UNIVERSITY OF CALIFORNIA, SAN DIEGO

VSL#3 Stimulates TCPTP and Attenuates IFN-γ Induced Epithelial Barrier Permeability

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EPIGRAPH

“Everything in moderation, except moderation.”

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TABLE OF CONTENTS

LIST OF FIGURES

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I would like to thank my lab and my family for their help on this project.

ABSTRACT OF THE THESIS

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In Inflammatory Bowel Disease (IBD), the colon and small intestine are chronically inflamed due, at least in part, to increased epithelial permeability and an inappropriate immune response to luminal antigens. IFN-γ is a prominent pro-inflammatory cytokine that increases permeability of the intestinal epithelium and T-Cell Protein Tyrosine Phosphatase (TCPTP) is a negative regulator of IFN-γ signaling. This research explores the effects of a probiotic preparation on TCPTP abundance, resolution of inflammatory signaling, and intestinal epithelial permeability by studying a probiotic blend called VSL#3. Research showed that in T_{84} colonic epithelial cells, VSL#3 was able to increase TCPTP protein levels by 150 ± 3% and enzymatic activity by 100 ± 5% (10^6 CFU/mL for 9 hours). Moreover, VSL#3 was able to decrease epithelial monolayer permeability by 25 ± 2% over 9 hours (10^6 CFU/mL). When IFN-γ and VSL#3 were co-incubated, VSL#3 attenuated the increase in permeability that resulted from 24 hours pre-treatment of T_{84} cells with IFN-γ by 120 ± 2%.

Moreover, levels of phosphorylated STAT-1 (a downstream effect of IFN-γ signaling) decreased when VSL#3 and IFN-γ were co-incubated and TCPTP levels showed an increase as VSL#3 doses were increased. Results were inconclusive as to whether VSL#3's effects on transepithelial resistance in IFN-γ pre-treated cells were TCPTP-mediated because the TCPTP inhibitor failed to display its expected action. Overall, the findings of this thesis indicate that VSL#3 can repair epithelial permeability defects caused by IFN-γ and this may be due in part to upregulation of a negative regulator of IFN-γ signaling, TCPTP.

I. INTRODUCTION

Background: Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) affects over 1.4 million people in the United States and there are over 362 cases of IBD per 100,000 people in the world [1]. Common symptoms include: diarrhea, vomiting, weight loss, abdominal pain, and rectal bleeding [1, 21]. Due to the high prevalence of IBD and its painful symptoms, a treatment option for IBD is a high priority. IBD is segmented into two major disease types: Crohn's disease (CD) and ulcerative colitis (UC). The incidence for CD is 11.2 / 100,000 and 5.9 / 100,000 for UC [2, 17]. Both subset diseases are characterized by chronic inflammation, but also have distinct features. UC is isolated only to the rectum and the colon, has continuous lesions, only affects the intestinal epithelial lining, and has IL-13 as the predominating cytokine [3]. CD can occur anywhere throughout the GI tract (mouth to anus), presents "skip lesions" that are not continuous, damages the entire intestinal bowel wall (transmural), and features IFN-γ as a primary pro-inflammatory cytokine [3].

Inflammatory Bowel Disease: Pathogenesis

In IBD, the intestinal epithelial lining is damaged making it more permeable. The epithelium of the gastrointestinal tract is needed for proper absorption of nutrients, electrolyte homeostasis, and to form a barrier between the intestinal commensal flora and the sub-mucosa [21]. In IBD, the increased permeability of the intestinal epithelial barrier allows commensal bacteria that naturally inhabit the lumen to come in contact with the immune system and trigger an inappropriate inflammatory response [4]. The inappropriate immune response allows luminal antigens (including commensal bacteria

to permeate through the sub mucosa and trigger a response from antigen-presenting cells (APCs). The APC's then recruit T-helper cells (T_H) to signal an inflammatory response. T_H cells trigger the inflammatory immune response by secreting pro-inflammatory cytokines. Cytokines are signaling proteins that can serve as immune-modulating agents and can be pro or anti-inflammatory [5]. In the context of IBD, primary cytokines secreted by T_H cells include IFN-γ and Tumor Necrosis Factor alpha (TNF-α). These cytokines play a key role in causing inflammation and increasing intestinal epithelial barrier permeability. In IBD, this condition of a damaged epithelial barrier and an inappropriate immune response leads to chronic inflammation because of the cyclical nature of the disease. It is cyclical, at least in part, because the luminal antigens that trigger the inappropriate immune response are naturally present in the colon and small intestine, however the exact mechanisms responsible for the waxing and waning of symptoms are still unknown [6]. What is known is that as cytokines are released, the permeability increases and the luminal antigens that are naturally present trigger the response that further weakens the barrier.

In Crohn's disease, increased concentrations of IFN-γ over other cytokines are seen due to an overactive T_{H1} and T_{H17} cell response [8]. The mechanism for the decrease in barrier integrity with pro-inflammatory cytokines such as IFN-γ has been shown to be through the formation of a leak pathway that is characterized by activation of myosin light-chain kinase (MLCK). When activated, MLCK phosphorylates myosin light-chain causing the contraction of the actinmyosin ring around the cell. Simultaneously, cytokine signaling causes an internalization of proteins such as ZO-1, occludin, and junctional adhesion molecule A (JAM-A) [9]. Occludin and ZO-1 are tight-junction proteins, so if

they are internalized permeability is increased because the proteins that tightly seal cells to their neighbors are reduced at the cell surface. Combined, the actinmyosin ring contraction and internalization of ZO-1, occludin, and JAM-A cause gaps in the areas between adjacent cells allowing solutes, ions, and luminal antigens to permeate through. This problem of increased barrier permeability is resolved when levels of pro-inflammatory cytokines are decreased and/or their effects are terminated [10]. To accomplish this, an increase in activity or expression of agents that can act as negative regulators of pro-inflammatory cytokine signaling pathways is required, in addition to decreased expression of the cytokines themselves.

IFN-γ is a pro-inflammatory cytokine that is involved in many different signaling cascade events from increases in phosphorylation of STAT-1 to ultimately affecting the localization of the tight-junction proteins. Mechanisms also exist to negatively regulate IFN-γ signaling. This includes the activity of phosphatase enzymes that dephosphorylate signaling intermediates downstream of the IFN-γ receptor. One enzyme that was of particular interest for this project was T-cell protein tyrosine phosphatase (TCPTP), which forms from a gene called TCPTP. TCPTP acts as a negative regulator of the IFN-γ signaling cascade.

Role of TCPTP in Crohn's disease

TCPTP chemically modifies its substrate by removing a phosphate group. TCPTP is a member of the protein tyrosine phosphatase (PTP) family and possesses the conserved familial motif of a cysteinyl residue [6]. This residue executes an SN1-type nucleophilic attack on the phosphate group, which then gets cleaved from the substrate

[11]. Protein tyrosine phosphatases play a wide role that includes affecting cell growth, differentiation, mitotic cycle, and oncogenic transformation [12] Additionally, *PTPN2* is an established disease candidate gene in in IBD, Type-I diabetes, and celiac disease.

TCPTP can form as two splice variants: a 45 KD version that can exist in the nucleus and a 48 KD version that exists in the cytoplasm near the endoplasmic reticulum. The 48 KD form localizes to the endoplasmic reticulum since it has a localization signal on its N-terminus [6, 9]. On the other hand, the 45 KD version of TCPTP has a nuclear importation signal instead of the endoplasmic reticulum localization signal. This allows the 45 KD splice variant to translocate from the cytosol into the nucleus [6]. Due to its ability to translocate to and from the nucleus, the 45 KD form exerts its phosphatase activity on a group of IFN-γ signaling molecules called signal transducers and activators (STATs). The STATs can translocate to the nucleus, bind DNA, and cause upregulation or inhibition of various genes [13].

As previously mentioned, IFN-γ increases the amount of the STAT-1 that is phosphorylated. This is accomplished when IFN-γ binds to the IFN-γ receptor causing the recruitment of JAK kinase. JAK kinase ultimately phosphorylates the IFN-γ receptor tails that recruit the STAT proteins (including STAT-1) and phosphorylates them [14]. The increase in phosphorylated STAT-1 leads to STAT dimerization and translocation to the nucleus where it can bind to STAT binding sites on target genes and initiate transcription. Increased STAT-1 phosphorylation in response to IFN-γ is correlated with increased permeability of the intestinal epithelial barrier [6]. Dephosphorylation of STATs (including STAT-1) causes inactivation and termination of STAT-mediated

transcription. TCPTP acts as a negative regulator of IFN-γ signaling in part through dephosphorylation of STAT-1 [6].

When TCPTP expression is lost, it facilitates prolonged STAT-1 nuclear activity and enhances STAT-1 transcriptional effects. Therefore, an important role of TCPTP is to decrease IFN-γ signaling by decreasing the amount of phosphorylated STAT-1. Two *in-vivo* studies demonstrated the aforementioned importance of TCPTP. These studies showed that TCPTP knockout mice develop hematopoietic defects, diarrhea, and anemia [15]. Moreover, genome wide association studies (GWAS) indicated that individuals with single nucleotide polymorphisms in the TCPTP locus have an increased risk of developing Crohn's disease and ulcerative colitis [16]. Moreover, our group has previously shown that TCPTP protects against intestinal epithelial barrier defects caused by IFN-γ [6, 11]. These data serve to link an IBD candidate gene (PTPN2), which is believed to be affected by loss of function mutations in a percentage of IBD patients, with a major pathophysiological event in the onset of IBD, loss of barrier function.

With the aforementioned information, it is of significant interest to find an agent that can increase the activity and expression of TCPTP and thereby reduce the deleterious effects of IFN-γ on the intestinal epithelial barrier in patients with Crohn's disease. One promising approach that is under investigation for therapeutic efficacy in IBD is modification of the microbial environment in the intestine through administration of "beneficial bacteria" or probiotics.

Commensal Bacteria and Intestinal Inflammation

A normal digestive tract contains over 400 types of bacteria that can be pathogenic or non-pathogenic. The gastrointestinal tract contains about 10^{14} CFU bacteria, with small numbers in the stomach increasing to 10^{12} CFU in the colon. The intestinal bacterial flora contributes to digestion of nutrients, metabolism of carcinogens, and serves as an important barrier function against pathogens [17, 22]. One example of a benefit the intestinal flora provides is short-chain fatty acids. They are produced by anaerobic bacterial fermentation of luminal carbohydrates and proteins and provide energy for intestinal epithelial cells, as well as contributing to fluid absorption in the colon. Additionally, the commensal bacteria secrete chemicals such as peptidoglycan and lipopolysaccharides that are immunostimulants [17, 19]. Thus, it can be concluded that the intestinal flora is crucial for the proper functioning of the GI tract and has significant effects on the immune system. In fact, chronic intestinal inflammation seems to be dependent on the presence of luminal microbiome [17]. In IL-10 (pro-inflammatory cytokine) deficient laboratory mice where flora was removed with antibiotics, IBD-like intestinal inflammation did not occur [17]. Since the intestinal flora's presence aids in digestion, the key to proper GI function is increasing the ratio of beneficial bacteria compared to harmful bacteria or other pathogens (yeasts, viruses) via addition of probiotics, which may have therapeutic value in conditions such as IBD [8, 17].

Probiotics and Resolution of Inflammation

Probiotics are non-harmful bacteria that, when added to the intestine, can be beneficial to the host by increasing the ratio of non-harmful bacteria compared to pathogens (yeasts, parasites, viruses, and pathogenic bacteria) in the intestine and thus

improving indigenous microbiome [18, 19, 22]. Examples of adverse changes to intestinal microme are virus spread, natural bacterial death, and antibiotics that kill both good and bad bacteria [17].

It was a logical choice to examine probiotic bacteria's effects on TCPTP because probiotics studies showed that bacterial DNA is able to reduce the secretion of pro-inflammatory cytokine IFN-γ from the colon [25]. TCPTP is a negative regulator of IFN-γ signaling, so probiotics might affect TCPTP levels, in addition to stopping IFN-γ secretion, to decrease inflammation. Furthermore, *in-vivo* studies using lactobacillus strains showed probiotics could both prevent or treat colitis induced by pathogenic bacteria (*Citrobacter rodentium*) [18]. The studies showed that pre-administration and co-administration of probiotics abated the pathogenic bacteria-induced barrier dysfunction, epithelial hyperplasia, and binding of pathogens to colonocytes (intestinal epithelial cells) [18]. The probiotics suppressed pro-inflammatory cytokines IFN-γ and TNF-α transcript upregulation with pre-treatment and suppressed IL-17 transcripts after treatment [18]. Finally, the studies showed that pathogenic bacteria (*Citrobacter rodentium*) negatively altered the fecal microbiome, but the introduction of probiotics was able to reduce those effects [18].

To aid in tipping the intestinal flora scale in favor of non-pathogenic bacteria, a non-pathogenic bacterial strain called *Lactobacillus acidophilus* can be used. For this project, we decided to examine if TCPTP, and its protective effects on barrier function, could be enhanced by a product called VSL#3, which is a proprietary blend of 8 different probiotic lactic acid producing bacteria strains. The strains include: *Bifidobacterium*

breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus bulgaricus, and *Streptococcus thermophilus* [23]. Thus, we tested T_{84} cells VSL#3 to determine its effects on TCPTP, attenuation of IFN-γ signaling, and intestinal epithelial barrier integrity in T_{84} cells.

