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IL-36/IL-36R Signaling Promotes CD4+ T Cell-Dependent Colitis via Pro-Inflammatory Cytokine Production

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by

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Under the Direction of Timothy L. Denning, Ph.D.

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ABSTRACT

Inflammatory bowel disease (IBD) is a multifactorial, chronic disease that affects approximately 1.5 million people in the United States [1]. It presents with inflammation of the intestine with unknown etiology. Its two main forms are Crohn's disease (CD), which can affect any part of the GI tract, and ulcerative colitis (UC), which impacts primarily the colon. Several important factors are implicated in the pathogenesis of IBD, one factor being dysregulation of the immune system. This dysregulation results in the accumulation and stimulation of innate and adaptive immune cells and subsequent release of soluble factors, including pro-inflammatory cytokines. One of these cytokines is a member of the IL-36 cytokine family, IL-36γ, which is overexpressed in human IBD and experimental models of colitis. In this study, we explored the role of IL-36γ in promoting CD4+ T cell activation and cytokine secretion. We found that IL-36γ stimulation of naïve CD4+ T cells significantly induced IFNγ expression *in vitro* and was associated with augmented intestinal inflammation in vivo using the T cell transfer model of colitis. Using IFNγ-/- naive T cells, we observed a dramatic decrease in the ability of these cells to produce TNFα and IL-12. Moreover, the transfer of these cell did not cause robust colitis. These data not only suggest that IL-36γ is a master regulator of a pro-inflammatory cytokine network involving IFNγ, TNFα and IL-12, but also highlight the importance of targeting IL-36γ and IFNγ as therapeutic approaches. Our studies have broad implications in relation to targeting of specific cytokines in human IBD.

INDEX WORDS: experimental colitis model, T cell mediated colitis, adoptive transfer, IL-36γ, IFNγ Signaling, Crohn's disease, ulcerative colitis, inflammatory bowel disease

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DEDICATION

I firstly dedicate my dissertation work to myself; and next, to my parents who have been fully supportive of everything I have done throughout the entirety of my academic journey and especially during the duration of my PhD. Accomplishing my goals would not have been possible without them being by my side. I would next like to dedicate this to my brothers, family members, and friends who always comforted me during any stressful situations that I faced.

Lastly, I dedicate my dissertation work to IBD patients and to the IBD research community. My dad, being a colon cancer survivor and a stage 4 liver cirrhosis patient, has only pushed me forward to continue my research. I hope this little contribution helped highlight new possible therapeutic venues.

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1.	IBD	Inflammatory Bowel Disease
2.	CD	Crohn's Disease
3.	Gastrointestinal	GI
4.	UC	Ulcerative Colitis
5.	IEC	Intestinal Epithelial Cells
6.	NF-κB	Nuclear Factor Kappa B
7.	MAPK	Mitogen-activated Protein Kinases
8.	GWAS	Genome-wide Association Studies
9.	TJ	Tight Junctions
10.	AMP	Anti-microbial Peptides
11.	APC	Antigen Presenting Cells
12.	DC	Dendritic Cells
13.	Th	T Helper Cells
14.	IL-	Interleukin
15.	TF	Transcription Factor
16.	IFNγ	Interferon Gamma
17.	TNFα	Tumor Necrosis Factor Alpha
18.	JAK	Janus Kinase

1. INTRODUCTION

1.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a multifactorial, chronic condition that remains incurable until this day. Its two main forms are Crohn's disease (CD) which affects any part of the GI tract, and ulcerative colitis (UC) which impacts the colon only. Symptoms of IBD include abdominal pain, loss of appetite, diarrhea, and weight loss. To date, the types of therapeutics currently available primarily work to reduce and suppress inflammation, without curing the disease [2]. Unfortunately, the exact cause of IBD remains ambiguous. Several factors play role in the pathogenesis of IBD, including genetics, the microbiota, environmental factors, and immunological dysregulation. A disruption in the mucosal barrier increases intestinal permeability [3], resulting in immune dysregulation with a large number of innate and adaptive leukocyte infiltration. These events lead to intestinal inflammation and IBD.

The intestinal epithelial barrier acts as a defense barrier between the intestinal mucosa and the lumen, composed of a layer of intestinal epithelial cells (IECs) [4]. Should this intestinal epithelial barrier be disrupted, intestinal permeability increases allowing for luminal contents to pass through. In return, an immune response is triggered. During a primary immune response, the priming of T cells occurs in the mesenteric lymph nodes, where naïve $CD4^+$ T cells interact with the antigen and differentiate into activated effector cells. These effectors can polarize into any of the different subsets, Th1, Th2, Th17, or T regulatory cells, depending on the microbial challenge to be eliminated [5], and this is accompanied by cytokine imbalance.

Some IL-36 cytokine family members like, IL-36〈 and IL-36γ, function at barrier tissues such as the intestines and are considered to be pro-inflammatory. Binding to their receptor, IL-

36R, activates nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) [6], causing inflammation. However, IL-36 cytokines are regulated by their natural inhibitor, the antagonist IL-36Ra, which inhibits the inflammatory signaling pathway [7].

1.2 Factors Involved in IBD Pathogenesis

The etiology of IBD is unknown, but multiple factors can cause intestinal inflammation and contribute to IBD pathogenesis. Such factors include genetics, environmental factors, the microbiota, and the immune system. Only 15% of IBD patients, specifically Crohn's disease, have family members with IBD. There are some rare genetic disorders that could cause an early-onset of IBD. However, studies have identified approximately 50 genetic disorders that are associated with IBD [8]. Genome-wide association studies (GWAS) analyzed the entire genomes of individuals and have identified over 200 loci and genetic variations associated with IBD. One of the first CD risk genes identified was *NOD2,* in which variants of this gene increase the risk of developing CD. One study successfully identified 25 new susceptible loci for IBD [9], and ongoing research still continues to identify genes associated with a higher risk of developing IBD.

Environmental factors that could lead to IBD include, but are not limited to, stress, diet, smoking, urbanization, air pollution, history of chronic diseases, use of antibiotics, and even geographic variation. The use of antibiotics in early life has particularly shown an increased risk for IBD because it can cause microbial dysbiosis [10]. This is explained by the hygiene hypothesis, which suggests that childhood exposure to certain infectious agents helps build a stronger immune system, while limited exposure to microorganisms during childhood increases the risk of developing diseases, such as CD [11].

Disruption of the gut microbiota, also referred to as dysbiosis, is a common feature seen in IBD patients. This includes alterations in the gut microbiota and changes in diversity and abundance of certain microbial species. Dysbiosis can then further drive intestinal inflammation by disrupting the integrity of the intestinal barrier, microbial-host interactions, and disrupting gut metabolic processes. This will cause an imbalance of the gut microbiota and trigger an immune response. Healthy gut bacterial phyla are 99% composed of Firmicutes, Bacteriodetes, Proteobacteria, and Actinobacteria. Specifically, IBD patients are found to have reduced microbial diversity [12]. This change can reduce key functions necessary to maintain intestinal homeostasis and intestinal barrier integrity.

Environmental factors, genetics, diet, and dysbiosis together can contribute to the immune dysregulation seen in IBD and trigger an inflammatory immune response. This response is due to an infiltration of innate and adaptive immune cells that produce more pro-inflammatory than anti-inflammatory cytokines. Immune dysregulation causes an abnormal immune response, regulatory T cell function, imbalanced cytokine production, and an impaired intestinal barrier function. Such pro-inflammatory cytokines include: IFN-γ, TNFα, and IL-17A.

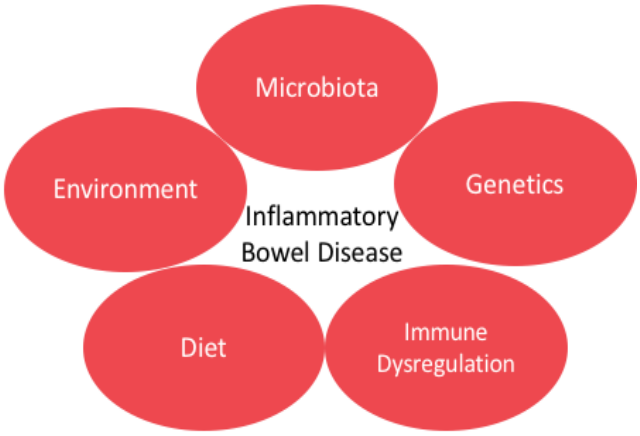

Figure 1: Factors that influence IBD pathogenesis.
A combination of environmental factors, genetics, diet, and dysbiosis play a big role in immune dysregulation and IBD pathogenesis.

1.3 Intestinal Epithelial Cells

The intestinal epithelial barrier acts as a primary layer of defense in the gut [3]. Healthy and stable gut permeability allows for selective molecules to pass through tight junctions Between IECs are proteins called tight junctions (TJ). Tight junctions are composed of transmembrane proteins, that function to control the paracellular passage of molecules. They also play a role in cell proliferation and differentiation and help maintain mucosal homeostasis and barrier integrity and permeability. On one side of the epithelial barrier, is the lumen, which is composed of a mucus layer covering the IECs. Goblet cells produce mucus proteins, and Paneth cells are responsible for antimicrobial peptide (AMP) production. On the other side of the intestinal epithelial barrier is the lamina propria, where innate immune cells reside [13]. IBD is characterized by a compromised intestinal epithelial barrier. Any dysregulation within the intestinal barrier causes this barrier to become “leaky”. Luminal content can then leak into the lamina propria, where antigens are met by innate immune cells that also act as antigen presenting cells (APCs). Some of the predominant APCs in the lamina propria include dendritic cells (DCs) and macrophages [4], which then present the luminal contents to naïve CD4+ T cells.

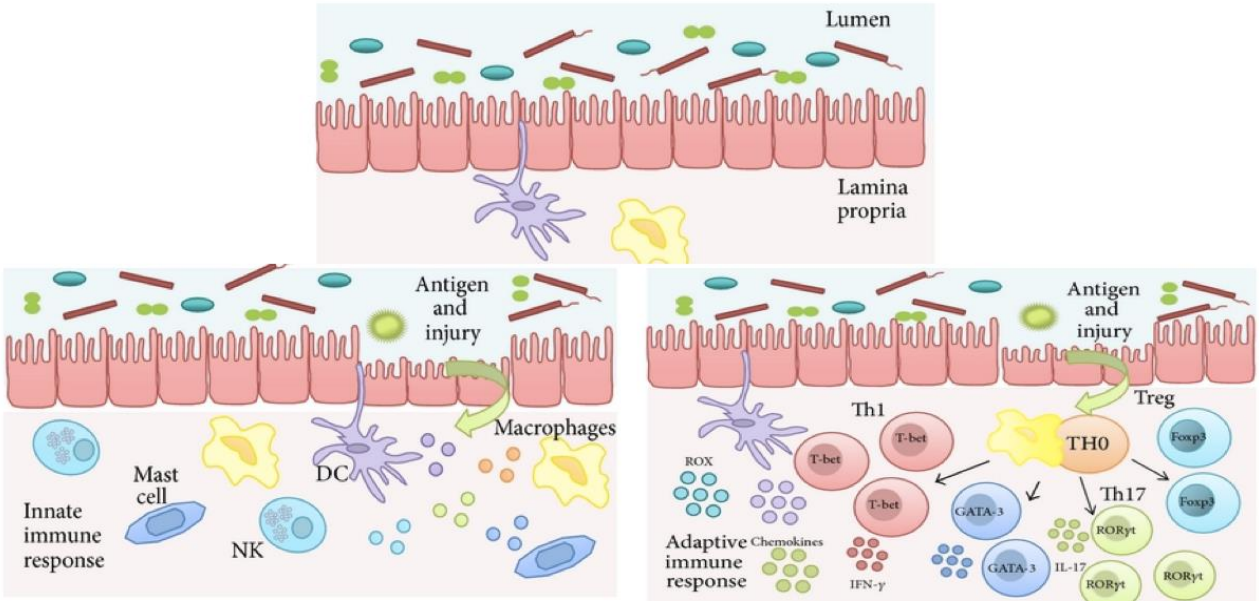

Figure 2: Pathophysiology of IBD.
The lumen is on one side of the intestinal epithelial barrier, and the lamina propria is on the other side. The luminal side is where the microbiota and food antigens reside. The lamina propria is where innate immune cells and adaptive immune cells reside. In IBD, barrier disruption causes luminal content to leak into the lamina propria. APCs present these antigens to naïve CD4+ T cells (Th0), and cytokine production aids in T cell polarization to different Th subsets. Therefore, causing cytokine imbalance and progression to IBD. This image was obtained from [14].

1.4 T Cell Signaling and Cytokine Imbalance in IBD

IBD is heavily mediated by T cell responses, specifically, CD4+ T cells. Once luminal content is presented by APCs to naïve CD4+ T cells, cytokines are produced that allow for T cell differentiation into inflammatory T helper (Th) subsets [15]. The different Th subsets help eliminate different microbial challenges. Th1 cells, especially seen in CD patients, function by producing IFNγ, TNFα, and IL-12. On the other hand, UC is thought to be more of a Th2 mediated response, producing IL-4, IL-5, and IL-13. Th17 cells are also found in IBD patients. This subset plays a role in eliminating extracellular pathogens and maintaining commensal microbiota by primarily producing IL-17A and IL-17F [15]. Th17 differentiation is induced by IL-6 and the transcription factor (TF) TGF. Another IBD-associated Th subset are Th22 cells. These cells are known for their production of IL-22 and expression of the TF T-bet [16]. All the

mentioned subsets and cytokines are classified as pro-inflammatory. Regulatory T cells (T regs) produce anti-inflammatory cytokines. They are known for their inhibitory effects and ability to dampen immune responses. These cells function by producing IL-10 and expressing the transcription factor Foxp3 [13].

