The Dissertation Committee for Feidi Chen Certifies that this is the approved version of the following Dissertation

Neutrophil Regulation of Colitis

Committee:

Yingzi Cong, Ph.D., Supervisor or Mentor

Jere W. Mcbride, Ph.D

Iryna Pinchuk, Ph.D.

Jiaren Sun, MD, Ph.D.

Haining Shi, Ph.D

Neutrophil Regulation of Colitis

by

Feidi Chen, M.D.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

November 2017

Dedication

My doctoral dissertation is dedicated to my mum, Xiaomin Zhou

Acknowledgements

I would like to thank my mentor Dr. Yingzi Cong for his guidance and support. Also I would like to thank my thesis committee Dr. Jere W. Mcbride, Dr. Iryna V. Pinchuk, Dr. Jiaren Sun and Dr. Haining Shi for all their helpful discussions and guidance. I thank the chairman of department of Pathology, Dr. Michael Laposata, my program advisors, Dr. Jere W. Mcbride, along with the experimental program coordinator, Paula L. Gabriles, for their continued support and encouragement. I would like to acknowledge current and past members of the Cong laboratory, particularly Dr. Anthony Cao, as well as the Soong laboratory, the Sun laboratory, and the Dann laboratory. Lastly, I would like to thank my friends and family for their support.

Neutrophil regulation of colitis

Publication No._____

Feidi Chen, Ph.D

The University of Texas Medical Branch, 2017

Supervisor: Yingzi Cong

The delicate intestinal homeostasis is regulated by interactions between microbiota, intestinal epithelial cells (IECs), and the intestinal immune system. As a crucial component of the innate immune system, neutrophils are the first responders to sites of inflammation when the intestinal epithelial barrier is breached and gut microbiota invade. Although accumulation of neutrophils in the intestine is correlated with inflammatory bowel disease (IBD), the relative contributions of neutrophils to the pathogenesis of IBD are still controversial.

It has been shown that neutrophils are active producers of both IL-22 and IL-17, which differentially regulate the pathogenesis of IBD. Our current studies indicate that IL-22-producing neutrophils play a crucial role in colitis. We showed that IL-23 can promote neutrophil production of IL-17 and IL-22. IL-23 signals through its receptors and further upregulates its own receptors. Subsequently, IL-23 activates the PI3K-mTOR pathway, which mediates the expression of retinoid acid receptor–related orphan receptor g t (RORγt) and aryl-hydrocarbon receptor (AhR). Neutrophils can also produce TGFβ to regulate IEC

functions. Neutrophil-derived TGFβ activates MEK1/2 signaling in IECs enhancing the production of amphiregulin (AREG), a member of EGFR ligand family. Upon the induction of an intestinal inflammatory state, AREG signals through the EGFR pathway to induce epithelial junction formation, as a way to restore epithelial function and ameliorate colitis.

Taken together, our studies reveal a previously unappreciated immune regulatory role of neutrophils in coordination with IECs to maintain and/or restore gut homeostasis. Given further research, these findings could ultimately lead to the development of novel targeted treatments for IBD and other autoimmune diseases.

TABLE OF CONTENTS

List of Figures

List of Abbreviations

APC	Antigen-presenting cell
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
AREG	Amphiregulin
BM	Bone marrow
BMI	Body mass index
C2	Acetic acid
C3	Propionic acid
C4	Butyric acid
CBA	Cecal bacterial antigens
CGD	Chronic granulomatous disease
CM	Neutrophil conditioned media
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
FACS	Fluorescence-activated cell sort
FBS	Fetal bovine serum
fMLF	Formyl-methionyl-leucyl phenylalanine
Foxp3	Forkhead box protein p3
FUT2	Fucosyltransferase 2
GALT	Gut-associated lymphoid tissue

GF	Germ free
GPR	G-protein coupled receptors
HDAC	Histone deacetylases
HIF	Hypoxia-inducible factor
i.v.	Intravenous
i.p.	Intraperitoneal injection
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin-
ILC	Innate lymphoid cell
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MMP	Matrix metalloproteases
MUC2	Glycoprotein mucin-2
mTORC1	mTOR complex 1
NETs	Neutrophil extracellular traps
NOD2	Nucleotide-binding oligomerization domain-containing protein
PCR	Polymerase chain reaction

pIgR	Polymeric immunoglobulin receptor
PMN	Neutrophil
RA	Retinoic acid
Rag	Recombination activating gene
RNA	Ribonucleic acid
RORγt	Retinoic acid-related orphan receptor gamma t
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SFB	Segmented filamentous bacteria
SNP	Single-nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Tg	Transgenic
TGF	Transforming growth factor
Th-	T helper cell
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulphonic acid
TNF	Tissue necrosis factor
Treg	regulatory T cell
WT	Wild type

INTRODUCTION

Chapter 1: Overview of the Intestinal Homeostasis

The gastrointestinal tract represents the largest mucosal surface in the body and harbors trillions of diverse microorganisms, namely microbiota [1]. Tight regulation of the mutualistic interaction between microbiota and host is required for proper digestion, provision of energy and nutrition, as well as shaping of the mucosal immune system. A single layer of intestinal epithelial cells (IECs) constitutes the first line barrier defense, sequestering microbiota in the lumen and preventing the over activation of immune responses. While microbiota and its metabolites can signal through various receptors on IECs to affect mucosal microenvironment [2], our host employs multiple mechanisms to regulate microbiota in return [2-7]. Both innate and adaptive immune responses are involved in regulation of host response against microbiota to maintain homeostasis of the gut.

MICROBIOTA

To date, over 1000 strains of microbiota have been identified to reside in the human gut. Over the course of coevolution, microbiota and host achieved mutualistic symbionts through a tightly regulated crosstalk. The presence of normal gut microbiota benefits the host for proper nutrient absorption, xenobiotic metabolism as well as immune adaption and modulation [8]. In healthy individuals, Bacteroidetes and Firmicutes are the dominate bacterial species in the gut along with other less abundant species such as Proteobacteria, Actinobacteria, Verrucomicrobia and Fusobacteria [9]. The composition of the gut microbiome is affected by multiple factors including age, genetics, lifestyle, body mass index (BMI), health state and antibiotic treatment [10].

In the context of intestinal disorders such as inflammatory bowel disease (IBD), dysbiosis has a major impact on the host immune system resulting in the perpetuation of intestinal inflammation. IBD-associated dysbiosis is characterized by loss of commensal microbiota, loss of diversity and occupation of pathobionts [11].

The gut microbiome is indispensable in for host nutrition. Synthesis of a variety of vitamins require the presence of microbiota [12]. Neonates lacking the colonization of commensal bacteria are susceptible to intracranial hemorrhage due to vitamin K deficiency. Microbiota also help digests food, turning them into products, such as short chain fatty acids (SCFAs). Among all microbiota-derived metabolites, SCFAs have been intensively studied in the past few decades. Acetate (C2), propionate (C3), butyrate (C4) are the three major subtypes of SCFAs. Derived from fiber, C4 is one of the most abundant metabolites found in the colon (3 fold higher than C2 and C3) [13]. SCFAs can modulate host immunity through several pathways. Their lipophilic nature allows for direct diffusion into cells to inhibit histone deacetylases (HDAC) [14]. Accumulating evidence shows that the microbiota can mediate regulatory T cell (Treg) differentiation and through its actions on HDAC [15]. SCFAs can also activate G-protein coupled receptors (GPRs) [16]. We have demonstrated that SCFAs promote IEC barrier defense through GPR43.

IEC

The intestinal epithelium covers a surface area of over 300 m^2, which is adapted to the constant insult from external stimulants as well as enteric microbiota. Breach of epithelial barrier leads to microbial translocation into the mucosa and submucosal areas, and excessive uptake of microbial antigens can induce exaggerated immune responses. Rapid turnover of IECs (3–4 days in mice, 7-10 days in human) allows for quick restoration of intestinal integrity by replacing the

damaged cells [17]. Goblet cells produce a thick layer of mucus to prevent the microbiota from attaching to IECs. Also, the mucus layer provides necessary energy for commensal microbiota. Notably, the invaginated structure of villi and the consumption of microbial metabolites by colonocytes limit the microbial signals as a way to restrict butyrate suppressive effect on intestinal epithelial stem cells [18]. IECs and Paneth cells can also produce antimicrobial peptides (AMPs) to control luminal microbiota. Several distinct families of AMPs have been identified including defensins, cathelicidins, C-type lectins (such as the regenerating islet-derived protein (REG) family). AMPs function to kill or inactivate microorganisms rapidly through various mechanisms, such as disrupting membranes, digesting specific cell wall structures, and sequestering nutrients.

INTESTINAL IMMUNE SYSTEM

Accumulating evidence indicates that the host immune system can sense gut bacterial metabolites in addition to TLR ligands. Recognition of these small molecules greatly impacts host immune response [13]. Th17 cells can upregulate polymeric Ig receptor (pIgR) on IECs as a way to promote intestinal IgA response to microbiota, thus, contributing to maintenance of intestinal homeostasis [4]. Innate lymphoid cells (ILCs) are capable of producing IL-22 to exert beneficial effects on IECs. Recently, type 2 ILCs has been demonstrated to produce amphiregulin (AREG) in response to IEC alarm signals [6, 19]. It is becoming clear that neutrophils are indispensable in intestinal homeostasis as evidenced by the massive infiltration of neutrophils in the inflamed site of the intestines [20].

CHAPTER 2: INFLAMMATORY BOWEL DISEASE

As one of the five most prevalent gastrointestinal disease burdens in the United States, inflammatory bowel disease (IBD) affects more than 1.6 million people in the country [21], and is becoming a global disease with increasing incidence and prevalence rate over time [22]. To date, IBD is incurable with few efficacious treatments; even with current treatments, IBD largely compromises patients' quality of life and causes tremendous economic loss. IBD represents a group of immunological disorder characterized by idiopathic chronic and relapsing inflammation of the gastrointestinal tract. Besides the systemic symptoms such as low-grade fever, weight loss, sweats, malaise and fatigue, patients exhibit both intestinal symptoms (diarrhea, abdominal cramping, bloating, urgency and rectal bleeding) [23] and extra-intestinal manifestations affecting musculoskeletal, cardiovascular, ocular and hepatobiliary systems [24]. Based on the distribution, endoscopic, radiological and histopathological features of the inflammatory lesions, the diagnosis of IBD falls into two major categories, Crohn's Disease (CD) and ulcerative colitis (UC) [25]. The two diseases are considered distinct entities, with different etiologies and pathogenesis which are not completely understood. Inflammation in CD patients is discontinuous, which is characterized by cobble stone-like lesions affecting any part of gastrointestinal tract, and most frequently found in the ileum and colon. The lesions involve whole layers of intestinal tube which explain the typical complications such as fistulae, fissures and strictures [26]. Increased expression of IL-17 and IL-23 has been found in CD lesions [27, 28]. IL-22 was also increased in the active lesions of CD, and increased serum levels of IL-22 has been shown to correlate with disease activity [29]. Conversely, lesions of UC are confined to mucosal and submucosal layer, affecting descending colon and rectum. IL-17 and IL-22 were also found to be increased in UC patients compared to

healthy controls, although to a lesser degree than in CD lesions [27, 28]. Importantly, patients who do not meet either of diagnostic criteria are classified as a third subtype, IBD-undetermined type.

PATHOGENESIS

The pathogenesis of IBD involves complex interplay between genetic, environmental, microbial and immunological factors. It is generally believed that environmental triggers compromise the integrity of intestinal epithelial barrier, resulting in translocation of microbiota into mucosa and submucosa area. In addition, altered commensal microbiota composition induces susceptibility of IBD [30]. Exaggerate microbial exposure as well as aberrant immune responses may perpetuate inflammation in genetic susceptive individual [31]. Based on genome-wide association studies (GWAS), various polymorphisms in genes have been identified associated with IBD. Among the 48 IBD risk genes that related to commensal microbiota, NOD2 was the first gene identified as a susceptibility gene conferring risk of IBD, especially CD [9]. NOD2 knockout mice are more susceptible to colitis, with lower levels of AMPs and higher bacterial load compared with control mice [32]. NOD1, on the other hand, is more closely associated with UC [33]. Moreover, CLEC7A encoding C-type lectin Dectin-1 has been linked to refractory UC [34]. Single-nucleotide polymorphisms (SNPs) of autophagy-related genes for example NOD2, ATG16L1 and IGRM1 disrupt the delicate host-microbiota balance, affecting microbiota composition, which predispose host to dysbiosis. Polymorphisms in genes related to normal IEC functions are also associate with the development of IBD. Glycoprotein mucin-2 (MUC2) is critical for mucus production, and SNPs in this gene results in thinner mucus layer and subsequent increased pathogen invasion [35]. HNF4α, which induces the formation of junction proteins has been identified to associate with an increased risk of IBD [36]. IECs also express EGFR, and

increased intestinal ERRFI1 inhibiting EGFR pathway has been shown in both CD and UC patients [37]. Finally, GWAS have linked IL-23 and TGFβ pathways to IBD, including IL-23R, RORC, AHR, STAT3, SMAD3 and SMAD7 [38].

CHAPTER 3: NEUTROPHILS IN IBD

Preserving the delicate balance between microbiota and host requires rapid elimination of invading microbes. As a crucial component of innate immunity, neutrophils play a fundamental role in the successful first line defense against pathogens. Neutrophils are usually absent from intestinal mucosa under physiological conditions. In the setting of acute inflammatory state, however, neutrophils immediately present to the inflamed sites, where they aid in antimicrobial activity through direct phagocytosis, generation of phagocytose reactive oxygen species (ROS), production of different elastase, MMPs and cytokines. Furthermore, neutrophils are capable of liberating neutrophil extracellular traps (NETs) to imprison larger microbes. It is conceivable that neutrophils may play a crucial role in the development, remission and relapsing of IBD. Neutrophils represents the most abundant leukocyte in the circulation (50-70% in humans, 10-25% in mice) [39]. They are considered short-lived cells with an average of half-life of 8-20 h in human circulation. Interestingly, recent data suggests that their circulatory lifespan can be up to 5 days in healthy state [40].

NEUTROPHIL FUNCTIONS

Reactive oxygen species (ROS), Matrix metalloproteases (MMPs) and Elastase

Pathogen invasion and translocation leads to the acute colitis in the intestines. Without proper elimination of the microbial insults, inflammation perpetuate to chronic and persistent state which contributes to the development of IBD. It has been reported that 80% patients with CD have detectable *Escherichia coli* DNA in the granulomas biopsy [41]. Neutrophils almost exclusively contain enzymes for ROS generation. ROS are reactive oxygen metabolites which are capable of

dysrupting membrane-associated targets and degrading microbial DNA. Defect in ROS pathway can have detrimental outcomes and predispose individual to severe infection. Patient with CGD are more prone to catalase positive microbes due to lack of NADPH oxidase, an critical enzyme in the ROS pathway [42]. MMP-9 (gelatinase B) is found in the granules of neutrophils, which aids in chemokine and cytokine shedding. Furthermore, ADAM17 is known for releasing membrane-bound tumor necrosis factor α (TNFα) or EGFR ligands to soluble mature forms. Increased ADAM17 has been found in the inflamed tissue of individuals with active CD, and positively correlates with colitis severity [43]. Elastase is also a potent antimicrobial molecule. However, studies showing that anti-elastase treatment render protection to chemical-induced colitis indicates that dysregulated elastase activity might contribute to the intestinal damage in IBD [44].

Neutrophil extracellular traps (NETs)

Neutrophils are equipped with a variety of cytotoxic armamentarium for microbial killing. Besides direct phagocytosis of invasive pathogen, neutrophils can liberate NETs as a way to capture and imprison the microbes in larger size. Neutrophils can also secret pentraxin 3, in the process of NETs. Pentraxin 3 is an antimicrobial protein, which has been implicated in crypt abscesses formation in UC [45].

Cytokine

Neutrophils are potent cytokine producers secreting both pro-inflammatory cytokines (e.g. TNFα, IFNγ and CXCL8) and anti-inflammatory cytokines (e.g. IL-10) during intestinal inflammation [46]. **IL-23.** Myeloid dendritic cells (DCs) was originally considered the major

source of IL-23, contributing to IL-23 signaling[47]. Not until recently, however, newest findings suggest that tissue infiltrating neutrophils largely contribute to local IL-23 level in the inflamed tissue [48]. Monoclonal antibody against IL-23 receptor showed promising outcomes treating patients with CD [49]. Il-23 can also induce other cytokine production in neutrophils, which indicates a positive feedback loop in the setting of colitis. **IL-17.** Although IL-17 is regarded as the signature cytokine of Th17 cells, accumulating data suggests that neutrophils remain the critical source of IL-17. Previous studies reported a pro-inflammatory role of IL-17 in the pathogenesis of IBD [50, 51]. Conversely, clinical trial attempting using either anti-IL-17 or anti-IL-17RA antibodiesto treat CD fail to yield positive results [52]. The discrepancy indicates a sophisticated role of IL-17 in intestinal inflammation. IL-17 promotes host antimicrobial defense. IL-17-producing neutrophils have been demonstrated confer protection in *Aspergillus* infection [53, 54]. IL-17 also induces IEC expression of different antimicrobial peptide as well as promotes intestinal IgA response against pathogen [4]. Last but not least, IL-17 enhances intestinal barrier integrity by enhancing junction protein expression. IL-17 knockout mice reveal dysregulated intestinal barrier function with increased epithelial permeability [55]. However, studies in salivary revealed a pathologic role of IL-17 weakening tight junction proteins [56]. In addition, IL-17 can have detrimental effect on surrounding tissue by inducing fibrosis. **IL-22.** Studies using various murine models of colitis have demonstrated a protective role for IL-22 signaling in IBD. IL-22 knockout mice present more exacerbated intestinal damage upon DSS insult compared to wide type control. Additionally, blockade of IL-22 signaling in mice leads to more severe colitis with impaired barrier integrity, evidenced by increased bacterial translocation in spleen and liver [57]. It has been demonstrated that IL-22 efficiently control luminal microbiota through induction of different antimicrobial peptides (defensin family, S100 protein family, Reg family and lipocalin-

2) by IECs [38]. Consistent with the *in vitro* finding of human IECs that IL-22 increases expression of MUC4 expression, administration of exogenous IL-22 rescues TCRαKO mice and alleviated the chronic inflammation [58]. Nevertheless, opposite role of IL-22 has been demonstrated in psoriasis, in which IL-22 inhibits the differentiation and cornification in keratinocytes [59].

Interaction with other cell types

It has been reported that neutrophils direct binding to IECs through ICAM-1 prevents neutrophil apoptosis, at the same time induces IEC protective functions [60]. Neutrophil transepithelial migration can also regulate expression of epithelial ADAM17. Importantly, neutrophil itself express ADAM17 on the surface [61]. Therefore, it is interesting to speculate that neutrophil contribute to the local shedding of cytokines and EGF ligands from various cellular sources. Neutrophils might even function as antigen present cell (APC) expressing major histocompatibility complex-II (MHC-II) and CD86 under certain circumstances [62]. These findings highlight the potential mechanisms neutrophil regulation of adaptive immune system.

RESULTS

Chapter 4: Neutrophil production of IL-17 and IL-22

Originally published in *The Journal of Immunology*. Feidi Chen, Anthony Cao, Suxia Yao, Heather L. Evans-Marin, Han Liu, Wei Wu, Eric D. Carlsen, Sara M. Dann, Lynn Soong, Jiaren Sun, Qihong Zhao and Yingzi Cong. 2016. mTOR Mediates IL-23 Induction of Neutrophil IL-17 and IL-22 Production. J. Immunol. Vol 196: 4390-4399.

INTRODUCTION

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), represent chronic or recurring inflammation in the gastrointestinal tract. The pathogenesis of IBD is considered multifactorial, involving compromise of the intestinal epithelial barrier and dysregulation of immune responses to commensal bacteria [3]. As an essential component of the innate immune system, neutrophils are implicated in the pathogenesis of IBD by virtue of the high neutrophilic influx in the inflamed intestinal tissue of IBD patients [63]. Emerging evidence highlights the greater value of neutrophil-derived products such as fecal calprotectin (S100A8/S100A9) than the traditional inflammatory marker C-reactive protein (CRP) in the assessment of disease activity in clinical practice [50, 64, 65].

Recently, neutrophils have been identified as an important source of both IL-17 and IL-22 [53, 66]. IL-22, a member of the IL-10 cytokine family, specifically targets epithelial cells based on the expression pattern of the IL-22 receptor (IL-22R) [67]. IL-22 has been shown to promote barrier defense and wound healing by inducing intestinal epithelial cell secretion of antimicrobial

proteins, epithelial cell differentiation, and goblet cell activation [68]. IL-17 is a critical component of mucosal immune defense [51, 69]. Although detrimental effects of IL-17 have been implied during colitis [70, 71], its protective function has also been demonstrated recently [4, 55]. Notably, IL-22-producing neutrophils are reported protective during acute colitis, in that colitis was alleviated in DSS-treated IL-22$^{-/-}$ mice receiving neutrophils from wide-type mice, whereas neutrophils from IL-22$^{-/-}$ mice exhibited little effect on disease progression [66]. On the other hand, IL-17-producing neutrophils aid in bactericidal defense during fungal infection [54, 72]. However, it is unclear how neutrophil production of IL-22 and IL-17 are regulated and their roles in the pathogenesis of chronic colitis must yet be clarified. We report here that IL-23 primarily promotes neutrophil production of IL-17 and IL-22 by regulating RORγt and AhR through activation of mTOR. Furthermore, neutrophils protect the intestines from chronic intestinal inflammation, possibly by producing IL-22.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice were obtained from the Jackson Laboratory and bred in the Animal Facilities at University of Texas Medical Branch and maintained there. All animal work was reviewed and approved by the Institutional Animal Care and Use Committees of the UTMB.

Antibody and reagents

IFNγ (XMG1.2), and IL-4 (11B11) were purchased from Bio X Cell (West Lebanon, NH). Thioglycollate broth was purchased from Sigma-Aldrich (St. Louis, MO). RORγt inhibitor was provided by Bristol Myers Squibb. AhR inhibitor, CH-223191 was purchased from Sigma-

Aldrich, and mTOR inhibitor rapamycin and AZD8055 from Selleck Chemicals (Houston TX). PI3K inhibitor LY294002, was purchased from Sigma-Aldrich. STAT3 inhibitor HJC0152 was kindly provided Dr. Jia Zhou at University of Texas Medical Branch. Antibodies against phosphorylated mTOR (S2448), phosphorylated 4E-BP1 (T70), phosphorylated STAT3 (P65), phosphorylated NF-kB (Y705), β-actin and HRP-conjugated anti-rabbit secondary antibody were obtained from Cell Signaling Technology (Danvers, MA) for western blot analysis. The following antibodies were used for flow cytometry: PerCP/Cy5.5-IL-17 (TC11-18H10.1), FITC- CD11b (M1/70), PE/Cy7- Ly6G (1A8), were from Biolegend (San Diego, CA). PE- IL-22 (1H8PWSR), APC- IL-10 (JES5-16E3), from eBioscience (San Diego, CA). Foxp3 Perm/ Fix Kit for intracellular permeabilization from eBioscience and Live/Dead Fixable Dead Cell Stain Kit from Life Technologies (Carlsbad, CA). Golgi Stop was purchased from BD Biosciences (San Diego, CA).

Neutrophil isolation and culture

The neutrophils were prepared from the peritoneal cavity as we previously described [73]. Briefly, C57BL/6 mice were injected with 1ml of 3% thioglycollate broth, and the peritoneal cavity was washed with cold PBS buffer containing 5% FBS 5 hours later. Neutrophils were separated from other cell types in the peritoneal exudate by using 50% Percoll (Sigma-Aldrich). Cell viability (>95%) was validated by trypan blue staining, and flow cytometry was performed to confirm neutrophil purity (>95%). For bone marrow neutrophil collection, murine femurs were obtained under aseptic conditions and flushed by cold PBS. Neutrophils were then isolated from bone marrow cells by using anti-mouse Ly6G-magnetic sorting (Miltenyi Biotec).