VSL#3 represents an attractive agent for study due to a study indicating promising evidence of clinical efficacy in reducing the disease activity index in UC patients compared with placebo [20, 24]. The goal of our project was to determine the effects of different VSL#3 doses and incubation times on TCPTP expression and activity. Additionally, we sought to examine whether VSL#3 affects TCPTP regulation of IFN-γ signaling and downstream effects on barrier integrity. This information could potentially provide insights into the mechanisms by which probiotics are able to treat IBD, the appropriate doses for probiotics, the time required for probiotics to exert an effect, and a mechanism to support VSL#3's administration to counter IFN-γ driven inflammatory events.

II. MATERIALS & METHODS

Materials

VSL#3 packets with 450 billion bacteria per sachet (Sigma-Tau Pharmaceuticals, Gathersburg, MD), human recombinant IFN-γ (Roche, Mannheim, Germany), monoclonal mouse anti-TCPTP antibody CF-4, which detects the 45-kilodalton and the 48- kilodalton isoforms (Calbiochem, San Diego, CA), anti-phospho-STAT1 (Tyr701), anti-STAT1, anti-phospho-STAT3 (Tyr705), anti-STAT3 (Cell Signaling Technologies, Danvers, MA), and monoclonal mouse anti-β-Actin (Sigma) were obtained from the sources noted. Millicell culture plate inserts were purchased from Millipore Corporation (Millipore, Bedford, MA). All other reagents were of analytical grade and acquired commercially.

Cell Culture

The human colonic epithelial cell line, T_{84}, was used for all experiments in this study. This cell line was developed from a lung metastasis of a human colonic carcinoma [29]. T_{84} cells display the functional properties of colonic crypt epithelial cells, and are capable of forming polarized monolayers with tight junctions. Cells were grown in DMEM/F12 media with 5% newborn calf serum in 75 cm^2 flasks. The cells were trypsinized and passaged at 70-90% confluency, and split 1:5 or 1:3. For *in vitro* experiments, T_{84} cells were seeded onto 12mm Millicell-HA culture plate inserts (filter membranes) and grown for 12-20 days before study, at which time they had stable values of transepithelial electrical resistance ranging from 1000 to 2000 Ω・cm2. IFN-γ (1000U/mL) was added basolaterally, whereas VSL#3 (10^2, 10^4, 10^6, 10^8 CFU/mL) was

added apically.

RNA Isolation and Real-Time Polymerase Chain Reaction

Total RNA was isolated from T_{84} cells and DNA was removed from T_{84} cells using the Direct-zol RNA MiniPrep kit (Zymogen, Irvine, CA) according to the manufacturer's instructions. RNA purity and concentration were assessed by absorbance at 260 and 280 nm. Complementary DNA (cDNA) synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative Reverse-Transcriptase Polymerase Chain Reaction was performed using MESA GREEN qPCR MasterMix Plus for SYBR Assays (Eurogentec, San Diego, CA on a StepOnePlus Real-Time PCR sytem using Step One Software v2.0 (Applied Biosystems). Measurements were performed in triplicate, human GAPDH was used as an endogenous control, and results were analyzed by the ΔΔCT method.

Preparation of Cytoplasmic Lysates

On the day of the experiment, cells from inserts containing T_{84} monolayers were suspended in ice cold lysis buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 20µM NaF, 1mM EDTA, 1µg/ml antipain, 1µg/ml pepstatin, 1µg/ml leupeptin, 1mM NaVO3,100µg/ml phenylmethylsulfonyl fluoride), vortexed thoroughly, and further subjected to lysis using a 22 gauge needle. Cells were centrifuged at 10,000 rpm for 10 min to remove insoluble material, and an aliquot was removed from each sample to determine protein content (Bio-Rad protein assay according to the manufacturer's instructions). Samples were resuspended in loading buffer (50mM Tris, pH 6.8, 2% SDS, 100 mM dithiothreitol, 0.2% bromophenol blue, 20% glycerol) and boiled for 5 min.

Western Blotting

Samples suspended in loading buffer were loaded onto a 4-15% gradient polyacrylamide gel in order to resolve proteins. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was incubated in blocking buffer (5% BSA in 0.1% TBST) for 1 hr followed by overnight incubation of the membrane in blocking buffer containing primary antibody diluted 1:1000. This was followed by four 10-min washes with 1% TBST. After washes, secondary antibody (goat-anti-rabbit, Cell Signaling, Danvers, MA, or goat-anti-mouse IgG conjugated to horseradish peroxidase, BD Pharmigen, San Diego, CA) diluted 1:5000 was added to the membrane and incubated for 30 min. This was followed by four more 10-min washes with wash buffer. The membrane was then treated with chemiluminescent solution according to the manufacturer's directions (Thermo Scientific, Rockford, IL) for 10 min and exposed to film. Densitometric analysis of the blot was performed using Image J software (NIH).

Immunoprecipitation

1×10^6 T_{84} cells were prepared per well on 6-well plates. Following stimulation, cells were scraped with cell scrapers and suspended in 1 mL of ice-cold PBS. Cells were centrifuged for 5 min at 5,000 rpm. The pellet was fully resuspended and lysed in 500 µl of ice-cold lysis buffer (5% IGEPAL CA-360, 750 mM NaCl, 250 mM Tris-Cl, pH 8.0,1µg/ml antipain, 1µg/ml pepstatin, 1µg/ml leupeptin, 100µg/ml phenylmethylsulfonyl fluoride). Lysed cells were centrifuged for 10 minutes at 10,000 rpm to pellet DNA and small insoluble particles. 1 µg of monoclonal mouse anti-TCPTP CF-4 antibody was added per sample and placed on a rotating platform at 4°C for 1 hr. This was followed by

the addition of 30 µl of the 50% (v/v) protein A-sepharose beads to the cell lysate/antibody mix and allowed to incubate on a rotating platform at 4°C for 1 hr. Samples were then centrifuged briefly to pellet the protein A-Sepharose-antibody-antigen complexes, and the complex was washed three times with cold lysis buffer followed by three more washes with phosphatase reaction buffer (25 mM Hepes, 50 mM NaCl, 1 mM dithiothreitol). Beads were resuspended in phosphatase reaction buffer and allowed to warm to room temperature before the initiation of the phosphatase assay.

Phosphatase Activity Assay

Phosphatase activity was assessed using the EnzChek Phosphatase Assay Kit (Molecular Probes, Eugene OR) according to manufacturer's instructions using the fluorescent phosphatase substrate, 6,8-difluoro-4- methylumbelliferyl phosphate (DiFMUP). Upon dephosphorylation, DiFMUP fluoresces at an excitation/emission wavelength of 360/460. Therefore, the greater the fluorescence units, the larger the amount of dephosphorylated DiFMUP. TCPTP was first immunoprecipitated from whole cell lysates using anti-TCPTP antibody (see above). Immunoprecipitates were incubated for 15 minutes with DiFMUP after which fluorescence was detected with a SpectraMax M2 Fluorescence Microplate reader using SoftMax Pro v5 Software (Molecular Devices, Sunnyvale, CA). Fluorescence was measured every 15 minutes for the first hour and every 30 minutes thereafter. Measurements were performed in triplicate. A sample from each immunoprecipitation was run on SDS-PAGE and probed for TCPTP to account for equal protein loading. To account for any differences in overall phosphatase amounts, fluorescence activity units gathered from each assay were compared to TCPTP densitometric values obtained from Western blotting. Thus, values

represent the specific activity of TCPTP rather than total quantities of the enzyme.

Transepithelial Electrical Resistance

Transepithelial electrical resistance (TER) across T_{84} monolayers was assessed by voltohmeter (WPI, Sarasota, FL) and companion electrodes (Millipore, Bedford, MA). An electrode was placed in the apical compartment and an electrode was placed in the basolateral compartment. The readout measures the integrity of the cell monolayer on the porous membrane. Measurements were calculated in Ω · cm2 and expressed as a percentage of the baseline measurement (Figure 7).

Statistical Analysis

All data are means for a series of experiments. Statistical analysis was performed by Student's unpaired t-test or analysis of variance (ANOVA) and Student-Newman-Keuls post-test using Graph Pad Instat software (Graph Pad Software, La Jolla, CA). P values < 0.05 were considered significant.

III. RESULTS

VSL#3 does not affect levels of TCPTP mRNA

To determine whether VSL#3 affected TCPTP mRNA levels in intestinal epithelial cells, T_{84} cells were grown to confluence monolayers on semi-permeable membrane supports were exposed to increasing doses of VSL#3. The VSL#3 doses were 10^2, 10^4, 10^6, and 10^8 CFU/mL. Each dose of VSL#3 was incubated with the cells for 3 hours, 9 hours, and 24 hours. VSL#3 was added to the physiologically-relevant apical side of the epithelial monolayers in a concentrated dose that did not require a media change. After being exposed to VSL#3, the permeable supports were put into Trizol. Experiments were performed in triplicate, with each as a trial number of one. Real-time PCR analysis showed no significant changes in mRNA levels for TCPTP when RT-PCR was done. These results were compared to the untreated levels for each dose and for each incubation time of 3, 9, and 24 hours.. There was no statistical significance between the various doses and incubation time conditions of probiotic (VSL#3) treatments and untreated conditions on mRNA levels (n = 3, Figure 8).

VSL#3 increases expression levels of cytoplasmic TCPTP

To determine whether VSL#3 affects levels of TCPTP protein in intestinal epithelial cells, T_{84} cells were grown as monolayers on permeable supports. A similar dose-response and time-course for the RT-PCR data was performed to see the effects on TCPTP. T_{84} cells were treated with increasing concentrations of VSL#3 (10^2, 10^4, 10^6, 10^8 CFU/mL) and each concentration of VSL#3 was incubated for 3, 9, and 24 hours. The VSL#3 probiotic cocktail was added to the apical compartment of the permeable

support system. Western blotting indicated that cytoplasmic TCPTP protein levels in 10^4 CFU/mL ($n = 3$, $p<0.01$) and 10^6 CFU/mL ($n = 3$, $p<0.05$) concentrations of VSL#3 increased 31 ± 4 % over untreated cells following a 3-hour incubation (Figure 7). As expected based on previous studies, IFN-γ increased TCPTP expression above untreated conditions [6] ($n = 3$, $p<0.05$). Additionally, it was consistently noted that the 10^8 CFU/mL dose of VSL #3 ($n = 3$, $p<0.05$) led to significant decreases in TCPTP levels with a 3-hour incubation (Figure 9). Following a 9-hour incubation, a VSL#3 concentration of 10^6 CFU/mL most markedly increased TCPTP levels by 220 ± 10 % over the untreated condition ($n = 3$, $p<0.05$) (Figure 10). Finally when VSL#3 was incubated for 24 hours, the VSL#3 concentration of 10^4 CFU/mL ($n = 3$, $p<0.05$) showed the strongest increase of 159 ± 15 % over the untreated condition (Figure 11). Additionally at 24 hours, VSL#3 was also administered to the apical side of the T_{84} monolayer at a concentration of 10^8 CFU/mL, but that concentration resulted in significant decreases in TCPTP after the 24-hour incubation (Figure 9, Figure 11).

VSL#3 increases cytoplasmic TCPTP activity in intestinal epithelial cells

To investigate whether VSL#3 affects TCPTP enzymatic activity in T_{84} cells, a dose-response and time course with VSL#3 was performed and TCPTP enzymatic activity was determined with a phosphatase assay. The phosphatase assay was run after the addition of the substrate (DiFMUP) and enzymatic activity of TCPTP was measured for 120 minutes. T_{84} cell monolayers were dosed with VSL#3 at varying concentrations (10^2, 10^4, 10^6, 10^8 CFU/mL) and varying incubation times (3 hr, 9 hr, 24 hr). Following incubation for 9 hours with or without VSL#3, TCPTP was immunoprecipitated from whole T_{84} cell lysates. In the dose-response, VSL#3 strongly stimulated TCPTP

enzymatic activity seen by a 66 ± 9 % increase in measured TCPTP enzymatic activity with the 10^6 CFU/mL concentration of VSL#3 ($n = 4$, $p<0.05$) compared to the untreated condition after 15 minutes of the phosphatase activity assay (Figure 16). Additionally, 10^2 CFU/mL and 10^4 CFU/mL doses of VSL#3 showed a stimulatory effect on TCPTP activity above untreated with an 41 ± 12 % increase and 38 ± 5 % increase, respectively, after 15 minutes of the phosphatase activity assay (Figure 16).

To follow up on the VSL#3 dose-response findings, the VSL#3 incubation time-course results showed a similar stimulatory trend. Due to the fact that 10^6 CFU/mL showed the strongest effect on TCPTP enzymatic activity in the dose-response, all incubation times were conducted with 10^6 CFU/mL of VSL#3. The results of the activity assay showed that a 9-hour VSL#3 pre-treatment time with a 10^6 CFU/mL concentration of VSL#3 showed the strongest increase in activity of 65 ± 5.3 % compared to the untreated conditions ($n = 4$, $p<0.05$; Figure 17). With the 10^6 CFU/mL concentration of VSL#3, incubation time-points of 3 hours and 24 hours also showed a significant increase in TCPTP activity over the untreated condition, but not to the same extent as the 9-hour time point (Figure 17).

