UNIVERSITY OF CALIFORNIA, SAN DIEGO

Spermidine Stimulates Protein Tyrosine Phosphatase N2-Mediated Protection of Intestinal Epithelial Barrier Function

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Harrison M. Penrose

Committee in charge:

Professor Kim E. Barrett, Chair
Professor Kit J. Pogliano, Co-Chair
Professor Ananda W. Goldrath
Professor Declan F. McCole

2012

The Thesis of Harrison M. Penrose is approved and is acceptable in quality and in form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2012

EPIGRAPH

"I didn't have time to write a short letter, so I wrote a long one instead."

Mark Twain

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would first like to express my appreciation for Dr. Kim Barrett and Dr. Declan McCole for giving me the opportunity to join their lab. Kim and Declan have been exceptional mentors, and have always offered support and guidance whenever needed. Their intellectual and professional influence will follow me wherever I go. A special thanks to Declan for the basis of figure 1 in this discussion. Furthermore, I am grateful for both Kim and Declan's patience throughout the many edits of this script.

I would also like to thank Dr. Kit Pogliano and Dr. Ananda Goldrath for serving as additional members of my thesis committee.

Certainly, I am indebted to Dr. Ron Marchelletta, who over the past three years has taught me the techniques needed to succeed in the lab. Whether I needed help designing experiments, interpreting results, or just talking about life in general, Ron has always been there for me. I am thankful for his friendship and guidance.

Finally, I would not be where I am today without my family. I have amazing parents, who have sacrificed endlessly to give my sister and me the best possible shot at life. For their unconditional love and support I am grateful. And of course if I didn't mention my lovely sister, Annie, I would most certainly get an earful. In any case, I am a lucky brother. Looking forward to the experiences we will share in the years ahead.

ABSTRACT OF THE THESIS

Spermidine Stimulates Protein Tyrosine Phosphatase N2-Mediated Protection of Intestinal Epithelial Barrier Function

by

Harrison M. Penrose

Master of Science in Biology

University of California, San Diego, 2012

Professor Kim E. Barrett, Chair

Professor Kit J. Pogliano, Co-Chair

Genome-wide association-studies (GWAS) have revealed that single nucleotide polymorphisms (SNP) in the gene locus encoding a particular phosphatase, protein tyrosine phosphatase non-receptor type 2 (PTPN2), are associated with the chronic intestinal inflammatory condition, Crohn's disease. PTPN2 is ubiquitously expressed. However, its expression in intestinal epithelial cells (IEC) has been shown to play an important role in the protection of epithelial barrier function during periods of inflammation by acting as a negative regulator of the pro-inflammatory cytokine,

interferon-γ (IFN-γ). Therefore, agents that increase the activity of PTPN2 are of general interest as modifiers of inflammatory signaling events.

A recent study demonstrated that the small molecule, spermidine, is a selective activator of PTPN2 *in vitro*. Here, I describe the effects of spermidine on PTPN2 expression and activity, as well as its effect on IFN-γ signaling and barrier function in the human colonic epithelial cell line, T_{84}. My studies revealed that spermidine increased both PTPN2 protein levels and enzymatic activity, correlating with a decrease in the phosphorylation of the signal transducers and activators of transcription (STAT)1 and 3, downstream mediators of IFN-γ signaling, upon co-administration of spermidine to IFN-γ treated cells. Additionally, spermidine protected barrier function in the setting of inflammation, restricting the decrease in transepithelial electrical resistance (TER) induced by IFN-γ in co-incubation experiments. Spermidine's ability to increase PTPN2 levels and activity, as well as reduce IFN-γ signaling in IECs, implicate spermidine as a potential therapeutic agent for treating conditions associated with dysregulated IFN-γ signaling and a faulty mucosal barrier.

I. INTRODUCTION

Inflammatory Bowel Diseases Inflammatory bowel disease (IBD) is the umbrella term given to two separate but related conditions, Crohn's Disease (CD) and Ulcerative Colitis (UC), both of which are characterized by chronic inflammation of the gastrointestinal tract [1]. CD can affect all regions of the gastrointestinal tract from mouth to anus, whereas UC is confined to the colon. CD and UC also differ from each other by the type of ulceration present (transmural vs. superficial), the location of the ulcers (ileal/colonic skip lesions vs. continuous colonic ulcerations), and the predominating cytokine (interferon-γ (IFN-γ) vs. IL-13), respectively [2]. Both subtypes of IBD are idiopathic in nature and the pathogenesis of disease is a multi-factorial event. Disease progression has been attributed to genetic predisposition, environmental triggers, and an inappropriate immune response to normally harmless commensal flora in the gut [3].

The epithelium of the gastrointestinal tract is vital for proper intestinal function. It is responsible for the absorption of water and nutrients, the maintenance of ionic homeostasis, and the establishment of a protective layer between the intestinal flora and sub-mucosa. In many cases, inflammation seen in patients with IBD is due to a dysfunctional epithelial mucosal barrier, as a decrease in intestinal barrier function is predictive of disease onset and clinical relapse in humans and mouse models [4, 5]. Both CD and UC are characterized by a decrease in epithelial barrier resistance and an increased permeability, thought to be a contributing factor to the major clinical symptom of IBD, diarrhea [6, 7]. This defective mucosal barrier coupled with a dysfunctional

immune response to commensal flora is thought to drive the development of chronic intestinal inflammation [3].

