IL-27 MIMICS INTERFERON ACTIVATION OF MACROPHAGES TO CONTROL VIRAL, BUT NOT BACTERIAL, INFECTION

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Pathobiology- Doctor of Philosophy

2018

ABSTRACT

IL-27 MIMICS INTERFERON ACTIVATION OF MACROPHAGES TO CONTROL VIRAL, BUT NOT BACTERIAL, INFECTION

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Interleukin(IL)-27 is a heterodimeric type I cytokine in the IL-12 cytokine family with reported roles in cancer, infectious disease, and autoimmunity. However, the literature reports conflicting roles for IL-27 for either ameliorating or potentiating inflammatory conditions, especially within the gut. The majority of the literature describing the functions of IL-27 focuses on T cell biology, resulting in a paucity of knowledge on how IL-27 may act on its primary cellular source: myeloid cells such as macrophages. The objective of this study was to characterize the effect of IL-27 on murine macrophages and determine if macrophages may mediate any potential effects of IL-27 on the colon epithelium. Here we found that IL-27 activated murine macrophages in a manner similar to interferons (IFN). RNA sequencing followed by validation with real time RT-qPCR revealed that IL-27 induced the transcription of genes associated with interferon signaling and pathogen responses in macrophages. Using gene specific knockout mice, we demonstrated that this IL-27-induced gene expression was dependent on STAT1, independent of IL-27-induced IFN-γ, and selectively dependent on IL-27-induced type I interferons. IL-27 also increased macrophage secretion of TNF-α and IFN-γ, consistent with an activated phenotype. IL-27 increased surface expression of MHC II as determined by flow cytometry in a STAT1-dependent, IFN-independent manner. To determine the functional impact of the observed IL-27-induced gene and protein upregulation, we investigated the ability of IL-27 to promote viral or bacterial clearance

in macrophages. IL-27 reduced vaccinia virus infection of macrophages and, in a separate experiment, also reduced the release of mouse cytomegalovirus by infected macrophages to subsequently decrease secondary viral infection of a susceptible cell line. In contrast, IL-27 was ineffective in reducing macrophage infection by *Salmonella*, likely due to a lack of nitric oxide production. Co-cultures of macrophages and colon epithelial organoids were used to evaluate the ability of macrophages to mediate the effects of IL-27 on murine colon epithelial cells, which do not respond directly to IL-27. RNA sequencing of colonoids co-cultured with IL-27-stimulated macrophages detected increased expression of gene pathways crucial for pathogen responses in colon epithelial organoids. In conclusion, our data provide novel mechanistic and functional insights into the shared and divergent effects of IL-27 and interferons on both non-activated resident and classically activated macrophages.

This work is dedicated to the members of the Cancer and Inflammation Program at the Frederick National Laboratory for Cancer Research. It took a village, and you are an extraordinary village.

ACKNOWLEDGEMENTS

The support and advice of previous and current members of the Durum, McVicar, Young, Oppenheim, Wink, and Trinchieri laboratories, as well as numerous other members of the Cancer and Inflammation Program, Molecular Pathology Unit, and Comparative Biomedical Scientist Training Program, has been greatly appreciated over the duration of my dissertation research. An extra special thank you to John Hickerson for keeping us all organized! Many thank yous are due to my committee members for their time and effort: Dr Scott Durum, Dr Matti Kiupel, Dr Tom Mullaney, Dr Gisela Hussey, Dr Martha Mulks, and Dr Mark Simpson. We are also grateful for the scientific collaborations facilitated by Dr Heather Hickman, Dr Courtney Malo, Dr Sergio Martin Pontejo, Dr Tregei Starr, and Dr Olivia Steele-Mortimer. Due to her lack of artistic ability, C.A. is tremendously grateful for the talent and kindness of the members of the National Cancer Institute's Scientific Publications, Graphics, and Media department. Many thanks are also due to our patient, hard-working, and talented animal technicians: Kelli Czarra, Megan Karwan, and Tim Back. C.A. is a fellow in the NIH Comparative Biomedical Scientist Training Program supported by the National Cancer Institute in partnership with Michigan State University. This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

PREFACE

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KEY TO ABBREVIATIONS

ANOVA- analysis of variance

APC- allophycocyanin

C3G- cyanidin-3-glucoside

CD- cluster of differentiation

cDNA- complementary deoxyribonucleic acid

CIITA- MHC class II transactivator

CLIC5- chloride intracellular channel protein 5

CT- cycle threshold

DAB- diaminobenzidine

DAMP- danger associated molecular pattern

DCAMKLI- doublecortin-like kinase

DKK- dickkopf-related protein

DSS- dextran sodium sulfate

EBI3- Epstein-Barr virus-induced gene 3

EDTA- ethylenediaminetetraacetic acid

FBS- fetal bovine serum

FGF2- fibroblast growth factor 2

FGL- fibroleukin

FITC- fluorescein isothiocyanate

GBP- guanylate-binding protein

GFP- green fluorescent protein

HMGB- high mobility group box

HSP- heat shock protein

IAP- inhibitor of apoptosis

IBD- inflammatory bowel disease

ICAM- intercellular adhesion molecule

IFIT- interferon-induced protein with tetratricopeptide repeats

IFN- interferon

IFNG- interferon gamma

IL- interleukin

IL-27Rα- interleukin-27 receptor alpha

ILC- innate lymphoid cell

IRF- interferon regulatory factor

IRGB- interferon-gamma-inducible guanosine-5-triphosphate-ase

IRGM- immunity-related guanosine-5-triphosphate-ase family M member

JAK- Janus kinase

Lgr5- leucine-rich repeat-containing G-protein coupled receptor 5

LPS- lipopolysaccharide

M cell- microfold cell

M-CSF- macrophage colony stimulating factor

M1- classically activated macrophage

MCP- monocyte chemoattractant protein

MHC- major histocompatibility class

MLCK- myosin light-chain kinase

MOI- multiplicities of infection

mRNA- messenger ribonucleic acid

NADPH- nicotinamide adenine dinucleotide phosphate

NCI- National Cancer Institute

NF-κB- nuclear factor-kappa B

NFIL3- nuclear factor, interleukin-3 regulated

NIH- National Institutes of Health

NLRC-Nod-like receptor family caspase recruitment domain containing

NLRP- Nod-like receptor family pyrin domain containing

Non-act- non-activated

NOS- nitric oxide synthase

PAMP- pattern associated molecular pattern

PBS- phosphate buffered saline

PE- phycoerythrin

PGP- protein gene produce

pSTAT1- phosphorylated signal transducer and activator of transcription

R- receptor

Rag- recombination activating gene

RANKL- receptor activator of NF-κB ligand

RIPK1- receptor interacting protein kinase 1

ROS- reactive oxygen species

RT-qPCR- reverse transcription quantitative polymerase chain reaction

SD- standard deviation

SLC26A3- solute carrier family 26 member 3

SLCO3A1- solute carrier organic anion transporter family member 3A1

Smac- second mitochondrial activator of caspases

SOCS- suppressor of cytokine signaling

STAT- signal transducer and activator of transcription

TFF- trefoil factor

TGF- transforming growth factor

TGTP- T cell specific guanine nucleotide triphosphate-binding protein

Tim-3- T cell immunoglobulin and mucin domain-3

TLR- Toll-like receptor

TNBS- 2,4,6-trinitrobenzenesulfonic acid

TNF- tumor necrosis factor

Tr1- T regulatory type 1 cell

TSLP- thymic stromal lymphopoietin

TWEAK- tumor necrosis factor-related weak inducer of apoptosis

Untx- untreated

VEGF- vascular endothelial growth factor

WISP-1- Wnt1-inducible signaling protein 1

ZO- zonula occludens

CHAPTER 1: Introduction

Cytokine tuning of intestinal epithelial function

The intestinal epithelium separates the diverse and ubiquitous members of the intestinal luminal microbiome, virome, and mycobiome from the largest population of resident immune cells anywhere in the body, forming our largest single barrier to the external environment (Carding et al. 2017; Mowat and Agace 2014; Peterson and Artis 2014; Woo et al. 2017). As such, in addition to its critical role in digestion, the gut epithelium is also charged with mediating much of the interaction between luminal organisms and immune cells to ensure appropriate defensive reactions to pathogens versus tolerance of commensal microorganisms (Peterson and Artis 2014).

The physical intestinal barrier consists of a continuous single layer of columnar epithelial cells overlain by a variably thick layer of mucus. This mucus layer is embedded with antibodies and antimicrobial peptides and physically separates the epithelium from direct contact with much of the luminal microbiota (Mowat and Agace 2014). The majority of intestinal epithelial cells are absorptive enterocytes, but the epithelium also contains a number of more specialized cell types, including Paneth cells (in the small intestine only), goblet cells, hormone-secreting enteroendocrine cells, microfold (M) cells, and tuft cells (Blander 2016; Mowat and Agace 2014). Indeed, even these subtypes are too generalized to fully reflect the diversity of intestinal epithelial cells. Recent single cell sequencing data identified two subtypes of tuft cells and subclassified enteroendocrine cells beyond the eight subclasses previously reported (Haber et al. 2017).

The gut epithelium is continuously renewed by *Lgr5*$^{+}$ stem cells located in the base of the intestinal crypts. Newly-formed precursor cells differentiate as they migrate away from the crypt toward the villus tip in the small intestine or luminal surface in the large intestine, where they are expelled into the lumen approximately every four to five days. The exception to this is Paneth cells, which are long-lived and instead move toward the crypt base (Blander 2016; Mowat and Agace 2014). Each cell type plays critical and distinct roles in intestinal function. Mucus-secreting goblet cells are crucial for maintenance of the luminal mucus layer and increase in frequency moving distally along the intestine, peaking at a frequency of approximately 25% of total epithelial cells in the distal colon (Mowat and Agace 2014). Small intestinal Paneth cells produce antimicrobial peptides and also contribute to stem cell maintenance and function through the production of Wnt3, pro-epidermal growth factor, and Notch ligands (Mowat and Agace 2014). M cells overlie gut-associated lymphoid tissues and facilitate the transport of luminal antigens to lymphoid cells, while tuft cells coordinate type 2 immune responses to parasites (Blander 2016; Howitt et al. 2016; von Moltke et al. 2016). Much of intestinal epithelial research, including a portion of that presented herein, has focused on the use of colorectal cancer cell lines to elucidate gut epithelial function. However, due to the heterogeneity of the intestinal epithelium in vivo, observations made from cell lines, which are not representative of all gut epithelial cell types, may be misleading. Recent advances in three dimensional intestinal epithelial organoid cultures, which differentiate into the variety of epithelial cell subtypes seen in vivo, are improving our ability to more effectively characterize intestinal epithelial function, and many of these studies will be highlighted in this review (Takahashi et al. 2018).

The gut-associated lymphoid tissues, including Peyer's patches and isolated lymphoid follicles, are likely the most well-recognized portion of the intestinal immune system. However, the entire gut is armed with a diverse repertoire of immune cells, which vary in location and frequency throughout the length of the intestine (Mowat and Agace 2014). The majority of these cells function in the lamina propria or within the epithelium of the intestinal mucosa. The epithelium predominantly hosts T cells, while the lamina propria is home to cells of both the adaptive and innate arms of the immune system, including T cells, B cells, innate lymphoid cells, macrophages, dendritic cells, mast cells, and eosinophils (Mowat and Agace 2014). Immune cells may sense luminal antigens directly when the epithelial barrier is breached or by the extension of transepithelial dendrites, as has been observed in macrophages and dendritic cells. The intestinal epithelium is uniquely positioned and equipped with a cadre of pattern recognition receptors to sense luminal antigens and danger signals and relay this information to immune cells (Mowat and Agace 2014).

The intestinal epithelium faces the difficult challenge of permitting nutrient absorption and ion movement while maintaining an impermeable barrier to microorganisms and antigens in the gut lumen. The integrity of the intestinal mucosal barrier is critical for health; dysfunction of this barrier has been proposed to contribute to both intestinal and systemic disease, including inflammatory bowel disease (IBD) and multiple organ dysfunction syndrome (Mittal and Coopersmith 2014; Neurath 2014). Intestinal epithelial cells are linked by three types of specialized junctional complexes that attach adjacent cells and permit the selective paracellular movement of solutes and ions: desmosomes, adherens junctions, and tight junctions (Mittal and Coopersmith

2014; Neurath 2014). Desmosomes and adherens junctions predominantly serve as physical attachments between cells, while the more apically located tight junctions act as selective semipermeable barriers to intercellular spaces (Groschwitz and Hogan 2009). Tight junctions are composed of four types of transmembrane proteins: junctional adhesion molecules, claudins, occludin, and tricellulin. Claudins are a family of proteins that are differentially expressed between tissues and exert different effects on paracellular permeability. Claudins critically regulate the selectivity of the epithelial barrier by forming charge- and size-specific channels between epithelial cells (Groschwitz and Hogan 2009). The types of claudin proteins within tight junctions determine the permeability of these paracellular channels. For example, claudin-2 and claudin-6 have been shown to increase tight junction permeability. Intracellular zonula occludens proteins connect tight junction transmembrane proteins to cytoskeletal actin/myosin complexes, which facilitate opening of the tight junction under specific conditions (Groschwitz and Hogan 2009; Mittal and Coopersmith 2014).

Cytokines and chemokines, soluble protein mediators critical for intercellular communication, support intestinal mucosal homeostasis, but can also be key drivers of intestinal inflammation and inflammation-associated damage (Dinarello 2007; Neurath 2014; Peterson and Artis 2014). For example, the genetic deletion of interleukin(IL)-10 or IL-2 precipitated spontaneous colitis in mice, suggesting that these cytokines are essential for colon homeostasis. However, a number of other cytokines, including IL-6, TNF, IL-18, IL-1β, and IL-17, are overexpressed in the inflamed intestine and have been implicated as contributors to intestinal damage (Neurath 2014). Despite these seemingly clear-cut observations, there is strong evidence that the traditional labels of

pro- and anti-inflammatory are too simplistic and perhaps even deceiving when used to describe cytokine actions in the intestine. In support of this, clinical trials targeting cytokines thought to be predominantly proinflammatory in the intestine, such as IL-17, failed to induce remission in patients with inflammatory bowel disease (Neurath 2014; Targan et al. 2012). Additionally, the literature contains conflicting and often equally convincing evidence for both pro- and anti-inflammatory actions of specific cytokines in the gut (Andrews et al. 2016; Neurath 2014). There are a number of potential explanations for these conflicting data, such as the timing of cytokine action, model system used, cytokine concentration, and the method of cytokine administration or removal (Andrews et al. 2016; Bradford et al. 2017; Waddell et al. 2015). As such, cytokine actions should be interpreted on a situational basis to gain a more complete understanding of their diverse roles in health and disease.

Cytokines and chemokines can positively or negatively affect intestinal epithelial barrier integrity and may be derived from resident innate or adaptive immune cells, infiltrating inflammatory cells, or from intestinal epithelial cells themselves (Figure 1) (Groschwitz and Hogan 2009; Hyun et al. 2015; Jeffery et al. 2017; Judd et al. 2015; Mittal and Coopersmith 2014; Neurath 2014).

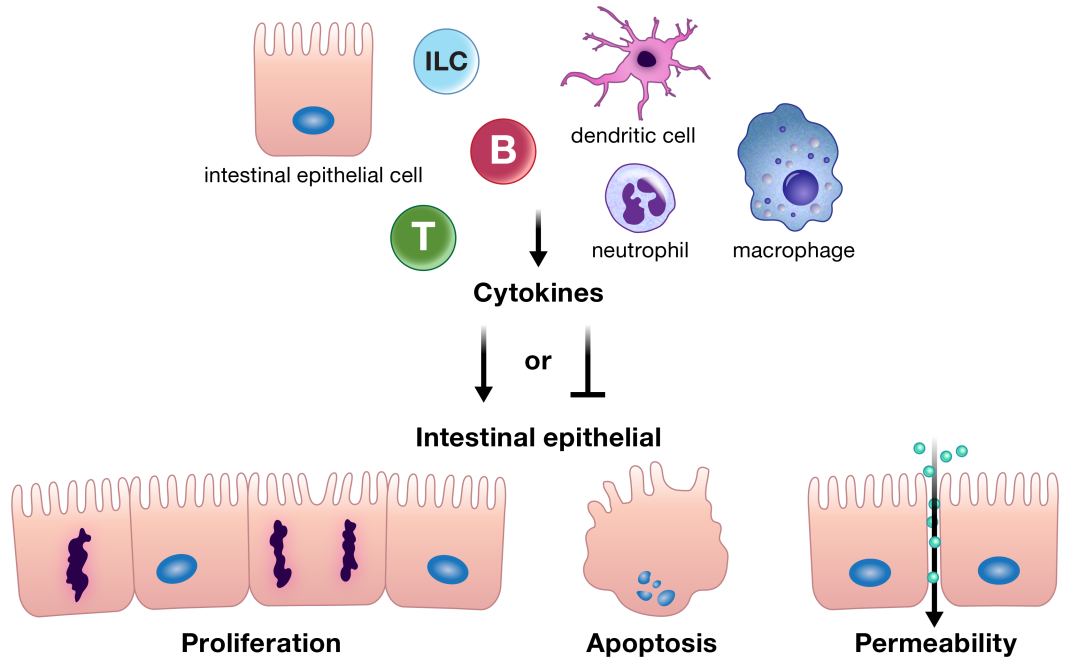

Figure 1. Cytokines can positively or negatively affect intestinal epithelial barrier integrity by driving or inhibiting critical epithelial cell functions such as proliferation, apoptosis, and appropriate epithelial barrier permeability. These cytokines can be derived from resident innate or adaptive immune cells, infiltrating inflammatory cells, or from intestinal epithelial cells themselves. T, T cell; B, B cell; ILC, innate lymphoid cell.

Intestinal epithelial proliferation and cell death can be induced or restricted by cytokines (Grabinger et al. 2017; McLean et al. 2014; Tinico-Veras et al. 2017). Concordantly, various cytokines help heal the epithelial erosions and ulcerations characteristic of severe intestinal inflammation, while others exacerbate these lesions (Neurath 2014). Specific cytokines have also been shown to regulate opposing epithelial functions under different circumstances, for instance proliferation or cell death (Bradford et al. 2017; Grabinger et al. 2017; Nava et al. 2010; Takahashi et al. 2014; Tang et al. 2014). Additionally, cytokines can directly alter intestinal epithelial permeability (Lee et al 2015; Ma et al. 2004). The permeability of epithelial tight junctions may be increased or decreased by cytokine modification of the expression or

localization of their protein components (Groschwitz and Hogan 2009; Lee et al. 2015; Mittal and Coopersmith 2014; Watari et al. 2017; Ye and Sun 2017). Cytokines can also drive phosphorylation of myosin light chains, resulting in contraction and opening of tight junctions (Mittal and Coopersmith 2014). Chemokine production by the intestinal epithelium recruits immune cells to areas of inflammation; however, whether this means epithelial suicide or survival depends on the inflammatory insult. Recruited immune cells may be crucial for defense against a pathogen, but can perpetuate inflammation in conditions such as IBD (Dent et al. 2014; Franzè et al. 2016; Regan et al 2013; Triner et al. 2017). Regardless of mechanism, cytokines and chemokines are critical players in the integrity of the intestinal epithelial barrier. The purpose of this review is to highlight recent advances in our understanding of how cytokines and chemokines, both those made by and acting on the intestinal epithelium, orchestrate many of the diverse functions of the intestinal epithelium and its interactions with immune cells in health and disease.

Cytokine actions on the intestinal epithelium

Cytokine stimulation of intestinal epithelial proliferation

Multiple cytokines regulate proliferation of the intestinal epithelium, a function that is crucial for both wound closure and replacing cells lost through homeostatic shedding (Figure 2) (Blouin and Lamaze 2013; Bradford et al. 2017; Chiriac et al. 2017; Gandhi et al. 2016; Gerbe et al. 2016; Griesenauer and Paczesny 2017; Guanghui et al. 2016; Howitt et al. 2016; Huang and Chen 2012; Jeffery et al. 2017; Kominsky et al. 2014; Kuhn et al. 2014; Lindemans et al. 2015; Mahapatro et al. 2016; Quiros et al. 2017;

Sabat et al. 2014; Scheibe et al. 2017; Sedger et al. 2014; Song et al. 2015; von Moltke et al. 2016).

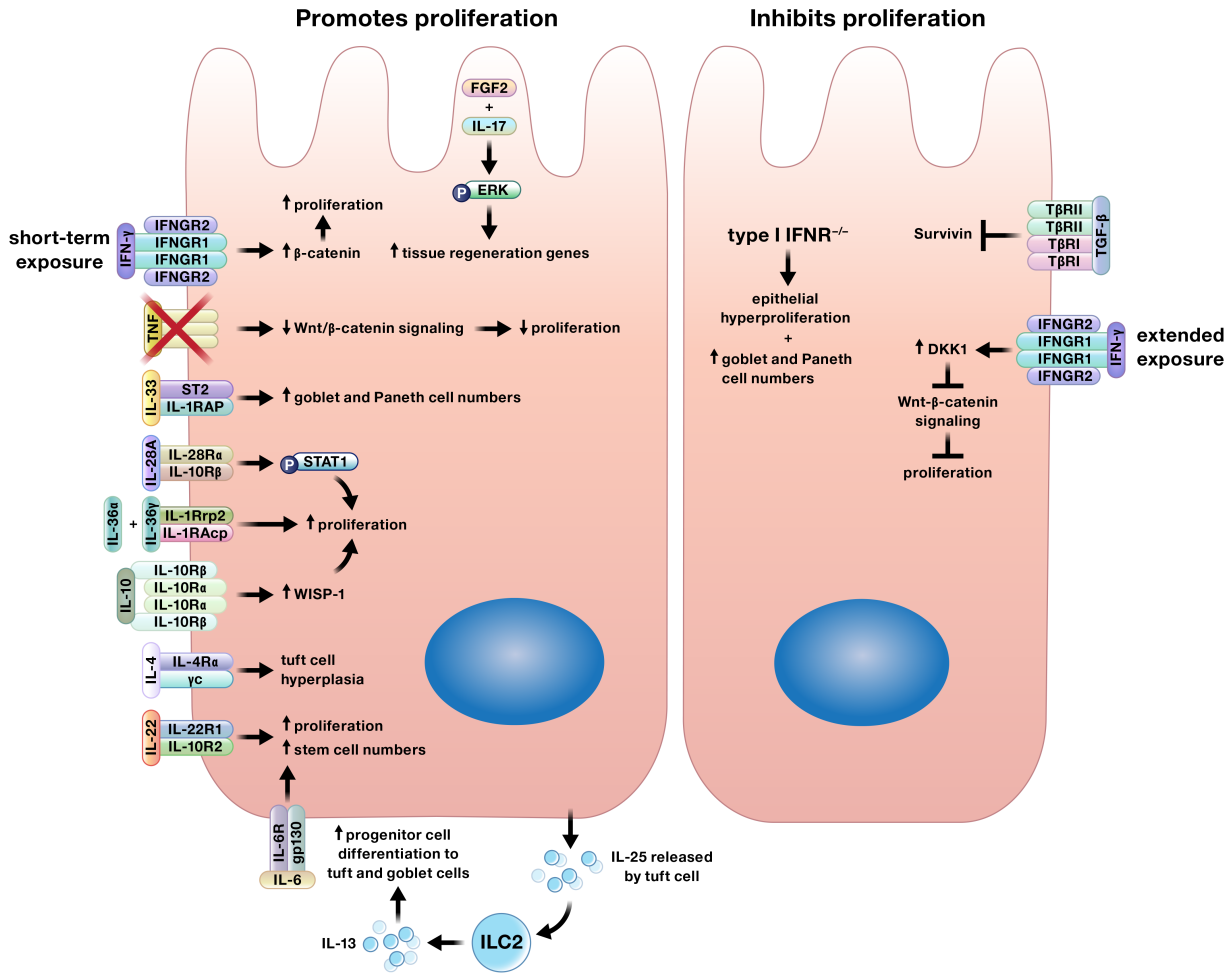

Figure 2. Cytokines may promote or inhibit proliferation of intestinal epithelial cells. Interferon (IFN)-γ may induce or limit intestinal epithelial proliferation based on duration of exposure. Additionally, specific cytokines may only induce proliferation of certain epithelial subtypes. For example, IL-4 increases tuft cell numbers, IL-13 signaling supports increases in tuft and goblet cells, and IL-33 stimulates the expansion of goblet and Paneth cells.

Although generally thought to contribute to the pathology of inflammatory bowel disease, recent studies have shown that tumor necrosis factor (TNF), IL-6, and IL-17

promote epithelial proliferation (Bradford et al. 2017; McLean et al. 2014; Song et al. 2015; Targan et al. 2012).

In murine models of T cell activation and chronic chemically-induced colitis, genetic ablation of either TNF or its receptor impaired Wnt/β-catenin signaling, resulting in reduced epithelial proliferation and delayed mucosal healing (Bradford et al. 2017). This result may seem curious in light of the success of anti-TNF therapy in IBD patients; however, the authors offer an explanation for this perceived conflict by highlighting the mechanism of action of efficacious versus ineffective anti-TNF therapies. Therapeutic anti-TNF antibodies reduce inflammation in IBD patients by inducing apoptosis in inflammatory cells expressing membrane-bound TNF (Waetzig et al. 2005). In contrast, treatment with a soluble TNF receptor, which was ineffective in treating Crohn's disease, binds soluble TNF, which the authors propose blocks the ability of TNF to promote mucosal healing (Bradford et al. 2017; Sandborn et al. 2001).

IL-6 increased proliferation and stem cell numbers in an in vitro model of murine small intestinal epithelial organoids, and the crypt epithelial cells also expressed IL-6, suggesting an autocrine signaling mechanism. Interestingly, the IL-6 receptor was only present on the basal membrane of crypt Paneth cells, making it unclear how IL-6 may affect epithelial cells in segments of the intestine lacking Paneth cells, such as the colon (Jeffery et al. 2017). However, Paneth cell metaplasia can be found in various types of colitis, in which case this mechanism of IL-6-facilitated epithelial repair could play a role (Elphick and Mahida 2005). Furthermore, Kuhn et al. demonstrated that the early inhibition of IL-6 in murine models of bacterial colitis and wounding by biopsy impaired colon wound healing by limiting epithelial proliferation. They also demonstrated by in

situ hybridization that IL-6 mRNA transcripts were enriched within the mucosa surrounding sites of intestinal perforation in human patients, suggesting that this IL-6-driven mechanism of wound healing may also be important in humans. These findings suggest that while Paneth cells may be crucial for IL-6-induced epithelial proliferation in the small intestine, other mechanisms exist for IL-6 to drive epithelial repair in the colon (Kuhn et al. 2014).

Similarly, genetic ablation of IL-17 reduced intestinal epithelial cell proliferation and worsened dextran sulfate sodium (DSS)-induced murine colitis (Song et al. 2015). Furthermore, IL-17 was shown to synergize with fibroblast growth factor 2 (FGF2) to promote intestinal healing in this study. FGF2 and IL-17 signaling synergistically activated ERK and induced genes related to tissue repair and regeneration in primary murine intestinal epithelial cells. The authors demonstrated that the mechanism of this synergy depended on Act1, an adaptor molecule that suppresses FGF2 signaling but is required for IL-17 signaling. When cells were co-stimulated with IL-17 and FGF2, Act1 was preferentially recruited to IL-17 receptors, preventing Act1-mediated suppression of FGF2 signaling (Song et al. 2015). These findings may offer one explanation for the unexpected results of a clinical trial investigating the inhibition of the IL-17 receptor as a therapy for active Crohn's disease, in which a disproportionate number of patients actually experienced worsening disease with treatment (Targan et al. 2012).

IL-22 increased growth in both human and murine intestinal organoids, both by inducing proliferation of the epithelial cells and facilitating stem cell expansion (Lindemans et al. 2015). IL-22 was also shown to be crucial for stem cell maintenance in vivo in the small intestine in a murine model of methotrexate-induced intestinal

damage (Aparicio-Domingo et al. 2015). During *Citrobacter rodentium* infection, IL-22 production by $CD4^+$ T cells was critical for colonic epithelial proliferation and resistance to infection-induced mucosal pathology (Bergstrom et al. 2015).

Induction of IL-36 receptor signaling through any one of its ligands, IL-36α, IL-36β, or IL-36γ, induced proliferation of intestinal epithelial cells in in vitro organoid cultures, and mice with genetic deletion of the IL-36 receptor were more susceptible to chemically-induced colitis, demonstrating higher disease activity, more severe colon pathology, greater bacterial translocation, and decreased survival. Furthermore, administration of a combination of IL-36α and IL-36γ accelerated wound healing in murine colons by increasing proliferation of epithelial cells adjacent to the experimental wounds (Scheibe et al. 2017).