While CD4+ T cells are strongly associated with IBD pathogenesis [17], it is the cytokine imbalance that allows the disease to progress [18]. Pro-inflammatory cytokines get produced and secreted in higher amounts than anti-inflammatory cytokines. Therefore, less T cells differentiate into T regulatory cells. Given that this cell population highly contributes to IBD progression, the adoptive T cell transfer model was developed and has been a valuable tool for studying IBD [19]. It mimics the immunological mechanisms seen in human IBD. In order for colitis to develop in this model, naïve CD4+ T cells are isolated from a donor mouse and transferred to an immunocompromised recipient mouse [20], and colitis should develop approximately within a period of 10-12 weeks. While there are chemically induced colitis models, we choose to proceed with the adoptive transfer model, since human IBD is not

chemically induced and does it happen over a short period of time.

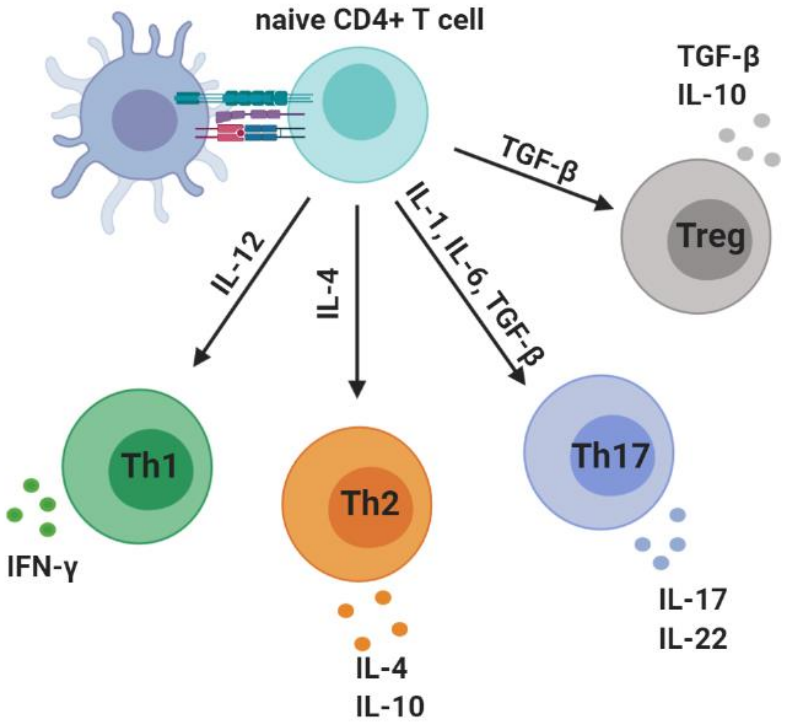

Figure 3: Polarization of naïve CD4+ T cells depends on cytokines secreted.
The production of different cytokines from naïve CD4+ T cells depends on the antigen that was presented. The cytokines produced help Th0 cells differentiate to different T helper subsets to activate different immune functions.
Taken from ImmunoBites.com (made using Biorender).

1.5 IL-36 Cytokine Family

The IL-1 superfamily of cytokines is important in innate and adaptive immunity. This superfamily consists of the following cytokines: IL-1α, IL-1β, IL-18, IL-33, and IL-36. IL-36 includes three agonists: IL-36α, IL-36β, and IL-36γ. These ligands and their receptor IL-36R are expressed mostly on epithelial cells [21], and some of their target cells include intestinal epithelial cells and naïve $CD4^+$ T cells [22]. The binding of IL-36 agonists to their receptor IL-36R causes its dimerization with the accessory protein, IL-1RacP [23], promoting inflammatory signaling in skin, lung, and kidney disease, in addition to IBD. In IBD patients, IL-36〈 and IL36γ levels were found to be elevated. IL-36 signaling promotes inflammation, making IL-36/IL-36R

potential therapeutic targets for IBD.

While IL-36 cytokines can be regulated by their antagonist IL-36Ra, they are also regulated via various mechanisms. IL-36 cytokines are upregulated by cytokines, TLR agonists, or by pathologic conditions in response to skin damage. They are constitutively expressed in normal skin at low levels, but RNAs from damaged cells will activate certain receptors and induce IL-36 expression. In bronchial epithelial cells, IL-36 cytokines can be induced by bacteria, cytokines, viral infections, and smoke. Some IL-36 ligands, like IL-36α and IL-36γ, are upregulated in an autocrine manner by IL-36 in human keratinocytes. In mice, IL-36α in lung tissue and IL-36β in neurons and glial cells are regulated by epidermal growth factor (EGF) [24]. In IBD, IL-36 ligands are expressed by gut cells like lymphocytes, macrophages, and intestinal epithelial cells. Neutrophils release proteases extracellularly to activate IL-36 ligands and make them biologically active, if locally present. Therefore, these enzymes can regulate the activity of these cytokines. The activation of IL-36R then induces NF-kappaB and MAPK in a MYD88 dependent manner, which induces other pro-inflammatory pathways by target cells in the colon, like fibroblasts, IECs, and lymphocytes [25].

Common ways to block IL-36R signaling include anti-IL-36R antibodies and receptor antagonists. Studies using the anti-IL-36R antibody led to the development of drugs like Spesolimab (a humanized monoclonal IgG1 antibody) [7]. This receptor blocking antibody prevents all IL-36 ligands from binding to the receptor [26]. Spesolimab showed clinical efficacy in patients who have the autoimmune disease, psoriasis, in phase I studies. It is currently being tested in CD patients and is in phase II trials in active UC patients [7]. A second common method to block IL-36R signaling is by receptor antagonist IL-36Ra. It is considered to be an

anti-inflammatory cytokine and is expressed in immune cells like DCs. IL-36Ra works by competing with the IL-36R agonists mentioned above and inhibiting NFκB activation. The significance of this antagonist's role was highlighted in several studies. Mutations in the IL-36Ra gene (IL36RN) were linked to several skin disorders. In IL-36rn-/- mice, wound healing was delayed, and an increase in IL-36γ levels was observed [27]. IL-36Ra was even shown to be produced by mucosal epithelial cells, which correlated to its high levels in IBD patients [28]. Moreover, studies show that IL-36 ligands are pathogenic and are characterized by the production of pro-inflammatory cytokines, such as IFNγ [29]. However, the role of IL-36 in IBD pathogenesis is complex and has yet to be fully understood.

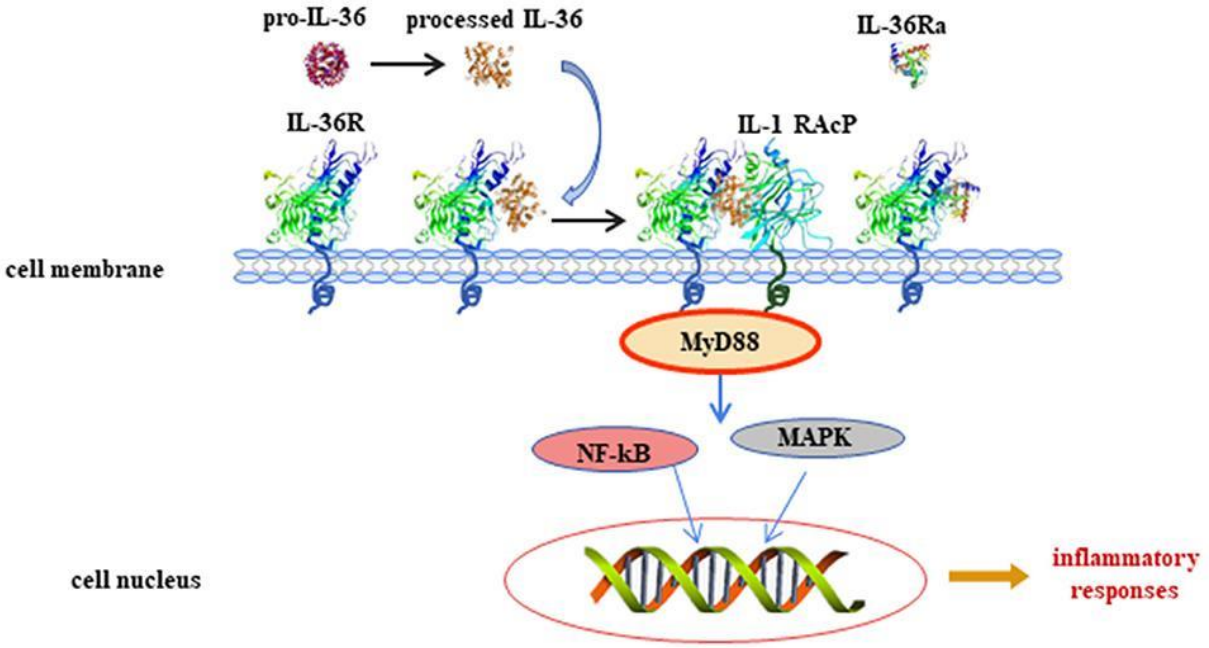

Figure 4: Processing of IL-36 cytokines.
IL-36 ligands are synthesized as precursor proteins indicated as pro-IL-36. Proteolytic cleavage is required to generate their active form. After processing, mature IL-36 can bind to the IL-36R expressed on distinct epithelial cells. The binding triggers intracellular signaling by the recruitment of the accessory protein IL-1RAcP, which activates downstream signaling pathways. This includes NF-κB and MAPK pathways. This figure was obtained from [29].

1.6 Role of IFNγ in Intestinal Inflammation

IBD is characterized by the production of many cytokines, one of the major ones being IFNγ. It is upregulated in CD and UC, as well as experimental murine colitis [29]. IFNγ is produced by several types of immune cells, such as T cells. An IFN signature, increased expression of several IFN-regulated genes, was observed in many autoimmune diseases [31]. In IBD, this pro-inflammatory cytokine contributes to vascular barrier disruption, therefore, worsening colonic inflammation [32]. Due to the disruption in epithelial and vascular barrier, more luminal antigens are translocated to the lamina propria; therefore, exacerbating the immune response. Targeting IFNγ signaling has been proposed as a potential therapeutic strategy for many autoimmune diseases, though its role in IBD pathogenesis needs further investigation. Studies that used IFNγ-/- mice in the DSS colitis models showed no signs of disease pathogenesis [33]. However, the IL-36/IFNγ pathway has not been assessed in the adoptive transfer model of colitis.

1.7 Other Immune Cells in IBD

While the adaptive immune system, specifically T cells, are the main drivers of IBD, the innate immune system is equally important. Macrophages play a vital role in IBD pathogenesis and have also been targeted as therapeutic approaches. These cells reside in intestinal tissue and contribute to innate and adaptive immune responses. When recruited to the site of inflammation, they secrete pro-inflammatory cytokines TNFα, IL-1 β, and IL-6. Together, they contribute to tissue damage and reactive oxygen species (ROS) production [34].

DCs are key players in the mucosal immune system. They act as a bridge between the innate and adaptive immune system by being antigen presenting cells. They present luminal

contents and antigens and present them to T cells, which triggers an immune response. These cells produce pro-inflammatory cytokines like IL-12 and TNF α. DCs also provide co-stimulatory signals and cytokines to enhance the proliferation and differentiation of T cells. There are current studies that target DCs as potential therapeutic strategies [35].

Neutrophils are innate immune cells that are first recruited to a site of injury or infection. They are also the most abundant leukocytes in the blood. Neutrophils are involved in IBD pathogenesis, but the exact mechanism is not fully understood yet. As mentioned previously, the dysregulation of the immune system plays a vital role in the progression of intestinal inflammation. In fact, impaired neutrophil function is involved in the initiation of intestinal inflammation [36]. In the inflamed tissue, neutrophils release ROS, cytokines, and proteases. All these factors contribute to tissue inflammation. Studies that used neutrophil depletion methods gave controversial results. Using neutrophil antibodies in the DSS and T cell transfer colitis models exacerbated disease, suggesting a protective role for these cells [37,38]. However, neutrophils ameliorated colitis symptoms in DSS colitis in another study [39]. Nonetheless, further research is needed to explain the role of neutrophils in colitis.