In IBD, pro-inflammatory cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α) are present at high concentrations, with elevated levels of IFN-γ predominating in CD [8]. While elevated levels of TNF-α are linked to an innate immune response and NFκB activation, the high levels of IFN-γ associated with CD are due to an overactive T-cell response, predominated by a T_H1 subpopulation. The T_H1 responses are mediated by IFN-γ, which is produced by antigen-presenting cells (APCs) in the intestinal mucosa in response to IL-12 stimulation [9]. Both IFN-γ and TNF-α have been shown to decrease barrier function [10]. These cytokines lead to increased epithelial permeability and decreased resistance through the leak pathway, which is characterized by the activation of myosin light-chain kinase (MLCK) leading to contraction of the actinomyosin ring around the cell body. At the same time, components of the tight junction such as junctional adhesion molecule A (JAM-A) are internalized [10, 11]. Both the contraction of the actinomyosin ring and the internalization of tight junction constituents lead to an increase in gaps between adjacent epithelial cells in the monolayer, thereby compromising the barrier [10]. Resolution of the barrier occurs when concentrations of pro-inflammatory cytokines fall through decreased production, or due to increased expression and/or activity of negative regulators of cytokine signaling. One of these negative regulators of IFN-γ cytokine signaling is protein tyrosine phosphatase non-receptor 2 (PTPN2).

The role of PTPN2 and mutations associated with CD PTPN2 is a member of the protein tyrosine phosphatase family (PTP) whose members share a highly conserved catalytic motif. All members of the PTP superfamily utilize the same basic catalytic mechanism, in which the cysteinyl residue in the signature motif executes a nucleophilic attack on the phosphate group in the substrate [12]. PTPs play an integral role in regulating a variety of basic cellular processes including cell growth and differentiation [13]. PTPN2, whose protein product is also referred to as T-cell protein tyrosine phosphatase (TCPTP), exists in two splice forms, a nuclear 45-kilodalton and a cytoplasmic 48-kilodalton variant that localizes to the endoplasmic reticulum via the ER localization signal at its N-terminus [14]. The ER localization signal of the 45-kilodalton variant is truncated and replaced instead with a nuclear localization sequence, allowing the 45 kDa isoform to traffic between the cytosol and the nucleus [14].

Among PTPN2 substrates are the phosphotyrosine residues on the receptors for epidermal growth factor [15] and insulin [16]. Additionally, PTPN2 negatively regulates phosphorylation of the IFN-γ receptor (IFNγR) and the IFN-γ signaling molecules, signal transducers and activators of transcription (STAT)1 and 3 [17, 18]. In the setting of inflammation, binding of IFN-γ to its receptor leads to receptor dimerization and recruitment of the JAK kinase. JAK induces phosphorylation of IFNγR tails, which is followed by STAT protein recruitment and phosphorylation. Phosphorylated STATs form homo- or heterodimers, which are able to translocate to the nucleus, bind DNA, and upregulate the expression of certain gene products [19]. Increased levels of phosphorylated STAT1 and 3 are seen in IBD, correlating with the elevated IFN-γ levels [20]. Dephosphorylation of STATs by PTPN2 leads to their inactivation and termination

STAT-induced transcription [17]. PTPN2, therefore, plays an integral role in regulating IFN-γ signaling and maintaining cellular homeostasis (Fig. 1).

Genome-wide-association-studies have revealed that individuals with single nucleotide polymorphic mutations in the PTPN2 locus have an increased susceptibility to developing CD and UC [21, 22]. Although PTPN2 expression is increased in CD intestinal biopsy specimens, the current hypothesis concerning the role of PTPN2 SNPs in IBD is that a loss of PTPN2 function may contribute to disease progression [7]. Loss of PTPN2 leads to prolonged IFN-γ induced STAT activation of transcription and increases in intestinal permeability [7]. While it has been shown that PTPN2 expression and activity is increased in response to IFN-γ, reduced PTPN2 expression leads to increased effects of IFN-γ on STAT signaling and a greater reduction in barrier function. Therefore, agents that increase the activity of PTPN2 are of general interest as modifiers of inflammatory signaling events.

The small polyamine, spermidine, as a stimulus of PTPN2 A recent study demonstrated that the small molecule, spermidine, is a selective activator of PTPN2 *in vitro* [23]. Small molecule activation of protein tyrosine phosphatases provides new insight into the potential pharmacologic regulation of certain cellular events. By investigating the effects of spermidine on PTPN2 in intestinal epithelial cells, my studies serve not only to promote a further understanding of intestinal epithelial barrier maintenance, but also to identify a possible therapeutic agent for conditions associated with dysregulated IFN-γ signaling and intestinal barrier defects.

Spermidine belongs to a class of molecules called polyamines, which are involved

in many physiological processes including cell growth and proliferation, immunity, protein and nucleic acid synthesis, and certain cellular signaling processes (Fig. 2) [24]. In addition to spermidine, two other major polyamines that are produced in the intestine include putrescine and spermine. Polyamines are low molecular weight amines that are water-soluble and fully protonated at physiological pH [25]. They therefore exhibit polycationic character, thought to play a critical role in their biological activity, including the interaction with polyanionic macromolecules such as DNA and RNA [26].

Polyamines are derived from a precursor molecule called ornithine. Putrescine is the first polyamine synthesized following an initial decarboxylation reaction facilitated by the enzyme ornithine-decarboyxlase (ODC) [25]. Spermidine is derived from putrescine, and spermine from spermidine. The formation of spermidine and spermine are derived from successive attachments of two propylamine groups by the action of the aminopropyl-transferases, spermidine and spermine synthetase [24].

In addition to *de novo* synthesis, polyamines are also taken up from extracellular sources. Transport is one of the main ways in which the intracellular polyamine concentration is regulated. The transport of polyamines has been well characterized in bacteria, but not in eukaryotes. In *Escherichia coli* (*E. coli*), multiple polyamine transport mechanisms have been determined, including two ATPase-dependent transporters, one particular to putresine, another to spermidine, and a third system specific to putrescine allowing for both its uptake and release [24]. In mammals, though, gene(s) encoding the transporter(s) have not been identified. Additionally, the uptake of polyamines in lung epithelial cells was shown to occur via diffusion [25].

Polyamines are known to play an important role in many biological processes.

