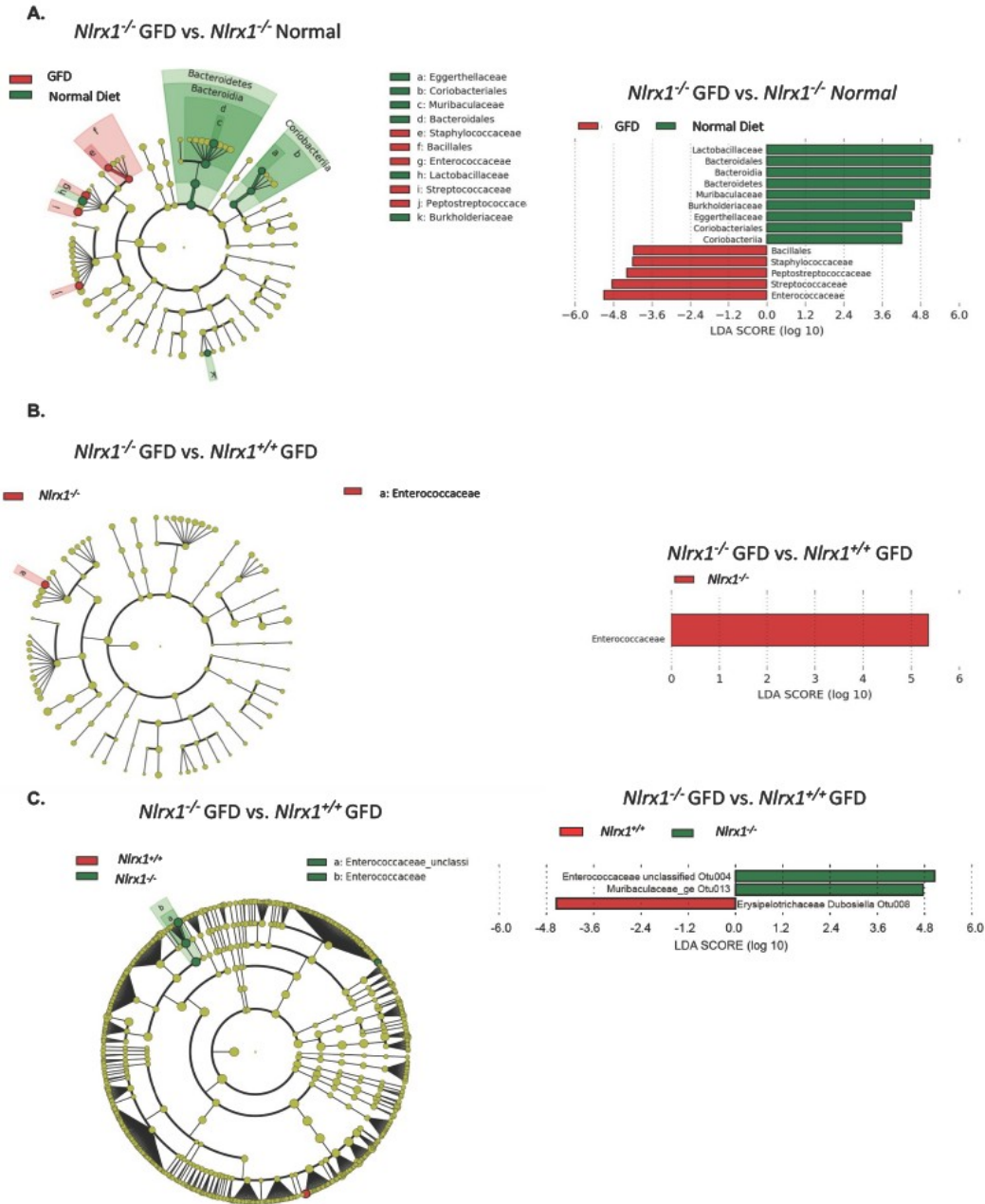


Figure 5. Comparison of the Effects of GFD on the Microbiome of NLRX1-deficient Mice. (A) Cladogram displaying differential bacterial abundance in *NLRX1^{-/-}* GFD (red) and normal diet (green). LEfSe differential analysis displaying bacterial abundance in *NLRX1^{-/-}* GFD (red) and normal diet reveals significant differences exist between the microbial populations at the family level. The graph was generated using the LEfSe program. *NLRX1^{-/-}* GFD (red). (B) Cladogram and LEfSe analysis displaying differential

bacterial abundance in *NLRX1*^{-/-} (green) and mice with intact NLRX1 signaling (red) following GFD at family level **(C)** Cladogram displaying differential bacterial abundance in *NLRX1*^{-/-} (green) and mice with intact NLRX1 signaling (red) following GFD at OTU level. LEfSe differential analysis displaying bacterial abundance in *NLRX1*^{-/-} (green) and wild-type mice (red) reveals miniscule differences exist between the microbial populations. The graph was generated using the LEfSe program. n = 7 mice per group.

Figure 6

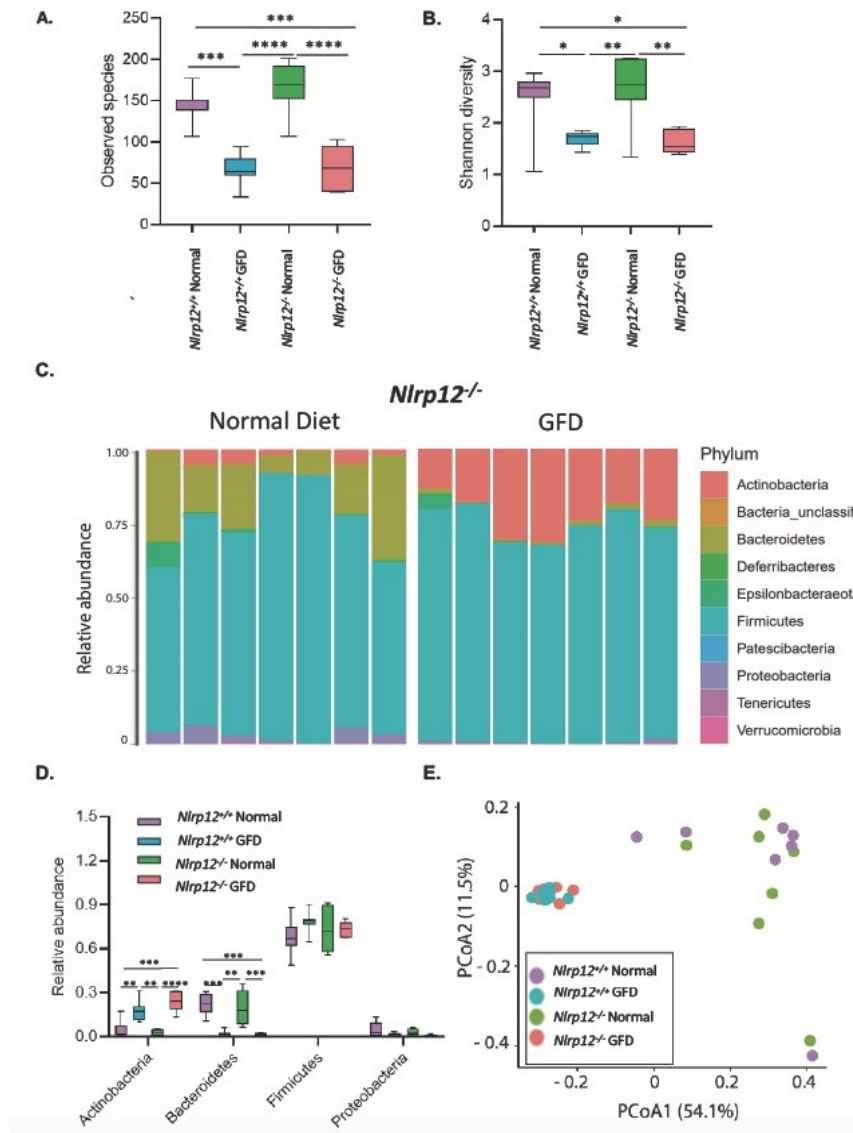


Figure 6: Absence of NLRP12 Has Minimal Effect on Gut Microflora. (A) There is no significant variation between *NLRP12*^{-/-} mice and wild-type mice in the observed species present, either for normal diet or GFD. (B) This trend is also observed in Shannon Diversity. (C) Upon assessment of the relative abundance of phyla in the *NLRP12*^{-/-} gut microbiome, the most abundant phyla present in the is Firmicutes. While the next most abundant phyla is dependent on diet type – Bacteroidetes is the second most abundant

phyla for mice adhering to a normal diet, while this phyla was significantly diminished in GFD mice. **(D)** There is no significant variation between *NLRP12*^{-/-} mice and wild-type mice in the observed species present, either for normal diet or GFD. **(E)** There is a clear clustering of the groups in PCoA analysis with clusters occurring based on diet-type. GFD, $n = 7$; normal diet, $n = 7$.

Figure 7

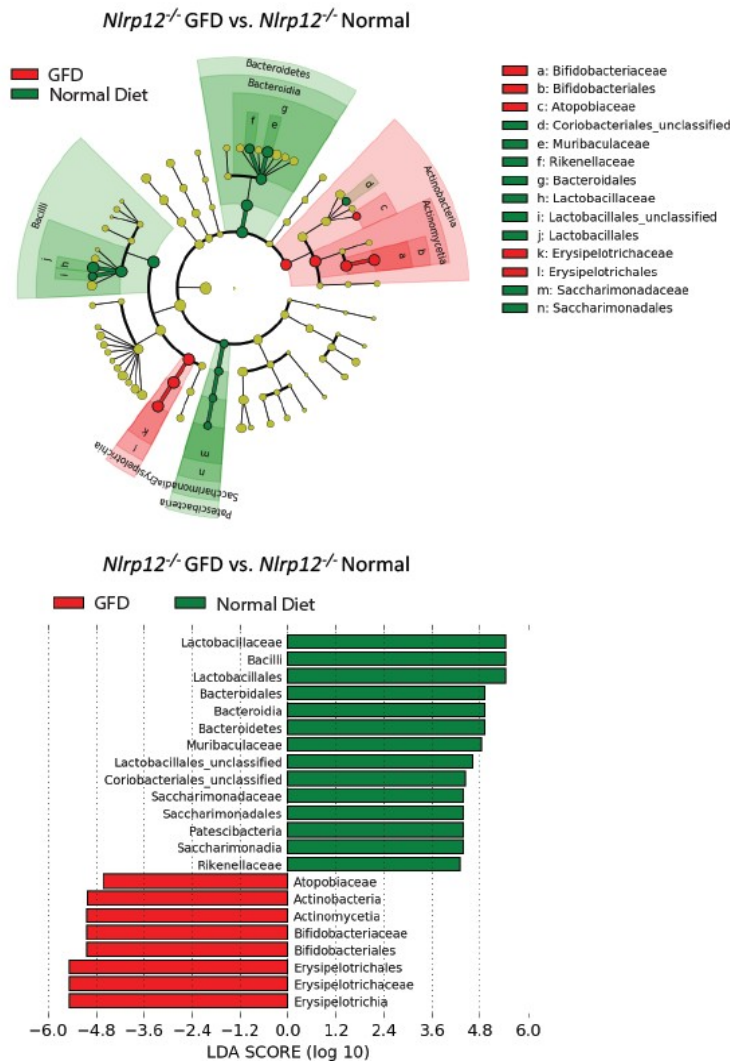


Figure 7: The Effects of GFD on the Microbiome of NLRP12-deficient Mice. (A) Cladogram displaying differential bacterial abundance in *NLRP12*^{-/-} mice fed GFD (red) and normal diet (green). **(B)** LefSe differential analysis displaying bacterial abundance in *NLRP12*^{-/-} mice fed GFD (red) and normal diet (green) reveals that significant differences exist between the microbial populations. The graph was generated using the LefSe program. GFD (red), *n* = 7; normal diet (green), *n* = 7.

Figure 8

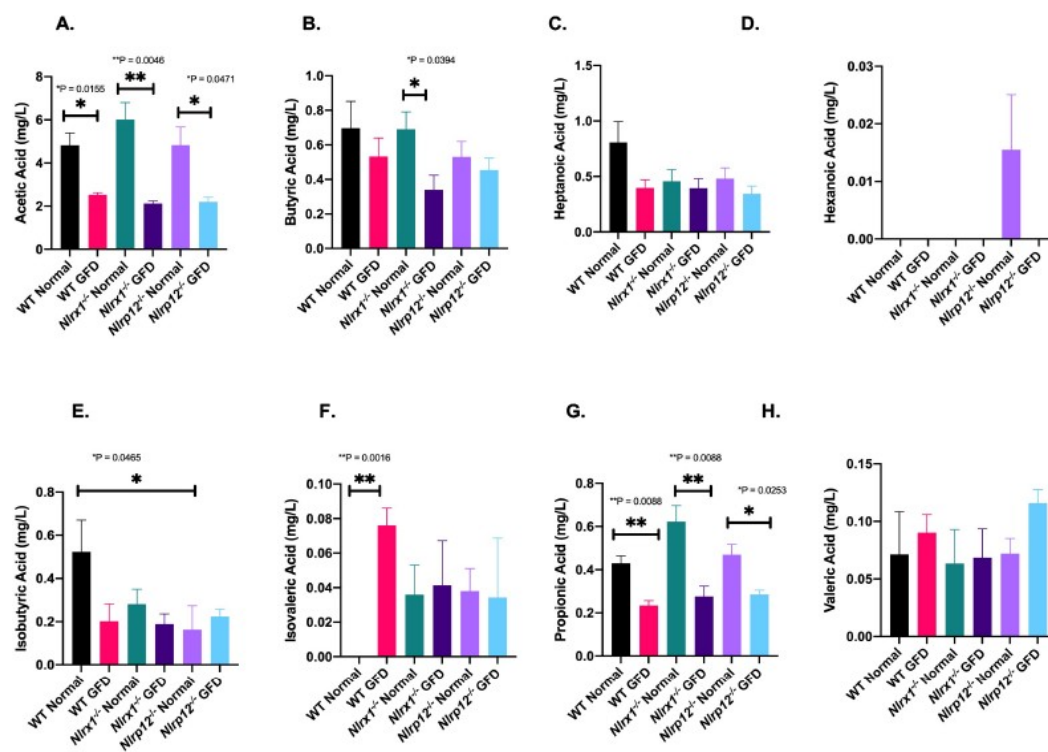
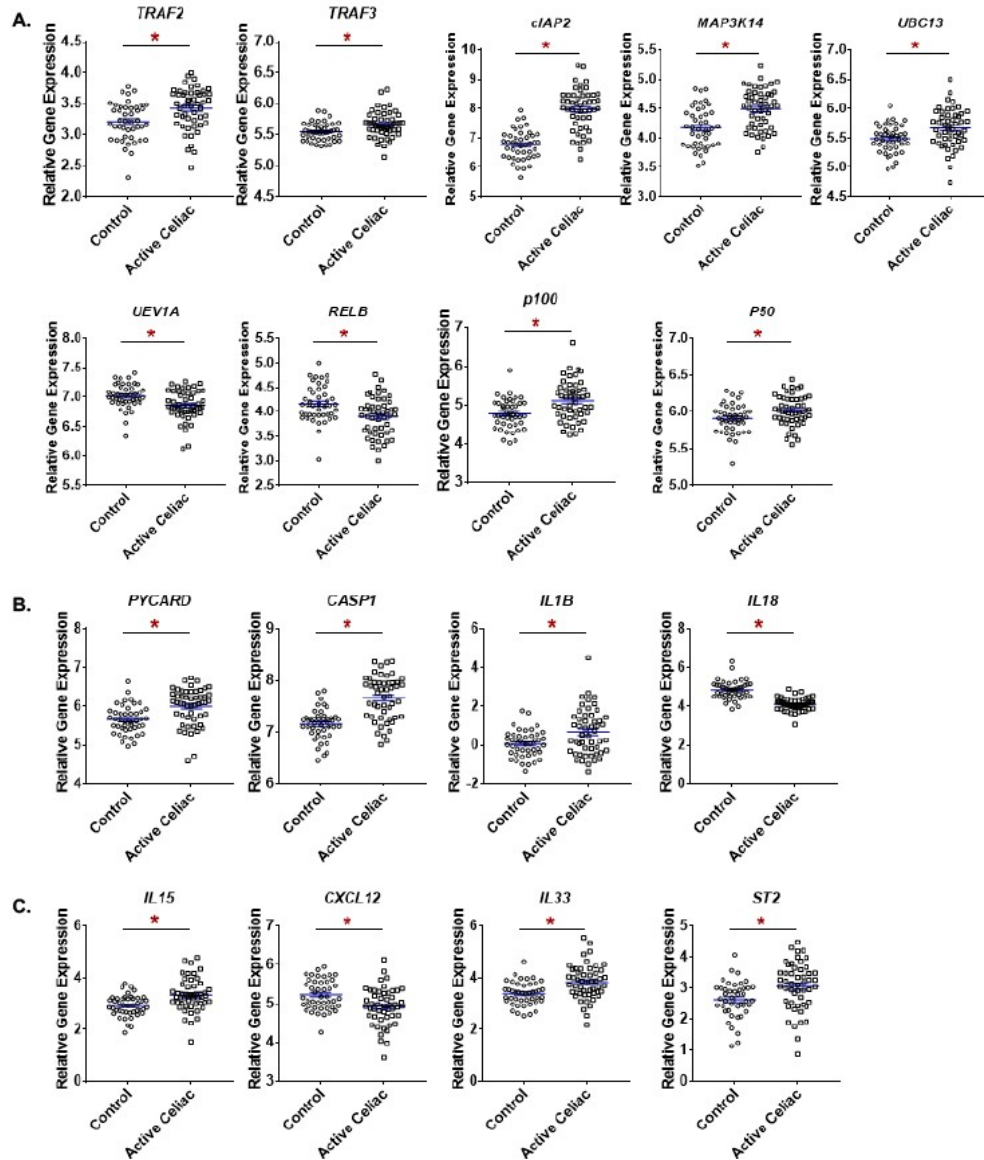


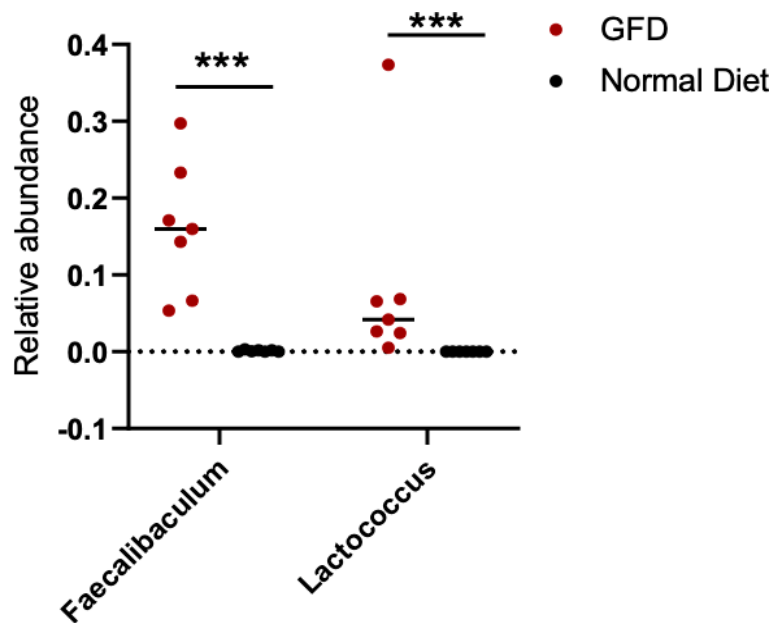
Figure 8: SCFA and MCFA Levels Significantly Vary Based on Diet. Abundance of the (A) acetic acid, (B) butyric acid, (C) heptanoic acid, (D) hexanoic acid, (E) isobutyric acid, (F) isovaleric acid, (G) propionic acid, and (H) valeric acid in mg/L of feces in wild-type, *Nlrp1*^{-/-}, and *Nlrp12*^{-/-} mice. *n* = 3-7. * *p* < 0.05, ** *p* < 0.005.

SUPPLEMENTARY MATERIAL

Supplemental Figure 1



Supplemental Figure 1: Potential Downstream Targets Implicated by Impaired NLR Signaling in CeD. The subset of genes evaluated here are commonly expressed upon pro-inflammatory signaling downstream of NLR signaling. The majority of genes were upregulated with the exception of *UEV1A*, *RELB*, *IL18*, and *CXCL12* in active CeD patients. * $p < 0.05$



Supplemental Figure 2. Diet Alters the Gut Microbiome of NLRX1-deficient Mice at the Genus Level. Relative abundance of two genera found at significantly increased levels in *Nlrp1*^{-/-} mice fed GFD. n = 7 mice per group. *** $p < 0.0001$

Supplemental Table 1: Ingredient List for GFD and Normal Diet

NORMAL DIET		GLUTEN-FREE DIET	
<i>Tekland Global 18% Protein Rodent Diet</i>		<i>Open Source Diets AIN-76A</i>	
Ingredient	g/kg	Ingredient	g/kg
<i>Ground wheat</i>	Proprietary information that cannot be disclosed by vendor	<i>Casein, Lactic, 30 mesh</i>	200.00
<i>Ground corn</i>		<i>Methionine, DL</i>	3.00
<i>Wheat middlings</i>		<i>Sucrose, Fine granulated</i>	500.00
<i>Dehulled soybean meal</i>		<i>Starch, Corn</i>	150.00
<i>Corn gluten meal</i>		<i>Solka Floc, FCC200</i>	50.00
<i>Soybean oil</i>		<i>Corn oil</i>	50.00
<i>Calcium carbonate</i>		S10001 Mineral Mix	35.00
<i>Dicalcium phosphate</i>		V10001 Mineral Mix	10.00
<i>Brewers dried yeast</i>		<i>Choline bitartrate</i>	2.00
<i>Iodized salt</i>			
<i>L-lysine</i>			
<i>DL-methionine</i>			
<i>Choline chloride</i>			
<i>Kaolin</i>			
<i>Magnesium oxide</i>			
<i>Vitamin E acetate</i>			
<i>Menadione sodium bisulfite complex</i>			
<i>Manganous oxide</i>			
<i>Ferrous Sulfate</i>			
<i>Zinc oxide</i>			
<i>Niacin</i>			
<i>Calcium pantothenate</i>			
<i>Vitamin A acetate</i>			
<i>Calcium iodate</i>			
<i>Vitamin B12 supplement</i>			
<i>Folic acid</i>			
<i>Biotin</i>			
<i>Vitamin D3 supplement</i>			
<i>Cobalt carbonate</i>			
Macronutrients	Kcal%	Macronutrients	Kcal%
Carbohydrate	58	Carbohydrate	67.7
Protein	24	Protein	20.8
Fat	18	Fat	11.5

Supplemental Table 2: AMOVA Analysis for NLRX1 PcoA Plot

<i>Nlr1</i> Comparisons	F_s	p-value
Nlr1+/+ Normal vs Nlr1 +/+ GFD	17.7	0.001
Nlr1+/+ Normal vs Nlr1 -/- Normal	3.39	0.004
Nlr1+/+ Normal vs Nlr1 -/- GFD	10.9	<0.001
Nlr1 -/- Normal vs Nlr1 -/- GFD	15.5	0.002
Nlr1 -/- Normal vs Nlr1 +/+ GFD	28.1	<0.001
Nlr1 +/+ GFD vs Nlr1 -/- GFD	9.9	0.001

Supplemental Table 3: AMOVA Analysis for NLRP12 PcoA Plot

<i>Nlrp12</i> Comparisons	F_s	p-value
Nlrp12+/+ Normal vs Nlrp12 +/+ GFD	16.5	0.002
Nlrp12+/+ Normal vs Nlrp12 -/- Normal	0.9	0.492
Nlrp12+/+ Normal vs Nlrp12 -/- GFD	16.7	<0.001
Nlrp12 -/- Normal vs Nlrp12 -/- GFD	18.6	0.001
Nlrp12 -/- Normal vs Nlrp12 +/+ GFD	18.5	0.001
Nlrp12 +/+ GFD vs Nlrp12 -/- GFD	3.9	0.004

CHAPTER FOUR

Negative Regulatory NLRs Mitigate Inflammation via NF- κ B Pathway Signaling in Inflammatory Bowel Disease

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ABSTRACT:

A subset of NLRs, the negative regulatory NLRs, function to mitigate overzealous pro-inflammatory signaling produced by NF- κ B activation. Under normal pathophysiologic conditions, proper signaling of these NLRs protect against potential autoimmune responses. These NLRs associate with several different proteins within both the canonical and noncanonical NF- κ B signaling pathways to either prevent activation of the pathway or inhibit signal transduction. Inhibition of the NF- κ B pathways ultimately dampens the production of pro-inflammatory cytokines and activation of other downstream pro-inflammatory signaling mechanisms. Dysregulation of these NLRs, NLRC3, NLRX1, and NLRP12 have been reported in human inflammatory bowel disease (IBD) and colorectal cancer patients, suggesting the potential of these NLRs as biomarkers for disease detection. Mouse models deficient in these NLRs also have increased susceptibility to colitis/colitis-associated colorectal cancer. While current standard of care for IBD patients and FDA-approved therapeutics function to remedy symptoms associated with IBD and chronic inflammation, these negative regulatory NLRs have yet to be explored as promising potential drug targets. In this review, we describe a comprehensive overview of recent studies that have evaluated the role of NLRC3, NLRX1, and NLRP12 in IBD, colitis, and colitis-associated colorectal cancer.