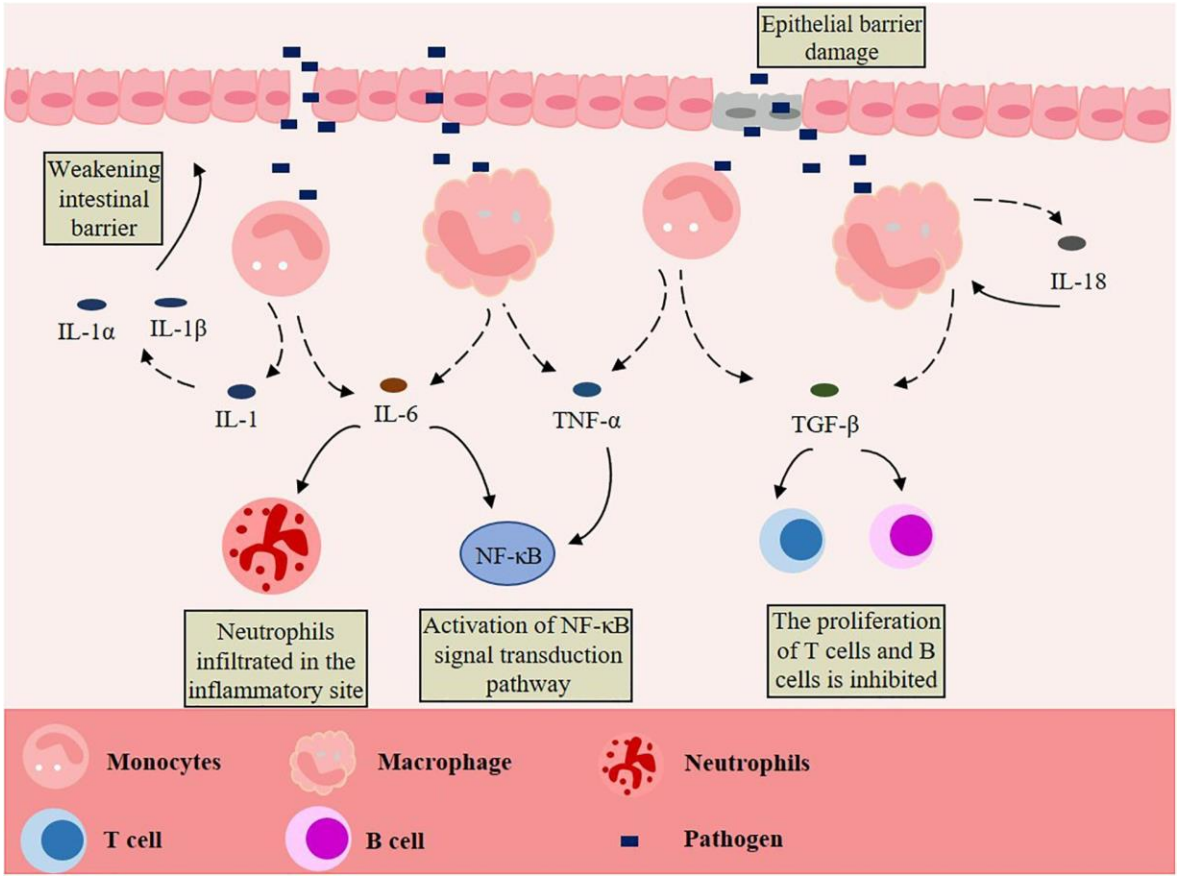

Figure 5: Processing of IL-36 cytokines.
Macrophages, neutrophils, and other innate and adaptive immune cells all contribute to the pathogenesis of IBD by secreting different cytokines. In turn, this promotes more epithelial barrier damage leading to inflammation. This image was obtained from [34].

1.8 Future Perspectives

While significant progress has been made towards managing IBD, understanding the role of IL-36 cytokine family in IBD pathogenesis is crucial, due to the fact that the antagonist IL-36γ is elevated in human IBD and experimental colitis, as well as other inflammatory diseases. While anti-TNfα therapy can induce clinical remission in CD patients [40], the disease ends up relapsing in more than 60% of patients [41]. Cytokine blockers and immunomodulators were also recently developed to treat intestinal inflammation. However, patients can still go into clinical relapse. Therefore, evaluation of IL-36/IL-36R signaling in T cell-mediated colitis will further narrow down therapeutic targets for intestinal inflammation.

2. IL-36γ/IFNγ PATHWAY EXACERBATES INTESTINAL INFLAMMATION DURING EXPERIMENTAL COLITIS

2.1 Introduction

Inflammatory Bowel Disease is characterized by chronic intestinal inflammation. The two types of IBD are Crohn's disease and ulcerative colitis. While CD causes prolonged inflammation to the GI tract, UC primarily affects the colon. Cytokine imbalance due to immune dysregulation plays a big role in the pathogenesis of IBD. There are several current studies that target cytokines and signaling molecules as therapeutics, but none currently exist that are known to cure IBD. For that reason, we continue the search for a therapeutic. In this study, we build on previous studies that tackle IL-36γ. This pro-inflammatory cytokine functions at epithelial barriers, one of them being the intestinal epithelial barrier. We plan on studying the influence of this cytokine on naïve CD4+ T cells and evaluating their role in colitis by using the adoptive transfer model, which involves the transfer of naïve CD4+ T cells from a donor mouse to a Rag-/- mouse that lacks a functional immune system.

2.2 Materials and Methods

2.2.1 Animal Models

The following mice were obtained from the Jackson Laboratory: Wild-type C57BL/6 (B6 WT), Rag-/-, and B6.129S7-*Ifng*^{tm1Ts}/J (IFNγ -/-). B6 and Rag-/- are also bred at GSU DAR. All animal procedures were performed according to the Guide for the Care of Use of Laboratory Animals, under *institutional animal care and use committee* (IACUC) guidelines. All mice involved in experimental procedures were eight to twelve weeks old, both females and males.

2.2.2 Cell Culture, RNA Isolation for QPCR, and Supernatant Harvesting for ELISA

Spleens and lymph nodes were collected from eight-week old wild-type and IFNγ -/- mice, both females and males. These organs were processed into cell suspensions. Splenocytes were resuspended in Ammonium-Chloride-Potassium (ACK) buffer for five minutes to lyse erythrocytes then washed once with PBS. Cells from splenocytes and lymph nodes are combined together to isolate naïve CD4+ T cells via EasySep™ Mouse Naïve CD4+ T Cell Isolation Kit (Stemcell). The enriched cells were checked for their purity by staining for CD4, CD45bRB, and CD25. The desired population is $CD4^{+}CD45RB^{hi}CD25^{-}$. These cells are resuspended in complete medium.

For cell culture, 96-well flat bottom plates are coated with 5 ug/mL αCD3 (BioLegend) and 1 ug/mL αCD28 (BioLegend) and left overnight at 4°C for maximum binding. The plate is washed three times with PBS the next day. Naïve CD4+ T cells are plated at 200,000 cells per well, in the presence or absence of IL-36α, IL-36β, and IL-36γ IL-36γ (R&D Systems) at a concentration of 100 ng/mL. In the latter experiments, IFNγ neutralizing antibody (referred to as αIFNγ) clone XMG.1 (Thermofisher) was used at a concentration of 2 ug/mL. The plate is then placed in the incubator for 48 hours at 37°C.

To harvest supernatant, the 96-well culture plate is spun down at 500 g, 5 minutes, and 4°C. The supernatant is aspirated into 1.5 mL Eppendorf tubes and frozen at -80°C, to be stored for long-term use. All ELISA kits were obtained from Invitrogen and followed according to the manufacturer's protocol; IFNγ, IL-2, IL-12p70, TNFα, and IL-23. For IL-17A ELISA, antibodies were purchased separately; IL-17A Monoclonal Antibody (eBio17CK15A5) as capture, biotinylated eBio17B7 antibody as detection, and recombinant IL-17A as the standard.

The cell pellets were resuspended in the lysis buffer RLT and frozen at -80°C until later use. Total RNA was isolated from cells, according to the QIAGEN RNeasy Mini Kit. After RNA isolation, cDNA was generated using SuperScript IV Reverse Transcriptase kit by Thermofisher.

2.2.3 Adoptive T Cell Transfer Colitis Model

Splenocytes and lymph node cells were harvested from eight-week old wild-type and IFNγ -/- mice, both females and males. Cells were processed and enriched for naïve CD4+ T cell ($CD4^{+}CD45RB^{hi}CD25^{-}$), as described in section 2.2.2 above.

After enrichment/sorting, cells were checked for their purity. This was done by staining for CD4, CD45bRB, and CD25. The desired population is $CD4^{+}CD45RB^{hi}CD25^{-}$. Cells are later resuspended in phosphate buffered saline (PBS) at a concentration of 2.5 million cells/mL. 500,000 cells are transferred to each Rag-/- mouse. Colitis then developed between ten to twelve weeks. Body weight and stool consistency were recorded weekly for all mice, until it was time to sacrifice them.

2.2.4 Hematoxylin and Eosin (H&E) Staining and Histological Score Evaluation

After termination of the diseased mice, their colons were harvested. Pieces of colonic tissue were fixed in 10% formalin at room temperature. Paraffin embedding, sectioning, and H&E staining was performed by HistoWiz.

Histological scoring was performed using a 0-4 scale related to inflammatory infiltrate.

2.2.5 Quantification of Fecal Lipocalin (LCN2) by ELISA

Fecal pellets were collected from mice during the course of disease and frozen at -80°C.To harvest fecal supernatants, the frozen fecal samples were resuspended in PBS at a concentration of 100 mg/mL late afternoon and left at 4°C overnight, to allow the feces to

soften. The next day, these samples were homogenized for 1 minute then centrifuged at maximum speed for 15 minutes at 4°C. The supernatant is aliquoted and frozen at -80°C.

To analyze lipocalin (LCN2) levels, Mouse Lipocalin-2/NGAL DuoSet ELISA kit by R&D Systems was used and followed according to the manufacturer's protocol. Fecal supernatants needed to be diluted with reagent diluent to fit under the standard curve.

2.2.6 Intracellular Cytokine Staining (ICCS)

After euthanizing the sick mice, their colons, spleens, and mesenteric lymph nodes were harvested. To process colon tissue, the tissue was cut for easier cleaning of the feces. Tissue was continuously washed in PBS. The tissue was predigested at 37°C by shaking. Then, enzymatic digestion of tissue pieces was done by adding collagenase and DNAse, also by shaking. This cell suspension is then centrifuged and filtered by running the cell suspension through glass wool.

Spleens were processed as mentioned previously, by lysing erythrocytes with ACK buffer for 5 minutes and washing with PBS. Mesenteric lymph nodes were processed the same way, without the need to lyse for erythrocytes.

The final cell suspensions are resuspended in complete medium and are now ready for ICCS. Desired cells are plated in a tissue culture plate to be stimulated for cytokine production. 50 ng/mL PMA, 1 ug/mL ionomycin, 1:1000 Brefeldin A, and 1:1000 monensin are added to each well and placed in the incubator at 37°C, for 4 hours. The cells are then spun down and stained with 1:1000 L/D dye for 5 minutes at room temperature. After a PBS wash, the cells will be stained for surface antigens CD4, CD44, and Ly6G for 20 minutes in the dark. The cells are spun down again and washed with PBS, then fixed by resuspending in Fixation/Permeabilization

Solution for 20-30 minutes in the dark at room temperature. The cells are spun and washed in PBS, then stained for cytokines for 20-30 minutes in the dark at room temperature. Cytokines fluorochrome antibodies should be diluted in Permeabilization buffer. The cells are later washed with PBS once and resuspended again in PBS for flow cytometer analysis.

2.2.7 qPCR Primers

All primers using for qPCR are listed below. Q-PCR was performed with SYBR Green using a StepOnePlus PCR system (Applied Biosystem). Gene expression was normalized to *GAPDH*.

Primers	5’ Forward 3'	5’ Reverse 3'
Gapdh	CGACTTCAACAGCAACTCCCACTCTTCC	TGGGTGGTCCAGGGTTTCTTACTCCTT
IFNγ	AAATCCTGCAGAGCCAGATTA	GTGGGTTGTTGACCTCAAACT
TNFα	CTACTCCCAGGTTCTCTTCAA	GCAGAGAGGAGGTTGACTTTC
IL-12p40	CAGAAGCTAACCATCTCCTGGTTTG	TCCGGAGTAATTTGGTGCTTCACAC
IL-2	CCTGAGCAGGATGGAGAATTACA	TCCAGAACATGCCGCAGAG

Table 1. Sequences of the QPCR primers used in the study.

2.2.8 Statistical Analysis

All statistical analyses were performed with GraphPad Prism software, version 9.0 (Graphpad Software). ONE-way ANOVA and Tukey’s Multiple Comparison Test or Student’s t test were used to determine significance. *P < 0.05, **P < 0.01, ***P < 0.001; ****P<0.0001; ns= not significant.

2.3 Results

2.3.1 IL-36*γ* Increases Naïve CD4+ T Cell Proliferation, Clustering, and IFN*γ* Production *In Vitro*

Previous studies have shown that IL-36β can increase naïve CD4+ T cell proliferation and induce pro-inflammatory cytokine production and Th1 polarization [42]. We choose to investigate the influence of the three IL-36 ligands, IL-36α, IL-36β, and IL-36γ on naïve CD4+ T cells. Splenocytes were harvested from B6 mice, and naïve CD4+ T cells were isolated. These cells were stimulated with αCD3/αCD28 for 48 hours in the presence or absence of any of the IL-36 ligands at 200,000 cells per well. As a negative control, some cells were left unstimulated on the plate. All other cells were stimulated with αCD3/αCD28, with or without cytokine. The cells were counted at 48 hours under a hemocytometer, where we observed an increasing cell number, especially in cultures that were stimulated with IL-36γ (Fig.8.1A).

Cells were collected for RNA isolation and supernatants were harvested at 48 hours. The Th1 cytokine IFNγ was evaluated in supernatants, and we observed significant induction of the cytokine from IL-36γ stimulated cells (Fig.8.1B), compared to the control sample (cells stimulated with αCD3/αCD28) and samples that received IL-36α or IL-36β. We chose to take a deeper look at our protein of interest, IL-36γ, and confirmed the robust IFNγ induction by measuring mRNA expression (Fig.8.1C).

To continue our focus on IL-36γ, we looked at these *in vitro* cultures under a microscope. After 48 hours, there was a notable T cell clustering in the control sample than the unstimulated cell sample. However, IL-36γ-stimulated cells particularly showed more T cell clustering and expansion (Fig.8.2A). This was also confirmed by checking for the activation

marker CD44 by flow cytometry. The plots show a major increase in CD4+CD44+ expression on the highly activated IL-36γ cells (Fig.8.2B).

To start testing the relationship between IL-36 signaling and IFNγ in naïve CD4+ T cells, IFNγ was blocked using a neutralizing monoclonal antibody at a concentration of 2 ug/mL for 48 hours. As a control, an isotype antibody was added to these cultures at the same concentration. Two additional controls were used: some cells only received the isotype (referred to as Isotype), and some only received the neutralizing antibody (referred to as αIFNγ). All cells were plated on wells pre-coated with αCD3/αCD28. These cultures were stimulated in the presence of IL-36α, IL-36β, or IL-36γ, and cell number was counted under a hemocytometer, as mentioned previously. Cytokines that were evaluated included TNFα, IL-12p70, and IL-23.