They have been shown to bind both DNA and RNA, leading to both increased expression of certain gene products, as well as the stabilization of mRNA transcripts [24]. Additionally, polyamines have been reported to be involved in cell signaling processes through interactions with various proteins [24]. Because of their polycationic character, polyamines interact strongly with membrane phospholipids, and therefore may play an important role in regulating the activity of membrane-linked enzymes. An activating effect on various protein tyrosine kinases and the GTPase activity of some G-proteins has been demonstrated [26, 27]. Additionally, a recent report demonstrated that the interaction of spermidine with PTPN2 stimulated the enzyme's activity [23]. While the proposed mechanism by which spermidine increases PTPN2 activity is not defined, one current hypothesis suggests that the polycationic character of spermidine is responsible for breaking an autoregulatory intramolecular bond between the carboxy-terminal and catalytic domain in PTPN2 [23]. This hypothesis is based on previous experiments showing that activation of PTPN2 was significantly increased in response to stimulation with the positively charged cytoskeletal protein, α_1-integrin [15]. This view is further supported by the fact that a truncated PTPN2, which lacks the carboxy-terminal region of the protein, has significantly greater activity than the wild-type protein [28]. Thus the carboxy-terminal sequence of PTPN2 is important for regulating its catalytic function, and keeps the phosphatase from being constitutively active. Moreover, breaking of this intramolecular bond through electrostatic interactions significantly stimulates its activity, correlating with increased dephosphorylation events.

In summary, my work here represents the first steps in understanding the effects of spermidine on PTPN2 in intestinal epithelial cells. Identifying the effects of

spermidine on PTPN2 is an important undertaking as increased activity of PTPN2 in IECs could lead to enhanced downregulation of inflammatory cytokine signaling. In addition, these findings, when applied to the setting of intestinal inflammation, could serve not only to promote a further understanding of intestinal epithelial barrier maintenance, but also to identify a possible therapeutic agent for conditions associated with intestinal barrier defects. Targets for the control of inflammation in IBD may be found by understanding the signaling pathways involved in disease progression. Moreover, researching the effects of spermidine on PTPN2 may contribute to discovering more about phosphatase function. This in turn could generate insights into the pharmacological development of PTPN2 agonists that could become clinically relevant therapies in the future.

II. MATERIALS AND METHODS

Materials Human recombinant IFN-γ (Roche, Mannheim, Germany), spermidine trihydrochloride (Sigma), spermine tetrahydrochloride (Sigma), cycloheximide (Sigma), monoclonal mouse anti-PTPN2 antibody CF-4, which detects the 45-kilodalton and the 48-kilodalton isoforms (Calbiochem, San Diego, CA), anti-phospho-STAT1 (Tyr^{701}), anti-STAT1, anti-phospho-STAT3 (Tyr^{705}), 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 95% confluency, and split 1:5. For *in vitro* experiments, T_{84} cells were seeded onto 12mm Millicell-HA culture plate inserts (filter membranes) and grown for 12-14 days before study, at which time they had stable values of transepithelial electrical resistance. IFN-γ (1000U/mL) was added basolaterally, whereas spermidine (10μM), spermine (10 μM), and cycloheximide (35.5 μM) were added bilaterally.

RNA Isolation and Real-Time Polymerase Chain Reaction Total RNA was isolated and DNA was removed from T_{84} cells using the Direct-zol RNA MiniPrep kit (Zymogen, Irvine, CA) according to 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, Fostery 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 perfored 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 $NaVO_3$, 100μg/ml phenylmethylsulfonyl fluoride), vortexed thoroughly, and 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 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 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 15-min washes with 0.1% TBST. After washes, secondary antibody (goat-anti-rabbit, Cell Signalling, Danvers, MA, or goat-anti-mouse IgG conjugated to horse radish perodixase, BD Pharmigen, San Diego, CA) diluted 1:2000 was added to the membrane and incubated for 30 min. This was followed by four more 15-min washes with wash buffer. The membrane was then treated with chemiluminescent solution according to the manufacturer's directions (Thermo Scientific, Rockford, IL) and exposed to film. Densitometric analysis of the blot was performed using Image J software (NIH).

Immunoprecipitation 6×10^6 T_{84} cells were prepared in 75 cm^2 flasks. Following stimulation, cells were scraped 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 nonsoluble particles. 1 µg of monoclonal mouse anti-PTPN2 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 ditheothreitol). 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 amount of dephosphorylated DiFMUP. PTPN2 was first immunoprecipitated from whole cell lysates using anti-PTPN2 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 PTPN2 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 PTPN2 densitometric values obtained from

Western blotting. Thus, values represent the specific activity of PTPN2 rather than total quantities of the enzyme.

Small Interfering RNA Transfection 2 x 10^6 T_{84} cells were seeded 3 days before transfection. For PTPN2, 100 pmol of 3 different annealed Silencer Pre-designed small interfering RNA (siRNA) oligonucleotides (Applied Biosystems, Foster City, CA) were transfected into T_{84} cells using the Amaxa nucleofector system (Amaxa, Gaithersburg, MD). After transfection, cells were cultured on filter membranes for 72 hours before treatment. Control siRNA SMARTpool (Upstat Biotechnology/Dharmacon, Chicago, IL) (100 pmol/transfection) was used as a negative control.

Transepithelial Electrical Resistance Transepithelial electrical resistance (TER) across T_{84} monolayers was assessed by voltohmeter (WPI, Sarasota, FL) and companion electrodes (Milipore, Bedford, MA). Measurements were calculated in $\Omega \cdot cm^2$ and expressed as a percentage of the baseline measurement.

Statistical Analysis All data are means ± SEM 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 3 software (Graph Pad Software, La Jolla, CA). *P* values < 0.05 were considered significant.