Similarly, IL-28A (also termed interferon λ2) induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) and proliferation in murine small and large intestinal epithelial organoid cultures (Chiriac et al. 2017). Mice with global knockout of the IL-28A receptor or intestinal epithelial cell specific knockout of STAT1 developed more severe oxazolone and DSS-induced colitis, and the administration of IL-28A or genetic ablation of the IL-28A receptor in mice with induced colon wounds improved or delayed wound healing, respectively. The authors went further to link their murine models to human patients with IBD, demonstrating that both IBD patients and mice with colitis showed increased expression of the IL-28A receptor on the colon epithelium, as well as higher expression of IL-28A by cells within the lamina propria of the colon mucosa. Co-labeling of lamina propria cells in IBD patients identified dendritic cells as a major source of IL-28A (Chiriac et al. 2017).

A separate study also highlighted innate immune cells as a crucial cytokine source for mucosal healing. In a murine model of biopsy-induced colon injury, macrophage derived IL-10 was crucial for optimal wound healing (Quiros et al. 2017). IL-10 mRNA and protein were increased at wound sites within one day of wounding, and IL-10 induced epithelial proliferation by stimulating synthesis of Wnt1-inducible signaling protein 1 (WISP-1). Interestingly, the absence of T and B cells in *Rag*1$^{-/-}$ mice also used in this study did not impair wound closure, further highlighting macrophages as the primary source of IL-10 in this model and suggesting that adaptive immune cells do not play a crucial role in this mechanism of wound healing (Quiros et al. 2017).

IL-13, IL-4, and IL-33 support the differentiation of specialized epithelial cells. Expansion of tuft cells, a specialized taste-chemosensory subtype of the intestinal epithelium, can also be induced by innate immune cells. During helminth infection, IL-25 secreted by tuft cells activates type 2 innate lymphoid cells to produce IL-13, which induces the differentiation of increased numbers of tuft and goblet cells from epithelial progenitor cells (von Moltke et al. 2016; Howitt et al. 2016). IL-4, which shares the common receptor subunit IL-4 receptor α with IL-13, can also induce tuft cell hyperplasia (Gerbe et al. 2016). Mahapatro et al. demonstrated that IL-33 also directly affected the differentiation of epithelial progenitor cells. The constitutive expression of IL-33 in the small intestine of mice increased goblet and Paneth cell numbers but did not promote the proliferation/differentiation of absorptive enterocytes. Challenge of IL-33$^{-/-}$ mice with *Salmonella Typhimurium* demonstrated that IL-33 was critical for microbial defense, as mice lacking IL-33 had more severe intestinal damage and a greater *Salmonella* burden associated with decreased numbers of goblet and Paneth

cells and reduced antimicrobial peptide production (Mahapatro et al. 2016). Similarly, mice with genetic deletion of IL-33 or its receptor had decreased numbers of goblet cells and more severe colitis in a model of oxazolone-induced intestinal inflammation (Waddell et al. 2015).

Cytokine-induced proliferation and carcinogenesis

In the absence of wound closure, cytokine-induced intestinal epithelial proliferation may prove to be more deleterious than healing. In fact, a number of studies have suggested that cytokines, including IL-17, IL-6, IL-22, TNF-α, IL-4, and IL-13, either alone or in combination, may promote carcinogenesis in intestinal epithelial cells (De Simone et al. 2015; Kirchberger et al. 2013; Kryczek et al. 2014; Liu et al. 2017; Wang et al. 2014). Wang et al. demonstrated that IL-17 receptor type A (IL-17RA) signaling promoted proliferation of transformed colon enterocytes. IL-17RA signaling also induced IL-6 expression, a cytokine previously associated with colitis-associated cancer development (Wang et al. 2014). The concurrent neutralization of either IL-6 and IL-22 or TNF-α and IL-17A inhibited NF-κB or STAT3 signaling, respectively, and reduced the mitogenic effects of these cytokines on human colorectal cancer cells (De Simone et al. 2015). Multiple studies have also shown that IL-22 alone can promote colorectal cancer progression (Kirchberger et al. 2013; Kryczek et al. 2014).

Furthermore, both IL-4 and IL-13 may contribute to colon cancer progression. IL-4 and IL-13 increased the expression of NADPH oxidase 1 in human colon cancer cell lines, which led to the production of reactive oxygen species and cellular proliferation. When examined in resected tissues from patients with colon cancer, the authors found increased active NADPH oxidase 1 in the tumor tissue relative to the adjacent normal

colon tissue, leading them to suggest that IL-4/IL-13-driven NADPH oxidase 1 expression may drive colon carcinogenesis (Liu et al. 2017).

Cytokine inhibition of intestinal epithelial proliferation

In complement to the plethora of proliferation-inducing cytokines detailed above, a smaller number of cytokines limit intestinal epithelial proliferation (Figure 2) (Ketlinskaya et al. 2016; Liu et al. 2017; Martini et al. 2016; Nava et al. 2010; Oshima et al. 2015; Tschurtschenthaler et al. 2014).

Transforming growth factor-β (TGF-β) suppressed expression of Survivin, a molecule critical for functional cell division in intestinal epithelial progenitor cells (Martini et al. 2016). Consistent with this finding, genetic disruption of TGF-β signaling in intestinal epithelial cells was sufficient for the development of invasive colon cancer in the face of chronic inflammation in mice (Oshima et al. 2015).

In a model of constitutive β-catenin signaling, Katlinskaya et al. demonstrated that type I interferons limit intestinal epithelial proliferation (Katlinskaya et al. 2016). Concordantly, Tschurtschenthaler et al. characterized mice with intestinal epithelial specific genetic deletion of the type I interferon receptor as having increased numbers of small intestinal goblet and Paneth cells, epithelial hyperproliferation, and increased tumor burden following tumor induction with azoxymethane and DSS (Tschurtschenthaler et al. 2014). Remarkably, the authors were able to eliminate the epithelial hyperproliferation and increase in tumors by cohousing the type I interferon receptor knockout mice with wildtype mice, demonstrating that these knockout-induced phenotypes were dependent on the gut microbiota (Tschurtschenthaler et al. 2014).

The effects of the type II interferon, interferon-γ, on the intestinal epithelium vary with length of exposure. The short-term incubation of the intestinal epithelial cell line T84 with interferon-γ activated β-catenin signaling and induced proliferation of the T84 cells, peaking at 24 hours. However, extended exposure of the T84 cells to interferon-γ induced expression of DKK1, which inhibited Wnt-β-catenin signaling and reduced proliferation. Interestingly, the addition of both TNF-α and interferon-γ enhanced these effects (Nava et al. 2010).

Damage control: cytokine regulation of apoptosis

While well-regulated apoptosis is essential for the homeostatic shedding of enterocytes, any perturbations to this process could quickly compromise the intestinal epithelial barrier. Indeed, increased apoptosis has been detected in the intestinal epithelium of IBD patients, although it is unclear if this is an initiating event in the disease, an effect of inflammation, or some combination of both (Blander 2016). Increased intestinal epithelial apoptosis is also a consistent feature in critically ill humans and animal models of critical illness, such as sepsis. This increase in apoptosis contributes to intestinal epithelial barrier compromise in critical illness, which has been implicated as a critical driver of multiple organ dysfunction syndrome (Mittal and Coopersmith 2014). Cytokines can induce or inhibit intestinal epithelial apoptosis (Figure 3) (Bradford et al. 2017; Grabinger et al. 2017; Jarry et al. 2016; Leppkes et al. 2014; Nava et al. 2010; Schuhmann et al. 2011; Tang et al. 2014; Takahashi et al. 2014; Tinico-Veras et al. 2017).

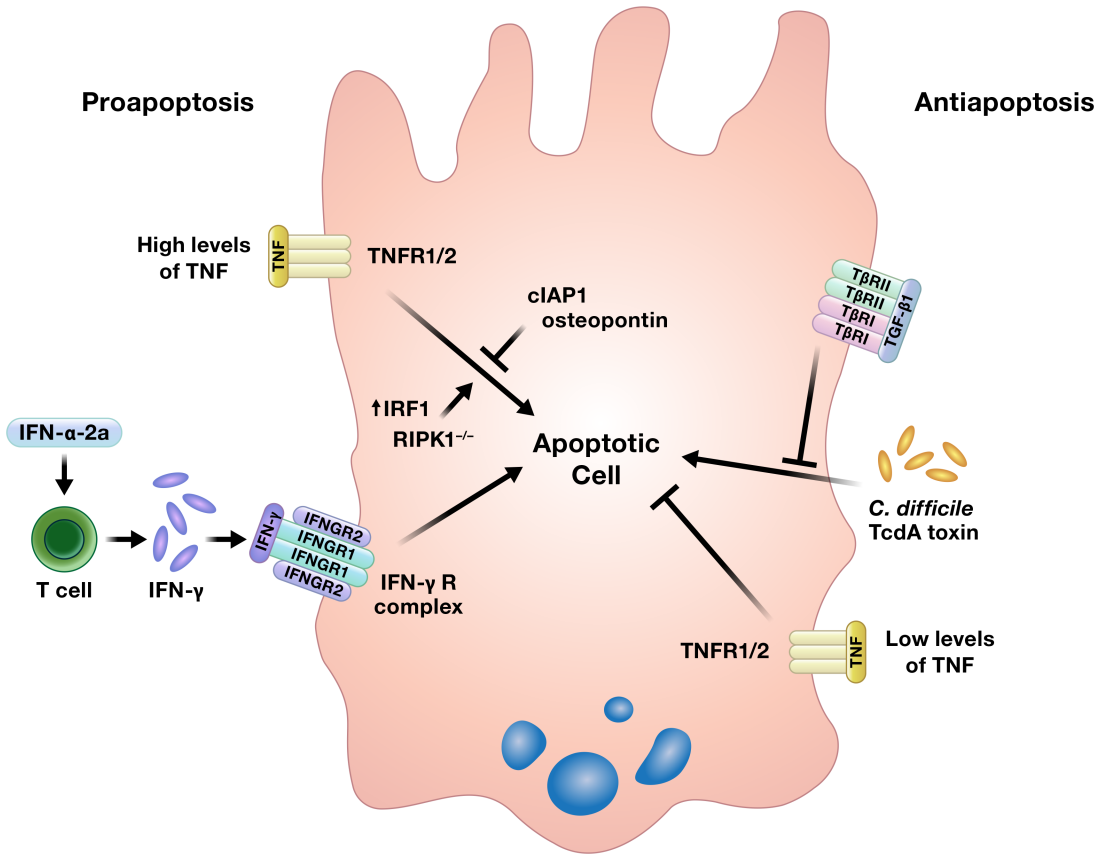

Figure 3. Cytokines can induce or prevent apoptosis in intestinal epithelial cells. TNF has been shown to either promote or inhibit intestinal epithelial cell apoptosis under different conditions. IAP, inhibitor of apoptosis protein; IRF1, interferon regulatory factor 1; RIPK1, receptor interacting protein kinase 1.

Interferons have been shown to induce apoptosis of intestinal epithelial cells. Using human colon explant cultures, Jarry et al. demonstrated that administration of interferon-α-2a rapidly induced interferon-γ production by lamina propria resident T cells and interferon-γ-dependent epithelial apoptosis, a direct effect of interferon-γ on the intestinal epithelium that has been reported previously (Jarry et al. 2016; Nava et al. 2010; Schuhmann et al. 2011). Katlinskaya et al. also demonstrated a role for type I interferon in promoting apoptosis of the intestinal epithelium in a model of constitutive β-catenin signaling (Katlinskaya et al. 2016).

In contrast to its ability to promote intestinal epithelial proliferation, one of the most well characterized actions of TNF in the intestine is its ability to induce epithelial cell death. Injection of mice with TNF results in increased apoptosis of both small and large intestinal epithelial cells within 6 hours, with a concentration of apoptotic cells in the intestinal crypts. Exposure of intestinal epithelial organoids derived from mice with genetic deletion of TNF receptors 1 and 2 revealed that while both receptors participated in TNF-mediated epithelial apoptosis, TNF receptor 1 signaling was predominantly involved. The authors further demonstrated that TNF-induced intestinal epithelial apoptosis is regulated by the inhibitor of apoptosis protein cIAP1. Inhibition of cIAP1 by second mitochondrial activator of caspases (Smac)-mimetic compounds, tumor necrosis factor-related weak inducer of apoptosis (TWEAK), or genetic deletion sensitized mice to TNF-induced intestinal epithelial apoptosis (Grabinger et al. 2017). A separate in vitro study using cancerous and noncancerous colon epithelial cell lines demonstrated that osteopontin reduced TNF-induced apoptosis, while the overexpression of interferon regulatory factor 1 increased TNF-mediated apoptosis (Tang et al. 2014). TNF was also implicated as contributing to the pathogenesis of intestinal inflammation in mice with conditional knockout of receptor interacting protein kinase 1 (RIPK1). Full RIPK1 knockout mice die perinatally, but the conditional RIPK1 knockout in intestinal epithelial cells in mice used in this study resulted in intestinal inflammation and early death associated with epithelial cell apoptosis. However, this phenotype was rescued by a deficiency in TNF receptor 1, and the lack of RIPK1 in in vitro cultured intestinal epithelial organoids sensitized the cultures to TNF-induced apoptosis (Takahashi et al. 2014).

In lieu of apoptosis, under certain circumstances cells may undergo the pro-inflammatory process of regulated necrosis termed necroptosis (Sarhan et al. 2018). In addition to its ability to drive apoptosis, TNF can also initiate necroptosis of intestinal epithelial cells under specific conditions. In a model of conditional knockout of caspase 8 in intestinal epithelial cells, Günther et al. demonstrated that necroptosis in gut epithelial cells was triggered by TNF-α produced by other cells upon bacterial lipopolysaccharide (LPS) stimulation, not direct LPS-induced Toll-like receptor 4 (TLR4) signaling in the epithelium. In contrast, gut epithelial necroptosis due to TLR3 ligation in the same model was cytokine-independent and directly initiated by TLR3 signaling (Günther et al. 2015).

In light of the strong evidence for a pro-apoptotic function of TNF in the gut, Bradford et al. curiously demonstrated an anti-apoptotic effect of TNF in the intestinal epithelium. In the murine model of T cell activation induced by anti-CD3 antibody injection used in this study, intestinal epithelial apoptosis is expected both acutely at the villus tips and later in the crypts around 24 hours post-injection. Interestingly, and perhaps counterintuitive to the evidence presented herein thus far, administration of anti-CD3 antibody in $TNF^{-/-}$ mice resulted in a seven-fold increase in crypt epithelial apoptosis, suggesting that TNF works to limit epithelial apoptosis in this model (Bradford et al. 2017). Other studies have also characterized an anti-apoptotic role for TNF in the intestinal epithelium, and the authors suggest that the level of TNF may determine whether it acts to promote or prevent apoptosis, with higher levels of TNF proposed to be pro-apoptotic (Bradford et al. 2017; Leppkes et al. 2014).

TGF-β1 can also inhibit intestinal epithelial cell death. TGF-β1 reduced apoptosis and prevented necrosis in rat jejunal crypt epithelial cells exposed to the TcdA toxin of *Clostridium difficile* (Tinico-Veras et al. 2017).

Cytokine reinforcement of intestinal epithelial barrier integrity

Appropriate permeability of the intestinal epithelium is crucial for the balance between nutrient absorption and pathogen exclusion, and a number of cytokines positively affect this epithelial function (Figure 4) (Groschwitz and Hogan 2009; Kominsky et al. 2014; Kuhn et al. 2017; Lee et al. 2015; Maxwell et al. 2015; Waddell et al. 2015; Wang et al. 2017).

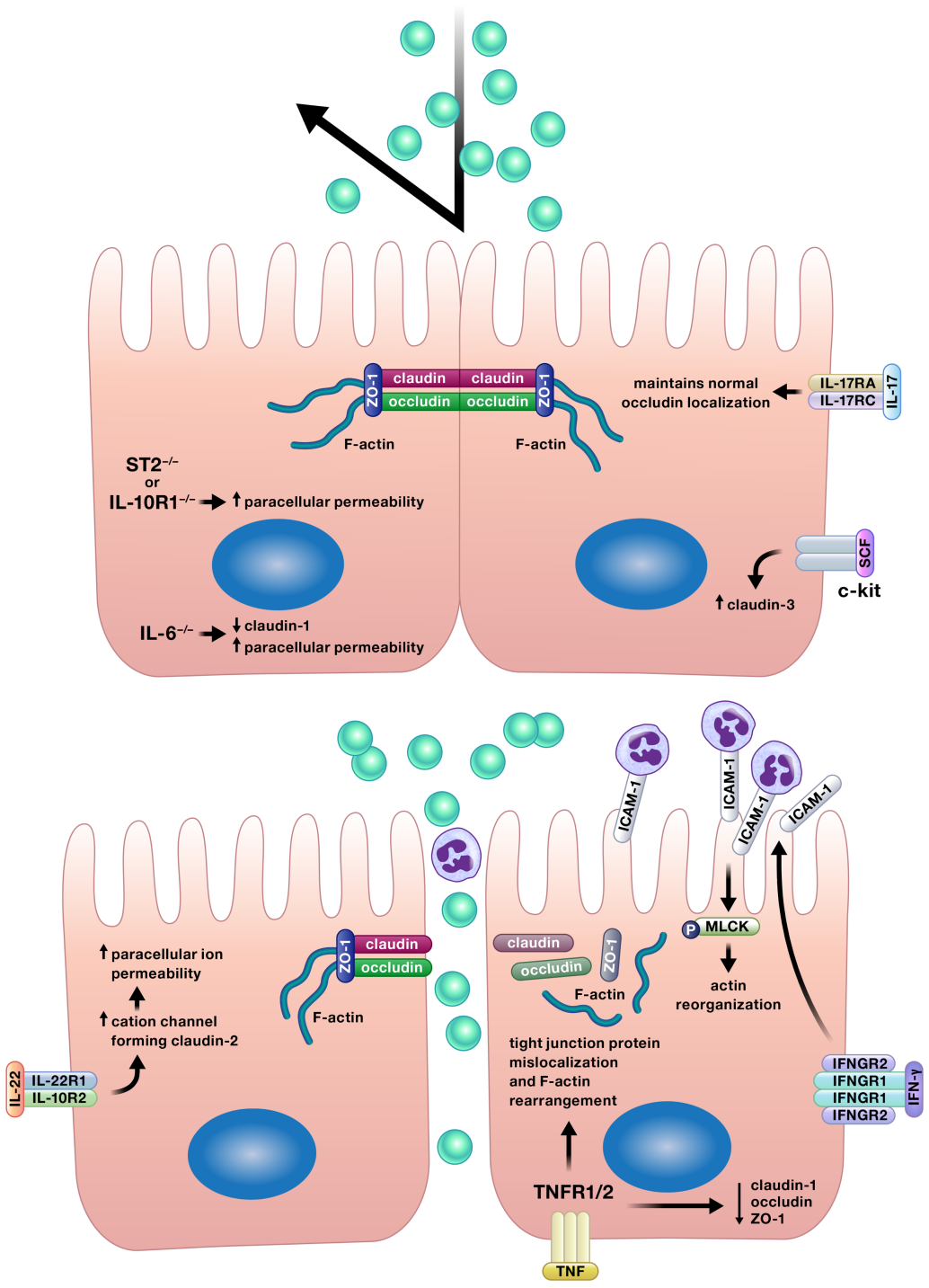

Figure 4. Appropriate permeability of the intestinal epithelium maintains balance between nutrient absorption and pathogen exclusion. Cytokines may reinforce or impair the intestinal barrier by altering permeability of the epithelium. Epithelial tight junction permeability may be increased or decreased by cytokine modification of the expression or localization of tight junction protein components, such as various claudins, occludin, or zonula occludens protein 1 (ZO-1). Cytokines can also drive

phosphorylation of myosin light chains, resulting in contraction and opening of tight junctions. Interferon(IFN)-γ increases intercellular adhesion molecule-1 (ICAM-1) expression, and subsequently, ICAM-1-mediated adherence of neutrophils to gut epithelial apical membranes. Neutrophil ligation of ICAM-1 drives the phosphorylation of myosin light-chain kinase (MLCK), resulting in actin reorganization leading to increased paracellular permeability and neutrophil transepithelial migration.

Inhibition of IL-17 receptor A by antibody neutralization worsened disease in the multidrug resistance-1a-ablated (*Abcb1a*$^{-/-}$) murine model of colitis and was associated with increased epithelial permeability as detected by increased serum concentrations of soluble CD14 and LPS binding protein and increased plasma concentrations of orally administered sucralose, lactulose, and mannitol (Maxwell et al. 2015). Lee et al. also demonstrated that a loss of IL-17 signaling increased intestinal epithelial permeability by showing increased amounts of orally administered fluorescein isothiocyanate (FITC)-dextran in the serum of mice with both chemically-induced and T cell transfer-induced colitis in which IL-17 was removed by antibody neutralization or genetic deletion (Lee et al. 2015). The authors attributed the increase in gut epithelial permeability in the absence of IL-17 to disruptions in the structure of tight junctions, junctional complexes which are critical to the selectivity inherent in appropriate gut barrier permeability. The absence of IL-17 resulted in the intracellular mislocalization of the tight junction complex protein occludin and a loss of co-localization of occludin with F-actin. To provide more support for this mechanism, the authors applied TNF-α, a cytokine previously reported to disrupt tight junctions and increase epithelial barrier permeability, to cultured Caco-2 cells with or without co-stimulation with IL-17A (Lee et al. 2015; Ma et al. 2004). Consistent with their observations in vivo, TNF-α altered the intracellular localization of occludin; however, co-stimulation with IL-17A reduced the TNF-induced occludin mislocalization (Lee et al. 2015). Along with the previously described ability of IL-17 to

induce intestinal epithelial regeneration, the ability of IL-17 to reinforce the intestinal epithelial barrier offers an additional potential explanation for the worsening of Crohn's disease observed in clinical trial patients treated with an antibody to inhibit IL-17 receptor signaling (Targan et al. 2012).

Multiple studies have shown the positive effects of IL-10 signaling in the gut epithelium for maintenance of appropriate epithelial permeability (Kominsky et al. 2014; Lorén et al. 2015; Zheng et al. 2017). Stimulation of T84 cell monolayers with IL-10 restored transepithelial electrical resistance disrupted by compromise of the monolayers by incubation with interferon-γ. Additionally, knockdown of the IL-10 receptor 1 in human intestinal epithelial cell lines impaired barrier formation as assessed by transepithelial electrical resistance and increased paracellular flux (Kominsky et al. 2014). These changes suggest alterations in the function of intercellular tight junctions owing to the lack of IL-10 signaling; however, this potential mechanism was not explored in this study. In the same study, mice with intestinal epithelial cell specific knockout of the IL-10 receptor 1 developed more severe chemically-induced colitis with increased epithelial permeability to FITC-dextran (Kominsky et al. 2014). The authors concluded that the more severe colitis in these mice was driven by increased barrier permeability due to a lack of IL-10 signaling in epithelial cells. However, as previously discussed, IL-10 can induce proliferation in intestinal epithelial cells (Quiros et al. 2017). As such, the inhibition of IL-10-induced epithelial restitution could have also contributed to the more severe colitis demonstrated in mice lacking intestinal epithelial expression of the IL-10 receptor 1 in this study.

In a separate study, Zheng et al. demonstrated how a cytokine, in this case IL-10, can interact with the intestinal microbiota to regulate epithelial function (Zheng et al. 2017). Butyrate, a short chain fatty acid made by the intestinal microbiota in vivo, induced the expression of both IL-10 receptor α subunit mRNA and protein in T84 and Caco-2 cells. Treatment of T84 cells with butyrate and IL-10 increased epithelial barrier integrity more than butyrate alone as determined by increased transepithelial electrical resistance. Based on the increased expression of the IL-10 receptor α subunit in the epithelial cells due to butyrate treatment, the mechanism for this increase in barrier integrity owing to butyrate and IL-10 could be hypothesized to be an increase in IL-10 signaling due to increased IL-10 receptor expression. However, the authors did not compare these data with the transepithelial electrical resistance induced by IL-10 in the absence of butyrate. As a result, it is unclear from these data whether butyrate and IL-10 synergistically increase transepithelial electrical resistance in intestinal epithelial cells, or if the level reported in this study could have been induced by IL-10 alone. The authors went further to demonstrate that butyrate reduced both the mRNA and protein expression of the pro-permeability tight junction protein claudin-2 in T84 cells in an IL-10 receptor α-dependent manner, providing a potential mechanism for the observed increases in epithelial barrier integrity in the presence of butyrate (Zheng et al. 2017). Interestingly, reductions in butyrate-producing bacteria have been reported in the microbiota of ulcerative colitis patients, suggesting a potential mechanism of epithelial barrier compromise due to dysbiosis as a contributing factor in this disease (Machiels et al. 2014).

A study by Lorén et al. demonstrated how IL-10 can increase the effectiveness of other therapies (Lorén et al. 2015). Previous work by this group correlated low IL-10 mRNA levels with poor glucocorticoid response in active Crohn's disease. In a later study, the authors discovered a possible mechanism for this observation, as treatment with a combination of IL-10 and glucocorticoids, but neither treatment alone, restored the transepithelial electrical resistance of Caco-2 cell monolayers following their disruption with TNF-α (Lorén et al. 2015).

A study by Kuhn et al. provided more evidence for the crucial relationship between the microbiota, immune system, and intestinal epithelial barrier (Kuhn et al. 2017). Bacteria in the order Bacteroidales were sufficient to induce localization of intraepithelial lymphocytes in the colons of mice, and these cells were an important source of IL-6. IL-6 supported epithelial barrier function, as IL-$6^{-/-}$ mice displayed reduced expression of the tight junction protein claudin-1, a thinner mucus gel layer, and augmented paracellular permeability, all defects which were resolved by the transfer of IL-$6^{+/+}$ intraepithelial lymphocytes to affected mice (Kuhn et al. 2017).

C-kit signaling has also been shown to promote intestinal epithelial barrier integrity through the regulation of a tight junction protein. The overexpression of c-kit or administration of its ligand stem cell factor increased expression of the tight junction protein claudin-3 in colorectal cancer cells in vitro, and decreased claudin-3 expression was observed in the colon epithelium of mice lacking functional c-kit (Wang et al. 2017).

Rectal biopsies from adult and pediatric patients with ulcerative colitis have increased IL-33 expression relative to specimens lacking inflammation (Waddell et al. 2015). To determine if this implicates IL-33 as a contributor to inflammation or an anti-

inflammatory response in these patients, Waddell et al. investigated the role of IL-33 in chemically-induced colitis in mice (Waddell et al. 2015). Mice with genetic deletion of ST2, the receptor for IL-33, had decreased colon transepithelial electrical resistance and increased permeability to FITC-dextran, suggesting that IL-33 promotes colon epithelial barrier function. In support of these data, genetic deletion of either ST2 or IL-33 precipitated more severe chemically-induced colitis in these mice (Waddell et al. 2015). However, the authors did not fully characterize the mechanism by which IL-33 promoted epithelial barrier integrity in these studies. The authors reported that intestinal epithelial proliferation and apoptosis were unaffected by the absence of IL-33 or ST2 in this model of colitis, but that goblet cell numbers and *Muc2* expression were decreased in these mice. This suggests that alterations in the mucus layer could have influenced epithelial barrier permeability in these mice, but the mucus layer itself was not evaluated. Additionally, potential effects of IL-33 on interepithelial junctional complexes were not assessed; however, the authors did demonstrate that IL-33-induced augmentation of transepithelial electrical resistance in T84 cell monolayers was dependent on ERK1/2 signaling (Waddell et al. 2015). This is particularly curious in light of a recent paper that reported reduced transepithelial electrical resistance and claudin-1 expression induced by IL-33-stimulated ERK signaling in human keratinocytes (Ryu et al. 2018). This discrepancy could be explained by the different cell types investigated; however, conflicting roles for IL-33 in intestinal inflammation have been reported. Other investigators have demonstrated exacerbation of multiple models of murine colitis and decreased intestinal epithelial barrier integrity due to the administration of IL-33 (Sattler et al. 2014; Sedhom et al. 2013). Waddell et al. suggest that these inconsistencies

could be due to differences in IL-33 concentrations among studies or the differing characteristics of inflammation in each colitis model, two reasonable explanations that warrant further investigation (Waddell et al. 2015). In support of the data reported by Waddell et al., Sattler et al. demonstrated the induction of protective IL-10-producing regulatory B cells by IL-33 (Sattler et al. 2014). The administration of IL-33 accelerated spontaneous colitis in IL-10 deficient mice but did not induce intestinal inflammation in wildtype mice. Additionally, the transfer of IL-33-induced, IL-10-producing regulatory B cells to IL-10 deficient mice reduced colitis severity and delayed disease onset (Sattler et al. 2014). As previously discussed, IL-10 promotes epithelial barrier integrity (Kominsky et al. 2014; Zheng et al. 2017). As such, reduced IL-10 production owing to genetic ablation of IL-33 signaling is a potential mechanism for the increased intestinal epithelial permeability observed by Waddell et al (Kominsky et al. 2014; Waddell et al. 2015; Zheng et al. 2017).