VSL#3 increases intestinal epithelial barrier integrity in a dose-dependent and time-dependent manner

To correlate VSL#3's effect on TCPTP protein level and enzymatic activity to a functional effect of regulation of intestinal epithelial barrier integrity, transepithelial electrical resistance (TER) was measured. TER is a common way of measuring the integrity of a T_{84} cell monolayer and a decrease in TER is associated with an increase in

paracellular permeability and thus a more "leaky" barrier [6, 18]. To determine how VSL#3 affected the integrity of the epithelial-cell monolayer, dose-response experiments with VSL#3 were run and resulting TER changes were recorded over 24 hours. VSL#3 used at all three doses (10^2, 10^4, 10^6 CFU/mL, n = 3) increased TER compared to untreated at the 9-hour and 24-hour time-points. The conditions that resulted in the strongest increase in TER over the untreated were the VSL#3 concentration of 10^6 CFU/mL with incubation times of 9 hours and 24 hours. At 9 hours, cells treated with 10^6 CFU/mL of VSL#3 had a TER that was 62 ± 10 % greater than untreated samples (n =3, Figure 19). At 24 hours, the condition with 10^6 CFU/mL of VSL#3 had a TER that was 38 ± 6 % greater than untreated samples (n = 3, Figure 19). Additionally, the 10^8 CFU/mL concentration of VSL#3 resulted in significant decreases in TER in all measurements past a 3-hour incubation (n = 3, Figure 19).

VSL#3 protects intestinal epithelial barrier integrity from *in vitro* effects of pro-inflammatory cytokine, IFN-γ

Given the strong effects on epithelial integrity seen for VSL#3, it was of interest to determine whether VSL#3 could exert a protective effect on intestinal epithelial barrier function following a challenge with IFN-γ. Again, TER was the appropriate readout since the goal was to see if VSL#3 could reduce the inflammatory effects of IFN-γ on epithelial monolayer permeability. One of the effects of IFN-γ is an increase in the permeability of the intestinal epithelial lining, so TER would measure how VSL#3 affects an increase in permeability caused by IFN-γ. IFN-γ at a concentration of 1000u/mL has been shown to decrease TER in T_{84} cells by as much as 50 ± 4 % after a 24-hour incubation period [6, 10] (Figure 20). For the IFN-γ and VSL#3 co-incubation experiments, IFN-γ was added

basolaterally (1000u/mL) once the cells had established a stable monolayer in which the baseline TER before treatment was greater than 900 $\Omega.cm^2$ (Figure 21). In the co-administration experiments, T_{84} cells were pre-treated with IFN-γ for 24 hours and then incubated with VSL#3 at 10^6 CFU/mL for 9 hours due the strongest effects on TCPTP activity and expression previously seen at that concentration and incubation time. After 33 hours (24 hours IFN-γ pre-treatment and 9 hours VSL#3), results showed that the IFN-γ and VSL#3 co-incubated condition had a final TER that was 212 ± 10 % than seen in cells treated with IFN-γ alone ($n = 3$, $p<0.05$, Figure 19). Additionally, the untreated condition showed a steady increase in TER showing that cells used the experiments were healthy (Figure 19).

VSL#3 attenuates IFN-γ signaling in intestinal epithelial cells

The next logical step was to delve further into the mechanism underlying VSL#3's effects on TER in the presence of IFN-γ. Since it was previously determined that VSL#3 stimulates TCPTP activity, it was important to see whether VSL#3 affects the IFN-γ signaling molecule STAT-1 in the co-incubation experiments. To test this, a pathophysiologically relevant dose of IFN-γ (1000U/mL) was added basolaterally to T_{84} monolayers [6]. Western blotting analysis revealed that IFN-γ increased STAT-1 phosphorylation by 31 ± 4 % following 3, 9, and 24 hours of treatment compared to untreated cells ($p<0.01$, $n = 4$). These data on induction of STAT-1 phosphorylation was sufficient to determine what concentration of IFN-γ was needed to activate downstream targets and ultimately cause increased permeability (and decrease in TER) (Figure 13).

After identifying the concentration and incubation time of IFN-γ needed to bring about loss of barrier integrity associated with a drop in TER, experiments could be conducted where VSL#3 and IFN-γ could be co-incubated and phosphorylated STAT-1 values could be measured. T_{84} cells were pretreated with IFN-γ (1000u/mL) for 24 hours, which had previously been showed to cause a drop in TER [6, 8]. Then, VSL#3 was then added for 9 hours at a concentration of 10^6 CFU/mL. This VSL#3 incubation time and concentration was chosen because, with these conditions, the strongest increase in expression and activity of TCPTP compared to untreated conditions was seen (Figure 11). The results showed that at the VSL#3 dose of 10^6 CFU/mL, the levels of phosphorylated STAT-1 in the co-incubation condition decreased by 43 ± 3 % compared to the condition with IFN-γ alone ($n = 3$, $p<0.05$) (Figure 13).

To confirm there was no stimulatory effect of VSL#3 alone on phosphorylation of STAT-1, dose-response experiments were conducted with phosphorylated STAT-1 as the readout. There was no STAT-1 phosphorylation as various concentrations of VSL#3 was added (10^2, 10^4, 10^6, 10^8 CFU/mL) to cells ($n = 2$, Figure 13).

Effects of VSL#3 and IFN-γ co-incubation with on TCPTP protein levels

Correlated with the drop in phosphorylated STAT-1 seen when VSL#3 and IFN-γ were co-incubated versus IFN-γ alone, TCPTP levels tended to increase as the dose of VSL#3 was increased in the presence of IFN-γ . 10^6 CFU/mL of VSL#3, which strongly decreased levels of phosphorylated STAT-1, caused a 30 ± 18 % increase in TCPTP protein levels over untreated conditions. However, this value did not achieve statistical significance ($n = 3$, Figure 14).

Effects of co-incubation with VSL#3 and IFN-γ on TCPTP enzymatic activity

In conjunction with the change in TCPTP protein levels in the presence of IFN-γ, it was important to examine effects on TCPTP enzymatic activity to fully understand whether VSL#3's effect on TCPTP might attenuate IFN-γ signaling. After 15 minutes of the phosphatase assay, the condition with IFN-γ alone and the condition with VSL#3 10^6 for 9 hours (after 24 hour IFN-γ pre-treatment) were 5 ± 0.3 % and 7 ± 0.5 % above the untreated condition, respectively. Additionally, the positive control and negative control reach expected levels.

VSL#3 Effects on IFNγ-signaling: TCPTP-dependent?

Previous studies showed VSL#3 increased TCPTP levels and activity while reducing IFN-γ signaling. Prior results also showed that when cells are incubated with VSL#3 (10^6 CFU/mL) as well as IFN-γ, phosphorylated STAT-1 decreases and an increasing trend of TCPTP protein levels is seen. These results suggest that VSL#3 increases TCPTP to combat phosphorylation of STAT-1 that would otherwise be caused by IFN-γ. The next step was to determine whether VSL#3's effects on IFN-γ signaling are mediated by TCPTP. A small organic molecule inhibitor of TCPTP enzymatic activity was used to see if VSL#3 could still reduce IFN-γ signaling if TCPTP's enzymatic activity was inhibited. To confirm efficacy of the inhibitor, we measured levels of phosphorylated STAT-1 compared to cells treated with IFN-γ alone. The results showed that cells incubated with IFN-γ in the presence of the TCPTP inhibitor showed a 24 ± 8 % decrease in STAT-1 phosphorylation levels compared to conditions with the TCPTP inhibitor alone. However, IFN-γ would be expected to increase phosphorylated

STAT-1 levels compared to conditions without IFN-γ . Thus, it is likely the TCPTP inhibitor was not able to inhibit TCPTP activity in these experiments and/or the inhibitor might have caused non-specific effects that prevented phosphorylation of STAT-1 (n = 2, Figure 15). Thus, unfortunately, these final experiments were inconclusive.

IV. DISCUSSION

Inflammatory Bowel Disease is characterized by chronic inflammation of the epithelial lining in the colon and small intestine. The inflammation stems from an inappropriate immune response to luminal antigens that gain access to the lamina propria due to increased permeability of intestinal epithelial lining. Our project focused on the permeability aspect of IBD inflammation via *in vitro* experiments to reduce the permeability of colonic epithelial cell monolayers. INF-γ is a pro-inflammatory cytokine present in IBD that has been shown to increase permeability [6]. The PTPN2 gene and its protein product, TCPTP, likely play an essential role in the regulation of inflammation in the gastrointestinal tract due to their regulatory effects on IFN-γ signaling [6]. Therefore, increases in TCPTP (levels and/or activity) might be expected to reduce permeability.

This project's aims were to see whether a probiotic formula (VSL#3) could positively affect TCPTP protein levels and enzymatic activity. As changes in TCPTP were examined, the project was also designed to examine how probiotic-induced changes in TCPTP altered levels of phosphorylated STAT-1 in an "inflammatory" setting. Finally, associated effects of various experimental treatments on epithelial monolayer integrity were noted using TER.

The first conclusion of our experiments was that VSL#3 increases TCPTP protein levels in T_{84} colonic epithelial cells after 3, 9, and 24 hours at doses of 10^4 and 10^6 CFU/mL. However, a high VSL#3 dose (10^8 CFU/mL) apparently led to cell death after 9

our project then focused on VSL#3's effects on TCPTP enzymatic activity. We found that in T_{84} cells, VSL#3 increases TCPTP enzymatic activity at 10^4 and 10^6 CFU/mL after 3, 9, and 24 hours incubation with VSL#3 compared with the control condition. It is worth noting that in all studies, VSL#3 at a dose of 10^8 CFU/mL led to cell death over all time points, so no further analysis on enzymatic activity was pursued. Additionally, this drop in activity for high 10^8 CFU/mL dose aligned with the drop in TCPTP protein levels noted via Western Blotting for .

These data on TCPTP protein levels and enzymatic activity are intriguing because in the intestine there is a bacterial concentration of 10^{12} CFU/mL, so it is hard to fathom that an increase of 10^8 CFU/mL would cause cell death. The prior data may suggest that an *in vitro* reductionist model, such as the one we have employed with an epithelial cell line, may be more sensitive to direct interaction with probiotics at a lower dose than the more complex environment in which intestinal epithelial cells exist *in vivo*. Our findings are nevertheless relevant for pre-clinical studies because they identify a specific dose of VSL#3 that evoked the strongest increase in TCPTP protein levels and the incubation time required to observe such an effect. They also help to identify a starting point for the administration of a dosing range of probiotics and the frequency of administration. For example, if TCPTP activity and levels are highest after 9 hours, then repeat dosing may be required at 9-12hr intervals.

On a physiologically-relevant level, we then showed that VSL#3 is able to strengthen the epithelial monolayer compared to untreated conditions. Over the course of 9 hours and 24 hours, the concentrations of 10^4 and 10^6 CFU/mL increased transepithelial

resistance (TER) over untreated conditions. Since VSL#3 was able to strengthen the barrier, the next step was to examine if VSL#3 could repair a barrier that had been compromised by pro-inflammatory cytokine effects. We examined the effects of VSL#3 on the TER of an "inflamed" monolayer following incubation with IFN-γ [6]. IFN-γ was added basolaterally for a pre-treatment of 24 hours. Then, VSL#3 at a concentration of 10^6 CFU/mL was added for 9 hours. The reason why this concentration and incubation time were chosen was because they showed the highest effect on protein levels and activity of TCPTP compared to the untreated condition (Figure 9).

TER measurements showed a resce effect of VSL#3 when cells were co-incubated with IFN-γ . The TER decreased in response to IFN-γ up to the 24-hour time point when VSL#3 was added. After 33 hours (24 hours IFN-γ and 9 hours VSL#3 10^6 CFU/mL), the TER in cells also exposed to VSL#3 was significantly greater than in those treated with IFN-γ alone. These data indicated that probiotics (VSL#3) are able to rescue, at least in part, the IFN-γ induced loss of barrier integrity. These data also lend strength to studies showing VSL#3 reduces IFN-γ secretion by the colon *in vivo* [25]. The studies suggest that probiotics DNA inhibit IFN-γ secretion and alter the way that epithelial cells react to inflammatory cytokine signaling [25].

We next chose to investigate potential mechanisms mediating the protective effect of VSL#3, and in particular we focused on potential modifications of TCPTP and/or IFN-γ signaling. Since we observed beneficial effects on TER when cells treated with IFN-γ were exposed to VSL#3, we wanted to determine whether IFN-γ -induced phosphorylation of STAT-1 was altered by VSL#3 as this might provide insights into the

mechanism for rescue of TER by VSL#3. VSL#3 in combination with IFN-γ showed the greatest decrease in phosphorylated STAT-1 at a VSL#3 dose of 10^6 CFU/mL compared to cells treated with IFN-γ alone. This effect provides insight into the mechanism for the increase in TER seen when VSL#3 is added to cells pre-treated with IFN-γ for 24 hours because increased phosphorylated STAT-1 levels have been correlated with increased expression of pore-forming proteins.