III. RESULTS

Spermidine shows no effect on PTPN2 mRNA transcription To determine whether spermidine affects intestinal epithelial PTPN2 expression, T_{84} cells grown as monolayers on permeable supports were treated with increasing concentrations of spermidine (0.1, 1.0, 10 μM) for 24 hours. Spermidine was applied to both the apical and basolateral compartment. RT-PCR analysis revealed no increase in PTPN2 messenger RNA (mRNA) levels in cells treated with spermidine compared to untreated T_{84} cells (n=6) (Fig. 3).

Spermidine increases PTPN2 protein levels in a dose dependent manner To investigate whether spermidine affects intestinal epithelial PTPN2 protein levels, T_{84} cells grown as monolayers on permeable supports were treated with increasing concentrations of spermidine (0.1, 1.0, 10 μM) for 24 hours. Spermidine was applied to both the apical and basolateral compartment. Western blotting indicated that cytoplasmic PTPN2 protein levels were increased in a dose-dependent manner compared to untreated cells following a 24-hour incubation with spermidine (Fig. 4). In T_{84} cells treated with 10 μM spermidine, PTPN2 levels were 2.2 ± 0.3-fold greater than those found in control cells ($p < 0.05$, $n = 4$) (Fig. 4). Spermidine was also administered to T_{84} monolayers at concentrations of 100μM and 1mM; however, these concentrations led to significant decreases in transepithelial electrical resistance following 24-hour incubation and were not pursued in further studies.

Spermidine may act to reduce PTPN2 protein degradation To further elucidate the mechanism by which spermidine increased PTPN2 protein levels, I used the ribosomal inhibitor, cycloheximide, to investigate whether spermidine prevented PTPN2 protein degradation, perhaps through post-translational modification(s). Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms. It is a small organic compound that works to inhibit protein elongation by interfering with the translocation of tRNA along the mRNA transcript, thus inhibiting amino acid addition. Previous studies employing the T_{84} cell line used cycloheximide at a final concentration of 35.5 μM to effectively inhibit protein synthesis [30]. Therefore, in my studies, T_{84} cells grown as monolayers on permeable supports were treated with cycloheximide (35.5 μM) for 30 minutes followed by the addition of media alone or media containing spermidine (10 μM) for 24 hours. Western blotting indicated an 18 ± 6% decrease in PTPN2 protein levels in T_{84} cells incubated with cycloheximide compared to the DMSO vehicle control ($p < 0.05$, $n = 4$) (Fig. 5). Co-administration of spermidine to cycloheximide-treated T_{84} cells restored PTPN2 to levels greater than those seen in the vehicle control (Fig. 5). Moreover, PTPN2 proteins levels were on average 26 ± 6% higher in cycloheximide-spermidine co-incubations compared to cycloheximide-treated T_{84} cells, indicating that spermidine appears to prevent PTPN2 degradation post-translationally ($p < 0.01$, $n = 4$) (Fig. 5).

Spermidine increases PTPN2 activity in intestinal epithelial cells Next, I investigated whether spermidine alters PTPN2 enzymatic activity in T_{84} cells. Following incubation with or without spermidine (10μM) for 30 minutes, PTPN2 was immunoprecipitated

from T_{84} whole cell lysates. Data acquired from the activity assays demonstrated that spermidine exhibited a stimulatory effect on PTPN2 activity for the first 45 minutes of the phosphatase activity assay, followed by a decline to basal levels thereafter (Fig. 6). PTPN2 activity in immunoprecipitates incubated with spermidine peaked at 45 minutes, and was 1.9 ± 0.2-fold greater than PTPN2 activity in immunoprecitates from unstimulated T_{84} cells ($p < 0.01$, $n = 3$) (Fig. 6). These data implicate spermidine as a bona fide stimulus of PTPN2 activity *in vitro* in the intestinal cell line, T_{84}.

Spermidine attenuates IFN-γ signaling in intestinal epithelial cells Having determined that spermidine stimulates PTPN2 activity, and given our laboratory's interest in PTPN2 regulation of IFN-γ signaling, it was of interest to establish whether spermidine had any effect on IFN-γ signaling and the phosphorylation state of the IFN-γ signaling molecules, STAT1 and 3. A pathophysiologically relevant dose of IFN-γ (1000 U/mL) was added basolaterally to T_{84} monolayers [7]. Western blot analysis revealed that IFN-γ induced STAT1 phosphorylation following 30 minutes of treatment compared to untreated cells ($p < 0.001$, $n = 5$) (Fig. 7). Co-administration of spermidine (10 μM) for this time significantly attenuated IFN-γ-induced phosphorylation of STAT1 by 26 ± 7 % ($p < 0.01$, $n = 5$) (Fig. 7). Likewise, IFN-γ induced STAT3 phosphorylation following 30 minutes of treatment compared to untreated cells ($p < 0.01$, $n = 4$) (Fig. 8). Co-administration of spermidine (10 μM) for this time attenuated IFN-γ-induced phosphorylation of STAT3 by 26 ± 7% ($p < 0.05$, $n = 4$) (Fig. 8). The decrease in STAT1 and 3 phosphorylation observed in cells co-administered spermidine with IFN-γ, coupled with the observed

spermidine-induced increase in PTPN2 phosphatase activity (Fig. 6), suggests that the increase in PTPN2 activity results in downregulation of IFN-γ signaling through a decrease in STAT1 and 3 phosphorylation.