Falling through the cracks: cytokine promotion of intestinal epithelial permeability

In contrast to the barrier reinforcing properties of the cytokines described above, a handful of cytokines can also disrupt the intestinal epithelium and promote barrier permeability (Figure 4) (Sumagin et al. 2014; Wang et al. 2017; Watari et al. 2017; Ye and Sun 2017).

A variety of the effects of TNF-α on the intestinal epithelium discussed herein could disrupt the epithelial barrier; however, TNF-α stimulation of intestinal epithelial cells has also been specifically demonstrated to decrease the protein expression of the tight junction proteins claudin-1, occludin, and zonula occludens protein 1, as well as to

induce cytoskeletal F-actin rearrangement and the mislocalization of occludin and zonula occludens protein 1 (Watari et al. 2017; Ye and Sun 2017). Multiple studies have identified mechanisms to reduce TNF-α-induced epithelial barrier compromise, including the overexpression of anterior gradient protein 2 homologue, rebeccamycin treatment, and the stimulation of muscarinic cholinoceptor-mediated signaling (Khan et al. 2015; Watari et al. 2017; Ye and Sun 2017).

IL-22 also increases gut epithelial permeability via manipulation of tight junction protein expression. IL-22 stimulation of Caco-2 cells in vitro and murine colon epithelial cells in vivo increased the expression of the tight junction protein claudin-2, which forms cation channels. Caco-2 monolayers treated with IL-22 displayed decreased transepithelial electrical resistance, indicating increased paracellular ion permeability, but no change in movement of uncharged macromolecules across the monolayers was observed (Wang et al. 2017).

The increase in intestinal epithelial permeability induced by interferon-γ described by Sumagin et al. provides an elegant example of the intricate relationships between cytokines, the epithelium, and immune cells (Sumagin et al. 2014). Using the T84 intestinal epithelial cell line for an in vitro model of transepithelial migration of neutrophils, the authors demonstrated that interferon-γ induced expression of the intercellular adhesion molecule-1 (ICAM-1) on the apical membrane of T84 cells and increased the number of neutrophils adherent to the apical epithelial membranes via ICAM-1 post-migration. The ligation of ICAM-1 by neutrophils resulted in the phosphorylation of myosin light-chain kinase and a subsequent increase in epithelial permeability characterized by actin cytoskeletal reorganization, paracellular FITC-

dextran flux, and a decrease in transepithelial electrical resistance. Notably in this model, this increase in epithelial permeability facilitated neutrophil transepithelial migration (Sumagin et al. 2014).

Additional cytokine effects on intestinal epithelial function

In addition to those detailed above, cytokines modulate a wide array of other intestinal epithelial functions. While endogenous type III interferon produced by intestinal epithelial cells does not restrict human rotavirus replication due to viral antagonism of the type III interferon response, treatment of human rotavirus-infected small intestinal organoid cultures with exogenous type I interferon, and to a lesser extent exogenous type III interferon, limits rotaviral replication (Saxena et al. 2017). However, other studies in mice have found that interferon-λ, a type III interferon, is more effective than type I interferons in limiting viral replication in the intestinal epithelium in models of reovirus and rotavirus infection (Mahlakõiv et al. 2015; Pott et al. 2011).

In a somewhat unexpected role, IL-22 production by neutrophils in chemically-induced murine colitis induced the expression of antimicrobial peptides by the colon epithelium and protected the epithelium from chemically-induced damage (Zindl et al. 2013). Epithelial signaling of the IL-17 receptor regulates colonization of the murine intestine with segmented filamentous bacteria through the epithelial expression of the apical NADPH oxidase *Nox1*, *polymeric immunoglobulin receptor* (*Pigr*), and α-defensins (Kumar et al. 2016). In addition to the functions previously discussed, TNF stimulation of the intestinal epithelium has also been shown to reduce expression of the Cl^-/HCO_3^- exchanging solute carrier family 26 member 3 (SLC26A3), which may represent a therapeutic target in IBD-associated diarrhea (Kumar et al. 2017). TNF also

augmented receptor activator of NF-κB ligand (RANKL)-induced M cell differentiation (Wood et al. 2016).

Talking back: intestinal epithelial-derived cytokines and chemokines

Pro- and anti-inflammatory functions of intestinal epithelial-derived cytokines

The intestinal epithelium is not simply beholden to respond to immune cell-derived cytokines, but is a rich source of cytokines and chemokines, which may ameliorate or promote inflammation. The colonic epithelium was found to be a larger source of trefoil factor 2 (TFF2) than colon leukocytes, and TFF2 was protective in both acute and chronic models of DSS-induced colitis (Judd et al. 2015). In models of helminth infection, production of IL-25 by intestinal epithelial tuft cells regulated the helminth-induced type 2 immune response and facilitated worm expulsion (Gerbe et al. 2016; von Moltke et al. 2016).

Intestinal epithelial cells also produce the anti-inflammatory cytokine IL-10, which likely contributes to tolerance to commensal bacteria. TLR4 ligation induced intestinal epithelial cell expression of IL-10, and this expression was enhanced by co-culture with macrophages (Hyun et al. 2015). As previously discussed, IL-10 has been shown to stimulate intestinal epithelial cell proliferation and reinforce the integrity of the epithelial barrier (Kominsky et al. 2014; Quiros et al. 2017; Zheng et al. 2017). Thus, the microbiota may contribute to intestinal epithelial integrity through epithelial TLR4 ligation and the subsequent autocrine action of epithelial-derived IL-10. If so, IL-10 would not be the only cytokine with an autocrine mechanism for promoting epithelial homeostasis. IL-6 production by the intestinal epithelium has also been detected, which was shown to act in an autocrine manner to regulate crypt homeostasis (Jeffery et al. 2017).

In contrast to these anti-inflammatory and homeostatic effects, intestinal epithelial products may also promote inflammation. The accumulation of visceral fat has been associated with chronic intestinal inflammation, and in support of this, co-culture of intestinal epithelial cells with differentiated adipocytes induced epithelial expression of TNF and matrix metalloproteinase-9 (Takahashi et al. 2017). IL-1α release by necrotic intestinal epithelial cells in a murine model of chemically-induced colitis induced cytokine production by mesenchymal cells and reactivated colon inflammation post-recovery when delivered via enema (Scarpa et al. 2015). The findings of Bersudsky et al. support these data, as genetic ablation of IL-1α ameliorated murine DSS-induced colitis (Bersudsky et al. 2014).

Intestinal epithelial cells also secrete IL-33; however, there is conflicting evidence in the literature regarding its role in both IBD (reviewed by Griesenauer et al.) and intestinal carcinogenesis (Griesenauer et al. 2017). IL-33 expression was found to be increased in epithelial cells of both murine and human intestinal tumors, and IL-33 promoted tumor development in *Apc*$^{Min/+}$ mice (He et al. 2017; Maywald et al. 2015). Similarly, the expression of IL-33 by intestinal epithelial cells was increased in the murine azoxymethane/DSS model of colon cancer, and the authors went further to demonstrate that the epithelial expression of IL-33 was driven by epidermal growth factor (Islam et al. 2016). In contrast, knockdown of the IL-33 receptor, ST2, in colon cancer cells from mice enhanced tumor growth, suggesting a potential antitumorigenic role for IL-33 (O'Donnell et al. 2016).

Calling in the troops: intestinal epithelial chemokine production

Intestinal epithelial-derived chemokines can contribute to both cellular defense and pathology. *Listeria monocytogenes* infection of an intestinal epithelial cell line induced expression of the chemokines IL-8, CCL1, and CCL20. Consistent with the epithelial invasiveness of *L. monocytogenes*, the high levels of CCL20 and IL-8 were likely induced by intracellular TLR10 signaling, the knockdown of which reduced chemokine levels more than silencing of TLR1 or TLR2 (Regan et al. 2013). IL-8, CCL1, and CCL20 are responsible for neutrophil, Th2 and regulatory T cell, and Th17 and dendritic cell trafficking, respectively, and would promote the infiltration of these cell types in the infected mucosa (Griffith et al. 2014). Interestingly, a separate study identified a non-chemotactic role for IL-8 in the intestine. Apically-secreted intestinal epithelial cell-derived IL-8 in response to TLR2 and TLR5 ligation was shown to act in an autocrine manner to promote gene expression related to cellular differentiation (Rossi et al. 2013).

Chemokines likely play a critical role in the perpetuation of intestinal inflammation in IBD patients. Dent et al. reported that co-cultured eosinophils and intestinal epithelial cells synergized to increase neutrophil chemotactic activity and CXCL5 production; however, the authors did not quantify the individual contributions of each cell type to this increase (Dent et al. 2014). As evidence of activated eosinophils has been detected in acute flares of IBD, this could contribute to excessive neutrophil recruitment to the intestine and increased tissue damage in active IBD (Dent et al. 2014). Production of the cytokine IL-34 is increased in the intestine of patients with active IBD, and Franzè et al demonstrated that production of the chemokine CCL20 was associated with IL-34

signaling in both the DLD-1 colon epithelial cell line and in mucosal explants from IBD patients (Franzè et al. 2016). CCL20 production could fuel the inflammatory response in active IBD patients through the recruitment of Th17 and dendritic cells. However, the potential consequences of increased CCL20 production are not so clear-cut. In fact, these cells could aid in restitution of the epithelial barrier in IBD patients. As noted previously, IL-17 can increase intestinal epithelial cell proliferation and reduce barrier permeability, and dendritic cells are a critical source of IL-28A in the gut, another cytokine shown to induce intestinal epithelial proliferation (Chiriac et al. 2017; Lee et al. 2015; Maxwell et al. 2015; Song et al. 2015). Conversely, this hypothesized cytokine-induced proliferation could be too much of a good thing. IL-17 has been shown to both induce the proliferation of transformed enterocytes and stimulate IL-6 production, a cytokine implicated in colitis-associated carcinogenesis (Wang et al. 2014). The neutrophil chemokine CXCL1 has also been shown to promote carcinogenesis. The upregulation of CXCL1 by colon tumor epithelium was dependent on hypoxia inducible factor-2α and contributed to colon carcinogenesis through neutrophil recruitment (Triner et al. 2017).

Intestinal epithelial responses to pathogens and commensals

The intestinal epithelium is uniquely located to be the ideal first line of defense or communication with intraluminal bacteria and viruses. A number of bacteria alter cytokine production by the gut epithelium (Figure 5) (Fiorentino et al. 2014; Kainulainen et al. 2015; Knodler et al. 2014; Tian et al. 2016; Wang et al. 2014; Zeuthen et al. 2008).

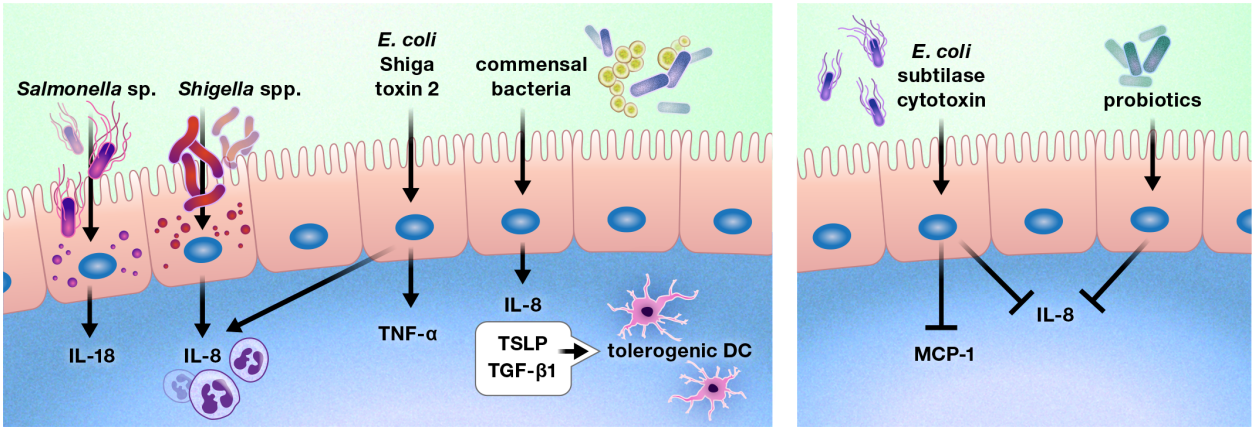

Figure 5. Pathogens, commensal bacteria, and probiotics can increase or diminish the production of cytokines and chemokines by the intestinal epithelium. These interactions may promote or deter immune cell infiltration of the gut, such as by increasing or reducing the production of chemokines, including IL-8 and MCP-1. In some cases, bacterial interactions with the gut epithelium may instruct the intestinal immune system. For example, intestinal epithelial cells produce TSLP and TGF-β1 in response to commensal bacteria, inducing a tolerogenic phenotype in dendritic cells.

Exposure of the colon epithelial cell line HCT-8 to Shiga toxin 2 produced by Shiga-toxigenic *Escherichia coli* increased protein expression of IL-8 and TNF-α. However, HCT-8 exposure to subtilase cytoxin produced by the same bacterium decreased protein expression of IL-8 and monocyte chemoattractant protein-1 (MCP-1) relative to unstimulated control cells, suggesting that these bacteria may use specific toxin production to differentially modulate host defenses (Wang et al. 2014). Infection of Caco-2 monolayers with *Shigella flexneri* 2a or *Shigella dysenteriae* 1 induced IL-8 secretion, which was predominantly released from the basolateral aspect of the epithelial cells, and *Salmonella enterica* serovar Typhimurium activated non-canonical inflammasome activity in murine and human intestinal epithelial cells, facilitating IL-18 secretion and bacterial clearance (Fiorentino et al. 2014; Knodler et al. 2014).

In contrast to these predominantly pro-inflammatory responses, stimulation of Caco-2 cells with commensal bacteria increased thymic stromal lymphopoietin (TSLP), IL-8, and TGF-β1 secretion, which resulted in the promotion of a tolerogenic dendritic cell phenotype by TSLP and TGF-β1 (Zeuthen et al. 2008). Additionally, probiotic bacterial strains have been shown to reduce gut epithelial production of IL-8 (Kainulainen et al. 2015; Tian et al. 2016).

Intestinal epithelial cytokine release prompted by viral infection can help clear infection or create pathology. Simian immunodeficiency virus infection of the intestinal epithelium of rhesus macaques induced IL-1β expression by Paneth cells before the induction of an antiviral interferon response. IL-1β expression was correlated with epithelial disruption characterized by the mislocalization and reduced expression of tight junction proteins, although these changes did not correspond to any aberrant responses to bacteria (Hirao et al. 2014).

Multiple studies have documented the production of interferon-λ by virus-infected intestinal epithelial cells, although the ability of this cytokine to limit viral infection varied between studies (Hernandez et al. 2015; Mahlakõiv et al. 2015; Saxena et al. 2017). A possible explanation for these discrepancies may be found in the work of Hernández et al., which demonstrated that group 3 innate lymphoid cell-derived IL-22 amplified interferon-λ signaling in intestinal epithelial cells, and synergistic signaling by the two cytokines was necessary for a reduction in viral replication and optimal stimulation of interferon-induced gene expression (Hernandez et al. 2015).

Dietary modulation of intestinal epithelial mediator release

Diet has been implicated as a possible contributing factor to IBD; however, research has failed to identify the “ideal” anti-inflammatory diet for IBD patients (Knight-Sepulveda et al. 2015). Nevertheless, recent studies have identified anti-inflammatory effects of specific dietary components on the intestinal epithelium. Pretreatment of Caco-2 cells with the plant-derived flavonoid cyanidin-3-glucoside (C3G) reduced TNF-α-induced gene expression of IL-8 and TNF-α. C3G also inhibited endothelial cell activation and subsequent leukocyte adhesion stimulated by co-culture with TNF-α-stimulated Caco-2 cells (Ferrari et al. 2017). Similarly, treatment of Caco-2 cells with the dietary fiber guar gum increased expression of the suppressor of cytokine signaling-1 (SOCS-1) and reduced TNF-α-induced IL-8 expression. Additionally, guar gum administration to mice with chemically-induced enteritis reduced disease activity and proinflammatory cytokine expression in the small intestine concurrent with an increase in SOCS-1 protein (Hung et al. 2017).

Concluding remarks

Cytokines and chemokines are critical for intestinal epithelial homeostasis and responses to disease. The ability of cytokines to directly facilitate or restrict intestinal epithelial proliferation, apoptosis, and permeability makes them key players in the maintenance, or at times destruction, of the intestinal epithelial barrier. Furthermore, the release of cytokines and chemokines by the intestinal epithelium in response to pathogens, commensal organisms, interactions with other cell types, and dietary compounds allows these cells to have critical input into their microenvironment. Despite our frequent tendency to classify cytokines as either pro- or anti-inflammatory, we must

realize that these labels fail to acknowledge the incredible diversity and situational basis of cytokine functions. While undoubtedly complex, the cytokine biology of intestinal mucosal immunology is a fascinating opportunity for investigations into both intestinal immunophysiology and potential translational approaches to modulate this physiology for much-needed novel therapies for intestinal disease.

IL-27 as a novel therapy for inflammatory bowel disease: a critical review of the literature

Inflammatory bowel disease (IBD) refers to a collection of idiopathic inflammatory disorders of the intestine, the most common of which are Crohn's disease and ulcerative colitis. IBD is thought to result from an abnormal inflammatory response to commensal organisms and other antigens normally confined to the intestinal lumen due to compromise of the mucosal epithelial barrier and subsequent inappropriate exposure of resident intestinal immune cells to luminal antigens. A single cause for IBD has not been identified. Development of the disease is likely multifactorial, as a variety of etiological factors, including hygiene status, previous gastrointestinal infection, genetics, diet, and various other lifestyle factors, have been implicated in its pathogenesis (Baumgart and Carding 2007). Geographically, the highest incidence of IBD has been reported in northern Europe and North America, where an estimated 329 per 100,000 people in the United States suffer from IBD (Baumgart and Carding 2007; Kappelman et al. 2007).

Treatment for IBD typically involves some form of immunosuppression, necessitating a balance between achieving remission and managing potential adverse effects. Additionally, likely due to the complex pathophysiology and multifactorial

etiology of IBD, rates for remission induction and maintenance with many of the current standard treatments for IBD remain modest and are often variable (Danese 2012; Peyrin-Biroulet and Lemann 2011). As a result, investigation into novel therapeutic targets is sorely needed. The success of anti-tumor necrosis factor-α treatment in a subset of IBD patients demonstrated that therapy targeting a single cytokine could be efficacious in IBD, and antibodies against a variety of cytokines, including interleukin (IL)-13, IL-18, and IL-21, have entered clinical trials (Danese 2012).

IL-27 is a relatively recently discovered type I cytokine with established roles in infectious disease, autoimmunity, and cancer in a variety of organs, including the central nervous system, lung, skin, and gastrointestinal tract (Pflanz et al. 2002; Yoshida and Hunter 2015). However, it has not been definitively determined whether IL-27 ameliorates or promotes intestinal inflammation, as seemingly contradictory roles for IL-27 have been reported in murine models of IBD (Yoshida and Hunter 2015). Several excellent reviews have recently discussed the biology of IL-27 (Aparicio-Siegmund and Garbers 2015; Kastelein et al. 2007; Meka et al. 2015; Yoshida and Hunter 2015). The purpose of this review is to highlight recent literature investigating the role of IL-27 in IBD, and to discuss possible explanations for the conflicting results of these studies. Evidence supporting IL-27 therapy as a potential treatment for IBD will also be discussed.

IL-27 and its receptor

IL-27 is a heterodimeric type I cytokine in the IL-12 cytokine family. It is composed of two subunits: Epstein-Barr virus-induced gene 3 (EBI3) and p28, so named due to its predicted molar mass by SDS-PAGE (Kastelein et al. 2007; Pflanz et

al. 2002). IL-27 is predominantly produced by myeloid cells, such as macrophages and dendritic cells, but can also be expressed by epithelial cells, plasma cells, and endothelial cells (Yoshida and Hunter 2015). A variety of signals can induce IL-27 expression, including CD40 ligation, type I and II interferon signaling, and Toll-like receptor signaling (Yoshida and Hunter 2015). IL-27 expression induced by Toll-like receptor signaling has been shown to require interferon-alpha in human macrophages and interferon regulatory factor 3 in murine dendritic cells (Molle et al. 2007; Pirhonen et al. 2007).

IL-27 signals through a heterodimeric receptor consisting of IL-27Rα, previously termed TCCR or WSX-1, and gp130 (Pflanz et al. 2004; Yoshida and Hunter 2015) Coexpression of both receptor subunits has been detected in T and B lymphocytes, mast cells, natural killer cells, natural killer T cells, monocytes, neutrophils, dendritic cells, intestinal epithelial cells, and endothelial cells (Diegelmann et al. 2012; Li et al. 2010; Pflanz et al. 2004; Villarino et al. 2005). Interestingly, expression of the receptor differs among T cell subsets. IL-27Rα is highly expressed by regulatory, effector, and memory T cells, and increases with T cell activation. In contrast, naïve T cells express low levels of IL-27Rα. Natural killer and natural killer T cells express high levels of IL-27Rα, but activation of these cells results in decreased expression of this receptor subunit (Villarino et al. 2005). Ligation of the IL-27 receptor induces signaling through the Jak/STAT pathway, resulting in the phosphorylation of STAT1, 3, 5, or 6 (Aparicio-Siegmund and Garbers 2015; Hall et al. 2012). In addition to the Jak/STAT pathway, the activated IL-27 receptor has been reported to induce p38MAPK, ERK, and Akt signaling. IL-27 signaling also induces SOCS3, a suppressor of IL-27 signaling that

directly binds to and inhibits the gp130 receptor subunit and associated Janus kinases and later facilitates their degradation (Aparicio-Siegmund and Garbers 2015).

IL-27 activities in the immune system

IL-27: the T cell police

IL-27 boasts a diverse repertoire of functions in both the adaptive and innate branches of the immune system; however, the most well-known and thoroughly investigated functions of IL-27 relate to its ability to regulate the adaptive immune system by modulating T cell function. When first discovered, IL-27 was reported to induce expansion of naïve murine and human $CD4^+$ T cells and to act synergistically with IL-12 to enhance interferon-γ production by both naïve T cells and NK cells (Pflanz et al. 2002). IL-27 blocks Th2 cytokine expression and the differentiation of Th2 cells by simultaneously increasing expression of the transcription factor T-bet while suppressing GATA-3 expression (Yoshimoto et al. 2007). Additionally, IL-27 can prevent the expression of both IL-17A and IL-17F by suppressing RORγt, a transcription factor necessary for Th17 cell differentiation (Diveu et al. 2009). IL-27 also inhibits the production of Th17-polarizing cytokines by human dendritic cells. To demonstrate the functional consequences of this inhibition, Murugaiyan et al. showed that human memory $CD4^+$ T cells cocultured with dendritic cells stimulated with both toll-like receptor (TLR) agonists and IL-27 produced significantly less IL-17 than those exposed to dendritic cells activated by TLR ligands alone (Murugaiyan et al. 2009). Additional noteworthy effects of IL-27 on T cells include its ability to prevent Fas-mediated activation-induced cell death (Kim et al. 2013) and to induce IL-10 production (Yoshida and Hunter 2015). The ability of IL-27 to stimulate the expression of IL-10 by T

cells is perhaps one of its most extensively characterized immunoregulatory functions, having been demonstrated in models of parasitic, viral, and autoimmune disease (Yoshida and Hunter 2015). IL-27 induces IL-10 and T cell immunoglobulin and mucin domain-3 (Tim-3) in both $CD4^+$ and $CD8^+$ T cells by expression of the transcription factor nuclear factor, interleukin-3 regulated (NFIL3) (Zhu et al. 2015), and also induces the production of IL-10 by Th1 and Th2 cells in a STAT1 and STAT3 dependent manner (Stumhofer et al. 2007). Even under Th17-polarizing conditions, generally thought to drive primarily proinflammatory cytokine production, the addition of IL-27 elicits significant increases in IL-10 production by human $CD4^+$ T cells (Murugaiyan et al. 2009). Multiple studies have also demonstrated a critical role for IL-27 in the development of IL-10-secreting T regulatory type 1 (Tr1) cells in both humans and mice (Awasthi et al. 2007; Jeon et al. 2012; Mishima et al. 2015; Murugaiyan et al. 2009; Pot et al. 2009; Wang et al. 2011). Interferon-γ and IL-27 have both been shown to promote T-bet^+ $CXCR3^+$ regulatory T cells specialized to modulate Th1 responses; however, the development of this regulatory response in the gut-associated lymphoid tissue required IL-27 (Hall et al. 2012). While IL-27 could play a role in the regulation of T regulatory cells by suppressing IL-2, the significance of this potential negative regulatory function has not been characterized in wild type animals (Artis et al. 2004).

IL-27's role in innate immunity

In addition to its impressive repertoire of regulatory functions in T cell biology, IL-27 has been demonstrated to have a number of both modulatory and complementary roles in innate immunity. After identifying the receptor responsible for IL-27 signaling, Pflanz and colleagues showed that IL-27 induced proinflammatory gene expression in

both human mast cells and monocytes, but that this increase in expression occurred in notably fewer genes and at a later time point in monocytes (Pflanz et al. 2004). In contrast, in murine *Trichuris muris* infection and a murine model of mast cell-dependent passive cutaneous anaphylaxis, mice with genetic deletion of IL-27Rα had enhanced mast cell responses, suggesting that IL-27 may also negatively regulate mast cell activities under certain circumstances (Artis et al. 2004). IL-27 also acts on dendritic cells. IL-27 can induce expression of the regulatory molecule CD39 on dendritic cells (Mascanfroni et al. 2013), and when applied to monocyte-derived dendritic cells during their differentiation, improved antigen processing and subsequent T cell stimulation (Jung et al. 2014).

A critical role for IL-27 in the suppression of neutrophil and monocyte function has been identified that demonstrated strikingly different outcomes in models of infectious disease based on the timing of IL-27 signaling (Liu et al. 2014; Wirtz et al. 2006). Mice with genetic deletion of IL-27Rα had more severe pulmonary histopathology and greater mortality following influenza infection than wild type mice, suggesting that IL-27 signaling is protective in influenza infection. In support of this finding, IL-27 treatment of mice in the late stage of influenza infection decreased both clinical signs of disease and lung histopathology scores without reducing clearance of the virus (Liu et al. 2014). This improvement in pathology corresponded to significantly lower pulmonary infiltrates of both neutrophils and monocytes, which were proposed by the authors to be due to IL-27-mediated suppression of the chemokines CXCL1, CCL5, and CCL4. However, in sharp contrast to this beneficial function of IL-27, the reduction of neutrophil and monocyte infiltrates due to administration of IL-27 early in influenza infection

prevented viral clearance and worsened the clinical signs of disease. Accordingly, the highest endogenous levels of the IL-27 p28 subunit in wild type mice were measured in the late phase of influenza infection as viral load was declining (Liu et al. 2014), suggesting that endogenous release of IL-27 can be precisely timed for limiting collateral damage from the inflammatory response while not hindering pathogen clearance.

However, data from a model of septic murine peritonitis provide more evidence for the sometimes deleterious effects of the direct suppression of innate responses by IL-27 (Wirtz et al. 2006). Mice lacking the EBI3 subunit of IL-27 had reduced mortality associated with greater bacterial clearance during septic peritonitis induced by cecal ligation and puncture. The authors further demonstrated that these mice lacking functional IL-27 had more robust intraperitoneal granulocytic infiltrates following cecal puncture, and that IL-27 directly inhibited the bacterial lipopolysaccharide-induced production of reactive oxygen intermediates by both granulocytes and macrophages/monocytes from wild type mice (Wirtz et al. 2006). Wild type mice were shown to upregulate the expression of IL-27 within 6 hours of cecal puncture (Wirtz et al. 2006), further suggesting that, when applied early in infection, the immunosuppressive effects of IL-27 may result in reduced pathogen clearance and more severe disease. Complementary to these findings in mice, IL-27 has also been shown to suppress human neutrophil adhesion and bacterial lipopolysaccharide-induced reactive oxygen species production in vitro (Li et al. 2010).

Collectively, these data inform critical stipulations for the use of IL-27 or anti-IL-27 as a therapy. As suggested by Wirtz et al., the inhibition of IL-27 signaling may prove

effective in conditions such as sepsis in which infection control is key (Wirtz et al. 2006). Alternatively, the administration of IL-27 could be a novel tool for limiting immunopathology in autoimmune diseases and later in the course of infections when dampening inflammation becomes a larger priority than controlling pathogens. However, for both of these potential applications, the timing of therapy and the presence of primary or secondary infections would be crucial factors in determining treatment regimens.