Real-Time Quantitative Reverse Transcription PCR

RNA was extracted with TRIzol (Life Technologies; Carlsbad, CA) and quantified for cDNA synthesis. Quantitative PCR reactions were performed by using TaqMan Gene Expression Assays (Life Technologies). Predesigned primers and probes for *il17* (Mm00439618_m1), *il22* (Mm00444241_m1), *il23r* (Hs00372324_m1), *rorc* (Mm01261022_m1), *ahr* (Mm00478932_m1) and *gapdh* (Mm99999915_g1) were ordered from Applied Biosystems and data were normalized to *gapdh* mRNA expression. Aliquots of PCR products were visualized under UV by electrophoresis on 1.5% agarose gels.

Statistical analysis

To assess an effect of a given sample size, power analysis was performed by using preliminary data sets. For comparison between samples, levels of significance were determined by Student's *t* test, and one -way ANOVA for multiple comparisons. P values < 0.05 were considered to be statistically significant. All statistical analysis was performed in Prism 5.0 (Graphpad Software; San Diego, CA). Results are shown as mean ± s.e.m. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

RESULTS

IL-23 stimulates neutrophil production of IL-17 and IL-22

IL-23 has been consistently shown able to stimulate IL-22 production in γδ T cells, dendritic cells (DCs) and type 3 innate lymphoid cells (ILC3s). IL-6, IL-21, TNFα, and IL-1β have also been able to stimulate IL-17 and/or IL-22 production at different levels in ILCs and several $CD4^+$ T cell subtypes [53, 74]. Although IL-23 itself induces little IL-17 or IL-22 in $CD4^+$ T cells,

it can greatly augment IL-17 and IL-22 production in Th17 cells [75]. In a recent report, colonic neutrophils isolated from mice with DSS-induced colitis mice have been shown to produce both IL-17 and IL-22 after IL-23 treatment. Colon tissue of IL-23a$^{-/-}$ mice revealed a significant decrease in IL-22 levels compared to those in wide-type mice [66]. However, the cytokines, which stimulate neutrophil production of IL-17 and/or IL-22, have not been carefully elucidated. The short half-life of neutrophils makes it hard to isolate neutrophils from intestines, as it requires a long processing time, and thus most neutrophils isolated would have died by the end of preparation. We thus use peritoneal neutrophils as they are ready to be prepared in large quantities in our *in vitro* studies [73]. We stimulated peritoneal neutrophils with IL-23, IL-6, IL-21, TNFα, or IL-1β to assess which cytokine(s) trigger IL-17 and IL-22 production in neutrophils. A portion of cells was stained for CD11b and Ly6G to assure that the purity of neutrophils population (CD11b^{+}Ly6G^{+}) is higher than 95% (**Figure 1**). The levels of IL-17 and IL-22 in the supernatant were determined via enzyme-linked immunosorbent assay (ELISA). Consistent with a previous report, treatment with IL-23 alone markedly enhanced production of IL-17 and IL-22 at levels of both protein and mRNA (**Figure 2A-B**). In contrast, IL-1β, IL-6, IL-21, and TNFα had little or no effect on the production of IL-17 and IL-22 when administered either alone or in combination with IL-23 (**Figure 2A**), indicating a critical role of IL-23 in inducing IL-17 and IL-22 production in neutrophils. As recent evidence indicates that there are different subsets of neutrophils, to evaluate whether the same or different neutrophils produce IL-17 or IL-22, we used flow cytometry to identify the subsets of IL-17- and IL-22-producing neutrophils after IL-23 stimulation. As shown in **Figure 2C**, increased amount of IL-22-producing CD11b^{+}Ly6G^{+} neutrophils were found relative to untreated cells. Interestingly, we found a slight increased number of IL-10^{+} neutrophils in the presence of IL-23.

IL-23 induces IL-23R expression on neutrophils

IL-23 receptor (IL-23R) mRNA has been recently found to be expressed on naïve neutrophils in bone marrow (BM) [53]. Neutrophils also appear to express IL-23R after migration into the inflamed colon [66]. The isolated neutrophils are more activated than those from BM after migrating from blood vessels to peritoneal cavity in response to thioglycollate, while BM neutrophils are naïve. To understand the regulation of neutrophil IL-23R expression, we examined the expression of IL-23R in peritoneal neutrophils. As shown in Fig. 2A and B, IL-23R was expressed in the peritoneal neutrophils at a higher level compared to that in BM cells (**Figure 3A-B**). The peritoneal neutrophils can further respond to stimulants and elicit more exaggerated response. Accordingly, we found that IL-23 further stimulated IL-23R expression in the peritoneal neutrophils (**Figure 3C**). Together, these data suggest that, unlike T cells, naïve neutrophils constantly express a basal amount of IL-23R and the expression of IL-23R increases as the cells become activated.

RORγt and AhR differentially regulate IL-17 and IL-22 production in neutrophils

Retinoid-acid Receptor-related Orphan Receptor gamma t (RORγt) is an essential transcription factor mediating IL-17 production in Th17 cells [76]. Moreover, it has been shown that RORγt is indispensable for IL-22 expression in ILC3 [77]. The ligand-dependent transcription factor Aryl-hydrocarbon Receptor (AhR) also contributes to IL-17 and IL-22 expression in lymphoid cells [77-79]. We then investigated whether RORγt and AhR are involved in IL-23-driven IL-17 and IL-22 production in neutrophils. As shown in **Figure 4A**, addition of IL-23 upregulated expression of transcripts encoding RORγt (*rorc*) and AhR (*ahr*) in neutrophils,

indicating that IL-23 is capable of inducing both transcription factors. To investigate the role of RORγt and AhR in neutrophil IL-17 and IL-22 production, we treated peritoneal neutrophils with small molecule inhibitors against RORγt or AhR in the presence of IL-23. As shown in **Figure 4B**, inhibition of RORγt compromised both IL-17 and IL-22 production in IL-23-treated neutrophils, indicating that induction of both cytokines is RORγt-dependent (**Figure 4B**). Interestingly, AhR inhibition only decreased IL-22 production with no detectable impact on IL-17 expression. Addition of both RORγt and AhR inhibitors further reduced IL-17 and IL-22 production, suggesting that RORγt and AhR act in a synergistic manner. Collectively, the data indicate that RORγt and AhR differentially regulate neutrophil production of IL-17 and IL-22.

mTOR regulates IL-23-driven IL-17 and IL-22 production in neutrophils

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is crucial for cell growth and proliferation [80]. Previous data indicate that mTOR is expressed in neutrophils and modulates neutrophil function [81], and therefore we investigated whether mTOR is involved in IL-23 induction of IL-17 and IL-22 in neutrophils. IL-23 treatment led to the phosphorylation of mTOR (**Figure 5A**). Additionally, 4E-BP1, a major downstream effector molecule of mTOR complex 1 (mTORC1) was also activated after the treatment of IL-23, further confirming that IL-23 activates mTOR pathway. We then applied rapamycin, a well-established mTORC1 inhibitor to neutrophils in combination with IL-23. As expected, rapamycin greatly attenuated IL-23-induced phosphorylation of 4E-BP1 (**Figure 5B**). To confirm these results, we adopted another mTOR inhibitor (AZD8055), which targets both mTORC1 and mTORC2 to neutrophils [82-84]. Consistent with rapamycin, AZD8055 inhibited IL-23-induced phosphorylation of 4E-BP1

(**Figure 5B**). To determine whether mTOR regulates IL-23-driven cytokine production in neutrophils, we first examined IL-17 and IL-22 gene transcription. The qRT-PCR showed markedly decreased IL-17 and IL-22 mRNA expression after blockade of mTOR signaling by rapamycin. We also demonstrated a similar effect of AZD8055 on IL-23-treated neutrophils, in that both IL-17 and IL-22 transcription were dramatically inhibited (**Figure 5C**). Additionally, ELISA revealed that production of IL-17 and IL-22 under IL-23 stimulation was inhibited by both mTOR inhibitors respectively (**Figure 5D**). In order to illustrate the molecular mechanisms as to how mTOR regulates IL-17 and IL-22 production, transcription of RORγt and AhR was examined in the presence of mTOR inhibitors. We found that the induction of both *rorc* and *ahr* by IL-23 was inhibited by rapamycin (**Figure 5E**). Similarly, AZD8055 remarkably decreased *rorc* and *ahr* mRNA expression in IL-23-treated neutrophils. Taken together, these data demonstrated that IL-23 signals through the mTOR pathway to positively regulate the production of both IL-17 and IL-22 by upregulating RORγt and AhR expression in neutrophils.

PI3K regulates mTOR pathway to promote IL-17 and IL-22 production in neutrophils

PI3K has been shown to positively regulate mTOR activation [85]. Based on the data that mTOR and its downstream proteins facilitate IL-23 signaling, we postulated that IL-23 regulates mTOR pathway through induction of PI3K. As shown in **Figure 6A**, IL-23 treatment enhanced the phosphorylation of PI3K in neutrophils. Addition of the PI3K antagonist LY294002 inhibited IL-23-induced PI3K phosphorylation and activation of 4E-BP1, suggesting that IL-23 activates the mTOR pathway through activation of PI3K. Moreover, treatment of neutrophils with LY294002 in the presence of IL-23 inhibited IL-17 and IL-22 production, indicating that stimulation of IL-17 and IL-22 production is mediated by PI3K pathway (**Figure 6B-C**).

LY294002 treatment also greatly decreased the transcription level of both *rorc* and *ahr* (**Figure 6D**). Notably, IL-23 treatment also led to p38 mitogen-activated protein kinase (MAPK) phosphorylation, which was unaffected by treatment with either mTOR or PI3K inhibitor, confirming the specific inhibition of the selected pathways without compromising neutrophil viability (data not shown). Collectively, these data indicate that IL-23 stimulates neutrophil production of IL-17 and IL-22 through the activation of PI3K-mTOR pathway.

Signal transducers and activator of transcription 3 (STAT3) has been implicated as a transcriptional regulator in T cell IL-17 and IL-22 production [86, 87]. Earlier studies showed that STAT3 regulated neutrophil chemotaxis and migration [88]. We then questioned whether STAT3 could also be involved in IL-23-induced IL-17 and IL-22 production in neutrophils. We cultured neutrophils with IL-23 in the presence or absence of STAT3 inhibitor HJC0152 [89]. As expected, IL-23 was able to induce phosphorylation of STAT3, which was abrogated by HJC0152 (**Figure 6E**). IL-17 and IL-22 production downstream of IL-23 signaling were reduced by addition of HJC0152 (**Figure 6F**). IL-23 also activated NF-κB in neutrophils (**Figure 6E**), and addition of NF-κB inhibitor inhibited IL-23-induced IL-17 and IL-22 (data not shown). Collectively, these data indicated that STAT3-NF-κB pathway also mediates IL-23 induction of IL-17 and IL-22 in neutrophils.

DISCUSSION

Neutrophils have been demonstrated recently to produce both IL-17 and IL-22 under various inflammatory/infectious conditions [53, 54, 66, 90]. However, it is still unclear how neutrophil production of these cytokines is regulated. In the present study, we demonstrated that

IL-23 induced neutrophil cytokine production by upregulating RORγt and AhR, which was mediated by the mTOR pathway.

In agreement with previous studies [66, 91], our results showed that in response to stimulation of IL-23, but not IL-6 or other cytokines tested, neutrophils produce IL-17 and IL-22. Notably, we found a large subset of IL-22$^+$ neutrophils after stimulation of IL-23, which corresponds to large amounts of IL-22 and IL-17 in the neutrophil culture supernatant, as well as the increased expression of *il22* and *il17* mRNA expression. Also, a small proportion of neutrophils are also IL-10$^+$. As IL-10 is a well-established anti-inflammatory cytokine, whether these IL-10$^+$ neutrophils function as regulatory cells to inhibit colitis warrants further investigation. The neutrophils are notable for their unique secretion of pre-made granules containing cytokines and other molecules. Indeed, a recent study reported that neutrophils release IL-17 via a neutrophil extracellular trap (NET) [92]. However, the signal pathways that regulate IL-17 and IL-22 production in neutrophils remain unknown. We demonstrated that unlike CD4$^+$ T cell subtypes and ILC3, neutrophil production of IL-17 and IL-22 is strictly IL-23-dependent. Interestingly, IL-23 promotes the expression of its own receptor by neutrophils, likely indicating a positive feedback to stabilize the neutrophil phenotype and contributing to constant IL-17 and IL-22 production. Furthermore, IL-23 induces the expression of RORγt and AhR, which differentially regulate neutrophil production of IL-17 and IL-22. Inhibition of RORγt inhibited IL-23-induced IL-17 and IL-22 production, whereas AhR inhibitor only elicited effect on IL-22 production in neutrophils. Blockade of both RORγt and AhR signaling led to complete abrogation of IL-17 and IL-22 production. Taken together, our results indicate that neutrophils utilize pathways similar to that of ILC3, in that AhR synergizes with RORγt for the induction of IL-22 but not IL-17. In addition, IL-

23 activates STAT3 and NF-kB in neutrophils, which is also contributed to IL-23-induction of IL-17 and IL-22.

The critical role of the PI3K/mTOR pathway has been implicated in various cellular functions [93-95]. Our data indicated that the mTOR pathway mediates IL-23 induction of the neutrophil production of IL-17 and IL-22, in that IL-23 activated mTOR pathway, and inhibition of mTOR severely compromised IL-17 and IL-22 production. It has recently been shown that mTOR induces NETosis after lipopolysaccharide (LPS) exposure. Thus, mTOR may facilitate neutrophil cytokine production in multiple aspects.

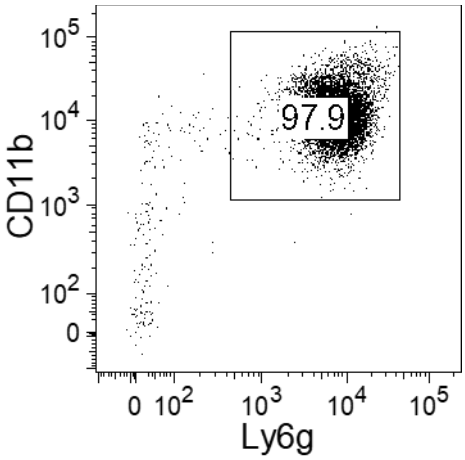

Figure 1. Peritoneal neutrophils purity
Isolated peritoneal neutrophils were stained for neutrophil surface marker and the purity of the cells was determined by flow cytometry. FACS plot is representative of 3 independent experiments.

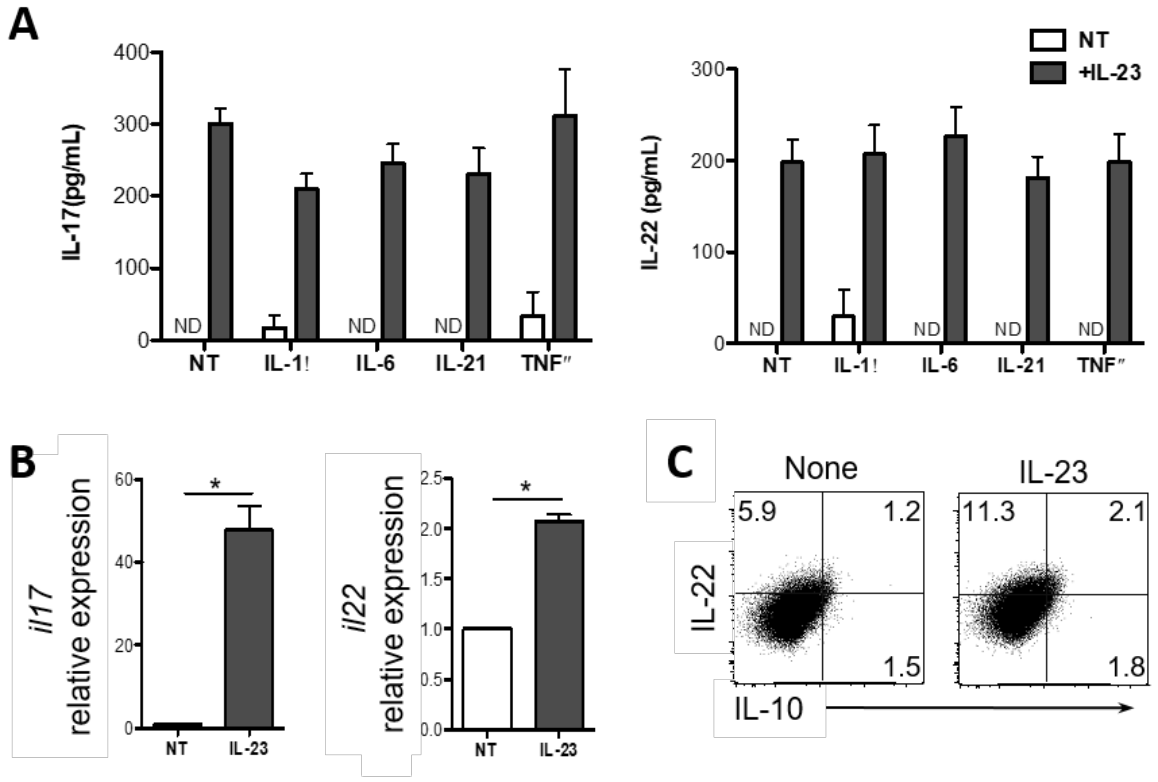

Figure 2. IL-23 stimulates neutrophil production of IL-17 and IL-22
(**A**) Peritoneal neutrophils were treated with IL-1β (20ng/ml), IL-6 (30ng/ml), IL-21 (20ng/ml), or TNFα (20ng/ml), alone or in combination with IL-23 (20ng/ml) for 24 hours. IL-17 and IL-22 production were measured from the supernatant by ELISA. (**B**) Neutrophils were stimulated with IL-23 for 6 hrs. mRNA for *il17* and *il22* were determined by qRT-PCR and normalized against *gapdh*. The relative expression of *IL-17 and IL-22* untreated neutrophils was arbitrarily set to 1.0. IL-17 and IL-22 expression was compared between the IL-23-treated and untreated neutrophils. *$p<0.05$. NT, no treatment. Data are representative of 3 independent experiments. (**C**) Neutrophils were stimulated with IL-23 (20ng/ml) in the presence of Golgi Stop for 12 hours. Cytokine expression was measured by flow cytometry. FACS plots are representative of 3 independent experiments.

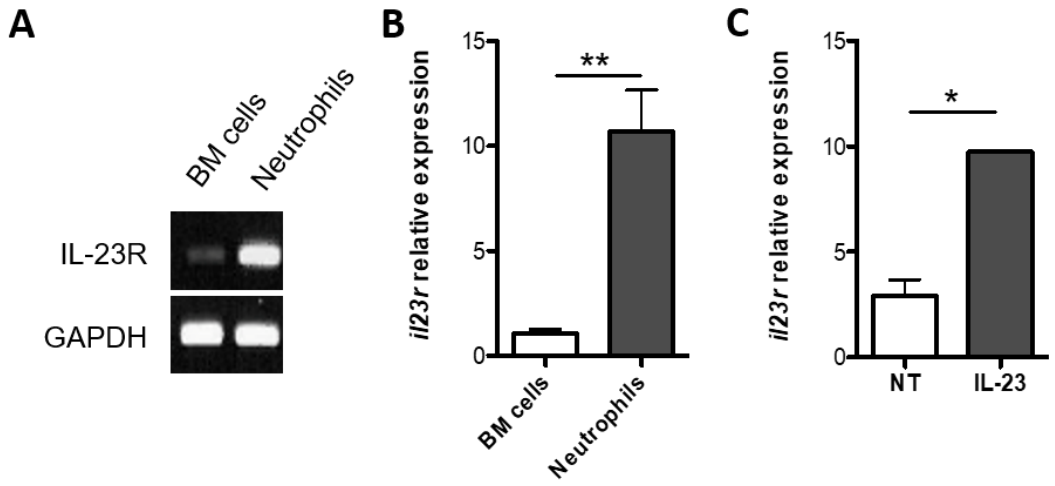

Figure 3. IL-23 induces IL-23R expression on neutrophils
2μg RNA from freshly isolated peritoneal neutrophils and bone marrow cells was used to analyze IL-23R expression by reverse transcription–PCR (RT–PCR) (**A**) and real-time PCR (B). *Gapdh* was used as a housekeeping gene. *Il23r* expression values were normalized to *Gapdh* expression. The relative expression of *il23r* of BM cells was arbitrarily set to 1.0. *il23r* expression was compared between the BM cells and peritoneal neutrophils. **$p<0.01$. (**C**) Neutrophils were treated with 20ng/ml IL-23 for 6 hours.

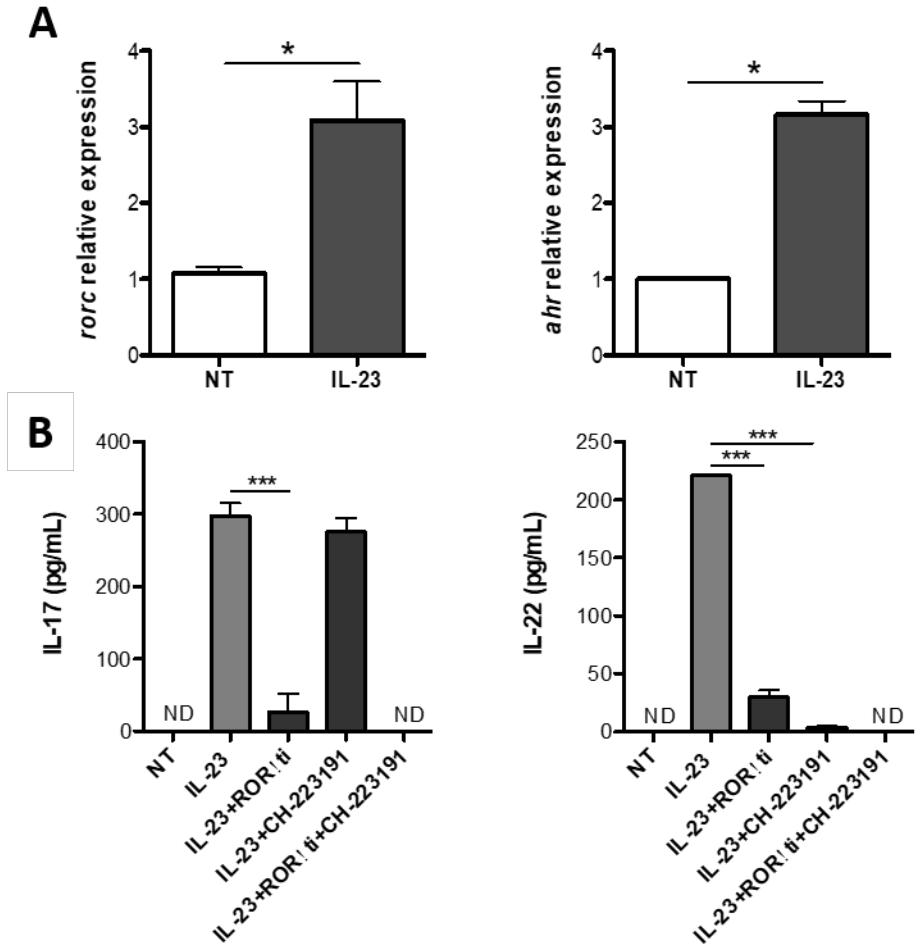

Figure 4. RORγt and AhR differentially regulate IL-17 and IL-22 production in neutrophils

(**A**) Neutrophils were treated with IL-23 (20ng/ml) for 6 hours. mRNA for *Il23r* was determined by qRT-PCR and normalized against *gapdh*. The relative expression of *rorc* and *ahr* of BM cells was arbitrarily set to 1.0 respectively. *rorc* and *ahr* expression was compared between the untreated and IL-23-treated neutrophils. *p<0.05; NT, no treatment. (**B**) Neutrophils were treated with IL-23 (20ng/ml), in addition to a RORγt inhibitor (1μM), and/or AhR inhibitor (3μM) respectively. IL-17 and IL-22 production were measured from supernatant by ELISA. Data are reflective of 4 independent experiments. *p<0.05, **p<0.01, ***p<0.001. NT, no treatment.