We next examined whether the anti-inflammatory effects of VSL#3 were involved with TCPTP. We first investigated if there was a correlation between decreased STAT-1 phosphorylation levels and the increase in TCPTP protein levels when VSL#3 was added. Our previous results showed that with the VSL#3 dose of 10^6 CFU/mL (greatest reduction in phosphorylated STAT-1), there was an increase in TCPTP protein levels over untreated conditions. There was also a trend towards increased TCPTP when VSL#3 was added to cells treated with IFN-γ, but again this did not reach significance. With these experiments, future efforts could focus on increasing the experimental numbers to gain statistical significance. The increasing TCPTP level trend suggests that if the experimental trials were increased, the data might reach statistical significance.

After identifying the changes in TCPTP protein levels caused by VSL#3 to combat IFN-γ signaling, we looked at changes in TCPTP enzymatic activity. The data showed a significant increase in TCPTP activity when compared to untreated samples after 15 minutes of the activity assay. These data, combined with the increasing trend in TCPTP levels with the INF-y and VSL#3 co-incubation experiments, provides further

elucidation to the mechanism by which VSL#3 is able to rescue TER following a reduction occasioned by IFN-signaling.

The final step we took was to examine if the ability of VSL#3 to reduce epithelial permeability was dependent on TCPTP. In these studies, we used a small organic molecule that has been reported to inhibit the activity of TCPTP. The readout used to identify whether the TCPTP inhibitor worked was to see if the TCPTP inhibitor co-incubated with IFN-γ increased phosphorylated STAT-1 compared to cells treated with IFN-γ alone. The results showed a small decrease with the TCPTP inhibitor plus IFN-γ versus the TCPTP inhibitor alone. This shows the opposite result from what we expected with the TCPTP inhibitor, as one would predict elevated STAT-1 phosphorylation levels in the INF-y and the inhibitor co-incubation over just the inhibitor alone; this is because IFN-γ causes increased STAT-1 phosphorylation and if TCPTP (a negative regulator of IFN-γ signaling) is inhibited it should increase even more. Thus, it is likely that the TCPTP inhibitor did not function correctly. Future related experiments include using the inhibitor to assess if it can inhibit TCPTP activity via a phosphatase activity assay instead of the readout of changes in phosphorylated STAT-1 levels. Furthermore, incorporation of TCPTP siRNA would likely represent a cleaner approach to interrogate a potential role for TCPTP as a mediator of the effect of VSL#3 on intestinal epithelial barrier permeability. Finally, the TCPTP inhibitor is a small organic molecule that is dissolved in DMSO. Future experiments should repeat the prior experimental conditions (untreated, INF-y, VSL#3, IFN-γ and VSL#3, the inhibitor, the inhibitor and IFNy, and the inhibitor, IFN-γ, and VSL#3), but also add the conditions of the inhibitor and VSL#3, DMSO, DMSO and IFN-γ, DMSO and VSL#3, and DMSO, the inhibitor, IFN-γ, and In

conclusion, our project results were able further to elucidate a mechanism by which VSL#3 acts to regulate IFN-γ signaling. It seems likely that there is some involvement of TCPTP, but the data cannot be seen as conclusive due to the lack of statistical significance and possibly the failure of inhibitor to act as advertised. Currently, the literature on probiotics has associated them clinically with reducing inflammation, but these findings have not consistently been noted [22, 23, 25]. Additionally, probiotics have been shown to reduce IFN-γ secretion by the colon [25]. Our project's data has shown that probiotics have the potential *in vitro* to attenuate inflammatory signaling. Our data also suggests that the ability to attenuate inflammatory IFN-γ signaling may involve TCPTP. The key leap that our data cannot make to tie into clinical studies on probiotics and inflammation is we do not know how much of chronic inflammation in IBD patients is attributed to increased permeability. Our data is not able to address much of the aggressive immune response to luminal antigens that aids in producing inflammation of the intestinal epithelial barrier. Ultimately, our data strengthen published studies on probiotic ability to decrease permeability *in vivo*, with *in vitro* studies that show beneficial effects. This leads in the right direction to first address the effects of probiotics on known factors involved with inflammation, so progress is being made. VSL#3 in combination with IFN-γ showed the greatest decrease in phosphorylated STAT-1 with the VSL#3 dose of 10^6 CFU/mL compared to cells treated with IFN-γ alone. This effect provides insight into the mechanism for the increase in TER seen when VSL#3 is added to cells pre-treated with IFN-γ for 24 hours because increased phosphorylated STAT-1 levels have been correlated with increased expression of pore-forming proteins.

We next examined whether the anti-inflammatory effects of VSL#3 were involved with TCPTP. We first investigated if there was a correlation between decreased STAT-1 phosphorylation levels and the increase in TCPTP protein levels when VSL#3 was added. Our previous results showed that with the VSL#3 dose of 10^6 CFU/mL (greatest reduction in phosphorylated STAT-1), there was an increase in TCPTP protein levels over untreated conditions. There was also a trend towards increased TCPTP when VSL#3 was added to cells treated with IFN-γ, but again this did not reach significance. With these experiments, future efforts could focus on increasing the experimental numbers to gain statistical significance. The increasing TCPTP level trend suggests that if the experimental trials were increased, the increasing data would reach statistical significance.

After identifying the changes in TCPTP protein levels that VSL#3 causes to combat IFN-γ signaling, we then looked at changes in TCPTP enzymatic activity. The data showed a significant increase in TCPTP's activity when compared to untreated samples after 15 minutes of the activity assay. This data, combined with the increasing trend in TCPTP levels with the INF-y and VSL#3 co-incubation experiments, provides further elucidation to the mechanism by which VSL#3 in able to rescue TER from effects of IFN-signaling.

The final step we took was to examine if the previously seen phenomenon of VSL#3's reduction in membrane permeability was dependent on TCPTP. In these studies, we used a small organic molecule that was known to inhibit the activity of TCPTP. The readout used to identify if the TCPTP inhibitor worked was to see if the

TCPTP inhibitor co-incubated with IFN-γ increased phosphorylated STAT-1 compared to cells treated with IFN-γ alone. The results showed a small decrease in the condition with the TCPTP inhibitor and IFN-γ versus the TCPTP inhibitor alone. This shows the opposite result from what we expected with the TCPTP inhibitor, as one would predict elevated STAT-1 phosphorylation levels in the INF-y and the inhibitor co-incubation over just the inhibitor alone; this is because IFN-γ causes increased STAT-1 phosphorylation and if TCPTP (a negative regulator of IFN-γ signaling) is inhibited it should increase even more. Thus, it is likely that the TCPTP inhibitor did not function correctly. Future related experiments include using the inhibitor to assess if it can inhibit TCPTP activity via a phosphatase activity assay instead of a readout as changes in phosphorylated STAT-1 levels. Furthermore, incorporation of TCPTP siRNA would likely represent a cleaner approach to interrogate a potential role for TCPTP as a mediator of the effect of VSL#3 on intestinal epithelial barrier permeability. Finally, the TCPTP inhibitor is a small organic molecule that is dissolved in DMSO. Future experiments should repeat the prior experimental conditions (untreated, INF-y, VSL#3, IFN-γ and VSL#3, the inhibitor, the inhibitor and IFNy, and the inhibitor, IFN-γ, and VSL#3), but also add the conditions of the inhibitor and VSL#3, DMSO, DMSO and IFN-γ, DMSO and VSL#3, and DMSO, the inhibitor, IFN-γ, and VSL#3. This crucial control to show that DMSO was not throwing off any changes we saw in the TCPTP inhibitor data should be incorporated.

In conclusion, our project results were able to further elucidate a mechanism by which VSL#3 acts on regulating IFN-γ signaling. It seems likely that there is some involvement of TCPTP, but the data cannot be seen as conclusive due to the lack of

statistical significance and likely defunct inhibitor. Currently, the literature probiotics on probiotics has associated them clinically with reducing inflammation, but these findings have not been consistently noted [22, 23, 25]. Additionally, probiotics have been shown to reduce IFN-γ secretion by the colon [25]. Our project's data has shown that probiotics have the potential *in vitro* to attenuate inflammatory signaling. Our data also suggests that the ability to attenuate inflammatory IFN-γ signaling is involved with TCPTP. The key leap that our data cannot make to tie into clinical studies on probiotics and inflammation is we do not know how much of chronic inflammation in IBD patients is attributed to increased permeability. Our data is not able to address much of the aggressive immune response to luminal antigens that aids in producing inflammation of the intestinal epithelial barrier. Ultimately, we see that our data strengthens the published studies on probiotics ability to decrease permeability *in vivo*, with *in vitro* studies that show beneficial effects. This leads in the right direction to first address the effects of probiotics on known factors involved with inflammation, so progress is being made.

V. FIGURES

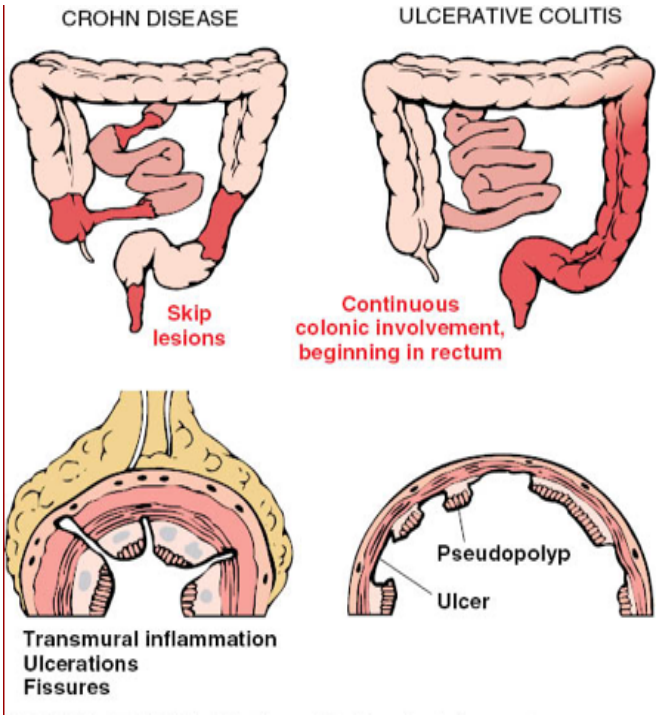

Figure 1 | Differences Between Crohn's Disease and Ulcerative Colitis

Inflammatory Bowel Disease has two subsets: Crohn's Disease and Ulcerative Colitis. Crohn's Disease can be present anywhere throughout the gastrointestinal tract (mouth to anus), presents "skip lesions" that are not continuous and damages through the entire intestinal bowel wall (transmural). Ulcerative Colitis is isolated only to the rectum and the colon, presents lesions in a continuous manner, and only damages on the intestinal epithelial lining.

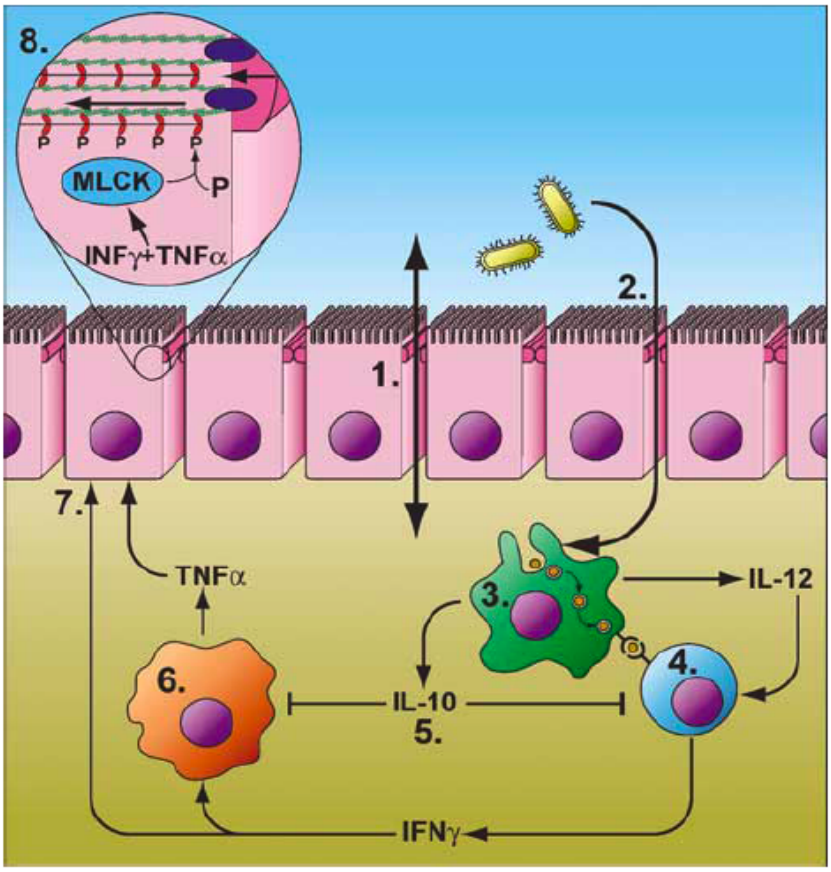

Figure 2 | Inflammatory Bowel Disease: Pathogenesis

(1) The permeability of the intestinal epithelial barrier allows commensal bacteria that are naturally in the lumen to come in contact with the immune response system and trigger an inappropriate inflammatory response. (2) The inappropriate immune response consists of luminal antigens (commensal bacteria) permeating to the sub mucosa and triggering a response from an antigen-presenting cell (APC). (3) The fact that the antigen presenting cells signals for an inflammatory response demonstrates that the antigens are seen as foreign. (4) The APC's then recruit T-helper cells (T_H) to signal an inflammatory response. T_H cells trigger the inflammatory immune response by secreting pro-inflammatory cytokines. (5, 6) Cytokines are inflammatory-signaling proteins that can also serve as immune-modulating agents. In the context of IBD, primary cytokines secreted by T_H cells include IFN-γ and TNF-a. (7, 8) These cytokines play a role in causing inflammation and increased intestinal epithelial barrier permeability.