IL-27 as a therapy for IBD: evidence from human patients and mouse models

IL-27 polymorphisms and mutations in IBD and cancer

Multiple studies have implicated *IL-27* as a candidate gene for IBD (Imielinski et al. 2009; Li et al. 2009; Wang et al. 2014). A genome wide association study in early-onset IBD identified *IL-27* within a susceptibility locus in a North American-European cohort. In support of this conclusion, the authors also demonstrated that healthy individuals with two copies of the risk allele expressed significantly less IL-27 relative to individuals with two copies of the nonrisk allele and that colonic expression of IL-27 was significantly lower in early-onset Crohn's disease patients than in healthy controls (Imielinski et al. 2009). *IL-27* polymorphisms have also been associated with risk for IBD in both Chinese and Korean populations (Li et al. 2009; Wang et al. 2014).

The IL-27 receptor, either wild type or mutated, can contribute to hematopoietic cell transformation (Lambert et al. 2011; Pradhan et al. 2007). Although IBD predisposes to colorectal cancer and *IL-27* polymorphisms affect risk for IBD, thus far the potential role of *IL-27* polymorphisms in colorectal cancer is unclear, as studies evaluating their

association with the risk for colorectal cancer produced conflicting results (Huang et al. 2011; Lyu, et al. 2015; Xu et al. 2014; Zhang et al. 2015).

IL-27 ameliorates colitis induced by T cell transfer or chemical-induction in mice Multiple studies have demonstrated the ability of IL-27 to ameliorate colitis in mice, either through lessening of induced colonic inflammation by IL-27 administration or demonstration of more severe colitis in mice deficient in IL-27 due to either antibody-mediated neutralization or genetic knockout (Dannet al. 2014; Hanson et al. 2014; McAleer et al. 2011; Sasaoka et al. 2011; Troy et al. 2009). Mucosal administration of IL-27 synthesized in situ by a food-grade bacterium improved survival and significantly decreased disease activity, colon and small intestine histopathology scores, and proinflammatory gene expression within the intestine in a mouse model of enterocolitis induced by T cell transfer (Hanson et al. 2014). The treatment effects in this study were both T cell- and IL-10-dependent; however, mucosal delivery of IL-27 was found to be more efficacious than direct mucosal delivery of IL-10 by the bacteria. A possible explanation is that IL-27 induces higher levels of endogenous IL-10 in the intestine and mesenteric lymph nodes than could be achieved by the bacteria producing IL-10 in situ. Interestingly, mucosal delivery of IL-27 was also more effective than systemic administration of recombinant murine IL-27 in this study, which had no detectable therapeutic effect (Hanson et al. 2014). Consistent with previous literature (Diveu et al. 2009), IL-27 treatment significantly decreased the expression of RORγt in the colons of enterocolitic mice in this study, and likely as a result, decreased the expression of both IL-17A and IL-17F as well. This study went further to show that IL-27 treatment also

reduced disease activity in dextran sulfate sodium (DSS)-induced colitis, a widely used chemically-induced model of colitis (Hanson et al. 2014).

Subcutaneous treatment with IL-27 in an acute chemically-induced model of colitis using 2,4,6-trinitrobenzenesulfonic acid (TNBS) was also reported to be protective (Sasaoka et al. 2011), with improved colonic macroscopic and histopathology scores and reductions in several of the same proinflammatory cytokines previously reported (Hanson et al. 2014), including IL-6, TNF-α, IL-17A, and IL-1β (Sasaoka et al. 2011). The ability of subcutaneous IL-27 treatment to protect the mice in this study from TNBS-induced colitis contrasts with the previously discussed findings of Hanson et al., in which systemically administered IL-27 by intraperitoneal injection showed no therapeutic effect (Hanson et al. 2014). However, in addition to a different route of injection, the most beneficial effects of subcutaneous IL-27 treatment were at a higher dose than that administered intraperitoneally in the other study, and these two studies evaluated IL-27 treatment in two mechanistically very different models of colitis (acute chemically-induced versus chronic T cell transfer), potentially explaining this discrepancy. The ability of IL-27 to reduce intestinal inflammation by multiple routes of administration could be an advantage clinically, making it a more versatile therapy (Hanson et al. 2014; Sasaoka et al. 2011).

Ablation of IL-27Rα is proinflammatory in murine models of colitis

Additional studies have indirectly demonstrated the anti-inflammatory effects of IL-27 in intestinal inflammation by documenting the impact of its absence. Antibody neutralization of IL-27 in mice infected with *Citrobacter rodentium* precipitated more severe colitis and increased production of IL-6 (Dann et al. 2014). In another study, a

more proinflammatory phenotype was observed in regulatory T cells lacking IL-27Rα, which produced more IL-17 and less IL-10 than regulatory T cells from wild type mice (McAleer et al. 2011). The consequences of this altered phenotype due to IL-27Rα deletion were reported by another laboratory using the T cell transfer model of enterocolitis. In this model naïve $CD4^+$ T cells are transferred into an immunodeficient recipient mouse, resulting in enterocolitis. However, the cotransfer of regulatory T cells can prevent the development of enterocolitis in this model. Interestingly, Do et al demonstrated that $Foxp3^+$ regulatory T cells lacking IL-27Rα cotransferred with naïve $CD4^+$ T cells were, unlike their wild type counterparts, unable to prevent the development of colitis. This group further showed that IL-27 stimulation of both human and murine regulatory T cells enhanced their ability to suppress inflammation and induced the expression of Lag3, a surface receptor critical for the suppressive function of regulatory T cells (Do et al. 2016).

Complementary to these findings, the induction of regulatory T cells by IL-10-secreting B cells is dependent on IL-27 signaling in T cells. Genetic deletion of IL-27Rα on $CD4^+$ T cells or neutralization of IL-27 blocked the induction of regulatory T cells by IL-10-secreting B cells. This loss of IL-27 signaling also limited the ability of these B cells to suppress proinflammatory cytokine production by both T cells lacking IL-27Rα and wild type T cells in the presence of antibody-neutralized IL-27. Remarkably, while wild type B cells co-transferred with wild type $CD4^+$ T cells reduced intestinal pathology due to T cell transfer, this effect was absent when the transferred T cells lacked IL-27Rα, demonstrating that IL-27 signaling is critical for the suppression of T cell-driven intestinal inflammation by IL-10-secreting B cells (Mishima et al. 2015).

In the DSS chemically-induced model of colitis, mice lacking the IL-27 receptor had significantly increased ratios of Th17 to Th1 cells in the mesenteric lymph nodes and colon both before and after DSS treatment compared to wild type controls (Troy et al. 2009). These mice developed more severe colitis more quickly when administered DSS compared to wild type controls and were significantly less likely to survive DSS exposure (Troy et al. 2009).

IL-27 suppresses the innate inflammatory response

Recent experiments have shown that mucosally-administered IL-27 improves histopathology scores in both the DSS (Andrews, unpublished data) and TNBS models of acute chemically-induced colitis (McLean et al. 2013). In the TNBS model, this IL-27 treatment significantly reduced neutrophil infiltrates in inflamed segments of colon (McLean et al. 2013). These data complement the findings of Li et al, in which IL-27 treatment of human neutrophils suppressed their adhesion capability in vitro. Furthermore, IL-27 treatment of neutrophils in this study reduced gene and protein expression of the integrin Mac-1, which the authors proposed as a possible mechanism for the suppression of neutrophil adhesion by IL-27 (Li et al. 2010). Additionally, a separate study found that IL-27 treatment of neutrophils stimulated with a TLR ligand in vitro significantly reduced the production of the proinflammatory cytokines IL-6 and IL-12/IL-23p40 (Troy et al. 2009).

Further supporting a suppressive role for IL-27 on the innate immune system, deletion of IL-27Rα in $RAG^{-/-}$ mice lacking T and B cells made them more sensitive to DSS-induced colitis (Troy et al. 2009). These findings suggest that while IL-27 treatment may depend on modification of T cell function under certain conditions, its effects on the

innate immune system alone may be sufficient for protection against some types of intestinal inflammation, widening its potential therapeutic indications. Collectively, these data lend further support for an immunosuppressive role of IL-27 in both innate and adaptive immunity in the intestine.

Additional protective functions of IL-27 in the intestine

In addition to the studies described above, which directly investigated the role of IL-27 in mouse models of intestinal inflammation, a number of studies have uncovered immunomodulatory and protective roles for IL-27 in the intestine while examining other aspects of intestinal biology. IL-27 promotes oral tolerance (Shiokawa et al. 2009) and mediates the ability of *Bifidobacterium infantis* to suppress IL-17 expression (Tanabe et al. 2008). Additionally, IL-27 was elevated in Fat-1 mice in chronic DSS-induced colitis in association with improved histopathology scores and decreased expression of Th17 cell cytokines (Monk et al. 2012). IL-27 can also promote intestinal epithelial barrier integrity. Intestinal epithelial cells have been shown to upregulate the IL-27 receptor in inflammation and bacterial infection, and IL-27 signaling increased intestinal epithelial cell proliferation and antibacterial peptide production (Diegelmann et al. 2012). IL-27 clearly demonstrates a variety of protective functions in the intestine, and therefore may prove to be an effective therapy for IBD.

The other side: proinflammatory effects of IL-27 in mouse models of intestinal disease

Ablation of IL-27Rα signaling can attenuate intestinal inflammation due to T cell transfer, DSS administration, and IL-10 deficiency

In contrast to the evidence presented above, a collection of studies have reported deleterious effects of IL-27 in intestinal inflammation based on inhibiting IL-27 receptor signaling (Cox et al. 2010; Honda et al. 2005; Visperas et al. 2013; Villarino et al 2008). Genetic deletion of IL-27Rα on either transferred T cells or in recipient mice effectively prevented the intestinal inflammation typical of the T cell transfer model of enterocolitis (Cox et al. 2010; Visperas et al. 2013). Increased numbers of transferred T cells lacking IL-27Rα became $Foxp3^+$, suggesting that IL-27 may negatively regulate the development of $Foxp3^+$ regulatory T cells (Cox et al. 2010). The second study also noted that inhibition of IL-27Rα in recipient mice prevented Th17 cell development by decreasing production of IL-1β and IL-6 by antigen presenting cells (Visperas et al. 2013). Similarly, IL-27Rα knockout mice reportedly develop less severe DSS-induced colitis than wild type mice, characterized by reduced expression of IL-6, TNF-α, and IFN-γ in intestinal lamina propria mononuclear cells (Honda et al. 2005). IL-10 deficient mice, which spontaneously develop intestinal inflammation, also reportedly have both delayed pathology and a survival advantage when IL-27Rα is concurrently genetically deleted (Villarino et al. 2008). Interestingly, a number of these findings attributed to IL-27Rα deletion, including reduced inflammation in both the T cell transfer and DSS models of colitis and reductions in IL-1β, IL-6, and TNF-α, have also been reported as

beneficial effects of IL-27 treatment, leaving more questions than answers for the interpretation of these aspects of IL-27 function.

IL-27 and inflammatory bowel disease: clarity amid the confusion

Conflict resolution

While a few studies suggest that inhibition of IL-27 receptor signaling can ameliorate intestinal inflammation, most of the literature supports IL-27 as a suppressor of intestinal inflammation caused by a variety of insults (Figure 6).

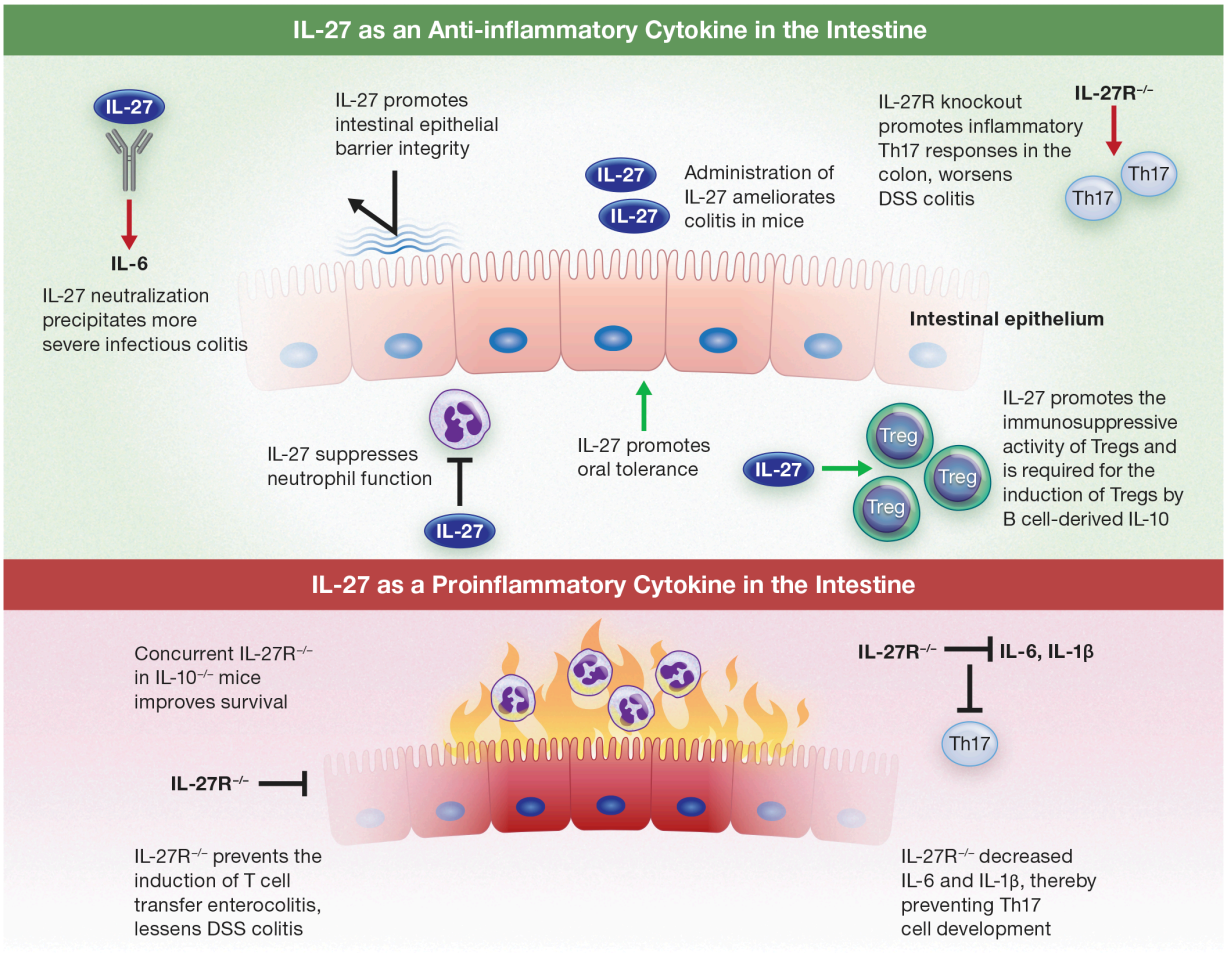

Figure 6. Evidence for IL-27 as an anti-inflammatory versus proinflammatory cytokine in the intestine. Results from conflicting studies are included.

It is important to note that all of the studies favoring a proinflammatory role for IL-27 in the intestine examined specific consequences of genetic deletion of the IL-27 receptor. In contrast, to our knowledge no report to date has demonstrated deleterious effects of exogenous IL-27 treatment on intestinal inflammation. Additionally, based on the complex biology of IL-27 discussed above, it is not hard to imagine how complete inhibition of IL-27 signaling could eliminate subtle checks and balances inherent to IL-27 function in wild type animals, as IL-27 signaling elicits distinct responses based on cell type and timing. For example, genetic deletion of IL-27Rα predicted that IL-27 would be protective in influenza infection. However, while the administration of IL-27 late in the course of influenza infection was beneficial, IL-27 therapy early in the infection resulted in more severe disease (Liu et al. 2014), an outcome impossible to predict by complete genetic inhibition of IL-27 signaling. Based on the ability of IL-27 to inhibit both Th2 and Th17 cell differentiation and promote Tr1 cell development (Awasthi et al. 2007; Diveu et al. 2009; Jeon et al. 2012; Mishima et al. 2015; Murugaiyan et al. 2009; Pot et al. 2009; Wang et al. 2011; Yoshimoto et al. 2007), it is also reasonable to speculate that genetic ablation of IL-27 signaling could alter the differentiation and subsequent phenotype of multiple cell types, thereby creating an inflammatory microenvironment that is not representative of that of wild type animals.

Additionally, the timing of IL-27 signaling and the evaluation of its result are critical. In human mast cells, IL-27 rapidly elicits transcription of inflammatory cytokine genes but elicits a slow response in human monocytes (Pflanz et al. 2004). As a result, conclusions taken at a single or limited time points may not fully illustrate the results of IL-27 therapy or elimination and may contribute to discrepancies between studies.

Finally, different concentrations of IL-27 have been shown to elicit distinct patterns of cytokine expression (Mishima et al. 2015). Different methods of IL-27 administration may produce different concentrations within the intestine and may be an explanation for differing results between some studies. Furthermore, the different patterns of cytokine expression induced by different concentrations of IL-27 suggest that genetic deletion of IL-27 signaling in an entire animal or subsets of its cells may create a misleading “all or nothing” microenvironment unrepresentative of physiologic reality.

When interpreting literature describing the biology of IL-27, it is critical to acknowledge that each subunit of the heterodimeric IL-27 cytokine has unique biologic activities of its own (Stumhofer et al. 2010; Wirtz et al. 2011). As a result, gene and/or protein expression of either of these subunits, even if co-expressed, is not necessarily indicative of the presence of the complete IL-27 dimer and may in fact represent production of these subunits independently or some combination of complete IL-27 and free subunits.

Interestingly, the existence of a soluble form of IL-27Rα has been described that is able to antagonize IL-27 and is thought to originate by cleavage of the membrane-bound receptor (Dietrich et al. 2014). Dietrich et al. reported that varying levels of soluble IL-27Rα were produced by human $CD4^+$ and $CD8^+$ T cells, B cells, monocyte-derived dendritic cells, and monocytes (Dietrich et al. 2014). While only speculation, it is interesting to question whether genetic deletion of IL-27Rα in only specific cells, such as the transferred T cells or recipient cells in the studies detailed above, could also eliminate or reduce this negative regulator of IL-27, resulting in dysregulated IL-27 signaling in remaining cells with functional IL-27Rα. The discovery of this endogenous,

soluble antagonist for IL-27 is intriguing, and further research into its function and regulation could impact concepts of IL-27 biology and potential therapeutic manipulation.

Lessons learned from targeting IL-17 to treat IBD

IL-23 promotes the development of Th17 cells (Aggarwal et al. 2003; Harrington et al. 2005), and a genome wide association study demonstrated a significant association between variants in the *IL-23R* gene and both Crohn's disease and ulcerative colitis (Duerr et al. 2006). However, while multiple studies have associated Th17 cells with the pathogenesis of IBD, the literature remains conflicted over whether their signature cytokines, IL-17A and IL-17F, drive intestinal inflammation or are protective (or both) (Fuss 2011; McLean et al. 2013). In contrast to the efficacy of anti-tumor necrosis factor-α antibody treatment in a subset of IBD patients (Danese 2012), antibody neutralization of IL-17A was surprisingly inefficacious, and in some cases, deleterious in patients with moderate to severe Crohn's disease (Hueber et al. 2012). Similarly, a clinical trial investigating antibody blockade of IL-17 receptor A in moderate to severe Crohn's disease was terminated due to a lack of efficacy and worsening of disease in some patients (Targan et al. 2012). However, two other therapies known to inhibit Th17 responses have shown promise as treatments for Crohn's disease and ulcerative colitis (Herrlinger et al. 2013; Sandborn et al. 2012). Vidofludimus, an inhibitor of the enzyme dihydroorotate dehydrogenase known to also inhibit the production of both IL-17A and IL-17F, induced steroid free remission in at least 50% of both Crohn's disease and ulcerative colitis patients in a clinical trial. Furthermore, of the remaining patients in the trial, partial remission was achieved in an additional 28.6% and 41.7% of

patients with Crohn's disease or ulcerative colitis, respectively (Herrlinger et al. 2013). Two trials have investigated the use of tofacitinib in IBD, demonstrating that it may be an effective therapy for ulcerative colitis, but not Crohn's disease (Sandborn et al. 2012; Sandborn et al. 2014). Tofacitinib inhibits Janus kinases 1, 2 and 3, and in doing so also blocks the differentiation of Th17 cells. Patients with moderately to severely active ulcerative colitis showed dose dependent clinical responses and clinical remission with tofacitinib therapy. Among patients receiving the highest dose, 78% showed a clinical response and 41% achieved clinical remission after 8 weeks of treatment (Sandborn et al. 2012). A shorter 4 week study found no significant reductions in Crohn's disease activity index in patients with moderate to severe Crohn's disease given tofacitinib (Sandborn et al. 2014); however, whether this lack of efficacy is due to differences between the two diseases or the shorter time of treatment (or both) is yet to be determined. Additionally, patients receiving placebo treatment in this study had a higher rate of response and remission than anticipated, leaving the authors to question whether this could have contributed to the nonsignificant difference in treatment results (Sandborn et al. 2014).

Interestingly, a recent study reported that antibody neutralization of both IL-17A and IL-17F, but neither cytokine alone, reduced colon histopathology scores in the murine T cell transfer model of enterocolitis (Wedebye et al. 2013). This could explain why vidofludimus and tofacitinib were more successful in clinical trials for IBD than antibody blockade of IL-17A alone, but offers little insight as to why anti-IL-17 receptor A treatment, which should inhibit signaling of both IL-17A and IL-17F (Russell et al. 2010), was inefficacious. This is particularly relevant to the discussion herein, as IL-27 is able

to block the expression of both IL-17A and IL-17F through inhibition of the transcription factor RORγt (Diveu et al. 2009). This potential therapeutic effect has been demonstrated experimentally in murine enterocolitis, in which decreased expression of RORγt, IL-17A, and IL-17F was reported in the colons of mice following mucosal administration of IL-27 (Hanson et al. 2014). IL-27 also inhibits the development of Th17 cells by inducing expression of programmed death ligand 1 on naïve T cells, which when cultured with naïve $CD4^+$ T cells, prevents their differentiation into Th17 cells (Hirahara et al. 2012). However, it is unclear what effect, if any, IL-27 may have on the secretion of IL-17 by immune cells other than T cells in the inflamed intestine, such as neutrophils and mast cells, which have been shown to be important sources of IL-17 in arthritis and psoriasis (Lin et al. 2011; Katayama et al. 2013). In contrast to antibody neutralization of a cytokine or its receptor, it is interesting to question whether the administration of another cytokine, potentially more subject to endogenous regulation, could create a more physiologically relevant anti-inflammatory microenvironment that might be able to maintain balance in the pro- and anti-inflammatory effects of the immune mediators it regulates. For example, the elimination of cytokine signaling by antibody neutralization or genetic deletion could block both its beneficial and harmful effects, while modulating a cytokine with one of its physiologic regulators could potentially preserve its beneficial functions.

Potential roles for IL-27 as a therapy for both Crohn's disease and ulcerative colitis

Although grouped together as IBD, Crohn's disease and ulcerative colitis are distinct conditions that vary both clinically and immunopathologically (Bouma and

Strober 2003). The lesions of Crohn's disease may be located anywhere throughout the gastrointestinal tract and are characterized by transmural infiltrates of macrophages and lymphocytes that in many patients multifocally organize to form granulomas. In contrast, ulcerative colitis is limited to the colon and features histopathologic changes of the mucosa only, including infiltrates of granulocytes and lymphocytes. Mucosal ulceration may be present in both conditions. Based on characterizations of cytokine expression and signaling, Crohn's disease is considered to be driven by Th1 responses, while ulcerative colitis is a Th2-mediated disease (Bouma and Strober 2003).

Despite differences in their immunopathology, there are abundant mechanisms by which IL-27 administration could be an effective therapy for both Crohn's disease and ulcerative colitis. Perhaps the most obvious mechanism by which IL-27 could reduce inflammation in IBD is through its stimulation of the immunoregulatory cytokine IL-10, which can be induced by IL-27 signaling in $CD8^+$ and regulatory, Th1, Th2, and Th17 cells (Awasthi et al. 2007; Jeon et al. 2012; Mishima et al. 2015; Murugaiyan et al. 2009; Pot et al. 2009; Stumhofer et al. 2007; Wang et al. 2011; Yoshida and Hunter 2015; Zhu et al. 2015). IL-10 boasts an incredible number of anti-inflammatory functions, including blocking Th1 and Th2 responses; inhibition of inflammatory cytokine and chemokine production by monocytes and neutrophils; limiting the recruitment of dendritic cells, T cells, neutrophils, and monocytes; inducing anergy in activated T cells; stimulating the production of interleukin-1 receptor antagonist and soluble tumor necrosis factor receptor; and reducing monocyte activation of T cells (Moore et al. 2001; Saraiva and O'Garra 2010). Complementing these functions of IL-10, IL-27 itself also limits tissue infiltration by neutrophils and monocytes, suppresses chemokine

production, and inhibits the generation of reactive oxygen intermediates by both macrophages/monocytes and granulocytes (Liu et al. 2014; Wirtz et al. 2006). As demonstrated in a mouse model of experimental autoimmune encephalomyelitis, IL-27's ability to induce CD39 on dendritic cells could contribute to the suppression of pathogenic T cell responses in IBD (Mascanfroni et al. 2013). As ulcerative colitis has been shown to be driven by Th2 immunopathology, IL-27's capacity to block Th2 cell differentiation and cytokine expression makes it well suited to treat this condition (Bouma and Strober 2003; Yoshimoto et al. 2007). Additionally, while the treatment of Th1-driven Crohn's disease with a cytokine known to promote Th1 responses sounds counterintuitive (Bouma and Strober 2003; Yoshida and Hunter 2015), IL-27 is also critical for the development of T-bet^{+} CXCR3^{+} regulatory T cells in the gut-associated lymphoid tissue that are specialized for regulating Th1 cells (Hall et al. 2012). IL-10 induced by IL-27 treatment could further contribute to the regulation of exuberant Th1 immunity in Crohn's disease (Awasthi et al. 2007; Jeon et al. 2012; Mishima et al. 2015; Murugaiyan et al. 2009; Pot et al. 2009; Stumhofer et al. 2007; Wang et al. 2011; Yoshida and Hunter 2015; Zhu et al. 2015).

How IL-27's ability to curb Th17 responses may influence its efficacy as a treatment for IBD is uncertain. However, in contrast to the failed trials investigating direct targeting of IL-17 signaling alone as a treatment for Crohn's disease, inhibition of Th17 responses is only one of many anti-inflammatory functions IL-27 exerts on adaptive and innate immunity. In this way IL-27 treatment would be more analogous to vidofludimus and tofacitinib therapy, which were effective in treating ulcerative colitis and/or Crohn's disease (Herrlinger et al. 2013; Sandborn et al. 2012), and, like IL-27,

exert diverse anti-inflammatory functions in addition to the blockade of IL-17A and IL-17F.

IL-27 and IL-27R expression in IBD

Both subunits of the IL-27 receptor, IL-27Rα and gp130, are expressed at low levels in normal intestinal epithelial cells, but are upregulated in inflammation in both epithelial cells and infiltrating leukocytes (Diegelmann et al. 2012). Increased expression of IL-27 in inflamed segments of the intestinal mucosa has been demonstrated in both Crohn's disease and ulcerative colitis (Leon et al. 2009; Diegelmann et al. 2012). Patients with active Crohn's disease have also been shown to have both significantly increased serum IL-27 and soluble IL-27Rα relative to healthy controls; however, despite an overall positive correlation between these two values, the ratio of cytokine to soluble receptor varied widely among patients (Dietrich et al. 2014).

The question remains whether elevations of IL-27 in the inflamed intestinal mucosa and serum of IBD patients are contributing to inflammation in these patients or represent an anti-inflammatory response. However, based on the evidence presented herein, it seems most likely that these elevations in IL-27 represent an (inadequate) anti-inflammatory response in diseased segments of intestine. While IL-27 signaling in the intestine presumably acts on inflammatory cells, inflamed intestinal epithelial cells upregulate the IL-27 receptor and are therefore also capable of responding to IL-27.

Concluding remarks

While complex, the majority of the literature investigating the role of IL-27 in mouse models of IBD complements knowledge gained from human patients and supports an anti-inflammatory role for IL-27 in IBD. However, the studies suggesting a

more pro-inflammatory role for IL-27 should not be ignored, but should rather inform future investigations into the complex physiology of this cytokine. While it's unlikely that a single "silver bullet" treatment for all cases of IBD will ever be found, IL-27 is a promising potential therapy that warrants further investigation.