BACKGROUND:

Inflammatory bowel disease (IBD) is a complex, multifactorial disorder that develops secondary to genetic, environmental, microbiological, and immunological factors (1). IBD is an umbrella term that encompasses two chronic clinical syndromes known as Crohn's Disease (CD) and ulcerative colitis (UC). Both variations of the disease can manifest with clinical signs that range from abdominal pain, rectal bleeding, diarrhea, and weight loss. CD is additionally associated with development of fistulas and rectal lesions, while pain associated with bowel movements is commonly described by UC patients. Anatomically, UC is mostly limited to the colonic and rectal mucosa, whereas CD can impact any part of the GI tract. However, CD is commonly localized to the ileum and colon (1). Patients suffering from the aforementioned clinical conditions are at increased risk for the development of colorectal cancer due to the chronic inflammation associated with both disease processes (2). IBD is characterized by immune

dysregulation in the gut and is frequently associated with dysbiosis, an imbalance in commensal bacteria.

In addition to dysbiosis, a mechanism of immune dysregulation that has been identified in IBD pathogenesis deals with pattern recognition receptors (PRRs). These are receptors found within the cell cytoplasm and lipid bilayer that are crucial for the maintenance of immune functionality in the gut. Specifically, PRRs detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) to initiate downstream signaling pathways that modulate inflammation, cell death, proliferation, tissue repair and remodeling. Dysregulation of PRR functionality in the GI tract commonly results in enhanced or dampened immune responses, as well as changes in the gut microbiome leading to dysbiosis (3). Nucleotide-binding and leucine-rich repeat-containing receptors (NLRs) are a specific group of receptors within the PRR family that are located in the cell cytoplasm that bind to PAMPs and DAMPs in the cell cytosol that also initiate or regulate downstream signaling cascades that manifest in inflammatory cytokine production.

Both positive and negative regulatory NLRs exist, with the negative regulatory NLRs encompassing NLRC3, NLRX1 and NLRP12. The role of NLRC3 has been investigated in association with colorectal cancer tumorigenesis. Karki et. al demonstrated that mice lacking NLRC3 exhibit increased susceptibility to colorectal cancer tumorigenesis. Similar to NLRC3, NLRP12 also plays a critical role in protection against the development of enteritis and colitis. For example, it has been demonstrated that mice lacking NLRP12 exhibit enhanced inflammatory signaling through NF- κ B and ERK pathways within macrophages (4). This is worth noting as regulation of NF- κ B is necessary for the maintenance of homeostasis associated with the gut microbiome. The dysregulation of NF- κ B may be a significant factor that contributes to the development of inflammatory diseases such as colitis and colorectal cancer. This relationship is important because many of the aforementioned NLRs can activate or inhibit the NF- κ B signaling pathway. This pathway can be activated via two separate arms known as the canonical and noncanonical pathways. The canonical pathway contains downstream RelA and p50 heterodimers which are regulated in the cytoplasm prior to activation by a category of inhibitors of NF- κ B (IkBa). Activation of this pathway is modulated through an IkB

kinase complex. Within this kinase complex are I κ B kinase γ , NF- κ B essential modulator (NEMO) and the catalytic units I κ B kinase α (IKK α) and I κ B kinase β (IKK β).

Following kinase activity, the IKK complex phosphorylates I κ B α , resulting in the release of the RelA/p50 heterodimer. The RelA/p50 heterodimer then translocates to the nucleus where transcription of inflammatory mediator genes occurs. The canonical NF- κ B pathway is responsible for modulating the inflammatory response under homeostatic conditions. Similar to the canonical pathway, a heterodimer of NF- κ B is present in the form of p52 and RelB, alternative to RelA and p50, culminating in signaling via the noncanonical pathway. The NF- κ B-inducing kinase (NIK) is responsible for downstream modulation of p100, as liberation of NIK from its inhibitor, TRAF3, is the first step in initiating this process. Once NIK is liberated into the cell cytosol, it phosphorylates downstream IKK α , which in turn phosphorylates and ubiquitinates p100, allowing for partial processing into p52. P52 then translocates into the nucleus with assistance from the nuclear localization sequence (NLS) to initiate gene transcription. The noncanonical NF- κ B pathway is relatively understudied however it is associated with production of cytokines associated with cancer and inflammation, recruitment of immune cells, cell proliferation, and development of secondary lymphoid structures in the GI tract. Human patients with colorectal cancer have been found to have significant downregulation of the noncanonical NF- κ B pathway within their colonic epithelial cells. This suggests that the noncanonical pathway plays a role in protecting against the formation of colorectal cancer through regulating the immune response in the gut. Therefore, further characterization of the role NF- κ B signaling has in IBD pathogenesis, especially given dysregulation of these canonical and noncanonical NF- κ B pathways via negative regulatory NLRs like NLRC3, NLRX1, and NLRP12, highlights an emerging interest for clinical relevancy. In this review, the culmination of data from recent literature illuminates a previously overlooked role of the negative regulatory NLRs in IBD and how previous work bolsters foundational data that may be useful in detecting new biomarkers, as well as targets for therapeutics.

NLRC3 – The dark horse in IBD pathogenesis

NLRC3 is one of the least characterized NLRs. This negative regulatory NLR was first discovered in 2005 as the cytoplasmic protein CATERPILLER 16.2 (CLR16.2)(5). The Conti *et al.* group first found that NLRC3 attenuates T cell activation and subsequently decrease NF- κ B by inhibiting the degradation of I κ B α . This subsequently blocked the NLS necessary for translocation of NF- κ B heterodimers to the nucleus (5). Further mechanistic studies found that NLRC3 negatively regulates NF- κ B signaling by directly associating with TRAF6 of the canonical NF- κ B signaling pathway to limit its activating ubiquitination (6). NLRC3 limits this critical step in signal transduction by associating with TRAF6 to prevent its auto-ubiquitination. This association between an NLR and TRAF protein is termed “TRAFasome” formation and renders subsequent signaling inactive. Of the TRAF proteins, these interactions were specific for TRAF6 in canonical NF- κ B signaling, whereas the TRAF2 and TRAF3 proteins of the noncanonical NF- κ B pathway were unaffected (6). Physiologic relevancy of these *in vitro* findings was also consistent with a murine model deficient in NLRC3 (*Nlrc3*^{-/-}). Upon stimulation of LPS, peritoneal macrophages collected from *Nlrc3*^{-/-} mice had increased TRAF6 protein at a resting basal level. *Nlrc3*^{-/-} mice were also shown to be more susceptible to a model for endotoxic shock following injection with LPS, as they had more severe weight loss, decreased body temperature, and significantly higher production of IL-6 and TNF. These findings were specific for the canonical NF- κ B pathway and were not shown to impair other pro-inflammatory signaling pathways, including mitogen-activated protein kinases p38, Erk, and Jnk. Early studies characterized the involvement of NLRC3 in immune function, where it was found to be a critical negative regulator in pro-inflammatory NF- κ B signaling. This soon reinforced NLRC3’s involvement in shaping the chronic inflammatory microenvironment of colitis.

Given its role as a negative regulator for NF- κ B-induced inflammation, NLRC3 has also been reported to have direct implications in colitis and colitis-associated CRC. A retrospective screening conducted with bioinformatics analysis found that *NLRC3* gene expression was downregulated in all five Oncomine ® Platform datasets for colorectal cancer (7). Validation with case-matched tumor and healthy formalin-fixed paraffin-

embedded (FFPE) human colorectal cancer biopsies also found decreased gene expression, highlighting NLRC3's potential as a candidate biomarker for CRC (7). Mechanistic insight from the Karki *et al.* 2016 demonstrates that *Nlrc3*^{-/-} mice have a heightened susceptibility to colitis and colitis-associated CRC (8). They found that *NLRC3* expression in enterocytes protects against tumorigenesis by inhibiting mTOR activation and subsequently inhibiting proliferation of this cellular compartment. Specifically, association with NLRC3 inhibits PI3K-dependent AKT activation following binding to a growth factor receptor or Toll-like receptor 4 to inhibit downstream mTOR signaling. Chemical induction of inflammation-induced tumorigenesis in the colon was used in this well-established AOM/DSS model in mouse models to show NLRC3-deletion resulted in increased susceptibility as evidenced by significantly increased number of tumors and weight loss. NLRC3-deficient mice also had increased inflammatory damage, circulation of pro-inflammatory mediators (IL-6, GCSF, KC, and MIP-1 α) in the sera compared to healthy controls, and levels of I κ B α and STAT3 phosphorylation. They found increased counts of macrophages, neutrophils, and natural killer cells in the *Nlrc3*^{-/-} mice. Interestingly, no significant differences in IFN γ ⁺ or TNF⁺CD4⁺ T cell population levels collected from splenocytes stimulated with CD3 and CD28 were observed. Bone marrow chimera studies with *Nlrc3*^{-/-} mice receiving *Nlrc3*^{-/-} mice bone marrow were most susceptible to tumorigenesis. Data yielded from this study provides further insight into the cellular compartment responsible for tumorigenesis using conditional knockouts. Conditional-deletion in intestinal epithelial cells developed the highest number of tumors followed by hematopoietic deletion. *Ex vivo* organoid culture from colonic epithelial stem cells further demonstrated that cells containing *Nlrc3*-deletions were more inclined to form organoids with larger diameters due to increased colony forming capacity. Molecular assays indicate that increased phosphorylation levels of mTOR targets including AKT at Thr308 was observed in organoids and colon tissue that likely contribute to increased NF- κ B signaling. Together, these data suggest that NLRC3 interacts with PI3K p85 and p111 α subunit to interfere with this association and therefore subsequent activation of the PI3K-AKT-mTOR pathway. A follow-up study conducted by this group in 2017 further found that NLRC3 functions to protect against CRC by directly associating with both TRAF6 and mTOR, which thereby promotes

cellular apoptosis and inhibits proliferation (9). *Nlrc3*^{-/-} mice in an AOM/DSS model had increased susceptibility to CRC and markers for proliferation. This group concludes that NLRC3 regulates the transcriptional activity of c-Myc (9). This was indicated by increased downstream expression of the intestinal stem cell markers *Bmi1* and *Olfm4*, and increased IHC staining of cells for Ki67 (proliferation marker) and cyclin D1 (proto-oncogene) (9). Comparatively, the colons of *Nlrc3*^{-/-} mice also had decreased apoptosis as a result of deregulated AKT-mediated phosphorylation of the tumor suppressor proteins FoxO3a and FoxO1 and decreased caspase-3 and caspase-7 (9). Taken together, these data reveal a critical role for NLRP3 in maintaining gastrointestinal homeostasis. Loss of this negative regulatory NLR culminates in not only dysregulation of pro-inflammatory signaling mechanisms, but also impaired functionality of enterocytes by increasing proliferation and decreasing apoptosis that ultimately impairs this cell population's capacity to cope with inflammatory damage.

Typically, chronic inflammation of the gut, such as in IBD, is resultant of dysbiosis of the gut, causing opportunistic colitogenic bacteria populations to expand. Karki *et al.* 2017 was one of the first groups to assess the gut microbiome in NLRC3-deficient mice using rt-PCR to assess 11 major bacterial species in stool samples. They found no significant variation in these levels between WT and *Nlrc3*^{-/-} mice. However, segmented filamentous bacteria, which are associated with IL-17 and IL-22-mediated chronic inflammation, was found to be increased in *Nlrc3*^{-/-} mice. This group concluded that because bacterial composition was not significantly varied at the species level for this selection of bacteria, CRC pathogenesis is not driven by the gut flora (9). However, this was a biased assessment of the microbiome as only a small selection of bacterial species was measured. A comprehensive analysis of the gut microbiome using metagenomics in the future may reveal more subtle shifts in the population, particularly difficult to measure organisms such as anaerobes and how they may contribute to dysbiosis and subsequent inflammatory disorders of the gut. More recent work conducted in 2018 by Cheng *et al.* found that the microbiome of a murine model for diabetes had decreased expression of *Nlrc3* in colonic tissue and reduced epithelial barrier integrity (10). Although not a direct assessment of the microbiome, this work further suggests that loss of NLRC3 may increase intestinal permeability, perturbing the composition of the

microbiota and ultimately creating permissive niches for the expansion of proinflammatory bacteria populations. Moreover, other work characterized NLRC3's involvement in host-pathogen immune responses, including PAMPs (11), bacteria (12), and viruses (13), which may also suggest the possible involvement of NLRC3 in maintaining homeostasis of the gut microbiome.

In summary, presence of NLRC3 within the gut is largely protective while maintaining gastrointestinal homeostasis and absence of this negative regulatory NLR confers susceptibility to various enteropathies, including colitis and cancer development. Previous work has suggested NLRC3 to be a promising biomarker for the diseased colon and perhaps a promising target for drug discovery. However, further evaluations of this NLR are necessary to decipher its direct involvement in the proinflammatory microenvironment of Inflammatory Bowel Disease, particularly its potential role in maintaining the microbiome, prior to further targeting as an IBD therapeutic.

NLRX1 - A multitasking NLR in the context of IBD

NLRX1, a widely expressed regulatory NLR has also been implicated in the development of colitis and IBD. Although NLRX1 does not have a fully characterized N-terminal domain, it is nonetheless involved in various anti-inflammatory actions and cellular functions. These range from attenuating NF- κ B signaling, blunting RIGI-MAVS signaling, promoting autophagy, and enhancing ROS production (14). Each unique and specific role has been characterized in neoplastic processes, traumatic brain injury, viral infection, and, most relevant to this review, colitis and IBD (15-21). Therefore, to understand the role that NLRX1 plays in IBD and ulcerative colitis, the specifics of its various functions must be understood to elucidate its protective role in the GI tract.

NLRX1 acts as a regulator of canonical NF- κ B signaling. Via TLR binding with subsequent MYD88 activation, NLRX1 can inhibit NF- κ B signaling in one of two ways. The first is through ubiquitination and dissociation from TRAF6, allowing NLRX1 to bind IKK α and disrupt downstream signaling. Conversely, through unknown mechanisms NLRX1 can instead remain associated with TRAF6 to form the "TRAFsome" complex. Ultimately both processes result in decreased IL-6 production, a potent pro-inflammatory cytokine. Decreased IL-6 therefore manifests in dampened innate immune signaling that confers protection on the GI mucosa (14, 21). NLRX1 is

also important for regulating the production of other inflammatory cytokines including $\text{TNF}\alpha$, $\text{IL-1}\beta$ and regulating ERK and STAT signaling pathways in enterocytes, which is necessary for development of these clinical signs. Inflammatory leukocytes do not appear to be essential for this process, as demonstrated via the generation of chimeric mice (21). Overall, these relationships are important to characterize as dysregulated inflammatory signaling with resultant cytokine production is associated with development of IBD and ulcerative colitis (21).

Additionally, NLRX1 is also implicated in regulating adaptive immune response within the GI tract. This has been demonstrated in a dextran sodium sulfate (DSS) induced colitis model. In the study, *Nlrp1*^{-/-} mice treated with DSS had greater numbers of Th17 and Th1 cells infiltrating the colon when compared to their wildtype (WT) counterparts, and *Nlrp1*^{-/-} Th1 and Th17 cells exhibited increased proliferative potential when compared to WT Th17 and Th1 cells (22). Furthermore, *Nlrp1*^{-/-} T cells also had altered metabolism and inflammatory cytokine production when compared to WT counterparts while also exhibiting resistance to PD-1 treatment (22). Overall, this study demonstrated that NLRX1 is crucial for T cell polarization and metabolism, which in turn attenuates a pro-inflammatory adaptive immune response that confers stronger gut health in the host.

Despite inflammatory signaling and T cell polarization, NLRX1 also influences individual enterocytes and the associated microbiome, a biological niche that can affect overall gut health and homeostasis. Studies involving mice in which NLRX1 has been knocked out in the enterocytes (*Nlrp1*^{fl/fl} *Villin*^{Cre+}), demonstrated similar histologic patterns and colitis scores comparable to whole body *Nlrp1*^{-/-} mice treated with DSS (20). Furthermore, WT mice housed with *Nlrp1*^{-/-} littermates exhibited skewed microbiome profiles similar to those characterized in *Nlrp1*^{-/-} mice. When mice were administered antibiotics, housed together, and challenged with DSS, WT mice exhibited similar disease and histopathologic scores comparable to *Nlrp1*^{-/-} mice. These findings demonstrate the synergistic role NLRX1 has on enterocyte health and the microbiologic niche, suggesting that dysregulated NLRX1 signaling may contribute to poor gut health and the imbalanced microbiome documented in IBD cases. Additionally, this study also characterized roles for NLRX1 in enhancing enterocyte proliferation, affecting glutamate

metabolism, decreasing enterocyte adhesion, and affecting sirtuin levels, a protein that can be broadly categorized as regulating enterocyte homeostasis (20). Taken together, these results demonstrate the effects NLRX1 has on GI tract health at the cellular, hormonal, and microbiologic level, suggesting that ineffective NLRX1 signaling in the aforementioned processes may also contribute to development of Crohn's disease and ulcerative colitis. These initial discoveries were further studied in the context of celiac disease (CeD) and consumption of a gluten free diet (GFD). For example, transcriptomic analysis revealed significantly downregulated NLRX1 signaling in CeD patients, suggesting that dysregulated NLRX1 signaling is associated with gluten sensitivity (23). Additionally, *Nlrp1*^{-/-} mice fed a normal diet exhibited greater microbiome diversity, however the niche was dominated by increased numbers of *Enterococcaceae* species. These are opportunistic bacteria that can exacerbate inflammation in patients with CeD and IBD (23). Conversely, *Nlrp1*^{-/-} mice fed a gluten free diet (GFD) exhibited decreased numbers of bacterial species within the intestinal tract. Together these data suggest that genotype and diet affect the composition of the gut microbiome (23). Perhaps most interesting of all, differences in metabolite composition in the murine model were also characterized with butyrate levels being significantly decreased in *Nlrp1*^{-/-} mice fed a GFD (23). Because low butyrate levels have been shown to incur adverse effects on overall gut health, it becomes obvious that microbiome composition, diet, and metabolic byproducts generated from such interactions all play a synergistic role in maintaining gut health.

Finally, NLRX1 may also be helpful in combating lipopolysaccharide (LPS) induced inflammation. LPS is a gram-negative cell wall constituent that initially signals through upstream TLR4. When *Nlrp1*^{-/-} murine macrophages were challenged with LPS, increased IL-6 production and decreased phosphorylation of I κ B α was demonstrated (18). Overall, these results suggest that TRAF6 mediated activation of NF- κ B signaling is attenuated via interaction with NLRX1 (18). This interaction becomes important when we consider gut health because LPS is a component of many pathogenic and non-pathogenic gram-negative bacteria that can reside in the GI tract. If gut wall integrity is compromised and there is dysbiosis characterized by overgrowth of gram-negative bacteria, LPS translocation from the gut into the bloodstream induces sepsis, a life-

threatening condition. Therefore, it can be postulated that this mechanism attempts to combat a cascade of inflammation initiated by LPS that could become clinically disastrous if left unchecked. Overall, the relationship between NLRX1 and LPS mediated inflammation should be considered when we discuss Crohn's disease and ulcerative colitis, two diseases in which gut wall integrity is compromised.

In summary, NLRX1 is a regulatory NLR that modulates inflammatory signaling associated with viral infections, TBI, tumorigenesis, and IBD. Because NLRX1 plays many roles in regulating gut health, further study is warranted to elucidate other roles for NLRX1 in attenuation or exacerbation of Crohn's disease and ulcerative colitis. If further mechanisms are characterized within the context of these disease processes, these mechanisms may serve as promising targets for solitary or supplementary treatments that can be utilized for patients suffering from IBD in the future.

NLRP12 – The well characterized NLR in colitis and colitis-induced tumorigenesis

Perhaps the most well-characterized negative regulatory NLR in colitis, NLRP12 was first discovered in 2003 as *Monarch-1*, where it was expressed by monocytic-myeloid cells and shown to regulate MHC class I genes (24). Further work characterized NLRP12 as a negative regulator of pro-inflammatory NF- κ B signaling by interfering with upstream TLR and TNFR signal transduction via MyD88, IRAK-1, and TRAF6, which activate canonical NF- κ B signaling (25). Here, NLRP12 was found to block TLR2 and TLR4 signaling by directly interacting with IRAK-1 and inhibiting its hyperphosphorylation (25). In addition to regulating canonical NF- κ B signaling, NLRP12 has also been shown to negatively regulate noncanonical NF- κ B signaling by directly associating with and inducing proteasomal degradation of the NF- κ B-inducing Kinase (NIK) (26-28). This kinase is critical for further signal transduction of this pathway. Together, these data reinforce the involvement of NLRP12 in holding pro-inflammatory signaling in check, whereas absence of this negative regulatory NLR results in an imbalance in both canonical and noncanonical NF- κ B signaling. This aberrant hyper-activation of NF- κ B contributes to increased transcription of pro-inflammatory cytokines, immune infiltration, inflammatory damage, and predisposition to various chronic inflammatory disorders, particularly those in the gastrointestinal tract.

More mechanistic studies have begun to delve out the involvement of NLRP12 in colitis and colitis-induced tumorigenesis using mouse models to assess its physiologic relevancy. NLRP12-deficient mice had increased susceptibility to developing colitis and colitis-induced tumorigenesis (29, 30). Work conducted by Zaki *et al.* 2011 found that NLRP12-deficient mice exposed to a chemical induced AOM/DSS model for colorectal cancer had increased infiltration of immune cells throughout the entire extent of the diseased colon, with further assessment indicating increased myeloid cell infiltration (29). Further, they attributed increased production of proinflammatory cytokines (IL-1 β , IL-6, TNF- α , IL-17, IL-15) observed in the colon to myeloid cells that likely elicit further pro-inflammatory and tumorigenic signaling in this microenvironment. As a result, increased downstream activation of NF- κ B, ERK, and STAT3 are associated with the increased proliferation of intestinal epithelial cells that contribute to hyperplasia. Summarizing their work, Zaki *et al.* measured canonical and noncanonical NF- κ B from *ev vivo* bone marrow-derived macrophages and found increased phosphorylated canonical p105 and increased RelA in the nucleus, while the noncanonical NF- κ B p100 and RelB proteins were found to be unchanged. This reinforces previous work that NLRP12 regulates canonical NF- κ B signaling and expands upon this finding in asserting that increased canonical NF- κ B drives inflammation-induced tumorigenesis in the colon. However, a similar study conducted by Allen *et al.* 2012 also assessed the susceptibility of NLRP12-deficient mice to an AOM/DSS model and concluded that loss of NLRP12 increases noncanonical NF- κ B signaling thus driving cancer development (30). This seminal work was also the first to demonstrate the multiprotein TRAFasome formation between NLRP12, NIK, and TRAF3, as NLRP12 directly binds to NIK and TRAF3 to stabilize TRAF3 and promote its degradation of NIK. This TRAFasome formation ultimately decreases the cytosolic levels of NIK necessary to further propagate the signal transduction of noncanonical NF- κ B signaling, rendering the pathway inactive. Further, this group found *ex vivo* dendritic cells had increased noncanonical NF- κ B and activation of MAP kinase (MAPK). Comparatively, they observed a modest increase in canonical NF- κ B activation with no significant increase in phosphorylated p65 in NLRP12-deficient and wild-type cells. Of the noncanonical NF- κ B produced chemokines, they

found >38X increase in CXCL13 levels in NLRP12-deficient colons. Consistent with the Zaki *et al.* group, this group also found increased activation of ERK in the colons. This further demonstrates that NLRP12 regulates not only noncanonical NF- κ B but also ERK signaling, and dysregulation of these pathways drives the colitogenic microenvironment that precedes cancer development. However, the role of autoinflammatory T cell responses had been previously overlooked in relation to colitis. NLRP12-deficient mice were found to have increased CD4⁺ and CD8⁺ T cell populations more skewed towards an inflammatory phenotype, producing increased IFN- γ and IL-17 upon restimulation (31). An adoptive transfer of CD4⁺CD45RB^{hi} T cells transferred from NLRP12-deficient mice to Rag1^{-/-} mice were more susceptible to colitis and modeled a more biologically relevant model for autoimmune-induced IBD (31). This model was characterized by a robust T cell population, with increased T cell counts accumulating in the spleen and production of pro-inflammatory IL-6, TNF- α , and GM-CSF. This work concludes by finding that NLRP12 regulates both canonical and noncanonical NF- κ B signaling, as NLRP12-deficient colon lysates had increased phosphorylation of I κ B α and processing of p100 to its active form p52. Unlike previous work, they did not observe altered ERK, p38, or STAT3 signaling in this model.

As the gut microflora is highly sensitive to perturbations and such fluctuations in microbial composition drives colonic inflammation, the role of NLRP12 in modulating the microbiome has also been investigated. Recent work found that NLRP12-deficient mice had colon inflammation at a resting state that is attributed to a genetically inherent dysbiotic microbiome lacking diversity (32). This microbiome was characterized by loss of commensal Lactospiraceae and enrichment by Erysipelotrichaceae, which is consistent with the microbiome of IBD patients. Interestingly, administration of the protective Lactospiraceae in colitis mice was found to ameliorate this condition and decrease levels of phosphorylated p65 (canonical NF- κ B), nuclear p52 (noncanonical NF- κ B), ERK, and STAT3 (32). Further work evaluating the microbiome of the NLRP12-deficient mice also found that this genetic predisposition confers susceptibility to obesity in a murine high-fat diet model with increased adipose deposition, blood glucose, and activation of NF- κ B and MAPK signaling (33). The translational relevancy of NLRP12-deficient mice to both a model of colitis and obesity is an intriguing finding.

Recent epidemiologic studies are revealing an association between changing environmental factors as obesity is often linked to Westernized diets, which may also alter the intestinal microbiome and contribute to IBD pathogenesis.