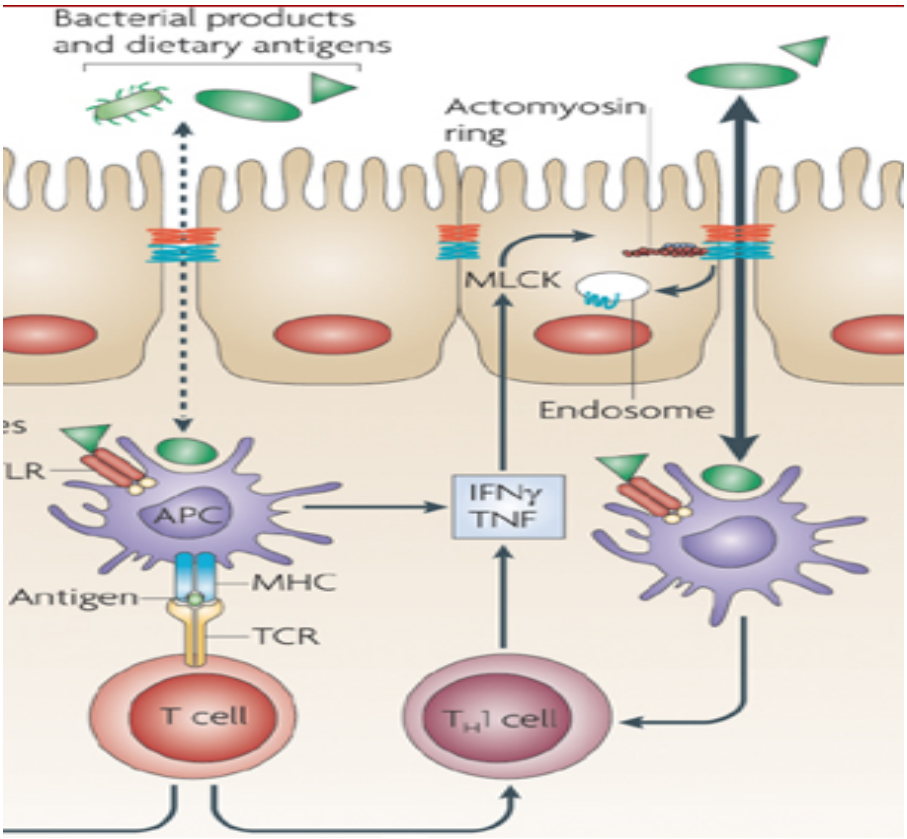

Figure 3 | IFN-Signaling Effects on Intestinal Epithelial Barrier Permeability

The mechanism for this decrease in barrier integrity with pro-inflammatory cytokines (IFN-γ) has been shown to be through the formation of a leak pathway that is characterized by activation of myosin light-chain kinase (MLCK). When activated, MLCK phosphorylates myosin light-chain causing the contraction of the actinmyosin ring around the cell. This contraction results in the internal localization of tight junction proteins like ZO-1, Occulin, and junctional adhesion molecule A (JAM-A). Combined, the actinmyosin ring contraction and internal localization of JAM-A cause gaps in the areas between adjacent cells allowing solutes, ions, and luminal antigens to permeate through.

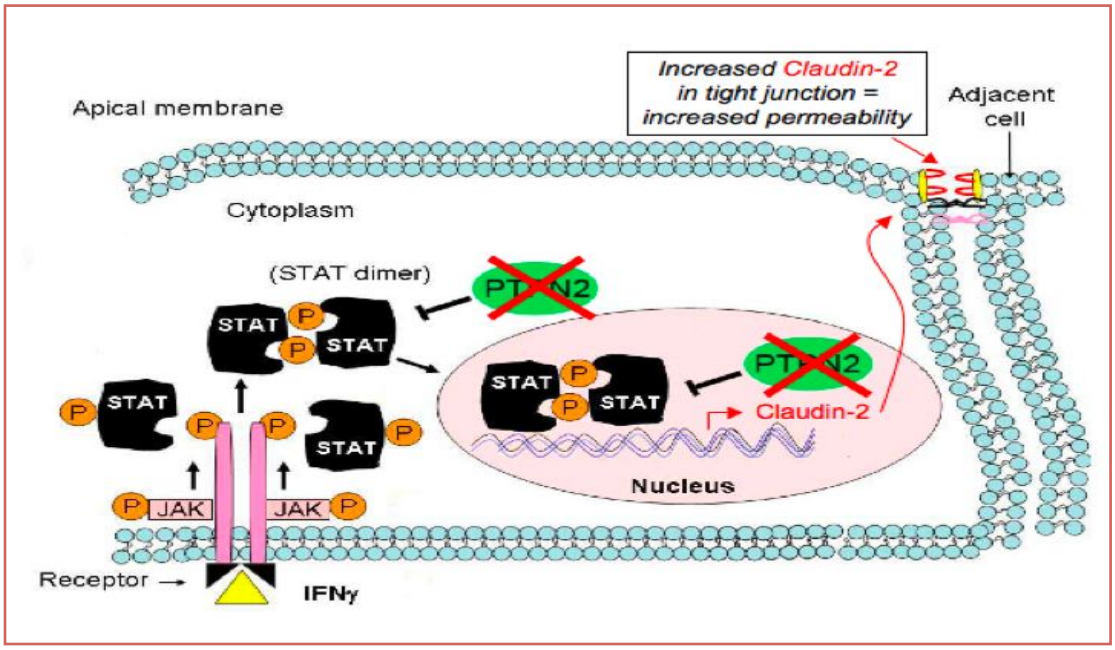

Figure 4 | TCPTP on IFN-γ Signaling

IFN-γ increases the amount of the STAT-1 that is phosphorylated. This is accomplished when IFN-γ binds to IFN-γ receptor causing the recruitment of JAK kinase. JAK kinase ultimately phosphorylates the IFN-γ receptor tails that recruit the STAT proteins (including STAT-1) and phosphorylate them [14]. The increase in phosphorylated STAT-1 is correlated with increased permeability of the intestinal epithelial barrier. Dephosphorylation of STATs (including STAT-1) causes inactivation and stoppage of STAT mediated transcription. TCPTP acts as a negative regulator for phosphorylation of STAT-1.

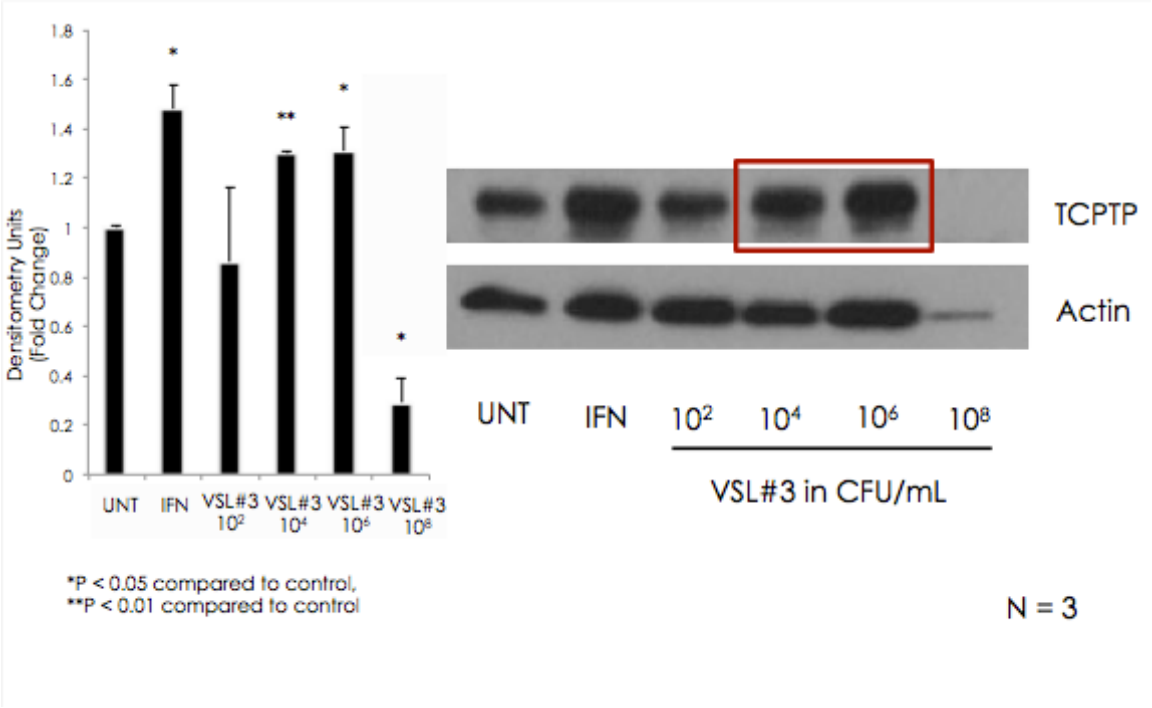

Figure 5 | RT-PCR of TCPTP mRNA Levels with VSL#3 Dose-Response and Time-Course

Results were compared to the untreated levels and each dose was administered for 3 hours, 9 hours, and 24 hours (n = 3). Experiments were performed in triplicates, with each as a trial number of one. There was no statistical significance between the various dose and incubation time conditions of probiotic (VSL#3) and mRNA levels.

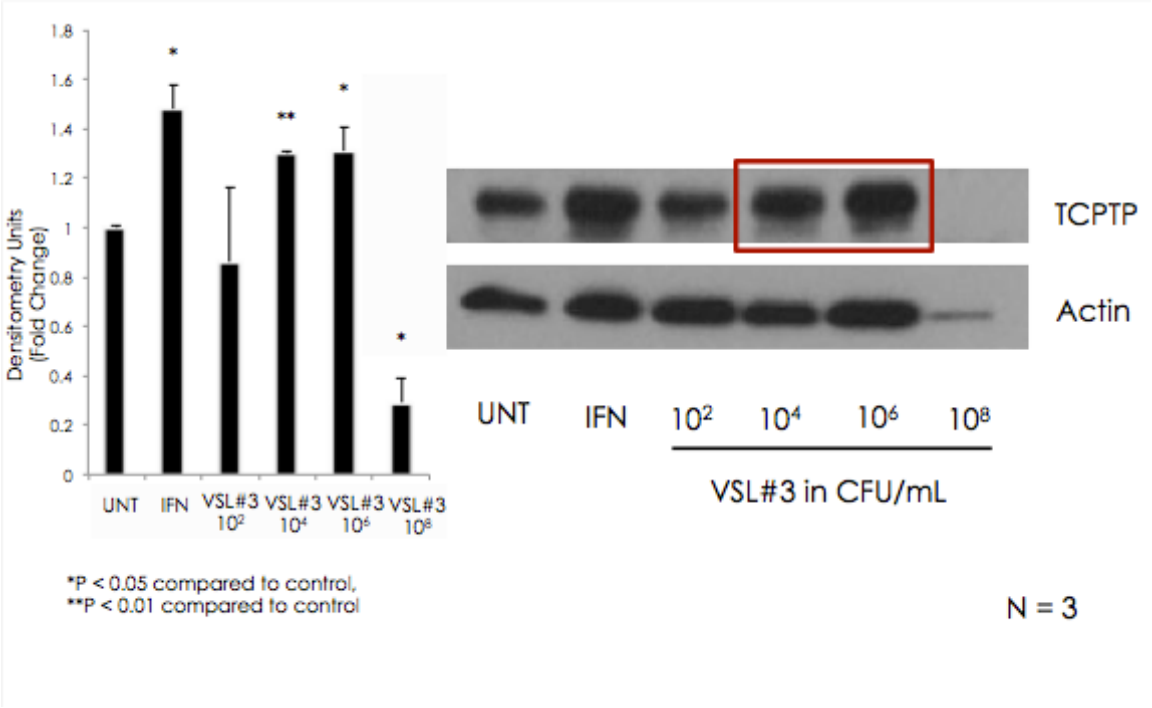

Figure 6 | TCPTP Protein Levels with VSL#3 Dose-Response with 3-Hour Exposure to VSL#3

Western blotting indicated that cytoplasmic TCPTP protein levels increased compared to untreated cells in a 10^4 CFU/mL ($n = 3$, $p<0.01$) and 10^6 CFU/mL ($n = 3$, $p<0.05$) concentrations of VSL#3 following a 3-hour incubation. As expected IFN-γ increased above untreated conditions due to TCPTP's function of regulating IFN-γ signaling in the presence of IFN-γ ($n = 3$, $p<0.05$). Additionally, it was noted with consistency that 10^8 CFU/mL ($n = 3$, $p<0.05$) lead to cell death.