The cell counts revealed that IL-36γ induced more cell proliferation than IL-36α or IL-36β in the presence of the isotype, even when IFNγ was neutralized (Fig.8.3A). While the cell number was slightly higher in the IL-36γ culture that received the neutralizing antibody, that change was not statistically significant.

Because the IFNγ neutralizing antibody used was clone XMG.1, the efficacy of the neutralizing antibody was assessed by an ELISA for another IFNγ clone, AN-18. The results showed that the antibody was effective in neutralizing any secreted IFNγ, when compared to cultures where the isotype antibody was added (data not shown).

In the presence of the isotype, IL-36α and IL-36β showed modest induction of TNFα; while IL-36γ induced TNFα in significant amounts. However, blocking IFNγ reduced the secretion of TNFα (Fig.8.3B). Lastly, IL-12p70 and IL-23 induction was assessed via ELISA. While blocking IFNγ reduced the levels of IL-12p70 produced by stimulated naïve CD4+ T cells in the

presence or absence of IL-36α and IL-36β, IL-36γ actually increased IL-12p70 concentrations with αIFNγ (Fig.8.3C). IL-23 was also secreted at low levels, but the results show that the stimulation with any of the IL-36 ligands suppresses IL-23 production (Fig.8.3D). Nonetheless, the concentrations of IL-12p70 and IL-23 observed were both very low and basal.

Overall, this data suggests that IL-36γ induces a pro-inflammatory pathway that involves IFNγ and TNFα, where IFNγ is the upstream driver of this pathway.

2.3.2 Transfer of IL-36*γ* Stimulated Naïve CD4+ T Cells to Rag-/- Mice Exacerbates T Cell-Mediated Colitis

Next, we evaluated the impact of the robust IFNγ secretion by IL-36γ on T cell-mediated colitis. The activated cells, in the presence or absence of IL-36γ, were transferred to Rag-/- mice. As a control, unstimulated naïve CD4+ T cells were transferred to Rag-/- mice. Weight, activity, stool consistency, and fecal LCN2 levels were monitored over the span of 10 weeks. The mice that were transferred with activated cells displayed much lower activity than the mice that received the unstimulated cells. Mice that received cells stimulated with IL-36γ cells also developed softer stool and secreted the highest fecal LCN2 levels, when checked by ELISA (Fig.8.4A). At time of sacrifice, the spleens, mesenteric lymph nodes, and colons were collected for analysis. Colons from the IL-36γ group were shorter in length than the other two groups and appeared to be more thickened and inflamed. Moreover, it is obvious that the unstimulated group colons portray distinct fecal pellets, the stool progressed to a softer consistency in the other two groups, especially in the group that was treated with IL-36γ-stimulated cells (Fig.8.4B). The latter also developed a visibly enlarged spleen (Fig.8.4C). H&E staining showed

that the same group had the most leukocyte infiltration (Fig.8.4D), and therefore, the highest histological score (Fig.8.4E).

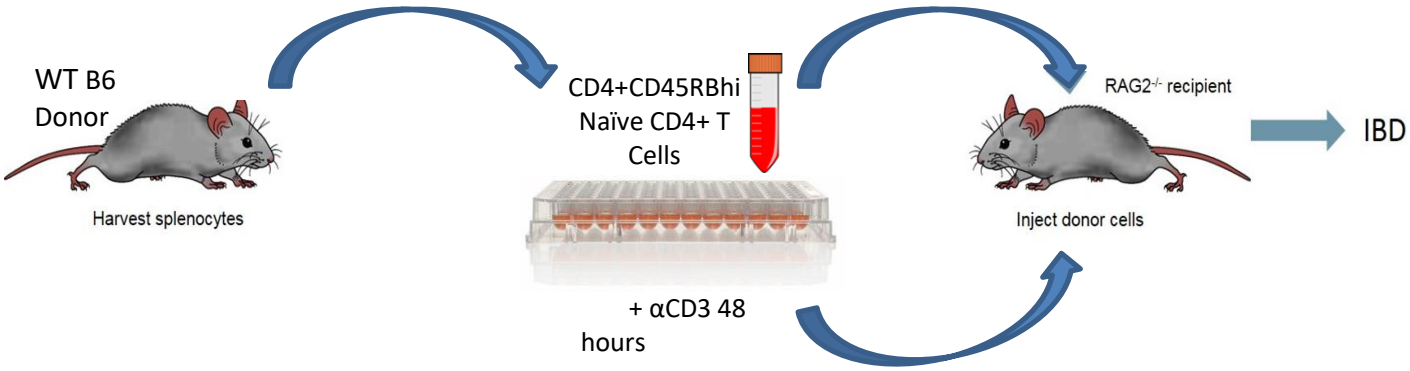

Figure 6: Stimulating T cells in vitro before their transfer to Rag-/- mice.

2.3.3 IFNγ-/- Naïve CD4+ T Cells Stimulated with IL-36γ Produce Less TNFα and IL-12

To test the link between IL-36γ and IFNγ, IFNγ-/- mice were used. Their splenocytes were harvested, and naïve CD4+ T cells were isolated. The cells were also stimulated *in vitro* with αCD3/αCD28 in the presence or absence of IL-36γ, as mentioned in the conditions above. For a control sample, some cells were left unstimulated. After 48 hours, the cells were counted. The cell number corresponded with the data collected using αIFNγ. Cells proliferated the most when stimulated with IL-36γ (Fig.8.5A). TNFα and IL-12p40 mRNA expression was evaluated to check whether the absence of the IFNγ gene influences pro-inflammatory cytokine production. Whether with or without IL-36γ stimulation, IFNγ-/- cells suppressed the production of TNFα and IL-12p40 (Fig.8.5B and Fig.8.5C). The data suggests that IFNγ, TNFα, and IL-12p40 work in a chain, and IFNγ is upstream of that pro-inflammatory pathway.

2.3.4 The Transfer of IFNγ-/- Naïve CD4+ T Cells Does Not Induce Robust Colitis

Next, we determined the influence of IFNγ on intestinal inflammation by transferring IFNγ-/- naïve CD4+ T cells to Rag-/- mice. Prior to the transfer, these cells were stimulated with

αCD3/αCD28 *in vitro* in the presence or absence of IL-36γ. As a control, WT naïve CD4+ T cells were also stimulated in the same conditions. The mice were monitored over the span of 12 weeks for their activity, weight, stool consistency, and LCN2 levels. Over time, it was obvious that mice transferred with WT cells +/- IL-36γ became less active and physically weaker, some even developing lethargy. On the other hand, the mice that received IFNγ-/- cells, in the presence or absence of IL-36γ, remained active. Mice that received WT+IL-36γ cells lost the most weight, followed by WT group. On the other hand, mice that received the knockout cells had minimal weight loss (Fig.8.6A). Stool samples were checked for LCN2 levels. WT+IL-36γ group had the highest LCN2 concentrations. Surprisingly, the group with the second highest LCN2 levels was IFNγ-/- +IL36γ. The other two groups, WT and IFNγ-/-, had mild fecal LCN2 (Fig.8.6B). Knowing that LCN2 is produced by neutrophils in colitis models [32], we stained for Ly6G, a neutrophil surface marker. Flow cytometry results showed higher neutrophil infiltration in the WT+IL-36γ and IFN-/- +IL-36γ spleens (Fig.8.7A). While that trend was not the same in colonic tissue, WT+ IL-36γ colons still had the most neutrophil infiltration (Fig.8.7B), confirming that IL-36γ in the presence of the IFNγ gene causes the most robust colitis.

After sacrificing the mice, their colons and spleens were harvested for further analysis. The colons of WT receiving groups were noticeably shorter in length, especially WT+IL-36γ (Fig.8.6C). This was accompanied by a visibly enlarged spleen (Fig.8.6D). Histological staining showed the most thickening of the submucosa and leukocyte infiltration in WT+IL-36γ colons out of the four groups (Fig.8.7A). The same colon tissue was stained for CD4 by immunohistochemistry staining, where both groups that received IL-36γ-stimulated cells had

the most CD4 infiltration (Fig.8.7B). Therefore, WT+IL-36γ colons received the highest histological score (Fig.8.7C).

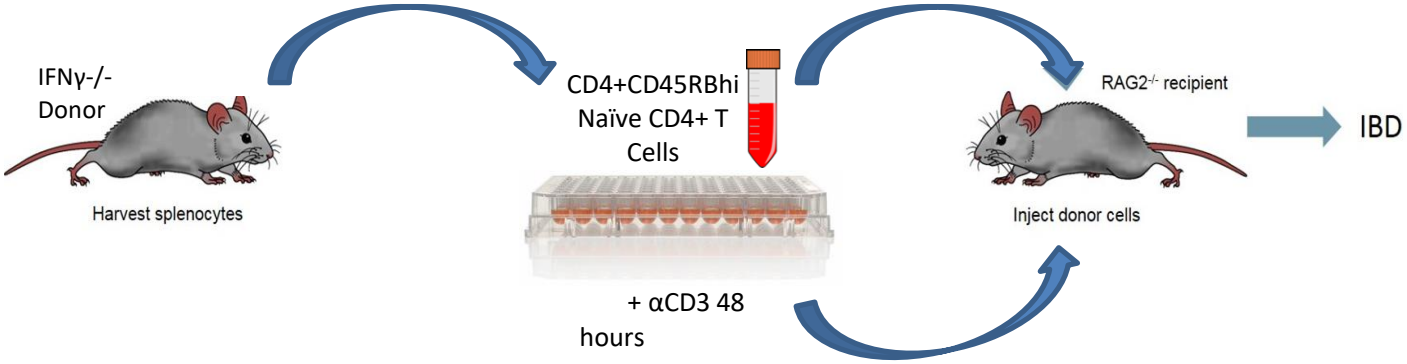

Figure 7: Stimulating IFNγ-/- T cells in vitro before their transfer to Rag-/- mice.

2.3.5 IL-36γ Stimulated IFNγ-/- Cells Induce Robust IL-17A Responses in T cells

After confirming that neutrophils exist at higher frequencies in the spleens of IL-36γ stimulated cell transfers, we checked for other Th lineages that produce cytokines, that induce neutrophil-recruiting chemokines, such as Th17. We checked for IL-17A production in naïve CD4+ T cells *in vitro* for WT and IFNγ-/- cultures. While IL-17A was produced in significant amounts in IL-36γ stimulated cells, but IL-17A concentration was exceptionally high from IFNγ-/- + IL-36γ cells (Fig.8.9A). Further research needs to be done to validate these results.

2.4 Discussion

Cytokines are signaling molecules that play a crucial role in the pathogenesis in IBD. They are secreted by various immune cells in the gut to regulate different functions of the immune system. One cytokine that is observed to be elevated in IBD and experimental colitis is IL-36γ, which is a ligand in the IL-36 family. This cytokine is found to be elevated in other diseases where epithelial cells are impacted, such as the skin condition psoriasis [45,46]. When it comes to IL-36γ signaling, some studies show a pathogenic role, while other studies found that IL-36γ can aid in healing of the disease. A study from our lab suggests a protective role for IL-36γ in

the DSS colitis model by helping in barrier restoration [16]; however, another study from our lab suggests a pathogenic role for the cytokine in a more T cell mediated colitis model, using oxazolone [47]. Since IBD is heavily mediated by T cell responses, we investigate the influence of IL-36γ on naïve CD4+ T cells and in the adoptive transfer model, which more closely resembles IBD seen in humans.

When T cells are stimulated with antigens, they produce cytokines that can either be pro-inflammatory or anti-inflammatory. During IBD, there are more pro-inflammatory than anti-inflammatory signals being secreted. Therefore, this worsens and progresses inflammation in the colon. The cytokines secreted by T cells determine T cell differentiation. They can differentiate to Th1, Th2, Th17, or T reg cells. These subsets help eliminate different microbial challenges. Two main subsets are observed during IBD, Th1 and Th17. Th1 responses are characterized by the production of IFNγ and IL-12; while Th17 responses can be identified by the production of IL-17 and IL-23. These cytokines are classified as pro-inflammatory and contribute to intestinal tissue damage in IBD, as well as immune cell recruitment.

Our results demonstrate that stimulating naïve CD4+ T cells with IL-36γ significantly induced cell proliferation and expansion. IL-36γ-stimulated cells also produced high concentrations of IFNγ. We investigate the IL-36γ/IFNγ axis by transferring naïve CD4+ T cells stimulated with IL-36γ to immunocompromised Rag-/- mice. These mice lost weight over time, and their stools had a looser consistency, in addition to elevated levels of fecal LCN2. Their colons were shorter in length, had a thicker epithelium, higher histological scores, and more leukocyte infiltration. Since this suggests a critical for IFNγ in T cell mediated colitis, we continued the investigation by using naïve CD4+ T cells from IFNγ deficient mice, using the

same method as WT mice. These cells were also stimulated *in vitro*, under similar conditions as WT cells, with IL-36γ. Two other pro-inflammatory cytokines were looked at, IL-12 and TNFα. When comparing these cytokine secretion levels as well as their mRNA expression, IFNγ deficient cells produced very little IL-12 and TNFα. This suggests a cytokine network of IFNγ/TNFα/IL-12, in the presence of IL-36γ.