Clinical relevancy of NLRP12 to IBD has been previously reported in human IBD patients. IBD patients unresponsive to standard of care anti-TNF therapeutics have significantly downregulated *NLRP12* gene expression and reciprocally increased levels of noncanonical NF- κ B signaling, whereas these levels are restored in patients responsive to anti-TNF biologics (34). Although IBD pathogenesis has been oftentimes attributed to the canonical NF- κ B pathway, this work illuminates the involvement of the previously overlooked noncanonical NF- κ B pathway in human colitis. A recent case-study conducted in 2020 reported a homozygous p.Leu412Phe variant for the toll-like receptor 3 (*TLR3*) gene and a frameshift-causing deletion in the *NLRP12* gene that was predicted to contribute to this patient's presentation of concomitant HSV esophagitis and Crohn's disease (35). Likewise, miR-372 was found to be upregulated in blood samples and colonic mucosa collected from human ulcerative colitis patients (36). They also found a conserved miR-372 binding site in the 3' untranslated region of NLRP12 and speculated that this miRNA likely drives UC disease development by interacting with and suppressing NLRP12's activity (36). Consistently, a metadata analysis found decreased expression of *NLRP12* in UC patients, which also included 10 sets of monozygotic twins among the cohort (32). Together, work culminated from these studies highlights the involvement of NLRP12 in IBD pathogenesis. However, validation of NLRP12 as a biomarker and FDA-approved biologics targeting this protein and downstream pathways like NF- κ B, ERK, and STAT3 have yet to be fully evaluated.

Conclusion: NLR-targeted Therapeutics for IBD Treatment

To date, IBD treatments conventionally remedy symptoms through the administration of aminosalicylates, corticosteroids, immunomodulators, and biologics as a means of pharmaceutical intervention (37). Specifically, corticosteroids have been shown to mitigate proinflammatory signaling by reducing the recruitment of transcription factors such as NF- κ B to the nucleus (38, 39). Immunomodulators include thiopurines, methotrexate, calcineurin inhibitors, and JAK inhibitors (37). Anti-TNF biologics,

including Certolizumab, Infliximab and Adalimumab use monoclonal antibodies to inhibit TNF and subsequent downstream pro-inflammatory signaling (40-43). Although at first proving to be a promising biologic for IBD treatment by demonstrating long-term remission in patients, a major limitation to anti-TNF biologics emerged as a subset of the IBD population showed unresponsiveness or lose responsiveness to these drugs (34, 44). Work published by Nguyen *et al.* demonstrated that lack of responsiveness is associated with increased noncanonical NF- κ B signaling, including downregulated gene expression of its negative regulator NLRP12. Therefore, additional anti-biologics have undergone phase 2 and phase 3 clinical trials, such as anti-IL-12/23 inhibitors (45-47) and anti-integrin inhibitors (48-51). Other treatments in development include small molecule therapy of JAK inhibitors (52-54) and sphingosine-1-phosphate receptors (55, 56) are also being evaluated.

Aside from these more conventional pharmacological interventions, novel treatments are currently being investigated to improve the microenvironment of the diseased intestine. These treatments include administration of antibodies that target inflammatory cytokine receptors, such as IL-6R (21). Although promising in a murine model, its utility in human patients has yet to be fully elucidated. A second novel treatment option is the administration of antibiotics and probiotics to mitigate the dysbiosis (37). Fecal microbiota transfers have also been performed to substantially shift the gut microbiota and have been associated with clinical remission without the administration of corticosteroids in metadata analyses (37). Given that negative regulatory NLRs have been associated with PRR signaling, host-pathogen interactions, and gut dysbiosis, fecal microbiota transplants suggest a promising method to rescue a genetically inherent pro-inflammatory microbiota composition of the gut. However, the long-term efficacy of this treatment method has yet to be comprehensively investigated, suggesting that clinical remission may be short-lived. Another novel treatment method includes stem cell transplantation ranging from hematopoietic, mesenchymal, and intestinal stem cells to improve wound healing responses given inflammatory-mediated damage and defects in epithelial barrier integrity (37). Hematopoietic stem cell transplantation may also improve immunomodulatory responses by counteracting the overly robust autoimmune responses elicited by idiosyncratic immune cells deficient in

proper NLR signaling. Gene editing of these transplanted cells to restore proper NLR levels and maintain tightly regulated NF- κ B signaling may be a promising advancement in this therapeutic option.

To date, traditional treatment methods for IBD are limited in their long-term efficacy, while also skirmishing the potential they have to provide curative effects rather than remedying symptoms. In this review, we provide thorough insight to the evidenced role of the negative regulatory NLRs in IBD pathogenesis, while predicting its potential involvement given other studies that have further evaluated its molecular involvement in other diseases and conditions. As current pharmacological intervention has targeted pro-inflammatory signaling in a broader sense through the administration of anti-TNF and JAK inhibitors, negative regulatory NLRs upstream of NF- κ B signaling may prove to be a promising target with potential multifactorial effects.

CONFLICT OF INTERESTS:

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS:

H.A.M., B.T., A.R. and I.C.A. prepared figures and wrote the manuscript.

ACKNOWLEDGEMENTS:

The authors would like to thank Bettina Heid, Hannah Ivester, Khan Mohammad Imran, Juselyn Tupik, Tamalika Paul, Manali Powar, Madeline Mott, Benjamin Tintera, and Kacie Hoyt for their support and assistance with the generation of this manuscript. The Virginia Maryland College of Veterinary Medicine, The Virginia Tech Center for Drug Discovery, The Via College of Osteopathic Medicine, The Virginia Tech Carilion School of Medicine, and the Virginia Tech Institute for Critical Technology and Applied Science. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or any other funding body. The authors declare no competing financial interests.

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Figure Legends:

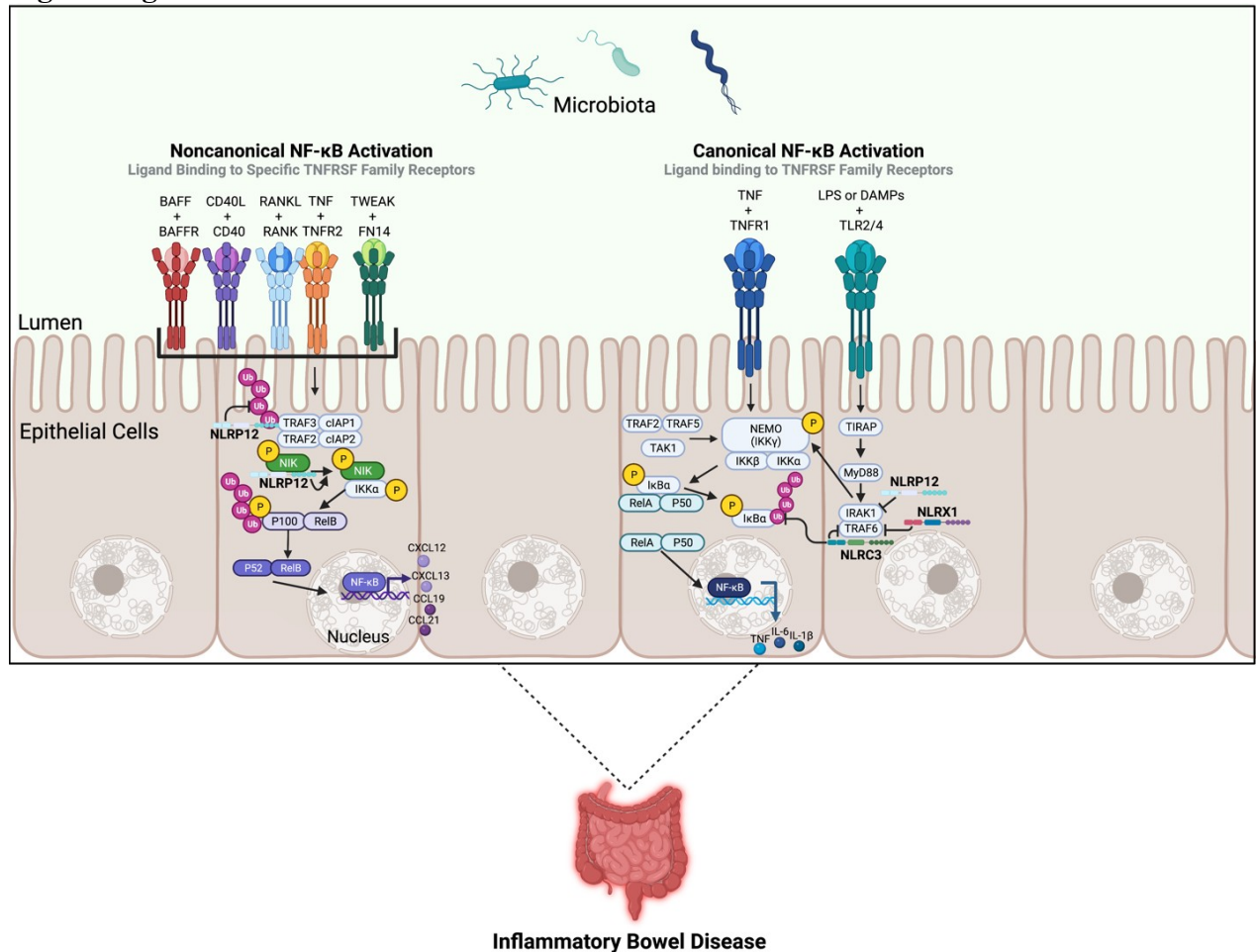


FIGURE 1: Regulation of canonical and noncanonical NF-κB signaling by negative regulatory NLRs. Activation of the NF-κB transcription factor occurs through canonical or noncanonical NF-κB signaling pathways. Negative regulatory NLRs, including NLRC3, NLRX1, and NLRP12, inhibit proper signal transduction of these pathways through protein interactions. NLRC3 and NLRX1 specifically dampen canonical NF-κB signaling by independently forming TRAFasome formations with TRAF6 to prevent its necessary auto-ubiquitination for further signal transduction. NLRP12 is demonstrated to inhibit canonical and noncanonical signaling. NLRP12 directly interacts with IRAK-1 to inhibiting its hyperphosphorylation and therefore preventing TLR2 and TLR4 signaling upstream of canonical NF-κB. NLRP12 also negatively regulates noncanonical NF-κB signaling in two manners: 1) by directly associating with NIK to promote its degradation or 2) by a TRAFasome formation between NIK and TRAF3 to promote degradation of NIK.

CHAPTER FIVE

Noncanonical NF- κ B Signaling Upregulation in Inflammatory Bowel Disease Patients is Associated with Loss of Response to Anti-TNF Agents

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ABSTRACT

Objectives: Targeting tumor necrosis factor (TNF) with biologic agents, such as infliximab and adalimumab, is a widely used and effective therapeutic strategy in inflammatory bowel disease (IBD). Unfortunately, a significant number of patients fail to respond or lose response over time to these agents. Previous studies have defined multiple complex roles for canonical NF- κ B signaling in the pathogenesis of IBD. However, preliminary evidence suggests that the lesser defined noncanonical NF- κ B signaling pathway also contributes to disease pathogenesis and response to anti-TNF agents. The objective of this study was to evaluate this hypothesis in Crohn's disease (CD) and ulcerative colitis (UC) patients.

Design: A total of 27 subjects with IBD (19 with CD and 8 with UC) and 15 control subjects were tested. Clinical criteria, patient history, and endoscopic disease activity were factors used to categorize patients and define therapeutic response. Biopsy specimens were collected during colonoscopy and expression was determined for 88 target genes known to be associated with noncanonical NF- κ B signaling and IBD.

Results: Noncanonical NF- κ B signaling was significantly upregulated in IBD patients and was associated with increased gastrointestinal inflammation, epithelial cell death, lymphocyte migration, and Nod-like receptor signaling. Furthermore, noncanonical NF- κ B signaling was further upregulated in patients unresponsive to anti-TNF agents and was suppressed in responsive patients. MAP3K14, NFKB2, CCL19, CXCL12, and CXCL13 were significantly dysregulated, as were genes that encode pathway regulators, such as CYLD, NLRP12, and BIRC2/3.

Conclusion: Our study identifies a previously uncharacterized role for the understudied noncanonical NF- κ B signaling pathway in the pathogenesis of IBD and anti-TNF therapy responsiveness. The genes and pathways identified may ultimately prove useful in IBD management and could potentially be used as biomarkers of drug response.

INTRODUCTION

Inflammatory bowel diseases (IBD) – Crohn's disease (CD) and ulcerative colitis (UC) - are characterized by chronic inflammation of the gastrointestinal tract, driven by elements of both the innate and adaptive immune systems in genetically susceptible

individuals. Together these diseases afflict approximately over 6 million people worldwide, which makes them a significant global health and economic burden (1). The nuclear factor kappa B (NF- κ B) family of transcription factors are master regulators of inflammation and drive a diverse spectrum of biological processes (2). This signaling cascade is commonly found dysregulated in IBD patients, leading to dysfunctional cytokine and chemokine production in the gastrointestinal tract. NF- κ B signaling occurs through two distinct pathways, defined as the canonical pathway and the noncanonical (or alternative) pathway.

In the context of IBD, the overwhelming majority of studies have focused on the canonical NF- κ B signaling cascade. This cascade relies on the proteins RelA (p65) and p50 which form the heterodimer RelA/p50. Upon activation of this pathway, this heterodimer translocates into the nucleus, where it functions as a transcription factor. In this pathway, signaling is rapid and constitutive. Activation of the cascade results in the transcription of a wide range of well characterized inflammatory mediators such as IL-1 β , TNF, and IL-6 (3). TNF in particular plays a central role in the pathogenesis of IBD (4) as shown by the clinical efficacy of anti-TNF medications such as infliximab (IFX) or adalimumab (ADA)-mainstay therapies for both CD and UC. These therapies have been shown to reduce the risk of hospitalization, surgery, and improve quality of life (5). In addition, they have been shown to prevent CD relapse after surgery (6). However, up to 40% of IBD patients do not respond to initial treatment with anti-TNF agents [primary nonresponse (PNR)], and a similar proportion of patients lose response over time [secondary loss of response (SLR)] after initially achieving clinical remission (7, 8). The mechanism of PNR and SLR to anti-TNF agents is not completely understood and likely to be multifactorial. Several explanations have been suggested, including the immune system switch to an alternative inflammatory pathway, immunogenicity with production of drug neutralizing antibodies and medical unresponsiveness due to advanced disease (9-12). While one mechanism does not exclude others and they might all play a role in different circumstances, the possibility of alternative pathways driving inflammation in IBD may provide insight into why medications targeting molecules different from TNF might be effective in SLR and PNR.

Unlike the canonical pathway, there is minimal data on the role of this signaling cascade in IBD. Noncanonical NF- κ B signaling is highly regulated at the post-transcriptional and post-translational level, resulting in a slower, more controlled, and chronic signaling response. The noncanonical pathway relies on a heterodimer different from that of the canonical pathway and is comprised of RelB and 100, which is later cleaved into its active form of RelB and p52 (13). In the noncanonical signaling cascade, NF- κ B initiates the transcription of a limited repertoire of target genes, including those coding for the chemokines CXCL12, CXCL13, CCL19, and CCL21 (**Supplementary Figure S1**). Prior research from our group using a pre-clinical mouse model found that mice lacking negative regulators of noncanonical NF- κ B signaling - such as the Nod-like receptor NLRP12-demonstrated increased noncanonical pathway activation and susceptibility to models of IBD and inflammation-driven colon tumorigenesis (14). These findings suggest a role of noncanonical NF- κ B signaling in IBD pathogenesis. In this study, we examine the hypothesis that this pathway may be involved in anti-TNF agents response in IBD patients.

MATERIALS AND METHODS

Protection of Human Subjects

All studies were conducted following the regulations, policies, and guidelines set forth by the National Institutes of Health for research involving human subjects. All studies were conducted under the approval of the Institutional Review Board of Carilion Clinic and Virginia Tech Carilion School of Medicine.

Patient Selection

A total of 27 established IBD patients (19 with CD and 8 with UC) and 15 control subjects were prospectively recruited from the IBD Center at Carilion Clinic from 2015 to 2018. Patients were ≥ 18 years of age, without other gastrointestinal or systemic disorders, without recent NSAIDs use, and scheduled for colonoscopy for clinical indications. Pregnant patients were excluded.

Sample Collection

Tissue samples from each patient were obtained through biopsies of both visible lesions and non-affected areas in the colon and/or the terminal ileum. Tissue samples were stored in RNAlater at -80°C . Patients were separated into different groups based on diagnosis

(CD or UC), type of medication use, and response to therapy. Tissue from controls was obtained from patients undergoing colonoscopy for reasons other than IBD (e.g., rectal bleeding) and included in the study only after endoscopy and histology proved to be normal.

Data Collection

Clinical data were abstracted from the patient medical charts. Demographics variables collected included age, gender, and smoking status. For IBD patients, disease diagnosis, and phenotype were determined at the time of study enrollment based on the Montreal disease classification (15). IBD medications and response to anti-TNF agents were recorded. Anti-TNF agents consisted of IFX and ADA in this patient cohort. Loss of response to anti-TNF therapy was classified as PNR or SLR. PNR was defined as complete lack of response to initiation of anti-TNF therapy whereas patients with SLR were those who initially responded to an anti-TNF agent - at least through the induction phase - but subsequently lost response. Clinical symptoms, inflammatory markers, imaging, and/or endoscopy findings were used to determine response.

Tissue Processing and Data Analysis

Biopsy samples were removed from RNAlater, finely minced, and homogenized in RLT buffer (Qiagen) with 2-mercaptoethanol. Tissues were processed using an AllPrep Kit (Qiagen) following the manufacturer's protocol. RNA was analyzed for quality and concentration using a NanoDrop™. Pools were made using equal concentrations of RNA from each patient sample for each experimental group. Patient samples were assigned to pools randomly and at least three pools were generated for each experimental group. The total amount of RNA used for each cDNA reaction was 600 ng and cDNA was generated using a First Strand Kit (Qiagen) following manufacturer's protocols. Following cDNA preparation, gene expression was evaluated using a custom Qiagen RT2 Superarray following manufacturer's protocol. A list of 88 candidate genes for the custom Superarray platform is provided in **Supplementary Table 1**. For each array, gene expression was determined utilizing the $\Delta\Delta C_t$ method, using a panel of five housekeeping genes and internal controls provided on each array. Gene expression was determined using a 7,500 Fast Block Real-Time PCR System (ABI). Data was analyzed

using SA Biosciences Data Analysis Center and Ingenuity Pathway Analysis (IPA). IPA images were then generated using BioRender.

In addition to the analysis of pooled samples, quantitative rtPCR was also performed in select genes of interest for each individual specimen. Complimentary DNA was generated from each specimen using an ABI High Capacity cDNA kit, in accordance with the manufacturer's protocol and 1µg amplified using the Taqman-based rtPCR platform (ThermoFisher) on the ABI system. Gene expression was determined using the $\Delta\Delta C_t$ method. All data were normalized to 18s and the fold change in gene expression was determined.

Statistical Analysis

For gene expression analysis, data was analyzed using GraphPad Prism, version 6 (GraphPad Software, Inc., San Diego, CA). Student's two-tailed t test was used for comparison between experimental groups. Multiple comparisons were conducted using one-way and two-way ANOVA where appropriate followed by Tukey post-test for multiple pairwise examinations. Correlation was computed using GraphPad Prism. Changes were identified as statistically significant if p-value was less than 0.05. Mean values were reported together with the SEM. The number of patients were determined based on a power analysis conducted using findings from a retrospective metadata analysis of publicly accessible gene expression data from dataset GSE16879. Gene expression was determined for a single marker of noncanonical NF- κ B signaling (*CXCL13*) and the average and standard deviation of expression for this gene was used to determine the number of patients to target in the clinical study presented here.

RESULTS

Noncanonical NF- κ B is Upregulated in IBD

Tissue samples were obtained from 27 IBD patients and 15 controls. In the IBD group, 19 patients had CD and eight patients had UC (**Table 1**). The mean age of IBD patients was 41 years, 44% were females and 15% were active smokers. Of this patient pool, 33% of IBD patients were anti-TNF responders while 44% were not on medications or were taking medications other than anti-TNF agents. All anti-TNF non-responders in this study experienced SLR. The majority of CD and UC patients had ileocolonic and extensive colonic involvement respectively.

First, we examined the association of the noncanonical NF- κ B pathway in IBD patients compared to controls. We found a significant upregulation of 39 genes and downregulation of three genes associated with noncanonical NF- κ B signaling in the pooled untreated CD patients (**Figure 1A**). These dysregulated genes encode for proteins with crucial roles in the signaling cascade. We also conducted individual real-time PCR analysis on a selection of these genes to validate the findings of the gene expression arrays. Individual qPCR was conducted on five genes that were significantly dysregulated in the custom gene expression arrays and are critical points in the noncanonical NF- κ B signaling pathway (Supplementary Figure S1). *TNF* is one of the activating molecules; NIK is a critical kinase required for perpetuation of this activating signal; and *CXCL12*, *CXCL13*, and *CXCR4* are products of the signaling pathway. The individual gene expression was consistent with custom arrays: *TNF*, *MAP3K14* (NIK), *CXCL12*, *CXCL13*, and *CXCR4* genes were all significantly upregulated in biopsies collected from inflamed areas compared to biopsies collected from non-inflamed areas in the same individual patients (**Figure 1B**).

Noncanonical NF- κ B Signaling is Downregulated in Anti-TNF Responsive Patients

We then examined the role of the noncanonical NF- κ B pathway in IBD patients in relation to their responsiveness to anti-TNF agents. Compared to controls, in IBD patients not responding to anti-TNF therapy, we identified 42 upregulated genes and only two downregulated genes (**Figure 2A**). Notably, *CXCL13* was significantly upregulated (+18.77 fold) in nonresponders.

Conversely, in IBD patients responding to anti-TNF agents we identified 20 upregulated genes and seven downregulated genes involved in noncanonical NF- κ B signaling (Figure 2B). Upregulated genes included *NLRP12*, a negative regulator of noncanonical signaling, while downregulated genes included noncanonical chemokine *CXCL13* and noncanonical receptor for *CXCR4*. Pathway analysis revealed significant downregulation of noncanonical NF- κ B signaling in IBD patients responsive to anti-TNF α therapy compared to untreated IBD patients and IBD patients treated with other (non-anti-TNF) medications (**Figures 2C–F**). This decreased expression was observed in several genes throughout the signaling pathway, and specifically included reduced expression of *MAP3K14*, *NFKB2*, *TRAF3*, *STAT3*, *CXCL12*, *CXCL13*, *CXCR4*, and

CCR7 that were not observed in the other IBD patient groups. Interestingly, in IBD patients unresponsive to anti-TNF therapy, noncanonical NF- κ B signaling was upregulated compared to untreated IBD patients. In these patients, upregulation of noncanonical NF- κ B signaling was strongly associated with the downregulation of *NLRP12* and upregulation of *MAP3K14*, *CXCL12*, and *CXCL13*. Together, these data demonstrate that noncanonical NF- κ B signaling was significantly decreased in anti-TNF responders and increased in anti-TNF nonresponders.

CXCL12 and CXCL13 Expression is Correlated to Anti-TNF Responsiveness

Currently, only four major chemokines have been associated with the noncanonical NF- κ B signaling cascade, *CCL19*, *CCL21*, *CXCL12*, and *CXCL13*. We examined these chemokine expression patterns in relation to anti-TNF responsiveness. In untreated IBD patients and in those treated with other (non-anti- TNF) therapies, *CCL19*, *CCL21*, *CXCL12*, and *CXCL13* expression were upregulated. IBD patients responding to anti- TNF therapy demonstrated a significant decrease in *CXCL12*, *CXCL13*, and *CCL21* compared to those in the untreated IBD group (**Figure 3A**). By contrast, *CXCL12* and *CXCL13* were significantly upregulated in IBD patients not responding to anti- TNF therapy while *CCL19* and *CCL21* were downregulated in these patients. Both *CXCL12* and *CXCL13* were strongly correlated with anti-TNF responsiveness, whereas *CCL19* and *CCL21* only demonstrated a weak correlation. Based on the expression profile associated with these chemokines, it appears that migration of naïve lymphocytes was the signaling network most strongly altered in patients not responding to anti-TNF agents (**Figure 3B**). Hence, by contrast with nonresponders, in IBD patients responding to anti-TNF therapy, the downregulation of *CXCL12* and *CXCL13* could result in reduced migration of lymphocytes to inflamed tissues.

DISCUSSION

Anti-TNF therapy acting through the canonical NF- κ B signaling pathway has been the cornerstone of IBD treatment for the past several years. Despite its success, a significant proportion of IBD patients may not respond or lose response over time to anti-TNF therapy. This population of anti-TNF nonresponders has an increased risk of disease complications but might respond to newer biologic medications such as ustekinumab or vedolizumab (16, 17). The mechanisms of PNR and SLR to anti-TNF agents are poorly

understood. One possible explanation is that the driving mechanism of inflammation in these IBD patients may have shifted to an alternative signaling pathway.

The noncanonical NF- κ B pathway has been recognized as a key regulator and promoter of the adaptive immune system, particularly in the development of secondary lymphoid organs. Prior studies from our group have demonstrated the role of this signaling pathway in mouse models of colon inflammation and eosinophilic esophagitis (14, 18).

In this study, we found significant upregulation of the genes involved in the noncanonical NF- κ B pathway in IBD patients compared to healthy controls. Furthermore, the main chemokines in this pathway, namely *CXCL12* and *CXCL13*, were significantly downregulated in IBD patients who responded to anti-TNF agents and were upregulated in nonresponders. These findings suggest that the activation of the noncanonical NF- κ B signaling pathway may contribute to the mechanism underlying the lack/loss of response to anti-TNF therapy. The upregulated genes in the noncanonical NF- κ B pathway involved in lack/loss of response to anti-TNF therapy may involve leukocyte migration to the affected tissue as predicted by IPA analysis.

The relatively small sample size and single center nature are limitations of our study. In addition, it is unclear how many of our anti-TNF nonresponders would respond to additional anti-TNF agents (19, 20). Whether the expression pattern of the noncanonical genes differs in patients who respond to a second anti-TNF agent compared to those who do not respond to any anti-TNF medication remains unknown. Also, our study does not clarify the role of this pathway in PNR – since all of our patients experienced SLR.