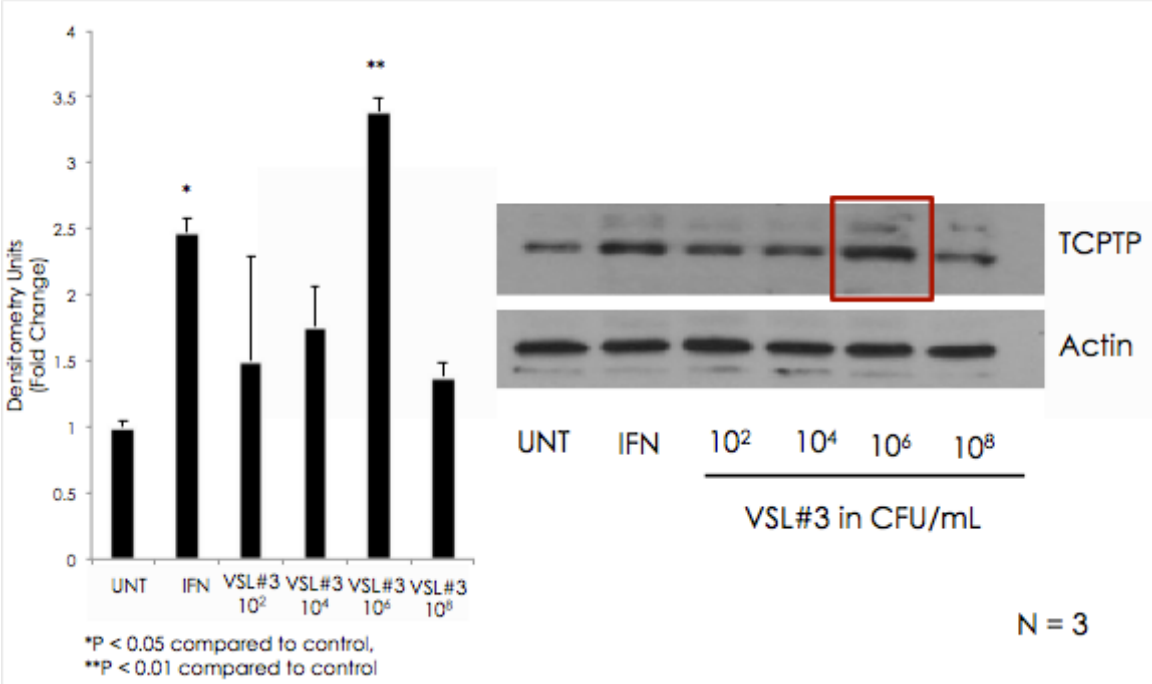

Figure 7 | TCPTP Protein Levels with VSL#3 Dose-Response with 9-Hour Exposure to VSL#3

Following a 9-hour incubation, a VSL#3 concentration of 10^6 CFU/mL showed the strongest increase over the untreated condition ($n = 3$, $p<0.05$) (Figure 10). Additionally, conditions were as expected because IFN-γ increased compared to untreated ($n = 3$, $p<0.05$).

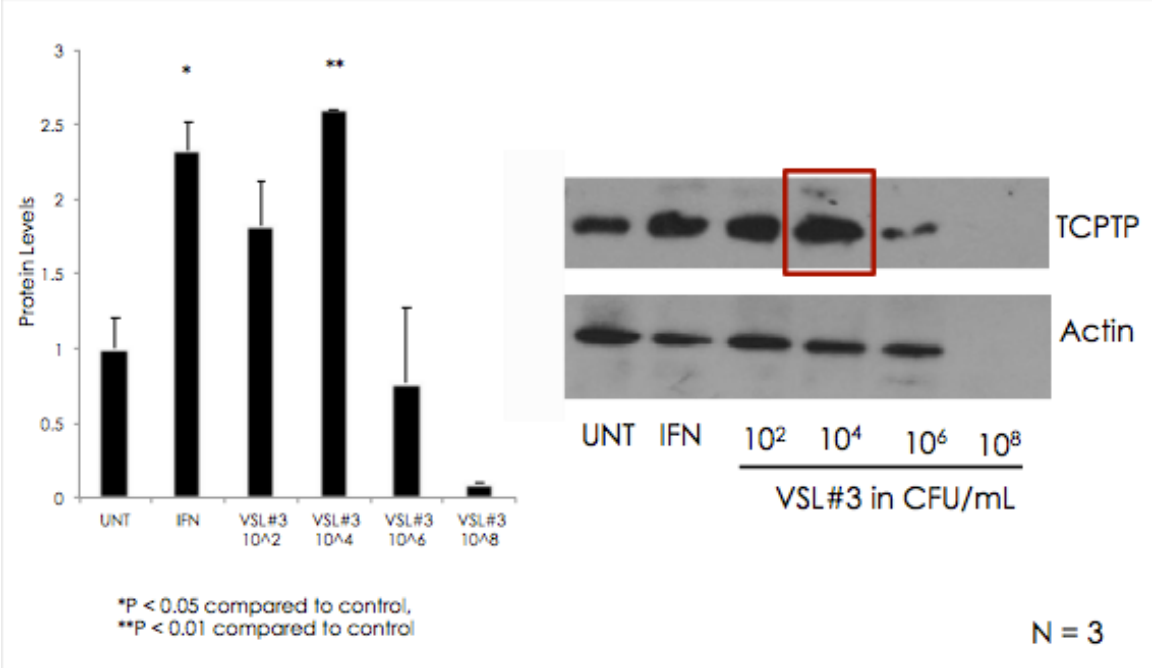

Figure 8 | TCPTP Protein Levels with VSL#3 Dose-Response with 24-Hour Exposure to VSL#3

Finally, 10^2 CFU/mL (n = 3, $p<0.05$) and 10^4 CFU/mL (n = 3, $p<0.05$) concentrations showed the strongest increase compared to the untreated condition in the 24-hour incubation with VSL#3. Additionally, IFN-γ was noted as increased over the untreated conditions.

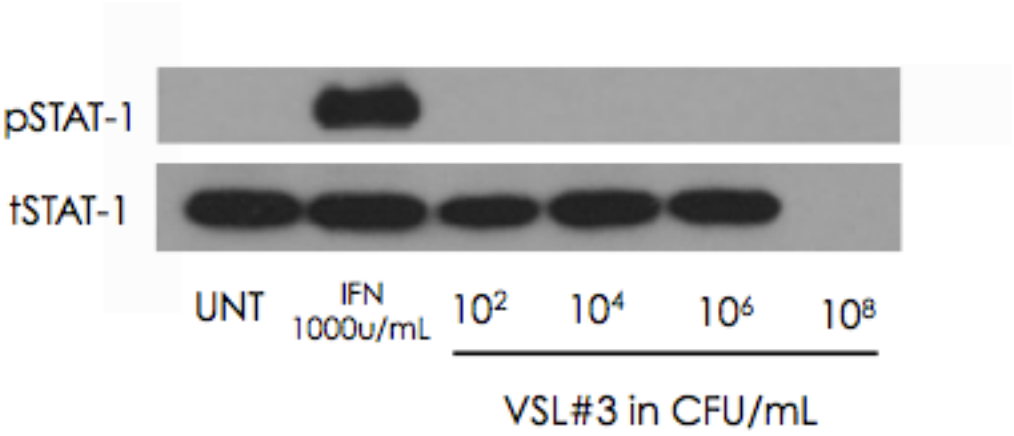

Figure 9 | VSL#3 Exhibits No Stimulatory Effects for Phosphorylating STAT-1

There were no stimulating effects of VSL#3 on phosphorylation of STAT-1, experiments were conducted as a dose-response on VSL#3 with phosphorylated STAT-1 as the readout. Experiments showed there was no pSTAT-1 stimulation as various concentrations of VSL#3 was added (10^2, 10^4, 10^6, 10^8 CFU/mL) to cells (n = 2).

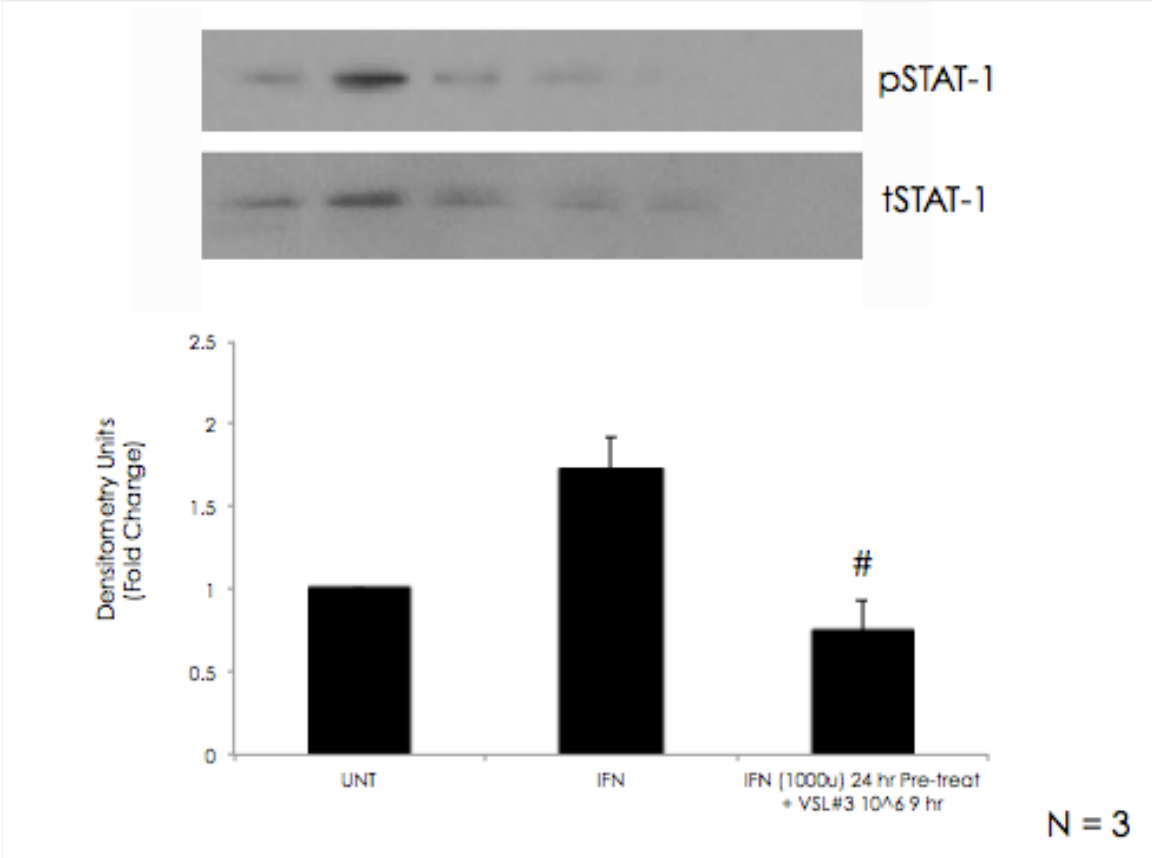

Figure 10 | Effects of VSL#3 and IFN-γ Co-Incubated on Phosphorylated STAT-1 Levels

After 24 hours with IFN-γ, VSL#3 was added for 9 hours at a concentration of 10^6 CFU/mL since that incubation time and concentration showed the strongest expression and activity of TCPTP compared to untreated conditions (Figure 10 and Figure ?). The results showed that as VSL#3 dose increased in the co-incubations, the levels of STAT-1 that was phosphorylated decreased compared to the condition with just IFN-γ ($n = 3$, $p<0.05$).

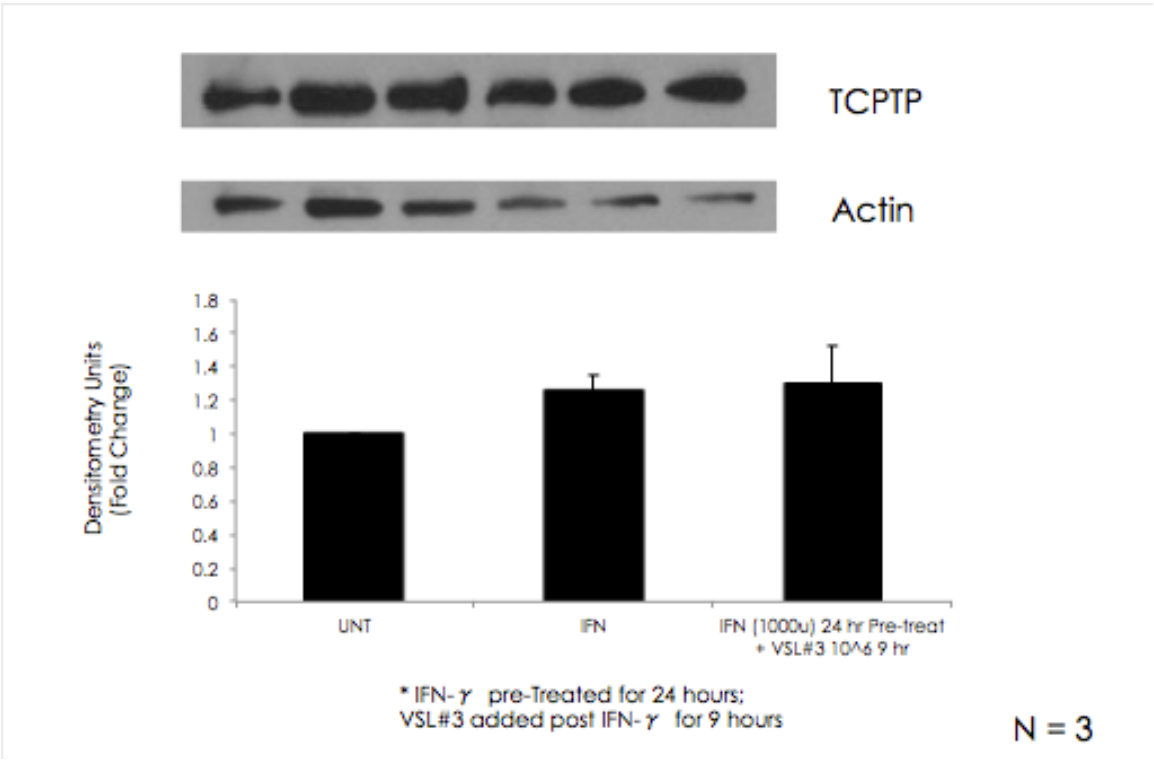

Figure 11 | Effects of VSL#3 and IFN-γ Co-Incubated on TCPTP Protein Levels

There was an increase in TCPTP was the VSL#3 dose increased in the presence of IFN-γ. Results showed that 10^6 CFU/mL of VSL#3, which showed strong decrease of phosphorylated STAT-1, had a 33% increase in TCPTP protein levels over untreated conditions. This value as well as the 61% increase in TCPTP protein levels over untreated conditions seen with 10^8 CFU/mL VSL#3 was not conclusive because there was no statistical significance.