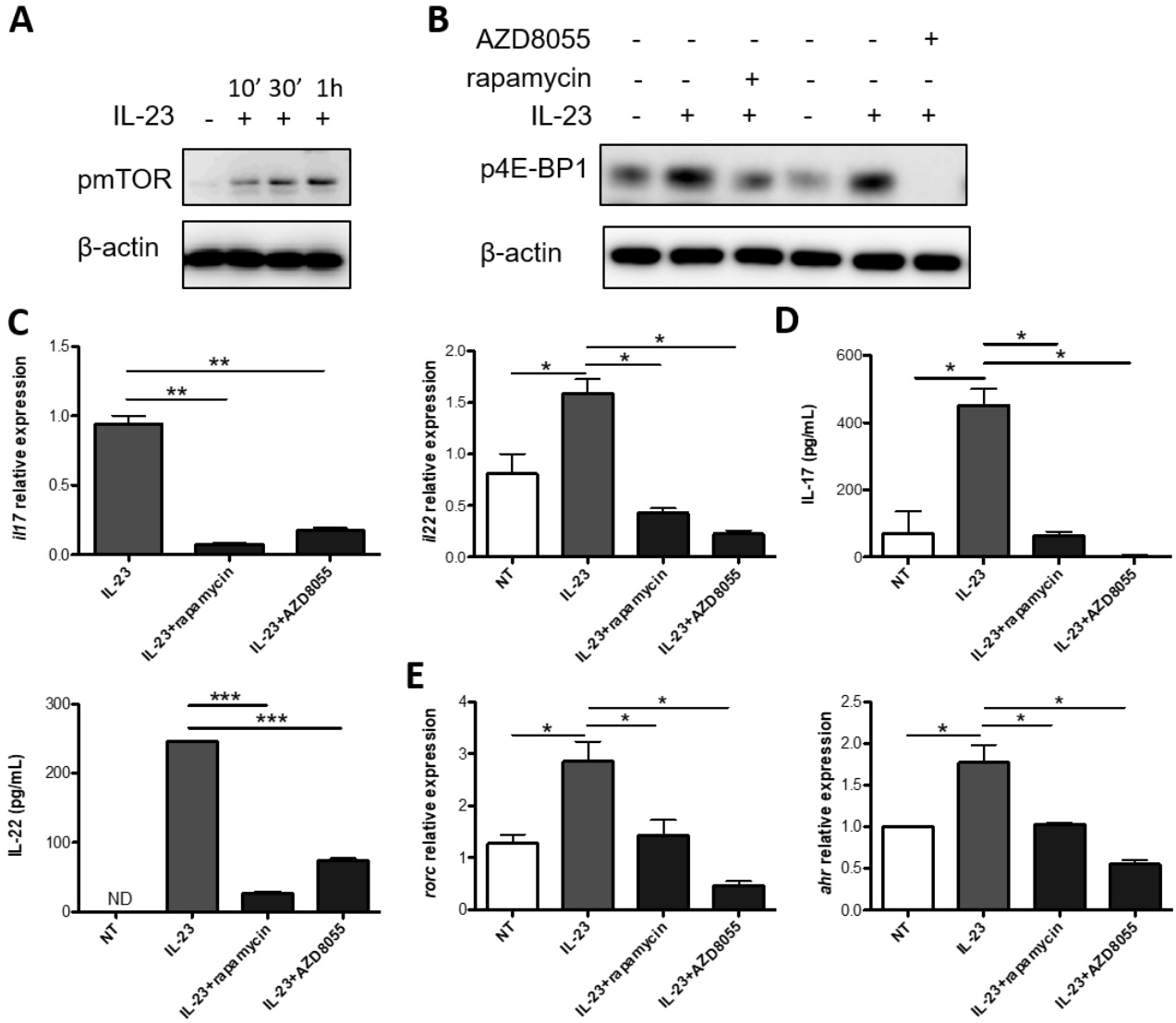

Figure 5. mTOR regulates IL-23-driven IL-17 and IL-22 production in neutrophils
(**A**) Peritoneal neutrophils were treated with IL-23 (20ng/ml) for the indicated time points. mTOR phosphorylation (Ser2448) was determined by Western blot. (**B**) Neutrophils were treated with IL-23 (20ng/ml) for 1 hour, in addition to rapamycin (2μM) or AZD8055 (1μM) respectively. Phosphorylated 4E-BP1 was detected by western blot, with β-actin as a loading control. (**C**) Neutrophils were treated with IL-23 (20ng/ml) for 6 hours. mRNA for *Il17* and *il22* were determined by qRT-PCR and normalized against *gapdh*. *p<0.05, **p<0.01. (**D**) Neutrophils were treated with IL-23 (20ng/ml), in the presence or absence of rapamycin (2μM,), or AZD8055 (1μM). IL-17 and IL-22 production were measured from supernatant by ELISA. *p<0.05, ***p<0.001. (**E**) mRNA for *rorc* and *ahr* were determined by qRT-PCR and normalized against *gapdh*. Data are reflective of 4 independent experiments. *p<0.05. NT, no treatment.

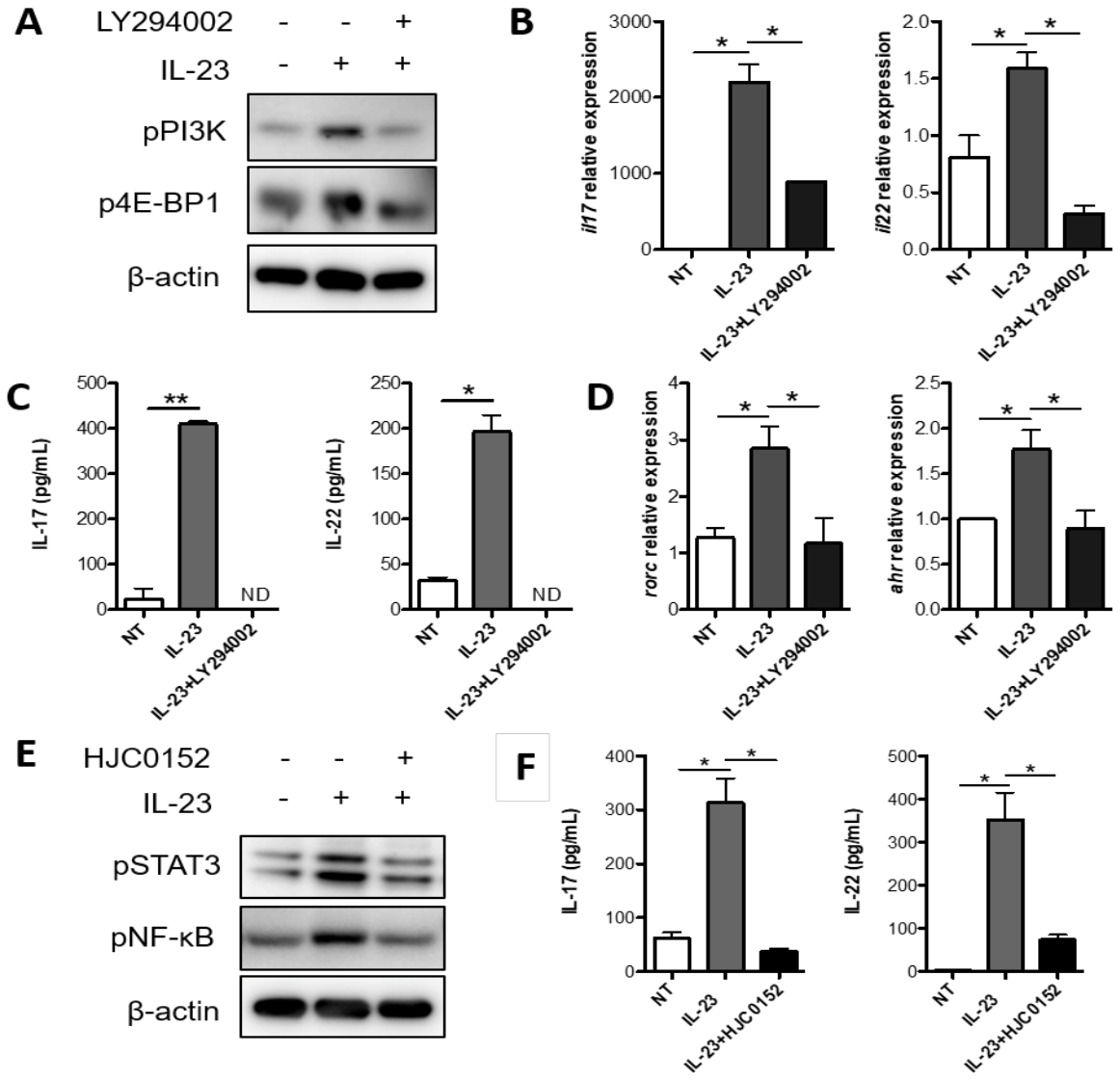

Figure 6. PI3K regulates mTOR activation and IL-17 and IL-22 production in neutrophils
(**A**) Neutrophils were treated with IL-23 (20ng/ml) for 1 hour with or without Ly294002 (10μM). Phosphorylation of PI3K and 4E-BP1 were detected by western blot, with β-actin as a loading control. (**B**) Neutrophils were treated with IL-23 (20ng/ml) for 6 hours. *p<0.05. (**C**) Neutrophils were treated with IL-23 (20ng/ml) with or without Ly294002 (10uM) for 24 hrs. IL-17 and IL-22 production were measured from supernatant by ELISA. *p<0.05, **p<0.01. (**D**) mRNA for *rorc* and *ahr* were determined by qRT-PCR and normalized against *gapdh*. (**E**) Neutrophils were treated with IL-23 (20ng/ml) for 1 hour and phosphorylation of STAT3 and NF-kB were detected by western blot, with β-actin as a loading control. (**F**) Neutrophils were treated with IL-23 (20ng/ml) with or without HJC0152 (5uM) for 24 hrs. IL-17 and IL-22 production were measured from supernatant by ELISA. *p<0.05. Data are reflective of 4 independent experiments. *p<0.05. NT, no treatment.

Chapter 5: The role of IL-22 producing Neutrophil during colitis

Originally published in *The Journal of Immunology*. Feidi Chen, Anthony Cao, Suxia Yao, Heather L. Evans-Marin, Han Liu, Wei Wu, Eric D. Carlsen, Sara M. Dann, Lynn Soong, Jiaren Sun, Qihong Zhao and Yingzi Cong. 2016. mTOR Mediates IL-23 Induction of Neutrophil IL-17 and IL-22 Production. J. Immunol. Vol 196: 4390-4399.

INTRODUCTION

Although intense efforts have been made to elucidate the role of neutrophils in IBD, conflicting data have been reported in different animal models that mimic IBD. In the dextran sodium sulfate (DSS)-induced colitis model, the ablation of neutrophils accentuated colitis, likely indicating a potential beneficial role of neutrophils [96, 97]. Furthermore, the mice with chronic granulomatous disease contain neutrophils with impaired functions, and these mice showed poor inflammatory resolution and succumbed rapidly during TNBS-induced colitis [96, 97]. This finding has been elaborated as a protective function of neutrophils by generating the "inflammatory hypoxia" microenvironment in the inflamed tissue as a way to promote intestinal epithelial healing through HIF-1 induction [98]. In contrast, it has also been reported that specific inhibition of neutrophils by monoclonal antibody attenuates DSS- induced colitis in rats [96], and the influx of neutrophils into the intestines leads to massive migration of neutrophils across intestinal epithelium into the intestinal lumen, thereby weakening the cell-to-cell junctions and increasing epithelial permeability, which results in subsequent bacteria translocation and water influx that worsens inflammation [66, 67]. Notably, all animal models used in previous reports were acute

colitis models, i.e. DSS- or TNBS-induced colitis, and thus, it is still unclear how neutrophils regulate chronic colitis with characteristics like those found in human patients with IBD.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice and Rag2$^{-/-}$ mice were obtained from the Jackson Laboratory. CBir1 flagellin-specific TCR transgenic (CBir1-Tg) mice [99] were bred in the Animal Facilities at University of Texas Medical Branch and maintained there. All animal work was reviewed and approved by the Institutional Animal Care and Use Committees of the UTMB.

Antibody and reagents

Neutralizing antibodies to Ly6G (1A8) was purchased from Bio X Cell (West Lebanon, NH). Neutralizing antibody to mouse IL-22 (8E11.9) was provided by Genentech (South San Francisco, CA). Thioglycollate broth was purchased from Sigma-Aldrich (St. Louis, MO). The following antibodies were used for flow cytometry: FITC- CD11b (M1/70), PE/Cy7- Ly6G (1A8), were from Biolegend (San Diego, CA). Foxp3 Perm/ Fix Kit for intracellular permeabilization from eBioscience and Live/Dead Fixable Dead Cell Stain Kit from Life Technologies (Carlsbad, CA). Golgi Stop was purchased from BD Biosciences (San Diego, CA).

Real-Time Quantitative Reverse Transcription PCR

RNA was extracted with TRIzol (Life Technologies; Carlsbad, CA) and quantified for cDNA synthesis. Quantitative PCR reactions were performed by using TaqMan Gene Expression Assays (Life Technologies). Predesigned primers and probes for *Hnf4α* (Mm01247712_m1) and

gapdh (Mm99999915_g1) were ordered from Applied Biosystems and data were normalized to *gapdh* mRNA expression.

Induction of colitis

$CD4^+$ T cells were isolated from the spleens of CBir1-Tg mice by using anti-mouse CD4-magnetic beads (BD Biosciences) as previously described [100]. To generate Th17 cells, $0.2x10^6$ $CD4^+$ CBirl-Tg T cells were cultured with the same number of irradiated splenocytes in the presence of 10ng/ml TGFβ1, 20ng/ml IL-6, 10μg/ml anti-IFNγ (XMG1.2), and 10μg/ml anti-IL-4 (12B11) for 5 days. The polarized Th17 cells were validated by FACS staining. Then, $2x10^6$ Th17 cells were transferred i.v. into the recipient $Rag^{-/-}$ mice. Mice were monitored by weight weekly and sacrificed either when body weight reached 80% of initial weight or at the end of 6 weeks. As previously described [4], 2% DSS (MP Biomedicals) was administrated to $Rag^{-/-}$ mice for seven days, followed by three days of fresh water. Mice were monitored by weight daily and sacrificed either when body weight reached 80% of initial weight or at the end of day 10.

Preparation of lamina propria cells

To isolate lamina propria cells, intestines were opened and cleaned of feces. Intestines were chopped and incubated with 50mM EDTA for 40 minutes. Remaining tissue was incubated with 0.5g/ml Collagenase IV and 5 μg/ml DNase I (Sigma-Aldrich) for 2 rounds of 30 minutes. Lamina propria cells were isolated from the interface of a 40%/75% Percoll interface.

Ex vivo colon organ culture and ELISAs

Colons were open and cleaned of fecal matter and contaminates. Then, 3mm biopsies from the ascending colon were placed into RPMI (10% FBS, HEPES, penicillin–streptomycin, 2-mecapto- ethanol, and sodium pyruvate) (Invitrogen, Carlsbad, CA, USA) cultured at 37°C with 5% CO_2. Supernatants were harvested after one day, and the concentration of cytokine was determined by ELISA as previously described [4]. Mouse IL-6, IL-17(A), IL-22, TNFα and IFNγ ELISA kits (BD Pharmingen; San Diego, CA) were used in the present study according to the manufacturer's protocols.

Histopathologic assessment

At necropsy, cecum and colon were harvested and washed with PBS. Swiss rolls were prepared. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Then, 5μm sections were sliced, stained with H&E, and histological scoring was blindly performed by an experienced pathologist using a modified scoring system reported previously [101]. The scoring includes the following aspects: lesion in intestinal crypts; goblet cell condition; crypt exudate; inflammatory cell infiltration; and tissue inflammatory condition. The damages were estimated and scored from 0 to 3, representative of intensity, i.e., absent, mild, moderate, or severe, respectively. The final colitis severity score was calculated by taking both intensity and extent of lesion into account.

Statistical analysis

To assess an effect of a given sample size, power analysis was performed by using preliminary data sets. The nonparametric Mann-Whitney U-test was used for assessing pathology scores. Levels of significance were determined by Student's *t* test, and one -way ANOVA for

multiple comparisons (Graphpad). P values < 0.05 were considered to be statistically significant. Results are shown as mean ± s.e.m. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

RESULTS

Neutrophils play a protective role in the pathogenesis of chronic colitis

Thus far, our results *in vitro* have demonstrated the molecular mechanisms involved in the regulation of neutrophil cytokine production. We sought to determine the role of neutrophils in regulating the pathogenesis of chronic colitis. We previously established a microbiota antigen-specific model of colitis by adoptive transfer of $CD4^+$ T cells from CBir1 TCR transgenic mice, which are specific for an immunodominant microbiota antigen CBir1 flagellin, into $Rag^{-/-}$ mice. The recipient mice developed colitis 4-6 weeks after cell transfer [102]. A neutrophil-specific Ly6G-depleting antibody was administered i.p. twice per week to deplete neutrophils. Six weeks later, the histopathology was evaluated in large bowel (LB) and cecum. Additionally, FACS staining confirmed that neutrophils were almost completely depleted in the mice administered with anti-Ly6G antibody (**Figure 7A**). Overall, in the absence of neutrophils, mice presented much more severe colitis than did the neutrophil-sufficient control group (**Figure 8A**), as evidenced by destroyed colon structure and loss of goblet cells. This finding corresponded to the higher pathology score of both large bowel (LB) and cecum in mice with neutrophil depletion (**Figure 8B**). The worsened disease was also associated with significantly decreased expression of hepatocyte nuclear factor 4alpha (Hnf4α), a critical regulator of tight junction proteins [103] (**Figure 8C**) and increased amounts of pro-inflammatory cytokines in colon organ cultures (**Figure 8D**). However, surface and intracellular staining of lamina propria cells of colitic mice revealed no significant difference of T cells in percentage and cytokine production (**Figure 9A-B**).

Collectively, these data demonstrated that neutrophils protect the intestines from chronic inflammation in response to microbiota.

Il-22-producing Neutrophils ameliorates intestinal disease severity

As IL-22 has been shown to be an important cytokine that contributes to intestinal protection and tissue repair of the epithelial layer, we then investigated whether IL-22 protected the intestine in CBir1 T cell-mediated colitis. We administered a neutralizing antibody against IL-22 from the same day of T cell transfer and twice per week thereafter for 6 weeks. As shown in **Figure 10A-B**, blockade of IL-22 increased the severity of colitis in comparison with the mice giving control antibody. T cells and ILCs have been shown as cell sources of IL-22, in addition to neutrophils [104, 105]. To elucidate the role of neutrophil-derived IL-22, independently of T cells or ILCs, we induced colitis in $Rag^{-/-}$ mice, which do not have T cells but with intact ILC3 and neutrophils, upon DSS insult. We then depleted ILCs by giving $Rag^{-/-}$ mice anti-Thy1.2 antibody (**Figure 11**), thus, the only known resource of IL-22 will be neutrophils. Depletion of ILCs worsened the colitis in $Rag^{-/-}$ mice after feeding DSS. When treated with anti-IL-22 antibody, those mice developed even more severe colitis (**Figure 10C-D**), as well as increased intestinal permeability as evidenced by FITC-dextran migration into the serum (**Figure 10E**), demonstrating that neutrophil production of IL-22 at least partially protects the mice from colitis.

DISCUSSION

As an essential component of the innate immune system, neutrophils are implicated in the pathogenesis of IBD. Neutrophils have been demonstrated recently to produce IL-22 under various inflammatory/infectious conditions [53, 54, 66, 90]. However, the role of neutrophils and their

production of these cytokines in the pathogenesis of chronic colitis remain unclarified. In the present study, we demonstrated that neutrophils protected the intestines from inflammation, possibly via the production of IL-22.

With more than 10^{14} commensal microbiota coexisting in the gut, the host immune system employs multiple strategies to maintain intestinal homeostasis [106]. Among them, neutrophils are critical components of the mucosal immune defense. The infiltration of neutrophils in chronically inflamed tissues has long been considered to exacerbate inflammation and perpetuate disease. However, recent studies have also demonstrated a beneficial effect by the accumulated neutrophils for limiting inflammation, which may outweigh their drawbacks during acute colitis [98]. Consistent with these findings, we demonstrated that the depletion of neutrophils leads to more severe inflammation and exacerbated colitis during chronic intestinal inflammation, indicating a protective role for neutrophils in chronic colitis. In a recent GWAS study, the gene encoding HNF4α has been reported as a susceptibility gene for UC [36]. Our finding that decreased expression of HNF4α in intestines of neutrophil-depleted mice indicates that the absence of neutrophils is correlated to the enhanced gut epithelial permeability. Since neutrophils share features with Myeloid-derived suppressor cells (MDSC), Ly6G-depleting antibody may also abolish the potential suppressive role of MDSCs. However, the concept and functional mechanisms of MDSCs are not yet clear. Thus, it is warranted to investigate the functional relationship between neutrophils and MDSCs, and their relative contributions to the intestinal homeostasis.

Previous studies elaborated the protective effects of IL-22 on host bactericidal response, tissue regeneration, and wound healing. IL-22-producing neutrophils have been reported to promote antimicrobial peptide production of intestinal epithelial cells [66]. Consistently, our data

demonstrated a worsened chronic colitis in mice after blockade of IL-22, indicating that neutrophils produce IL-22 as a possible way to resolve chronic colitis. T cells and ILCs have been implicated as cell sources for IL-22, in addition to neutrophils. Our data indicated that neutrophil production of IL-22 at least partially contributes to protection of the intestines from inflammation. Upon DSS insult, depletion of ILCs partially protected the $Rag^{-/-}$ mice, which do not have T cells, from colitis. This could be attributed to their production of IL-22, among other possible mechanisms. Interestingly, blockade of IL-22 in ILC-depleted $Rag^{-/-}$ mice further worsened the colitis, indicating that neutrophil-derived IL-22 protected the intestines from inflammation.

Despite the pro-inflammatory role of IL-17 in the pathogenesis of IBD highlighted in previous reports [50, 51], recent studies have also demonstrated a protective role of IL-17 in the maintenance of intestinal homeostasis through induction of intestinal epithelial cell expression of antimicrobial peptide and promoting intestinal IgA response against microbiota [4]. The ubiquitous expression of IL-17 receptors on a variety of cell types suggests that neutrophils can also "cross talk" with lymphoid, myeloid, and stromal/mesenchymal cells, and exhibit effector function [91]. Thus, in the intestines, IL-17 may also contribute to the protective effects of neutrophils [55]. In other systems, while the beneficial role of IL-17-producing neutrophils been demonstrated during *Aspergillus fumigatus* infection [53, 54], neutrophil-derived IL-17 were not detectable in certain *Candida albicans* infection [72], indicating that neutrophil IL-17 production may be limited to certain conditions or infections.