In conclusion, this is the first study in humans that has explored the role of the noncanonical NF- κ B signaling in the pathogenesis of IBD. More importantly, our findings provide a potential mechanistic insight into the lack/loss of response to anti-TNF agents in IBD. If our observations are confirmed, targeting the noncanonical NF- κ B signaling pathway could significantly impact IBD management and therapy outcomes.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept an article that does not adhere to our open data policies.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB Carilion Clinic. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VN was responsible for investigation and writing (review and editing); HM, KE, KK, SS, RB, and DG were responsible for investigation, DS and IA were responsible for conceptualization, investigation, methodology, formal analysis, supervision, and writing (initial draft, review and editing).

FUNDING

Carilion Research Advancement Program (RAP) Grant 13 Role of the Non-Canonical NF- κ B Inflammatory Cascade in Therapeutic Response and Pathogenesis of IBD.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at:

<https://www.frontiersin.org/articles/10.3389/fphar.2021.655887/full#supplementary-material>

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TABLES

Table 1. Patient characteristics.

	IBD	Controls
N	27	15
Age (years)	41 ± 15	55 ± 14
Female	12 (44%)	6 (40%)
Smoker	4 (15%)	2 (13%)
CD	19 (70%)	
CD extent		
L1 (ileal)	5 (26%)	
L2 (colonic)	7 (37%)	
L3 (ileocolonic)	7 (37%)	
UC extent		
E1 (rectum)	2 (25%)	
E2 (left sided)	2 (25%)	
E3 (extensive)	4 (50%)	
IBD medications		
Anti-TNF agents	15 (56%)	
Non-anti-TNF therapies	10 (37%)	
No therapy	2 (7%)	
Anti-TNF responsive	5/15 (33%)	

Age is reported as mean ± SD.

FIGURE LEGENDS

Figure 1

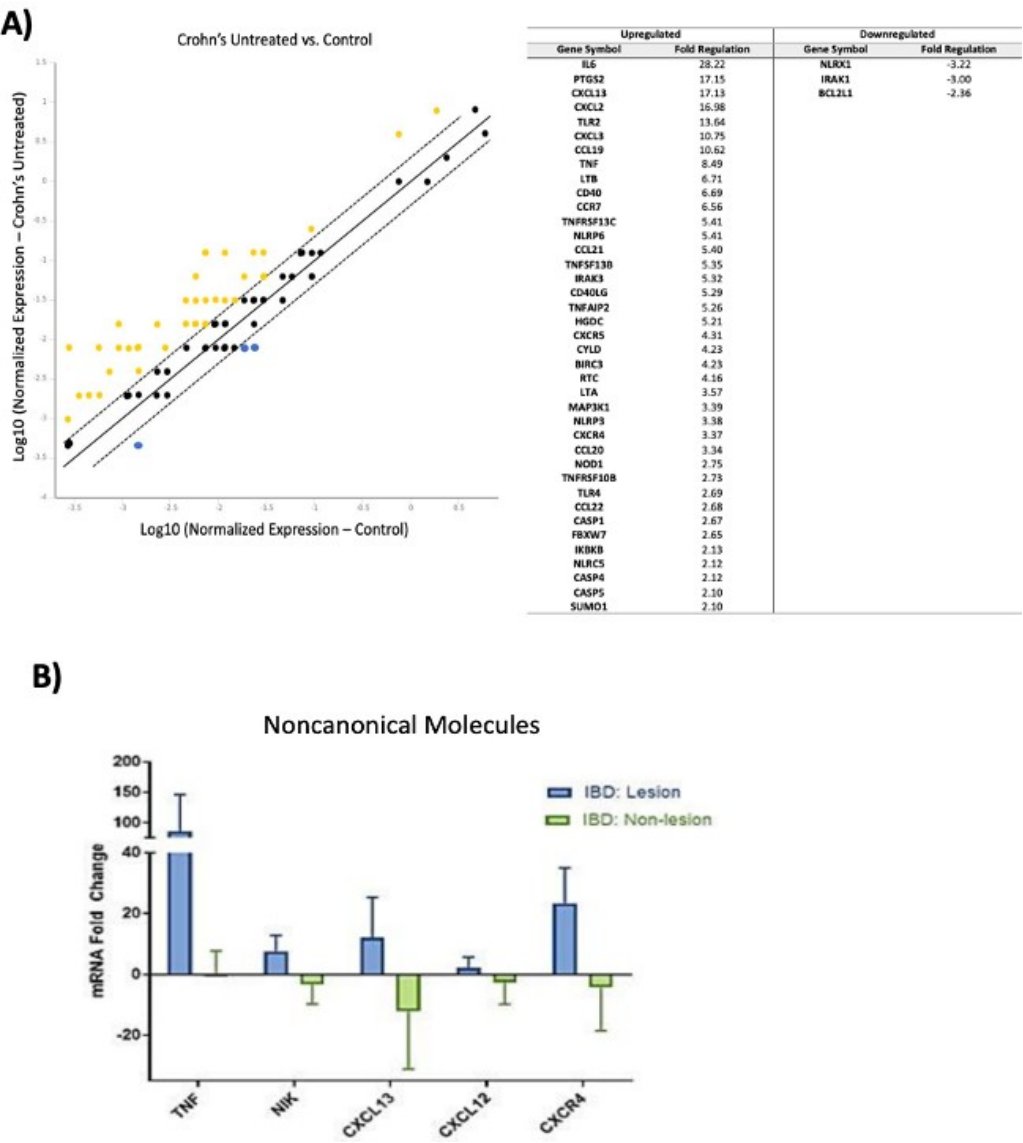


Figure 1. Noncanonical NF- κ B Signaling is Upregulated in Inflammatory Bowel

Disease Patients (A) Left: Compared to gene expression from intestinal tissues of controls (n=15, 3 pools), 39 genes related to noncanonical NF- κ B pathway were upregulated in intestinal tissues obtained from untreated CD patients (n=2, 1 pool) with yellow indicating upregulation and blue indicating downregulation (lines represent 1, 0, and -1 fold change; genes outside of dashed lines are considered significantly dysregulated). Housekeeping genes used for normalization include *ACTB*, *B2M*, and *GADPH*; Right: list of 39 upregulated genes and three downregulated genes and fold change values. **(B)**. Using real-time PCR of individual patient samples, the main noncanonical NF- κ B pathway components *TNF*, *NIK*, *CXCL12*, *CXCL13*, and *CXCR4* were found to be upregulated in inflamed areas compared to non-inflamed areas obtained from the same individual patients (inflamed lesions n=4; non-inflamed tissue n=4).

Figure 2

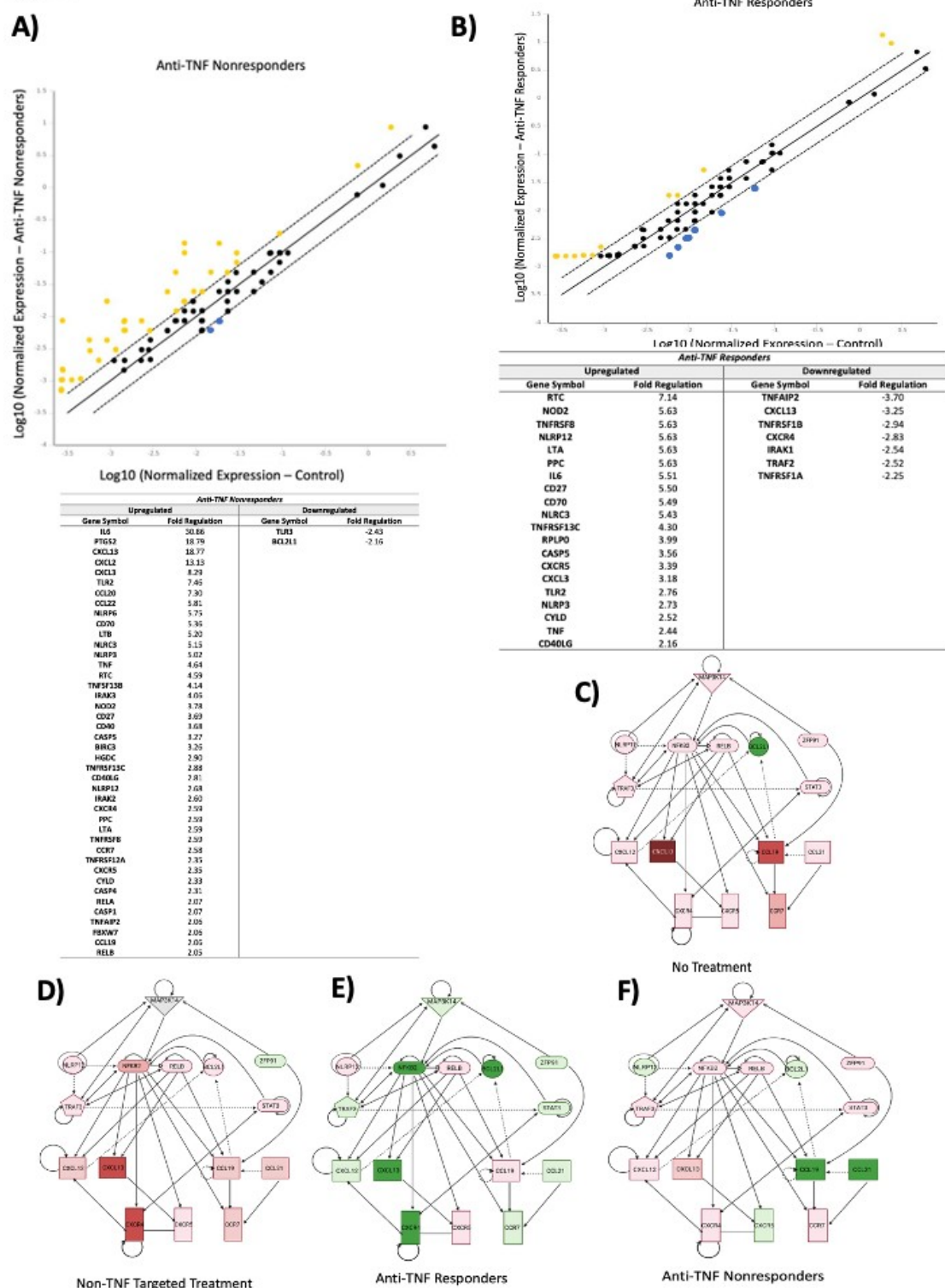


Figure 2. Noncanonical NF- κ B is Downregulated in IBD Patients Responding to Anti-TNF Therapy. (A): Inflamed tissues from anti-TNF nonresponders (n=10, two pools, CD patients and UC patients) showed 39 upregulated genes related to the noncanonical NF- κ B signaling, compared to only 19 genes in anti-TNF responders (n=5, two pools) (B), with yellow indicating upregulation and blue indicating downregulation (lines represent 1, 0, and -1 fold change; genes outside of dashed lines are considered significantly dysregulated). A list of the involved genes is provided next to each graph. Housekeeping genes used for normalization include *ACTB*, *B2M*, and *GADPH* (C–F): Ingenuity Pathway Analysis revealed that noncanonical NF- κ B was a significant signaling hub that was downregulated in patients responding to anti-TNF therapy. Expression levels of the main noncanonical genes NIK (*MAP3K14*), *CXCL12*, *CXCL13*, and *CCL21* were upregulated in both untreated patients (n=2, one pool) (C) and patients treated with non-anti-TNF medications (n=10, two pools). (D) However, anti-TNF responders (n=5, two pools) (E), showed a distinct attenuation of expression of these genes. Meanwhile, anti-TNF non-responders showed upregulation of *CXCL12* and *CXCL13* and downregulation of *CCL21*. Note: green represents downregulation; red represents upregulation.

Figure 3

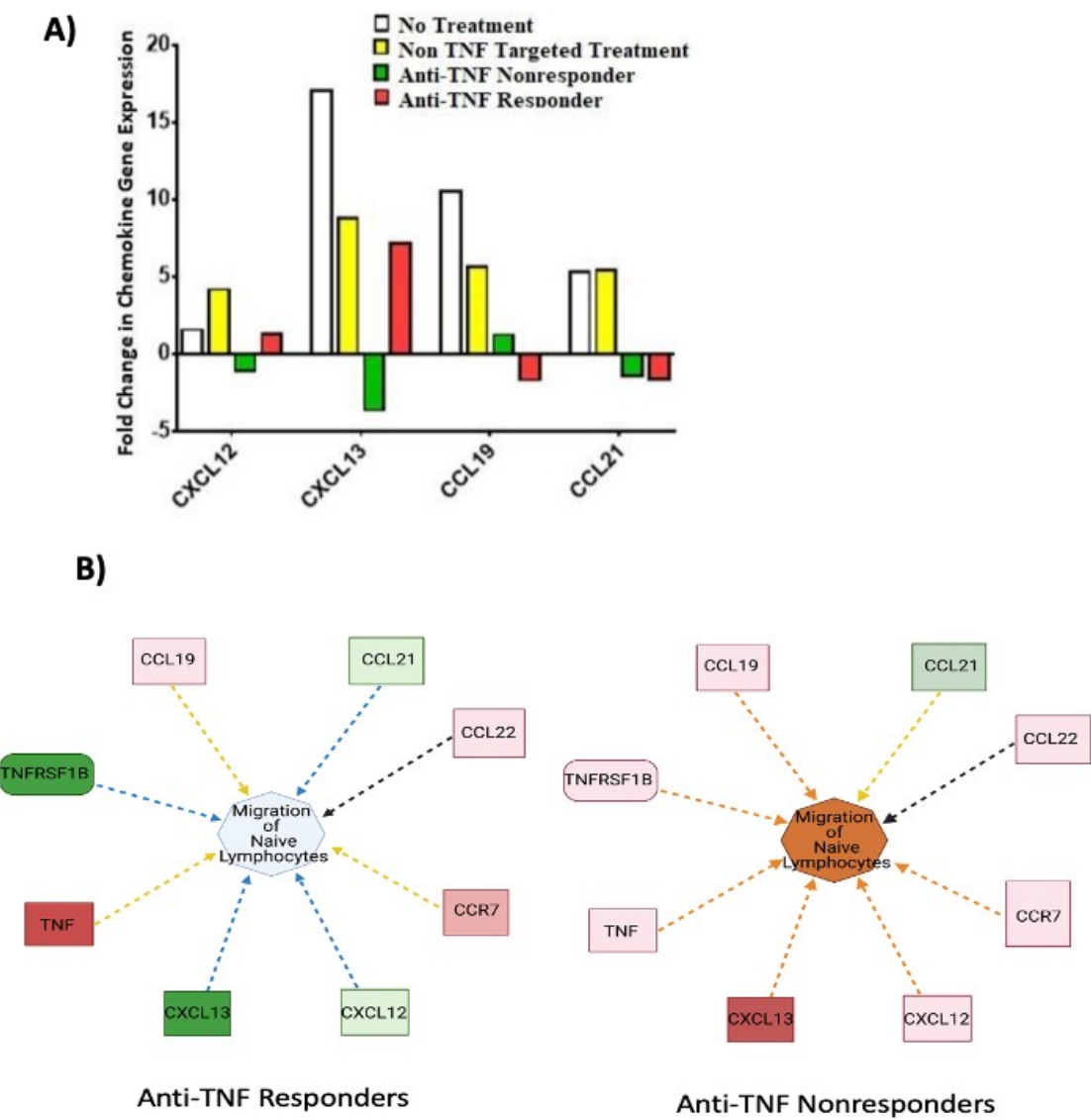
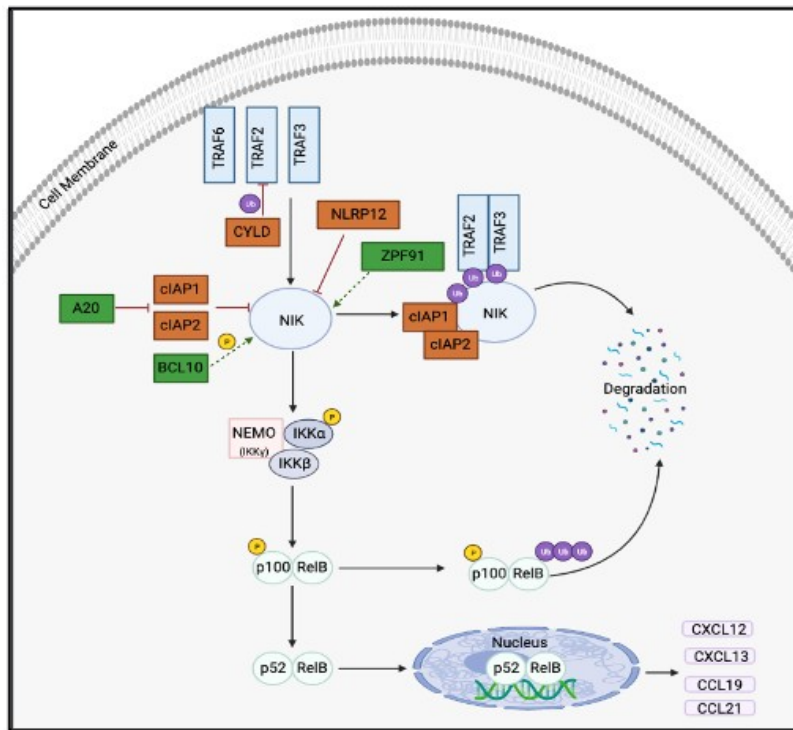


Figure 3. *CXCL12* and *CXCL13* are Downregulated in IBD Patients Responding to Anti-TNF α Therapy. (A) *CXCL12*, *CXCL13*, *CCL19*, and *CCL21* were significantly upregulated in both untreated IBD patients (n=2, one pool) and IBD patients treated with non-anti-TNF medications (n=10, two pools). *CXCL12*, *CXCL13*, and *CCL19* displayed opposite expression profiles based on anti-TNF responsiveness, with upregulation in anti-TNF nonresponders (n=10, two pools) and downregulation in responders (n=5, two pools) for *CXCL12* and *CXCL13*. *CCL21* remained downregulated regardless of response to anti-TNF agents. (B) Predicted effect on the migration of naïve lymphocytes to inflamed tissue based on changes of the noncanonical chemokines which are involved in lymphocyte trafficking. In anti-TNF responders, lymphocyte trafficking is reduced. By contrast anti-TNF nonresponders demonstrate increased lymphocyte trafficking. In general, the increase in noncanonical signaling molecules results in a large increase in predicted lymphocyte migration. Note: Blue and orange dashed lines with arrows indicate indirect inhibition and activation, respectively. Yellow and black dashed lines with arrows depict inconsistent effects and no prediction, respectively.

SUPPLEMENTARY MATERIAL

Supplemental Figure 1



Supplementary Figure 1. Schematic Illustration of Key Components of Noncanonical NF- κ B Signaling Significantly Dysregulated in IBD Patients. Our data revealed a significant increase in chemokines associated with noncanonical NF- κ B signaling, including CXCL12, CXCL13, CCL9, and CCL21. Additional pathway analysis revealed that these changes were associated with increased expression of genes encoding a variety of proteins that regulate the non-canonical NF- κ B signaling pathway through modulation of NIK, including the up-regulation of cIAP1, cIAP2, CYLD, and NLRP12 and the down-regulation of BCL10, ZPF91, and A20. Created with BioRender.com.

Supplementary Table 1: Custom Gene Array to Evaluate Noncanonical NF- κ B Signaling in IBD Patients. The gene list for the custom superarray platform is provided with RefSeq Number and Gene Symbol. These 88 gene candidates serve a role in the noncanonical NF- κ B pathway and dysregulated transcription levels may have implications on IBD pathogenesis and drug responsiveness. For each array, gene expression was determined utilizing the delta-deltaCt method, using a panel of five housekeeping genes and internal controls provided on each array.

Well	GeneBank	Symbol	Description	Gene Name
A1	NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1	AKT, CWS6, PKB, PKB-ALPHA, PRKBA, RAC, RAC-ALPHA
A2	NM_003921	BCL10	B-cell CLL/lymphoma 10	CLAP; mE10; CIPER; IMD37; c-E10; CARMEN
A3	NM_000633	BCL2	B-cell CLL/lymphoma 2	Bcl-2; PPP1R50
A4	NM_001191	BCL2L1	BCL2 like 1	BCLX; BCL2L; Bcl-X; PPP1R52; BCL-XL/S
A5	NM_001166	BIRC2	Baculoviral IAP repeat containing 2	API1, HIAP2, Hiap-2, MIHB, RNF48, c-IAP1, ciAP1
A6	NM_001165	BIRC3	Baculoviral IAP repeat containing 3	AIP1, API2, CIAP2, HAIP1, HIAP1, MALT2, MIHC, RNF49, c-IAP2
A7	NM_033292	CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	ICE, IL1BC, P45
A8	NM_001225	CASP4	Caspase 4 (human homolog of mouse casp11)	ICE(rel)II, ICEREL-II, ICH-2, Mih1/TX, TX
A9	NM_001136109	CASP5	Caspase 5, human homology of mouse casp11)	ICH-3; ICEREL-III; ICE(rel)III
A10	NM_006274	CCL19	C-C motif chemokine 19	ELC; CKb11; MIP3B; MIP-3b; SCYA19
A11	NM_004591	CCL20	Chemokine (C-C motif) ligand 20	CKb4, Exodus, LARC, MIP-3-alpha, MIP-3a, MIP3A, SCYA20, ST38
A12	NM_002989	CCL21	chemokine (C-C motif) ligand 21	ECL; SLC; CKb9; TCA4; 6Ckine; SCYA21
B1	NM_002990	CCL22	Chemokine (C-C motif) ligand 22	A-152E5.1, ABCD-1, DC, B-CK, MDC, SCYA22, STCP-1

Well	GeneBank	Symbol	Description	Gene Name
B2	NM_002985	CCL5	Chemokine (C-C motif) ligand 5	D17S136E, RANTES, SCYA5, SIS-delta, SISd, TCP228, eoCP
B3	NM_001301714	CCR7	chemokine (C-C motif) receptor 7	BLR2; EBI1; CCR-7; CD197; CDw197; CMKBR7; CC-CKR-7
B4	NM_001242	CD27	CD27 molecule	T14; S152; Tp55; TNFRSF7; S152. LPFS2
B5	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5	Bp50, CDW40, TNFRSF5, p50
B6	NM_000074	CD40LG	CD40 ligand	IGM; IMD3; TRAP; gp39; CD154; CD40L; HIGM1; T-BAM; TNFSF5; hCD40L
B7	NM_001252	CD70	CD70 molecule, tumor necrosis factor (ligand) superfamily, member 7	CD27L; CD27LG; TNFSF7; TNLG8A
B8	NM_001278	CHUK	Conserved helix-loop-helix ubiquitous kinase	IKBKA, IKK-alpha, IKK1, IKKA, NFKBIKA, TCF16
B9	NM_000609	CXCL12	chemokine (C-X-C motif) ligand 12	IRH; PBSF; SDF1; TLSF; TPARI; SCYB12
B10	NM_001142523	IRAK3	interleukin 1 receptor associated kinase 3	ASRT5; IRAKM
B11	NM_006419	CXCL13	chemokine (C-X-C motif) ligand 13	BLC; BCA1; ANGIE; BCA-1; BLR1L; ANGIE2; SCYB13
B12	NM_001114182	IRAK4	interleukin 1 receptor associated kinase 4	IPD1; REN64; IRAK-4; NY-REN-64
C1	NM_002089	CXCL2	Chemokine (C-X-C motif) ligand 2	CINC-2a, GRO2, GROb, MGSA-b, MIP-2a, MIP2, MIP2A, SCYB2
C2	NM_002090	CXCL3	Chemokine (C-X-C motif) ligand 3	CINC-2b, GRO3, GROg, MIP-2b, MIP2B, SCYB3

Well	GeneBank	Symbol	Description	Gene Name
C3	NM_001008540	CXCR4	Chemokine (C-X-C motif) receptor 4	FB22; HM89; LAP3; LCR1; NPYR; WHIM; CD184; LAP-3; LESTR; NPY3R; NPYRL; WHIMS; HSY3RR; NPY3R; D2S201E. Receptor for cxcl12/13
C4	NM_001716	CXCR5	Chemokine (C-X-C motif) receptor 5	BLR1; CD185; MDR15. Receptor for cxcl12/13
C5	NM_001042355	CYLD	Cylindromatosis (turban tumor syndrome)	EAC; MFT; SBS; TEM; BRSS; CDMT; MFT1; CYLD1; CYLDI; USPL2
C6	NM_005228	EGFR	Epidermal growth factor receptor	ERBB, ERBB1, HER1, NISBD2, PIG61, mENA
C7	NM_001013415	FBXW7	F-box and WD repeat domain containing 7	AGO; CDC4; FBW6; FBW7; hAgo; FBX30; FBXW6; SEL10; hCdc4; FBXO30; SEL-10
C8	NM_006597	HSPA8	Heat shock cognate protein 70	Hsc70 or Hsp73, HSPA8
C9	NM_001556	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	IKK-beta, IKK2, IKBK, IMD15, NFKBIKB
C10	NM_003639	IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	AMCBX1, FIP-3, FIP3, Fip3p, IKK-gamma, IMD33, IP, IP1, IP2, IPD2, NEMO, ZC2HC9
C11	NM_000600	IL6	Interleukin 6 (interferon, beta 2)	BSF2, HGF, HSF, IFNB2, IL-6
C12	NM_001569	IRAK1	Interleukin-1 receptor-associated kinase 1	IRAK, pelle
D1	NM_001570	IRAK2	Interleukin-1 receptor-associated kinase 2	IRAK-2