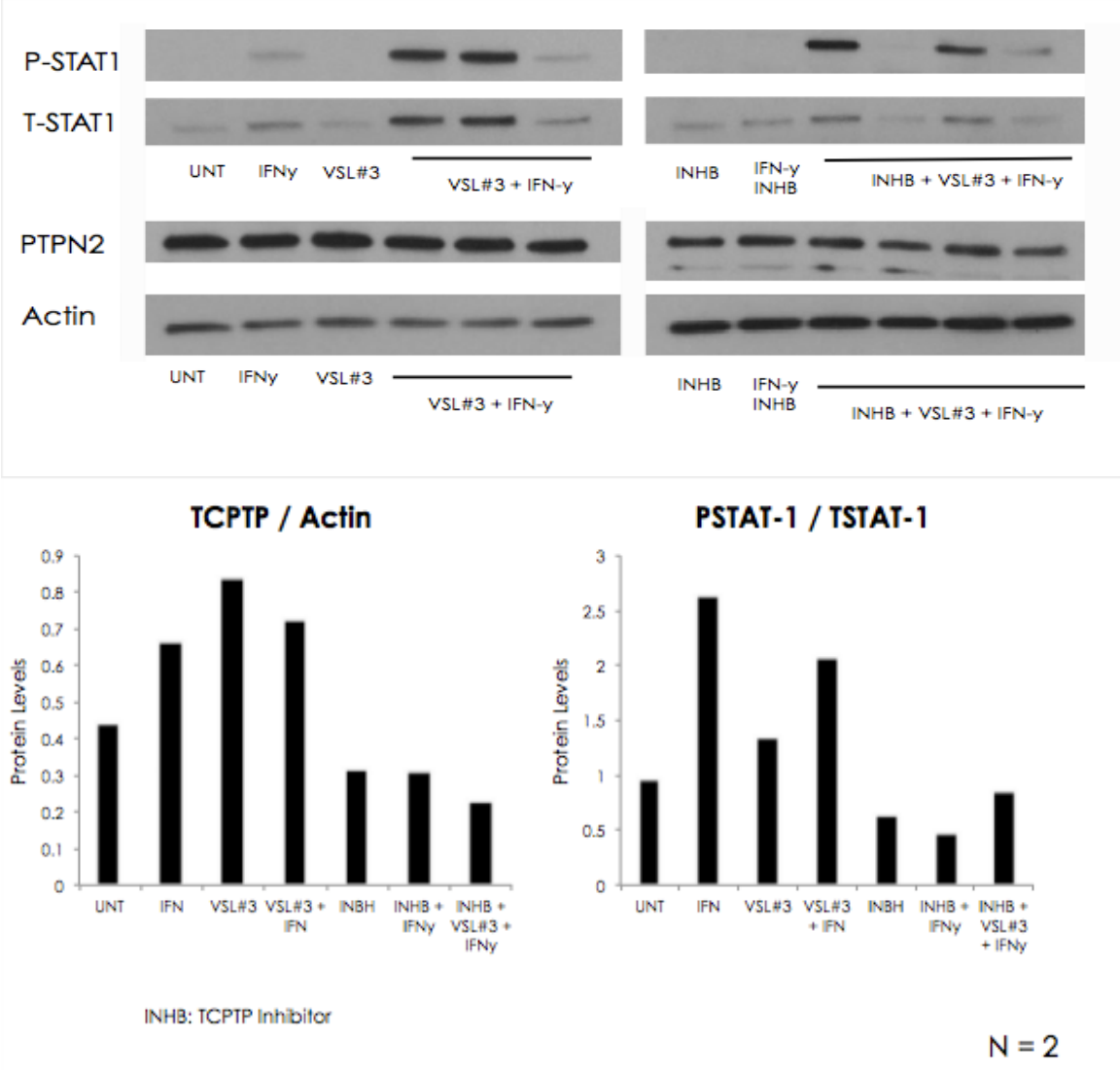

Figure 12 | VSL#3 Effects TCPTP Mediated?

An inhibitor of TCPTP enzymatic activity was used to see if VSL#3's effects on IFN-γ signaling if TCPTP's enzymatic activity is inhibited. The readout was increased phosphorylated STAT-1 compared to cells treated with IFN-γ alone. The results showed that the inhibitor of TCPTP alone showed a decrease of 34% in phosphorylated STAT-1 levels, so that was noted. Ultimately, all conditions with the TCPTP inhibitor showed drastically reduced levels of phosphorylated STAT-1 compared to IFN-γ alone and IFN-γ with VSL#3. The phosphorylated STAT-1 of the IFN-γ alone condition decreased 67% for the condition with IFN-γ, the TCPTP inhibitor, and VSL#3 (106 CFU/mL). It is likely the TCPTP inhibitor was not able to inhibit TCPTP activity or the inhibitor might have caused external effects that prevented phosphorylation of STAT-1.

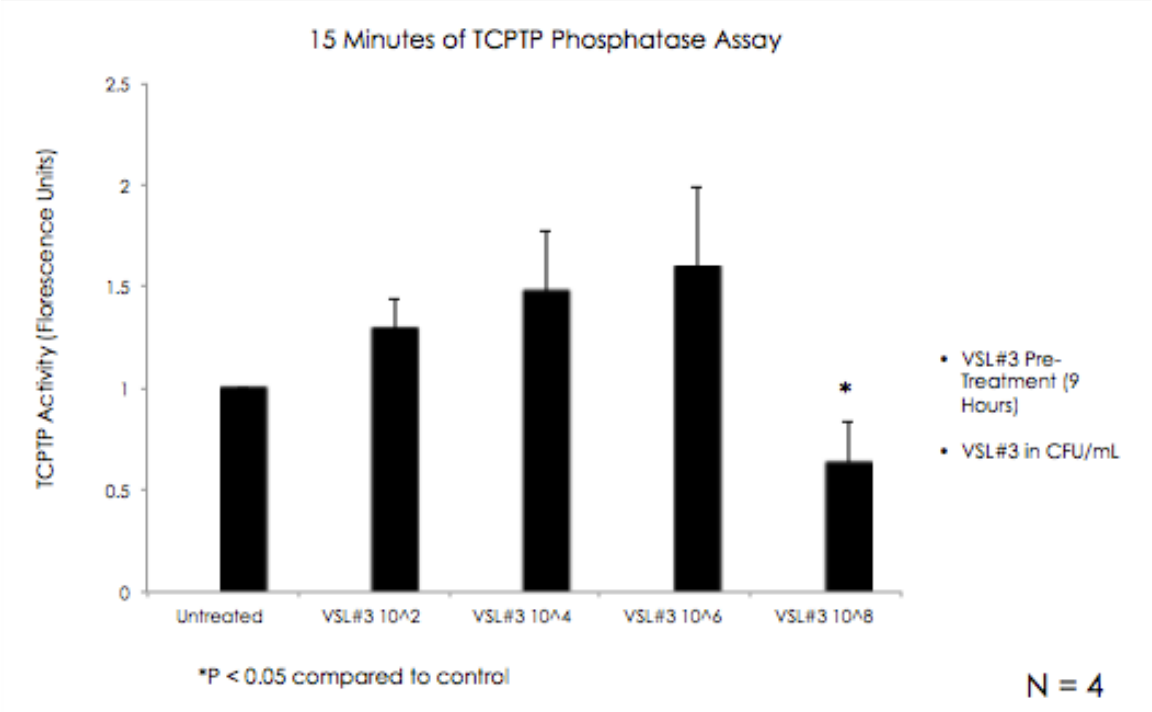

Figure 13 | VSL#3 Effects on Cytoplasmic TCPTP Enzymatic Activity – Dose Response

Data acquired from the VSL#3 dose-response activity assays demonstrated that VSL#3 exhibited the strongest stimulatory effect of a 100% increase on TCPTP enzymatic activity compared to untreated with a 10^6 CFU/mL of VSL#3 ($n=5$, $p<0.05$) after 120 minutes of the phosphatase activity assay (Figure 13). Additionally, 10^2 CFU/mL and 10^4 CFU/mL doses of VSL#3 showed a stimulatory effect on TCPTP activity above untreated at an 85% increase after 120 minutes of the phosphatase activity assay (Figure 13).

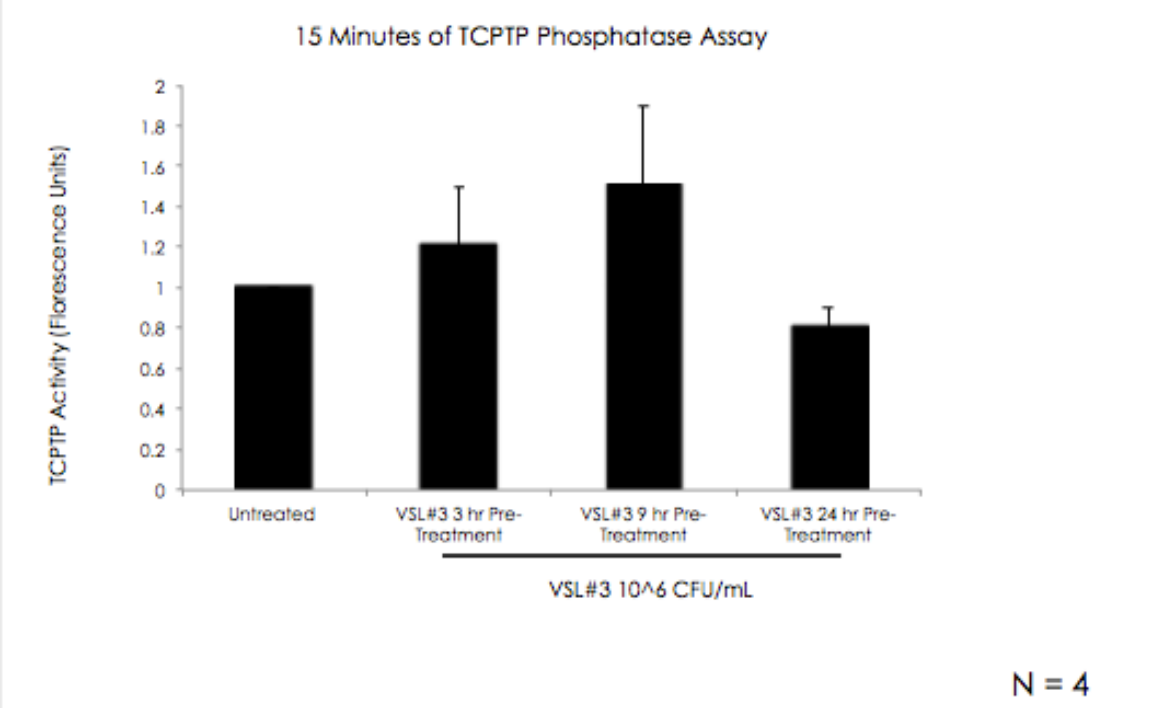

Figure 14 | VSL#3 Effects on Cytoplasmic TCPTP Enzymatic Activity – Time Course

VSL#3 incubation time-course results showed a similar stimulatory trend as the dose-response. Due to the fact that 10^6 CFU/mL showed the strongest effect on TCPTP enzymatic activity in the dose-response, all incubation times were does with 10^6 CFU/mL of VSL#3. The results of the activity assay showed that a 9-hour VSL#3 pre-treatment time with a 10^6 CFU/mL concentration of VSL#3 showed the strongest increase in activity compared to the untreated conditions ($n=5$, $p<0.05$). The demonstrated increase was a 100% increase (Figure 14). With a 10^6 CFU/mL concentration, incubation time-points of 3 hours and 24 hours also showed an increase in TCPTP activity over the untreated, but were less stimulatory.