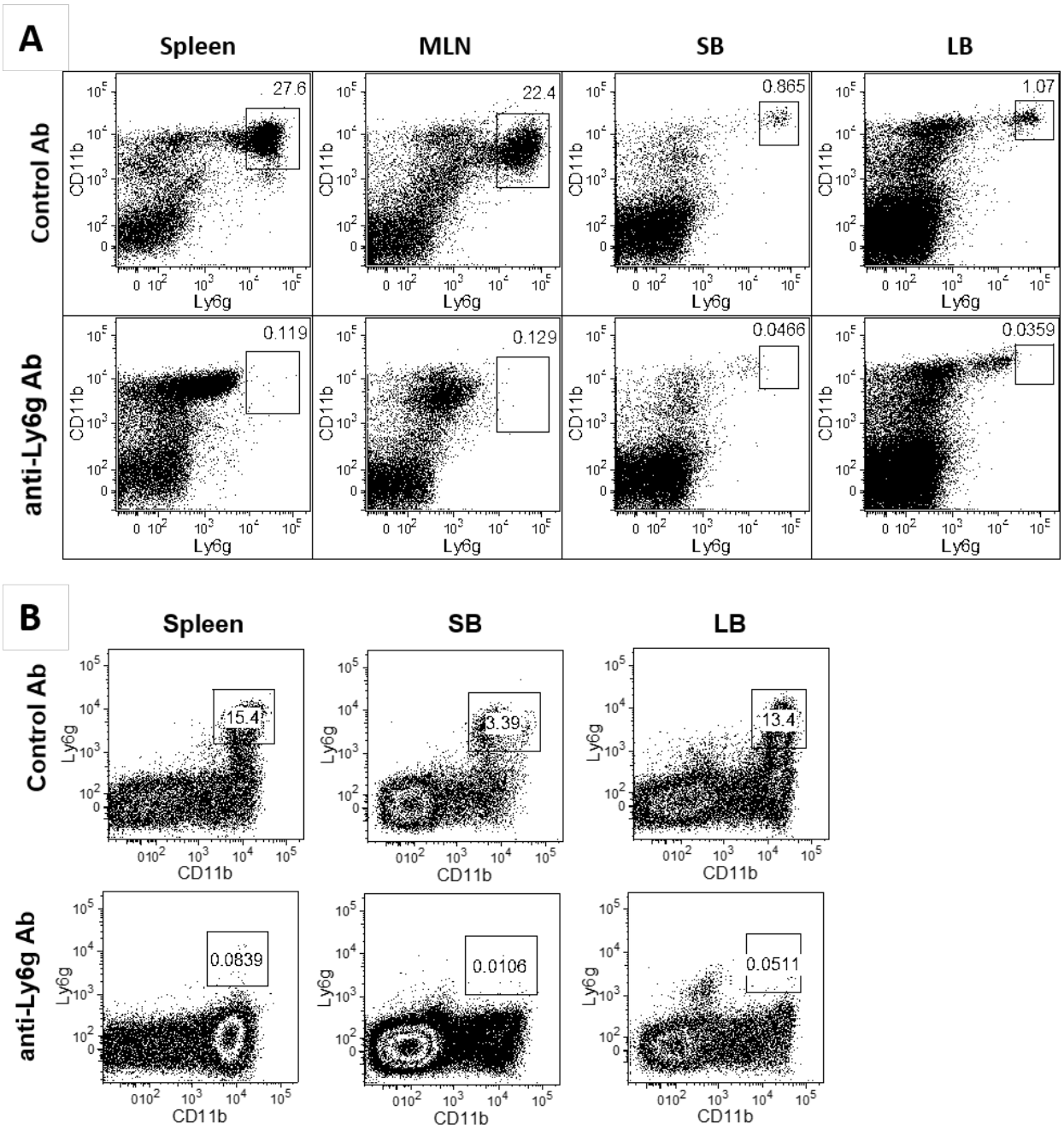

Figure 7. Depletion of neutrophils in T cell-mediated colitis
Neutrophil depletion in the spleen, mesenteric lymph node (MLN), small bowel (SB), and large bowel (LB) was measured by flow cytometry. (**A**) $2x10^6$ CBir1 Th17 cells were i.v. transferred into $Rag^{-/-}$ mice. Recipient mice were treated with anti-Ly-6g depleting antibody (1A8) or control Ab twice per week. (**B**) DSS was administrated to the drinking water of mice for 7 days followed by 3 day of water. Recipient mice were treated with anti-Ly-6g depleting antibody every 3 days. FACS plots are representative of 2 independent experiments.

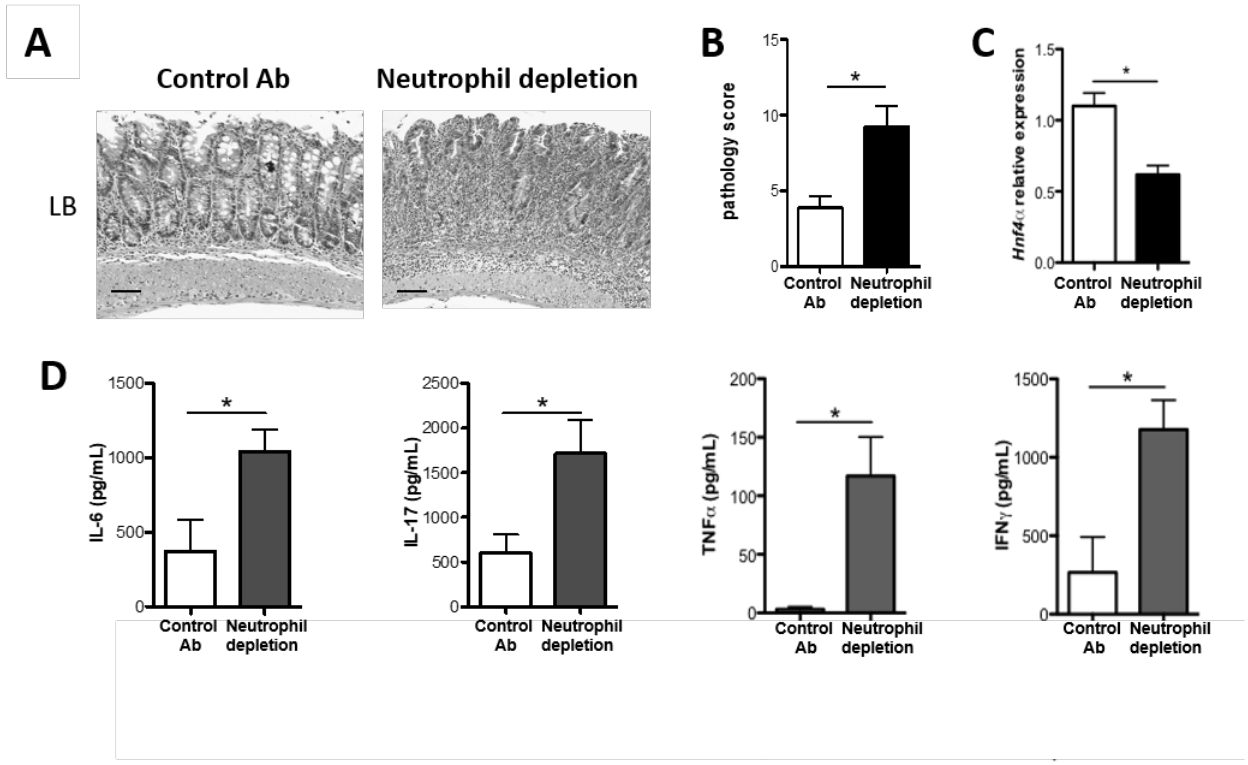

Figure 8. Neutrophils protect the intestines from chronic inflammation
$2x10^6$ CBir1 Th17 cells were i.v. transferred into $Rag^{-/-}$ mice. (A-C) Recipient mice were treated with anti-Ly-6G depleting antibody (4mg/kg) or control mAb twice per week. At week 6 post-Th17 cell transfer, (**A**) H&E staining was performed on colonic histopathology of control antibody-treated mice (left), and neutrophil-depleted mice (right) for (**B**) blinded histological scoring. N=4 mice per group. *p<0.05. (**C**) Tissue RNA were isolated from large intestines (LB). mRNA for *Hnf4α* was determined by qRT-PCR among two groups of mice and normalized against *gapdh*. *p<0.05. (**D**) IL-6, IL-17, TNFα, and IFNγ production were measured from the supernatant of colon tissue cultures from 4 mice per group by ELISA. Data are reflective of 3 independent experiments. *p<0.05.

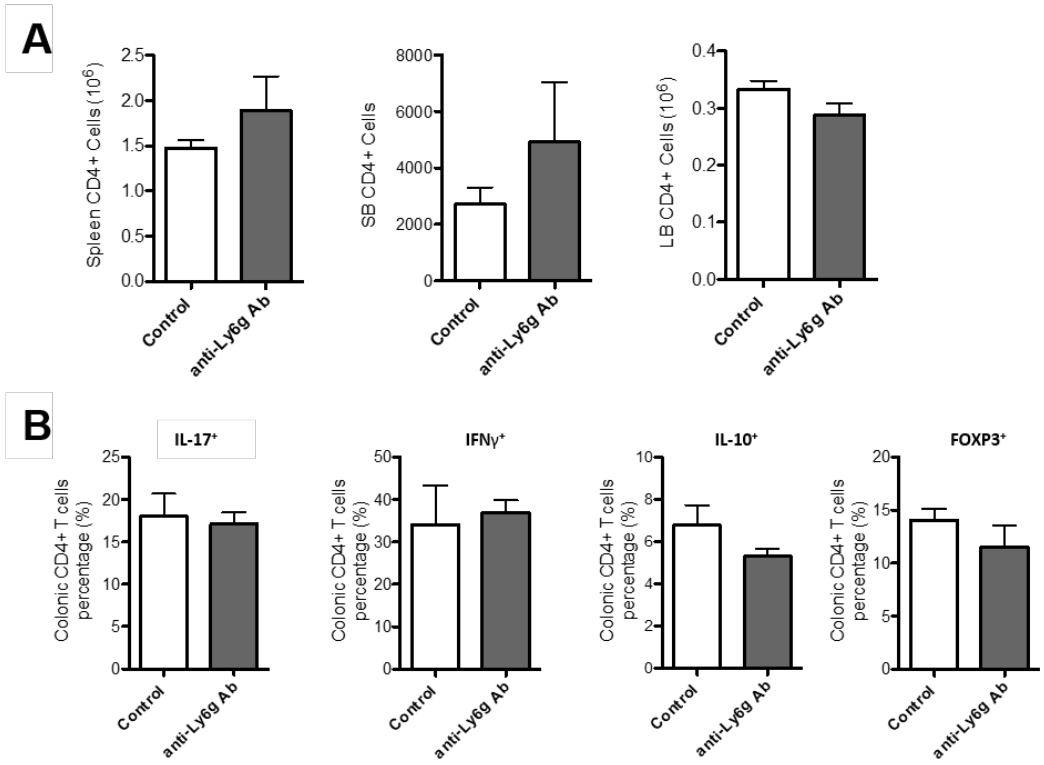

Figure 9. Depletion of neutrophils does not affect LP T cells
(**A**) Lamina propia cells were collected from mice of neutrophil depletion study, and total number of CD4+ T cell was determined in spleen, small bowel (SB), and large bowel (LB). (**B**) Intracellular staining of IL-17, IFNγ, IL-10 and Foxp3 revealed no difference of $CD4^+$ T cell cytokine production between mice with or without the presence of neutrophils.

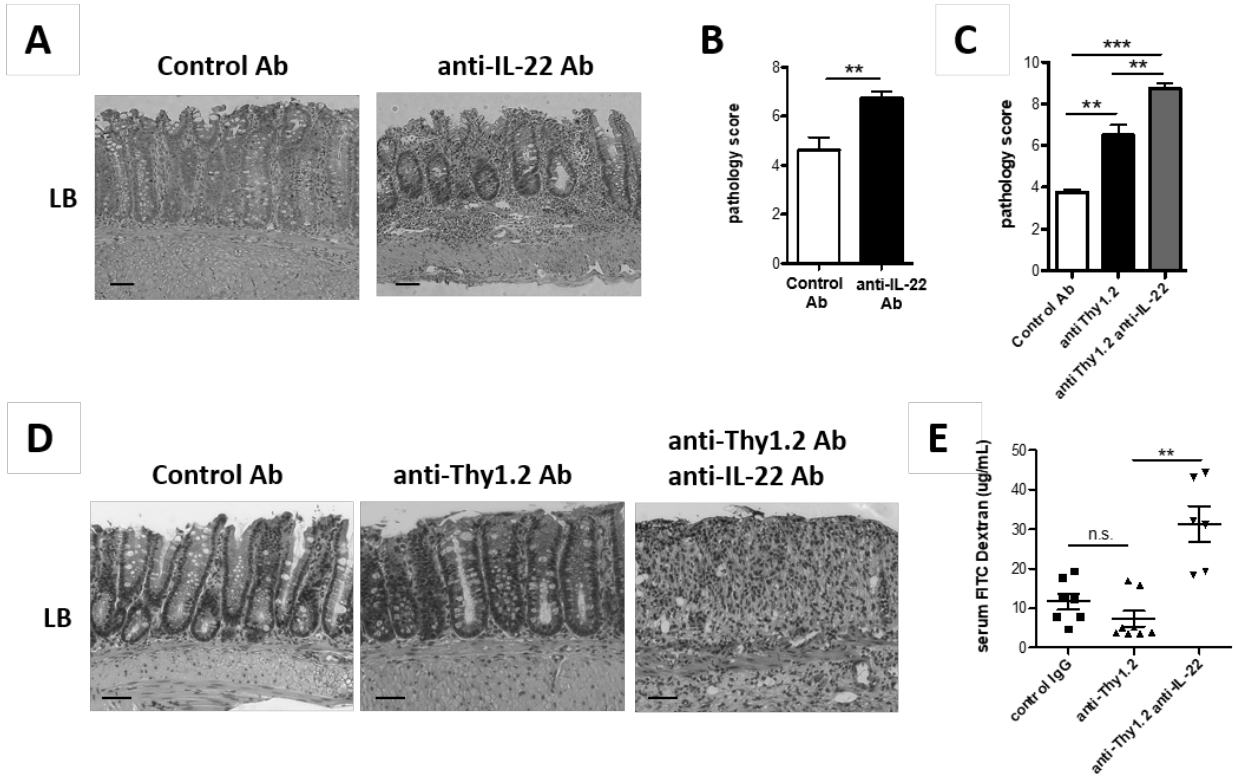

Figure 10. IL-22 producing neutrophils protect the intestines from chronic inflammation
(A-B) CBir1 T cell recipient mice were treated with 6mg/kg of anti-IL-22 or control mAb twice per week. On week 6 post-Th17 cell transfer, H&E staining was performed and colonic histopathology determined. (**A**) Histopathology of control antibody-treated mice (left) and anti-IL-22 antibody-treated mice (right), and (**B**) histological scores. N=4 mice per group. Data are one representative of 2 independent experiments with similar results. *p<0.05, **p<0.01. (C-E) Rag$^{-/-}$ mice were fed in drinking water with 2% DSS for seven days, followed by three days of fresh water, and injected IgG control mAb, 20mg/kg anti-Thy1.2 mAb with or without 6mg/kg anti-IL-22 mAb, respectively. (**C**) Blinded histological scoring was examined, and (**D**) H&E staining was performed. (**E**) FITC-dextran level in plasma was determined. N=4 mice per group, Data are one representative of 2 independent experiments with similar results. n.s. indicates no significant difference *p < 0.01.

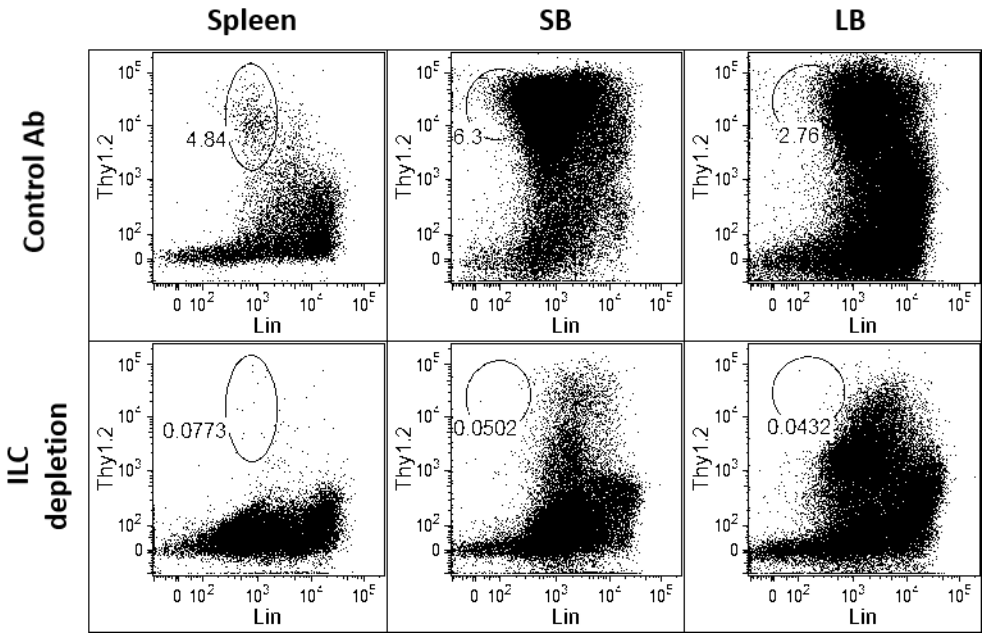

Figure 11. Depletion of innate lymphoid cells in Rag$^{-/-}$ mice
Rag$^{-/-}$ mice were fed in drinking water with 2% DSS for seven days, followed by three days of fresh water, and treated with IgG control mAb or 20mg/kg anti-Thy1.2 mAb. innate lymphoid cell (ILC)depletion in the spleen, small bowel (SB), and large bowel (LB) was measured by flow cytometry. FACS plots are representative of 2 independent experiments.

Chapter 6: Neutrophils promote amphiregulin production in intestinal epithelial cells through TGFβ and contribute to intestinal homeostasis

INTRODUCTION

As the largest mucosal surfaces in the body, the intestinal epithelium serves as a critical relay station between microbiota and mucosal immune cells. Multiple pattern recognition receptors allow intestinal epithelial cells (IECs) to sense microbes and translate the signals to mucosal immunity [2]. Meanwhile, thick layer of mucus and deep invaginated intestinal crypt structure buffer the microbial signals preventing excessive exposure of antigens and subsequent over activation of the immune system [18]. IECs capacity for rapid self-renewal protects the host from continuous exposure to microbial stimuli and environmental insults [17]. On the other hand, tissue resident immune cells closely interact with IECs to support barrier function and regulate luminal microbiota. We previously reported that Th17 cells upregulate polymeric Ig receptor (pIgR) on IECs as a way to promote intestinal IgA responses to microbiota, thus, contributing to maintenance of intestinal homeostasis [4]. Accumulating evidences also show that innate lymphoid cells (ILCs) are capable of producing IL-22 and amphiregulin (AREG) to exert beneficial effects on IECs [6, 19]. IEC dysfunction has detrimental effects on the host, resulting in increased bacterial translocation and risk of developing inflammatory bowel disease (IBD) and other inflammatory diseases. It has been reported that individual with altered IEC gene expression are more susceptible to IBD [107]. Moreover, investigations in patients with IBD showed increased intestinal permeability, much of which was attributed to compromised IEC function [108]. However, the cells and factors that regulate IEC function are still not completely understood.

The role of AREG has been emerging for regulation of the homeostasis at epithelial interfaces [109]. As a member of the epidermal growth factor family, AREG is essential in regulating cell differentiation and proliferation. It has been reported that AREG-deficient ($AREG^{-/-}$) mice spontaneously develop gastric tumors [110]. In the context of intestinal injury after dextran sodium sulfate (DSS) administration, $AREG^{-/-}$ mice develop more severe colitis compared to wild-type B6 (WT) mice, which suggest a crucial role for AREG in wound healing and tissue repair [6]. Additionally, AREG is thought to play a role in type 2-mediated immune resistance and tolerance. Lung resident Th2 cells and group 2 ILCs produce AREG against pathogens, allowing for increased barrier protection against infection [111, 112]. Regulatory T cells (Treg) have also been identified as an important source of AREG during early influenza infection [5]. Conversely, AREG has been reported to promote the suppressive capacity of Treg during inflammation [113]. Among various hematopoietic and non-hematopoietic AREG producers, IECs remain a critical source for paracrine AREG responses. Within the lung, epithelial-derived AREG can enhance barrier defense against pathogen [114]. Despite these advances, relatively little is known about the factors that regulate IECs production of AREG and the functional significance of IEC-derived AREG in regulating tissue protection.

The association between massive infiltration of neutrophils into the intestines and compromised IEC function found in IBD patients suggests a central role for dysregulated neutrophil-IEC interaction in the pathogenesis of IBD [46]. Neutrophils are the most abundant leukocyte in circulation and are pertinent in responding to microbial invasion at epithelial surfaces. At sites of microbial invasion, neutrophils perform several functions to control inflammation, including direct phagocytosis of invading pathogens, secretion of neutrophil extracellular traps (NETs), production of matrix metalloproteases (MMPs), elastase and other proteolytic enzymes

[20]. Neutrophils are also potent cytokine producers. We have previously reported that neutrophils protect the intestines from inflammation, and neutrophil-derived IL-22 ameliorated colitis by promoting epithelial integrity [115, 116]. In addition, IL-22-producing neutrophils have been shown to enhance IEC antimicrobial peptide production, which aids in barrier defense [66]. The infiltration of neutrophils can rapidly deplete O_2 levels in the microenvironment via reactive oxygen species (ROS) production, which also contributes to barrier protection by stabilizing IEC hypoxia-inducible factor (HIF) expression [98]. Despite the protective functions of neutrophils, excessive or prolonged neutrophil activity causes collateral tissue damage. Dysregulated neutrophil transepithelial migration results in altered expression of tight junction proteins in IECs, and neutrophil-derived pro-inflammatory mediators affect IEC viability [39, 46]. Hence, proper crosstalk between IECs and neutrophils is crucial for a balanced immune response and to maintain intestinal homeostasis.

In the current study, we demonstrated that neutrophils induce AREG expression from primary IECs through production of TGFβ. Upon DSS insults, depletion of neutrophils results in more severe colitis due to decreases in AREG production by IECs. Administration of AREG to mice after depletion of neutrophils alleviates intestinal injury. Thus, our study demonstrates that neutrophil augment epithelial protection through induction of AREG by IECs and reveals a novel pathway of neutrophil-mediated tissue protection.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice and $Rag^{-/-}$ mice were obtained from the Jackson Laboratory, and bred and maintained in the Animal Facilities at the University of Texas Medical Branch

(UTMB). Littermates of WT and Egfr^{wa5} mice were obtained from Dr. Fang Yan of Vanderbilt University. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the UTMB.

Antibodies and reagents

Neutralizing Antibody against Ly6G (1A8) was purchased from Bio X Cell. Recombinant AREG was purchased from Leinco Tecnologies. Thioglycollate broth and percoll were purchased from Sigma-Aldrich. Culture medium RPMI 1640, HEPES, penicillin/streptomycin, β-Mercaptoethanol (β-ME), sodium pyruvate, L-glutamine and ITS were purchased from Life Technologies. HBSS and DMEM were purchased from Corning. DMEM/F12 media (12634-010), L-Glutamine (25030) and B27 (12587-010) were purchased from Invitrogen. Mouse recombinant cytokines were purchased from Biolegend. Retinoic acid was purchased from Sigma-Aldrich. Matrigel was purchased from BD Bioscience. Recombinant EGF (2028-EG), Noggin (1967-NG/CF), R-spondin (3474-RS), Wnt3a (35036-WN/CF) and N2 supplement (AR009) were purchased from R&D Systems. SMARTpool siRNAs specific for murine MEK1, MEK2 and non-targeting siRNA were purchased from Dharmacon. Inhibitor U0126 and PD98059 were purchased from Promega. Western blot antibodies against phosphorylated ERK1/2, β-actin, and anti-rabbit secondary antibody conjugated with HRP were purchased from Cell Signaling Technology.