Spermidine attenuates IFN-γ signaling in a PTPN2-dependent manner Next, I wanted to investigate whether the attenuation of STAT1 phosphorylation observed in T_{84} cells co-administered spermidine with IFN-γ was reversed by PTPN2 knockdown. T_{84} cells were transfected with either PTPN2-specific siRNA or nonspecific control siRNA, seeded on permeable supports, and allowed to grow for 72 hrs. Nonspecific effects of the siRNA transfections were not observed, as shown by equivalent levels of the loading control, β-actin, and total STAT1 in each experiment. Following the addition of IFN-γ (1000 U/mL) to cell monolayers, Western blotting revealed that IFN-γ induced STAT1 phosphorylation following 30 minutes of treatment and that this level was increased in the PTPN2 knockdown samples (n = 3) (Fig. 9). As expected, a decrease in STAT1 phosphorylation was observed in control siRNA transfected cells co-administered spermidine (10μM) with IFN-γ (Fig. 9). Interestingly, the decrease in STAT1 phosphorylation caused by spermidine was not observed in PTPN2 knockdown cells compared to control siRNA-treated cells (Fig. 9). Together, these data indicate that PTPN2 is likely the target by which spermidine reduces IFN-γ-induced STAT1 phosphorylation. While these data did not achieve statistical significance, they show a meaningful trend. The lack of significance is likely due to a low number of replicates, and the study should be repeated.

Activation of PTPN2 is specific for the chemical nature of spermidine There are other polyamines that have similar chemical structures to spermidine. Therefore, it was of interest to determine whether or not other structurally similar polyamines had any effect on IFN-γ signaling events in T_{84} cells. One of these chemically relevant polyamines is spermine (Fig. 10). As mentioned earlier, spermine is produced by the addition of a propylamine group to spermidine, facilitated by the enzyme spermine transferase. To study the effects of spermine on IFN-γ signaling, IFN-γ (1000 U/mL) was added basolaterally to T_{84} monolayers. Western blot analysis revealed that IFN-γ induced STAT1 phosphorylation following 30 minutes of treatment ($p < 0.01$, $n = 5$) (Fig. 11). Co-administration of spermine had no significant effect on IFN-γ-induced phosphorylation of STAT1, revealing that activation of PTPN2 seems to be specific for the chemical nature of spermidine (Fig. 11).

Spermidine protects intestinal epithelial barrier function in the setting of inflammation Finally, it was of interest to determine whether spermidine exhibited a protective effect on IEC barrier function following challenge with IFN-γ. A conventional way to determine barrier function in a cell model that establishes tight junctions is by measuring transepithelial electrical resistance (TER). Decreases in TER correlate with increases in paracellular permeability and a 'leaky' barrier. IFN-γ has been reported to decrease TER in T_{84} cells by as much as 40% as early as 24 hours after treatment [31]. To determine whether spermidine protects barrier function in the setting of inflammation, IFN-γ (1000 U/mL) was added basolaterally to T_{84} cells once they had established a stable monolayer in which the baseline TER before treatment was $\geq 1000\ \Omega \cdot cm^2$.

Following 24-hour treatment with IFN-γ, TER decreased on average by 35 ± 5% ($p < 0.001$, $n = 4$) (Fig. 12). Co-administration of spermidine (10 μM) for this time lessened the effects of IFN-γ. Following a 24-hour co-incubation, TER was reduced by only 20 ± 4%, and was significantly higher than TER in IFN-γ-treated cells ($p < 0.05$, $n = 4$) (Fig. 12).

IV. DISCUSSION

PTPN2 has been shown to play an essential role in regulating inflammatory cytokine signaling by inactivating the IFN-γ signaling molecules, STAT1 and 3. This view is supported by the fact that PTPN2 knockout mice die within 3-5 weeks from systemic inflammation characterized by elevated levels of IFN-γ and TNF-α [32]. IFN-γ has been demonstrated to increase PTPN2 expression and activity; moreover, activated PTPN2 alleviates IFN-γ-induced STAT1 and 3 activation [7]. These results indicate that PTPN2 is involved in a negative feedback mechanism that is ultimately responsible for limiting IFN-γ-induced effects.

Both STAT1 and 3 have been shown to stimulate very different biological effects in response to IFN-γ. STAT1 is associated with mediating IFN-γ-dependent changes in the immune response, cell proliferation, as well as in antiviral effects [33]. Conversely, STAT3 is more closely associated with anti-apoptotic effects, which have been associated with colon cancer pathogenesis [34]. It can therefore be seen that tight regulation of STAT signaling is critical in the maintenance of intestinal epithelial cell homeostasis. PTPN2 is a key regulator of STAT1 and 3 phosphorylation, and therefore plays an integral role in limiting their activity.

SNPs in the PTPN2 locus have been associated with CD and it is thought that these mutations could potentially be linked with its loss of function. While this has not been formally proven, it is thought that loss of PTPN2 function may contribute to IBD progression, due in part to prolonged IFN-γ-induced STAT activation of transcription and subsequent increases in intestinal permeability [7]. Therefore, agents like spermidine that

increase PTPN2 activity are of general interest as modifiers of inflammatory signaling events.

My experiments with spermidine were designed to examine its effects on PTPN2 expression and enzymatic activity, as well as inflammatory cytokine signaling. Using the human colonic epithelial cell line, T_{84}, as my model I have shown that spermidine increases both PTPN2 protein levels and enzymatic activity. Importantly, I demonstrated that the increase in enzymatic activity observed in an *in vitro* activity assay correlates with downregulation of IFN-γ-induced STAT1 and 3 phosphorylation in a cell setting. Moreover, the attenuation of IFN-γ signaling is specific for the chemical nature of spermidine and occurs through a PTPN2-dependent mechanism. Finally, I have shown that spermidine protects intestinal epithelial barrier function in cells challenged with IFN-γ.