Project goals

In this project, we used in vitro culture systems to characterize the effect of IL-27 on murine macrophages and how these potential effects may impact the colon epithelium. Our ultimate goal is to further illuminate the mechanism of how exogenously administered IL-27 may work in the intestine and if it may be a suitable therapy for IBD or other intestinal diseases. However, we chose a reductionist, in vitro approach to focus our investigation solely on macrophages and colon epithelial cells. We studied the impact of IL-27 treatment on gene and protein expression in both non-activated resident and induced, classically activated, proinflammatory (M1) peritoneal macrophages. We discovered that IL-27 both induces the expression of and mimics the action of interferons in macrophages, and we used gene specific knockout mice to further elucidate the mechanisms of IL-27 activation of macrophages. Moving forward from what we learned in our inquiry of gene and protein expression due to IL-27, we evaluated the ability of IL-27 to facilitate viral or bacterial pathogen clearance in macrophages. Finally, we assessed transcriptional changes in colon epithelial cells subsequent to co-culture with IL-27-stimulated macrophages to more fully understand how IL-27 treatment of macrophages could alter the function of murine colon epithelial cells, which do not directly respond to IL-27.

Hypothesis

IL-27 activates macrophages, which mediate the effects of IL-27 on the colon epithelium.

CHAPTER 2: Interleukin-27 mimics interferon activation of macrophages to modulate viral, but not bacterial, infection

Introduction

IL-27 is a heterodimeric type I cytokine in the IL-12 cytokine family with reported roles in cancer, infectious disease, and autoimmunity (Kastelein et al. 2007; Pflanz et al. 2002; Yoshida et al. 2015). Multiple studies have demonstrated that the administration of IL-27 effectively ameliorates multiple experimental models of colitis in mice (Hanson et al. 2014; McLean et al. 2017; Sasaoka et al. 2011); however, the role of IL-27 in inflammatory disease is not so clear-cut. A collection of studies using genetic deletion of the IL-27 receptor demonstrated a potential pro-inflammatory, potentiating function of IL-27 in models of intestinal inflammation (Cox et al. 2010; Honda et al. 2005; Villarino et al. 2008; Visperas et al. 2013). Additionally, conflicting effects of IL-27 have been reported in murine models of viral infection. Genetic deletion of the IL-27 receptor resulted in greater mortality and lung pathology due to influenza infection relative to wildtype mice, while the administration of IL-27 early in influenza infection worsened disease and reduced viral clearance (Liu et al. 2014). In contrast, Harker et al. demonstrated that IL-27 signaling was essential for the control of both the early innate and later adaptive immune responses to lymphocytic choriomeningitis viral infection (Harker et al. 2018). As a result, further research is necessary to fully characterize the situational mechanisms of action of IL-27 before this cytokine can be considered as a potential therapy for human disease.

While a protective role for IL-27 in multiple models of murine colitis has been published, the mechanism of action and target cell of IL-27 differs between models

(Hanson et al. 2014; McLean et al. 2017). To identify which cells in the colon respond to IL-27 in vivo, McLean et al. evaluated the phosphorylation of STAT1, a signaling molecule in the IL-27 pathway, in mice treated with mucosally-delivered IL-27. In colitic mice treated with IL-27, phosphorylated STAT1 was observed in cells morphologically consistent with colon enterocytes, macrophages, and neutrophils in microscopic sections; however, it was not clear if this phosphorylation of STAT1 was a direct result of IL-27 signaling or another mediator induced by IL-27, as STAT1 phosphorylation is not unique to the IL-27 signaling pathway. This study further demonstrated that infiltrating neutrophils, but not macrophages, were reduced in number in the colons of colitic mice treated with IL-27 (McLean et al. 2017). As such, these macrophages could play a role in mediating the effects of IL-27 administration in the gut.

Myeloid cells such as macrophages are the predominant source of IL-27 in vivo (Yoshida and Hunter 2015); however, the majority of the literature investigating this cytokine has focused on its ability to modulate T cell biology rather than any potential effects on macrophages. The objective of this study was to characterize the effect of IL-27 on murine macrophages and determine if macrophages may mediate any potential effects of IL-27 on the colon epithelium. Here we found that IL-27 activated murine macrophages in a manner similar to interferons; however, this activation conveyed only antiviral, not antibacterial, resistance. Furthermore, IL-27-activated macrophages induced the expression of genes critical for immune and pathogen responses in the colon epithelium.

Results

IL-27 induced the transcription of genes associated with interferon signaling and pathogen responses in macrophages

To characterize the response of macrophages to IL-27, peritoneal macrophages were harvested from experimentally unmanipulated mice (non-activated macrophages) or mice injected with thioglycolate 5 days prior to macrophage harvest. Thioglycolate-induced macrophages were then driven to a M1 phenotype in culture, defined as classically activated, proinflammatory macrophages (Martinez and Gordon, 2014; Mosser and Zhang 2008). In vitro M1-driven macrophages expressed increased amounts of TNF-α, IL-1β, and IL-6 relative to non-activated macrophages, consistent with the published phenotype of these cells (Appendix A) (Martinez and Gordon, 2014). Harvested cells (both non-activated and thioglycolate-induced) were ≥90% $F4/80^{+}CD11c^{-}$ macrophages (Figure 7A). Both M1 and non-activated macrophages expressed the IL-27 receptor alpha (IL-27Rα) gene, and this gene expression was unchanged by IL-27 treatment (Figure 7B). M1 and non-activated macrophages phosphorylated STAT1 in response to IL-27 (Figure 7C). mRNA sequencing of M1 macrophages cultured with 100 ng/ml IL-27 for 24 hours identified 40 genes that were upregulated owing to IL-27 treatment (Figure 7D).

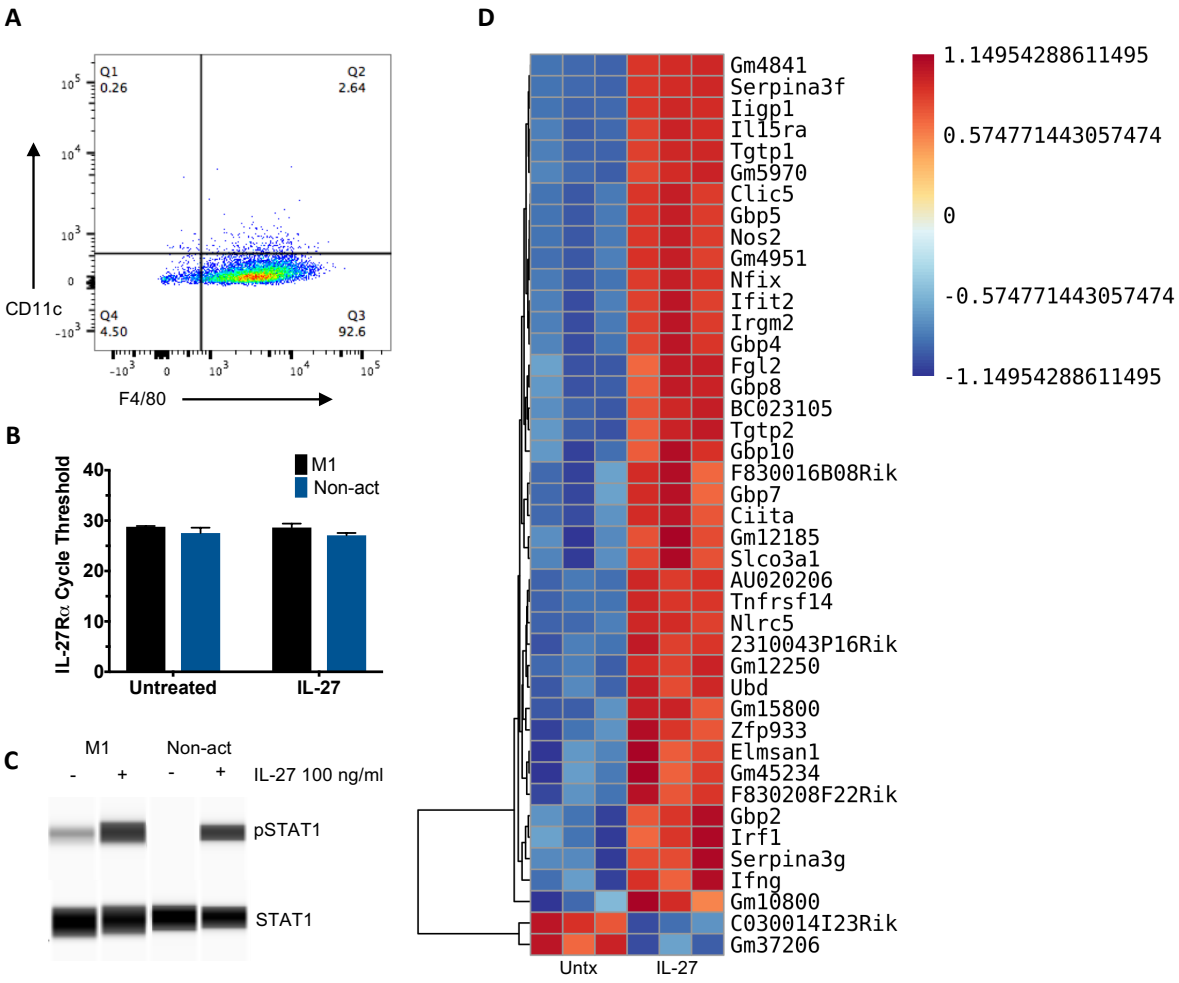

Figure 7. IL-27 induced the transcription of genes associated with interferon signaling and pathogen responses in macrophages. (A) Representative flow cytometry plot showing the phenotype of both non-activated and thioglycolate-induced peritoneal macrophages used in this study. (B) Expression of the IL-27Rα gene in both untreated and IL-27-stimulated non-activated and thioglycolate-induced, M1-activated peritoneal macrophages. (C) Capillary western blot for phosphorylated STAT1 in both non-activated and M1 macrophages stimulated with IL-27. (D) Differentially expressed genes detected by RNA sequencing in thioglycolate-induced M1 macrophages cultured with IL-27 for 24 hours. All data are representative of a minimum of at least three independent experiments. P<0.05. Graph depicts mean + SD.

Of these 40 genes, 17 genes related to interferon signaling or pathogen responses were chosen for validation by real time RT-qPCR and further investigation (Table 1) (Ingram et al. 2017; Rusinova et al. 2013).

Gene			
IFNG	*	#	Interferon gamma
CLIC5	*		Chloride intracellular channel protein 5
GBP2	*	#	Guanylate-binding protein 2
GBP5	*	#	Guanylate-binding protein 5
GBP7	*	#	Guanylate-binding protein 7
GBP8	*	#	Guanylate-binding protein 8
GBP10	*	#	Guanylate-binding protein 10
CIITA	*	#	MHC class II transactivator
NOS2	*	#	Nitric oxide synthase, inducible
NLRC5	*	#	NLR family CARD domain containing 5
GM12250/IRGB10	*	#	Interferon-gamma-inducible p47 GTPase
IRF1	*	#	Interferon regulatory factor 1
IL15RA	*		Interleukin-15 receptor subunit alpha
IFIT2	*		Interferon-induced protein with tetratricopeptide repeats 2
FGL2	*		Fibroleukin
SLCO3A1	*		Solute carrier organic anion transporter family member 3A1
TGTP1/2	*	#	T-cell-specific guanine nucleotide triphosphate-binding protein 1/2

Table 1. RNA sequencing revealed significant increases in 17 genes of interest due to IL-27 stimulation for 24 hours in thioglycolate-induced M1 macrophages. Many of these genes are associated with interferon signaling (*) or pathogen responses (#).

The upregulation of all 17 genes of interest due to treatment with IL-27 was validated by real time RT-qPCR in non-activated macrophages (Figure 8A and B). In M1 macrophages, IL-27 increased the expression of all investigated genes except *GBP8*,

GBP10, SLCO3A1, and NLRC5, for which the IL-27-induced increases in expression did not reach statistical significance (Figure 8C and D). As a number of these genes have been shown to be upregulated earlier than 24 hours after treatment with various stimuli, expression of these 17 genes was also evaluated after 1, 4, and 6 hours of IL-27 treatment (Figure 8A-D). While multiple genes were upregulated after only 1 hour of IL-27 stimulation, *IRGB10* and *IRF1* were highly upregulated in non-activated macrophages by IL-27 at this timepoint, nearly reaching their peak expression for the timepoints investigated. Of note, all of the studied genes peaked in expression after 24 hours of IL-27 stimulation in M1 macrophages, while many of these genes were more highly upregulated at 4 and/or 6 hours of IL-27 stimulation in non-activated macrophages.

Because a number of the IL-27-induced genes we identified by RNA sequencing of macrophages have been associated with IFN-γ-driven killing of intracellular bacteria, we investigated the expression of additional genes in this pathway, as well as the cytokines *IL6, TNF, IFNA2*, and *IFNB1*, in both non-activated and M1 macrophages stimulated with IL-27 for 1, 4, 6, or 24 hours (Table 2).

Gene	
IL6	Interleukin-6
TNF	Tumor necrosis factor
IFNB1	Interferon beta 1
IFNA	Interferon alpha 2
GBP3	Guanylate-binding protein 3 #
NLRC4	NLR family CARD domain containing 4 #
NLRP3	NLR family, pyrin domain containing 3 #
IRGM1	Immunity-related GTPase family M member 1 #

Table 2. Additional genes representing proinflammatory cytokines or (#) factors associated with IFN-γ-driven killing of intracellular bacteria investigated.

IL-27 increased the expression of 7/8 of the additional genes investigated in non-activated macrophages, but only 3/8 of these genes were further elevated by IL-27 in M1 macrophages (Figure 8E and F).

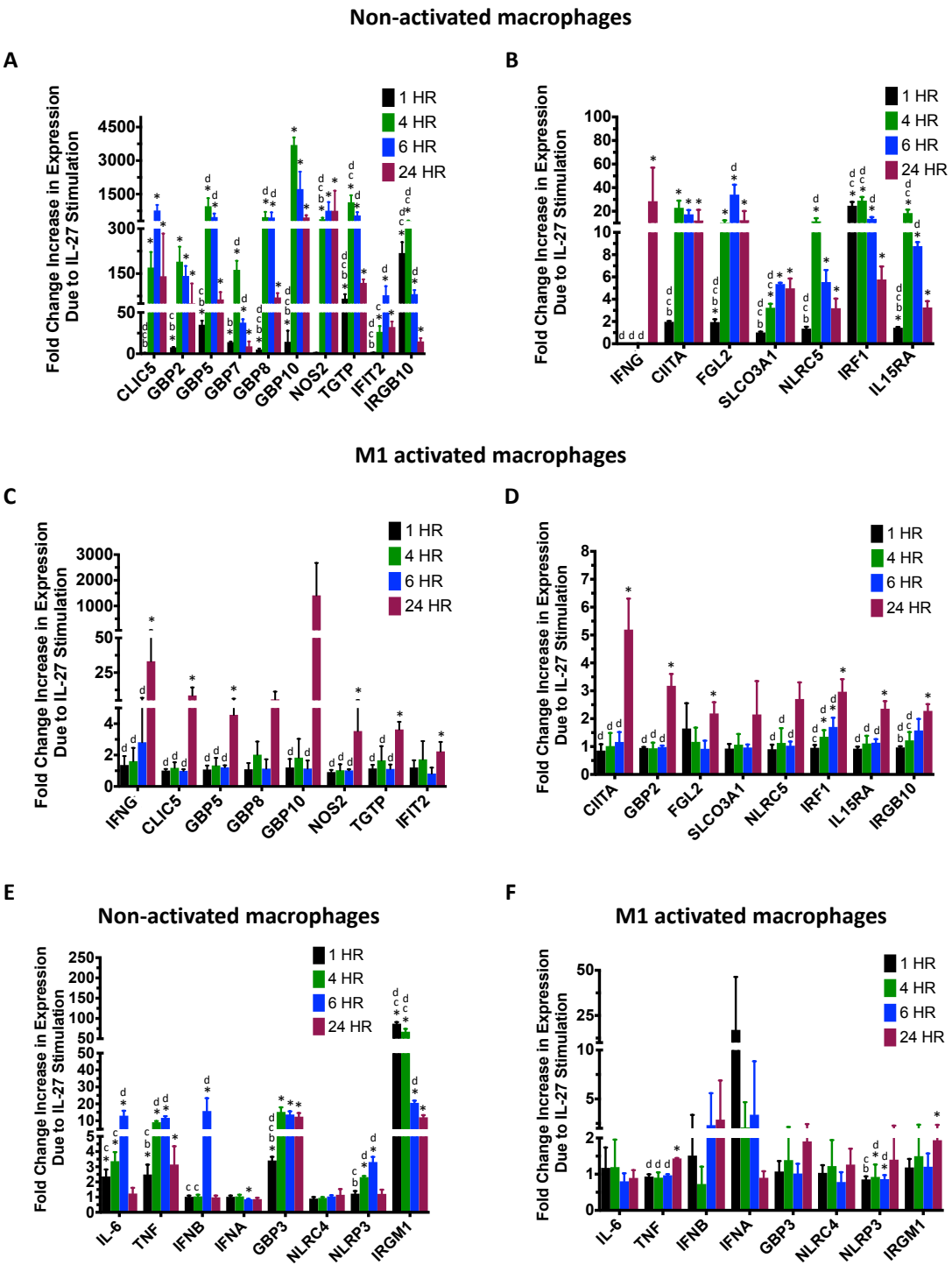

Figure 8. (A-D) Real time RT-qPCR validation of genes identified as upregulated by IL-27 by RNA sequencing of M1 macrophages in both non-activated (A and B) and M1 (C and D) macrophages. (E-F) Gene expression induced by IL-27 as determined by real time RT-qPCR for the additional genes listed in Table 2 in both non-activated and M1 macrophages. *p<0.05 by T-test of log-transformed delta cycle threshold values between untreated and IL-27-treated macrophages for each timepoint. Letters indicate

p<0.05 between gene expression timepoints by one-way ANOVA of log-transformed delta delta cycle threshold values. a=different versus expression at 1 hour. b=different versus expression at 4 hours. c=different versus expression at 6 hours. d=different versus expression at 24 hours. Graphs depict mean + SD. Data representative of three independent experiments.

IL-27-induced gene expression was dependent on STAT1, independent of IL-27-induced IFN-γ, and selectively dependent on IL-27-induced type I interferons

Due to the overlap of IL-27-induced genes in the macrophages of this study with IFN-induced gene signatures and the induction of *IFNG* by IL-27 in this study, we sought to determine if the observed changes in gene expression were due to IL-27 or IL-27-induced IFNs. To this end, peritoneal macrophages were harvested from *IFNG*$^{-/-}$, type I IFNR$^{-/-}$, or *STAT1*$^{-/-}$ mice. Macrophages from *IFNG*$^{-/-}$, type I IFNR$^{-/-}$, and *STAT1*$^{-/-}$ mice express the IL-27Rα subunit similarly to wildtype mice (Figure 9).

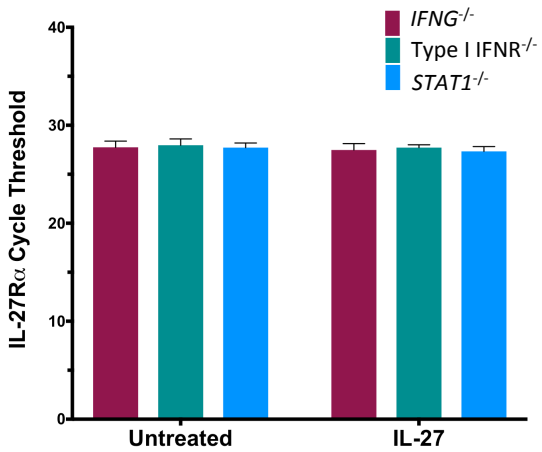

Figure 9. Expression of the IL-27Rα subunit in macrophages from *IFNG*$^{-/-}$, type I IFNR$^{-/-}$, and *STAT1*$^{-/-}$ mice in both untreated and IL-27-stimulated, non-activated macrophages. Graph depicts mean + SD. Data representative of three independent experiments.

Real time RT-qPCR analysis of IL-27-stimulated macrophages with genetic deletion of *IFNG* confirmed that all genes of interest were not induced by IL-27-induced IFN-γ (Figure 10A). The majority of genes of interest were similarly not induced by IL-

27-induced type I IFNs, as evidenced by similar expression of the majority of these genes between IL-27-stimulated wildtype macrophages and IL-27-stimulated macrophages lacking the type I IFNR. However, the expression of *IFNG* was nearly eliminated in IL-27-stimulated type I IFNR$^{-/-}$ macrophages relative to wildtype macrophages (Figure 10B). As such, it appears that the IL-27-induced expression of *IFNG* in macrophages depends on IL-27-induced type I IFN expression. In contrast, the genetic deletion of *STAT1* in macrophages reduced or nearly ablated the IL-27-induced increases seen in wildtype mice in 11/15 of the investigated genes, indicating that the IL-27-induced expression of the majority of the genes of interest is dependent on STAT1 signaling (Figure 10C).

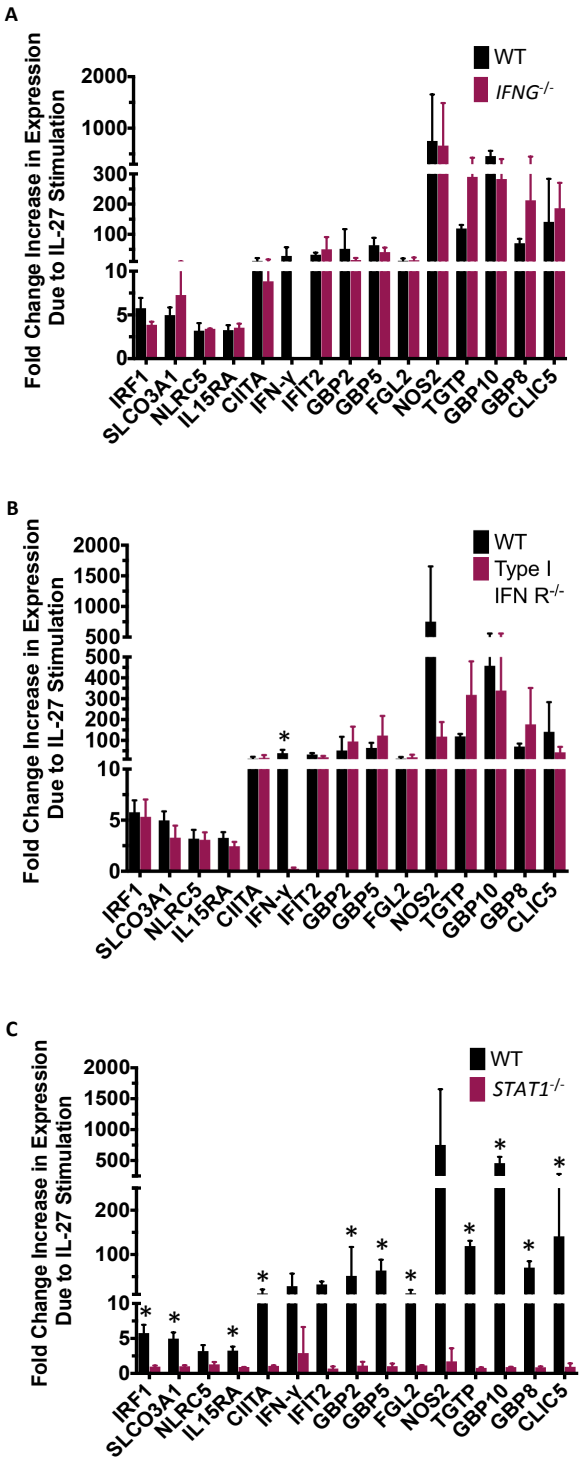

Figure 10. IL-27-induced gene expression was dependent on STAT1, independent of IL-27-induced IFN-γ, and selectively dependent on IL-27-induced type I interferons. IL-27-induced gene expression detected by real time RT-qPCR in wildtype macrophages relative to those with genetic deletion of (A) *IFNG*, (B) type I IFNR, or (C) *STAT1*. Graphs depict mean + SD. Data representative of 3 independent experiments.

*p<0.05 by T-test of delta cycle thresholds of the gene expression in wildtype and the respective knockout strain.

IL-27 induced protein expression of TNF-α and IFN-γ in macrophages

Due to the induction of *IFNG*, *TNF*, and *IL6* gene expression by IL-27 in macrophages, we investigated the expression of these proteins in culture supernatants of M1 and non-activated macrophages. IL-27 increased the production of IFN-γ by both M1 and non-activated macrophages after 24 hours of stimulation (Figure 11D), but was unable to increase IFN-γ protein expression after only 6 hours of IL-27 treatment (Figure 5A). Furthermore, the IL-27-induced increases in IFN-γ protein were no longer apparent after 48 hours of IL-27 treatment (Figure 11G). IL-27 increased macrophage production of TNF-α after 6 hours of stimulation (Figure 11C); however, this effect was lost at 24 and 48 hours of culture with IL-27 (Figure 11F and I). There was a trend for increased IL-6 after 6 and 24 hours of IL-27 stimulation of non-activated macrophages (Figure 11B and E), but these changes did not reach statistical significance. Notably IL-27 was not able to further increase expression of these proteins at the majority of timepoints in M1 macrophages. These data suggest that while IL-27 activates macrophages, this action is not universally additive to the M1 activation of macrophages by IFN-γ and LPS.

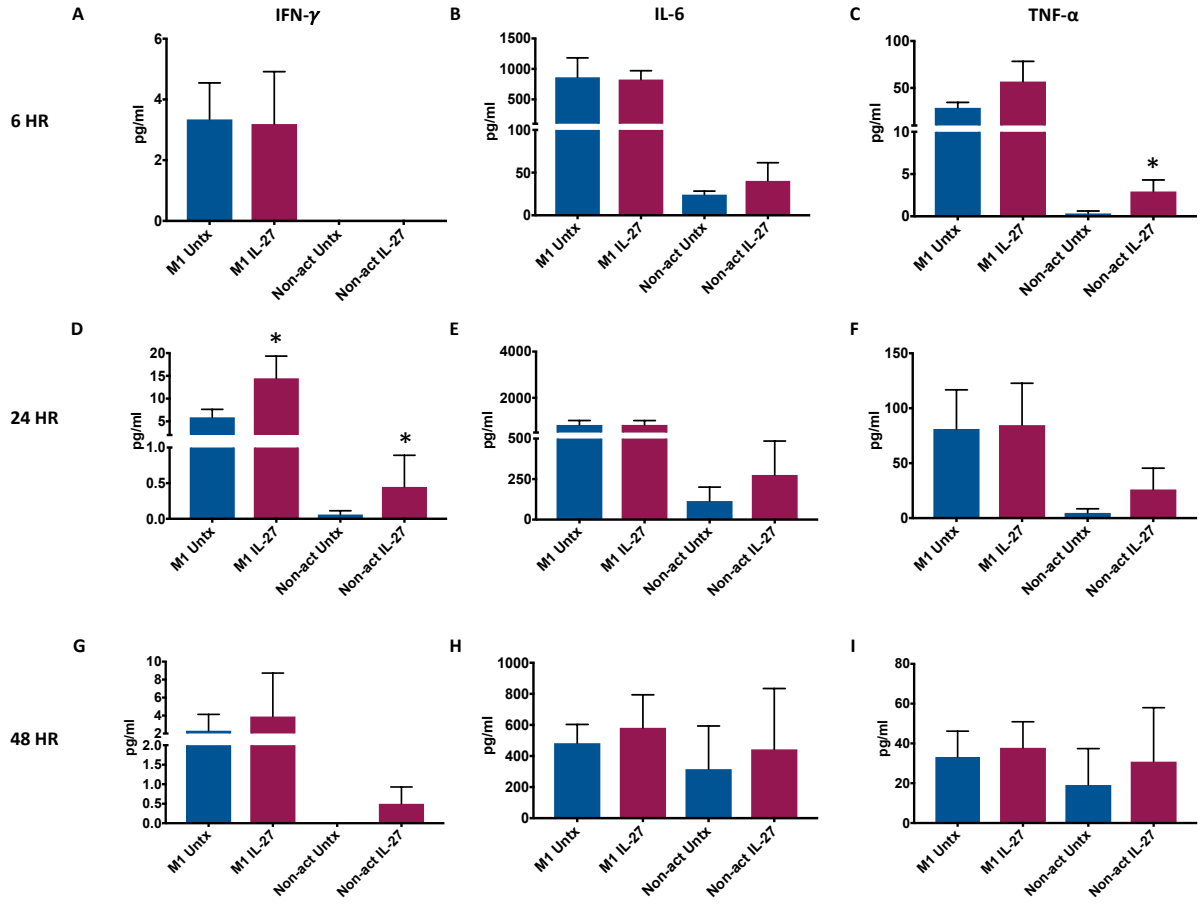

Figure 11. IL-27 induced protein expression of TNF-α and IFN-γ in macrophages. Protein production of IFN-γ, TNF-α, and IL-6 detected by Meso Scale immunoassay by M1 or non-activated macrophages after 6 (A-C), 24 (D-F), or 48 hours (G-I) of IL-27 stimulation. Graphs depict mean + SD. Data representative of 3 independent experiments. *p<0.05 by T-test of log transformed protein concentrations in untreated (untx) versus IL-27-stimulated M1 or non-activated macrophages.