Well	GeneBank	Symbol	Description	Gene Name
D2	NM_000595	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	LT, TNFB, TNFSF1
D3	NM_002341	LTB	Lymphotoxin beta (TNF superfamily, member 3)	TNFC, TNFSF3, p33
D4	NM_002342	LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)	CD18, D12S370, LT-BETA-R, TNF-R-III, TNFCR, TNFR-RP, TNFR2-RP, TNFR3, TNFRSF3
D5	NM_173844	MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1	IMD12, MLT, MLT1
D6	NM_005921	MAP3K1	Mitogen-activated protein kinase kinase kinase 1	MAPKKK1, MEKK, MEKK 1, MEKK1, SRXY6
D7	NM_003954	MAP3K14	mitogen-activated protein kinase kinase kinase 14	NIK
D8	NM_002468	MYD88	Myeloid differentiation primary response gene (88)	MYD88D
D9	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	EBP-1, KBF1, NF-kB1, NF-kappa-B, NF-kappaB, NFKB-p105, NFKB-p50, NFkappaB, p105, p50
D10	NM_002502	NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	CVID10, H2TF1, LYT-10, LYT10, NF-kB2, p100, p52
D11	NM_020529	NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	IKBA, MAD-3, NFKBI
D12	NM_004556	NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	IKBE
E1	NM_178844	NLRC3	NLR family, CARD domain containing 3	NOD3; CLR16.2
E2	NM_032206	NLRC5	NLR family, CARD domain containing 5	NOD4; NOD27; CLR16.1

Well	GeneBank	Symbol	Description	Gene Name
E3	NM_001033053	NLRP1	NLR family, pyrin domain containing 1	NAC; CARD7; CIDEA; NALP1; SLEV1; DEFCAP; PP1044; VAMAS1; CLR17.1; DEFCAP-L/S
E4	NM_001277126	NLRP12	NLR family, pyrin domain containing 12	RNO; PAN6; RNO2; FCAS2; NALP12; PYPAF7; CLR19.3
E5	NM_001079821	NLRP3	NLR family, pyrin domain containing 3	ALI; AVP; FCU; MWS; FCAS; CIAS1; FCAS1; NALP3; C1orf7; CLR1.1; PYPAF1; AGTAVPRL
E6	NM_001276700	NLRP6	NLR family, pyrin domain containing 6	AVR; NAVR; PAN3; NALP6; PYPAF5; CLR11.4; NAVR/AVR
E7	NM_001282143	NLRX1	NLR family member X1	NOD5; NOD9; NOD26; DLNB26; CLR11.3
E8	NM_006092	NOD1	Nucleotide-binding oligomerization domain containing 1	CARD4, CLR7.1, NLRC1
E9	NM_001293557	NOD2	nucleotide binding oligomerization domain containing 2	CD; ACUG; BLAU; IBD1; NLRC2; NOD2B; CARD15; CLR16.3; PSORAS1
E10	NM_000963	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	COX-2, COX2, GRIPGHS, PGG, HS, PGHS-2, PHS-2, hCox-2
E11	NM_002908	REL	V-rel reticuloendotheliosis viral oncogene homolog (avian)	C-Rel
E12	NM_021975	RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	NFKB3, p65
F1	NM_006509	RELB	V-rel reticuloendotheliosis viral oncogene homolog B	I-REL, IREL, REL-B

Well	GeneBank	Symbol	Description	Gene Name
F2	NM_003804	RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	RIP, RIP1
F3	NM_003150	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	ADMIO, APRF, HIES
F4	NM_001293197	STUB1	STIP1 homology and U-box containing protein 1, E3 ubiquitin protein ligase	CHIP
F5	NM_001005781	SUMO1	small ubiquitin-like modifier 1	DAP1; GMP1; PIC1; SMT3; UBL1; OFC10; SENP2; SMT3C; SMT3H3
F6	NM_003264	TLR2	Toll-like receptor 2	CD282, TIL4
F7	NM_003265	TLR3	Toll-like receptor 3	CD283, IIAE2
F8	NM_138554	TLR4	Toll-like receptor 4	ARMD10, CD284, TLR-4, TOLL
F9	NM_000594	TNF	Tumor necrosis factor	DIF, TNF-alpha, TNFA, TNFSF2
F10	NM_006291	TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	B94, EXOC3L3
F11	NM_006290	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	A20, OTUD7C, TNFAIP2
F12	NM_003844	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	APO2, CD261, DR4, TRAILR-1, TRAILR1
G1	NM_003842	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	CD262, DR5, KILLER, KILLER, DR5, TRAIL-R2, TRAILR2, TRICK2, TRICK2A, TRICK2B, TRICKB, ZTNFR9
G2	NM_003839	TNFRSF11A	Tumor necrosis factor receptor superfamily member 11a	RANK
G3	NM_016639	TNFRSF12A	Tumor necrosis factor receptor superfamily member 12A	FN14

Well	GeneBank	Symbol	Description	Gene Name
G4	NM_052945	TNFRSF13C	Tumor necrosis factor receptor superfamily member 13C	BAFFR
G5	NM_001065	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	CD120a, FPF, MS5, TBP1, TNF-R, TNF-R-I, TNF-R55, TNFAR, TNFR1, TNFR1-d2, TNFR55, TNFR60, p55, p55-R, p60
G6	NM_001066	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	
G7	NM_001243	TNFRSF8	tumor necrosis factor receptor superfamily member 8	CD30; Ki-1; D1S166E
G8	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	APO2L, Apo-2L, CD253, TL2, TRAIL
G9	NM_001145645	TNFSF13B	Tumor necrosis factor superfamily member 13b	BAFF
G10	NM_003789	TRADD	TNFRSF1A-associated via death domain	
G11	NM_021138	TRAF2	TNF receptor-associated factor 2	MGC:45012, TRAP, TRAP3
G12	NM_003300	TRAF3	TNF receptor-associated factor 3	CAP-1, CAP1, CD40bp, CRAF1, IIAE5, LAP1
H1	NM_004620	TRAF6	TNF receptor-associated factor 6	MGC:3310, RNF85
H2	NM_003809	TNFSF12	tumor necrosis factor superfamily member 12	TWEAK
H3	NM_001167	XIAP	X-linked inhibitor of apoptosis	API3, BIRC4, IAP-3, ILP1, MIHA, XLP2, hIAP-3, hIAP3
H4	NM_001197051	ZFP91	zinc finger protein 91	Associates with NIK, causes stabilization and activation of NIK. PZF; DMS-8; DSM-8; FKSG11; ZFP-91; ZNF757

Well	GeneBank	Symbol	Description	Gene Name
H5	NM_001101	ACTB	Actin, beta	BRWS1, PS1TP5BP1
H6	NM_004048	B2M	Beta-2-microglobulin	-
H7	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD, GAPD, HEL-S-162eP
H8	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT, HPRT
H9	NM_001002	RPLP0	Ribosomal protein, large, P0	L10E, LP0, P0, PRLP0, RPP0
H10	SA_00105	HGDC	Human Genomic DNA Contamination	HIGX1A
H11	SA_00104	RTC	Reverse Transcription Control	RTC
H12	SA_00103	PPC	Positive PCR Control	PPC

CHAPTER SIX

NF- κ B Inducing Kinase Attenuates Colorectal Cancer by Regulating Noncanonical NF- κ B Mediated Intestinal Epithelial Cell Regeneration

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ABSTRACT:

BACKGROUND & AIMS: Increased proliferative capacity within the intestinal epithelial cell (IEC) compartment drives tumorigenesis in colorectal cancer and is associated with impaired stem cell regulation within the crypt. We demonstrate that NF- κ B-inducing kinase (NIK) and subsequent noncanonical NF- κ B signaling mitigates colitis-associated colorectal cancer by maintaining the regeneration/differentiation signaling axis within IECs.

METHODS: Inflammation-induced tumorigenesis was induced in conditional-knockout mice with IEC-specific NIK deletion following chemical administration of azoxymethane and dextran sulfate sodium (AOM/DSS). Further mechanistic studies evaluated crypt morphology/functionality, capacity to generate organoids, transcriptome profiles, and microbiome assessments.

RESULTS: At a resting baseline, whole-body NIK knockout mice display an altered microbiome characterized by colitogenic microbiota. Conditional knockout mouse models containing deletions of NIK in IECs had increased tumor formation following chemical induction (AOM/DSS) of colitis-associated colorectal cancer in the colon. Mechanistic studies found loss of NIK further revealed an imbalance of proper apoptosis and proliferation signaling, as colonic crypts have increased accumulation of mature, non-dividing IECs. Further, organoids derived from isolated ISCs exhibit stunted growth indicating diminished stem cell potential. Transcriptomic analysis reveals gene expression signature following loss of NIK alters differentiation-regeneration profile, including differentially expressed gene signatures related to stem cells, epithelial cell development/differentiation, apoptosis, cancer, and wound healing.

CONCLUSIONS: Our findings were validated using human colorectal cancer biopsies, in which we also found a significant decrease in gene expression levels related to NIK and the noncanonical NF- κ B signaling pathway. Culminating our results, we conclude that loss of NIK and subsequent loss of noncanonical NF- κ B signaling in IECs alters the regeneration-differentiation signaling axis, which ultimately increases susceptibility to colorectal cancer and drives tumorigenesis.

SYNOPSIS: NF- κ B-inducing kinase (NIK) is a critical regulator of intestinal epithelial cell (IEC) regeneration and loss of NIK predisposes cells to malignant transformation.

During colorectal cancer, NIK attenuation results in the accumulation of mature epithelial cells, which are more susceptible to mutation and transformation.

INTRODUCTION:

The transcription factor nuclear factor kappa B (NF- κ B) is an essential regulator of biological processes in the gastrointestinal system. The NF- κ B signaling pathways have been well studied in a variety of gastrointestinal diseases (1-11). NF- κ B signaling proceeds through two distinct pathways, the canonical and noncanonical pathway (12- 14). To date, the majority of studies have focused on canonical NF- κ B signaling in the gut. In canonical NF- κ B signaling, (RelA/p65)/p50 heterodimers are maintained in an inactive state in the cytoplasm by a family of inhibitors of NF- κ B (I κ B α). Upon activation, the large I κ B kinase complex consisting of the I κ B kinase γ (NEMO), I κ B kinase α (IKK α) and I κ B kinase β (IKK β) phosphorylates I κ B α , resulting in its degradation and release of the RelA/p50 heterodimer. This heterodimer rapidly translocates into the nucleus, driving the transcription of a large repertoire of mediators that control diverse biological processes, including inflammation, cell death, and proliferation.

Unlike canonical NF- κ B, there is a relative lack of data associated with noncanonical NF- κ B signaling, as noncanonical NF- κ B was originally considered an auxiliary signaling mechanism to canonical NF- κ B. Noncanonical NF- κ B signaling is associated with the p52/RelB heterodimer. p52 is maintained in a precursor form as p100 and acts as an I κ B-like molecule that restricts RelB to the cytoplasm. Upon receptor stimulation, p100 is degraded by the proteasome and processed to p52, which unmask the nuclear localization sequence and facilitates nuclear translocation. Processing of p100 to p52 is tightly regulated by the essential NF- κ B-inducing kinase (NIK). Under normal conditions, NIK is constitutively degraded via a multiprotein regulator complex consisting of TNF-receptor associated factor 3 (TRAF3), TRAF2, and cellular inhibitor of apoptosis 1 and 2 (cIAP1/2), which prevents basal activation of noncanonical NF- κ B signaling. In the gut, most studies have focused on TNF-mediated noncanonical NF- κ B signaling. Upon recognition of a TNF family signaling molecule, TRAF3 is degraded and NIK becomes stabilized, allowing it to phosphorylate IKK α . IKK α subsequently phosphorylates p100, resulting in ubiquitination and proteasome cleavage into active p52.

The p52/RelB heterodimer initiates the transcription of a relatively limited set of target genes that are distinct from those targeted by the canonical NF- κ B subunits. Several chemokines are well defined targets of noncanonical NF- κ B signaling, including CXCL12, CXCL13, CCL19, and CCL21 (5, 15).

Both conventional and cell-type specific NIK knockout mice have significant phenotypes in the gastrointestinal system. For example, the most defined biological function attributed to noncanonical NF- κ B signaling is the development of secondary lymphoid structures, such as Peyer's patches in the gut (16-20). In murine models containing a mutation in NIK to inhibit noncanonical NF- κ B signaling or lacking the lymphotoxin β receptor (LT β R) upstream of NIK, lymph nodes and Peyer's patches are significantly underdeveloped or lacking entirely (20). In addition, disruption of NIK has also been linked to a hypereosinophilic syndrome (HES)-like disease with progressive eosinophilia, tissue destruction, and premature death (4). Interestingly, this syndrome appears to progress through an IKK α -independent mechanism. During the early stages of this disorder, mice develop upper-gastrointestinal phenotypes that effectively model key aspects of EoE, including clinically relevant esophageal-specific eosinophil recruitment and remodeling (3). This phenotype is limited to the upper-GI tract prior to HES development.

In addition to its defined role in secondary lymphoid development, noncanonical NF- κ B signaling can also modulate adaptive immune responses including T-cell differentiation, B cell maturation, and interferon signaling (21-28). These mechanisms have been best defined using mice carrying cell-type specific disruptions of NIK (16). For example, transgenic mice with conditionally deleted NIK in CD11c⁺ dendritic cells have normal lymphoid organs but are defective in antigen cross-priming of naïve CD8⁺ T cells and secretion of IL-12p40 (29). These data reveal that NIK and noncanonical NF- κ B signaling regulate the capacity of dendritic cells to cross-present antigens and prime CD8⁺ T cell responses following either anti-CD40 blockade or TLR3 signaling. Similarly, NIK was found to have a cell-intrinsic role in regulating the homeostasis of peripheral T cells and T regs NIK using CD4⁺ cre mice to delete NIK in both CD4⁺ and CD8⁺ cells (30). Mice have also been generated with conditional deletion of NIK in B cells. Consistent with the decrease in B cell populations described in the spleen and

lymph nodes of conventional *Nik*^{-/-} mice, B cells from animals with the B cell specific NIK deletion fail to respond to BAFF stimulation, exhibit significant decreases in germinal center B cells, and attenuated serum IgA levels (31). In the gastrointestinal tract, NIK has been conditionally deleted in intestinal epithelial cells (IECs), using Cre recombinase under the control of the *Villin* promoter (27). These mice were found to be more susceptible to local and systemic inflammation in IBD and sepsis models. The mechanism associated with this susceptibility was found to be associated with improper maintenance and differentiation of microfold cells (M-cells) in the epithelial cell barrier. Loss of epithelial cell NIK resulted in decreases in IL-17 expression and IgA coating of colitogenic bacteria, resulting in increased gastrointestinal inflammation.

Expanding the role of NIK and noncanonical NF- κ B signaling beyond inflammatory diseases, this pathway has also been evaluated in the context of cancer in a few limited studies. For example, as discussed above, studies using *Nik*^{-/-} mice revealed a role for NIK and noncanonical NF- κ B signaling in HES (4). HES is a myeloproliferative disorder characterized by the hyperproliferation and dysfunctional migration of eosinophils in the *Nik*^{-/-} mice. Additional studies using mice with T cell specific NIK deletions have revealed critical roles in metabolic fitness in anti-tumor immunity. NIK deficiency was shown to impair glycolysis induction, impairing CD8⁺ effector T cell function in the tumor microenvironment (32). Conversely, overexpression of NIK enhanced CD8⁺ T cell function and improved the efficacy of T cell adoptive therapy (32). NIK prevents autophagic degradation of hexokinase 2, which is a rate-limiting enzyme in the glycolytic pathway (32). Therefore, prior work highlights the critical role of NIK in further activation and signal transduction of the noncanonical NF- κ B signaling, as well as its emerging role in maintaining health beyond its involvement in lymphoid organogenesis.

Despite the studies detailed above, the role of NIK and noncanonical NF- κ B signaling in cancer is complex and remains enigmatic. This is especially true in the gastrointestinal system, where very few studies have been conducted beyond those discussed above. Here, we show that NIK attenuates colorectal cancer through the regulation of noncanonical NF- κ B mediated maintenance of IEC regeneration and differentiation. In novel genetically modified mice, we illustrate that noncanonical NF- κ B signaling is essential for protection against colorectal cancer and functions through the epithelial cell

compartment. Conversely, canonical NF- κ B signaling in the myeloid cell compartment augments colorectal cancer progression through contributing to tumor promoting inflammation. In the absence of NIK, improper IEC differentiation and regeneration allows damage to accumulate, promoting the transformation of these cells and potentially via “top-down” tumorigenesis in the colon.

RESULTS:

Noncanonical NF- κ B signaling is attenuated in human colorectal cancer patients

Noncanonical NF- κ B signaling is differentially regulated in human patients with gastrointestinal disorders (2, 3). Therefore, we first sought to evaluate noncanonical NF- κ B signaling in human colorectal cancer patients. Using Oncomine®, GEO datasets, CompBio, and Ingenuity Pathway Analysis (IPA) we conducted a series of retrospective metadata analyses of gene expression profiles in CRC patients (**Figure 1**). Here, in an unbiased approach, we evaluated 80 known components or modifiers of the noncanonical NF- κ B signaling pathway at multiple levels, including ligand-receptor interactions, pathway components, regulators, and effectors (**Figure 1K**). The most comprehensive data analysis utilized the largest dataset available across platforms, The Cancer Genome Atlas (TCGA), which is composed of 215 CRC biopsy samples and 22 control tissues (**Figure 1**). All 80 genes evaluated were significantly dysregulated in the CRC patients, with 11 genes (13.75%) identified as being significantly dysregulated across all platforms and analyses (**Figure 1**). B-cell activating factor (*BAFF*), its receptor *BAFFR*, the noncanonical receptor cluster of differentiation 40 (*CD40*) and its ligand *CD40L*, and lymphotoxin beta (*LTB*), which are all potent stimulators of noncanonical NF- κ B signaling, were all significantly downregulated in biopsy samples from CRC patients (**Figure 1A-E**). However, the gene encoding the LTB receptor, *LTBR*, was the single gene consistently found significantly upregulated in CRC patients (**Figure 1F**). In the signaling pathway, NIK, encoded by the gene *MAP3K14*, is also significantly downregulated in CRC patients (**Figure 1G**). We next investigated the expression of downstream chemokines of noncanonical signaling not only within the TCGA dataset, but across multiple colorectal cancer datasets available on Oncomine® where these four genes were expressed and included in the datasets. Here, we observed the four most common chemokines associated with noncanonical NF- κ B signaling, *CXCL12*, *CXCL13*,

CCL19, and *CCL21*, consistently and significantly downregulated across datasets, including different subsets of colorectal cancer (**Figure 1H**).

Validating the metadata analysis, we measured RNA collected from formalin- fixed paraffin-embedded tissue of colonic biopsies from 6 human CRC patients and 6 control (non-inflamed, non-neoplastic) biopsies. 80 genes were validated from the metadata analysis using pooled RNA (**Figure 1, Figure 2**). Consistent with the metadata analysis, significant differences between CRC patients and controls were observed for all 80 genes, with the majority being downregulated (**Figure 1I-J**). *NIK* and the four noncanonical chemokines were all significantly downregulated in CRC patients compared to the control specimens (*NIK*, -2.59 fold; *CXCL12*, -3.67 fold; *CXCL13*, -7.34 fold; *CCL19*, -59.28 fold; *CCL21*, -30.02 fold). Using Ingenuity Pathway Analysis (IPA), we confirmed that noncanonical NF- κ B signaling was significantly decreased in the CRC patients, including *NIK*, *RELB*, and all four chemokines (**Figure 1J; Figure 2B**). *CXCR4* and *CXCR5*, respective receptors for *CXCL12* and *CXCL13*, were found upregulated. Consistently, *NLRP12*, a negative regulator of noncanonical NF- κ B, was also found to be upregulated (**Figure 1J; Figure 2D**). In addition to *NLRP12*, *A20* (*TNFAIP3*) and several additional members of the Nod-like receptor signaling family were also identified as critical signaling hubs/regulators of both canonical and noncanonical NF- κ B that were significantly dysregulated in our CRC patients (**Figure 2C-D**). Based on the global changes in gene expression, IPA also predicted 3 pharmacological agents, romidepsin, S-nitrosoglutathione, and methotrexate, that may impact key aspects of noncanonical NF- κ B signaling that were significantly altered in our CRC patient populations (**Figure 2E**). These findings were further confirmed using individual real-time PCR for *NIK*, *CXCL12*, *CXCL13*, *CCL19*, and *CCL21* (**Figure 1I**).

NIK Functions to Attenuate Experimental Colitis and Colon Inflammation

Given *NIK* was found to be the most significantly downregulated gene expressed in the human CRC biopsies, we next assessed the *in vivo* role of *NIK* in maintaining gastrointestinal homeostasis in the colon. Due to the prior phenotypes described for the *Nik*^{-/-} mice (33-35), we verified that the resting colon was phenotypically similar between the *Nik*^{-/-} and wild-type animals between 8-12 weeks of age (**Figure 3A-B**). Histologically, colons collected from wild-type mice and *Nik*^{-/-} mice were similar, as there

appeared to be normal distributions of enterocytes with no evident inflammatory reaction (**Figure 3A-B**).

A previous study has shown that negative regulators of NIK can suppress colon inflammation through attenuating noncanonical NF- κ B signaling (5). Conversely, NIK has been shown to attenuate experimental colitis and sepsis progression through the IEC compartment (27). Thus, we sought to directly evaluate these findings using the *Nik*^{-/-} mice in a model of DSS-induced experimental colitis (36). Male *Nik*^{-/-} and littermate *Nik*^{+/+} mice were used in all studies. Chemical induction of colitis was performed by administering 5% DSS in their drinking water for 4 days. The next 4 days, mice were rested on normal drinking water to allow epithelial cell regeneration (**Figure 3C-J**). Clinical scores, which are composite scores of activity levels, fecal consistency, blood in stool, and weight loss, were generated daily for each mouse in the study (5). *Nik*^{-/-} mice had significantly increased clinical scores at all monitoring points, indicating that NIK plays a critical function in attenuating experimental colitis progression (**Figure 3C**). Colons collected from *Nik*^{-/-} mice were significantly shortened and contained poorly formed, loose fecal material (**Figure 3D-E**). Histologically, wild-type colons contained significant ulceration, inflammation, and the beginnings of crypt reformation (**Figure 3F- G**). However, we observed significantly increased colon inflammation and IEC ulceration in the *Nik*^{-/-} mice (**Figure 3H**), which were histopathologically assessed and quantified using a composite score, as previously described (37). Additionally, the systemic effects of this ulceration and inflammation were more evident in the *Nik*^{-/-} mice, as indicated by increased IL-6 in blood sera and serum endotoxin (**Figure 3I-J**).

To evaluate the impact of dysbiosis on the increased inflammation and epithelial cell barrier damage, feces were collected from *Nik*^{-/-} and wild-type littermates prior to induction of colitis. *Nik*^{-/-} mice displayed distinct microbiomes with abundant presence of bacterial taxa commonly associated with inflammation, which is indicative of dysbiosis (**Figure 4**). The *Nik*^{-/-} gut microbiome was characterized by two genera of *Clostridium* and a *Clostridiaceae* family member. Presence of Clostridial bacteria is important in colonic homeostasis; however, whether they exert a protective or proinflammatory effect is dependent on the species level. Additionally, the *Nik*^{-/-} colon is also characterized by *Helicobacteraceae*. *Helicobacter* is commonly identified within the dysbiotic microbiome

of various enteropathies, namely *Helicobacter pylori* and gastric cancer. Bacteria belonging to the *Campylobacteriales* order was also increasingly abundant in the *Nik*^{-/-} microbiome, which is associated with colonic inflammation (**Figure 4B-C**). The phylum *Proteobacteria*, which is associated with colitis and compromised colonic mucous barriers (38), including *Moryella*, *Candidatus arthromitus*, *Rikinella*, and *Epsilonproteobacteria* are also elevated in *Nik*^{-/-} microbiomes (**Figure 4B-C**). Culminating these results, the microbiota assessment indicates that *Nik*^{-/-} mice have an inherently dysbiotic gut microbiome that predisposes their gastrointestinal tract towards the elevated pro-inflammatory signaling levels and susceptibility to inflammation- induced tumorigenesis.

NIK Attenuates Colitis-associated Colorectal Cancer Through Intestinal Epithelial Cells (IECS)

As we demonstrated above, mice lacking NIK and noncanonical NF-κB signaling are more sensitive to DSS induced experimental colitis. These data are consistent with prior studies using conditional-knockout mice, where epithelial NIK was shown to protect against colon inflammation and sepsis through the maintenance of M cells (27). These prior studies utilized conditional-knockout mice, where *Nik* deletion was driven by cre under the control of the epithelial *Villin* promoter. Based on these data and our findings in human cancer patients, we next defined the role of NIK in colorectal cancer using cell-type specific conditional-knockout animals in evaluating tumorigenesis via the AOM+DSS model. We generated a novel mouse line carrying a *Nik* locus that is sensitive to disruption by cre recombinase (**Figure 5L**). Briefly, key regions of the *Map3k14* (*Nik*) gene were flanked by loxP sites (*Nik*^{fl/fl}), resulting in the deletion of exons 7 and 8 in cells where cre recombinase is expressed (**Figure 5L**). Based on the findings from our studies with the *Nik*^{-/-} mice and prior studies focused on epithelial cells, we generated mice lacking *Nik* in the IEC compartment by crossing our *Nik*^{fl/fl} mice with transgenic *Villin*^{cre} mice. It is also well established that the hematopoietic compartment, specifically monocytic cells, also significantly contribute to the progression of experimental colitis and colitis associated cancer. Thus, we crossed our *Nik*^{fl/fl} mice with transgenic *LysM*^{cre} mice. In addition to *Nik*^{fl/fl} mice, we also evaluated canonical NF-κB signaling using previously described *RelA*^{fl/fl} mice, which were also crossed with the *Villin*^{cre} and *LysM*^{cre}

animals (39). Cell-type specific deletion of both *Nik* and *RelA* was verified for all mouse lines prior to studies. All studies were conducted using littermate controls.