My initial experiments characterized the effect of spermidine on PTPN2 expression. RT-PCR data revealed that spermidine had no effect on the levels of PTPN2 mRNA. However, interestingly, PTPN2 protein levels were increased in a dose-dependent manner compared to untreated cells following 24-hour incubation with the polyamine. This implies that spermidine may be stabilizing PTPN2 at the protein level. In an effort to further elucidate the mechanism by which spermidine increased PTPN2 protein levels, I studied spermidine's effect on PTPN2 following translational inhibition using the ribosomal inhibitor, cycloheximide. Following 24-hour treatment with cycloheximide, PTPN2 protein levels were significantly reduced compared to the vehicle control. The addition of spermidine alleviated this decrease. The results of this experiment suggest that the effect of spermidine on PTPN2 protein does not involve

protein synthesis. While it cannot be concluded definitely from this particular experiment, the increase in PTPN2 protein may be due to spermidine physically interacting with PTPN2 and shielding it from ubiquitination, thus preventing its degradation. This speculation could be tested by measuring the amount of ubiquitin associated with PTPN2 following stimulation with or without spermidine. Additionally, the increase in PTPN2 protein could also be an indirect result of spermidine interfering with the activity of the proteasome itself, or other effects on protein degradative pathways.

Spermidine also significantly increased PTPN2 enzymatic activity. Following incubation with spermidine, PTPN2 activity in immunoprecipitates was 1.9 times greater than that of PTPN2 immunoprecipitated from untreated cells. These data fall in line with a previous report implicating spermidine as a stimulus of PTPN2 [23]. Here, again, the mechanism by which spermidine increases PTPN2 activity is not fully understood; however, it is thought that the polycationic character of spermidine alleviates an autoregulatory bond between the c-terminus and catalytic domain of the phosphatase. The idea that electrostatic interactions may mediate PTPN2 activity is supported by previous work that established an increase in activity of the enzyme in response to increased ion concentration. In this *in vitro* study, it was demonstrated that increasing the KCl concentration to 100 mM stimulated the activity of TC45, the 45 kDa PTPN2 variant, about 76-fold [28]. Moreover, the increase in PTPN2 activity determined in the pull-down phosphatase assay was also observed in a cell setting. In my studies, co-administration of spermidine to IFN-γ-treated cells attenuated IFN-γ induced phosphorylation of STAT1 and 3. These data suggest that spermidine is effectively taken

up by T_{84} cells, either passively through diffusion and/or via a transport system, and is capable of interacting with and activating PTPN2, leading to increased dephosphorylation events. It is important, though, not to rule out the possibility that the effect of spermidine on PTPN2 activity may be due to a spermidine metabolite, because spermidine can be oxidized in serum-containing media [35]. Additionally, it can be speculated that the decrease in the phosphorylation levels of STAT1 an 3 are resultant of an overall increase in PTPN2 amount, rather than a spermidine-induced effect on PTPN2 activity. However, it is important to remember that increases in PTPN2 protein by spermidine were observed following 24 hours of treatment. Phospho-STAT1 and 3 levels in cells treated with IFN-γ and spermidine were determined following 30 minutes of treatment. The decrease in STAT1 and 3 phosphorylation observed in IFN-γ-treated cells co-administered spermidine at this time point indicates that it is likely due to a spermidine-induced effect on PTPN2 activity rather than an increase in total phosphatase amount. It would be interesting to look at the effect of spermidine on PTPN2 protein levels following 30 minutes of treatment. Increases in PTPN2 protein levels following 30 minutes of treatment with spermidine may then suggest that the decrease in STAT phosphorylation at this time is due to an overall increase in PTPN2 concentration and not activity.

Next, because there are other cellular protein tyrosine phosphatases present in intestinal epithelial cells, it was of interest to determine whether spermidine's ability to reduce STAT1 phosphorylation was occurring through a PTPN2-dependent mechanism. Following PTPN2 knockdown using small interfering RNA (siRNA), IFN-γ-induced STAT1 phosphorylation was no longer attenuated by spermidine. Importantly, STAT1 phosphorylation was reduced by spermidine in cells treated with a non-specific control

siRNA. As expected, phospho-STAT1 levels were increased in PTPN2 knockdown cells, reinforcing PTPN2 as the cellular phosphatase responsible for regulating IFN-γ-induced STAT1 phosphorylation. Moreover, attenuation of phospho-STAT1 observed in T_{84} cells co-administered spermidine with IFN-γ was reversed in response to PTPN2 knockdown. Although further experiments are needed, together these data indicate that PTPN2 is the cellular phosphatase by which spermidine limits IFN-γ signaling.

It was also of interest to determine whether downregulation of IFN-γ-induced STAT1 phosphorylation was specific for the chemical nature of spermidine. Spermidine is the precursor compound to spermine, a structurally similar polyamine, which is formed by the addition of a propyl amine group to spermidine. Spermine contains an extra amine group, and, therefore a greater net positive charge, which could play a role in the activation of PTPN2. Spermine was unable to reduce STAT1 phosphorylation in IFN-γ-treated cells. To a certain degree, the results of this experiment were surprising since the structures of both spermidine and spermine are so similar. The chemical nature of spermine may not have influenced PTPN2 activity in the same manner as spermidine for at least two reasons. First, the increased molecular weight and increased polycationic character of spermine may decrease its ability to diffuse across the membrane. More importantly, the size and structural nature of spermine may prevent it from directly interacting and influencing PTPN2 in the same manner as spermidine. The results of this experiment are encouraging as to the possible specificity of spermidine for *in vivo* use. Additionally, they shed light onto the chemical nature of PTPN2 activation and may serve as a starting point for those interested in the pharmacological design of more potent activators of this phosphatase.

Finally, I validated spermidine as a possible protective agent in conditions associated with a leaky epithelial barrier. Following 24-hour treatment with IFN-γ, TER measurements were reduced on average by 35%, which agrees with previously reported data demonstrating the effects of IFN-γ on T_{84} monolayers [31]. Co-administration of spermidine during this time significantly attenuated the IFN-γ-induced drop in TER. The mechanism by which spermidine protects barrier function certainly may be due to its increase in PTPN2 protein levels and enzyme activity, and, therefore, subsequent decreases in IFN-γ signaling. However, additional pathways could also be involved. Previous reports have suggested an important role for polyamines in the regulation of tight junction protein expression as well as intestinal epithelial barrier integrity under various physiological conditions [36]. In addition to downregulation of IFN-γ signaling, therefore, spermidine may activate other cellular mechanisms responsible for increasing barrier function in the setting of inflammation. It would be of interest to study the effects of spermidine on barrier function in PTPN2-deficient cells that are also subject to IFN-γ challenge. Any protective effect of spermidine under these conditions would suggest that it targets additional protective mechanisms.