IL-27 induction of surface MHC II expression in macrophages was independent of, but not as strong as, MHC II induction by IFN-γ

RNA sequencing identified the MHC II transactivator gene *CIITA* as being upregulated in macrophages by IL-27 (Figure 7D). However, there is conflicting evidence in the literature regarding IL-27 and MHC II expression, as it has been shown to both promote and inhibit MHC II expression in various types of dendritic cells (Jung et

al. 2015; Mascanfroni et al. 2013). Therefore, we sought to determine if IL-27 could induce greater surface expression of MHC II in macrophages and if this expression was attributable directly to IL-27 or to the IL-27-induced production of interferons. To this end, we stimulated wildtype non-activated, wildtype M1, *IFNG*$^{-/-}$, type I IFNR$^{-/-}$, and *STAT1*$^{-/-}$ macrophages with 100 ng/ml IL-27 for 48 hours and evaluated surface MHC II expression by flow cytometry (Figure 12). IL-27 more than doubled the number of wildtype non-activated, *IFNG*$^{-/-}$, and type I IFNR$^{-/-}$ macrophages expressing MHC II (Figure 12A, B, C). However, this increase was less than that induced by IFN-γ, which consistently induced MHC II surface expression in nearly 100% of each of these macrophage phenotypes. Concordantly, IFN-γ increased surface MHC II expression in M1 macrophages, while IL-27 did not induce additional MHC II expression in these cells (Figure 12E). The induction of MHC II by both IL-27 and IFN-γ is dependent on STAT1 signaling, as the genetic ablation of this transcription factor in macrophages eliminated the induction of surface MHC II by both of these cytokines (Figure 12D).

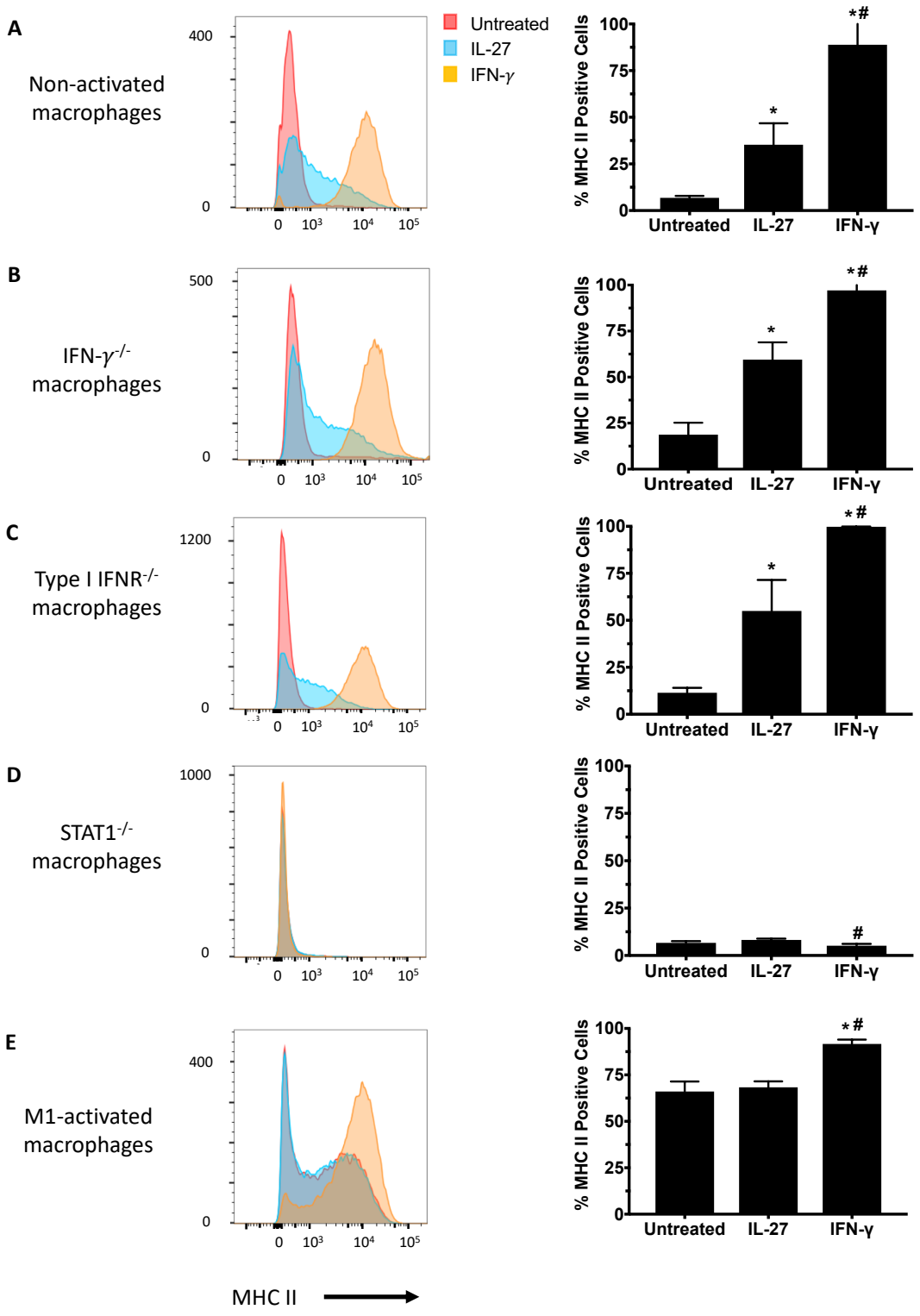

Figure 12. IL-27 induction of surface MHC II expression was independent of, but not as strong as, MHC II induction by IFN-γ. Surface MHC II expression determined by flow cytometry in wildtype non-activated (A), *IFNG*$^{-/-}$ (B), type I IFNR$^{-/-}$ (C), *STAT1*$^{-/-}$

(D), or wildtype M1 macrophages cultured with 100 ng/ml IL-27 or 10 ng/ml IFN-γ for 48 hrs. Graphs depict mean + SD. Data representative of three independent experiments. *p<0.05 by one-way ANOVA versus untreated macrophages. #p<0.05 by one-way ANOVA in IFN-γ-treated versus IL-27-treated macrophages.

IL-27 activated antiviral, but not antibacterial, resistance in macrophages

Due to the number of genes associated with responses to both viral and bacterial pathogens induced by IL-27 in the macrophages of this study, we investigated the potential for IL-27 to induce viral or bacterial resistance in macrophages. To investigate the ability of IL-27 to prevent viral infection of macrophages, non-activated peritoneal macrophages were infected for 6 hours with six multiplicities of infection (MOI) of GFP-expressing vaccinia virus following overnight treatment with IL-27. Consistent with our hypothesis, IL-27 reduced viral infection of macrophages at 0.5 and 1 MOI (Figure 13). However, IL-27 did not reduce viral infection at the higher ratios of infection of 5 and 10 MOI.

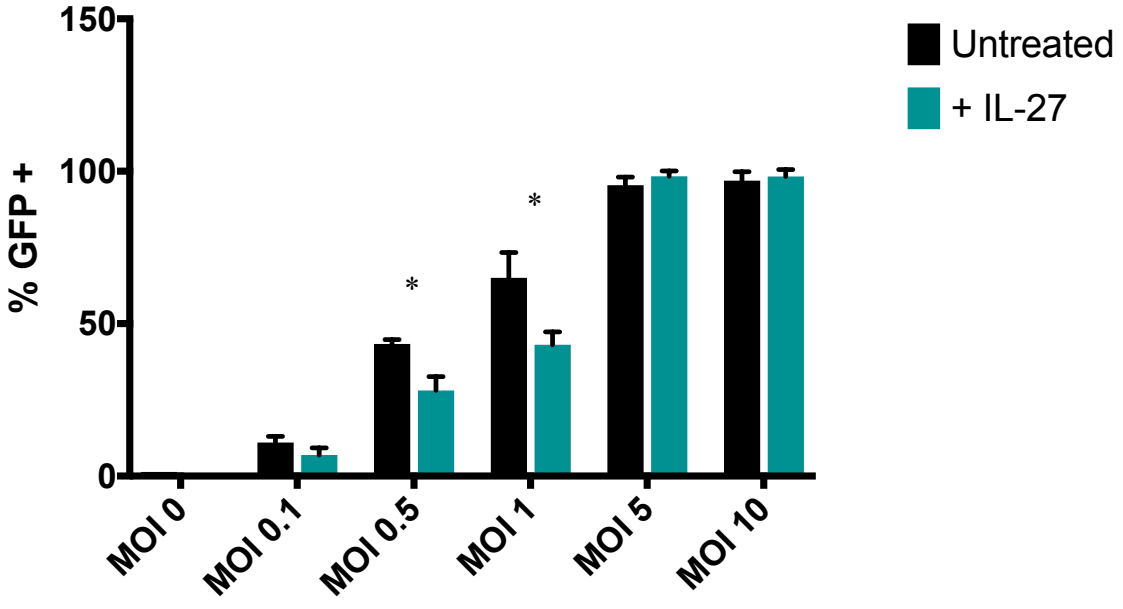

Figure 13. IL-27 reduced viral infection of macrophages. Infection of wildtype non-activated macrophages with GFP-expressing vaccinia virus at six multiplicities of

infection (MOI). IL-27 reduces viral infection of macrophages at 0.5 and 1 MOI. Graph depicts mean + SD of values from triplicate samples.

We then sought to determine if IL-27 could reduce the number of active viral particles and subsequent infection of susceptible cells. To investigate this, non-activated wildtype peritoneal macrophages were treated with IL-27 or IFN-γ for six hours and infected with mouse cytomegalovirus. Supernatants from these cultures were removed at 24, 48, and 72 hours post-infection and used to infect a susceptible line of fibroblasts. We observed a dose dependent IL-27-induced reduction in active viral particles (Figure 14). 100 ng/ml of IL-27 24 hours post-infection of macrophages reduced active viral particles to the extent that infection of fibroblasts with these supernatants was significantly reduced compared to fibroblast infection with untreated, infected macrophage supernatants (Figure 14). At 72 hours post-infection of macrophages, all tested concentrations of IL-27 reduced active viral particles such that infection of fibroblasts with infected, IL-27-treated macrophage supernatants was lower than infection of fibroblasts with infected but untreated macrophage supernatants (Figure 14).

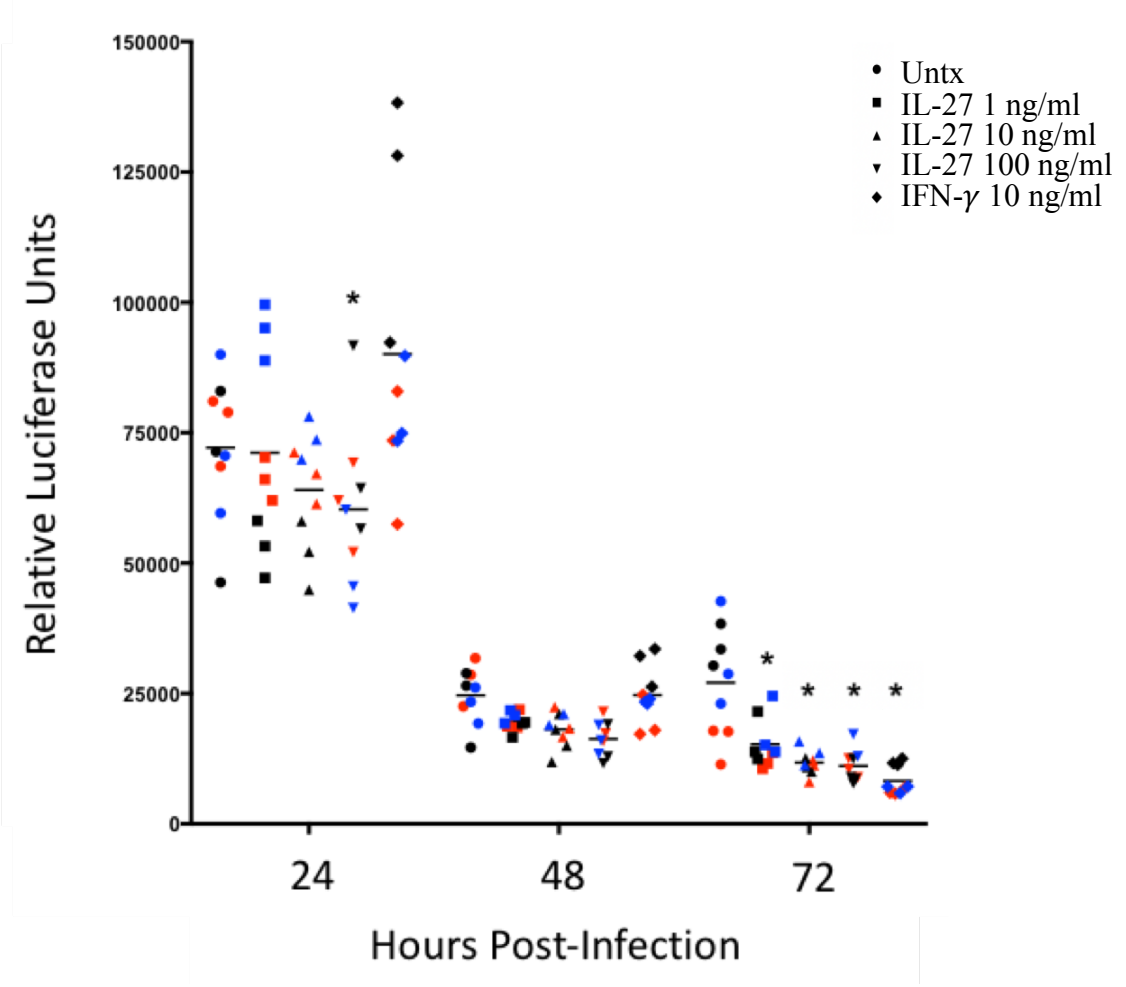

Figure 14. IL-27 reduced active viral particles and subsequent viral infection of susceptible cells. Viral infection detected by luciferase in fibroblasts infected with mouse cytomegalovirus via supernatants taken at 24, 48, or 72 hours post wildtype macrophage infection from mouse cytomegalovirus-infected macrophages +/- IL-27 or IFN-γ. Luciferase read at 18 hours post fibroblast infection with infected macrophage supernatants. Data representative of three independent experiments. *p<0.05 by T-test between treated and untreated controls. Each data point color represents triplicates from each independent experiment.

Because interferons are known to have antiviral properties and our data indicates that IL-27 induces both type I and type II IFN production by macrophages, we repeated this experiment in mice with genetic deletion of the type I IFN receptor. Based on our data presented herein, this would eliminate any effect of IL-27-induced type I interferon

and IL-27-induced IFN-γ, which is dependent on IL-27-induced type I interferon. Similar to the experiment with wildtype macrophages, 100 ng/ml of IL-27 treatment of type I IFNR$^{-/-}$ macrophages reduced active viral particles to the extent that subsequent infection of fibroblasts with supernatants from these macrophages 24 hours post-infection was reduced. A reduction in fibroblast infection was also noted with 100 ng/ml IL-27-treated, infected macrophage supernatants taken 48 hours post-infection. However, in contrast to the data from wildtype macrophages and IFN-γ-treated type I IFNR$^{-/-}$ macrophages, fibroblast viral infections obtained from infected type I IFNR$^{-/-}$ macrophage supernatants taken 72 hours post macrophage infection rebounded to the highest infection levels seen in either experiment. As a result, it can be concluded that IL-27-induced production of type I and/or II IFN is necessary for the longer term (>48 hours) reduction in active viral particles by IL-27, while IL-27 itself reduces active virions within 48 hours of infection (Figure 15).

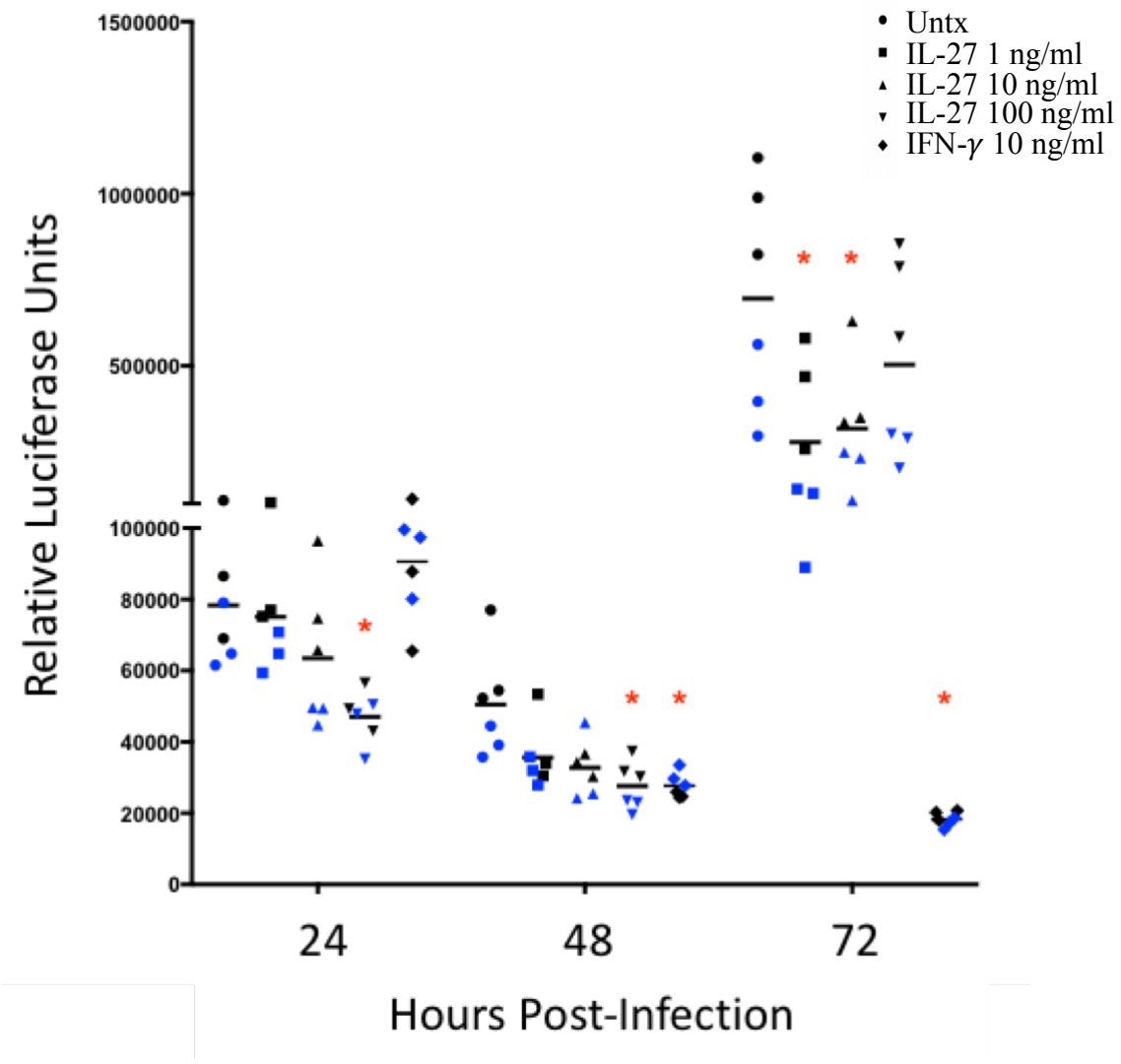

Figure 15. IL-27 reduced active viral particles acutely but required IL-27-induced interferon production for sustained viral inhibition. Viral infection detected by luciferase in fibroblasts infected with mouse cytomegalovirus via supernatants taken at 24, 48, or 72 hours post type I IFNR$^{-/-}$ macrophage infection from mouse cytomegalovirus-infected type I IFNR$^{-/-}$ macrophages +/- IL-27 or IFN-γ. Luciferase read at 18 hours post fibroblast infection with infected macrophage supernatants. Data representative of two independent experiments. *p<0.05 by T-test between treated and untreated controls. Each data point color represents triplicates from each independent experiment.

A number of the genes upregulated by IL-27 in the macrophages of this study are specifically implicated in the clearance of intracellular *Salmonella*, including the various *GBP*s, *IFNG, IRGM1, IRF1, NLRP3*, and *IRGB10* (Ingram et al. 2017). IL-27 also

strongly induced the expression of *NOS2* in the macrophages of this study, further suggesting it may have an antibacterial function (Alam et al. 2002). As such, we hypothesized that IL-27 may promote macrophage clearance of *Salmonella* infection. However, instead of promoting the clearance of *Salmonella*, the pretreatment of macrophages for 24 hours prior to infection or the addition of IL-27 at the time of infection actually inhibited *Salmonella* clearance (Figure 16A). Concordantly, despite the induction of *NOS2*, no evidence for nitric oxide production due to IL-27 treatment was detected (Figure 16B).

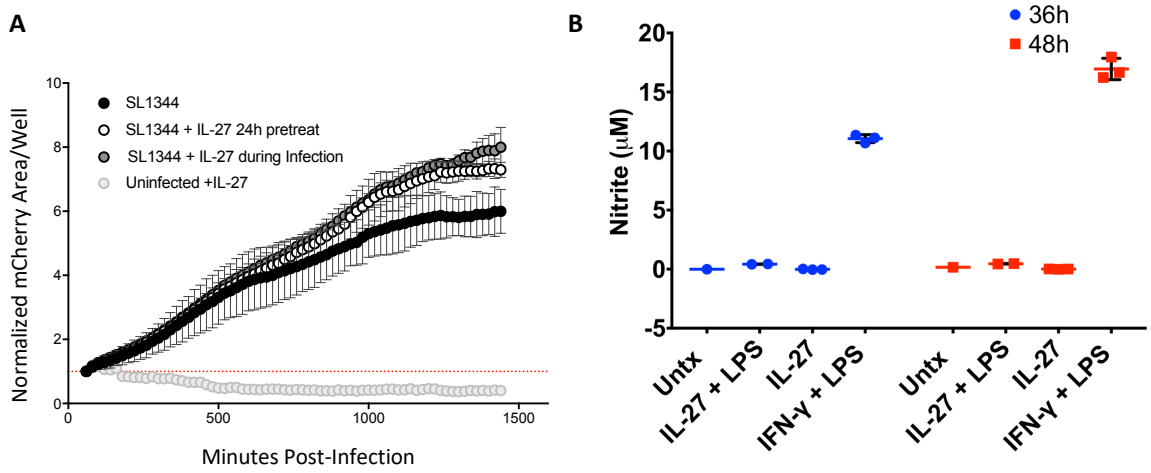

Figure 16. IL-27 inhibited macrophage clearance of *Salmonella*. (A) Macrophages infected with mCherry-expressing *Salmonella enterica* serovar Typhimurium strain SL1344 +/- treatment with IL-27 24 hours prior to or at the time of infection. (B) Detection of nitrite as an indicator of nitric oxide production in macrophages activated with IL-27 +/- LPS or the positive control of IFN-γ with LPS.

Co-culture of IL-27-stimulated macrophages induced gene expression crucial for pathogen responses in colon epithelial organoids

To identify how the IL-27 activation of macrophages may affect the colonic mucosal epithelium and influence its potential as a treatment for intestinal disease, we

co-cultured macrophages and 3-dimensional colon epithelial organoids (colonoids) in Transwell plates. Colonoids were chosen for these experiments because, unlike intestinal epithelial cell lines, they differentiate into all of the cellular components of the normal colon mucosal epithelium (Appendix B and C) in vitro. IL-27 does not directly induce phosphorylation of STAT1 in colonoids (Figure 17A) and has no effect on murine colon epithelial proliferation (Appendix D). Because this conflicts with data previously published in human colorectal cancer cell lines (Diegelmann et al. 2012), we repeated this experiment with the human colorectal cancer cell line HCT-116 to evaluate our culture systems and detection methods. In agreement with the previously published data, IL-27 induced phosphorylation of STAT1 in the HCT-116 cells (Appendix E). We next investigated if potential apical polarity of the IL-27 receptor in colon epithelial cells was preventing interaction between the colonoid epithelium and in vitro administered IL-27. To investigate this possibility, intact crypts that had not yet developed into spheroids were stimulated with IL-27. Similar to the differentiated colonoids, IL-27 did not induce phosphorylation of STAT1 in the freshly isolated crypts (Appendix F). However, culture of the colonoids with the supernatants of IL-27-stimulated macrophages induced phosphorylation of STAT1 (Figure 17B), suggesting that a soluble mediator(s) produced by macrophages in response to IL-27 can act on colon epithelial cells.

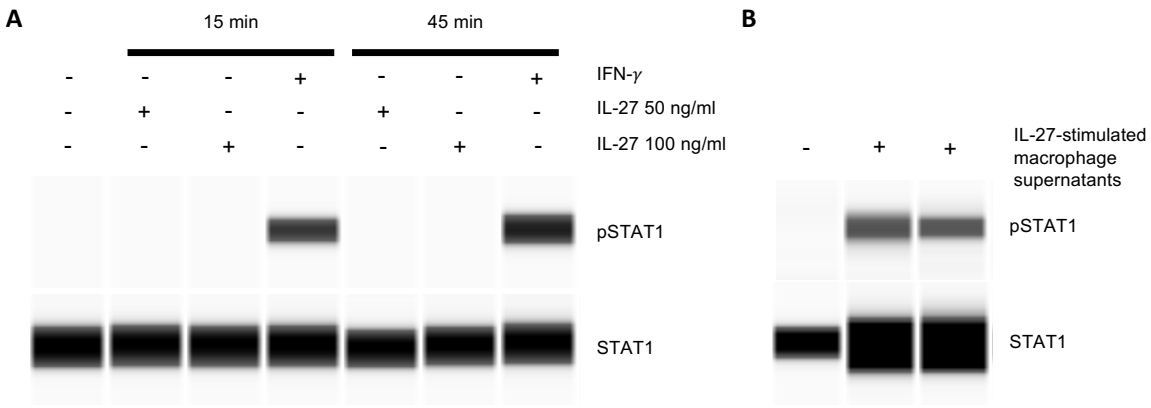

Figure 17. IL-27-stimulated macrophages, but not IL-27, induced signaling in the colon epithelium. Capillary western blot of STAT1 and phosphorylated STAT1 (pSTAT1) in colonoids stimulated with either IL-27 (A) or IL-27-stimulated macrophage supernatants (B). Data representative of at least three independent experiments.

To investigate the effects of these soluble mediators on the colon epithelium, colonoids were co-cultured in Transwell plates with IL-27-stimulated M1 macrophages such that the two cell types were not in contact but shared culture media. RNA sequencing was then performed on the colonoids to determine how IL-27-stimulated macrophages may affect colon epithelial gene expression. RNA sequencing of these colonoids revealed that co-culture with IL-27-stimulated M1 macrophages induced the upregulation of a number of pathways associated with immune and pathogen responses (Table 3).

	Pathway	pValue	FDR	Pathway Components
1	Immune response IFN-alpha/beta signaling via JAK/STAT	1.021E-11	1.367E-08	TAP1 (PSF1), OAS1, MIG, GBP1, RSAD2, IP10, GBP4, Oas1b, ISG15, IRF1, CCL2, I-TAC, ERAP140, ISG54, XAF1, IFI47, SOCS3, RIG-G, USP18
2	Immune response IFN-alpha/beta signaling via MAPKs	2.660E-09	6.229E-07	Ubiquitin, Axin2, TAP1 (PSF1), RSAD2, SMAD3, IP10, AKT(PKB), ISG15, Filamin B (TABP), Irgm2, p38 MAPK, ISG54, Oasl2, TCF7L2 (TCF4), FasR(CD95), RIG-G, Oas1g, TRIM6
3	Immune response HSP60 and HSP70/ TLR signaling pathway	3.846E-08	6.437E-06	Ubiquitin, IKK-gamma, I-kB, MEK1/2, IRAK1/2, NF-kB, iNOS, NF-kB1 (p105), ICAM1, MyD88, HSP70, p38 MAPK, TAB3, TNF-alpha
4	Immune response IL-1 signaling pathway	5.039E-08	7.497E-06	CCL5, p38alpha (MAPK14), I-kB, MEK1/2, EGR1, NF-kB, iNOS, NF-kB1 (p105), IP10, AKT(PKB), NF-kB1 (p50), IRF1, ICAM1, CCL2, ZFP36(Tristetraprolin), CCL7, TNF-alpha
5	Cooperative action of IFN-gamma and TNF-alpha on astrocytes in multiple sclerosis	2.447E-07	2.589E-05	CIITA, I-kB, NF-kB, iNOS, IP10, IRF1, ICAM1, CCL2, TNF-R1, NFKBIA, TNF-alpha
6	Immune response IL-9 signaling pathway	2.514E-07	2.589E-05	CCL5, MEK1/2, Shc, SOCS2, CISH, Scinderin, CCL13, AKT(PKB), CCL2, CITED2, Mucin 2, Pim-1, SOCS3, CCL7
7	Immune response IL-18 signaling	3.108E-07	2.972E-05	I-kB, MEK1/2, Shc, NF-kB, iNOS, AKT(PKB), c-Fos, IL-18, ICAM1, MyD88, CCL2, p38 MAPK, H-Ras, TNF-alpha

Table 3. Co-culture of IL-27-stimulated macrophages and colonoids drove colonoid gene expression crucial for immune signaling and pathogen responses. 25 of the most significantly upregulated pathways in colonoids co-cultured with IL-27-stimulated M1 macrophages. Data representative of three independent experiments.