To determine the role of NIK in myeloid cells during colitis-associated cancer, *Nik^{ΔMYE}* (*Nik^{fl/fl}* X *LysM^{cre+}*) and control (*Nik^{fl/fl}* X *LysM^{cre-}*) mice were treated with AOM+DSS. Both *Nik^{ΔMYE}* and control mice experienced increased clinical scores and histopathological features associated with the IBD index and CRC indexes consistent with increased DSS induced gastrointestinal inflammation and colon tumorigenesis, without any significant differences between genotype (**Figure 5A-B**). Likewise, at necropsy, we did not observe significant differences in polyp sizes or numbers between the *Nik^{ΔMYE}* and control mice (**Figure 5C-D**). Conversely, AOM+DSS studies were also conducted with the *Nik^{ΔIEC}* (*Nik^{fl/fl}* X *Villin^{cre+}*) and control mice (*Nik^{fl/fl}* X *Villin^{cre-}*) (**Figure 5E-J**). While both the *Nik^{ΔIEC}* and control mice demonstrated increased clinical scores with each round of DSS, the *Nik^{ΔIEC}* animals demonstrated significantly increased clinical parameters associated with disease progression at each round of DSS, including changes in behavior, fecal consistency, blood in stool, and weight loss (**Figure 5E**). Upon necropsy, histopathology assessments revealed that both genotypes of mice demonstrated similar severity of experimental colitis, in the form of the IBD Index score (**Figure 5F**). However, the *Nik^{ΔIEC}* mice demonstrated significantly increased histopathological features associated with colorectal cancer, including inflammation, edema, epithelial defects, crypt atrophy, hyperplasia, and dysplasia (**Figure 5F**). Consistent with the histology grading, we also observed significant increases in polyp number in the *Nik^{ΔIEC}* mice compared to the control animals with no significant differences in polyp size (**Figure 5G-H**). Grossly, in addition to the polyps being more numerous in the *Nik^{ΔIEC}* mice, they also extended up to the transverse colon from the cecum to the rectum (**Figure 5I**). Conversely, polyps in the control animals were localized in the distal, descending colon and rectal region (**Figure 5I**), which is more consistent with the AOM+DSS model (36). All the polyps were pathologically defined as adenocarcinomas that were composed of disorganized, arborizing dysplastic tubules separated by thick fibrovascular stroma, admixed with mononuclear cells (**Figure 5J**). Further, the colons of *Nik^{ΔIEC}* mice had markedly increased Ki67, a marker for cellular proliferation, which extended beyond the

typical localization within the stem cell niche at the base of the crypt, towards the terminally differentiated IEC niche at the top of the crypts (**Figure 5K**).

RelA Augments Colitis-associated Colorectal Cancer Through Myeloid Cells

As NIK and noncanonical NF- κ B signaling attenuates inflammation-driven tumorigenesis through IECs, we subsequently determined the unique, cell-intrinsic role of noncanonical NF- κ B in comparison to canonical NF- κ B. Prior studies evaluating the canonical NF- κ B signaling pathway using RelA conditional-knockout mice in spontaneous intestinal disease and in DSS based models of experimental colitis have also revealed that loss of epithelial RelA results in deregulated intestinal proliferation and increased inflammation (39). Here, we sought to expand these prior studies beyond the short-term, acute inflammation model and evaluate the cell-type specific role of canonical NF- κ B signaling in the context of inflammation-driven tumorigenesis. Thus, the *RelA* ^{Δ MYE} (*RelA* ^{Δ /fl} X *LysM*^{cre+}) and control (*RelA* ^{Δ /fl} X *LysM*^{cre-}) mice were treated with AOM+DSS. Unlike the *Nik* ^{Δ MYE} mice, the *RelA* ^{Δ MYE} mice were significantly protected from the development of colitis-associated cancer (**Figure 6**). These animals demonstrated significantly improved clinical scores throughout each round of DSS-driven colon inflammation compared to the control animals (**Figure 6A**). Histopathology evaluation revealed increased features consistent with colitis in both sets of mice; however, the *RelA* ^{Δ MYE} mice had a significantly attenuated IBD and CRC index compared to the control animals (**Figure 6B-C**). This was predominately associated with reduced inflammation in the colon. Gross assessments of the colons revealed significantly reduced numbers of polyps, which were also smaller in size in the *RelA* ^{Δ MYE} mice compared to the controls (**Figure 6D-F**). In both sets of mice, the polyps were defined as adenocarcinomas by histopathology (**Figure 6G**). Conversely, we did not observe any significant differences in clinical progression between the *RelA* ^{Δ IEC} and their respective control mice (**Figure 6H**). This was confirmed histopathologically in the IBD and CRC indexes (**Figure 6I**) and grossly with similar sizes and numbers of macroscopic polyps (**Figure 6J-K**).

Loss of NIK Compromises IEC Differentiation and Regeneration

Deletion of NIK in epithelial cells increased susceptibility to inflammation-induced colorectal cancer. To better characterize the transcriptional impact of the loss of

NIK in the IECs (**Figure 7**), we next performed ClariomTM S transcriptome analysis of lesion tissue (LT) and non-lesion “healthy” tissue (HT) following AOM+DSS (**Figure 5I**). We identified 19 genes related to NF- κ B signaling significantly upregulated and one gene (*Tnfrsf11A*) significantly down-regulated in the *Nik^{ΔIEC}* lesion compared to the adjacent healthy tissue collected from the same animals (**Figure 7A**). Beyond the NF- κ B specific pathway analysis, we also identified the top 25 up-regulated and down-regulated genes associated with NIK-deletion in *Nik^{ΔIEC}* lesion compared to healthy tissue (**Figure 7B-C**). These genes were generally found to contribute to biological functions associated with intestinal barrier function and host-bacteria interactions by Gene Ontology (GO) analysis (**Figure 7D**). Specifically, the data suggest impaired keratinocyte, epidermal cell, and epithelial cell differentiation when NIK is deficient in the IEC compartment. This is predicted to result in altered regulation of epithelial cell proliferation compared to the control lesion tissue (**Figure 7E**). We did not observe any significant differences in the transcription profiles between *Nik^{ΔIEC}* and control healthy tissues at baseline.

Further investigation into the role of NIK in regulating epithelial cell signaling revealed an imbalance in gene expression patterns associated with stem cell function and differentiation into epithelial cells. We observed an increase in markers associated with epithelial cell differentiation, including keratin expression, in the lesions from *Nik^{fl/fl}* control mice (**Figure 7F**). Transcriptome analysis further revealed altered gene expression related to Stem Cell Function (**Figure 7G**), Epithelial Cell Differentiation (**Figure 7H**), and Epithelial Cell Development (**Figure 7I**). These data may suggest that epithelial cells in the *Nik^{ΔIEC}* mice either fail to reach maturation given improper differentiation signaling or de-differentiate from IECs to become more “stem-like” to potentially compensate for the loss of pluripotency within the colonic crypt.

NIK and Noncanonical NF- κ B Signaling Regulates Intestinal Epithelial Cell Regeneration and Turnover

Next, we assessed the physiological implications of the transcriptomics observations detailed above and functionally define the impact of defective noncanonical NF- κ B signaling and loss of NIK in the epithelial cells. Here, we isolated and evaluated whole crypts from untreated *Nik^{-/-}* and wild-type mice to evaluate the role of NIK in maintain crypt development, including the balance of ISC proliferation and IEC

differentiation. Immunohistochemistry for Ki-67 revealed intense staining near the bottom of the crypts from wild-type mice (**Figure 8A**), consistent with the normally high levels of proliferation and regeneration in the crypt stem cell niche. However, Ki-67 staining was significantly decreased in the stem cell niche in crypts isolated from the *Nik*^{-/-} animals (**Figure 8A**). Indeed, quantification of Ki67 immunohistochemistry revealed a 4.375-fold decrease in Ki67 staining in the *Nik*^{-/-} crypts compared to those from wild-type mice (**Figure 8B**). We also observed a significant decrease in *Lgr5* expression, which is a colonic stem cell marker, in the *Nik*^{-/-} crypts compared to wild-type crypts (**Figure 8C**). Together, these data suggest reduced proliferation in the *Nik*^{-/-} crypts compared to wild-type, which initially seems contradictory to the increased tumorigenesis observed in the *in vivo* mouse models. However, morphological analysis consistently revealed significant elongation of the *Nik*^{-/-} crypts compared to wild-type crypts (**Figure 8A, D**). *Nik*^{-/-} crypts averaged almost twice the length of the wild-type crypts (**Figure 8D**). We next evaluated the expression of cytokeratin 20 (*Krt20*), which is a marker of mature enterocytes, and found significantly increased levels in *Nik*^{-/-} crypts (**Figure 8E**). The colonic crypts of *Nik*^{-/-} mice also had decreased expression of poly-ADP ribose (PARP1) and its cleaved product (cPARP1) (**Figure 8F**). These findings suggest that elongation of crypts is associated with the lack of regeneration and turnover of mature, non-dividing colonic epithelial cells, given a lack of normal apoptosis in these mature cells.

Consistent with the transcriptome analysis, we also observed diminished regulation of Cancer Pathways and apoptosis in the *Nik*^{AIEC} lesion tissue (**Figure 8 G-H**). Reciprocally, *Nik*^{AIEC} lesion tissue is characterized by upregulation of genes related to wound healing, including a 796.99 fold-change upregulation of the ECR remodeling enzyme gene *Mmp7* (**Figure 7I**). Together, these data may support a “top-down” tumorigenesis model in the *Nik*^{AIEC} mice, whereby the absence of NIK causes IECs to lack proper aging signaling given decreased senescence, inefficient apoptotic signaling to repopulate, and turnover damaged IECs, and increased wound healing responses to cope with the accrual of damage (**Figure 7I**). As a result, in the absence of NIK, the damaged IECs would be predicted to exhibit more stem-like characteristics to account for defective regenerative potential within the crypt.

If our postulated working model were true, then we would anticipate observing reduced intestinal epithelial stem cell proliferation. Thus, to evaluate this aspect of our model, we generated colon organoids from intestinal stem cells isolated from wild-type, *Nik*^{-/-}, and *Apc*^{min} animals (**Figure 9**). Culturing colonic organoids generates an epithelial-cell only population that arises from isolated stem cells and differentiates into all the various epithelial cell-types of the colonic mucosa including enterocytes, goblet cells, and Paneth cells. The *Apc*^{min} mice lack a negative regulator of the canonical Wnt/ β -Catenin signaling pathway, resulting in the upregulation of the WNT pathway. This mechanism is strongly associated with stem cell proliferation and self-renewal at the base of the intestinal crypts (40). Thus, they are used here as positive controls for stem-cell hyper-proliferation in the organoids. Colonic crypt stem cells from each mouse line were collected and cultured for 6 days with size measurements conducted over time. Wild-type organoids formed at a highly consistent rate and grew to a predictable size over the 6-day period (**Figure 9A**). Organoids from the *Apc*^{min} mice demonstrated significantly accelerated epithelial cell proliferation between days 3 and 4 (**Figure 9A**), consistent with the Wnt signaling dysregulation and subsequent stem cell hyperproliferation. The *Apc*^{min} organoids maintained a diameter that was approximately double that of the wild-type organoids throughout the remainder of the study (**Figure 9A**). Conversely, organoids from the *Nik*^{-/-} mice demonstrated significantly attenuated proliferation and growth beyond day 4 (**Figure 9A**). Diameter measurements revealed that the *Nik*^{-/-} organoids were significantly smaller, averaging approximately half of the size of the wild-type organoids (**Figure 9B**). Despite the smaller size, the *Nik*^{-/-} organoids were still composed of live cells, demonstrated similar morphology, and were composed of approximately equivalent populations of IEC subtypes when compared to the wild-type and *Apc*^{min} organoids (**Figure 9C**). The attenuated growth is consistent with the attenuated stem cell proliferation and reduced turnover described above. Together, these data suggest that the increased tumorigenesis in the *Nik*^{-/-} mice is associated with a deficiency in stem cell proliferation and self-renewal, ultimately resulting in dysfunctional enterocyte regeneration.

DISCUSSION:

Taken together, our data suggests that NIK attenuates colorectal cancer through the modulation of IEC regeneration and repair. Our human and mouse data shows that in the absence or down regulation of NIK and noncanonical NF- κ B signaling, the ISC pool within crypts lacks sufficient proliferative capacity. Without a steady pool of proliferative ISCs and minimal activity within amplifying zones of crypts, mature and damaged IECs accrue at the top of the crypt and throughout the villus. Compounding this lack of proliferation, we also observed reduced apoptosis in crypts isolated from *Nik*^{-/-} mice (**Figure 8F**). Controlled cell death is a critical event in IEC barrier regeneration and repair processes. The combination of reduced proliferation and apoptosis is predicted to promote the survival of mature enterocytes well beyond their typical life span. While the crypts significantly lack self-renewal capacity, this lack of IEC turnover is not clinically apparent under normal, homeostatic conditions. However, following inflammatory insult, given chronic inflammatory conditions or upon exposure to carcinogens, damage accrues in the mature IEC populations resulting in amplified disease states.

Historically, colorectal cancer has been studied from an ISC perspective. For example, most models of disease progression are based on well characterized mouse models, including constitutive activation of β -catenin/Wnt signaling in *Apc*^{min} mice (41, 42). These models are based on neoplasms arising from hyperproliferation within the ISC niche and tumorigenesis proceeding through a “bottom-up” mechanism (6, 43). However, our data suggests that the increased tumorigenesis observed in the *Nik*^{-/-} and *Nik*^{AIEC} mice is associated with a “top-down” mechanism. Top-down tumorigenesis is strongly associated with the accumulation of mutations in mature IECs that do not undergo the typical replenishment process in the gut (8, 44-47), which is consistent with the data we presented. Lack of proper IEC regeneration leads to the accumulation of mutations upon chronic damage and diminished apoptosis, resulting in a greater likelihood of cellular transformation. In the CAC models, this process is accelerated by the addition of the carcinogen AOM and the chronic inflammation-driven by DSS (**Figure 5**). This process is further compounded by attenuated ISC proliferation and down-regulation of stem cell markers. The top-down mechanism is characterized by terminally differentiated IECs de-differentiating to replenish the lack of Lgr5⁺ ISCs or to compensate for the attenuation in stem cell renewal. This mechanism is supported by our data demonstrating increased

mature IEC expansion, reduced proliferation in the stem cell niche, and reduced *Lgr5* in the crypt preps from the *Nik*^{-/-} mice (**Figures 8-9**). The reduced *Lgr5* expression observed in the *Nik*^{-/-} crypts is also consistent with prior studies that suggest *Lgr5* may be important in maintaining stemness and functions to prevent other cells, such as mature IECs, from gaining these abilities (48). In the absence or suppression of *Lgr5* and a range of other stem cell markers, mature enterocytes have been previously reported to gain plasticity. This newly acquired plasticity can result in terminally differentiated cells being reprogrammed to regain features of stemness and accelerated growth (49).

Our data suggests that IEC-associated NIK protects the colon from top-down tumorigenesis, through the regulation of noncanonical NF-κB signaling. Conversely, prior studies observed that activation of canonical NF-κB in the crypt stem cell niche enhances Wnt signaling to promote the de-differentiation of the IECs, thereby promoting tumorigenesis in the gut (8). This suggests that canonical and non-canonical NF-κB signaling have opposing functions in the maintenance of the ISC and IEC niches during colorectal cancer. Here, we included the *RelA*^{ΔIEC} and *RelA*^{ΔMYE} mice to contrast canonical and noncanonical NF-κB signaling in our CAC models. Previous studies have utilized the *RelA*^{ΔIEC} mice in the DSS model of experimental colitis (39). These animals were found to be more susceptible to a single round of acute DSS, with increased proliferation, sustained epithelial cell apoptosis, and decreased animal survival (39). The *RelA*^{ΔMYE} mice have also been previously evaluated in the DSS model (50). These animals demonstrate attenuated experimental colitis linked to altered expression of IL-6, Ccl11, and calprotectin in the DSS treated colons (50). While both prior studies utilized acute DSS to model experimental colitis in these animals, neither study evaluated relapsing remitting colitis or cancer development. Unlike the findings from the acute DSS study (39) and our observations with *Nik*^{ΔIEC}, we did not observe any significant phenotypes in the *RelA*^{ΔIEC} animals in the cancer model used here (**Figure 5**). However, our findings clearly show that loss of RelA in monocytes confers significant protection against tumor formation in the CAC model (**Figure 6**). These findings are consistent with the prior *RelA*^{ΔMYE} studies demonstrating attenuated colitis in the acute model (50). AOM alone typically requires an inflammatory insult for tumor formation in C57Bl/6 mice. Thus, it is likely that the reduced tumorigenesis in the *RelA*^{ΔMYE} mice is due to the overall

attenuation of DSS driven inflammation in the colon. However, regardless of the mechanism, our data provides an intriguing contrast between the contribution of NIK/noncanonical NF- κ B and RelA/canonical NF- κ B signaling in the IEC and monocyte compartments during CAC.

NIK contributes to a variety of disease processes, including cancer, through multiple mechanisms and cell-types. However, the role of NIK in the gut and its contribution to colorectal cancer is both complex and not yet well defined. Early studies by our research team found that loss of negative regulation of NIK, coupled with increased p100 processing to p52 and reduced TRAF3 signaling, increased gastrointestinal inflammation and tumorigenesis in CAC models (5). This is consistent with other findings associated with NIK signaling in mucosal dendritic cells where the loss of NIK signaling attenuated colitis induction (10). Thus, while most studies in the gut have linked NIK function with noncanonical NF- κ B signaling, which is also consistent with the data presented here in both the human and mouse studies, it is certainly possible that NIK may be contributing to CAC through other yet to be discovered mechanisms beyond non-canonical NF- κ B signaling in the IECs.

The increased tumor susceptibility in the *Nik*^{-/-} and *Nik*^{ΔIEC} mice is consistent with reduced IEC turnover in the top-down tumorigenesis model. Reduced IEC turnover has been reported in acute experimental colitis models using a different conditional NIK deficient mouse line, whereby loss of NIK is mediated by tamoxifen-inducible Cre recombinase driven by the villin promoter (*Nik*^{F/F;VilERT2Cre}) (27). Like our findings reported here, these IEC-deficient mice demonstrated enhanced inflammation following a single round of 2% DSS for 7 days. This was shown to be p52 dependent, indicating that the mechanism was associated with dysregulated noncanonical NF- κ B signaling, with additional studies revealing a role for RANK signaling up-stream of NIK. IL-17 mediated IgA production appears to be protective in this model and attenuated the observed phenotype. This same study found that constitutive expression of NIK and noncanonical NF- κ B signaling also increased susceptibility to inflammatory injury through inducing M-cell differentiation and chronic increases in IL-17A (27). Mechanistically, NIK was found to regulate Microfold cell (M-cell) maintenance and differentiation, impacting the function of the intestinal lymphoid follicles. M-cells are specialized IECs that regulate

luminal microbiota and dietary antigen sampling in the gut. While this study is one of the most comprehensive assessments of NIK and noncanonical NF- κ B signaling in the gut to date, the work is specifically focused on gastrointestinal inflammation over a short duration in an acute model. The cancer studies presented in the current work do not specifically evaluate the M-cells. However, it is likely that this mechanism contributes to the *in vivo* protective effects of NIK in the CAC studies. It is important to note that the crypt and organoid data included here indicate that NIK is contributing to other mechanism in the mature enterocytes, beyond the M-cells. For example, M-cells are not part of the organoids shown in **Figure 9**. Organoids require RANKL and other specific factors added exogenously, that were not included here, to differentiate and include M- cells (51). Thus, the truncated development of the *Nik*^{-/-} organoids is associated with an M-cell independent mechanism.

In conclusion, we show that NIK and non-canonical NF- κ B signaling plays a protective role in human patients and in mouse models of inflammation-driven colon tumorigenesis. Our studies using novel *Nik*^{AIEC} conditional-knockout mice show that this protective mechanism is driven through the IEC compartment. This contrasts with RelA and the canonical NF- κ B signaling pathway, which promotes CAC driven tumor development through the modulation of inflammation driven through the monocyte compartment. Mechanistically, our data supports a top-down tumorigenesis model in the *Nik*^{-/-} and *Nik*^{AIEC} mice, whereby IEC regeneration is attenuated, allowing elongated crypts that are the result of faulty IEC turnover, diminished apoptosis, and decreased proliferation/differentiation to contribute to tumorigenesis. The accumulation of mature enterocytes is predicted to be increasingly susceptible to damage and mutations associated with increased inflammation and environmental insults in the colon. NIK appears to protect against this process by maintaining the replicative potential of the stem cell niche in the crypts. Future work may include further investigation of de-differentiation of IECs following loss of NIK in contributing to top-down tumorigenesis.

METHODS:

Human Specimens

CRC biopsy samples were collected from 6 patients and 6 controls. Control biopsies were collected from patients presenting for conditions other than IBD/CRC from areas defined

as unaffected tissue during endoscopy/histology. All tissue specimens were further confirmed by a board-certified pathologist to be control (lack histological features) or CRC (neoplasia). RNA was extracted from CRC tissue preserved as 50µm sections of formalin-fixed paraffin-embedded tissue and RNAlater®-preserved control tissue. DNA/RNA/protein were extracted using the Qiagen AllPrep kit according to manufacturer's protocols.

Human Metadata Analysis

Using the Oncomine® Platform (www.oncomine.org), we identified microarray studies of human colorectal cancer patients to evaluate gene expression relevant to noncanonical NF- κ B in biopsy tissue. Relative gene expression was reported as log-transformed median-centered data used in the following datasets (n= CRC/control): Gaedcke (65/65) (52), Skrzypczak (81/24) (53), Hong (70/12) (54), TCGA colon cancer (215/22) (55), Kaiser (100/5) (56), Graudens (18/12) (57), Ki (50/28) (58), Gaspar (56/22) (59), Notterman (18/18) (60) and Sabates-Bellver (32/32) (61). Statistical significance was determined using the unpaired Mann-Whitney U test, where significance was set at $p \leq 0.05$.

Experimental Animals

Conventional *Map3K14* deficient mice (*Nik*^{-/-}) have been previously described and were graciously provided by Amgen and Dr. Vanessa Redecke (St. Jude) (4). The *Map3K14* flox mice (*Nik*^{fl/fl}) are novel and were generated by the PI and Ingenious Targeting Laboratory following general protocols as previously described and outlined in **Figure 5L** (62). The *RelA*^{fl/fl} mice have been previously described and were graciously provided by Dr. Al Baldwin (UNC Chapel Hill) (39). *Apc*^{min} mice (*C57BL/6J-ApcMin/J*) were commercially acquired (Jackson Laboratory). Mice carrying floxed alleles were crossed with transgenic mice that express Cre recombinase under the control of either the *Villin* promoter ((*B6.Cg-Tg(Vill-cre)1000Gum/J(Villin-Cre)*) or the *LysM* promoter ((*B6.129P2-Lyz2tm1(cre)Ifo/J(LysM-cre)*), resulting in IEC or myeloid cell specific deletions, respectively. *Villin-Cre* and *LysM-Cre* mice were commercially acquired (Jackson Laboratories). Gene deletion was confirmed prior to and/or following all studies using PCR genotyping and western blot for either NIK or RelA in selected mouse tissues. All animals were maintained as breeder colonies at Virginia Tech's vivarium under

specific pathogen free conditions for the duration of the study. All animals were maintained on the C57Bl/6 background. All animals were fed *ad libitum* with standard rodent chow (Research Diets) and maintained in a 12-hr light/dark cycle. For all studies/experiments, littermate controls were utilized. Control and mutant animals were housed separately.

Induction of Colitis and Inflammation-driven Tumor Progression

Acute experimental colitis was induced by exposing mice to a single cycle of 5% DSS (MP Biomedicals; Affymetrix) for 5 days and harvested on Day 8 as previously described (37). Relapsing, remitting experimental colitis was induced by exposing the mice to three cycles of 2.5% DSS (MP Biomedicals or Affymetrix) as previously described (63). To induce inflammation-driven colitis-associated cancer, mice were given 1 intraperitoneal injection (10 mg/kg body weight) of the mutagen AOM (Sigma Aldrich), followed by 3 DSS exposures (2.5%) as previously described (5). For all studies, mice were sacrificed and characterized at the planned end of the study (up to 60 days) or when the animals were moribund. Disease progression was determined by the change in body weight, the presence of rectal bleeding, and stool consistency. Parameters was scored and averaged to generate a semiquantative clinical score, as described (63, 64). Mice were euthanized by carbon dioxide narcosis followed immediately by cervical dislocation. Colon length was measured upon completion of each study. Due to well defined sex differences in response to DSS (65), only male mice were used in the colitis and colitis-associated cancer studies.

Macroscopic Polyp Analysis and Histopathology

The entire colon was dissected, excised, opened longitudinally, and removed of fecal pellets to observe for macroscopic polyp formation as previously described (5, 37, 63, 66). Polyps were identified and diameter width was measured using a ruler. One representative polyp and adjacent non-healthy tissue were collected in a sterile microcentrifuge tube and stored at -80°C for further downstream applications. The colons were then Swiss rolled and fixed in 10% buffered formalin. Tissue samples were then paraffin embedded, processed routinely, sectioned at 5µm and stained with H&E for histopathology. Grading was performed using clinical parameters consistent with human IBD (inflammatory indices) and CRC (hyperplasia parameters) (67).

Crypt Isolation and Generation of Colonic Organoid Culture

Colonic crypts were harvested from mice and subsequently used to generate organoids as previously described (68). Organoids were randomly selected for diameter measurements. For time course images, the same organoids were measured over time.

IHC and Quantification

Smears for immunocytochemistry were made by placing a drop of concentrated crypt suspension on a frosted microscope slide and allowed to air-dry. Slides were subsequently fixed in 4% paraformaldehyde for 10 minutes and rinsed with 1X PBS. Enzymatic antigen retrieval was performed using a 0.5% trypsin solution in distilled water. IHC was performed according to the manufacturer's protocol using the Pierce IHC kit and an anti-mouse Ki-67 antibody (CST Product #12202) at a 1:1000 dilution. ImageJ was used to quantify staining.