In summary, Crohn's Disease and Ulcerative Colitis are chronic inflammatory diseases of the intestines that cause significant morbidity and severely affect the quality of life. These two conditions are characterized by an elevated inflammatory cytokine profile and dysfunctional epithelial barrier. It is therefore of particular interest to develop therapeutic agents that could target the pathogenic mechanisms underlying these inflammatory conditions. Today, there has been success in the treatment and management of Crohn's Disease through the use of anti-TNF-α antibodies; however,

approaches to control elevated IFN-γ signaling are still being pursued. PTPN2, a non-receptor type cellular phosphatase, is present in intestinal epithelial cells and has been demonstrated to regulate IFN-γ signaling through the dephosphorylation of the IFN-γ signaling molecules, STAT1 and 3. SNPs in the PTPN2 gene locus have been associated with a greater susceptibility to Crohn's Disease, and it is thought that loss of functional PTPN2 may contribute to disease progression. Discovering or developing agents that increase PTPN2 protein levels and/or activity are of general interest as modifiers of inflammatory cytokine signaling. My experiments have shown that the small polyamine, spermidine, is capable of both increasing PTPN2 protein levels and stimulating its enzymatic activity *in vitro*. I have shown that this increase in enzyme activity translates to downregulation of IFN-γ signaling in a cell setting, and that regulation of IFN-γ signaling by spermidine seems to be occurring in a PTPN2-dependent manner. Importantly, I have further shown that spermidine protects barrier function following challenge with IFN-γ. These data implicate spermidine as a bona fide stimulus of PTPN2 and protective agent of intestinal epithelial barrier function. Moreover, small molecule activation of protein tyrosine phosphatases provides new insight into the potential pharmacologic regulation of certain cellular events and acts a starting point for those interested in developing more potent PTPN2 activators.

V. FIGURES

Figure 1 | PTPN2 Regulates IFN-γ Signaling in Intestinal Epithelial Cells

IFN-γ binds to its receptor causing receptor dimerization and phosphorylation. This leads to recruitment and phosphorylation of the signaling protein, JAK. JAK-induced phosphorylation of IFN-γ receptor tails is followed by STAT protein recruitment and phosphorylation. Phosphorylated STATs form homo- or heterodimers, translocate to the nucleus, bind DNA, and activate transcription of certain gene products. PTPN2 dephosphorylates JAK and STAT proteins in the cytoplasm. PTPN2 also dephosphorylates STAT dimers in the nucleus, thus promoting their nuclear export. This terminates STAT-induced transcription and subsequent increases in IEC permeability.

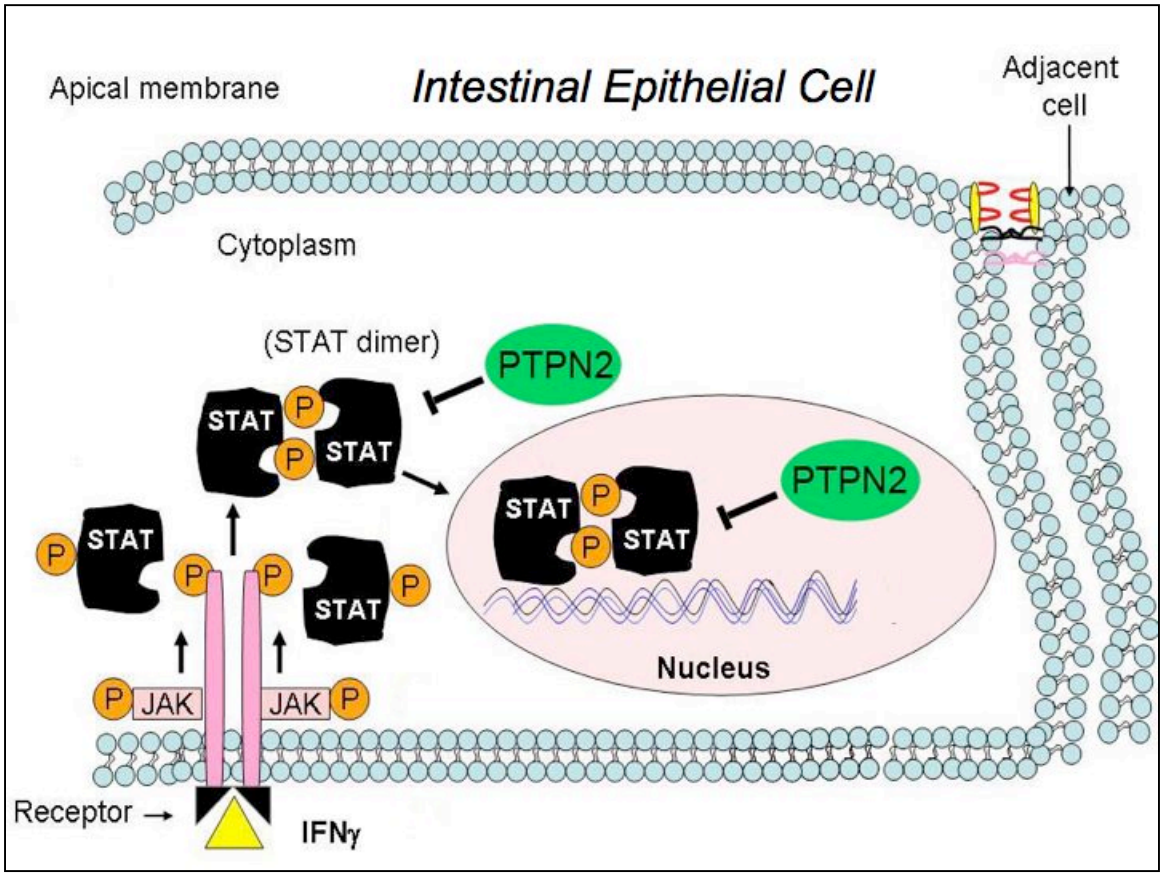

Apical membrane
Intestinal Epithelial Cell
Adjacent cell
Cytoplasm
(STAT dimer)
PTPN2
STAT
P
STAT
PTPN2
STAT
P
STAT
Nucleus
P
JAK
JAK
P
Receptor
IFNγ