Table 3 (cont'd)

	Pathway	pValue	FDR	Pathway Components
8	Immune response IL-3 signaling via JAK/STAT, p38, JNK and NF-kB	3.467E-07	3.095E-05	Cyclin D2, IKK-gamma, I-kB, CISH, NF-kB, AKT(PKB), c-Fos, ICAM1, CSF2RB, p38 MAPK, Pim-1, DPF3, H-Ras, C3aR, SOCS3, BMP2, SOCS1
9	Immune response TLR5, TLR7, TLR8 and TLR9 signaling pathways	5.982E-07	4.711E-05	IRAK2, Ubiquitin, IKK-gamma, I-kB, MEK1/2, NF-kB, NF-kB1 (p105), AKT(PKB), IRF1, MyD88, p38 MAPK, TAB3, TNF-alpha
10	Immune response TLR2 and TLR4 signaling pathways	1.015E-06	7.151E-05	IRAK2, Ubiquitin, IKK-gamma, I-kB, MEK1/2, NF-kB, iNOS, NF-kB1 (p105), AKT(PKB), MyD88, p38 MAPK, H-Ras, TAB3, TNF-alpha
11	Signal transduction NF-kB activation pathways	1.138E-06	7.617E-05	IRAK2, Ubiquitin, IKK-gamma, I-kB, NF-kB, NF-kB1 (p105), NF-kB1 (p50), MyD88, TNF-R1, RIPK1, TAB3, TNF-alpha
12	Immune response HMGB1/RAGE signaling pathway	1.768E-06	1.087E-04	NFKBIB, I-kB, MEK1/2, NF-kB, iNOS, AKT(PKB), ICAM1, HMGB1, PLAT (TPA), p38 MAPK, NFKBIA, TNF-alpha
13	Development VEGF signaling via VEGFR2 - generic cascades	1.787E-06	1.087E-04	IKK-gamma, I-kB, Shc, PLAUR (uPAR), Actin cytoskeletal, Calcineurin A (catalytic), iNOS, Calmodulin, PKC, AKT(PKB), CCL2, p38 MAPK, H-Ras, NF-AT2(NFATC1), TCF7L2 (TCF4), MEK2(MAP2K2)
14	Oxidative stress ROS-induced cellular signaling	3.056E-06	1.637E-04	AMPK alpha subunit, p38alpha (MAPK14), EGR1, NOXA, NF-kB, iNOS, GPX1, PKC, AKT(PKB), Isoform p66 Shc, Glutaredoxin 1, Catalase, FASN, p38 MAPK, NFKBIA, HSPA1A, TNF-alpha
15	Immune response histamine H1 receptor signaling in immune response	3.379E-06	1.740E-04	I-kB, Calcineurin A (catalytic), iNOS, Calmodulin, c-Fos, ICAM1, p38 MAPK, NF-AT2(NFATC1), NFKBIA, MEK2(MAP2K2), TNF-alpha

Table 3 (cont'd)

	Pathway	pValue	FDR	Pathway Components
16	Development ERBB-family signaling	3.891E-06	1.930E-04	IKK-gamma, I-kB, Shc, Amphiregulin, TGF-alpha, NF-kB, AKT(PKB), c-Fos, H-Ras, MEK2(MAP2K2)
17	Immune response IL-17 signaling pathways	7.059E-06	2.954E-04	IKK-gamma, I-kB, IL-17RC, NF-kB, iNOS, AKT(PKB), c-Fos, ICAM1, CCL2, p38 MAPK, MEK2(MAP2K2), CCL7
18	Immune response antiviral actions of Interferons	9.654E-06	3.854E-04	CIITA, OAS1, IDO1, OAS3, WARS, OAS2, eIF2S1, iNOS, IRF1, RNaseL, 2'-5'-oligoadenylate synthetase
19	Immune response HMGB1/TLR signaling pathway	1.495E-05	5.132E-04	Ubiquitin, IKK-gamma, p38alpha (MAPK14), I-kB, IRAK1/2, HMGB1, MyD88, TAB3, TNF-alpha
20	Apoptosis and survival NO synthesis and signaling	1.703E-05	5.524E-04	NF-kB p50/p50, PDE5A, Calcineurin A (catalytic), NF-kB, iNOS, Calmodulin, AKT(PKB), c-Fos, H-Ras, MEK2(MAP2K2), TNF-alpha
21	Immune response inhibitory action of lipoxins on pro-inflammatory TNF-alpha signaling	1.905E-05	5.932E-04	IKK-gamma, I-kB, TNF-R2, PPAPDC2, AKT(PKB), TNF-R1, RIPK1, SOCS1, TNF-alpha, 14-3-3
22	Immune response IL-5 signaling via JAK/STAT	2.038E-05	6.113E-04	SOCS2, Amphiregulin, CISH, E4BP4, NF-kB1 (p105), c-Fos, CSF2RB, Pim-1, NFKBIA, BLIMP1 (PRDI-BF1), SOCS1
23	Immune response C3a signaling	2.830E-05	7.727E-04	CCL5, MEK1/2, AKT(PKB), CCL2, KLF5, C3a, p38 MAPK, NFKBIA, C3aR, TNF-alpha
24	Immune response IL-33 signaling pathway	2.885E-05	7.727E-04	Ubiquitin, IKK-gamma, p38alpha (MAPK14), I-kB, MEK1/2, NF-kB, AKT(PKB), ICAM1, MyD88, CCL2, TNF-alpha
25	Immune response HMGB1 release from the cell	3.001E-05	7.727E-04	MEK1/2, TNF-R2, NF-kB, Calmodulin, HMGB1, MyD88, TNF-R1, HSPA1A, TNF-alpha

However, RNA sequencing was only able to identify upregulated pathways, and the expression of selected individual genes within these pathways was not found to be significantly changed via real time RT-qPCR (Appendix G). Because a number of these upregulated pathways facilitate responses to danger and pathogen associated molecular patterns (DAMPs and PAMPs, respectively), we hypothesized that exposure of colonoids to select DAMP or PAMPs following co-culture with IL-27-stimulated macrophages may increase their response to these stimuli. Contrary to our hypothesis, there was no difference in gene expression assessed by real time RT-qPCR or the secretion of selected cytokines (TNF-α, IL-6, IFN-γ; data not shown) in colonoids exposed to DAMPs or PAMPs after macrophage co-culture (Appendix H). Co-culture of IL-27-stimulated macrophages with colonoids also had no effect on IFN-γ-induced apoptosis of colonoids (Appendix I).

Discussion

Despite their role as a primary source of IL-27, there is a paucity of literature on the effect of IL-27 signaling in macrophages (Yoshida and Hunter 2015). In this study, we demonstrated that both non-activated peritoneal resident and induced, classically activated M1, F4/80^{+}CD11c^{-} macrophages express the IL-27Rα subunit and phosphorylate STAT1 in response to IL-27 treatment. Our RNA sequencing and subsequent PCR validation revealed that IL-27 increased the expression of 24 genes previously described in the literature as interferon regulated genes (Rusinova et al. 2013). Remarkably, a number of these genes were upregulated as early as one hour after the addition of IL-27 to the culture medium of non-activated resident macrophages, including *GBP2, GBP3, GBP5, GBP7, GBP8, GBP10, TGTP1/2, IRGB10, FGL2, IRF1,*

IL15RA, IL6, TNF, and *IRGM1*. Evaluating IL-27-induced changes in gene expression in both non-activated and M1 macrophages allowed us to detect expression changes due to IL-27 and its interaction with IFN-γ that we would have missed had we only evaluated M1 macrophages. The M1 macrophages received IFN-γ as part of the media to drive these cells to a M1 phenotype overnight. As such, it is not surprising that the majority of early fold increases in the examined genes were evident in the non-activated but not M1 macrophages, because many of these genes were likely already upregulated due to the previous IFN-γ exposure. In non-activated macrophages, the majority of examined genes peaked in expression within 6 hours of IL-27 treatment. In contrast, only two genes, *IRF1* and *NLRP3*, were upregulated by IL-27 prior to the 24 hour timepoint in M1 macrophages. A possible explanation for this delay in IL-27-induced gene expression in the M1 macrophages is the induction of the protein suppressor of cytokine signaling 3 (SOCS3) by the IFN-γ used to drive the M1 phenotype in these macrophages prior to IL-27 treatment (Bluyssen et al. 2010). SOCS3 inhibits IL-27 signaling by directly binding to and inhibiting the gp130 receptor subunit of the IL-27 receptor and its associated Janus kinases (Aparicio-Siegmund and Garbers 2015). Therefore, pretreatment of the cells with IFN-γ could delay IL-27 signaling in the M1 macrophages via degradation of its receptor. In complement to our data, Rolvering et al. discovered a STAT1-dependent, IFN-γ-like pattern of gene transcription in response to IL-27 stimulation in hepatocellular carcinoma cell lines. Additionally, pre-stimulation of the cell lines with IL-6, another inducer of SOCS3, inhibited the previously observed responses to IL-27 (Rolvering et al. 2017).

Of the interferon-associated genes upregulated by IL-27 in the macrophages of this study, *IFNG* was increased in both non-activated and M1 macrophages, and the type I interferons *IFNB1* and *IFNA2* were upregulated in non-activated macrophages. As such, it wasn't clear from our initial data if the gene expression changes we detected were due directly to IL-27, or if these changes were the result of IL-27-induced interferon signaling. Using knockout mice, we determined that none of the changes in gene expression observed were the result of IL-27-induced IFN-γ. Interestingly, repeating this experiment in mice lacking the type I IFN receptor revealed that all of the examined genes with the exception of *IFNG* were induced directly by IL-27. In contrast, the removal of type I interferon signaling abolished the induction of *IFNG*. This indicates that the increased *IFNG* transcription observed due to IL-27 stimulation is dependent on the production and signaling of IL-27-induced type I interferons. Our data also highlight that the observed changes in gene expression are dependent on STAT1 signaling, a feature shared with some IFN-γ-activated genes (Ramana et al. 2002).

IL-27 directly induced an increase in surface expression of MHC II in the macrophages of our study independent of IL-27-induced interferon, which is known to increase MHC II expression (Giroux et al. 2003). Interestingly, and in complement to our gene and protein expression data suggesting IL-27 is at times eclipsed by IFN-γ signaling, IL-27 consistently induced MHC II expression, but to a lesser extent than IFN-γ. As was also predicted by our gene expression data and previous literature (Ramana et al. 2002), the elimination of STAT1 signaling abolished the ability of both IL-27 and IFN-γ to upregulate MHC II expression. These data are particularly interesting in light of the conflicting reports in the literature regarding IL-27 and MHC II expression.

Concordant with our data, Jung et al. observed increased MHC II surface expression in human monocyte-derived dendritic cells differentiated in the presence of IL-27 (Jung et al. 2015). In sharp contrast to Jung et al.'s report, IL-27 pretreatment of conventional murine splenic dendritic cells prior to LPS stimulation reduced MHC II surface expression. The reason for this discord is unclear, but may be related to the different types of dendritic cells used or a potential difference in murine and human dendritic cells. In support of our data, upregulation of surface MHC II in response to IL-27 has been reported in a human promonocytic cell line; however, this study did not address whether this upregulation was directly due to IL-27 or potentially to some combination of IL-27 and IL-27-induced interferon (Feng et al. 2008).

IFN-γ is well known for its prowess in combatting both viral and intracellular bacterial infections such as *Salmonella* (Goodbourn et al. 2000; Ingram et al. 2017). Recent reports in the literature also describe the antiviral properties of IL-27, but its mechanism of action appears to differ between viruses. For example, IL-27 was found to be a critical regulator of both the early innate and chronic adaptive immune responses to lymphocytic choriomeningitis virus infection in mice. In this study, the ablation of IL-27 signaling decreased type I interferon production, compromising early control of the viral infection (Harker et al. 2018). In contrast, antibody neutralization of IFN-γ and type I interferons had no effect on the ability of IL-27 to inhibit the replication of human immunodeficiency virus in monocyte-derived macrophages (Imamichi et al. 2008). Notably, Imamichi et al. also observed a transcriptional profile of interferon-inducible genes in IL-27-stimulated monocyte-derived macrophages unique from the data presented herein (Imamichi et al. 2008). In the present study, we describe the

inhibitory effects of IL-27 on two viruses as of yet unreported to be inhibited by IL-27. Our data demonstrate the ability of IL-27 to inhibit replication of vaccinia virus in murine macrophages and to reduce the release of active viral particles of mouse cytomegalovirus following macrophage infection so as to reduce subsequent infection in a susceptible fibroblast cell line. These data also provide further evidence for the relationship between IL-27 and type I interferon in the control of viral infection. Our data indicate that in the case of mouse cytomegalovirus infection, IL-27 alone attenuates the release of active virions early in the infection; however, type I interferon signaling is necessary for the sustained control of the virus. IL-27 attenuation of virus release has also been demonstrated in multiple cell types in herpes simplex virus type 1 infection (Heikkilä et al. 2016).

Previous reports in the literature implicate IL-27 as detrimental to the clearance of multiple types of bacterial infections, including *Mycobacterium tuberculosis* and *Staphylococcus aureus* (Robinson et al. 2015; Sharma et al. 2014). However, our gene expression data showed the upregulation of a number of key mediators for IFN-γ killing of intracellular *Salmonella*. However, treatment of bone marrow-derived macrophages before or during *Salmonella* infection did not improve, but rather showed a trend to worsen, *Salmonella* clearance. The most likely explanation for this result in our study is the lack of detected nitric oxide in macrophages following IL-27 stimulation despite the strong induction of *NOS2* expression. While other pathways exist to combat intracellular *Salmonella*, Alam et al. demonstrated that *NOS2* deficient mice died even when infected with an avirulent strain of *Salmonella* to which wildtype mice were highly resistant (Alam et al. 2002).

Our RNA sequencing data from colonoids indicates that IL-27-stimulated macrophages produce products that drive gene expression important for pathogen responses in colon epithelial cells. While significant changes in individual genes were not detected in colonoids in this experiment, the pathways upregulated in the colonoids correlate nicely with the products detected due to IL-27 stimulation of macrophages in separate experiments. These pathways include: immune response IFN-alpha/beta signaling via JAK/STAT, immune response IFN-alpha/beta signaling via MAPKs, signal transduction NF-κB activation pathways, and immune response antiviral actions of interferons. Furthermore, pathways related to signaling in TLRs 2, 4, 5, 7, 8, and 9 were upregulated in these colonoids. So the question remains: why did this upregulation of gene expression not translate to enhanced responses to simulated pathogens in this study? There are a number of potential technical reasons for this, including the potential for missing the appropriate timepoints or inadequate cell numbers or ratios. Additionally, the biological variation in this experiment may have precluded the statistical detection of some critical factor(s) that might explain these unexpected results.

In conclusion, our data provide novel mechanistic and functional insights into the shared and divergent effects of IL-27 and interferons on both non-activated resident and classically activated macrophages. Furthermore, our data highlight the ability of immune cells, in this case macrophages, to mediate (or potentially derail) the intended effects of biologic therapies in target organs.

Methods

Mice. Animal studies were approved by the NIH Animal Care and Use Committee. The following mouse strains were obtained from the Frederick National Laboratory Core

Breeding Specific Pathogen Free Facility: C57BL/6NCr, B6.129S7-*Ifng*^{tm1Ts}/J, B6.129S2-*Ifnar1*^{tm1Agt}/Mmjax, and B6.129S(Cg)-*Stat1*^{tm1Dlv}/J.

Cell and tissue collection. Non-activated resident or thioglycolate-induced peritoneal macrophages were harvested from mice as previously described (Zhang et al. 2008). Prior to all tissue harvests, mice were humanely euthanized with CO_2 followed by cervical dislocation as a secondary method of euthanasia. Resident peritoneal macrophages were harvested from experimentally unmanipulated mice by lavage of the peritoneal cavity with 10 ml of sterile cold PBS (Corning). For thioglycolate induction of peritoneal macrophages, 1 ml of sterile 3% Brewer's thioglycolate medium (gift from the McVicar lab) was injected into the peritoneum. Induced peritoneal macrophages were harvested five days post-injection by peritoneal lavage with 10 ml of sterile cold PBS. For all studies except for the *Salmonella* clearance assays, macrophages were isolated by immunomagnetic sorting with the Macrophage Isolation Kit (peritoneum, mouse) (Miltenyi Biotec). Thioglycolate-induced peritoneal macrophages were driven to the classically activated M1 phenotype by overnight culture with 10 ng/ml IFN-γ (Peprotech), 100 ng/ml LPS (Sigma), and 10 ng/ml M-CSF (Peprotech) as previously described (Mosser and Zhang, 2008). For *Salmonella* clearance assays, bone marrow-derived macrophages were plated and differentiated for five days with M-CSF. Macrophages were selected by plate adherence.

For colon organoid preparation, colons were harvested following humane euthanasia described above, flushed with sterile cold PBS to remove feces, incised longitudinally, and placed in sterile cold PBS on ice until processed.

Cell culture. For RNA-sequencing, gene expression, protein expression, or flow cytometry experiments, non-activated or thioglycolate-induced M1 macrophages were cultured with 100 ng/ml IL-27 (R&D Systems) or 10 ng/ml IFN-γ for 1, 4, 6, 24, or 48 hours in DMEM cell culture media (Corning) containing 1% penicillin/streptomycin (Corning) and 10% fetal bovine serum. For co-culture experiments, approximately 1 x 10^6 macrophages were plated at the bottom of 24 well plates. Colonoids were prepared as described below and seeded in Matrigel within Transwell inserts (VWR). Co-culture experiments were performed in DMEM/F12 media (Corning) with 10% fetal bovine serum, 1% bovine serum albumin, 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM GlutaMAX (Gibco), and 10 mM hepes.

Flow cytometry. Single cell suspensions of macrophages were taken immediately after harvest or following culture by removal of the adherent macrophages by a two minute incubation at 37°C in 0.05% trypsin/0.53 mM EDTA (Corning) followed by gentle mechanical lifting from the plate with a sterile cell scraper. Cells were then washed with PBS and nonspecific binding was blocked by a ten minute incubation at 4°C with anti-mouse CD16/32 antibody (TruStain FcX, BioLegend). Cells were again washed with PBS and then incubated for 20 minutes at 4°C with the fluorochrome conjugated antibody of choice for that experiment: rat anti-mouse F4/80 APC (BD Biosciences clone T45-2342), APC rat IgG_{2a},κ isotype control antibody (BD Biosciences), hamster anti-mouse CD11c PE (BD Pharmingen clone HL3), PE Armenian hamster $IgG_1,\lambda 2$ isotype control antibody (BD Pharmingen), rat anti-mouse I-A/I-E PE (BD Pharmingen clone M5/114.15.2), or PE rat IgG_{2b},κ isotype control antibody (BD Pharmingen). Cells were then washed once more in PBS and resuspended in 1% paraformaldehyde for

analysis by the NIH Cancer and Inflammation Program Flow Cytometry Core. Data were analyzed with FlowJo software.

Gene expression analysis. RNA was extracted from macrophages or colonoids with either the Qiagen RNeasy Mini or Micro kit or TRIzol reagent (Invitrogen) as per the manufacturer's instructions. RNA was then quantified and evaluated for purity and contamination via Nanodrop Spectrophotometer (Thermo Scientific). RNA for RNA sequencing was evaluated by both Nanodrop and Agilent Bioanalyzer. RNA was reverse transcribed to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per the manufacturer's protocol. No reverse transcriptase controls were prepared by following the same cDNA transcription protocol with the omission of the reverse transcriptase. For real time RT-qPCR, Taqman gene expression assay primer/probe sets for the genes of interest and TaqMan Universal PCR Master Mix were purchased from ThermoFisher. Samples were analyzed via Applied Biosystems 7300 Real-Time PCR System. RNA sequencing was performed by the NCI Frederick Advanced Technology Research Facility on an Illumina HiSeq2500 instrument.

Viral infection assays. To investigate the ability of IL-27 to inhibit viral infection of macrophages, non-activated peritoneal macrophages were treated overnight with 100 ng/ml IL-27 and then infected with green fluorescent protein (GFP)-expressing vaccinia virus (six hour infection). Infected cells were quantified by flow cytometry. We were also interested in the potential ability of IL-27 stimulation of macrophages to reduce active virions and therefore reduce subsequent infection of other cells. To investigate this, non-activated peritoneal macrophages were treated with 1, 10, or 100 ng/ml of IL-27 or

10 ng/ml of IFN-γ for six hours. The media with the cytokines was removed, and the macrophages were infected with luciferase-expressing mouse cytomegalovirus. Supernatants from these cultures were removed at 24, 48, and 72 hours post-infection and used to infect a susceptible line of fibroblasts. The fibroblasts were evaluated for infection level at 18 hours post-infection.

Bacterial infection assays. Bone marrow derived macrophages were either pretreated for 24 hours or treated at the time of *Salmonella* infection with 100 ng/ml IL-27. Cells were infected with a MOI of 5-10 mCherry-expressing *Salmonella enterica* serovar Typhimurium strain SL1344 and monitored for viral replication via fluorescence with an IncuCyte Live-Cell Analysis System (Sartorius) for 24 hours.

Nitric oxide quantification. Nitrite as an indicator of nitric oxide was measured in cell culture supernatants via a Griess Reagent Kit (ThermoFisher) according to the manufacturer's protocol.

Protein analysis. Non-secreted proteins were evaluated by capillary western blot on the Protein Simple Wes instrument as per the manufacturer's protocol. Prior to analysis, cells were lysed in RIPA lysis buffer (ThermoFisher) for 10 minutes on ice. Samples were then centrifuged for 5 minutes at 2500 rpm, and protein samples were removed to another tube for freezing at -20°C for later analysis. A total of 4 μg of protein was analyzed for each target. The following antibodies were purchased from Cell Signaling Technologies for use in the Wes instrument: Cleaved Caspase-3 (Asp175) Rabbit Antibody #9661 (used at 1:50 dilution), Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb #9167 (used at 1:50 dilution), Stat1 Rabbit Antibody #9172 (used at 1:500 dilution), Caspase-3 Antibody #9662 (used at 1:50 dilution).

Secreted proteins were evaluated from cell culture supernatants via Meso Scale immunoassay as per the manufacturer's protocol (Meso Scale Diagnostics) on the Meso Scale Diagnostics QuickPlex SQ 120 instrument.

Colon organoid culture. Colon organoids (colonoids) were derived based on a previously published protocol (Sato and Clevers 2012). Longitudinally incised colons flushed free of feces were washed twice in cold sterile PBS. The colons were then laid flat with the mucosal surface facing upward and the mucus layer and luminal epithelium was gently scraped away with a sterile glass slide. The colons were then washed again, cut into approximately 1-2 mm pieces, and washed another 5-10 times until the supernatant was clear. Colon pieces were then incubated in 2 mM EDTA for 30 minutes at 4°C. The supernatant was then removed and replaced with PBS, the colon pieces were shaken by hand for 5 minutes, and then the supernatant was removed. The colon pieces were then resuspended in 20-30 ml PBS and pipetted up and down with a seropipette to further mechanically loosen the intact crypts. This procedure was repeated approximately 5-6 times, with collection of the supernatant each time. The supernatant was filtered with a 100 μm cell strainer and centrifuged for 5 minutes at 300 G. The crypts were then resuspended in DMEM/F12 (Corning) culture medium containing 1% penicillin/streptomycin (Corning) and 100 μg/ml Primocin (Invivogen). The crypts were then washed six times in this media with centrifuging for 2 minutes at 200 G to remove any contaminating single cells. Crypts were then resuspended in Matrigel (Corning) and plated in either standard or Transwell (VWR) 24 well plates (50 μl of Matrigel/crypt mixture per well; approximately 750 crypts per well). Crypts were then overlain with the following culture media (complete media): DMEM/F12 media

(Corning) containing 1% bovine serum albumin, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM GlutaMAX (Gibco), 10 mM hepes, 1X N2 and B27 supplements (Gibco), 1 mM N-acetylcysteine (Sigma), 50 ng/ml epidermal growth factor (ThermoFisher Scientific), 100 ng/ml Noggin (Peprotech), 1 μg/ml R-spondin (Peprotech), 100 ng/ml Wnt3a (R&D Biosystems), 10 mM nicotinamide (Sigma), 500 nM A83-01 (Tocris), 10 nM prostaglandin E2 (Sigma), 10 nM [Leu-15]-gastrin-1 (Sigma), 10 μM SB202190 (Sigma), 2.5 μM thiazovivin (Stemgent), and 100 μg/ml Primocin (Invivogen). 1μM Jagged-1 peptide (Anaspec) was embedded with crypts in Matrigel. When cultures were initiated, 2.5 μM CHIR99021 (Stemgent) and 10 μM Y-27632 dihydrochloride were included in media but were discontinued thereafter. Media was changed every other day.

For DAMP/PAMP response assays, colonoids were co-cultured for 24 hours with macrophages +/- IL-27, Transwell inserts were transferred to new plates containing fresh either plain or complete colonoid media, and colonoids were stimulated with one of the following: 100 ng/ml LPS (Sigma), 100 μg/ml poly I:C (Tocris), or 100 pg/ml IL-1α (R&D Systems).

Immunohistochemistry. Immunohistochemistry to identify cell types within colonoids was performed according to standard protocols by Histoserv, Inc (Gaithersburg, MD). Briefly, formalin-fixed paraffin-embedded sections were deparaffinized and hydrated by submersion in xylene followed by a series of ethanol dilutions (100%, 95%, 70%) ending in distilled water. Endogenous peroxidases were blocked with 3% hydrogen peroxide in methanol, and antigen retrieval was subsequently performed for 15 minutes in a steamer. Antigen retrieval was followed by a nonspecific protein and avidin/biotin block.

Sections were incubated with primary antibody for one hour and secondary antibody for 30 minutes, followed by avidin/biotin amplification and addition of the 3,3'-diaminobenzidine (DAB) substrate. Slides were then counterstained with hematoxylin and dehydrated in increasing ethanol solutions (70%, 95%, 100%) followed by submersion in xylene and coverslipping. The following primary antibodies were used: rabbit anti-mouse DCAMKLI at a 1:300 dilution (Cell Signaling Technology #62257) to label tuft cells and rabbit anti-mouse UCHL1 (PGP 9.5) at a 1:400 dilution (Cell Signaling Technology #13179) to label enteroendocrine cells.

Proliferation Analysis. After 60 hours of differentiation in complete media in culture, colonoids were stimulated with 100 ng/ml IL-27 for 24 hours in DMEM/F12 media (Corning) with either 10% or 0.5% fetal bovine serum, 1% bovine serum albumin, 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM GlutaMAX (Gibco), and 10 mM hepes. Colonoids were then washed gently with room temperature PBS and incubated with Corning Cell Recovery Solution for 30 minutes at 4°C with gentle shaking. Colonoids were then recovered by centrifugation, washed once with PBS, and fixed in 10% neutral buffered formalin. Colonoid cell pellets were resuspended in Histogel (ThermoFisher), processed, and paraffin embedded. Blocks of formalin-fixed paraffin-embedded colonoid pellets were then stained for argyrophilic nucleolar organizing regions as a measure of proliferation (Trerè et al. 1989) per standard protocols at the Michigan State University Veterinary Diagnostic Laboratory.