Real-Time Quantitative PCR

RNA quality and concentration was measured using Nanodrop. RNA was reverse transcribed to cDNA using the ThermoFisher High-Capacity cDNA Reverse Transcription Kit. Pathway focused gene expression was evaluated using custom noncanonical NF- κ B signaling from Superarray Platforms (Qiagen). Individual real-time PCR was performed using Taqman primers for specific human and mouse genes. 18S was used as an internal control. All studies were performed using an ABI 7500 Fast Block Thermocycler. Fold change was calculated using the $\Delta\Delta$ Ct method (69) and all changes were log-transformed. Statistical analysis was calculated using Graphpad Prism v. 7 and the unpaired Mann-Whitney U test. Significance was set at $p > 0.05$.

Clariom S Microarray

Mouse RNA extractions were prepared for ClariomTM S Assays following vendor guidelines (ThermoFisher). The Clariom S Assay is microarray based and provides coverage of >20,000 annotated genes. cDNA was reverse transcribed from RNA. cDNA quality and yield were validated after RNase H treatment, cDNA was then loaded onto GeneChipMouse Transcriptome Array 2.0. Cartridge array hybridization was conducted on the GeneChipTM Instrument, with target hybridization, washing, staining, and scanning. Data analysis was performed using the Transcriptome Analysis Console (TAC) 4.0. Additional pathway analysis was conducted using Ingenuity Pathway Analysis (IPA)TM (Qiagen) and CompBioTM (Canopy Bioscience) softwares. Findings were also

compared to results from publicly accessible microarray and RNAseq meta-analysis. All differentially expressed genes were reported as fold-change and considered significant if ≥ 2 , ≤ -2 .

ELISA and Immunoblots

All tissues were processed as previously described (5). All ELISAs utilized commercial kits and were conducted following manufactures procedures. For immunoblots, a total of 30µg of protein from each sample was separated by SDS-PAGE with 4%-12% NuPAGE Bis-Tris gels (Invitrogen), transferred, and incubated overnight with the respective antibodies. Anti-actin-HRP (sc-1615 HRP) (Santa Cruz Biotechnology) was used as a loading control in all studies.

16S rRNA Gene Sequencing and Bacterial Community Analysis

Fecal pellets were collected were collected aseptically from mouse colons. Pellets were stored in a sterile microcentrifuge tube and flash frozen on dry ice, until stored at -80oC prior to processing. Bacterial DNA was extracted using the QIAamp DNA Stool Mini Kit following manufacturer's protocols. 20ng DNA per sample was used to generate the libraries for 16s rDNA sequencing on an Illumina Miseq. Each sample had 200,000+ reads. Quality control was set at 99% percent of reads passing Q20. After QC and trimming the adaptors, mothur (<http://www.mothur.org/>) was used to analyze the data. Paired-end reads were joined and mapped to Greengenes 13.8. OTUs were picked against Greengenes database, setting a 97% similarity threshold. Samples were normalized to the same number of reads for Lefse analysis. Lefse (<https://huttenhower.sph.harvard.edu/galaxy/>) was used to compare the different bacteria abundance in *Nik*^{-/-} and control (wild-type) mice. Diversity was calculated with 95% CI. Heat map plots with bacterial OTU were created with 1000+ reads. **Graphical Illustrations**

Graphical illustrations and schematics were generated using Biorender.com.

Statistics

Data was analyzed using GraphPad Prism, version 7 (GraphPad Software, Inc., San Diego, CA). The appropriate statistical tests were applied as follows: student's two-tailed *t*-test for comparing two experimental groups; one-way and two-way ANOVA were employed as appropriate for multiple comparisons. Significance is set at $p \leq 0.05$. Mean

values were reported with the standard error of the mean (SEM) or standard deviation (SD), where appropriate. All studies were repeated at least 3 independent times with representative data from single experiments shown. All authors had access to the study data and had reviewed and approved the final manuscript.

Study Approval

All human studies were conducted following Duke University and Virginia Tech's IRB guidelines. Written informed consent was received from patients prior to participation. All animal studies were conducted in accordance with the Virginia Tech's IACUC guidelines and the NIH Guide for the Care and Use of Laboratory Animals

AUTHOR CONTRIBUTIONS:

H.A.M., K.E., and I.C.A. lead and designed studies, performed experiments, analyzed/interpreted data, prepared figures, and wrote the manuscript. A.J.R., C.M., M.C.S. performed experiments. D.E.R., Y.Q., P.A.W., S.L.B., K.M.H, and E.K.H. were responsible for designing/executing specific studies and providing key reagents and/or expertise. K.E. is a board-certified pathologist and was responsible for pathology characterization. E.K.H. collected and provided human specimens.

FUNDING INFORMATION: This work was financially supported by the NIH (R03DK105975; NCATS iTHRIVE UL1TR003015), The Virginia-Maryland College of Veterinary Medicine, The Via College of Osteopathic Medicine, and The Virginia Tech Carilion School of Medicine. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or any other funding body.

DATA AVAILABILITY: Data has been deposited in the GEO repository (Accession Number: GSE227993).

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE227993>

META-ANALYSIS STATEMENT: Followed HuGENet guidelines.

ACKNOWLEDGEMENTS:

The authors acknowledge Dr. Sheryl Coutermarsh-Ott, Bettina Heid, Hannah Ivester, Khan Mohammad Imran, Juselyn Tupik, Margaret Nagai-Singer, Brie Trusiano, Benjamin Tintera, and Kacie Hoyt for their technical support. We thank the College of Veterinary Medicine Teaching and Research Animal Care Support Service, Dr. Al Baldwin for providing the *RelA^{fl/fl}* mice, and Amgen and Dr. Vanessa Redecke for

providing the *Nik*^{-/-} mice. We also thank Drs. Jean Peccoud, Neil Adames for assistance conceptualizing the *Nik*^{fl/fl} targeting constructs. We appreciate the assistance of Drs. Dario Sorrentino and Vu Nguyen with human clinical specimen acquisition.

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FIGURE LEGENDS

Figure 1

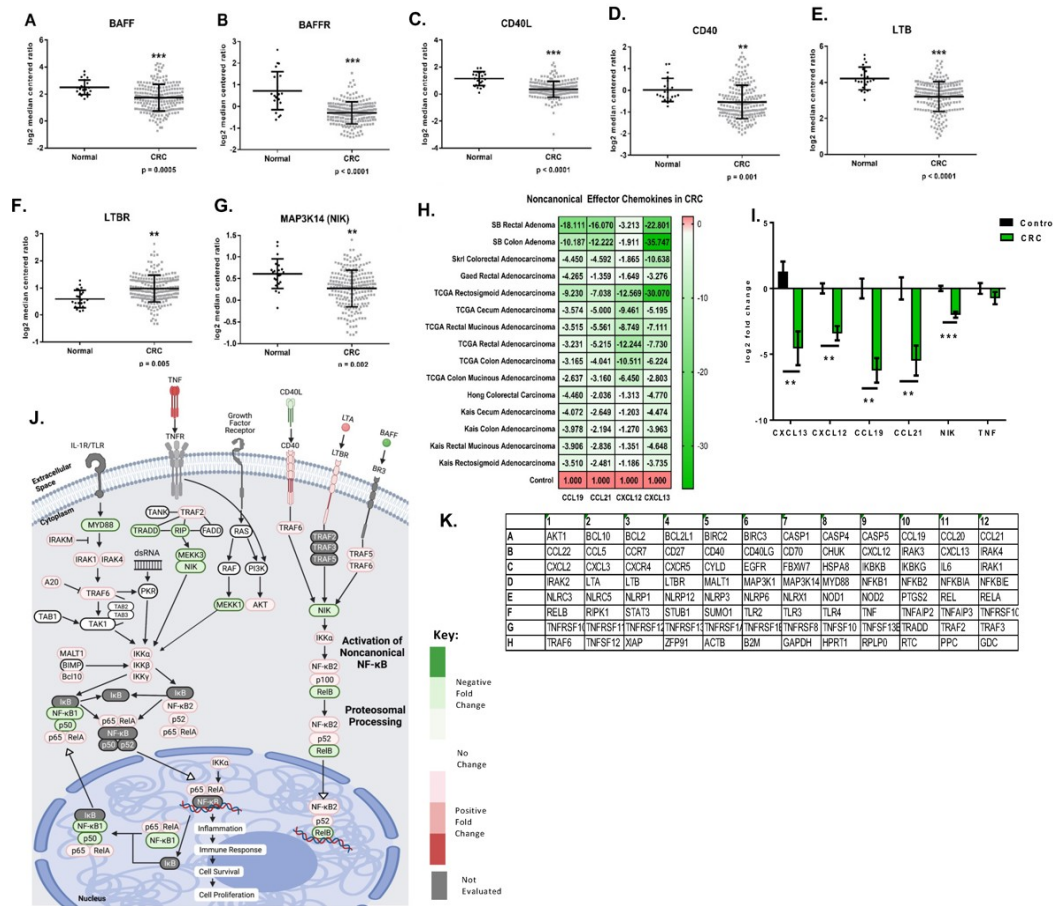


FIGURE 1: Noncanonical NF-κB Signaling is Attenuated in Human Colorectal Cancer Patients. **A-H)** Data from the TCGA datasets were analyzed and the expression of key genes associated with noncanonical NF-κB signaling were compared between normal healthy controls (n=22) and colorectal cancer (CRC) patients (n=215). Significant changes were observed in **(A) BAFF**; **(B) BAFFR**; **(C) CD40L**; **(D) CD40**; **(E) LTB**; **(F) LTBR**; and **(G) MAP3K14 (NIK)**. **H)** Using TCGA datasets, the gene expression levels of four effector chemokines produced by noncanonical NF-κB signaling

(CCL19, CCL21, CXCL12, and CXCL13), were analyzed across different types of colorectal cancers. Datasets were only included if they contained all four chemokines. Significance was defined as +/- 2-fold-change in expression. **(I)** Data from the retrospective studies were confirmed by rt-PCR from colon biopsies from CRC patients (n = 6 control, 6 CRC). **(J)** Gene expression data from the GEO dataset was evaluated using Ingenuity Pathway Analysis. Both canonical and noncanonical NF- κ B signaling were identified as being significantly dysregulated, with noncanonical NF- κ B signaling predicted to be significantly down-regulated in the CRC patient populations. **(K)** 80 known components or modifiers of the noncanonical NF- κ B signaling pathway at multiple levels, including ligand-receptor interactions, pathway components, regulators, and effectors **p<0.01; ***p<0.005

Figure 2

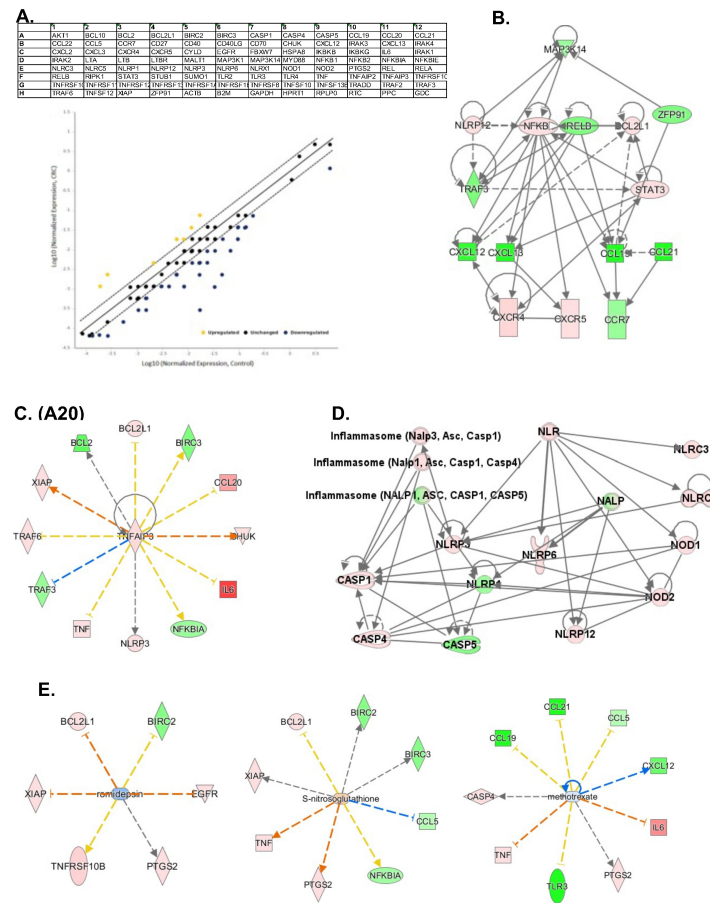


FIGURE 2: Noncanonical NF- κ B Signaling is Significantly Downregulated in Biopsies from CRC Patients. A) RNA was extracted from colon biopsies and gene expression was evaluated using custom NF- κ B signaling Superarray. Compared to control tissue, tissue from CRC patients showed a general downregulation of genes related to noncanonical signaling (A; yellow = upregulation; blue = downregulation). The solid line represents no change from control, and each dotted line marks the borders of likely physiologic significance (i.e. change in fold regulation of >2). **B-E)** Gene expression data was evaluated using Ingenuity Pathway Analysis (IPA). **B)** IPA confirmed that noncanonical NF- κ B signaling was significantly down-regulated and identified NIK (*MAP3K14*) as a critical driver of this dysregulation. **C)** A20 and **(D)** Nod-like receptor signaling were identified as significant regulatory hubs for the altered

NF- κ B signaling identified by IPA. **E)** Based on the gene expression changes identified by IPA, romidepsin, s-nitrosoglutathione, and methotrexate were predicted by IPA to potentially impact disease progression. n = 6 control; n = 6 CRC human patients used in Superarray. Significant values ≥ 2 (yellow/red) and ≤ 2 (blue/green) reported as fold-change.

Figure 3

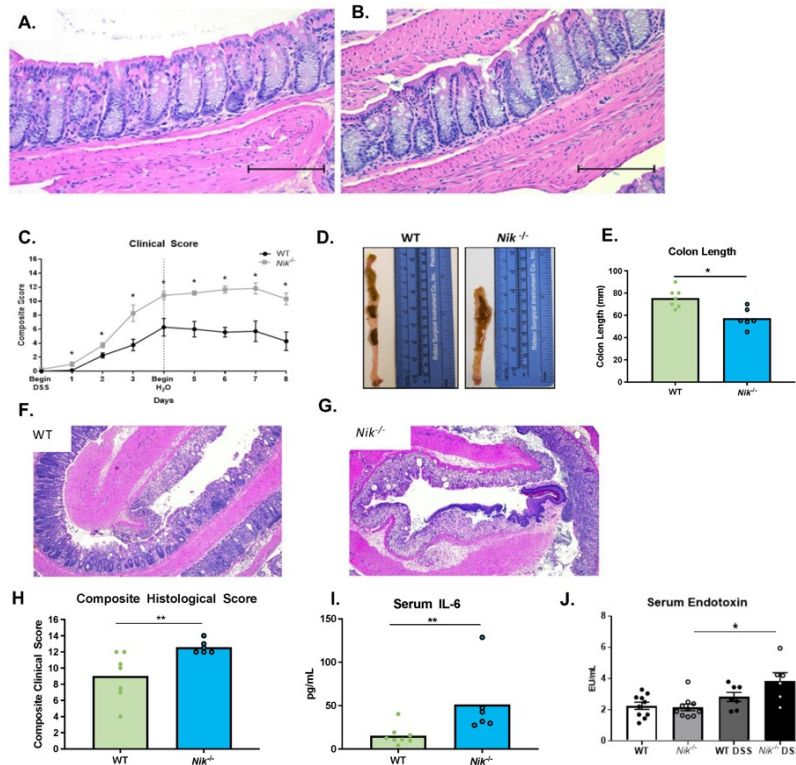


FIGURE 3: *Nik*^{-/-} Mice are Sensitive to DSS Induced Experimental Colitis. **A)** Wild-type mice and **(B)** *Nik*^{-/-} mice display almost identical architecture, with healthy crypts and lack of inflammation under unstimulated conditions at the time points evaluated in the AOM+DSS study. Scale bars represent 100 μm. **C)** *Nik*^{-/-} mice exposed to 5% DSS in the acute experimental colitis model demonstrated enhanced clinical features of disease progression (weight loss, blood in stool, stool consistency, body condition, and behavior) compared to wild-type mice. **D-E)** Colon length, a typical gross marker of inflammation and damage, was significantly decreased in the *Nik*^{-/-} mice. **F-G)** Histopathology assessments revealed increased inflammation in the *Nik*^{-/-} colons, including increased damage to the epithelial cell barrier, compared to wild-type. **H)** Semi-quantitative scoring of pathology, assessing inflammation, epithelial cell defects, dysplasia, hyperplasia,

which was translated into a composite score as previously described (1). **I)** Serum cytokine levels were evaluated by ELISA, with significant increases in IL-6 observed in the *Nik*^{-/-} mice compared to the wild-type animals. **J)** Serum endotoxin levels significantly increased in *Nik*^{-/-} mice following DSS exposure. n = 6; wild-type, n=7. *p ≤ 0.05; **p ≤ 0.01.

Figure 4

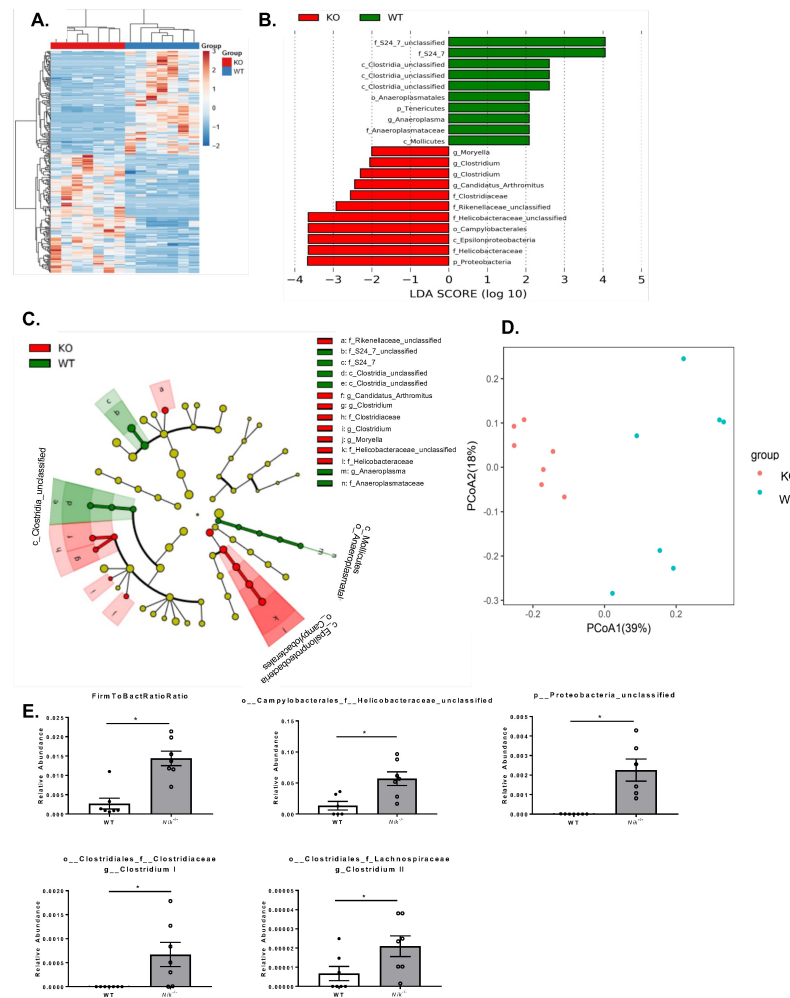


FIGURE 4: *Nik*^{-/-} mice display an altered microbiome with differential expression of species important to gut health. (A) The OTU heatmap shows a different expression pattern of bacteria taxa in the colonic contents of *Nik*^{-/-} mice versus wild-type littermates (KO = *Nik*^{-/-}, WT = wild-type). (B) The LDA score is a measure of bacterial species abundance. An LDA score of more than 2-fold-change is considered significant. *Nik*^{-/-} and wild-type mice exhibited different patterns of bacteria abundance, with particular emphasis on *Clostridia*, *Helicobacter*, and *Campylobacter*. (C) The phylogenetic tree reveals the relationships between different orders, families, and genera that were changed in the *Nik*^{-/-} and wild-type microbiomes. (D) PCA graph shows distinct clustering based on genotype. (E) Increased Firmicutes to Bacteroidetes ratio and unclassified bacteria in *Nik*^{-/-} mice. n = 7 mice per genotype. Significance was defined as p ≤ 0.05.

Figure 5

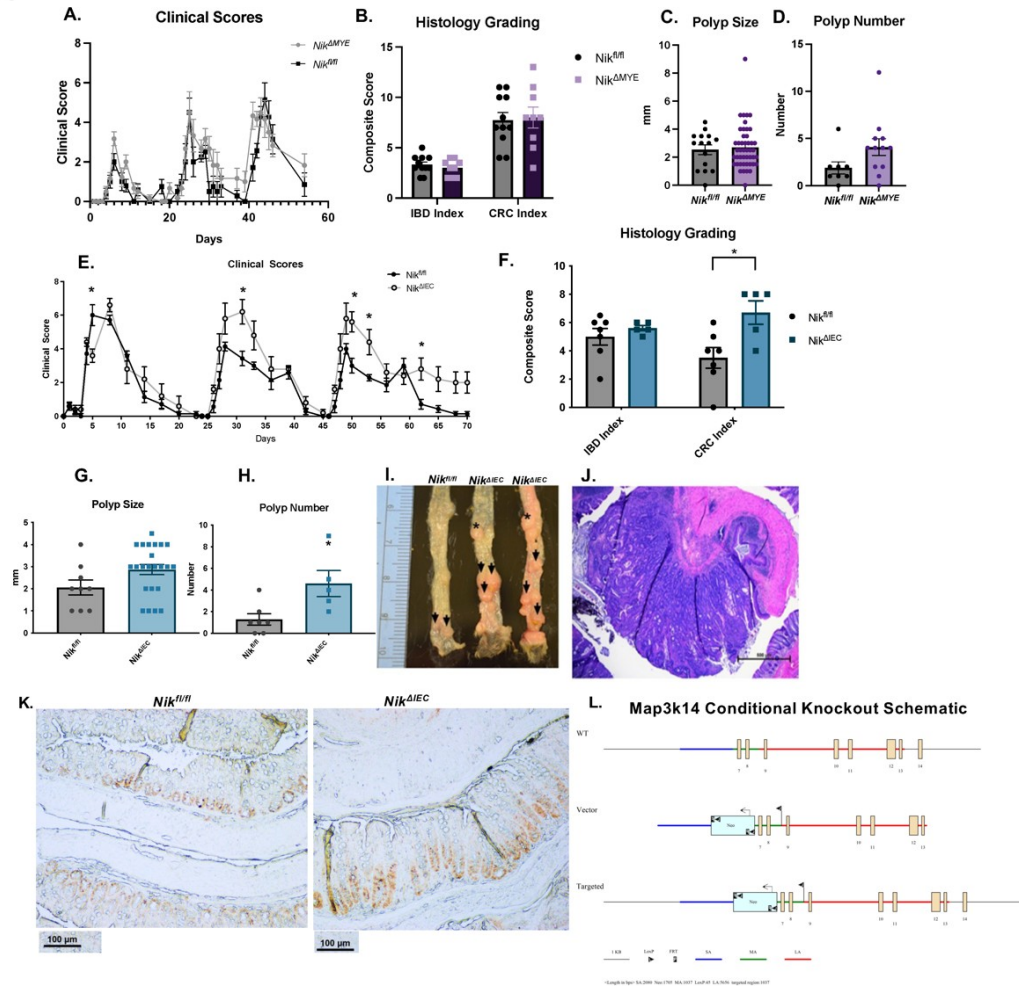


FIGURE 5: Intestinal epithelial cell specific NIK knockout (*Nik*^{ΔIEC}) mice display increased susceptibility to colorectal tumorigenesis. The mice were subjected to the AOM+DSS model of inflammation-driven tumorigenesis. **A)** Composite clinical scores were assessed throughout the AOM+DSS model for *Nik*^{ΔMYE} and *Nik*^{flx/flx} (littermate control) mice. **B)** Histopathology scoring of *Nik*^{ΔMYE} and control tissues at the completion of the model reflecting experimental colitis (IBD Index) and tumorigenesis (CRC Index). **C-D)** Gross assessments of macroscopic polyps from *Nik*^{ΔMYE} and control mice measuring **(C)** diameter and **(D)** number of polyps. **E)** Composite clinical scores from the *Nik*^{ΔIEC} and *Nik*^{flx/flx} (littermate control) mice. **F)** Histopathology scoring of *Nik*^{ΔIEC} and control tissues at the completion of the model. **G-H)** Gross assessments of macroscopic polyps from *Nik*^{ΔIEC} and control mice measuring **(G)** diameter and **(H)** number of polyps.

I) Gross examination of the colon further emphasized the increased tumor burden in the *Nik^{AIEC}* mice. Grossly, polyps were large, raised, smooth to slightly cauliflower-like projections from the mucosa (**arrowheads**) that were typically confined to the distal colon in the *Nik^{flox/flox}* littermates, but were found up to the level of the transverse colon in the *Nik^{AIEC}* mice (**asterisks**). **J)** Histologically, all polyps were determined to represent well-differentiated adenocarcinomas. Histology scale bar = 500µm. n = 5-7 mice per group. **K)** Representative images of immunocytochemistry staining for Ki67 in *Nik^{flox/flox}* (left) and *Nik^{AIEC}* (right) colons. Increased staining extending towards top of crypt observed in *Nik^{AIEC}* colons. Schematic illustrating the key components of the targeting construct used to insert loxP sites flanking key exons in the kinase domain of the *Map3k14* gene that encodes NIK, ultimately generating *Nik^{fl/fl}* mice. Mice carrying the floxed alleles appear to be phenotypically normal prior to crossing with cre expressing animals, with no detrimental effects noted to date. *p ≤ 0.05.