IFNγ-/- naïve CD4+ T cells stimulated with IL-36γ were also transferred to Rag-/- mice. Rag-/- mice that were transferred with IFNγ deficient naïve CD4+ T cells, whether with or without IL-36γ stimulation, did not lose a significant amount of weight. Their activity remained healthy throughout the course of the experiment, with no signs of illness of lethargy. Lipocalin levels were assessed at time of sacrifice. WT+IL-36γ had the highest fecal LCN2 concentrations. IFNγ-/- +IL-36γ cell recipients showed a low induction of fecal LCN2 levels, but these levels were minimal. Given that neutrophils are one of the main hematopoietic cell subtypes that are known to produce LCN2 [36] and knowing that Th17 is a major subset involved in IBD, we looked at IL-17A, which enhances neutrophilic chemokines [43]. We found that IL-36γ induces IL-17A production, and the absence of the IFNγ causes T cells to switch from a Th1 response to a Th17 response. In fact, an arthritis study that looked at the IFNγ/IL-17A axis used IFNγ-/- CD4+ T cells. Proteoglycan-induced arthritis (PGIA) depends on the production of IFNγ. Their results demonstrated that the absence of IFNγ caused a reduction in arthritis and also exacerbated the IL-17A response [47]. In colitis, a study found that IL-17 production could be behind altered immune responses [48].

When comparing the spleens and colonic lamina propria from our experimental groups,

the spleen. However, IL-36γ recruited more neutrophils to the WT colon than the IFNγ-/- colon.

Therefore, we conclude that the elevated fecal LCN2 levels from the WT+IL-36γ are due to a robust Th1 IFNγ response, in addition to IL-17A possibly recruiting neutrophils. Moreover, we suspect that the induced fecal LCN2 levels in IFNγ-/- +IL-36γ recipients is solely due to IL-17A production. However, further studies need to be done to fully understand the mechanism.

2.5 Figures

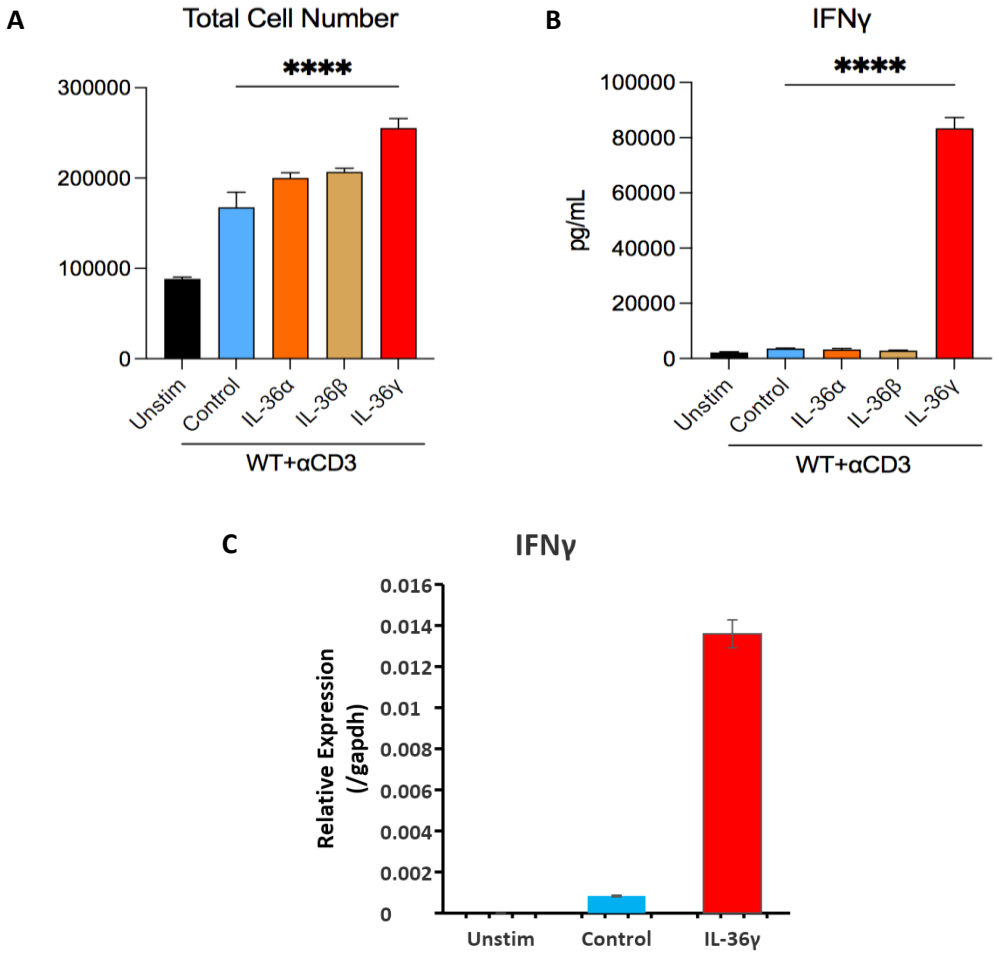

Figure 8.1: IL-36γ induces the most naïve CD4+ T cell proliferation and IFNγ production.
(A) Naïve CD4+ T cell clustering and expansion was compared among 5 conditions conditions: Unstimulated, αCD3, αCD3+IL-36α, αCD3+IL-36β, and αCD3+IL-36γ by counting their cell number under a hemacytometer. (B) Supernatants from these cultures were checked for the pro-inflammatory cytokine IFNγ by ELISA. (C) IFNγ expression was checked by qPCR to confirm the high induction observed by ELISA in IL-36γ cultures. All data are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001; ****P<0.0001; n.s.= not significant, one-way ANOVA with Tukey's multiple comparison test.

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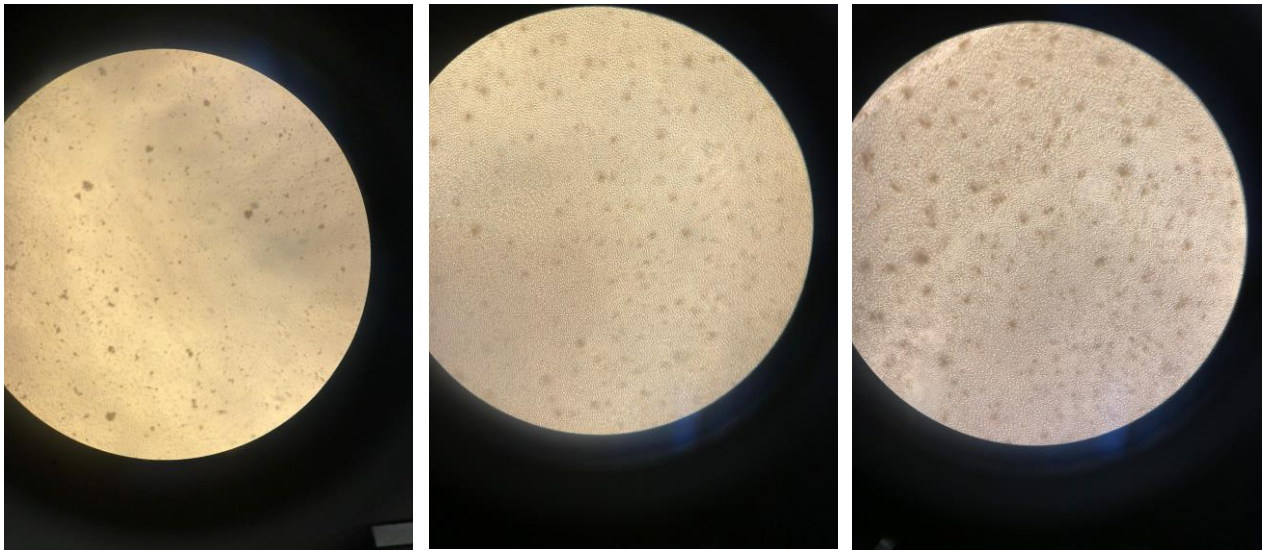

WT WT+αCD3 WT+αCD3/IL-36γ

B

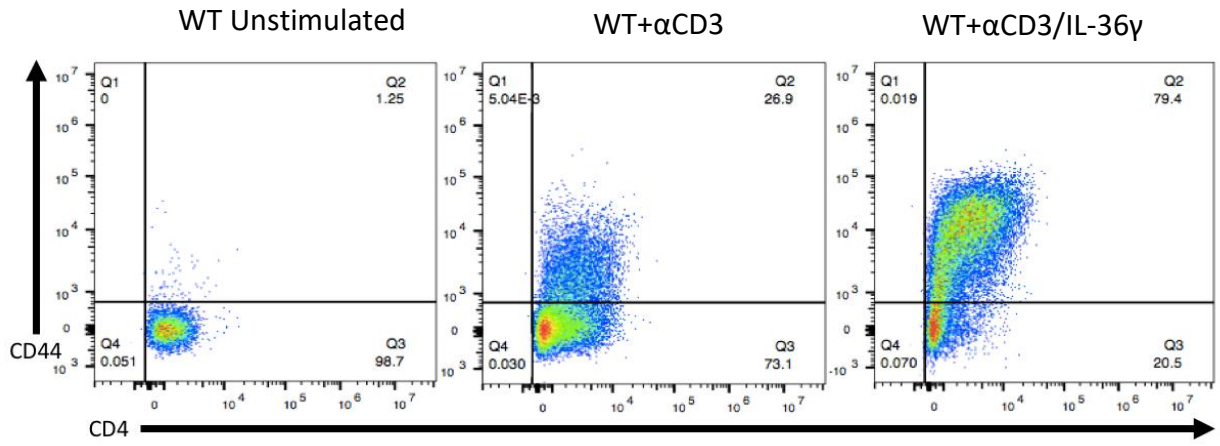

Figure 8.1: IL-36γ increases naïve CD4+ T cell expansion and activation.
(A) Naïve CD4+ T cells were checked under the microscope after 48 hours, where T cell clustering was clear in the presence of IL-36γ. (B) The expression of the activation marker CD44 was evaluated after 48 hours, which was found to be highly expressed on cells stimulated with IL-36γ.

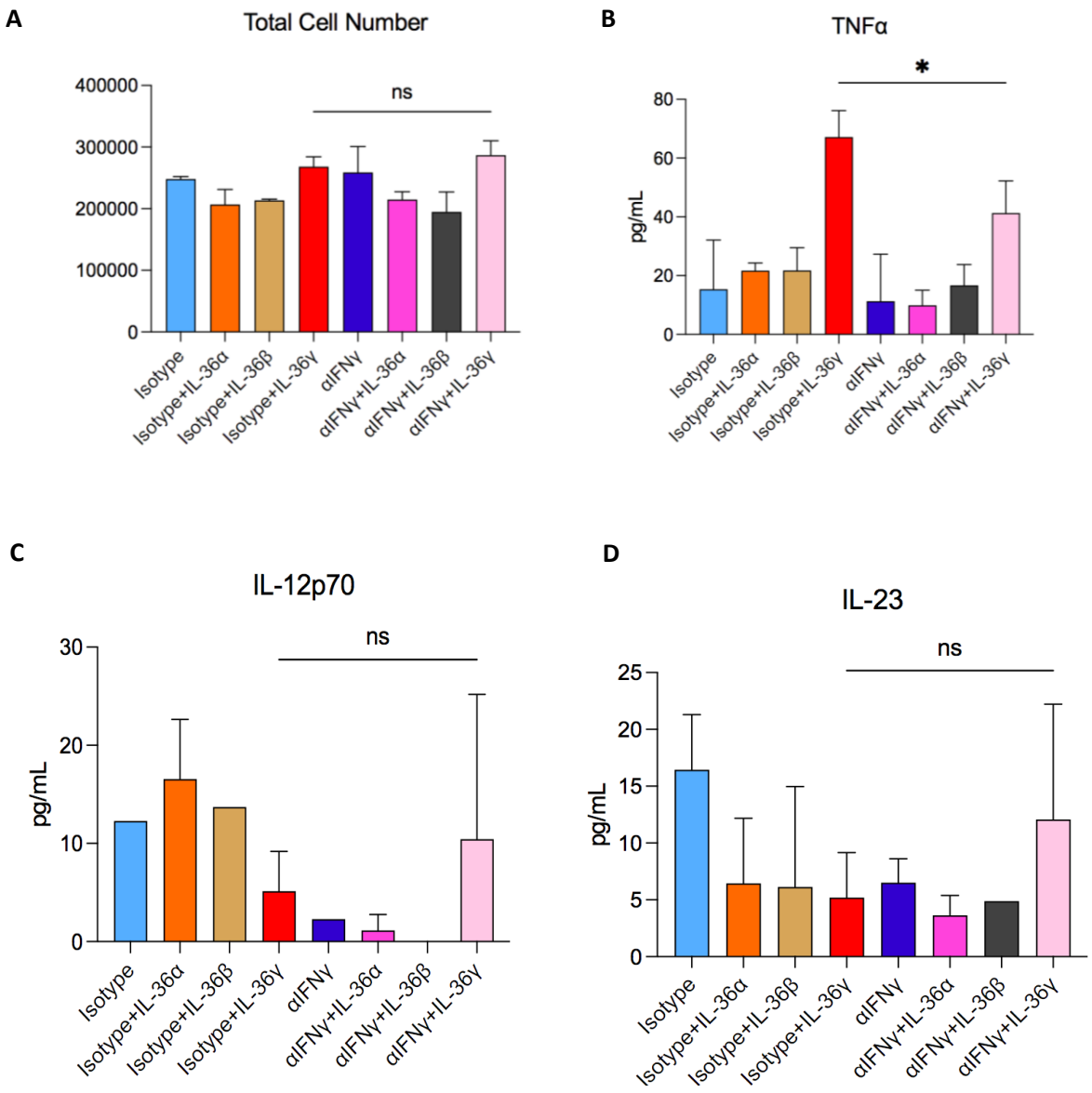

Figure 8.2: Neutralizing IFNγ reduces TNFα without significantly effecting cell proliferation. (A) The cell number remained approximately the same as the cultures that received the neutralizing antibody, except in IL-36γ-stimulated cells, where cell number actually slightly increased. (B) Cultures that received the neutralizing antibody had a reduction in TNFα production. (C) IL-12p70 concentrations checked from cell supernatants by ELISA. (D) IL-23 concentrations were evaluated by ELISA. All data are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001; ****P<0.0001; n.s.= not significant, one-way ANOVA with Tukey's multiple comparison test.