Statistical analysis. Real-time RT-qPCR data were analyzed via the 2^{-ddct} method to calculate fold change relative to untreated groups. Cycle threshold (CT) values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels

for each sample. Statistical analysis of real time RT-qPCR, secreted protein concentration data, and viral load data were analyzed with Prism 7 GraphPad Software. Protein concentration data were log transformed to approximate normality and untreated and IL-27-treated groups were compared by Student's T-test. For comparison of real time RT-qPCR gene expression data between untreated and IL-27-treated groups, delta CT data were log transformed to approximate normality and compared by Student's T-test. Comparison of gene expression between timepoints was performed by log transformation of the delta delta CT data to approximate normality followed by analysis by one-way ANOVA.

For RNA sequencing data analysis, the raw RNA-Seq fastq reads were aligned to mouse genome (mm10) using STAR (v. 2.5.2b) on 2-pass mode with mouse gencode (release 12) gtf (Dobin et al. 2013). Genes were subsequently counted using RSEM (v.1.3.0) (Li and Dewey 2011) and further normalized using TMM (edgeR) and analyzed for gene expression changes using limma-voom with quantile normalization (Charity et al. 2014). Batch removal was performed using ComBat (Leek et al. 2012).

APPENDICES

APPENDIX A: Macrophages driven to the M1 classically activated phenotype in culture display gene expression characteristic of the M1 phenotype

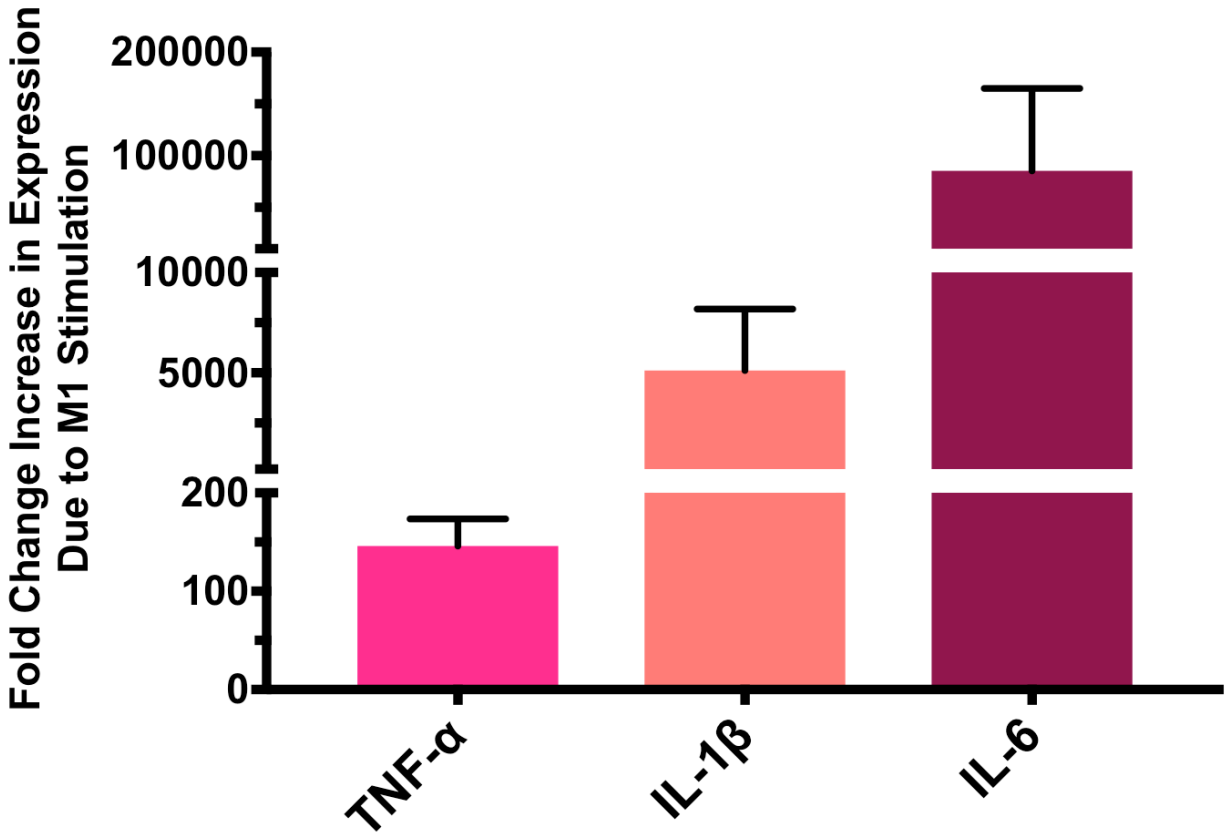

Figure 18. M1 activated, thioglycolate-induced macrophages express higher levels of TNF-α, IL-1β, and IL-6 than non-activated, thioglycolate-induced macrophages. Graph depicts the mean + SD for three independent experiments.

APPENDIX B: Colon epithelial organoid (colonoid) structure

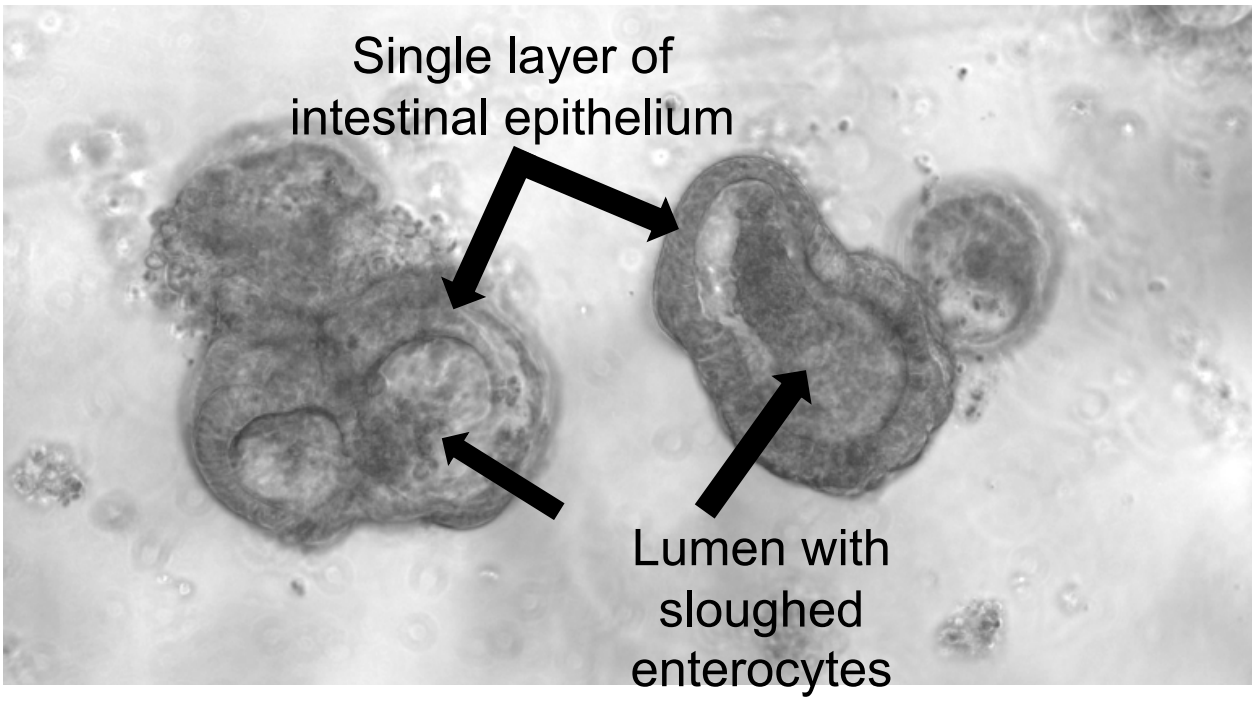

Figure 19. Brightfield microscopic image of two colonoids suspended in Matrigel approximately 60 hours after culture initiation. Colonoids consist of roughly spherical, luminal structures encompassed by a single layer of colon epithelium. Colonoid epithelial cells undergo apoptosis and slough into the colonoid lumen similar to normal intestinal epithelial cells in vivo.

APPENDIX C: Colon epithelial organoids (colonoids) differentiate into all of the cellular constituents of the normal colon mucosal epithelium

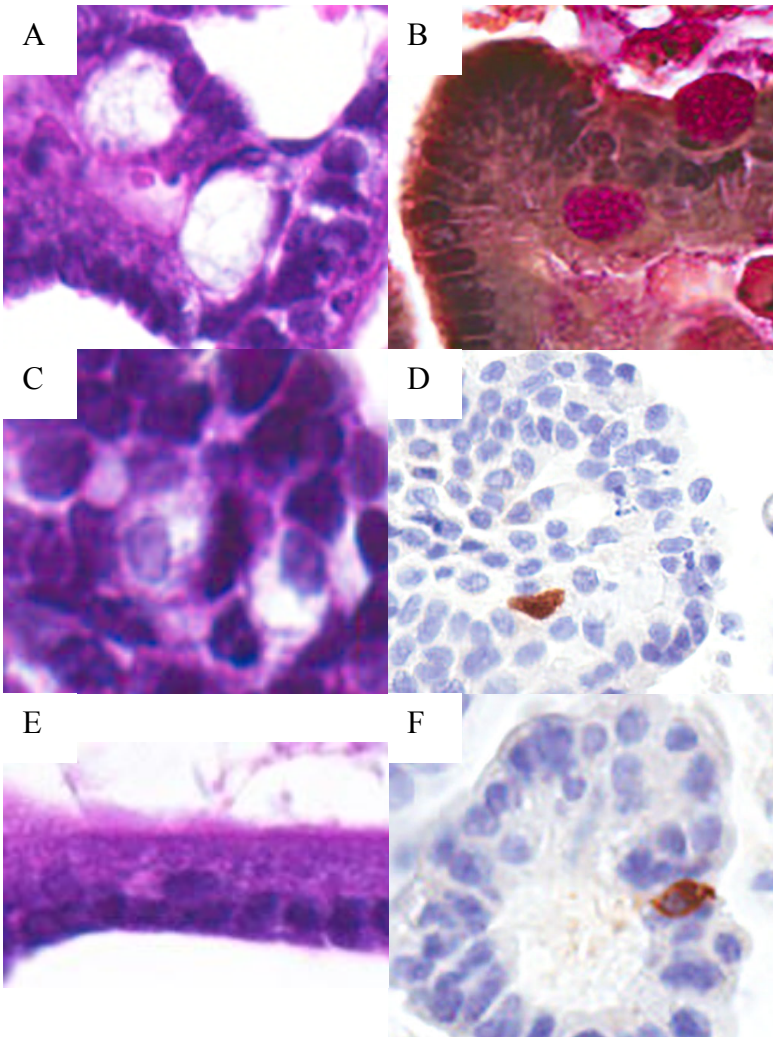

Figure 20. Colonoids derived in vitro from murine intestinal crypts differentiate into absorptive enterocytes, goblet cells, enteroendocrine cells, and tuft cells. (A) Hematoxylin and eosin (H&E) stained section of a colonoid highlighting two large, clear, vacuolated goblet cells. (B) Mucicarmine stain highlighting goblet cells (bright pink) in a colonoid. (C) H&E section illustrating enteroendocrine cells within a colonoid. (D) Immunohistochemistry for protein gene product (PGP) 9.5 labeling an enteroendocrine cell in a colonoid. (E) Single layer of enterocytes with a visible brush border from a colonoid. (F) Immunohistochemistry for doublecortin-like kinase 1 (DCAMKL1) labeling a tuft cell in a colonoid.

APPENDIX D: IL-27 does not affect murine colon epithelial proliferation

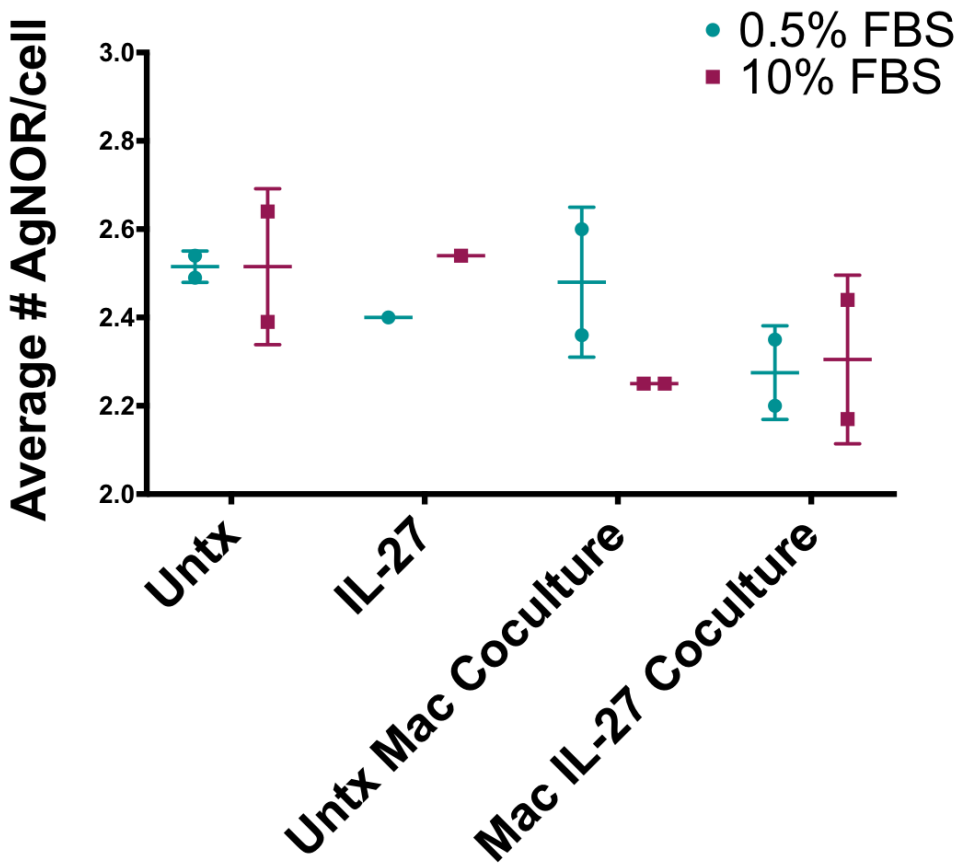

Figure 21. Culture of colonoids with IL-27 or IL-27-stimulated macrophages does not enhance colon epithelial proliferation as has been previously described in human colorectal cancer cell lines. Data representative of two independent experiments, with the exception of one IL-27-treated sample from each group that was lost in processing.

APPENDIX E: IL-27 phosphorylates STAT1 in a human colorectal cancer cell line

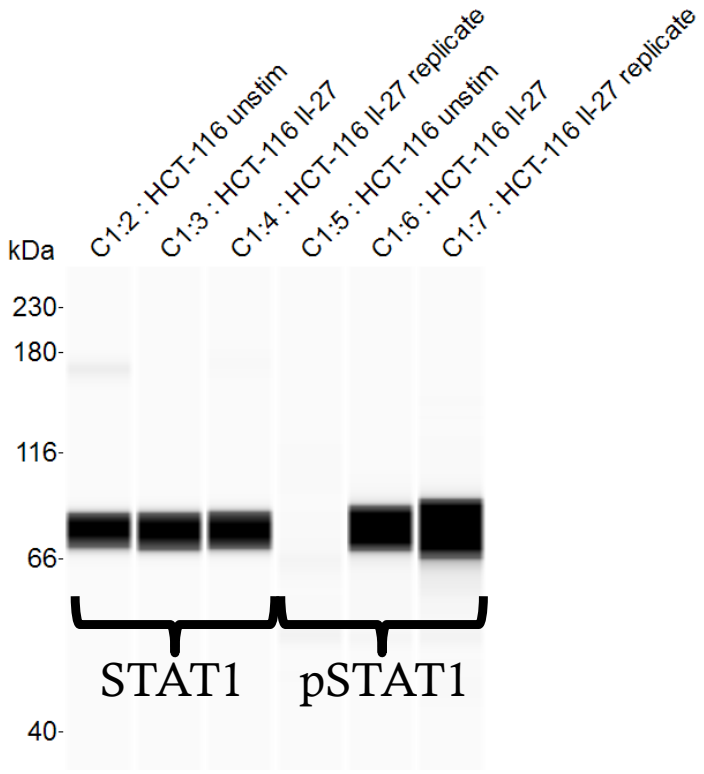

Figure 22. Capillary western blot for STAT1 and pSTAT1. To evaluate our culture systems and detection methods, we repeated a previously published experiment describing phosphorylation of STAT1 in the human colorectal cancer cell line HCT-116. In agreement with the previously published data, IL-27 induced phosphorylation of STAT1 in the HCT-116 cells.

APPENDIX F: Freshly isolated crypts do not respond to IL-27

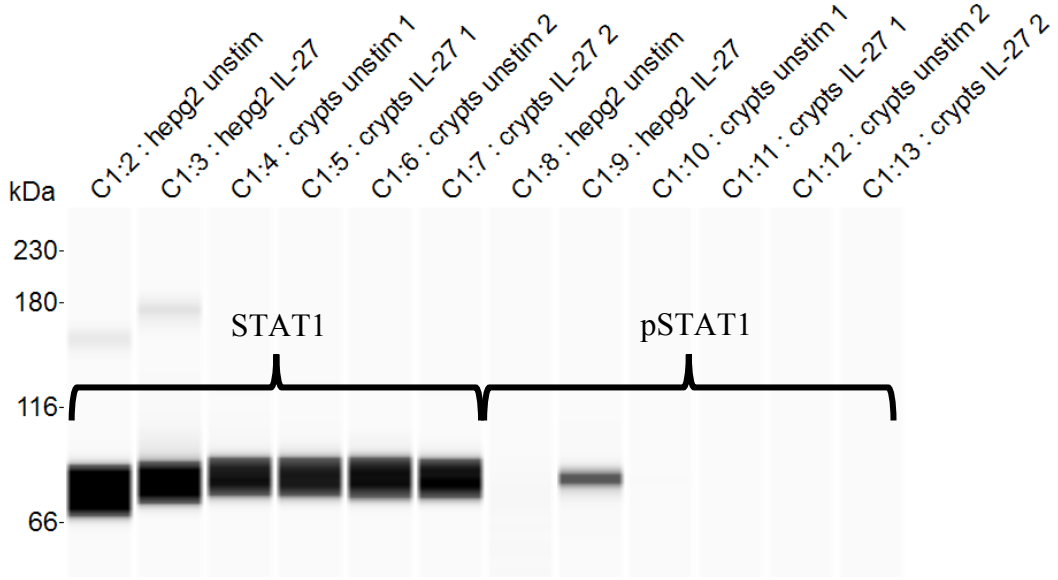

Figure 23. Capillary western blot for STAT1 and pSTAT1 in freshly isolated intestinal crypts +/- 100 ng/ml IL-27. HepG2 cells, which reliably respond to IL-27, were used as a positive control.

APPENDIX G: Upregulation of individual genes from pathways upregulated in colonoids by co-culture with IL-27-stimulated macrophages was not detected by real time RT-qPCR

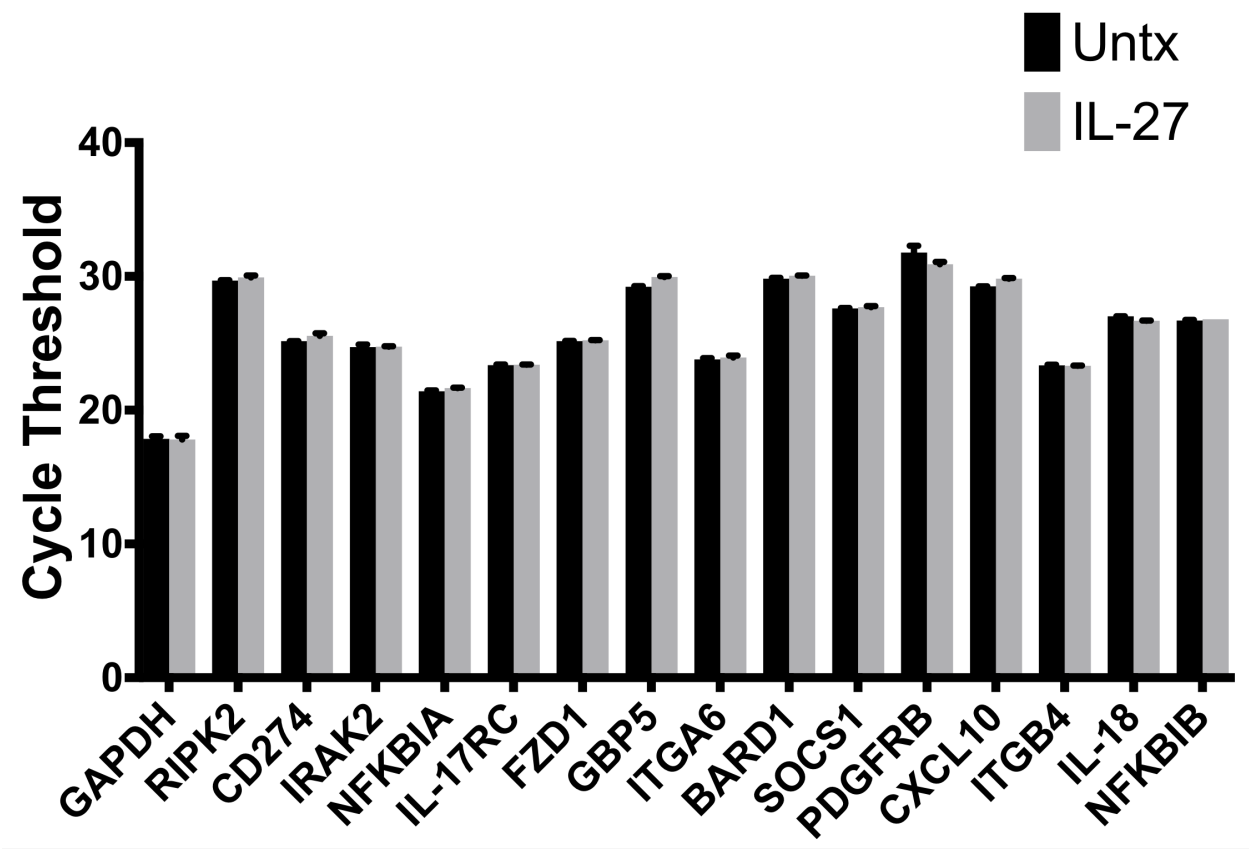

Figure 24. CT values from real time RT-qPCR of selected genes that are components of pathways upregulated in colonoids due to co-culture with IL-27-stimulated macrophages. No changes in expression of individual genes was observed. Graphs depict mean + SD of two independent experiments.

APPENDIX H: Prior exposure to IL-27-stimulated macrophages does not enhance responses to PAMPs or DAMPs in colonoids

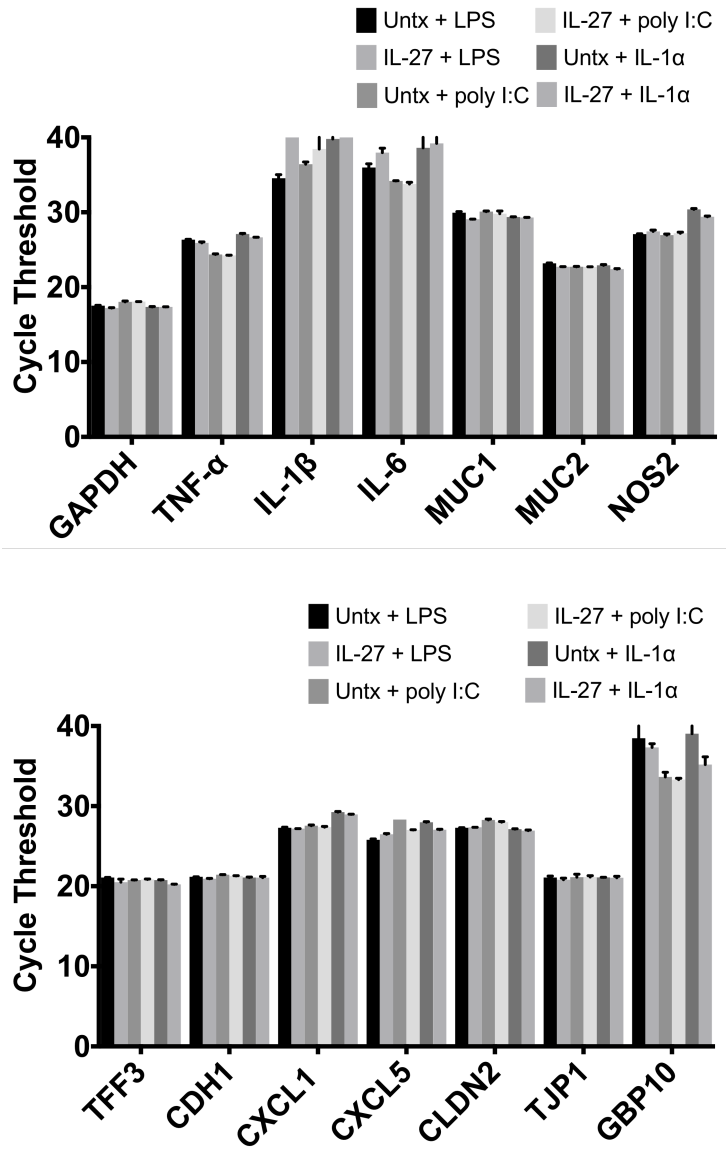

Figure 25. Expression of genes as determined by real time RT-qPCR in colonoids co-cultured with IL-27-stimulated macrophages for 24 hours followed by exposure to either LPS, poly I:C, or IL-1α.

APPENDIX I: Co-culture with IL-27-stimulated macrophages does not prevent or reduce IFN-γ-induced apoptosis in colonoids

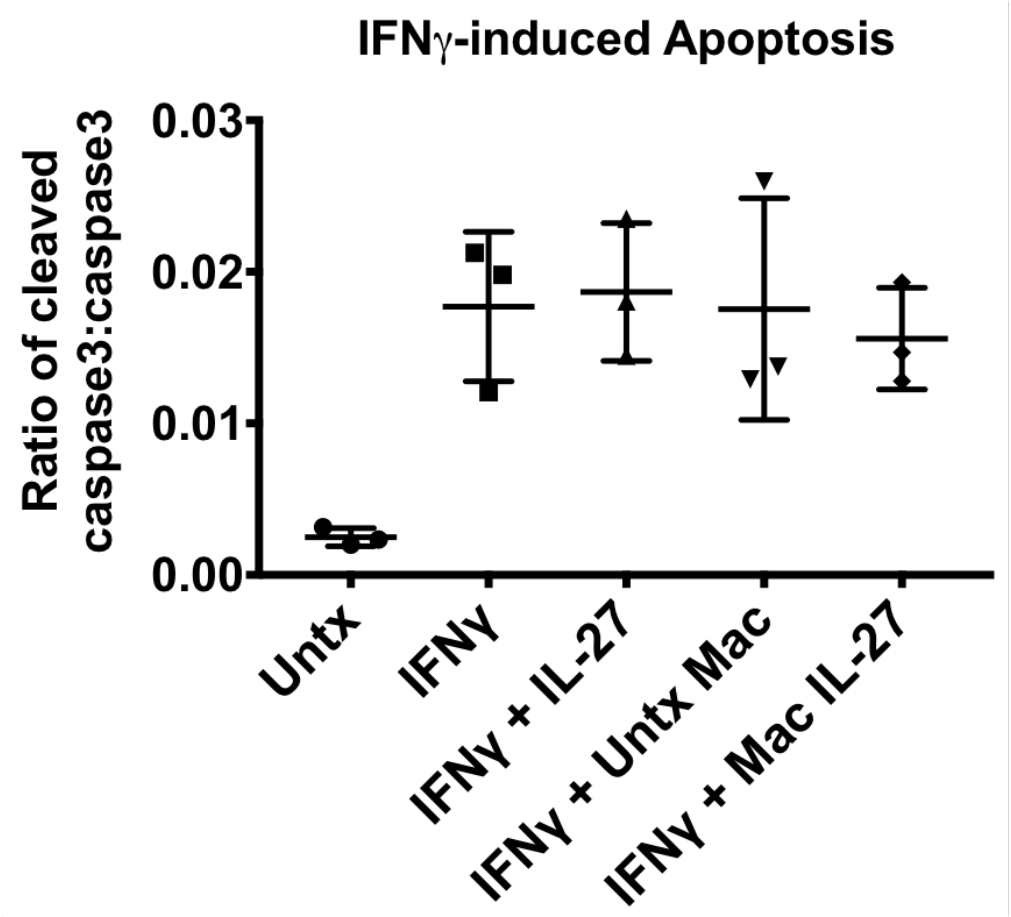

Figure 26. Ratio of cleaved caspase 3: caspase 3 indicative of apoptosis in colonoids as analyzed by capillary western blot. Apoptosis was induced by 20 ng/ml IFN-γ. To determine if IL-27-stimulated macrophages could reduce IFN-γ-induced apoptosis, colonoids were co-cultured for 24 hours with macrophages +/- IL-27. Data representative of three independent experiments.

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