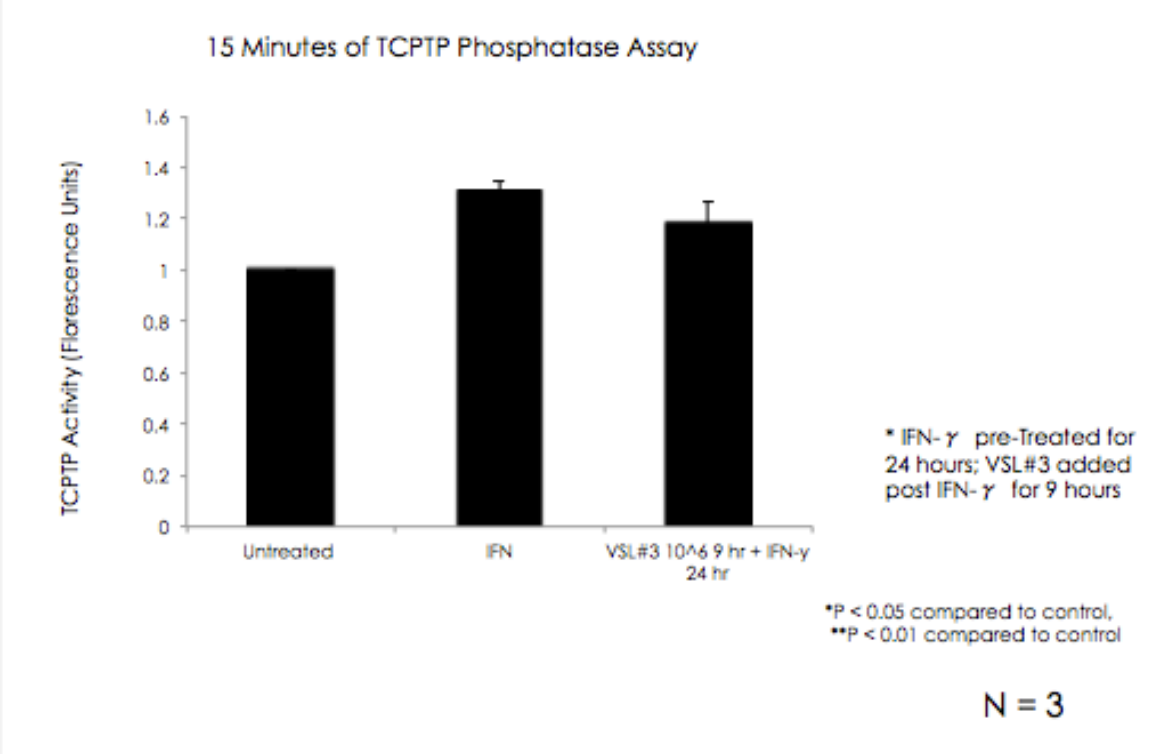

Figure 15 | VSL#3 and IFN-γ Effects on TCPTP Activity

Results here examined affects on TCPTP enzymatic activity to get a full scope of how VSL#3's effects on TCPTP exert an effect on IFN-γ signaling. There were significant fluctuations with the enzymatic activity in conditions with VSL#3. At the initial part of the phosphatase assay, the condition with IFN-γ alone and the condition with VSL#3 106 for 9 hours (after 24 hour IFN-γ pre-treatment) were only 300 units apart. After 120 minutes with the substrate for the assay added, the IFN-γ condition was 2000 units above the 9-hour co-incubated condition. Additionally, the beginning of the assay shows the 9-hour co-incubated condition 800 units above untreated, but after 120 minutes it is 1500 units below untreated. If the end of the assay was a better predictor of how TCPTP activity lasts in the cell over time, then there is a significant drop in activity of TCPTP when VSL#3 and IFN-γ are co-incubated.

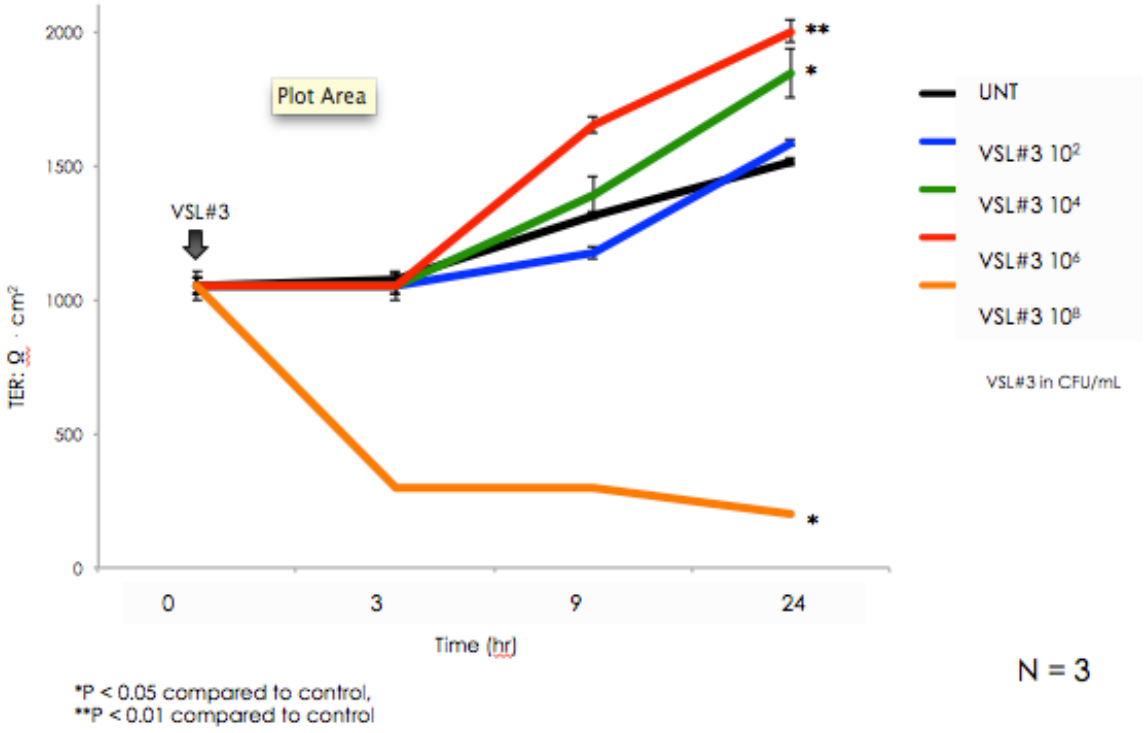

Figure 16 | VSL#3 Effects on TER – Dose Response and Time Course

TER increased in all doses of VSL#3 (10^2, 10^4, 10^6 CFU/mL, n = 3) compared to untreated at the 9 hour and 24 hour time-points. The 10^8 CFU/mL concentration of VSL#3 resulted in cell death in all measurements past 3 hour incubation. The combined condition that resulted in the strongest increase in TER over the untreated was the 10^6 CFU/mL with an incubation time of 9 hours and 24 hours. At 24 hours, the condition with 10^6 CFU/mL of VSL#3 had a TER that was 32% greater than untreated samples after 24 hours. Additionally, at 9 hours the condition with 10^6 CFU/mL of VSL#3 had a TER that was 25% greater than untreated samples after 9 hours

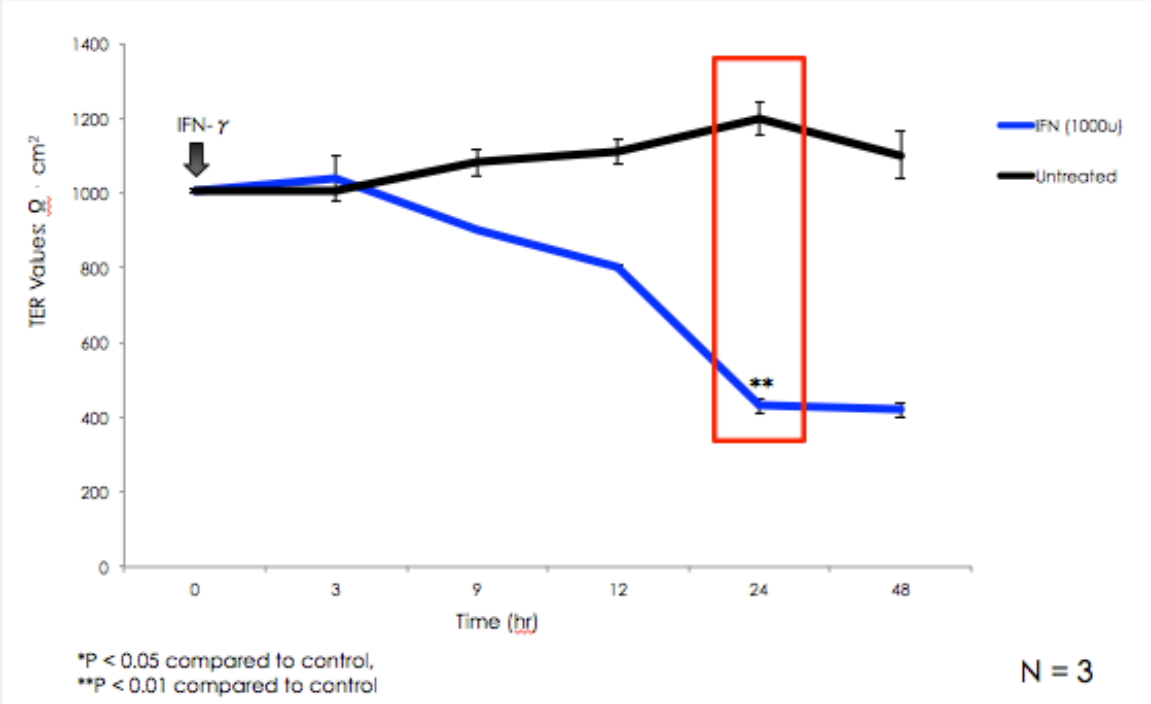

Figure 17 | IFN-γ Signaling Effects on TER

T_{84} cells were treated with 100u/mL and 1000u/mL. TER was recorded through a time course to see when TER is affected by IFN-γ signaling. This data would be used with Figure 19 to determine when addition of VSL#3 to repair damaged intestinal epithelial barrier (low TER) was needed. Results show that after 24 hours, a 66% decrease from untreated TER is seen with 1000u/mL of IFN-γ.

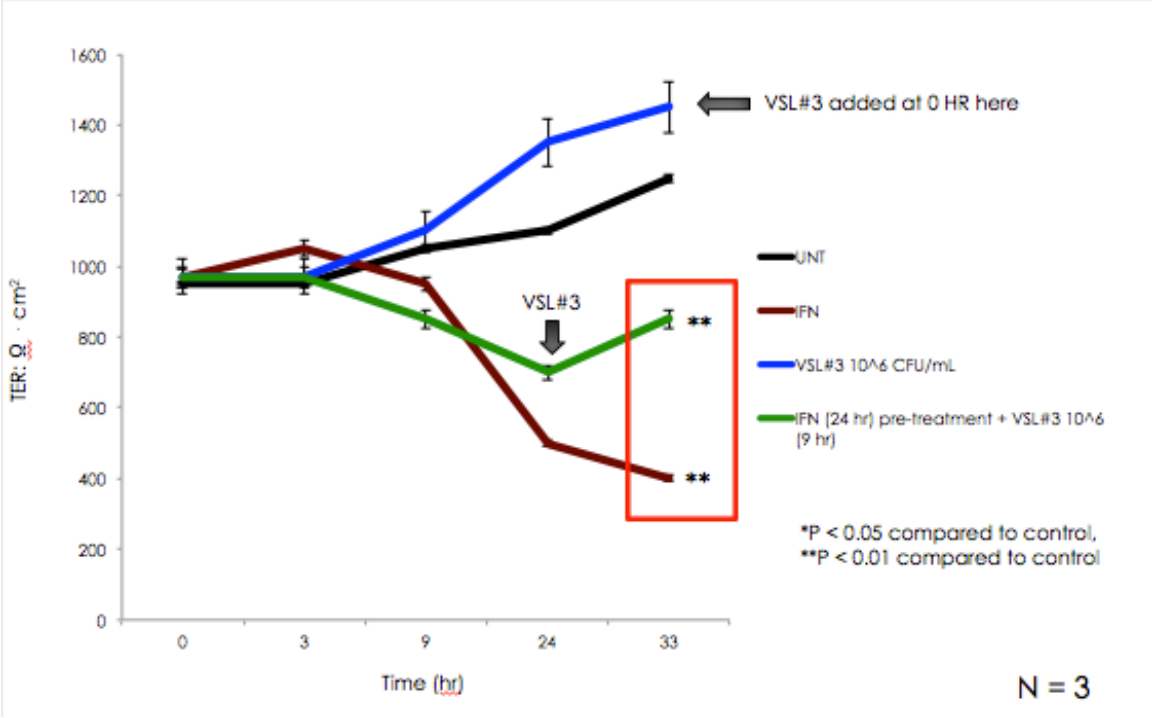

Figure 18 | Effects of VSL#3 on TER in Setting of Inflammation from IFN-γ

Following a 24-hour incubation with IFN-γ, TER decreased on average by 64% compared to untreated. Co-administration experiments included VSL#3 at 10^6 CFU/mL due the strongest effects on TCPTP activity and expression seen at that concentration. Co-incubation included a time-course along with all samples having 106 CFU/mL concentrations. The co-incubation with VSL#3 at 10^6 CFU/mL significantly reduced the effects of IFN-γ (1000u) (n = 3).

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