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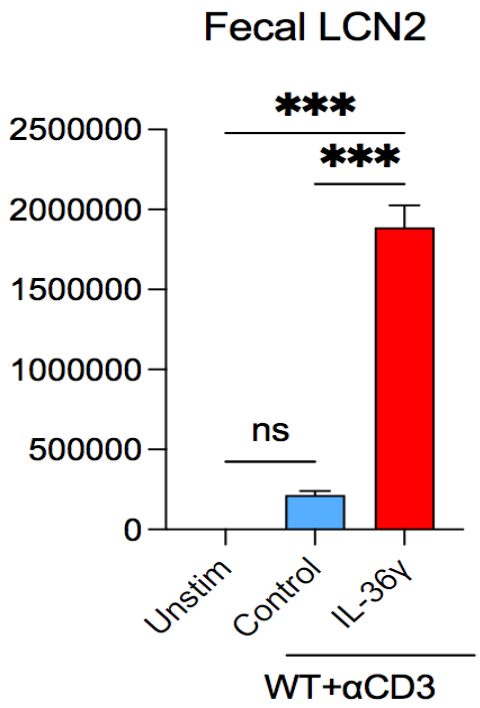
Fecal LCN2
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IL-36γ
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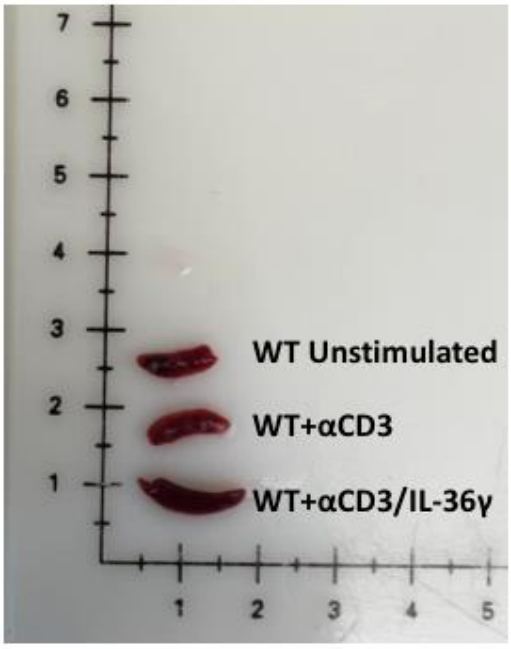
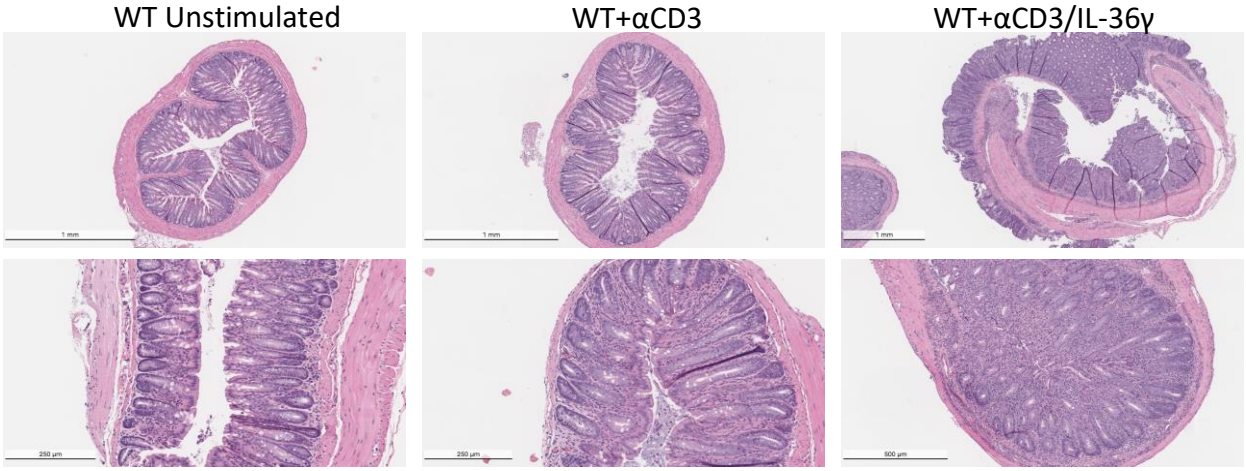
B


WT Unstimulated
WT+αCD3
WT+αCD3/IL-36γ

C


WT Unstimulated
WT+αCD3
WT+αCD3/IL-36γ

D



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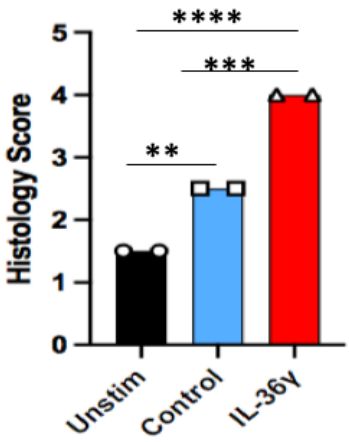

Figure 8.3: The transfer of IL-36γ stimulated naïve CD4+ T cells to Rag-/- mice induces robust colitis.

(A) Lipocalin 2 (LCN2), a colitis inflammatory biomarker, was measured by enzyme-linked immunosorbent assay (ELISA) from fecal samples at time of sacrifice from mice that were adoptively transferred with unstimulated naïve CD4+ T cells or naïve CD4+ T cells stimulated with αCD3 +/- IL-36γ. (B&C) Images of spleens and colons collected from the mice, upon euthanization, show an enlarged spleen and a shorter colon length, along with thickened tissue in mice that received naïve CD4+ T cells stimulated with IL-36γ. (D) The H&E staining of colon sections from the mentioned mice groups is shown. (E) Histology scoring of colon sections from mice that were treated as mentioned. A scale of 0-4 for inflammatory infiltrate was used (0=none, 1=low, 2=moderate, 3=high, 4=maximal). All data are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001; ****P<0.0001; n.s.= not significant, one-way ANOVA with Tukey's multiple comparison test.

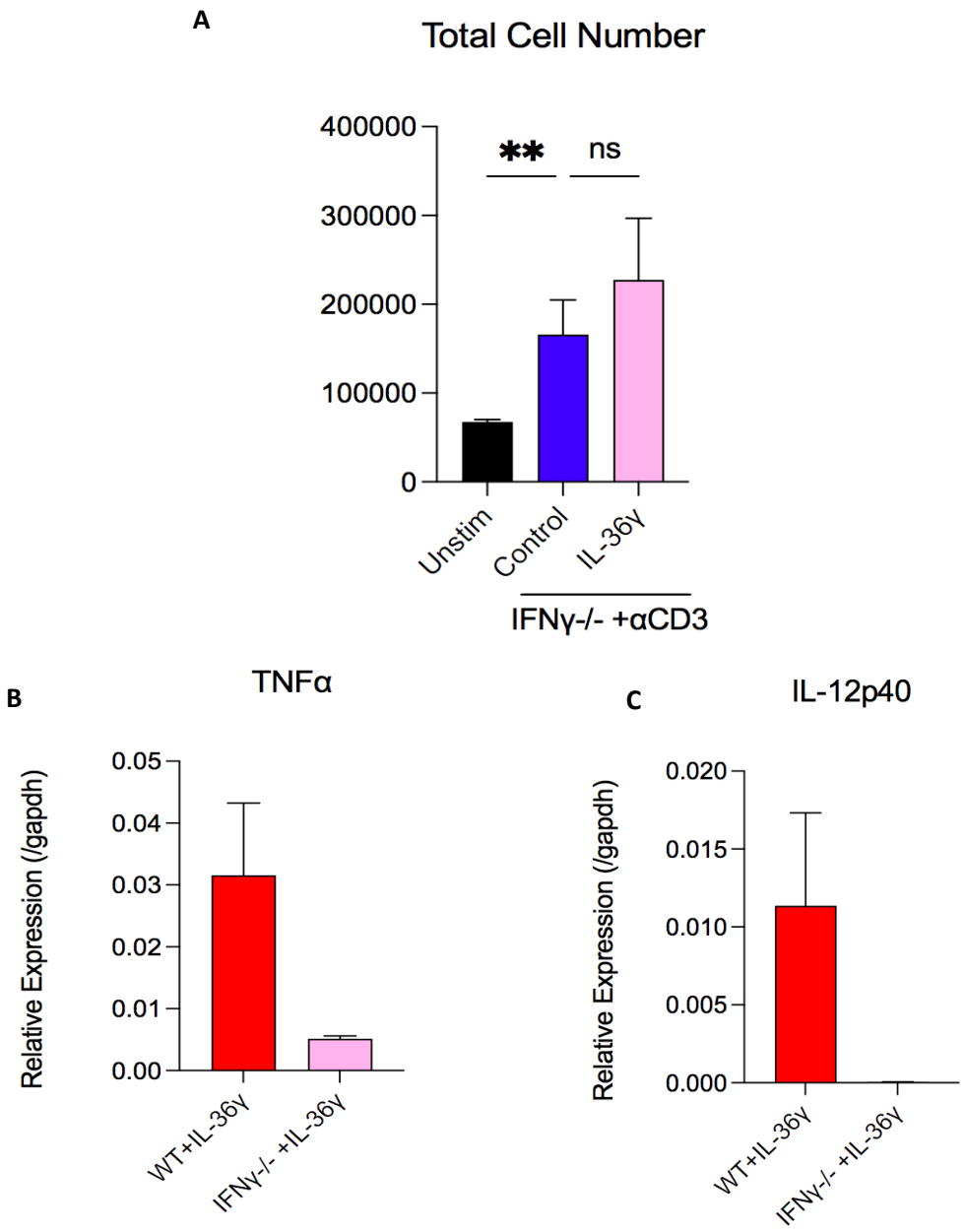

Figure 8.4: While IL-36γ still promotes cell proliferation even in IFNγ-deficient naïve CD4+ T cells, but the absence of IFNγ reduces the production of other pro-inflammatory cytokines.
(A) Cell numbers of IFNγ-/- naïve T cells (unstimulated or stimulated with αCD3 +/- IL-36γ for 48 hours) were counted under a hemocytometer, using Trypan Blue dye to check for viability. (B) To test the relationship between IL-36γ and IFNγ, we looked at WT and IFNγ-/- cells stimulated with IL-36γ and evaluated them for other pro-inflammatory cytokines. The absence of IFNγ decreased TNFα production, as well as IL-12p40 (C). All data are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001; ****P<0.0001; n.s.= not significant, one-way ANOVA with Tukey's multiple comparison test.

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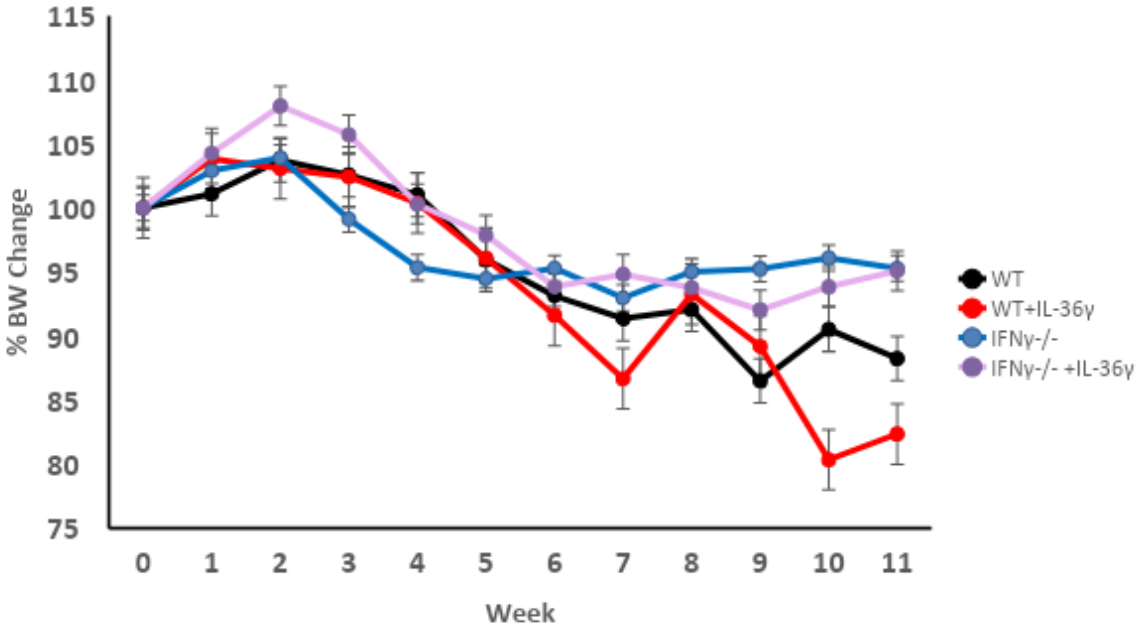
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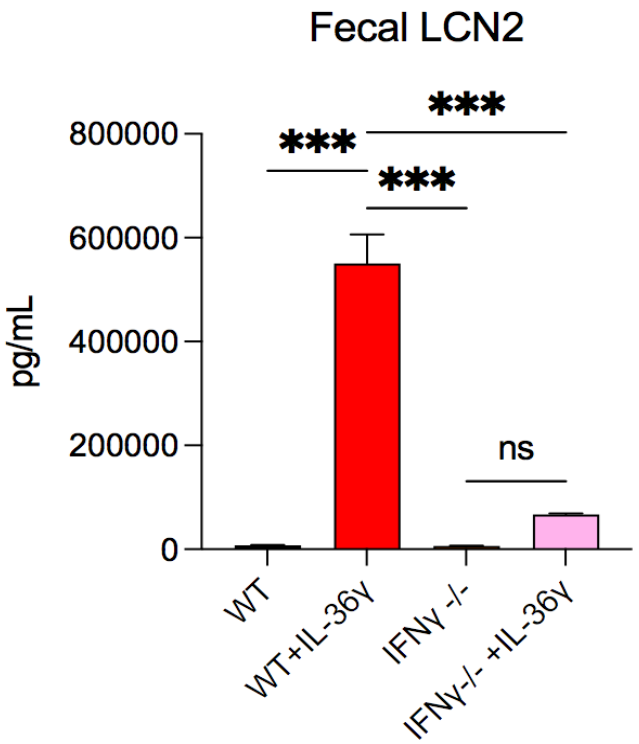
Fecal LCN2
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WT
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IFNγ-/- +IL-36γ