Neutrophil isolation

Neutrophils were collected from the peritoneal cavity as previously described [115]. Briefly, peritoneal cells were collected by lavage with 10ml PBS 5% FBS 5 h after 1ml 3%

thioglycollate broth i.p. injection. Neutrophils were separated from other cell types by using 50% Percoll. After spinning for 20 min at 1200 rpm without brake, neutrophils pellet was collected at the bottom of the tube. Neutrophil purity was >90% as tested by flow cytometry after CD11b and Ly6G staining. Neutrophil supernatant was harvest after 24 h of culture in complete culture media. Peripheral neutrophil

Enteroid culture

After cleaning, small intestines were removed from the euthanized mice, cut into small pieces (<0.5-cm pieces), and rocked in a Falcon tube with ice cold PBS for 15 min at 4°C. The intestinal tissues were treated with 2 mM EDTA for 30 min at 4 °C. The tissues were then transferred into a new tube with 5 ml cold PBS containing 43.3 mM sucrose and 54.9 mM sorbitol. After shaking for 2 min, the tissues were filtered through a 70-µm cell strainer and rinsed with 5 ml shaking buffer. Supernatant was collected and centrifuged at 150 g for 10 min at 4 °C. The resulting pellet containing detached crypts was re-suspended gently in Matrigel with 0.5 µg/ml recombinant EGF, 1 µg/ml recombinant Noggin, 5 µg/ml recombinant R-spondin and 1 µg/ml recombinant Wnt3a. Next, 50µl Matrigel with 500 crypts was plated in each well of the pre-warmed (37 °C) 24-well plate. After polymerization of Matrigel for 30 min, 500 µl of pre-warmed advanced DMEM/F12 media with 2 mM L-Glutamine, 1% penicillin/streptomycin, 10 µM HEPES, 1 x N2 supplement and 1 x B27 was gently added to each well. Enteroids were used after 5 days of culture and 250 µl of media was replaced with neutrophil supernatant for microarray study. For enteroid extraction, media were removed and gentle washed with ice cold PBS. 1ml cold PBS was added to each well, and the matrigel was scraped off using pipet tip at

the bottom of the well. After removing PBS and matrigel, enteroids were ready for subsequent study.

Microarray analysis

RNA expression analysis was conducted using Affymetrix Mouse transcriptome 1.0 assay kit (Affymetrix, PN 902919). Total RNA isolated using the TRIzol method (Invitrogen) was quantitated and qualified by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and only those total RNA with a RNA integrity number (RIN) >7.0 were included in microarray analysis (MA). Gene expression analysis (GEA) was done using the high-resolution array of Affymetrix GeneChip Mouse transcriptome array 1.0, which evaluates the expression of more than 66,100 different genes. Total RNA sample processing, labeling and hybridization were performed using the Affymetrix GeneChip WT PLUS with the WT Terminal Labeling Kit, according to the manufacturer's guidelines (Affymetrix). Scanning and data extraction of the microarray were followed by the transformation of fluorescence data into CEL files employing the Affymetrix GeneChip Command Console (AGCC) software. Microarray expression Data was furthermore analyzed using Transcriptome Analysis Console (TAC) 4.0 Software.

Epithelial cell culture

MSIE cells were cultured in RPMI 1640 medium with 5 U/ml murine IFNγ, ITS (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenous acid), 100 U/ml penicillin/streptomycin, and 5% FBS at 33°C. After reaching 80% confluence, cells were starved in RPMI 1640 medium with 100 U/ml penicillin/streptomycin and 0.5% FBS at 37°C for 16 h before subsequent experiments.

All subsequent experiments were performed at 37°C. Caco-2 cells were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO_2.

siRNA transfection

siRNA transfection in MSIE cells was performed by using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions. 2×10^5 MSIE cells were incubated with 30 pmol siRNA and 3 µl Lipofectamine RNAiMAX transfection reagent in OPTI-MEM medium (1 ml per well) for 6 h, followed by 1 ml normal medium per well for 24 h at 33°C. MSIE cells were then cultured in medium containing only 0.5% FBS for 16 h at 37°C before treatment. Transfection efficiency was determined at 24 h post transfection (**Figure S3**).

Knockout of MEK1 by using CRISPR

The CRISPR/Cas9 system that we used was the lentiCRISPR vector (Addgene, Cambridge, USA, plasmid#52961) established by the Zhang lab (Sanjana NE, Pubmed 25075903). The design and cloning of the target gRNA sequences was performed as recommended by the Zhang lab GeCKO website (http://www.genome-engineering.org/gecko/). Briefly, the suitable target sites for gRNA sequence design against MEK1 were identified using CRISPR design tool software at http://crispr.mit.edu. Cas9-target sites for the indicated genes were designed in http://crispr.genome-engineering.org. Then, synthetic gRNA (ITN) containing target sites were sub-cloned into the lentiCRISPR vector. The new constructed lentiCRISPR plasmids were then transfected into MSIE cells. After antibiotic positive selection, cells were established as a stable cell line. qRT-PCR and Western blot analysis were performed to confirm the deletion. Guide RNA

oligo sequences for MEK1: Forward 5' -CACCGCTTGTGCTTCTCCCGAAGAT-3' and Reverse 5' -AAACATCTTCGGGAGAAGCACAAGC-3'.

Quantitative Real-time PCR

RNA was extracted from homogenized tissue or cultured cells with TRI Reagent (Molecular Research Center) and followed by cDNA synthesis with qScript cDNA Supermix (Quanta BioSciences). Quantitative PCR was performed with SYBR Green Gene Expression Assays. Predesigned primers for were ordered from Life Technologies and normalized against *Gapdh* mRNA expression. The specific primer sequences are listed below:

PCR primers		
mGAPDH	Forward	5'-CCATGGAGAAGGCTGGGG-3'
	Reverse	5'-CAAAGTTGTCATGGATGACC-3'
mAREG	Forward	5'-CCTCCTTCTTTCTTCTGTTTCTCC-3'
	Reverse	5'-GTCACTATCTTTGTCTCTGCCA-3'
TGFβ	Forward	5'-TGACGTCACTGGAGTTGTACGG -3'
	Reverse	5'-GGTTCATGTCATGGATGGTGC-3'
HNF4α	Forward	5'-TGTTCTTGCATCAGGTGAGG-3'
	Reverse	5'-TCCAGTTCATCAAGCTCTTCG-3'
hGAPDH	Forward	5'-GAAGGTCGGAGTCAACGGATT-3'
	Reverse	5'-CGCTCCTGGAAGATGGTAAT-3'
hAREG	Forward	5'-CGAACCACAAATACCTGGCTA-3'
	Reverse	5'-TCCATTTTTGCCTCCCTTTT-3'

Dextran sulfate sodium model of colitis and neutrophil depletion

1.5-2% dextran sulfate sodium (DSS) w/v (Gojira FC) was dissolved into drinking water and administered *ad libitum* for 7 days, followed by 3 days of fresh water. Body weight was monitored daily. For neutrophil depletion, Ly6G-depleting antibody(1A8) (4 mg/kg) or control antibody were administered i.p. to DSS-treated mice every 3 days.

Histopathological assessment

At necropsy, individual colons were separated and Swiss rolls prepared. Colons were fixed in 10% buffered formalin and paraffin embedded. 5μm sections were sliced, stained with H&E, and blindly scored by an experienced pathologist. Histological scoring was performed using a modification of scoring system reported previously. Briefly, longitudinal sections were examined for epithelial damage based on hyperplasia, crypt degeneration, and loss; goblet cell loss; crypt exudate; LP and submucosal inflammatory cell accumulation; submucosal edema; mucosal ulceration; and transmural inflammation. Each lesion component was scored 0, 1, 2, or 3 for absent, mild, moderate, or severe, respectively (intensity), or 1, 2, 3, or 4 for 25, 50, 75, or 100% of the tissue affected, respectively (extent). The total lesion severity score was calculated by summation of the products of extent and intensity scores for each individual lesion component.

Isolation of IECs

After cleaning, small intestines and large intestines were removed from the euthanized mice, cut into small pieces, and rinsed thoroughly with cold PBS respectively. After 40 min incubation with 5mM EDTA in HBSS buffer containing 5% FBS at 37°C, IECs were collected by passaging supernatant through a 100 μM cell strainer (BD Falcon). After washing with PBS,

IECs were then underlayed with a solution of 20% percoll and 40% percoll. After spinning for 20min 2,000 rpm at 25°, IECs were collected at the 20/40 interface.

Ex Vivo Colonic Tissue Cultures

After cleaning, two pieces of colonic punctures (2mm) were obtained from proximal and distal colon respectively in each mouse. Tissue segments were cultured in 1640 RPMI medium supplemented with 100 U/ml penicillin/streptomycin, 10% FBS, 100 mM sodium pyruvate, 1M HEPES and 2-Mercaptoethanol in a 24-well culture plate for 24 h at 37 °C, 5% CO_2.

Statistics

Statistical significance was calculated by GraphPad (Prism 6.0) using paired or unpaired Student's t tests, and one -way ANOVA for multiple comparisons. The Mann–Whitney U test was used for assessing pathology scores. Where appropriate, mean ± SEM is represented on graphs. A p value < 0.05 was considered statistically significant and shown as asterisk (*).

RESULTS

Neutrophils induce AREG expression in IECs

Our previous studies have demonstrated that depletion of functional neutrophils in the mice led to increased intestinal permeability upon dextran sulfate sodium (DSS) insults [115]. As IECs have served as first line of defense against gut bacterial invasion, we thus hypothesized that tissue-infiltrating neutrophils can directly regulate IECs functions as a way to restore the homeostasis of the gut. To investigate whether neutrophils regulate IEC functions, we took advantage of using intestinal epithelial enteroids, a primary culture system for mouse intestinal cells. We first cultured

peritoneal neutrophils for 24 h and collected the supernatants to serve as neutrophil conditioned media, and then cultured the enteroids in the presence or absence of conditioned media for 6 h. RNA were extracted for microarray analysis using Affymetrix Mouse Gene 1.0 ST microarrays. Judging by the criteria >2-fold increase or decrease, we observed 579 genes with significant and altered expression between the two groups of enteroids (**Figure 12A**). To better understanding the functional interaction of these genes, we analyzed those 579 probes using Cytoscape software. Data analysis revealed that neutrophil conditioned media affected genes encoding regulators of IEC turnover and barrier functions. The heat map in **Figure 12A** demonstrated the differentially expressed transcripts associated with IEC proliferation (AREG, Anxa1 and Anxa2), differentiation (AREG, Skil, Id1, Cd44 and Nox1) and junction formation (Cldn4, Lamc2, Ace2 and Ace). AREG has been recently shown to be highly protective along the epithelial surfaces of both airway and intestine. We found that AREG transcripts were induced (3.25-fold upregulated) in primary IECs treated with neutrophil conditioned media compared to controls (**Figure 12A-B**). We further verified the finding through quantitative real-time PCR (**Figure 12C**). Using mouse small intestinal epithelium (MSIE) cells, a conditionally immortalized epithelial cell line established from the intestines of normal mice [117]. we reproduced the results, and demonstrated that neutrophil conditioned media were capable of inducing AREG expression in MSIE cells (**Figure 12D**). Taken together, these results demonstrated that neutrophils induce IEC production of AREG through production of soluble factors.

TGFβ induces AREG production in IECs

To identify the factors that potentially induce IEC AREG production, we treated the MSIE cells with different cytokines, including TGFβ1 (hereafter referred to as TGFβ), IL-17, IL-22, IL-

10, IL-1β, IL-4, TNFα, IFNγ and IL-6 for 6 h [116, 118-122]. We also treated the MSIE cells with retinoic acid (RA), Lipopolysaccharide (LPS) and IL-33, which been previously reported capable of inducing AREG in different cell types [6, 123, 124]. Among all the candidates, only TGFβ induced AREG expression in MSIE cells (**Figure 13A**). Similarly, we observed an increased AREG expression in primary IECs treated with TGFβ but no other cytokines tested (**Figure 14A**), indicating that TGFβ is an inducer of AREG in IECs. Next, we performed ELISA and confirmed the present of TGFβ in conditioned neutrophil media (**Figure 13B**). To determine the kinetics of TGFβ induction of AREG in IEC, we treated the MSIE cells with TGFβ and measured AREG expression with respect to time over the course of 48 h. The increase of AREG expression was starting from 3 h post-treatment with TGFβ, reached the peak at 24 h and then went down (**Figure 13C**). To determine whether TGFβ mediates neutrophil conditioned media induction of AREG production by IECs, we pre-treated neutrophil conditioned media with anti-TGFβ antibody to neutralize TGFβ or control antibody, and then implied them into cultures of MSIE. We demonstrated that anti-TGFβ antibody abrogated the effect of neutrophil conditioned media in induction of AREG by MSIE cells at both RNA and protein levels (**Figure 13D-E**), suggesting that neutrophil-derived TGFβ contributes to the AREG production in IECs.

TGFβ induction of AREG in IECs is MEK1/2-dependent

We next investigated the mechanisms underlying TGFβ-induced AREG production by IECs. Previous studies have suggested a positive feedback loop in TGFβ signaling through upregulating the expression of its own receptors in different cells [125, 126]. We first set out to assess whether the phenomenon was consistent in IECs as well. However, we observed no significant change in the expression of either TGFβ type I receptor (TGFβ-RI) or TGFβ type II

receptor (TGFβ-RII) in MSIE cells under TGFβ treatment (**Figure 14C**). In addition to activating the cascade of SMADs, it is known that the activation of TGFβ receptor complex can also lead to the subsequent activation of mitogen activated protein (MAP) kinase pathways [127]. We then sought to determine if MEK-ERK signaling regulates AREG production in IECs. Western blot analysis showed that TGFβ activated ERK1/2 in MSIE cells, as evidenced by increased phosphorylation of ERK1/2 after treated with TGFβ (**Figure 15A**). To determine if activation of ERK mediates TGFβ-induction of AREG in IECs, we used two selective inhibitors of MEK, an ERK-activating enzyme. PD98059 and U0126 are small molecules readily crossing cell membrane to block ERK activation. We showed that addition of MEK inhibitors greatly decreased AREG expression induced by TGFβ (**Figure 15B**). To further confirm these results, we used MEK siRNA, which specifically inhibits the expression of MEK. Transfection of MSIE cells with MEK1 or MEK2 siRNA, which are specific for MEK1 and MEK2 respectively, were characterized with a decreased expression of ERK-activating enzymes (**Figure 16A**). We demonstrated that transfection with either MEK1 or MEK2 siRNA decreased AREG expression in TGFβ-treated MSIE cells compared to non-targeting controls (**Figure 15C)**. Furthermore, a specific knockout of MEK1 using CRISPR caused marked decrease of AREG production induced by TGFβ in MSIE cells compared to WT controls (**Figure 16B-C**). Altogether, these results indicate that MEK1/2 is required for TGFβ-induction of AREG production by IECs.

AREG protects DSS-induced colitis

AREG is a growth factor capable of inducing proliferation, differentiation, and maturation of a number of mesenchymal and epithelial cell types [128]. Previous studies have also detailed the importance of AREG in the development of intestinal epithelial structure and integrity [110,

129]. Therefore, we sought to determine if administration of exogenous recombinant AREG conferring protection against colitis upon DSS insults. WT mice were fed with DSS in drinking water, and recombinant AREG was administered to one group of the mice every 2 days. After 7 days of DSS, followed by 2 days of water, the mice that received exogenous AREG suffered from a less severe wasting disease than control mice, as measured by change in body weight (**Figure 17A**). Consistent with changes in body weight, analysis of histopathology also showed improved disease with AREG treatment (**Figures 17B-C**). It has been shown that AREG has a potent effect upon maintaining epithelial barrier integrity and repair. As such, the mice given exogenous AREG had significantly improved pathology scores (**Figure 17B**), specifically with regards to epithelial damage after DSS colitis. As the severity of tissue damage in the underlying mucosa and submucosa regions did not improve greatly with AREG treatment, it appears that AREG confers the greatest benefit upon the epithelial layer.

Deficiency in AREG signaling increased susceptibility to intestinal damage upon DSS insults

AREG signals through the EGF receptors to exert effects on various tissues. To further determine the role of AREG in intestinal homeostasis, we challenged both WT and $Egfr^{wa5}$ mice with DSS and monitored disease outcome. It has been shown that $Egfr^{wa5}$ mice with disrupted EGF receptor structure, present significantly decreased EGF receptor activity in the intestine [130]. Under steady conditions, $Egfr^{wa5}$ mice did not demonstrate intestinal inflammation similar to those in WT mice (**Figure 18A**). Administration of DSS induced colitis in both WT and $Egfr^{wa5}$ mice. However, $Egfr^{wa5}$ mice exhibited exacerbated colitis with increased pathology score after DSS challenge (**Figure 18B**). Histological examination of the colon tissue revealed expansion of inflammatory infiltration into submucosa area, more severe crypt damage and increased immune

cells accumulation in Egfr^{wa5} mice. Collectively, these data suggest that deficiency in AREG signaling impairs intestinal homeostasis.

Neutrophils confer protection against colitis through the induction of AREG by IECs

To determine whether neutrophils protect the mice from intestinal inflammation through inducing IEC production of AREG, we first administrated a Ly6G-specific neutralizing antibody to deplete neutrophils in the Rag$^{-/-}$ mice. Both control and neutrophil-depleted mice were exposed to 2% DSS for 7 days and off to regular drinking water for additional 3 days. Weight loss was monitored daily over a period of 10 days. Treatment with Ly6G antibody almost completely depleted neutrophils (**Figure 7B**). Consistent with our previous report [115], in comparison with mice treated with control antibody, mice treated with Ly6G antibody exhibited more severe weight loss (**Figure 19A**), and higher pathology scores (**Figure 19B**). Hematoxylin/eosin (H&E)-stained colon sections demonstrated exacerbated colitis in mice treated with Ly6G antibody, as evidenced by loss of crypt architecture and dramatic cells infiltrations (**Figure 19C**). To determine if neutrophils produce TGFβ, which possibly regulates IEC production of AREG in the setting of inflammation, we isolated neutrophils to measure their TGFβ expression. Because neutrophils are extremely fragile and it is almost practically impossible to isolate purified neutrophils from intestinal lamina propria as almost all neutrophils are dead after long process time (normally over 10 h), we isolated neutrophils from the peripheral blood of mice and measured TGFβ expression by qRT-PCR. As it has been well-established that T regulatory (Treg) cells produce high levels of TGFβ, we also generated Treg cells from CD4^{+} T cells to serve as positive controls. As shown in **Figure 19D,** peripheral neutrophils from mice with DSS-induced colitis expressed higher levels of TGFβ compared to normal controls, at levels comparable to Treg cells. Moreover, the depletion

of neutrophils in the mice were associated with a significant decrease of colonic TGFβ mRNA expression (**Figure 19E**). Interestingly, lower level of soluble AREG was detected in the supernatants of *ex vivo* colon culture in neutrophil-depleted group (**Figure 19F**). Consistently, primary IECs isolated from neutrophil-depleted mice showed a decreased expression of AREG at both RNA and protein levels (**Figures 19G-H)**. It has been shown that AREG is first expressed as a transmembrane precursor in the cells [131]. Shedding of AREG requests ADAM17, a TNFα converting enzyme found on neutrophil surface [132]. We found that depletion of neutrophils resulted in decreased ADAM17 in the colonic tissue of mice upon DSS insult **(Figure 19I**). Together, these data indicate that neutrophils induce IEC expression of AREG as well as ADAM17, which sheds AREG, thus promote net AREG production by IECs. It also suggests a role for TGFβ-producing neutrophils in AREG-dependent tissue protective pathway in the intestine.

Neutrophils-elicited AREG production by IECs alleviates DSS-induced colitis and promotes IEC expression of HNF4α

To investigate the mechanisms by which neutrophil-induced AREG protects the intestine from colitis, we performed the rescue experiments by giving neutrophil-depleted mice exogenous AREG daily in the setting of DSS colitis. We treated $Rag^{-/-}$ mice with anti-Ly6G antibody to deplete neutrophils, and groups of the mice were given AREG or PBS every two days. Neutrophil-depleted mice received AREG treatment revealed less weight loss during the later course of DSS exposure (**Figure 20A**). In accordance with the improved pathology score (**Figure 20B**), H&E-stained colon sections showed less severe pathological changes in AREG-treated mice, as evidenced by the restoration of epithelial architecture and less infiltration of immune cells in the

inflamed sites (**Figure 20C**). Moreover, *ex vivo* culture of colonic tissues showed decreased production of pro-inflammatory cytokines, such as IL-6, TNFα and IFNγ in mice received AREG (**Figure 20D**), suggesting that AREG can, at least partially, compensate the loss of neutrophils. Importantly, gene expression analysis revealed an elevated expression of HNF4α in IECs (**Figure 20E**), a protein which plays critical role in epithelial integrity and has been associated with ulcerative colitis [36]. To investigate whether AREG improves epithelial barrier permeability, we fed different groups of mice with FITC-dextran and examined serum fluorescence following FITC-dextran gavage. The enhanced expression of HNF4α corresponded to the improved permeability in the gut in neutrophil-deficient mice with additional AREG (**Figure 20F**). Taken together, these data indicate that neutrophil-mediated IEC protection is possibly associated with AREG induction of HNF4α.

DISCUSSION

Tightly regulated intestinal barrier function is crucial in the maintenance of intestinal homeostasis. We demonstrated in this report that neutrophils protect the intestines from inflammation, at least partially through induction of AREG production by IECs. Administration of exogenous AREG mitigated tissue damage following depletion of neutrophils upon DSS challenge by restoring epithelial barrier function. Our findings, thus, reveal a previously unappreciated innate immune mechanism for coordinated epithelial response to microbiota through neutrophils.

Despite the many advances made in studying the interaction between epithelial cells and immune cells, the interplay between IECs and innate cells remains poorly defined. We previously reported that IL-22-producing neutrophils enhance intestinal barrier integrity in the context of

colitis, suggesting that neutrophil-IECs crosstalk plays a critical role during inflammatory state [115]. To further interrogate the mechanisms underlying neutrophil regulation of IEC, we took advantage of intestinal epithelial enteroids, which recapitulate the diverse composition and complexity of the intestinal epithelial cells. Analysis of microarray data showed that neutrophil conditioned media-treated enteroids were enriched in mRNA responding to wound healing and junction formation, indicating a role for neutrophils in tissue repair. We also observed an increase in mRNA encoding proteins for IEC proliferation and differentiation, in which AREG is essential in both processes [7]. We confirmed that neutrophil conditioned media induced AREG expression by MSIE cells, a mouse small intestinal epithelial cell line. AREG has been shown as a crucial EGFR ligand in protection of inflammation in the intestine [6, 7]. We showed that the increased level of AREG corresponded with improved disease outcome in the DSS-induced colitis model. Moreover, additional exogenous AREG administration ameliorated disease with the improvement in the epithelial layer in the mice. Given that IBD is rooted in chronic dysregulated immune responses against the commensal microbiota, any initial contact with the microbiota by the immune system would lead to overwhelming inflammation that perpetuates itself into a chronic diseased state. As such, the rule of thumb for preventing aberrant immune activation is to possess a strong intact epithelial barrier to prevent penetration by the microbiota and leave the immune system quiescent. We speculate that deficiency in AREG production leads to a less robust epithelial barrier, thereby conferring susceptibility to IBD. In supporting this notion, we showed that the deficiency of AREG-EGFR signaling results in exacerbated colitis upon DSS insult. This finding is in accordance with a recent transcriptomic microarray analysis showing an increased intestinal ERRFI1 in both Crohn's disease and ulcerative colitis patients. ERRFI1 mediates inhibition of the EGFR, thereby blocking signaling of AREG and other EGF family members [37].

Interestingly, IECs themselves also express EGFR [133]. As accumulating evidence shows that AREG enhances epithelial function [6, 7], we thus treated MSIE cells with exogenous AREG to investigate if AREG can further amplify its own production. However, there was no evidence for changes in expression of AREG in IECs (**Figure 14B**).