Figure 6

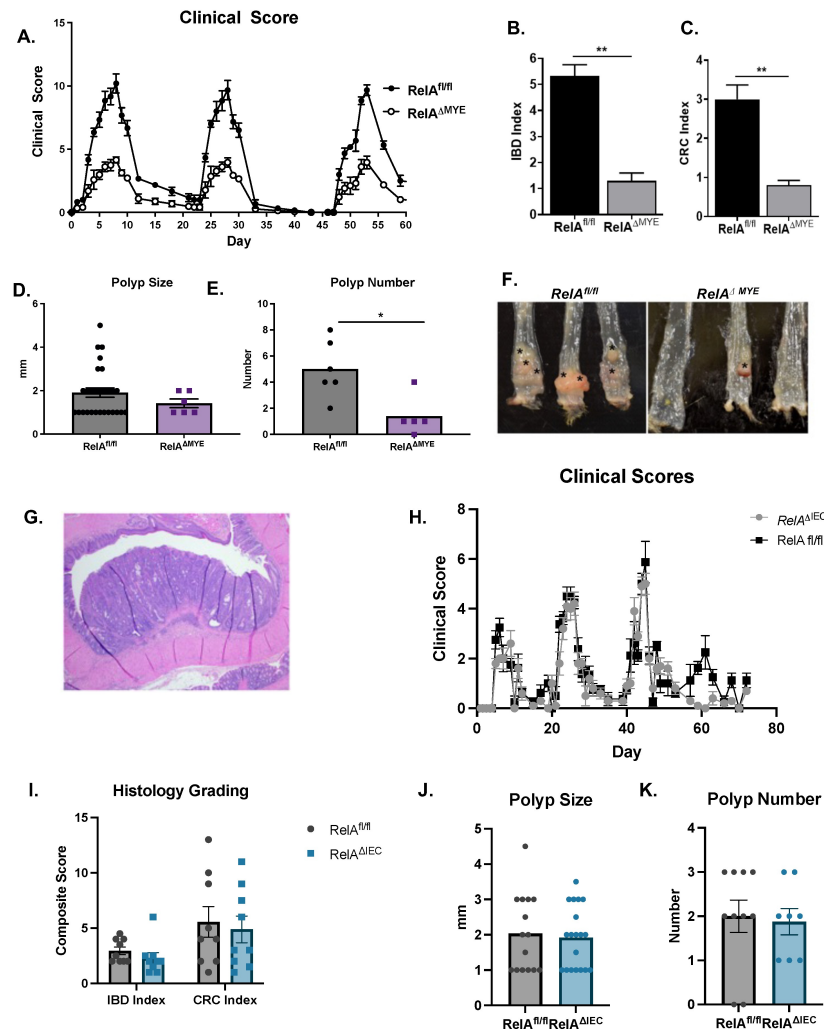


FIGURE 6: Myeloid cell specific RelA knockout (*RelA^{ΔMYE}*) mice display attenuated colorectal tumorigenesis. The mice were subjected to the AOM+DSS model of inflammation-driven tumorigenesis. **A)** Composite clinical scores comprised of weight loss, fecal consistency, rectal bleeding, and behavior evaluations were assessed throughout the AOM+DSS model for *RelA^{ΔMYE}* and *RelA^{fl/fl}* (littermate control) mice. **B-C)** Histopathology scoring of *RelA^{ΔMYE}* and control tissues at the completion of the model reflecting experimental colitis (IBD Index) and tumorigenesis (CRC Index). **D-F)** Gross assessments of macroscopic polyps from *RelA^{ΔMYE}* and control mice measuring **(D)** diameter and **(E)** number of polyps. **F)** Gross examination of the colon further emphasized the decrease in tumor burden in the *RelA^{ΔMYE}* mice. Grossly, polyps from the *RelA^{ΔMYE}* mice were seldom observed. All polyps in both genotypes were confined to the

distal colon (**asterisks**). **G**) Histologically, all polyps were determined to represent well-differentiated adenocarcinomas. **H**) Composite clinical scores from the *RelA^{ΔIEC}* and *RelA^{lox/lox}* (littermate control) mice. **I**) Histopathology scoring of *RelA^{ΔIEC}* and control tissues at the completion of the model reflecting IBD Index and CRC Index. **J-K**) Gross assessments of macroscopic polyps from *RelA^{ΔIEC}* and control mice measuring (**J**) diameter and (**K**) number of polyps. Histology scale bar = 500μm. n = 5-7 mice per group. *p ≤ 0.05.

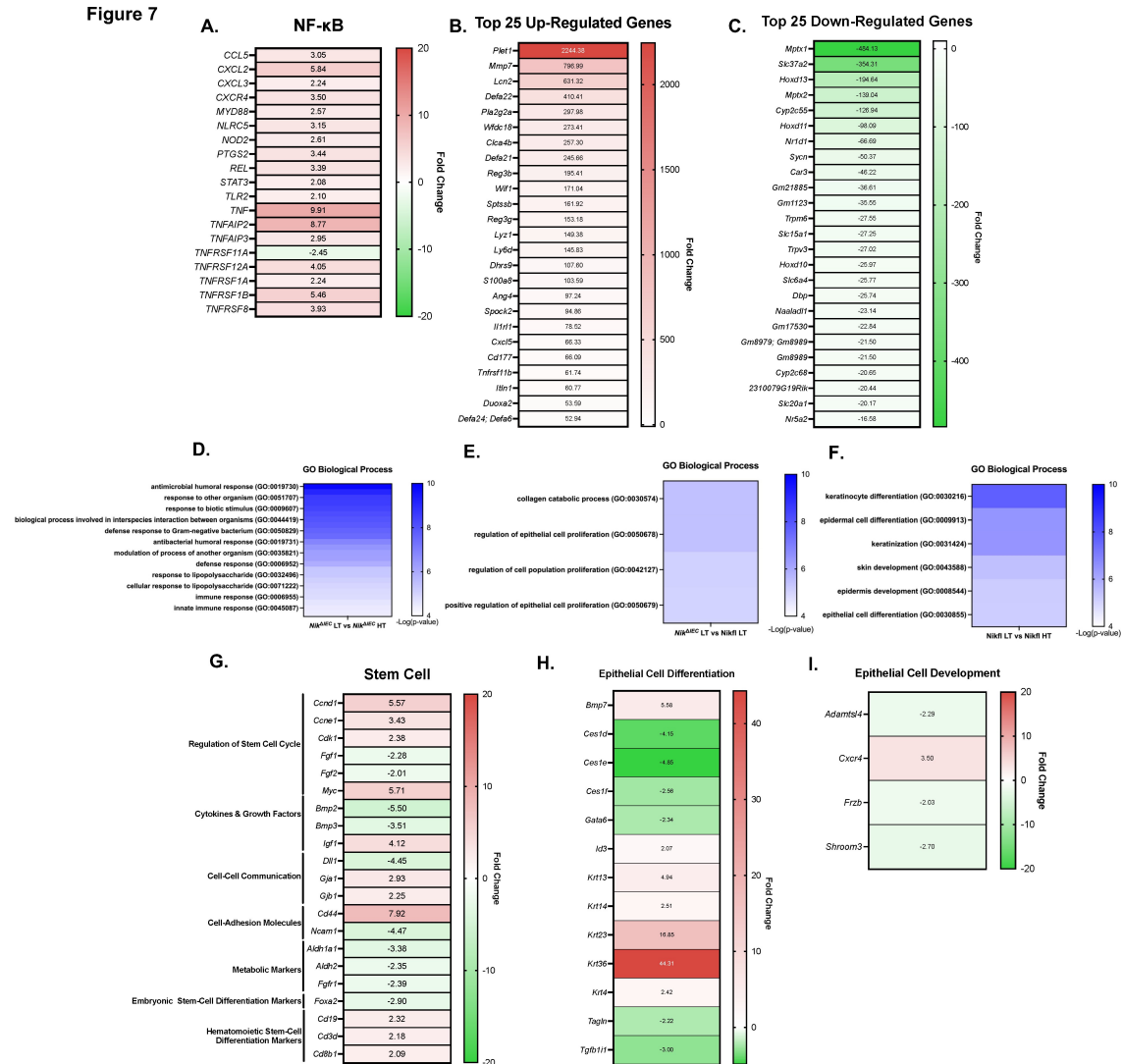


FIGURE 7. Gene Expression Signature Following Loss of NIK Reveals Altered Differentiation-Regeneration Profile. Gene expression profiles for **A)** NF- κ B signaling, **B)** Top-25 up-regulated genes, and **C)** Top 25 down-regulated genes. **D-F)** Altered biological functions predicted by Gene Ontology (GO) given unique transcriptome profiles. **D)** *Nik^{ΔIEC}* lesions are predicted to have increased defense responses to bacterial infection. **E)** *Nik^{ΔIEC}* lesion compared to *Nik^{ΔJ1}* lesion tissue predicted to have altered regulation of epithelial cell proliferation. **F)** *Nik^{ΔJ1}* lesion tissue collected from control mice is characterized by keratinocyte/epidermal cell/epithelial cell differentiation biological processes. Gene expression profiles for **G)** Stem Cell Functions, **H)** Epithelial Cell Differentiation, and **I)** Epithelial Cell Development. n = 3 mice/group selected

randomly from AOM+DSS study performed in **Figure 5**. Significant values ≥ 2 (red) and ≤ 2 (green) reported as fold-change. Not significant values (white) in heatmap.

Figure 8

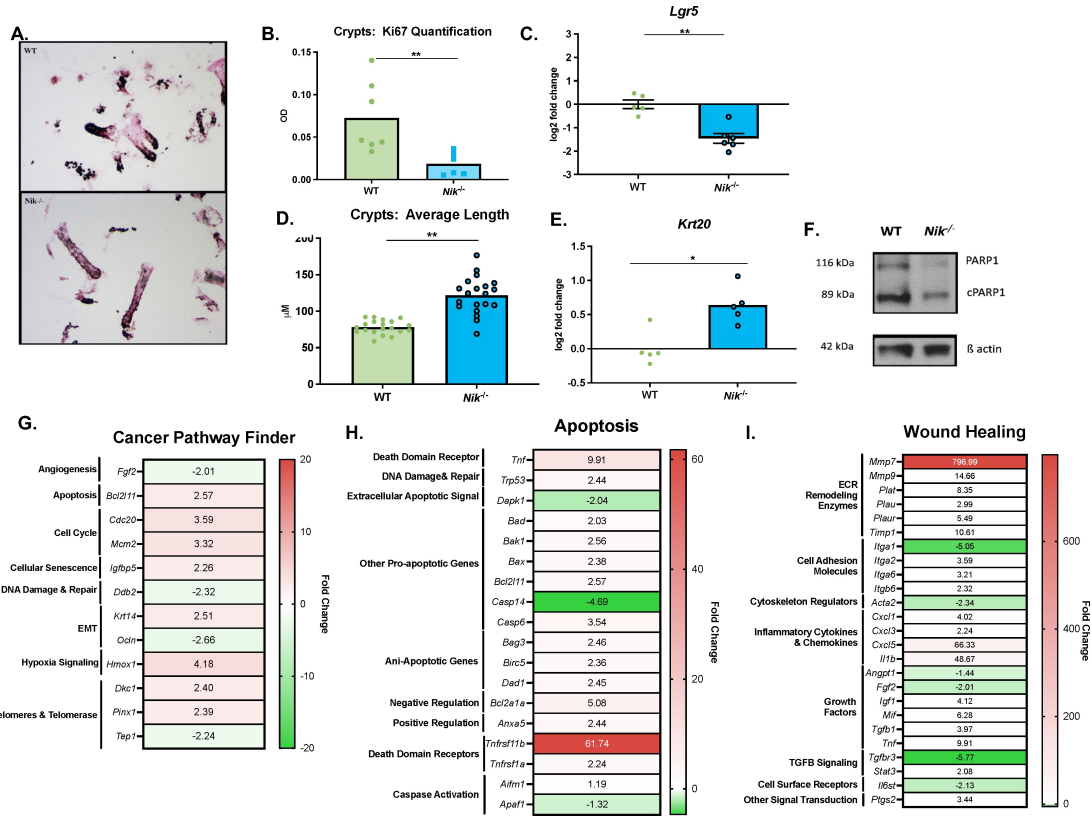


FIGURE 8: Colonic Crypts from *Nik*^{-/-} Mice Display Decreased Levels of Stem Cell Proliferation and Mature Intestinal Epithelial Cell Death. A-B) Immunocytochemistry of wild-type and *Nik*^{-/-} crypts revealed decreased Ki-67 expression in the crypt base. C) *Lgr5* gene expression was significantly attenuated in the *Nik*^{-/-} crypts. D) Crypts from the *Nik*^{-/-} animals were significantly elongated compared to those from wild-type mice. E) *Krt20* gene expression was significantly increased in the *Nik*^{-/-} crypts. F) Western Blot analysis of the crypt fractions revealed decreased mature and cleaved poly-ADP ribose (PARP) levels in the *Nik*^{-/-} crypts compared to wild-type. G-I) Transcriptome analysis reveals altered gene signature for G) Cancer Pathways, H) Apoptosis, and I) Wound Healing. n = 8 mice per group for crypt analysis. For Ki-67 quantification, each data point represents the average of 10 crypts from 8 mice of each

genotype. For Western Blot, image is representative of 4 animals of each genotype. * $p \leq 0.05$; ** $p \leq 0.01$. For transcriptomic data, $n = 3$ mice/group selected randomly from AOM+DSS study performed in **Figure 5**. Significant values ≥ 2 (red) and ≤ 2 (green) reported as fold-change. Values that did not reach significance (white) in heatmap.

Figure 9

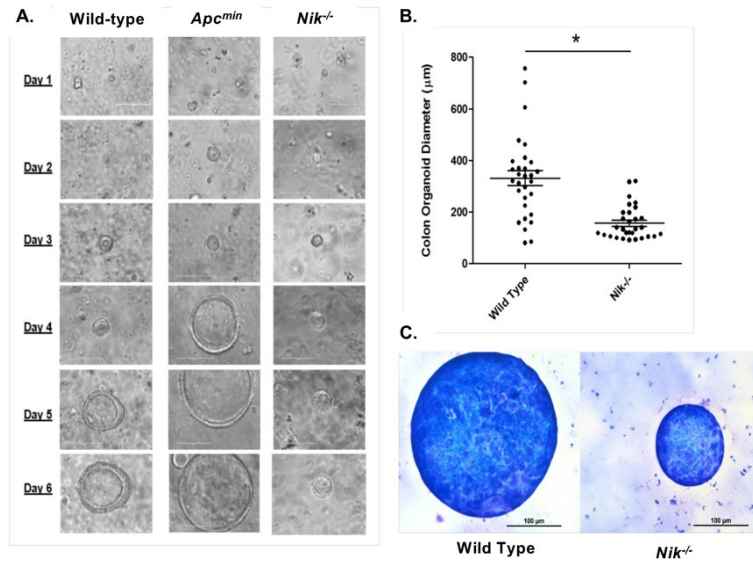


FIGURE 9: Colonic Organoids from *Nik^{-/-}* Mice Demonstrated Attenuated Growth and Proliferation. Colonic crypts were isolated and reduced to a single-cell suspension from wild-type, *Nik^{-/-}*, and *Apc^{min}* mice and grown in culture for 6 days, without passage. **A)** Organoids from *Apc^{min}* mice displayed typical overzealous proliferation, while wild-type organoid growth was steady and highly uniform. *Nik^{-/-}* organoids remained small compared to both wild-type and *Apc^{min}*. **B)** Blinded diameter measurement of randomly chosen organoids from wild-type and *Nik^{-/-}* mice. **C)** Organoids were manually disassociated from Matrigel and stained cytologically with Diff-Quik to evaluate morphology (wild-type/left; *Nik^{-/-}*/right) despite the difference in size. Data represents the

average of three independent trials. Measurements were taken from 30 randomly chosen organoids spread over a total of 12 wells. *p $\leq$ 0.05.

CHAPTER SEVEN

Conclusions and Future Directions

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Overcoming the long withstanding misconception that the noncanonical NF- κ B signaling pathway is merely an auxiliary or “alternative” pathway to its canonical counterpart, we provide evidence in this culmination of works that the noncanonical NF- κ B signaling pathway is a critical signaling axis in maintaining gastrointestinal health. Proper noncanonical NF- κ B signaling and stabilization of the NF- κ B-inducing Kinase (NIK) is integral to holding in check the balance of pro-inflammatory versus anti-inflammatory signaling, apoptotic versus proliferative signaling axis, and differentiation versus de-differentiation signaling mechanisms. Yet again, our laboratory has highlighted another “Goldilocks Conundrum” (2). Here, we describe the delicate balance in which NIK and noncanonical NF- κ B signaling must be regulated to prevent the development of various chronic inflammatory enteropathies. Within this dissertation, we found that the regulation of noncanonical NF- κ B signaling is both tissue-specific and cell-specific in maintaining gastrointestinal health.

In regard to tissue-specific regulation of the gastrointestinal system, dysregulated noncanonical NF- κ B signaling in the large intestine rather than the esophagus/small intestine contributes to disease development. In **Chapter Two**, we found that noncanonical NF- κ B signaling is not dysregulated at a gene expression level in human Eosinophilic Esophagitis (EoE) biopsy samples and therefore does not contribute to EoE, an inflammatory disorder of the upper gastrointestinal tract. This is contrary to earlier works which highlight a spontaneous hypereosinophilic syndrome-like mouse model bearing deletion of *Map3k14*, the gene that encodes for NIK, as a potential model for EoE (3, 4). Although similar characteristics of disease progression is apparent in the murine model following NIK-deletion, loss of the noncanonical NF- κ B pathway has yet to be previously evaluated in human EoE. Work here diminishes the potential of NIK/noncanonical NF- κ B signaling as a potential biomarker/drug target for this disease of interest. This finding is also consistent with the data presented in **Chapter Three**, which investigates the plausible roles of canonical and noncanonical NF- κ B in a mouse

model for Celiac Disease (CeD)/Non-Celiac Gluten Sensitivities (NCGS) following administration of a gluten-free diet (GFD). Contribution of specific NF- κ B pathways were evaluated in murine models bearing deficiencies in either the negative regulatory NLRs NLRX1 or NLRP12 that correspond to canonical and noncanonical NF- κ B respectively. In this study, the gut microbiomes of *Nlrp12*^{-/-} and *Nlrp12*^{-/-} mice were assessed for a genetically inherent dysbiosis. NLRX1-deficient mice were characterized as having a dysbiotic gut that is further exacerbated by GFD, while the microbiome of NLRP12-deficient mice was relatively unchanged compared to control animals. This work reveals overzealous canonical NF- κ B signaling may contribute to, if not only exacerbate, immune responses in CeD/NCGS, which is clinically relevant as *NLRX1* was the only NLR found to be downregulated in a metadata analysis of human CeD patients with active disease.

While noncanonical NF- κ B signaling was not found to be significant in diseases of the upper gastrointestinal tract and the small intestine, prominence of this pathway took precedence in chronic inflammatory disorders of the large intestine. We evaluate this in Inflammatory Bowel Disease (IBD) in **Chapters Four and Five** and Colitis-Associated Colorectal Cancer (CAC) in **Chapter Six**. Intriguingly, both over abundant NIK levels (i.e. IBD) and deficits in NIK levels (i.e. CAC) contribute to disease pathogenesis of the large intestine, further demonstrating the spatial involvement of this pathway in the GI system. In **Chapter Four**, we reexamined the role of negative regulatory NLRs in mitigating inflammation by inhibiting NF- κ B signaling and that dysregulation contributes to Inflammatory Bowel Disease. Fundamentally, current literature examines how diminished activity of negative regulatory NLRs results in increased downstream NF- κ B-mediated pro-inflammatory signaling. NLRC3 and NLRX1 negatively regulates canonical NF- κ B, while NLRP12 is uniquely poised to inhibit both canonical and noncanonical NF- κ B pathway. All three of these NLRs are diminished in colitis and colitis-induced tumorigenesis. This, therefore, confers dysregulation of immune responses, dysbiosis of the gut, and activation of further downstream pro-inflammatory and proliferative cellular signaling mechanisms like mTOR, STAT3, MAPK, and ERK (5-9). This is further reinforced in **Chapter Five**, where we found increased noncanonical NF- κ B signaling is associated with loss of drug

responsiveness to anti-TNF agents. In this gene expression analysis of human colonic biopsies, we found several genes related to noncanonical NF- κ B signaling to be upregulated in IBD patients, and even further exacerbated in patients unresponsive to anti-TNF therapeutics. This work concludes with the promise that *CXCL12* and *CXCL13*, two of four chemokines produced by noncanonical NF- κ B signaling, may be promising candidates for biomarkers in predicting drug responsiveness to anti-TNF therapeutics.

Given that a medical history of IBD predisposes patients to developing an inflammatory-mediated subtype of colorectal cancer termed “Colitis-Associated Colorectal Cancer” (CAC) and noncanonical NF- κ B is upregulated in colitis/IBD (8-13), NIK is poised as a potential candidate for targeting overzealous inflammation and inhibiting further transduction of the noncanonical NF- κ B signaling pathway. Foundational work by Allen *et al.* 2012 is precedent for this field, as they found that loss of NLRP12 in a murine model for colitis and colitis-induced tumorigenesis is driven by excessive noncanonical NF- κ B signaling. **Chapter Six** serves as a continuation of this study by assessing the effects of diminished noncanonical NF- κ B signaling following loss of NIK in a murine model for CAC. We originally hypothesized that loss of NIK protects against colitis/colitis-induced tumorigenesis. To our surprise, whole-body deletion of NIK increased susceptibility to tumorigenesis. We further assessed the cell compartment driving tumorigenesis and found that deletion of NIK in intestinal epithelial cells (*Nik^{fl/fl} x Villin^{Cre}*) had increased clinical severity and tumor burden in the colon, whereas deletion of NIK in myeloid cells (*Nik^{fl/fl} x LysM^{Cre}*) were unchanged. This functionality is unique to this pathway, as RelA in myeloid cells (*RelA^{fl/fl} x LysM^{Cre}*) drives tumorigenesis. In this work, we further provide more mechanistic studies through the evaluation of crypt morphology and capacity of intestinal stem cells to generate organoids. Crypts isolated from NIK-deficient mice were elongated and composed of mature enterocytes with significantly decreased Ki67 for proliferation and *Lgr5* expression (marker for intestinal stem cells). Although these mice were more predisposed towards inflammation-induced tumorigenesis, they were not inherently proliferative. This is further evidenced by their stunted organoid growth, indicating loss of NIK decreases proliferative intestinal stem cell activity. These findings are seemingly contradictory to traditional tumor biology, where cancer is characterized as a *hyper*proliferative disorder.

However, our work further reinforces this paradigm shift, in which cancer biology is not always rooted in a stem cell origin. Instead, there is an emerging interest in de-differentiation of terminally differentiated cells to a progenitor-state, which may be observed here (14-17). The third rendition of the Hallmarks of Cancer updated by Douglas Hanahan in 2022 features “Unlocking Phenotypic Plasticity” as an emerging hallmark, where they highlight colorectal cancer as an example where both hyperproliferative stem cells and dedifferentiation of not yet committed epithelial cells have been characterized as originating cell populations (18). Although further mechanistic studies are necessary to fully demonstrate that intestinal epithelial cells are indeed de-differentiating, we propose an elusive model for such studies suggesting loss of NIK driving this phenomena. Concluding this work, we highlight NIK as a promising biomarker for disease detection and also a candidate target for drug development in colitis and CAC.

However, an indiscriminate downregulation of NIK in all tissues and cell-types for targeting IBD and CAC, as we see in **Chapter Six**, would be a naïve assertion. Rather, evidence provided in this thorough investigation of NIK and noncanonical NF- κ B signaling in gastrointestinal health and related inflammatory diseases emphasizes a precise balance of such signaling is dependent on several factors, including tissue/organ, cell-type, and disease status. Regulation of NIK/noncanonical NF- κ B signaling is, therefore, contingent on the precise microenvironment exacerbating these chronic inflammatory disorders. Further, our work pushes this field of study forward by pinpointing where in the gastrointestinal tract such attention should be focused in harnessing the potential of regulated noncanonical NF- κ B signaling. Likewise, we demonstrate that this previously understudied signaling pathway has untapped potential - we are only skirmishing its contributions to regulating not only gastrointestinal health but also the various biological functions (esp. de-differentiation) that have yet to be associated with this pathway.

Having dedicated almost exactly five years of research to this under-appreciated, under-studied, and under-utilized signaling pathway, one of my ultimate goals during my PhD has been to unveil its hidden potential and reassess its overshadowed contributions to the assortment of previously well-defined diseases of the gastrointestinal system. I am

grateful for the opportunity to have contributed to this field of research by collaborating with medical doctors, who have also shared our enthusiasm for this research. By working with clinicians, we were able to harness this passion to publish these works in the hopes it will contribute to advancing patient care by improving biomarker development for diseases/drug responsiveness, pinpointing the tissues/cell-types affected by dysregulated signaling in various disease contexts, and highlighting NIK as a candidate target for drug development.

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APPENDIX A

Complete List of Published Works

1. **Morrison HA**, Trusiano B, Rowe AJ, Allen IC. Negative Regulatory NLRs Mitigate Inflammation via NF- κ B Pathway Signaling in Inflammatory Bowel Disease. *Biomed J.* 2023 Jun 13;100616. doi: 10.1016/j.bj.2023.100616. Epub ahead of print. PMID: 37321320.
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