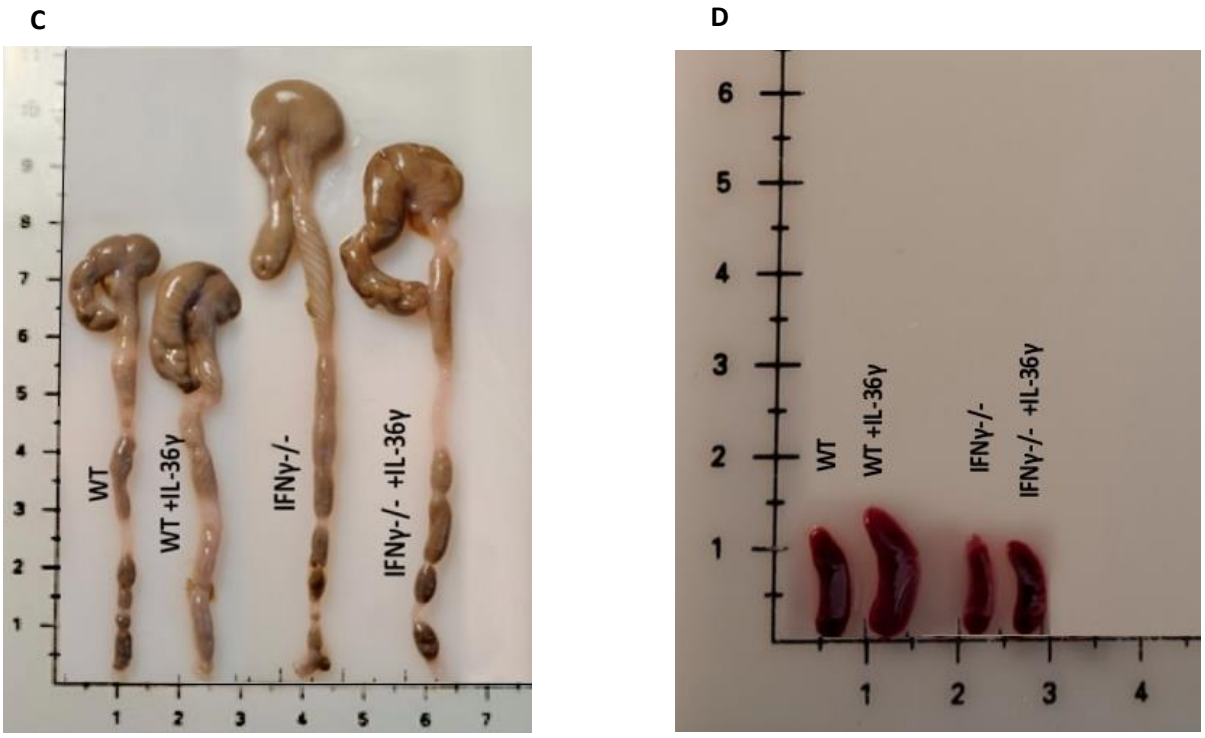

Figure 8.5: The transfer of IFNγ-/- naïve CD4+ T cells causes reduced colitis and fecal LCN2.
(A) Naïve CD4+ T cells were isolated from WT or IFNγ -/- and stimulated in vitro with αCD3, in the presence or absence of IL-36γ for 48 hours- before transferring them to Rag-/- mice. Their weights were monitored over the course of disease. (B) Levels of fecal LCN2 levels were evaluated at time of sacrifice by ELISA. (C) Image of the harvested colons show that mice that received cells stimulated with IL-36γ cause the most inflammation to the colon, making it the shortest in length. (D) Images of the harvested spleen, show an enlarged spleen for the IL-36γ group. All data are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001; ****P<0.0001; n.s.= not significant, one-way ANOVA with Tukey's multiple comparison test.

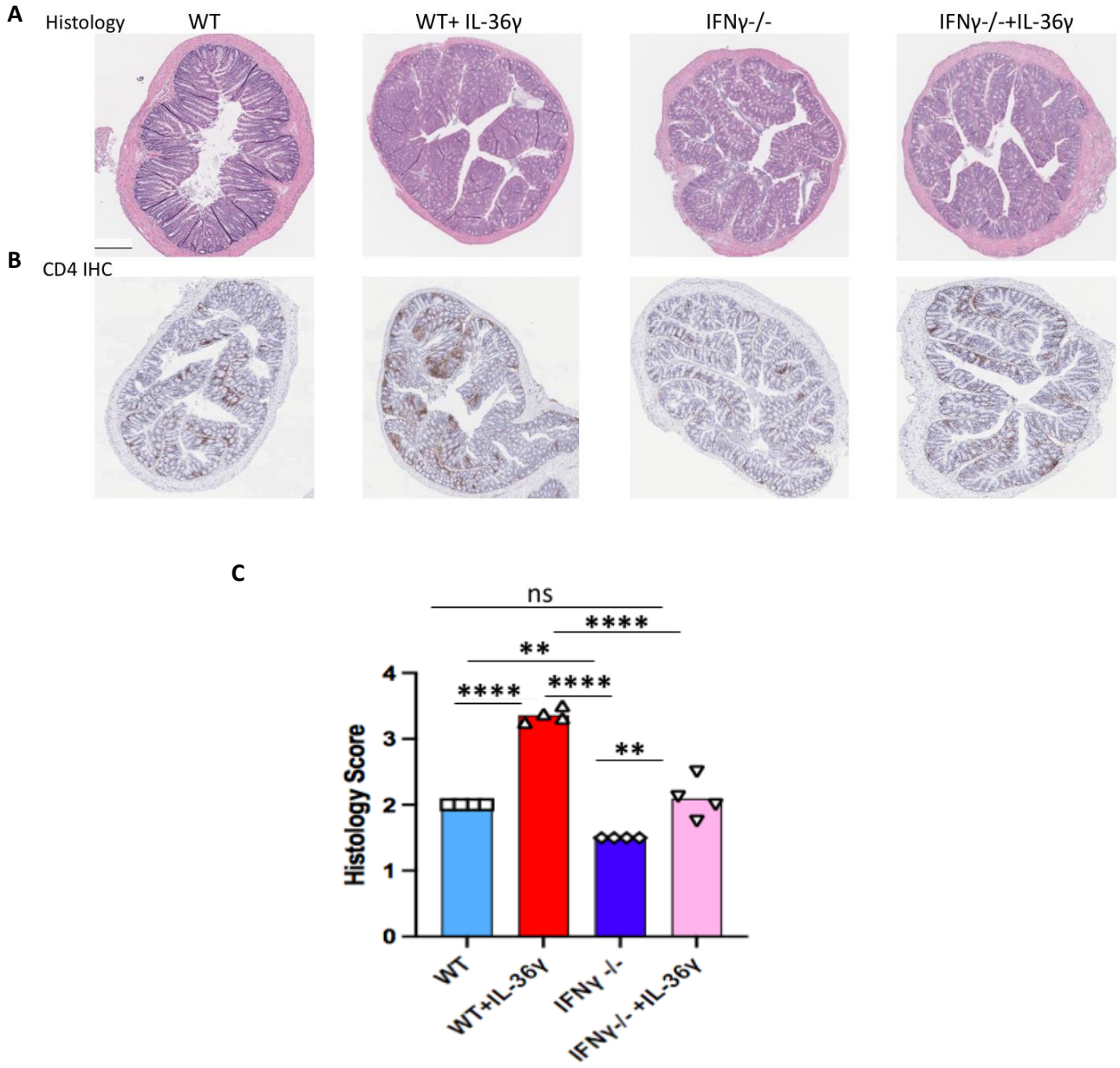

Figure 8.6: IL-36γ-stimulated naive CD4+ T cells induces robust leukocyte infiltration and IFNγ dependent T cell mediated colitis.

(A) H&E staining of colon sections from mice that received WT or IFNγ-/- naïve CD4+ T cells, in the presence or absence of IL-36γ. All cells were primarily stimulated with αCD3. (B) Immunohistochemistry staining for CD4, of the same colonic tissue samples. (C) Histology scoring of colon sections from the same mice. A scale of 0-4 for inflammatory infiltrate was used (0=none, 1=low, 2=moderate, 3=high, 4=maximal).

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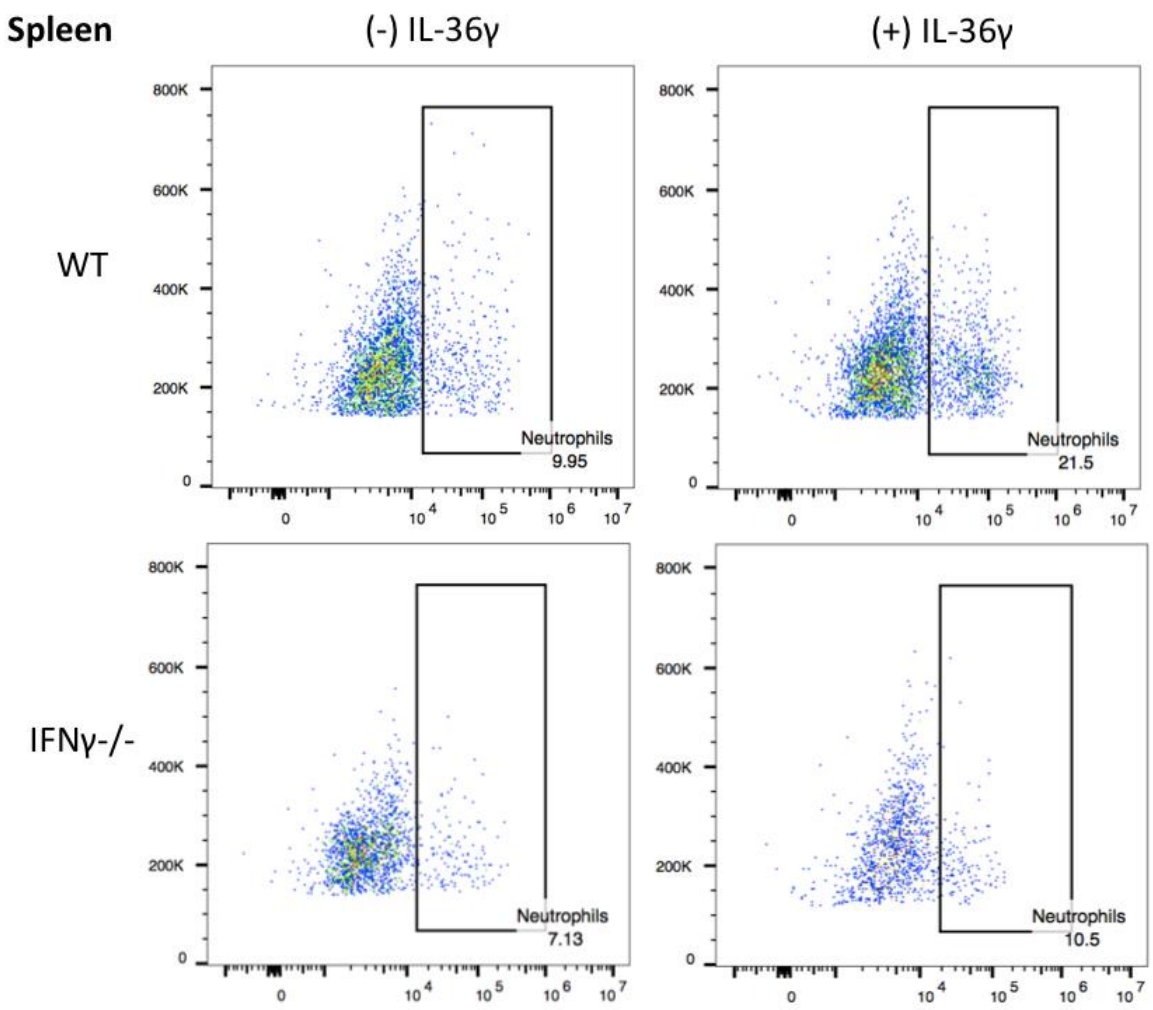
Spleen
(-) IL-36γ
(+) IL-36γ
WT
IFNγ-/-
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600K
400K
200K
0
Neutrophils
9.95
Neutrophils
21.5
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7.13
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10.5
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10^4
10^5
10^6
10^7