Consistent with our previous finding using T cell-induced colitis model, depletion of neutrophils led to more severe inflammation in DSS colitis model in $RAG^{-/-}$ mice. This data reinforces the idea of the innate immune components, especially neutrophils, as indispensable regulators in inflammation resolution. We found that the expression of TGFβ was impaired in the intestines of mice receiving neutrophil-depleting treatment. Similar to lung epithelium, we demonstrated that TGFβ induced robust expression of AREG in IECs. However, unlike innate lymphoid cell [6], Treg [5], cholangiocyte [123], epidermal cell [124] or fibroblast [57, 134], in IECs, TGFβ remains the only inducer among cytokines/factors tested capable of eliciting AREG production. Although the first report of neutrophil production of TGFβ was dated back to 1989 [135], research attention has been mainly focusing on neutrophil-derived products facilitating TGFβ activation [136]. Our data provide the evidence that that neutrophils produced TGFβ was elevated in the setting of inflammatory state. Correlation between impaired tissue TGFβ and decreased IEC production of AREG revealed a previous unappreciated role for neutrophil regulation of barrier function.

As an essential regulator of epithelial integrity, HNF4α has been identified as a susceptibility gene conferring risk of IBD [36]. We demonstrated that depletion of neutrophils impaired HNF4α in colonic tissue [115], whereas exogenous AREG treatment rescued HNF4α expression in the colon of neutrophil-depleted mice, restoring epithelial permeability. Additionally, neutrophil conditioned media induced Cldn4 encoding tight junction protein and

Lamc2 encoding extracellular matrix glycoproteins in the enteroids. As it has been reported neutrophils direct contacting IECs prevents neutrophil apoptosis, and meantime confer protection against tissue injury [60], the question whether direct physical interaction between neutrophils and IECs promote barrier function warranties a future investigation.

ADAM17, a transmembrane protease found on the surface of neutrophils, regulates shedding of various substrates including AREG [61]. Our finding that neutrophil depletion impaired tissue ADAM17 level indicates a potential protective role of infiltrating neutrophils regulation of inflammation through localized AREG induction. Furthermore, neutrophil-facilitated AREG production is not only limited to epithelial origin, but also other cellular sources as well. Given the chemotactic effect of IECs on neutrophils under mucosal injury [137, 138], it is intriguing to speculate that IECs recruit and retain neutrophils in the inflammatory site as a way to increase local TGFβ level and promote AREG secretion during rampant inflammation. Such an occurrence would be consistent with the role of neutrophils to maintain and/or restore intestinal homeostasis.

In summary, our studies demonstrated a novel pathway of neutrophil regulation of intestinal inflammation through induction of AREG by IECs, which is dependent on TGFβ. As TGFβ has been considered as one of the most important anti-inflammatory cytokines, our data provides a novel anti-inflammatory function of TGFβ in the intestines through induction of IEC production of AREG.

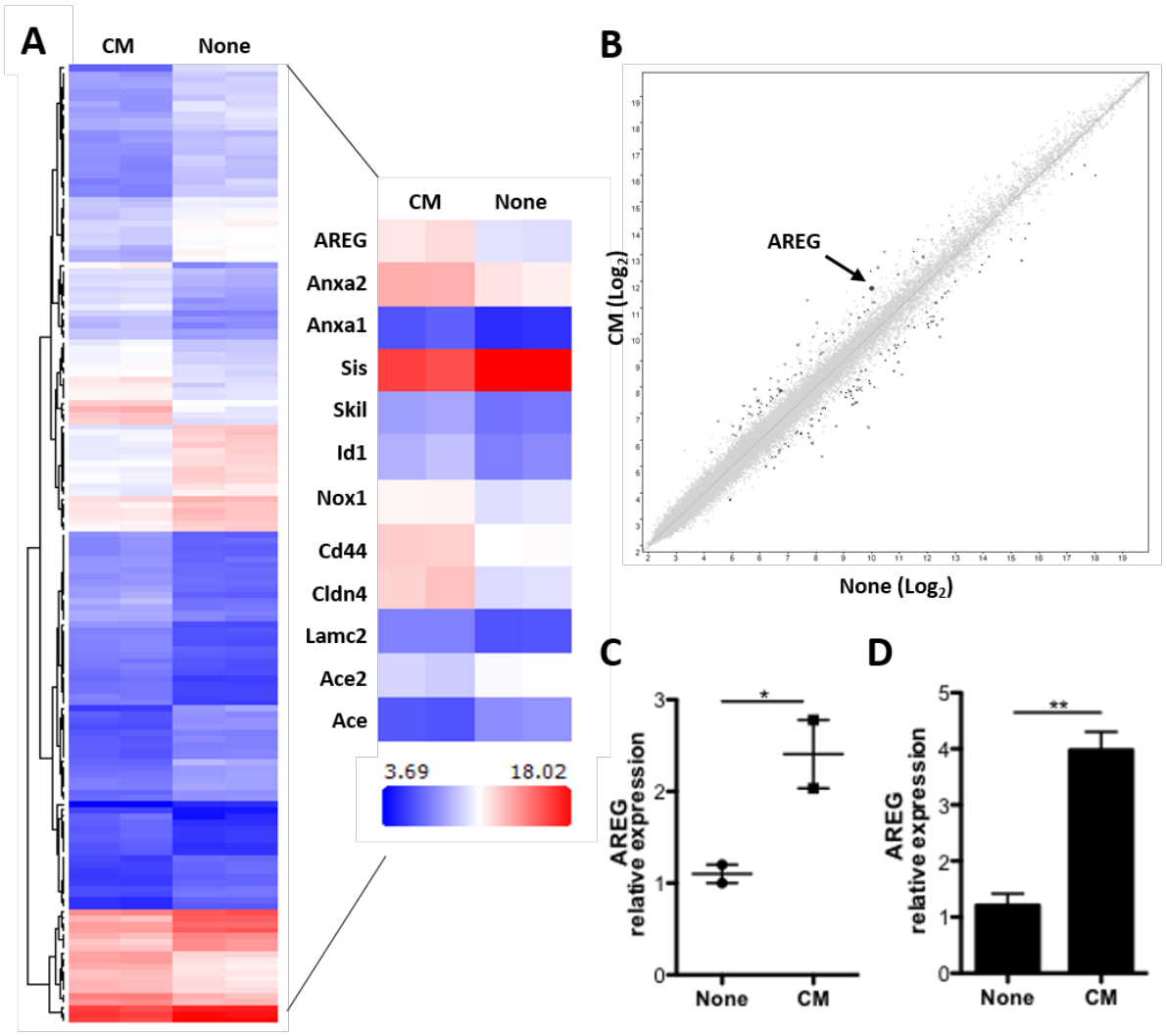

Figure 12. Neutrophil conditioned media induces AREG expression by IECs
Intestinal enteroids were cultured with or without neutrophil conditioned media for 6 h and the RNAs were applied to microarray analysis. (**A**) Hierarchical clustering of genes that were significantly ($P < 0.05$ one-way ANOVA) and differentially (|Fold change| > 2) expressed between the two groups (n = 2). Heat map of some differentially expressed probes between the two groups (n = 2). (**B**) Scatterplot displaying the log2 fold change in expression between the two groups (n = 2). (**C**) mRNA expression levels of AREG in the enteroids was measured by qRT-PCR and normalized to GAPDH. (**D**) MSIE cells were treated with or without neutrophil conditioned media for 6 h**,** and mRNA expression level of AREG measured by qRT-PCR and normalized to GAPDH. Data are presented as mean ± SEM of three independent experiments. CM, neutrophil conditioned media; None, media alone. *$P < 0.05$, **$P < 0.01$ Student's t test.

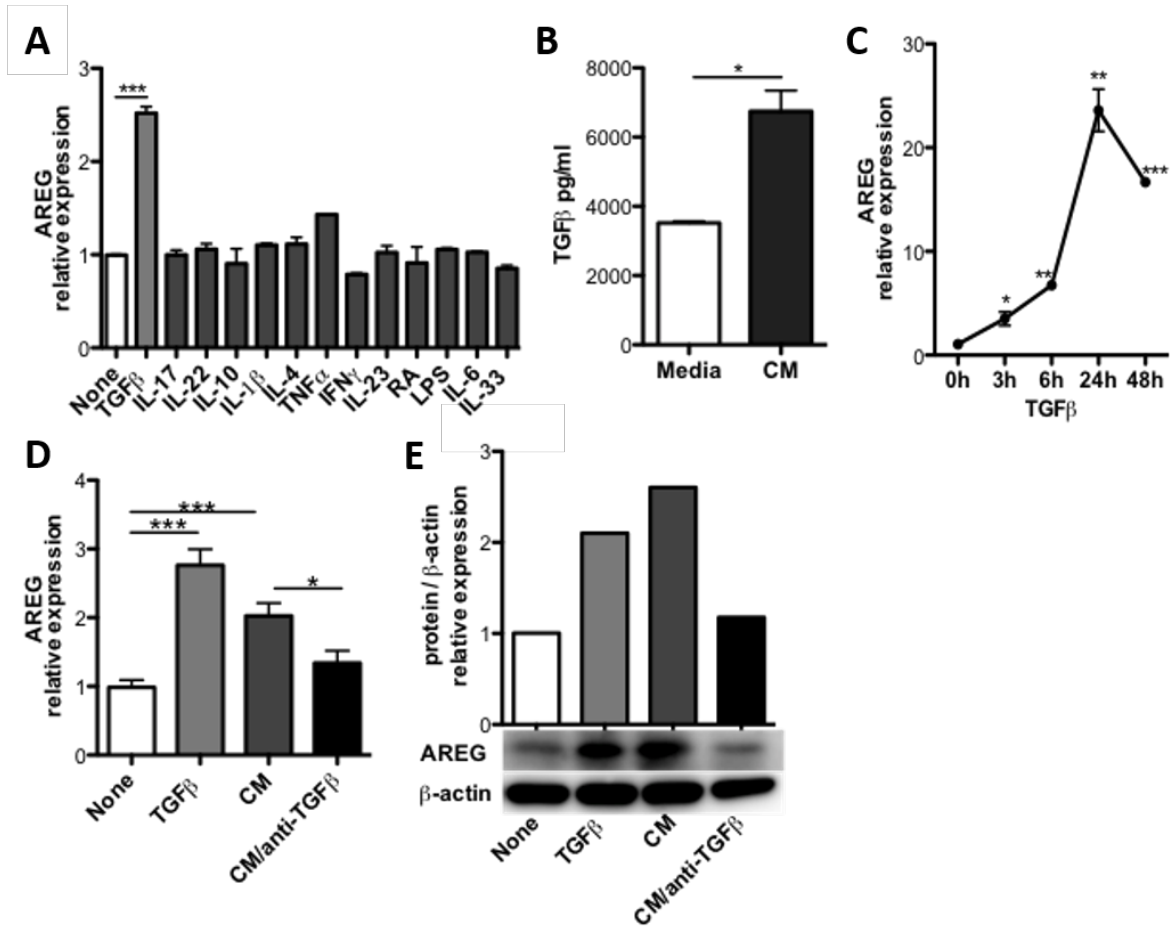

Figure 13. TGFβ induces AREG production in IECs
(**A**) MSIE cells were treated with different cytokines/factors for 6 h, and mRNA expression level of AREG was measured by qRT-PCR and normalized against GAPDH. (**B**) Level of TGFβ was determined in neutrophil conditioned media versus media control. (**C**) mRNA expression level of AREG in the MSIE cells treated with 20 μg/ml TGFβ over time, and normalized against GAPDH. (**D and E**) Neutrophil conditioned media was pre-treated with 10 μg/ml anti-TGFβ antibody and then added into MSIE cell cultures. (**D**) mRNA expression level (6 h) and (**E**) protein level of AREG (24 h) were measured by qRT-PCR and Western blot respectively. Data are presented as mean ± SEM of three independent experiments. CM, neutrophil conditioned media; None, media alone. *P < 0.05, **P < 0.01, ***P < 0.001 Student's t test, one-way ANOVA.

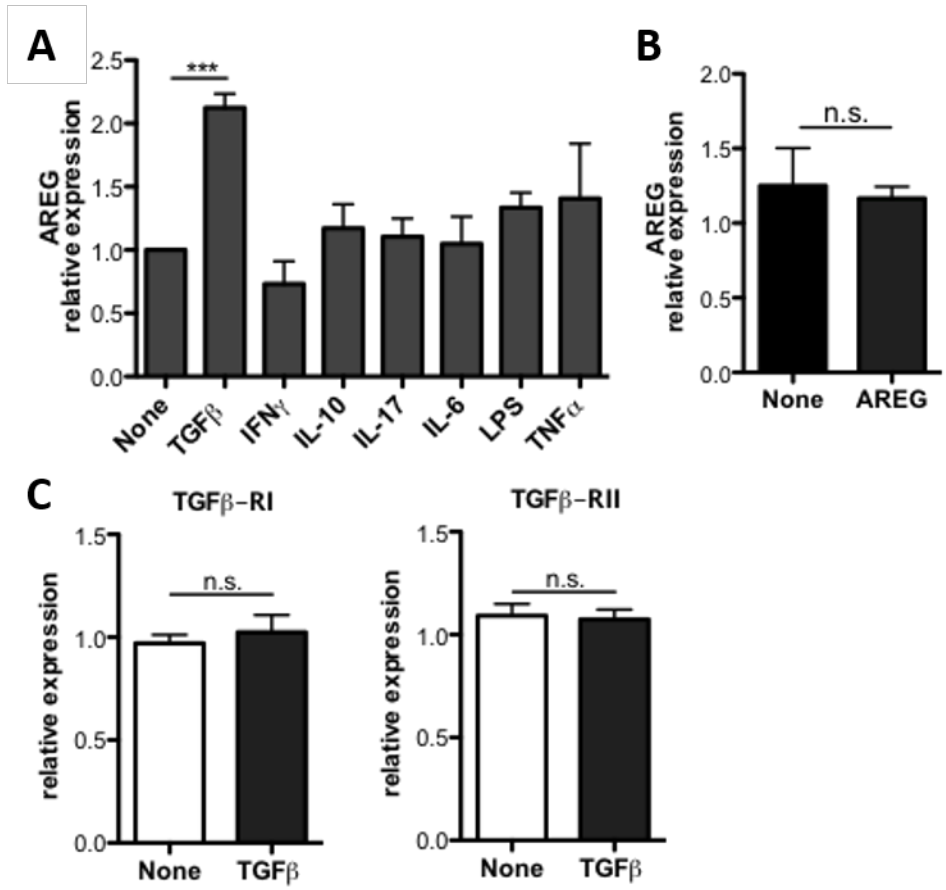

Figure 14. TGFβ induces AREG production in primary IECs
(**A and B**) mRNA expression level of AREG in isolated primary IECs treated with (**A**) different reagents, and (**B**) AREG. n.s., no significant difference; Data are presented as mean ± SEM of three independent experiments; ***P < 0.001 one-way ANOVA. (**C**) mRNA expression level of TGFβ receptor I and II in MSIE cells treated with TGFβ for 6 h. n.s., no significant difference; Data are presented as mean ± SEM of three independent experiments. Student's t test.

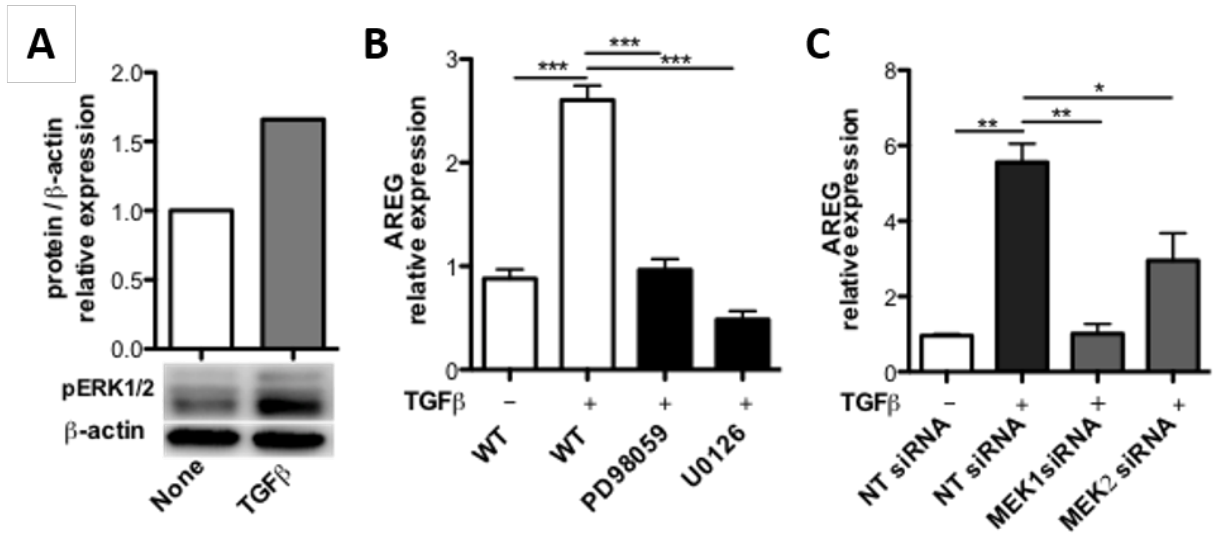

Figure 15. TGFβ induction of AREG in IECs is MEK1/2-dependent
MSIE cells were treated with or without TGFβ. (**A**) The phosphorylated ERK1/2 (pERK1/2) was determined by Western blot (1 h). (**B and C**) MEK1/2 was inhibited or knockdown in MSIE cells in the presence of TGFβ. mRNA expression level of AREG was measured by qRT-PCR in the MSIE cells treated with (**B**) specific inhibitors or (**C**) siRNA. Data are presented as mean ± SEM of three independent experiments; NT, no treatment; NT siRNA, non-targeting siRNA. *P < 0.05, **P < 0.01, ***P < 0.001 one-way ANOVA.

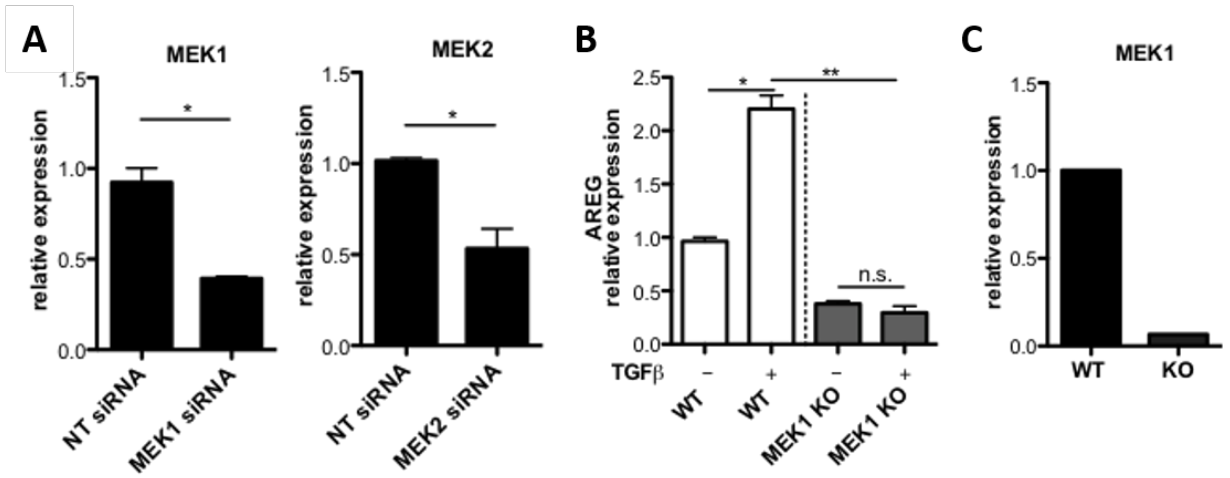

Figure 16. MEK1/2 regulates TGFβ-induced AREG production in IECs
(**A**) Transfection efficiency of siRNA targeted either MEK1 or MEK2 in MSIE cells. (**B and C**) MEK1 was knocked out in MSIE cells by CRISPR, and then treated with TGFβ for 6 h. (**B**) mRNA expression level of AREG in WT and MEK1 KO MSIE cells. (**C**) CRISPR knock out efficiency. n.s., no significant difference; Data are presented as mean ± SEM of three independent experiments. *P < 0.05, **P < 0.01 Student's t test, one-way ANOVA.

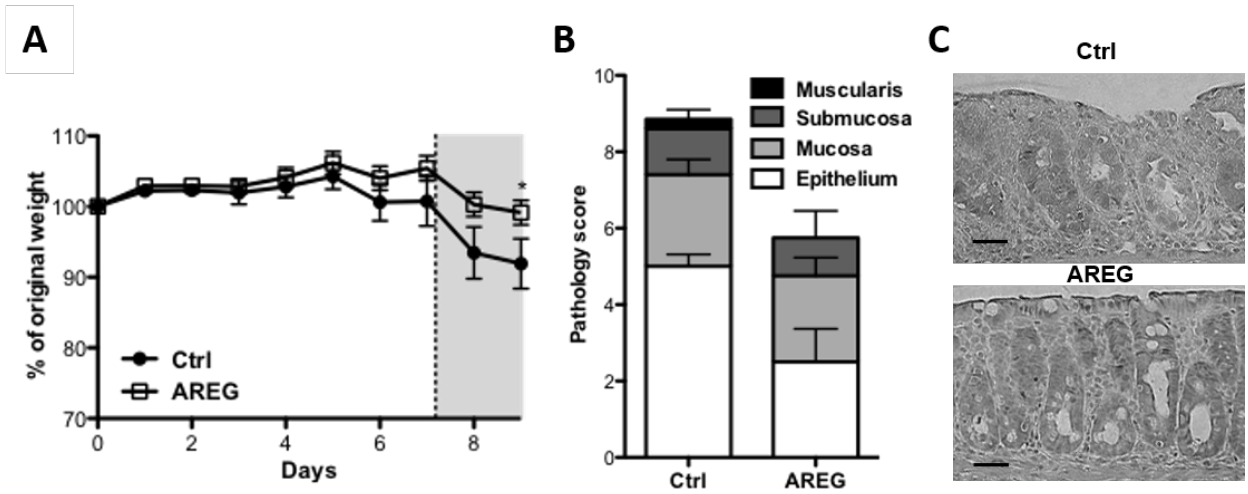

Figure 17. AREG protects the intestinal epithelium from DSS-induced injury
WT mice were administered 2% DSS in drinking water for 7 days, followed by 2 days of water. One group of mice received 15µg of AREG every 2 days. (**A**) Relative body weight change. (**B**) Blinded histopathological scoring. (**C**) Representative images of H&E-stained colon sections. (Scale bar, 100 µm) n = 4 per group per experiment. Data are representative of 3 independent experiments; *p<0.05 Student's t test.

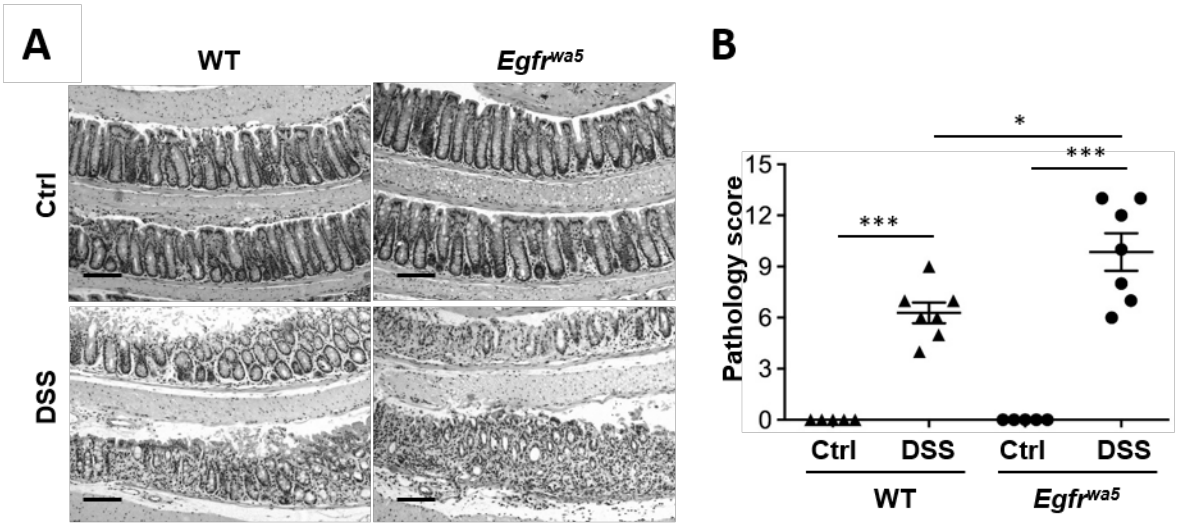

Figure 18. Deficiency in AREG signaling increases susceptibility to intestinal damage upon DSS insult

WT and Egfr^{wa5} mice received drinking water in the presence or absence of 3% DSS in drinking water. (**A**) Representative images of H&E-stained colon sections. (Scale bar, 200 μm) (**B**) Blinded histopathological scoring. n = 5-7 per group per experiment. Data are representative of 3 independent experiments; *P < 0.05, ***P < 0.001 one-way ANOVA.