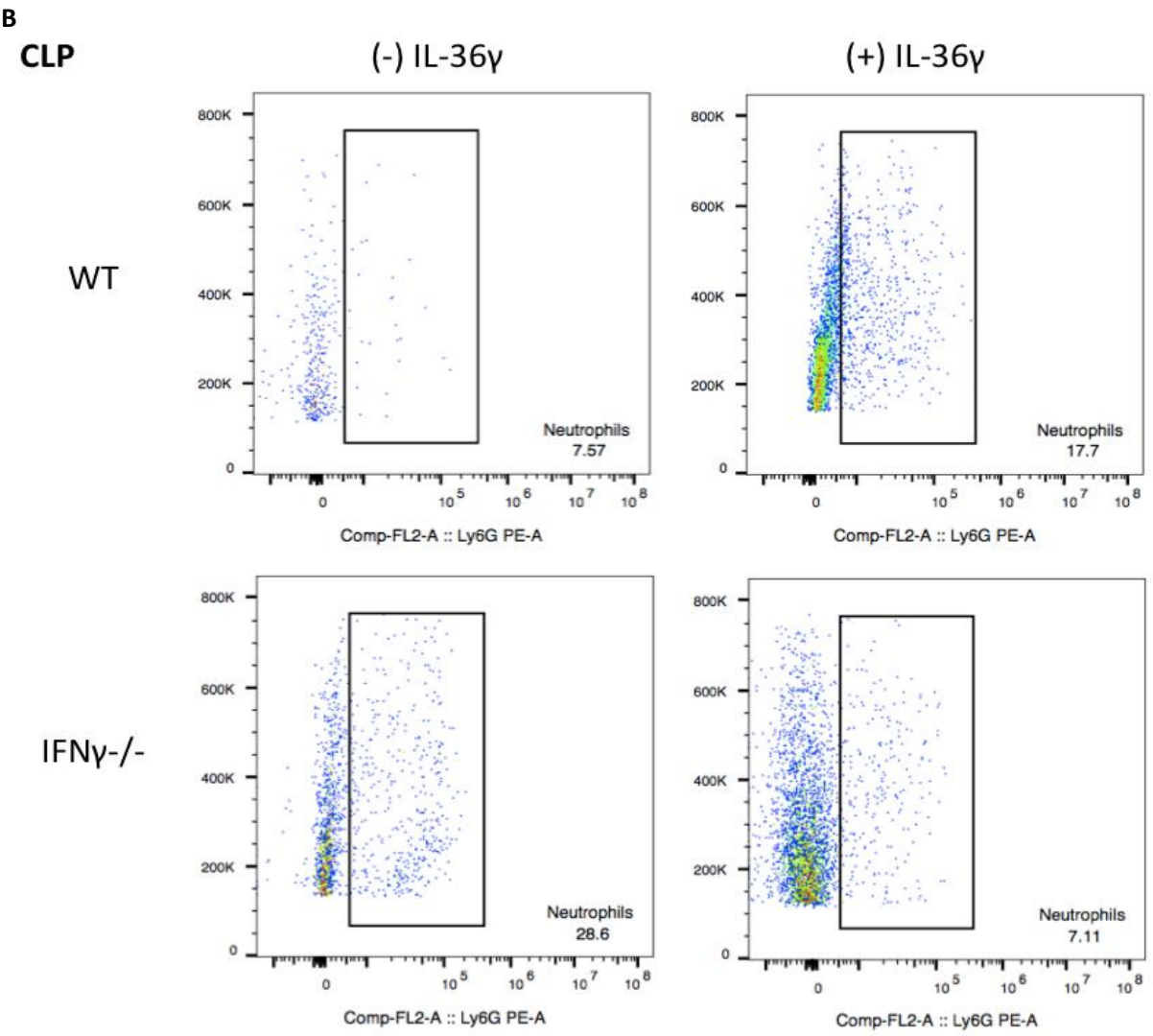

Figure 8.7: WT IL-36γ-stimulated cells transferred to Rag-/- mice recruited more neutrophils to the spleen and colonic lamina propria (CLP).

(A&B) Flow cytometry data of the spleen and colonic lamina propria stained for neutrophil marker Ly6G.

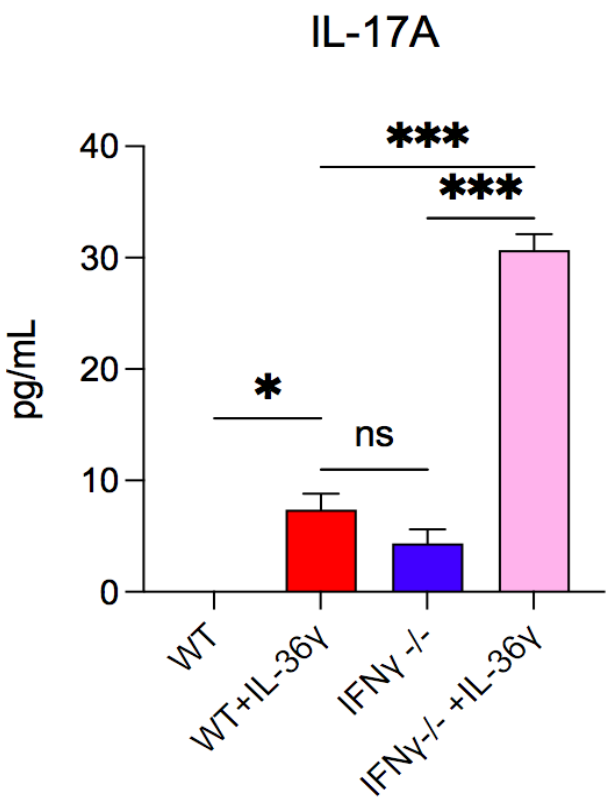

Figure 8.8: The absence of IFNγ induces a switch in T cells to a Th17 response, which is exacerbated when stimulated with IL-36γ.

(A) ELISA of IL-17A from supernatants of WT or IFNγ-/- cells stimulated with αCD3/αCD28 in the presence or absence of IL-36γ for 48 hours. All data are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001; ****P<0.0001; ns= not significant, one-way ANOVA with Tukey's multiple comparison test.

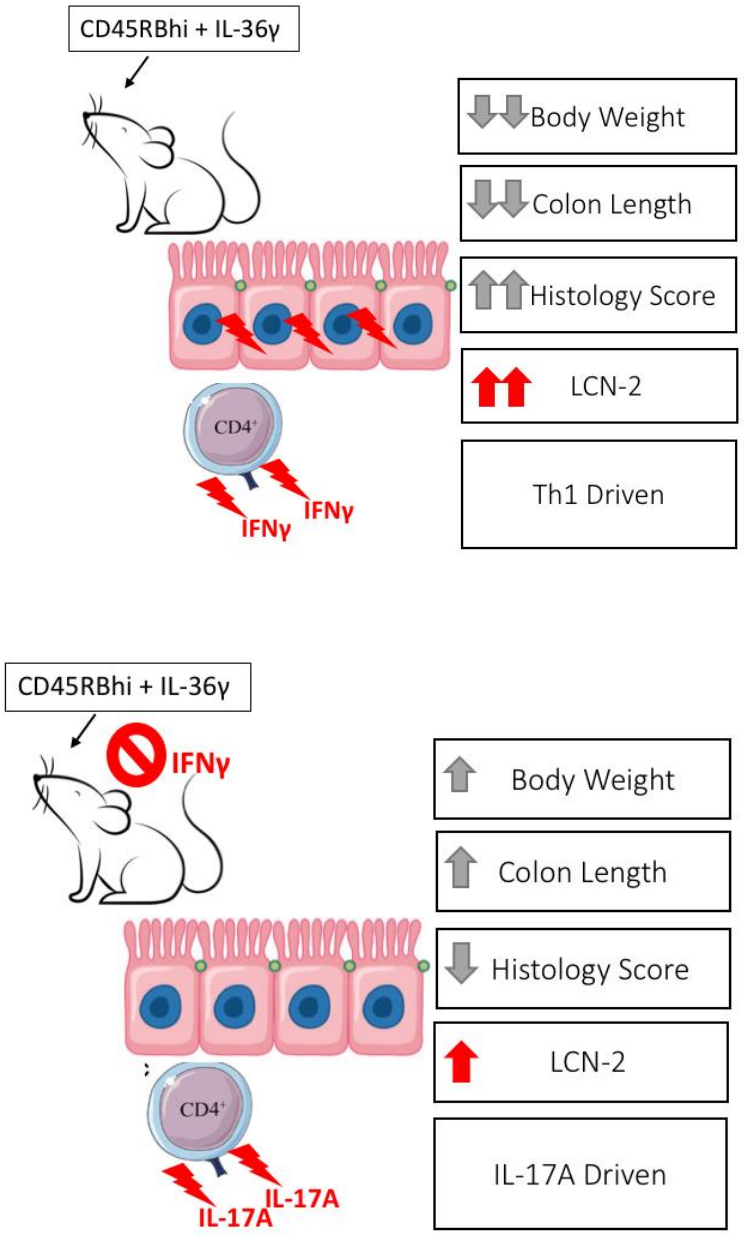

Figure 9: IFNγ deficiency dampens the immunological response in T cell-mediated chronic colitis.

Stimulating WT naïve CD4+ T cells with IL-36γ leads to a robust induction of IFNγ and promotes enhanced Th1 T cell mediated colitis. On the other hand, stimulating IFNγ-/- naïve CD4+ T cells with IL-36γ induces significant levels of IL-17A and causes milder intestinal inflammation**. Adapted from Biorender.com**

3. CONCLUSION

IL-36 cytokines are members of the IL-1 superfamily that include IL-36α, IL-36β, and IL-36γ. Their roles have been recently highlighted in inflammatory skin disorders. There is also evidence that IL-36 cytokines, especially IL-36γ, are overexpressed in human IBD and experimental chronic colitis. Targeting IL-36 signaling pathways has been proposed as a potential therapeutic strategy for IBD; however, their mechanism in IBD pathogenesis is not fully understood.

IL-36 cytokines promote the recruitment of immune cells, such as T cells; and they induce the secretion of other pro-inflammatory cytokines, such as TNFα. Our data showed that IL-36γ induced a notable expression and production of the Th1 pro-inflammatory cytokine IFNγ, by naïve CD4+ T cells compared with the other IL-36 family members. This was expected, as previous studies showed IL-36 cytokines induce the production of IFNγ [34]. We evaluate the relationship between IL-36 cytokines and IFNγ by neutralizing IFNγ *in vitro* in the presence of these cytokines. Our data suggests that blocking IFNγ can suppress the expression and secretion of the pro-inflammatory cytokine TNFα. When naïve CD4+ T cells were stimulated with IL-36γ and transferred to Rag-/- mice, the mice lost weight, developed looser stool consistency over time, and had the highest fecal LCN2 levels. The mice in this group also became visibly weaker over time. At time of sacrifice, the colon was also notably shorter in length and had thicker tissue. IFNγ deficient (IFNγ-/-) naïve CD4+ T cells were also stimulated with IL-36γ and transferred to Rag-/- mice. However, they did not lose a significant amount of weight. Their colons were longer and they had mild levels of fecal LCN2. This suggests that IL-36γ drives IFNγ-dependent T cell mediated colitis.

We briefly explored another T cell lineage, Th17, and assessed IL-17A secretion from stimulated naïve CD4+ T cells in these conditions WT, WT+IL-36γ, IFNγ-/-, and IFNγ-/- +IL-36γ. We observed IL-17A production from WT cells that were stimulated with IL-36γ, mild IL-17A induction from IFNγ-/-, but a very robust IL-17A induction from IFNγ-/- +IL-36γ cells. This correlates with a recent study that looks at an IFNγ-dependent arthritis model, which demonstrated that the IL-17A response was exacerbated when using IFNγ-/- CD4+ T cells [48].

Based on the available pre-clinical studies on pro-inflammatory cytokines in the T cell mediated colitis model, understanding the role of IL-36γ in intestinal inflammation will highlight new therapeutic approaches. In conclusion, our results demonstrate a potential for IL-36γ and IFNγ to be mutually targeted as therapeutics to alleviate colitis symptoms and perhaps prevent IBD, in the long run. Most of the available research targets downstream drivers of IBD, which narrows down the focus to specific molecules or pathways. Such downstream targets include TNFα, the IL-12/IL-23 axis, IL-23/IL-17 axis, JAK inhibitors, integrins and receptor modulators. Some antibodies and biological therapies were developed to interfere with the function of these molecules and pathways. Anti-TNF therapy, anti-IL-12/23, anti-integrins, and JAK inhibitors have been approved for treating UC or CD. Unfortunately, they are not universally effective [49]. Targeting upstream drivers, such as IL-36 cytokines, could have better therapeutic effects, as it would target cytokines downstream, such as TNFα and IFNγ.

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5. VITAE

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EDUCATION

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Ph.D., Translational Biomedical Science
Graduate Research Assistant analyzed the role of pro-inflammatory cytokines in IBD pathogenesis *in vitro* and *in vivo*.

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Kennesaw State University – Kennesaw, GA December 2018
B.S. Biology
Undergraduate Research Assistant worked on characterizing **Myxococcus** xanthus.

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ACCOMPLISHMENTS, ACTIVITIES, CONFERENCES ATTENDED

Guest lectures at Kennesaw State University- Kennesaw, GA (January 2020 – January 2023)

- Immunology lectures to undergraduate students

Digestive Disease Research Day Conference- Stone Mountain, GA (September 2022)

Duke Medical University- Regulatory Affairs Training Program Completion

MBIG Seminar at Kennesaw State University- Kennesaw, GA (September 2023)

- Immunology and IBD Research Seminar

Health Connect South Annual Conference at the Georgia Aquarium- Atlanta, GA (September 2029 and September 2022)

BIO Innovation Summit by GaBIO and CGHI at the Mercedez Benz Stadium - Atlanta, GA (October 2022)

Georgia State University Graduate Conference- Atlanta, GA (October 2022)

- Presented “IL-36γ is a master regulator of a pro-inflammatory cytokine network in experimental colitis”

Southeast Regional Clinical and Translational Science Conference- Atlanta, GA (October 2022)

- Oral talk about my research

Preprint Manuscript

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