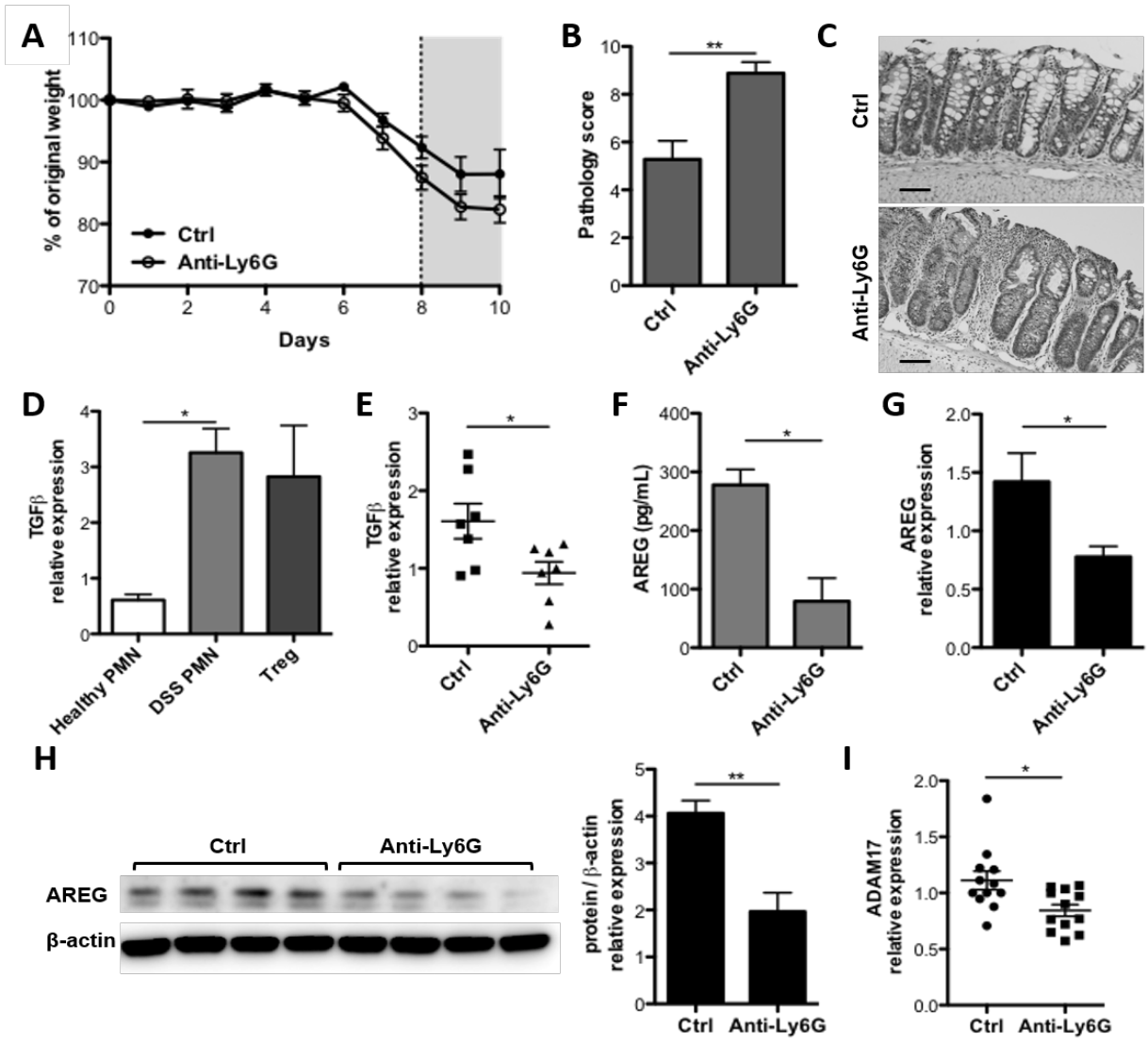

Figure 19. Neutrophils confer protection against DSS-induced colitis through the induction of AREG in IECs

Rag1$^{-/-}$ mice were administered 2% DSS in drinking water for 7 days, followed by 3 days of water. One group of mice received Ly6G-depleting antibody (4 mg/kg) every 3 days. (**A**) Relative body weight change. (**B**) Blinded histopathological scoring. (**C**) Representative images of H&E-stained colon sections. (Scale bar, 100 μm) (**D**) mRNA expression level of TGFβ in peripheral neutrophils isolated from healthy versus disease mice with Treg as positive control. (**E**) mRNA expression level of TGFβ in the colonic tissue of mice. (**F**) Soluble AREG was measured in colonic culture supernatant via ELISA. (**G and H**) IECs were isolated from the colon of mice, mRNA expression level (**G**) and protein level (**H**) of AREG were measured in IECs. (**I**) mRNA expression level of ADAM17 in the colonic tissues of mice. n = 4 per group. Data are representative of 3 independent experiments; PMN, neutrophil; *P < 0.05, **P < 0.01 unpaired Student's t test, one-way ANOVA, nonparametric Mann–Whitney U test.

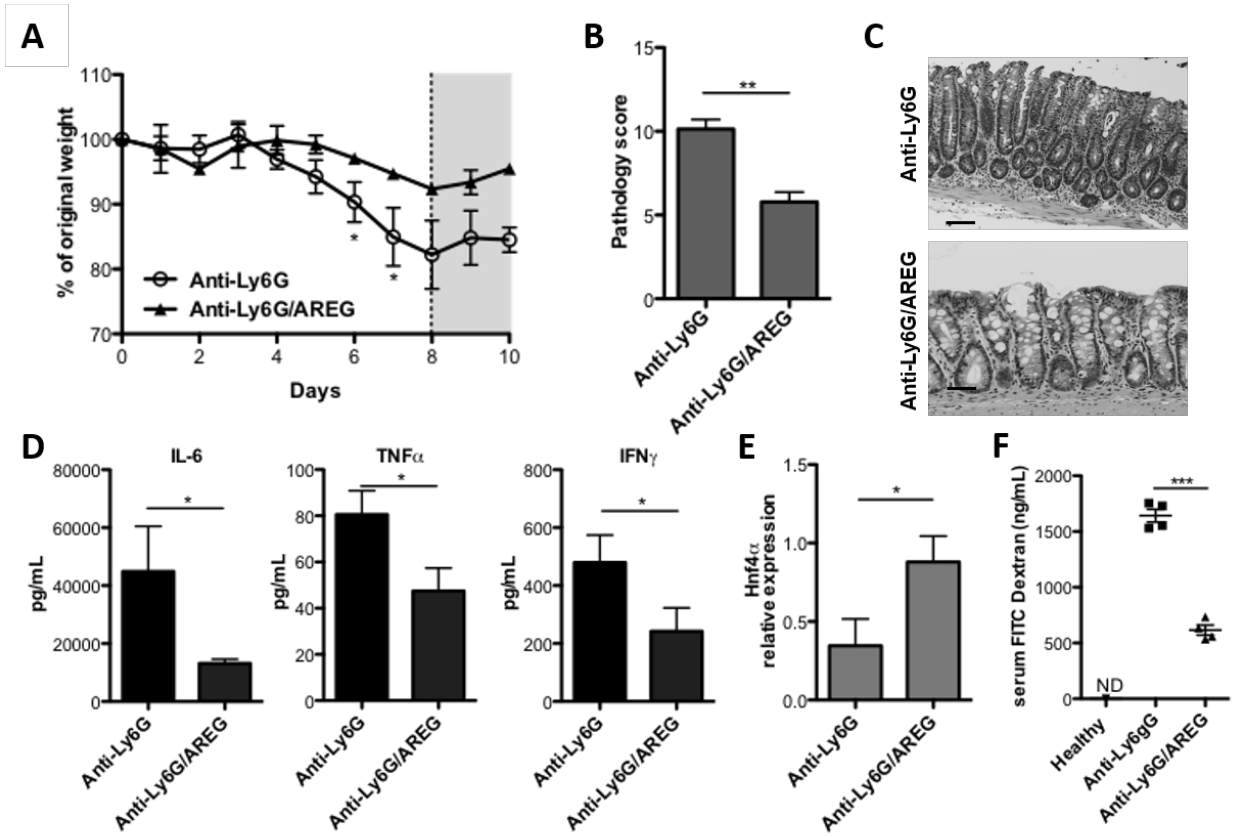

Figure 20. Neutrophil supernatant induces AREG expression in IECs
Rag1$^{-/-}$ mice were administered 2% DSS in drinking water for 7 days, followed by 3 days of water. The mice were given Ly6G-depleting antibody i.p. every 3 days. A group of mice received AREG every 2 days and the other group received PBS. (**A**) Relative body weight change. (**B**) Blinded histopathological scoring. (**C**) Representative images of H&E-stained colon sections. (Scale bar, 100 μm) (**D**) Protein level of inflammatory cytokines in colonic culture supernatant. (**E**) mRNA expression level of HNF4α in isolated IECs. (**F**) FITC-dextran level in plasma was determined. n = 4 per group. Data are representative of 3 independent experiments; ND, not detectable; *P < 0.05, ***P < 0.001 unpaired Student's t test, one-way ANOVA, nonparametric Mann–Whitney U test.

SUMMARY AND CONCLUSION

The intestines are the largest mucosal surface in the body, and are continually exposed to luminal microbiota and environmental insults. The mutualistic interaction between microbiota and the host requires an intact intestinal epithelial barrier and proper immunity. Chronic systemic inflammation due to the compromise of epithelial integrity or dysregulation of the subsequent immune responses predispose individuals to IBD. As an essential component of the innate immune system, neutrophils contribute to the maintenance and/or restoration of gut homeostasis. In my dissertation studies, I discuss how neutrophils contribute to AREG-mediated protection, and the mechanisms and functional implications underlying neutrophil production of both IL-17 and IL-22 in colitis.

It has been proposed that neutrophils are capable of producing both IL-17 and IL-22 under various inflammatory or infectious conditions [53, 54, 66, 90]. However, the underlying molecular mechanisms remain poorly defined. In the present study, I identified IL-23 as an active inducer of both IL-17 and IL-22 in neutrophils. Different from other cell types like $CD4^+$ T cell subtypes and ILC3, IL-23 remains the sole inducer of IL-17 and IL-22 in neutrophils. Notably, partially activated peritoneal neutrophils express higher amounts of IL-23 receptor compared to bone marrow naïve cells, and IL-23 further promotes the expression of its own receptor, likely suggesting a positive feedback loop to stabilize the neutrophil phenotype and contributing to constant IL-17 and IL-22 production. I showed that IL-23 induces the expression of two transcription factors, RORγt and AhR. RORγt regulates the production of both IL-17 and IL-22, whereas AhR only contributes to IL-22 production in neutrophils. The two transcription factors also work in a synergetic manner, as indicated by the depletion of cytokine production with pathway inhibition. Given the crucial role of the mTOR pathway in neutrophil metabolism and

functions [93-95], I tested PI3K-mTOR signaling in the presence of IL-23 treatment. Using specific inhibitors targeting mTOR, I found that IL-23 activates mTOR, specifically mTOR complex 1. Mechanistically, mTOR regulates the transcription of both RORγt and AhR as a way to mediate IL-17 and IL-22 production in neutrophils. In line with this, I observed that alternative activation pathways such as P38 MAPK pathway were unaffected by mTOR/PI3K inhibition. Finally, I showed that STAT3-NF-κB pathway is also involved in IL-23-induced cytokine production by neutrophils.

As one of the first responders to microbial invasion, neutrophils contribute to the resolution of inflammation during the early stages of colitis. However, the accumulation and insufficient clearance of neutrophils have been considered detrimental in the context of a chronic inflammation state [98]. In the present study, I challenged the conventional dogma by depleting neutrophils in mice in the setting of T cell adoptive transfer colitis. I demonstrated that without the presence of neutrophils, mice suffered from worse disease compared to control mice as evidenced by increased weight loss and increased disease score. In line with this finding, I observed similar outcomes in both lymphocyte-sufficient and lymphocyte-deficient mice with DSS-induced colitis. Together, my initial studies indicate a protective role for neutrophils in colitis.

Prevailing evidence has demonstrated a protective role of IL-22 on host antibacterial defense, tissue regeneration and wound healing [58]. This phenomenon has been observed in my studies in which blockade of IL-22 signaling led to exacerbated colitis in mice compared to control group. Given my data of a clear subset of IL-22^{+} neutrophils after stimulation of IL-23, and large amounts of soluble IL-22 detected in the neutrophil conditioned media, I speculated that IL-22-producing neutrophils remain an important source of IL-22, promoting colitis resolution. In fact, a recent report showed that IL-22-producing neutrophils promote antimicrobial peptide production

of intestinal epithelial cells, aiding host bactericidal response [66]. To isolate the function of IL-22$^+$ neutrophils, I depleted ILCs in lymphocyte-deficient mice to block other major IL-22 sources. I showed that IL-22$^+$ neutrophils mediated the expression of HNF4α, a susceptibility gene for UC reported in a recent GWAS study [36]. HNF4α facilitates the formation of epithelial junction proteins which is crucial for epithelial integrity. Consistent with these findings, I found that mice with functional IL-22-producing neutrophils have preserved intestinal permeability.

In light of the decrease HNF4α in the intestines of neutrophil-depleted mice, I questioned whether neutrophils interact with IECs upon an inflammatory state. By utilizing intestinal epithelial enteroid culture system, microarray data showed that neutrophil conditioned media-treated enteroids had up-regulated expression of the gene encoding AREG. AREG belongs to the EGF family, which plays important role in cell processes including cell proliferation, differentiation, survival and wound healing [6, 7]. Recently, it has been shown that TGFβ is capable of inducing AREG in lung epithelium [139]. In accordance with this finding, I proved that neutrophil-derived TGFβ attributes to the AREG production in IECs. Few reports show that human peripheral neutrophil produce TGFβ [135]. I detected soluble TGFβ in neutrophil conditioned media. Consistently, murine peripheral neutrophils showed increased TGFβ expression under inflammatory insult when compared to control neutrophils from healthy state. On the other hand, I observed impaired tissue expression of TGFβ in the intestines when neutrophils were depleted. Strikingly, decrease tissue TGFβ positively correlated with the production of AREG by IECs in the context of intestinal inflammation. Together, my data revealed a previously unappreciated role for neutrophils as a critical source of TGFβ and regulation of barrier function.

AREG-EGFR signaling has been implicated in the pathogenesis of IBD [37]. Deficiency in this pathway leads to increased susceptibility of chemical-induced colitis. Oppositely,

exogenous AREG protects mice from intestinal inflammation. I further demonstrated that administration of exogenous AREG following depletion of neutrophils renders mice resistance against DSS-induced colitis. The AREG-mediated protection was conferred by inducing HNF4α. Indeed, I revealed elevated expression of Cldn4 and Lamc2, which encode tight junction protein and extracellular matrix glycoproteins respectively, in enteroids treated with neutrophil conditioned media. Moreover, I found that neutrophil depletion resulted in decreased tissue ADAM17 levels in the intestines of mice. ADAM17 is a protease indispensable for AREG shedding [131]. Given that neutrophils express ADAM17 on the surface of membrane [140], it is intriguing to speculate that neutrophils not only directly induce IEC production of AREG, but also contribute to AREG production from other cellular sources.

Taken together, my studies demonstrated novel mechanisms of neutrophil regulation of intestinal colitis through interaction with IECs. In the context of microbial breach, injured IECs release chemokines and cytokines to recruit and retain neutrophils in the inflamed sites [137, 138]. Accumulation of neutrophils increases local IL-22 and TGFβ concentration attempting to promote epithelial barrier function and re-establish intestinal homeostasis. However, it is plausible that these repeated attempts fail to hamper the rampant inflammation, and over time end up pushing the system further out of equilibrium. Therefore, the role of neutrophils in IBD is more complicated than we originally thought, and should not be simply classified as either pro-inflammatory or anti-inflammatory. Several points have to be taken into considerations: 1) Infiltration of neutrophils ≠ pro-inflammatory role. Increasing evidence suggests that transmigrating neutrophils help shape the mucosal microenvironment contributing to tissue protection. 2) Neutrophils are critical sources of various cytokines. Influx of neutrophils enable replenishment of cytokines to maximally augment the anti-inflammatory effect. 3) Neutrophil targeted treatments may provide new insight

into IBD therapy. Our studies to better understand the mechanisms of neutrophil regulation of colitis may help direct therapeutic approaches for IBD through the regulation of neutrophils and their pathways.

References

1. Alexander, K.L., S.R. Targan, and C.O. Elson, 3rd, *Microbiota activation and regulation of innate and adaptive immunity.* Immunol Rev, 2014. **260**(1): p. 206-20.
2. Peterson, L.W. and D. Artis, *Intestinal epithelial cells: regulators of barrier function and immune homeostasis.* Nat Rev Immunol, 2014. **14**(3): p. 141-53.
3. Strober, W., I. Fuss, and P. Mannon, *The fundamental basis of inflammatory bowel disease.* J Clin Invest, 2007. **117**(3): p. 514-21.
4. Cao, A.T., et al., *Th17 cells upregulate polymeric Ig receptor and intestinal IgA and contribute to intestinal homeostasis.* J Immunol, 2012. **189**(9): p. 4666-73.
5. Arpaia, N., et al., *A Distinct Function of Regulatory T Cells in Tissue Protection.* Cell, 2015. **162**(5): p. 1078-89.
6. Monticelli, L.A., et al., *IL-33 promotes an innate immune pathway of intestinal tissue protection dependent on amphiregulin-EGFR interactions.* Proc Natl Acad Sci U S A, 2015. **112**(34): p. 10762-7.
7. Shao, J. and H. Sheng, *Amphiregulin promotes intestinal epithelial regeneration: roles of intestinal subepithelial myofibroblasts.* Endocrinology, 2010. **151**(8): p. 3728-37.
8. Jandhyala, S.M., et al., *Role of the normal gut microbiota.* World J Gastroenterol, 2015. **21**(29): p. 8787-803.
9. Hall, A.B., A.C. Tolonen, and R.J. Xavier, *Human genetic variation and the gut microbiome in disease.* Nat Rev Genet, 2017.
10. Rodriguez, J.M., et al., *The composition of the gut microbiota throughout life, with an emphasis on early life.* Microb Ecol Health Dis, 2015. **26**: p. 26050.
11. Levy, M., et al., *Dysbiosis and the immune system.* Nat Rev Immunol, 2017. **17**(4): p. 219-232.
12. Kau, A.L., et al., *Human nutrition, the gut microbiome and the immune system.* Nature, 2011. **474**(7351): p. 327-36.
13. Sun, M., et al., *Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases.* J Gastroenterol, 2017. **52**(1): p. 1-8.
14. Chen, J.S., D.V. Faller, and R.A. Spanjaard, *Short-chain fatty acid inhibitors of histone deacetylases: promising anticancer therapeutics?* Curr Cancer Drug Targets, 2003. **3**(3): p. 219-36.
15. Smith, P.M., et al., *The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis.* Science, 2013. **341**(6145): p. 569-73.
16. Bhutia, Y.D. and V. Ganapathy, *Short, but Smart: SCFAs Train T Cells in the Gut to Fight Autoimmunity in the Brain.* Immunity, 2015. **43**(4): p. 629-31.
17. van der Flier, L.G. and H. Clevers, *Stem cells, self-renewal, and differentiation in the intestinal epithelium.* Annu Rev Physiol, 2009. **71**: p. 241-60.

18. Kaiko, G.E., et al., *The Colonic Crypt Protects Stem Cells from Microbiota-Derived Metabolites.* Cell, 2016. **165**(7): p. 1708-1720.

19. Sonnenberg, G.F. and D. Artis, *Innate lymphoid cell interactions with microbiota: implications for intestinal health and disease.* Immunity, 2012. **37**(4): p. 601-10.

20. Kruger, P., et al., *Neutrophils: Between host defence, immune modulation, and tissue injury.* PLoS Pathog, 2015. **11**(3): p. e1004651.

21. M'Koma, A.E., *Diagnosis of inflammatory bowel disease: Potential role of molecular biometrics.* World J Gastrointest Surg, 2014. **6**(11): p. 208-19.

22. Saleh, M. and C.O. Elson, *Experimental inflammatory bowel disease: insights into the host-microbiota dialog.* Immunity, 2011. **34**(3): p. 293-302.

23. Baumgart, D.C. and W.J. Sandborn, *Inflammatory bowel disease: clinical aspects and established and evolving therapies.* Lancet, 2007. **369**(9573): p. 1641-57.

24. Vavricka, S.R., et al., *Extraintestinal Manifestations of Inflammatory Bowel Disease.* Inflamm Bowel Dis, 2015. **21**(8): p. 1982-92.

25. Lennard-Jones, J.E., *Classification of inflammatory bowel disease.* Scand J Gastroenterol Suppl, 1989. **170**: p. 2-6; discussion 16-9.

26. Gecse, K.B., et al., *A global consensus on the classification, diagnosis and multidisciplinary treatment of perianal fistulising Crohn's disease.* Gut, 2014. **63**(9): p. 1381-92.

27. Kobayashi, T., et al., *IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease.* Gut, 2008. **57**(12): p. 1682-9.

28. Fujino, S., et al., *Increased expression of interleukin 17 in inflammatory bowel disease.* Gut, 2003. **52**(1): p. 65-70.

29. Brand, S., et al., *IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration.* Am J Physiol Gastrointest Liver Physiol, 2006. **290**(4): p. G827-38.

30. Evans-Marin, H.L. and Y. Cong, *Microbiota regulation of inflammatory bowel disease.* Inflamm Allergy Drug Targets, 2014. **13**(1): p. 65-73.

31. Zhang, Y.Z. and Y.Y. Li, *Inflammatory bowel disease: pathogenesis.* World J Gastroenterol, 2014. **20**(1): p. 91-9.

32. Petnicki-Ocwieja, T., et al., *Nod2 is required for the regulation of commensal microbiota in the intestine.* Proc Natl Acad Sci U S A, 2009. **106**(37): p. 15813-8.

33. Oehlers, S.H., et al., *The inflammatory bowel disease (IBD) susceptibility genes NOD1 and NOD2 have conserved anti-bacterial roles in zebrafish.* Dis Model Mech, 2011. **4**(6): p. 832-41.

34. Iliev, I.D., et al., *Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis.* Science, 2012. **336**(6086): p. 1314-7.

35. Boltin, D., et al., *Mucin function in inflammatory bowel disease: an update.* J Clin Gastroenterol, 2013. **47**(2): p. 106-11.

36. Consortium, U.I.G., et al., *Genome-wide association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region.* Nat Genet, 2009. **41**(12): p. 1330-4.

37. Raine, T., et al., *Generation of primary human intestinal T cell transcriptomes reveals differential expression at genetic risk loci for immune-mediated disease.* Gut, 2015. **64**(2): p. 250-9.

38. Wu, W., et al., *Microbiota-specific Th17 Cells: Yin and Yang in Regulation of Inflammatory Bowel Disease.* Inflamm Bowel Dis, 2016. **22**(6): p. 1473-82.

39. Wera, O., P. Lancellotti, and C. Oury, *The Dual Role of Neutrophils in Inflammatory Bowel Diseases.* J Clin Med, 2016. **5**(12).

40. Pillay, J., et al., *In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days.* Blood, 2010. **116**(4): p. 625-7.

41. Ryan, P., et al., *Bacterial DNA within granulomas of patients with Crohn's disease--detection by laser capture microdissection and PCR.* Am J Gastroenterol, 2004. **99**(8): p. 1539-43.

42. Marks, D.J., et al., *Inflammatory bowel disease in CGD reproduces the clinicopathological features of Crohn's disease.* Am J Gastroenterol, 2009. **104**(1): p. 117-24.

43. Cesaro, A., et al., *Differential expression and regulation of ADAM17 and TIMP3 in acute inflamed intestinal epithelia.* Am J Physiol Gastrointest Liver Physiol, 2009. **296**(6): p. G1332-43.

44. Nagao, Y., et al., *[Effects of ulinastatin on experimental ulcerative colitis in rats].* Nihon Yakurigaku Zasshi, 1997. **109**(1): p. 41-52.

45. Savchenko, A.S., et al., *Long pentraxin 3 (PTX3) expression and release by neutrophils in vitro and in ulcerative colitis.* Pathol Int, 2011. **61**(5): p. 290-7.

46. Fournier, B.M. and C.A. Parkos, *The role of neutrophils during intestinal inflammation.* Mucosal Immunol, 2012. **5**(4): p. 354-66.

47. Sakuraba, A., et al., *Th1/Th17 immune response is induced by mesenteric lymph node dendritic cells in Crohn's disease.* Gastroenterology, 2009. **137**(5): p. 1736-45.

48. Kvedaraite, E., et al., *Tissue-infiltrating neutrophils represent the main source of IL-23 in the colon of patients with IBD.* Gut, 2016. **65**(10): p. 1632-41.

49. Kolls, J.K. and Z. Zhang, *Anti-interleukin-12 antibody for active Crohn's disease.* N Engl J Med, 2005. **352**(6): p. 627-8; author reply 627-8.

50. Gaffen, S.L., et al., *The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing.* Nat Rev Immunol, 2014. **14**(9): p. 585-600.

51. Shabgah, A.G., E. Fattahi, and F.Z. Shahneh, *Interleukin-17 in human inflammatory diseases.* Postepy Dermatol Alergol, 2014. **31**(4): p. 256-61.

52. Symons, A., A.L. Budelsky, and J.E. Towne, *Are Th17 cells in the gut pathogenic or protective?* Mucosal Immunol, 2012. **5**(1): p. 4-6.

53. Taylor, P.R., et al., *Activation of neutrophils by autocrine IL-17A-IL-17RC interactions during fungal infection is regulated by IL-6, IL-23, RORgammat and dectin-2.* Nat Immunol, 2014. **15**(2): p. 143-51.

54. Werner, J.L., et al., *Neutrophils produce interleukin 17A (IL-17A) in a dectin-1- and IL-23-dependent manner during invasive fungal infection.* Infect Immun, 2011. **79**(10): p. 3966-77.

55. O'Connor, W., Jr., et al., *A protective function for interleukin 17A in T cell-mediated intestinal inflammation.* Nat Immunol, 2009. **10**(6): p. 603-9.

56. Zhang, L.W., et al., *Interleukin-17 Impairs Salivary Tight Junction Integrity in Sjogren's Syndrome.* J Dent Res, 2016. **95**(7): p. 784-92.

57. Andoh, A., et al., *Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts.* Gastroenterology, 2005. **129**(3): p. 969-84.

58. Dudakov, J.A., A.M. Hanash, and M.R. van den Brink, *Interleukin-22: immunobiology and pathology.* Annu Rev Immunol, 2015. **33**: p. 747-85.

59. Sabat, R., W. Ouyang, and K. Wolk, *Therapeutic opportunities of the IL-22-IL-22R1 system.* Nat Rev Drug Discov, 2014. **13**(1): p. 21-38.

60. Sumagin, R., et al., *Neutrophil interactions with epithelial-expressed ICAM-1 enhances intestinal mucosal wound healing.* Mucosal Immunol, 2016. **9**(5): p. 1151-62.

61. Carnet, O., et al., *Mesenchymal Stem Cells Shed Amphiregulin at the Surface of Lung Carcinoma Cells in a Juxtacrine Manner.* Neoplasia, 2015. **17**(7): p. 552-63.

62. Ostanin, D.V., et al., *Acquisition of antigen-presenting functions by neutrophils isolated from mice with chronic colitis.* J Immunol, 2012. **188**(3): p. 1491-502.

63. Mumy, K.L. and B.A. McCormick, *The role of neutrophils in the event of intestinal inflammation.* Curr Opin Pharmacol, 2009. **9**(6): p. 697-701.

64. Zhang, R., et al., *Up-regulation of Gr1+CD11b+ population in spleen of dextran sulfate sodium administered mice works to repair colitis.* Inflamm Allergy Drug Targets, 2011. **10**(1): p. 39-46.

65. de Luca, A., et al., *IL-1 receptor blockade restores autophagy and reduces inflammation in chronic granulomatous disease in mice and in humans.* Proc Natl Acad Sci U S A, 2014. **111**(9): p. 3526-31.

66. Zindl, C.L., et al., *IL-22-producing neutrophils contribute to antimicrobial defense and restitution of colonic epithelial integrity during colitis.* Proc Natl Acad Sci U S A, 2013. **110**(31): p. 12768-73.

67. Bleicher, L., et al., *Crystal structure of the IL-22/IL-22R1 complex and its implications for the IL-22 signaling mechanism.* FEBS Lett, 2008. **582**(20): p. 2985-92.

68. Turner, J.E., B. Stockinger, and H. Helmby, *IL-22 mediates goblet cell hyperplasia and worm expulsion in intestinal helminth infection.* PLoS Pathog, 2013. **9**(10): p. e1003698.

69. Li, L. and V.A. Boussiotis, *The role of IL-17-producing Foxp3+ CD4+ T cells in inflammatory bowel disease and colon cancer.* Clin Immunol, 2013. **148**(2): p. 246-53.

70. Zhang, Z., et al., *Critical role of IL-17 receptor signaling in acute TNBS-induced colitis.* Inflamm Bowel Dis, 2006. **12**(5): p. 382-8.

71. Feng, T., et al., *Th17 cells induce colitis and promote Th1 cell responses through IL-17 induction of innate IL-12 and IL-23 production.* J Immunol, 2011. **186**(11): p. 6313-8.

72. Huppler, A.R., et al., *Neutrophils Do Not Express IL-17A in the Context of Acute Oropharyngeal Candidiasis.* Pathogens, 2015. **4**(3): p. 559-72.

73. Carlsen, E.D., et al., *Permissive and protective roles for neutrophils in leishmaniasis.* Clin Exp Immunol, 2015. **182**(2): p. 109-18.

74. Ouyang, W., *Distinct roles of IL-22 in human psoriasis and inflammatory bowel disease.* Cytokine Growth Factor Rev, 2010. **21**(6): p. 435-41.

75. Chung, Y., et al., *Critical regulation of early Th17 cell differentiation by interleukin-1 signaling.* Immunity, 2009. **30**(4): p. 576-87.

76. Ivanov, II, et al., *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-33.

77. Lee, J.S., M. Cella, and M. Colonna, *AHR and the Transcriptional Regulation of Type-17/22 ILC.* Front Immunol, 2012. **3**: p. 10.

78. Veldhoen, M., et al., *The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins.* Nature, 2008. **453**(7191): p. 106-9.

79. Abe, H., et al., *Aryl hydrocarbon receptor plays protective roles in ConA-induced hepatic injury by both suppressing IFN-gamma expression and inducing IL-22.* Int Immunol, 2014. **26**(3): p. 129-37.

80. Haidinger, M., et al., *A versatile role of mammalian target of rapamycin in human dendritic cell function and differentiation.* J Immunol, 2010. **185**(7): p. 3919-31.

81. Itakura, A. and O.J. McCarty, *Pivotal role for the mTOR pathway in the formation of neutrophil extracellular traps via regulation of autophagy.* Am J Physiol Cell Physiol, 2013. **305**(3): p. C348-54.

82. Hu, M., et al., *AZD8055 induces cell death associated with autophagy and activation of AMPK in hepatocellular carcinoma.* Oncol Rep, 2014. **31**(2): p. 649-56.

83. Willems, L., et al., *The dual mTORC1 and mTORC2 inhibitor AZD8055 has anti-tumor activity in acute myeloid leukemia.* Leukemia, 2012. **26**(6): p. 1195-202.

84. Li, S., et al., *The mTOR inhibitor AZD8055 inhibits proliferation and glycolysis in cervical cancer cells.* Oncol Lett, 2013. **5**(2): p. 717-721.

85. Davis, W.J., P.Z. Lehmann, and W. Li, *Nuclear PI3K signaling in cell growth and tumorigenesis.* Front Cell Dev Biol, 2015. **3**: p. 24.

86. Hirahara, K., et al., *Signal transduction pathways and transcriptional regulation in Th17 cell differentiation.* Cytokine Growth Factor Rev, 2010. **21**(6): p. 425-34.

87. Backert, I., et al., *STAT3 activation in Th17 and Th22 cells controls IL-22-mediated epithelial host defense during infectious colitis.* J Immunol, 2014. **193**(7): p. 3779-91.

88. Nguyen-Jackson, H., et al., *STAT3 controls the neutrophil migratory response to CXCR2 ligands by direct activation of G-CSF-induced CXCR2 expression and via modulation of CXCR2 signal transduction.* Blood, 2010. **115**(16): p. 3354-63.

89. Chen, H., et al., *Discovery of O-Alkylamino Tethered Niclosamide Derivatives as Potent and Orally Bioavailable Anticancer Agents.* ACS Med Chem Lett, 2013. **4**(2): p. 180-185.

90. Campillo-Gimenez, L., et al., *Neutrophils in antiretroviral therapy-controlled HIV demonstrate hyperactivation associated with a specific IL-17/IL-22 environment.* J Allergy Clin Immunol, 2014. **134**(5): p. 1142-52 e5.

91. Katayama, M., et al., *Neutrophils are essential as a source of IL-17 in the effector phase of arthritis.* PLoS One, 2013. **8**(5): p. e62231.

92. Keijsers, R., et al., *In vivo induction of cutaneous inflammation results in the accumulation of extracellular trap-forming neutrophils expressing RORgammat and IL-17.* J Invest Dermatol, 2014. **134**(5): p. 1276-1284.

93. Thomson, A.W., H.R. Turnquist, and G. Raimondi, *Immunoregulatory functions of mTOR inhibition.* Nat Rev Immunol, 2009. **9**(5): p. 324-37.

94. Cargnello, M., J. Tcherkezian, and P.P. Roux, *The expanding role of mTOR in cancer cell growth and proliferation.* Mutagenesis, 2015. **30**(2): p. 169-76.

95. Nardella, C., et al., *Differential requirement of mTOR in postmitotic tissues and tumorigenesis.* Sci Signal, 2009. **2**(55): p. ra2.

96. Natsui, M., et al., *Selective depletion of neutrophils by a monoclonal antibody, RP-3, suppresses dextran sulphate sodium-induced colitis in rats.* J Gastroenterol Hepatol, 1997. **12**(12): p. 801-8.

97. Kucharzik, T., et al., *Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins.* Am J Pathol, 2001. **159**(6): p. 2001-9.

98. Campbell, E.L., et al., *Transmigrating neutrophils shape the mucosal microenvironment through localized oxygen depletion to influence resolution of inflammation.* Immunity, 2014. **40**(1): p. 66-77.

99. Cong, Y., et al., *A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota.* Proc Natl Acad Sci U S A, 2009. **106**(46): p. 19256-61.

100. Cong, Y., et al., *Colitis induced by enteric bacterial antigen-specific CD4+ T cells requires CD40-CD40 ligand interactions for a sustained increase in mucosal IL-12.* J Immunol, 2000. **165**(4): p. 2173-82.

101. Iqbal, N., et al., *T helper 1 and T helper 2 cells are pathogenic in an antigen-specific model of colitis.* J Exp Med, 2002. **195**(1): p. 71-84.

102. Feng, T., et al., *Interleukin-12 converts Foxp3+ regulatory T cells to interferon-gamma-producing Foxp3+ T cells that inhibit colitis.* Gastroenterology, 2011. **140**(7): p. 2031-43.

103. Pastorelli, L., et al., *Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics.* Front Immunol, 2013. **4**: p. 280.

104. Goc, J., M.R. Hepworth, and G.F. Sonnenberg, *Group 3 innate lymphoid cells: regulating host-commensal bacteria interactions in inflammation and cancer.* Int Immunol, 2016. **28**(1): p. 43-52.

105. Chewning, J.H. and C.T. Weaver, *Development and survival of Th17 cells within the intestines: the influence of microbiome- and diet-derived signals.* J Immunol, 2014. **193**(10): p. 4769-77.

106. Putignani, L., et al., *The human gut microbiota: a dynamic interplay with the host from birth to senescence settled during childhood.* Pediatr Res, 2014. **76**(1): p. 2-10.

107. McCole, D.F., *IBD candidate genes and intestinal barrier regulation.* Inflamm Bowel Dis, 2014. **20**(10): p. 1829-49.

108. Bischoff, S.C., et al., *Intestinal permeability--a new target for disease prevention and therapy.* BMC Gastroenterol, 2014. **14**: p. 189.

109. Zaiss, D.M.W., et al., *Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair.* Immunity, 2015. **42**(2): p. 216-226.

110. Nam, K.T., et al., *Amphiregulin-deficient mice develop spasmolytic polypeptide expressing metaplasia and intestinal metaplasia.* Gastroenterology, 2009. **136**(4): p. 1288-96.

111. Monticelli, L.A., et al., *Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus.* Nat Immunol, 2011. **12**(11): p. 1045-54.

112. Zaiss, D.M., et al., *Amphiregulin, a TH2 cytokine enhancing resistance to nematodes.* Science, 2006. **314**(5806): p. 1746.

113. Zaiss, D.M., et al., *Amphiregulin enhances regulatory T cell-suppressive function via the epidermal growth factor receptor.* Immunity, 2013. **38**(2): p. 275-84.

114. Hall, O.J., et al., *Progesterone-Based Therapy Protects Against Influenza by Promoting Lung Repair and Recovery in Females.* PLoS Pathog, 2016. **12**(9): p. e1005840.

115. Chen, F., et al., *mTOR Mediates IL-23 Induction of Neutrophil IL-17 and IL-22 Production.* J Immunol, 2016. **196**(10): p. 4390-9.

116. Zhou, G., et al., *CD177+ neutrophils as functionally activated neutrophils negatively regulate IBD.* Gut, 2017.

117. Whitehead, R.H. and P.S. Robinson, *Establishment of conditionally immortalized epithelial cell lines from the intestinal tissue of adult normal and transgenic mice.* Am J Physiol Gastrointest Liver Physiol, 2009. **296**(3): p. G455-60.

118. Wright, H.L., R.J. Moots, and S.W. Edwards, *The multifactorial role of neutrophils in rheumatoid arthritis.* Nat Rev Rheumatol, 2014. **10**(10): p. 593-601.

119. Naegelen, I., et al., *Regulation of Neutrophil Degranulation and Cytokine Secretion: A Novel Model Approach Based on Linear Fitting.* J Immunol Res, 2015. **2015**: p. 817038.

120. Tecchio, C., A. Micheletti, and M.A. Cassatella, *Neutrophil-derived cytokines: facts beyond expression.* Front Immunol, 2014. **5**: p. 508.

121. Brandt, E., et al., *IL-4 production by human polymorphonuclear neutrophils.* J Leukoc Biol, 2000. **68**(1): p. 125-30.

122. Deguine, J., et al., *Local TNFR1 Signaling Licenses Murine Neutrophils for Increased TLR-Dependent Cytokine and Eicosanoid Production.* J Immunol, 2017. **198**(7): p. 2865-2875.

123. Trussoni, C.E., et al., *Lipopolysaccharide (LPS)-Induced Biliary Epithelial Cell NRas Activation Requires Epidermal Growth Factor Receptor (EGFR).* PLoS One, 2015. **10**(4): p. e0125793.

124. Rittie, L., et al., *Retinoid-induced epidermal hyperplasia is mediated by epidermal growth factor receptor activation via specific induction of its ligands heparin-binding EGF and amphiregulin in human skin in vivo.* J Invest Dermatol, 2006. **126**(4): p. 732-9.

125. Bauge, C., et al., *Modulation of transforming growth factor beta signalling pathway genes by transforming growth factor beta in human osteoarthritic chondrocytes: involvement of Sp1 in both early and late response cells to transforming growth factor beta.* Arthritis Res Ther, 2011. **13**(1): p. R23.

126. Menke, A., et al., *Transforming growth factor-beta-induced upregulation of transforming growth factor-beta receptor expression in pancreatic regeneration.* Biochim Biophys Acta, 1999. **1449**(2): p. 178-85.

127. Moustakas, A. and C.H. Heldin, *Mechanisms of TGFbeta-Induced Epithelial-Mesenchymal Transition.* J Clin Med, 2016. **5**(7).

128. Zaiss, D.M., et al., *Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair.* Immunity, 2015. **42**(2): p. 216-26.

129. Troyer, K.L., et al., *Growth retardation, duodenal lesions, and aberrant ileum architecture in triple null mice lacking EGF, amphiregulin, and TGF-alpha.* Gastroenterology, 2001. **121**(1): p. 68-78.

130. Wang, L., et al., *Activation of epidermal growth factor receptor mediates mucin production stimulated by p40, a Lactobacillus rhamnosus GG-derived protein.* J Biol Chem, 2014. **289**(29): p. 20234-44.

131. Sternlicht, M.D. and S.W. Sunnarborg, *The ADAM17-amphiregulin-EGFR axis in mammary development and cancer.* J Mammary Gland Biol Neoplasia, 2008. **13**(2): p. 181-94.

132. Mishra, H.K., J. Ma, and B. Walcheck, *Ectodomain Shedding by ADAM17: Its Role in Neutrophil Recruitment and the Impairment of This Process during Sepsis.* Front Cell Infect Microbiol, 2017. **7**: p. 138.

133. Frey, M.R., A. Golovin, and D.B. Polk, *Epidermal growth factor-stimulated intestinal epithelial cell migration requires Src family kinase-dependent p38 MAPK signaling.* J Biol Chem, 2004. **279**(43): p. 44513-21.

134. Liu, F.L., C.C. Wu, and D.M. Chang, *TACE-dependent amphiregulin release is induced by IL-1beta and promotes cell invasion in fibroblast-like synoviocytes in rheumatoid arthritis.* Rheumatology (Oxford), 2014. **53**(2): p. 260-9.

135. Grotendorst, G.R., G. Smale, and D. Pencev, *Production of transforming growth factor beta by human peripheral blood monocytes and neutrophils.* J Cell Physiol, 1989. **140**(2): p. 396-402.

136. Granot, Z. and J. Jablonska, *Distinct Functions of Neutrophil in Cancer and Its Regulation.* Mediators Inflamm, 2015. **2015**: p. 701067.

137. Ohtsuka, Y., et al., *MIP-2 secreted by epithelial cells increases neutrophil and lymphocyte recruitment in the mouse intestine.* Gut, 2001. **49**(4): p. 526-33.

138. Schuerer-Maly, C.C., et al., *Colonic epithelial cell lines as a source of interleukin-8: stimulation by inflammatory cytokines and bacterial lipopolysaccharide.* Immunology, 1994. **81**(1): p. 85-91.

139. Bauche, D. and J.C. Marie, *Transforming growth factor beta: a master regulator of the gut microbiota and immune cell interactions.* Clin Transl Immunology, 2017. **6**(4): p. e136.

140. Arndt, P.G., et al., *Leukocyte ADAM17 regulates acute pulmonary inflammation.* PLoS One, 2011. **6**(5): p. e19938.

Vita

Feidi Chen was born on October 20, 1989 in China to Xiaomin and Zubin Chen. Feidi attended the Guangzhou Medical University, where she received her M.D. in Clinical Medicine in 2003. Shortly after, she began her Ph.D studies at the University of Texas Medical Branch in Experimental Pathology. She was a McLaughlin predoctoral fellow from 2015 to 2017 and received a trainee abstract award from the American Association of Immunologists in 2015 and 2017 respectively.

Education

M.D. Clinical Medicine, June 2013, Guangzhou Medical University, Guangzhou, China

Publications

Chen, F., Cao, A., Yao, S., Evans-Marin, H.L., Liu, H., Wu, W., Carlsen, E.D., Dann, S.M., Soong, L., Sun, J., et al. (2016). mTOR Mediates IL-23 Induction of Neutrophil IL-17 and IL-22 Production. J Immunol 196, 4390-4399.

Zhao, Y.*, Chen, F.*, Wu,W., Sun, M., Bilotta, AT., Yao, S., Xiao, Y., Huang, X., Evans-Marin, H.L., Yan, F., Souza,W., et al. (2017). GPR43 mediates microbiota metabolite SCFA regulation of antimicrobial peptide expression in intestinal epithelial cells via activation of mTOR and STAT3. Mucosal Immunology (revision) *Co-first author

Wu, W.*, Chen, F.*, Liu, Z., and Cong, Y. (2016). Microbiota-specific Th17 Cells: Yin and Yang in Regulation of Inflammatory Bowel Disease. Inflamm Bowel Dis 22, 1473-1482. *Co-first author

Liu, H., Chen, F., Wu, W., Cao, A.T., Xue, X., Yao, S., Evans-Marin, H.L., Li, Y.Q., and Cong, Y. (2016). TLR5 mediates CD172alpha(+) intestinal lamina propria dendritic cell induction of Th17 cells. Sci Rep 6, 22040.

Wu, W., Liu, H.P., Chen, F., Liu, H., Cao, A.T., Yao, S., Sun, M., Evans-Marin, H.L., Zhao, Y., Zhao, Q., et al. (2016b). Commensal A4 bacteria inhibit intestinal Th2-cell responses through induction of dendritic cell TGF-beta production. Eur J Immunol 46, 1162-1167.

Xiao, Y., Huang, X., Zhao, Y., Chen, F., Sun, M., Chen, L., Yao, S., Peniche, A., Dann, S.M., Sun, J., Golovko, G., et al. (2017). Interleukin-33 Promotes REG3γ Expression in Intestinal Epithelial Cells and Controls Gut Microbiota. Cellular and Molecular Gastroenterology and Hepatology (submitted)

Evans-Marin, H.L., Cao, A.T., Yao, S., Chen, F., He, C., Liu, H., Wu, W., Gonzalez, M.G., Dann, S.M., and Cong, Y. (2015). Unexpected Regulatory Role of CCR9 in Regulatory T Cell Development. PLoS One 10, e0134100.

Sun, M., Wu,W., Chen, L., Huang, X., Ma, C., Chen, F., Xiao, Y., Zhao, Y., Ma, C., Yao, S., Carpio, V., et al. (2016). Microbiota metabolites short-chain fatty acids inhibit pathogenic function of T-helper (Th)1 cells in the intestines through induction of IL-10 which is mediated by GPR43. Gastroenterology (submitted)

Zhou, G., Yu, L., Fang, L., Yang, W., Yu, T., Miao, Y., Chen, M., Wu, K., Chen, F., Cong, Y., et al. (2017). CD177+ neutrophils as functionally activated neutrophils negatively regulate IBD. Gut.

Permanent address: Qiang Wei Street, Zhong Hai Kang Cheng Garden, Guangzhou, China 510663

This dissertation was typed by Feidi Chen.