Figure 2 | The Structural Formula of Spermidine

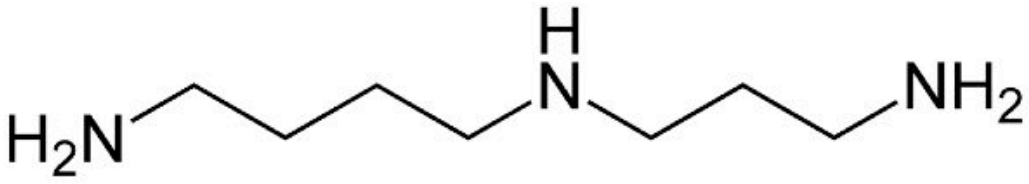

H
N
H2N
NH2

Figure 3 | The Effect of Spermidine on PTPN2 mRNA Levels in T_{84} Cells

Spermidine (SPD) had no effect on PTPN2 mRNA levels following 24 hour incubation with the respective concentrations compared to untreated controls (n = 6). PTPN2 mRNA expression was normalized to the housekeeping gene GAPDH. Measurements were performed in triplicates. Data are expressed as a percentage of the control ± SEM.

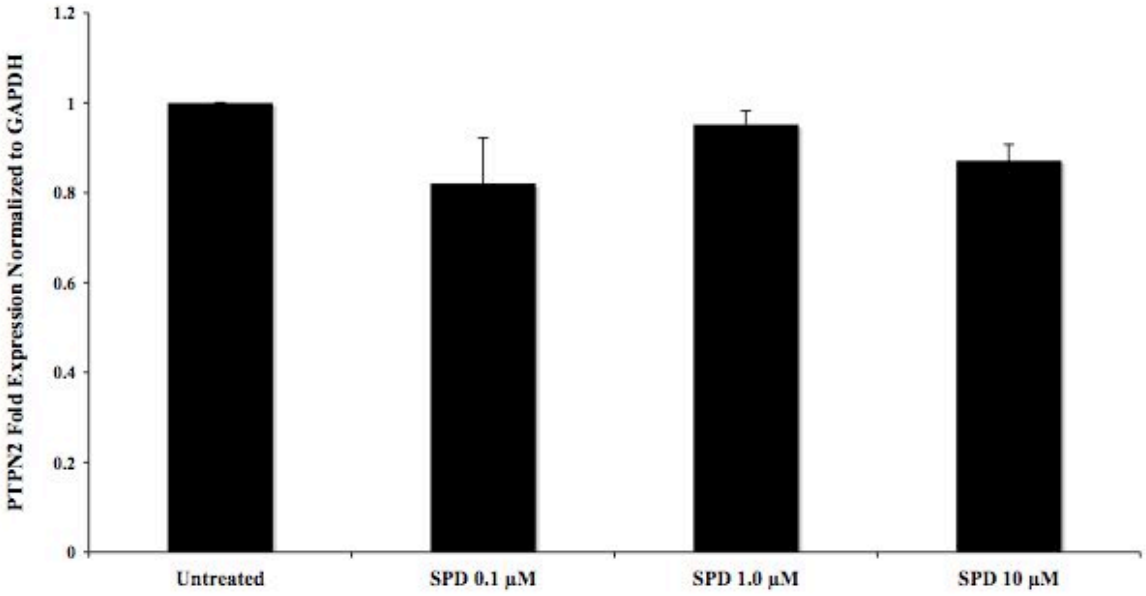
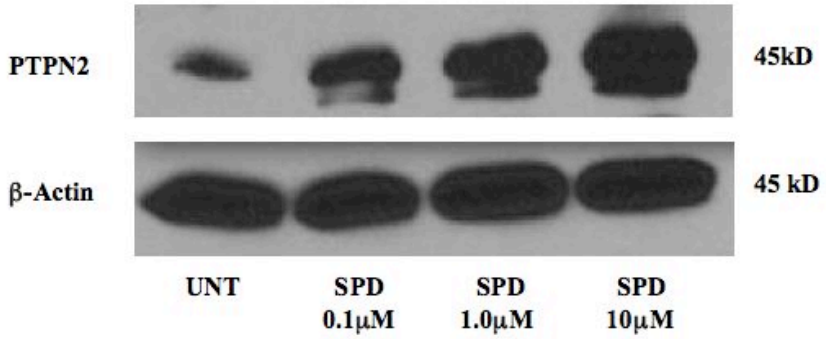

PTPN2 Fold Expression Normalized to GAPDH
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
SPD 0.1 µM
SPD 1.0 µM
SPD 10 µM

Figure 4 | The Effect of Spermidine on PTPN2 Protein Levels in T_{84} Cells

Spermidine (SPD) increased PTPN2 protein levels in T_{84} cells in a dose-dependent manner. Cytoplasmic PTPN2 protein in spermidine-treated T_{84} cells is shown by a representative Western blot in the upper panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 4). Data are expressed as a percentage of the control ± SEM. (*$P < 0.05$ compared to untreated control, using Anova and the Student-Newman-Keuhls post-hoc test).


PTPN2
45kD
β-Actin
45 kD
UNT
SPD 0.1μM
SPD 1.0μM
SPD 10μM

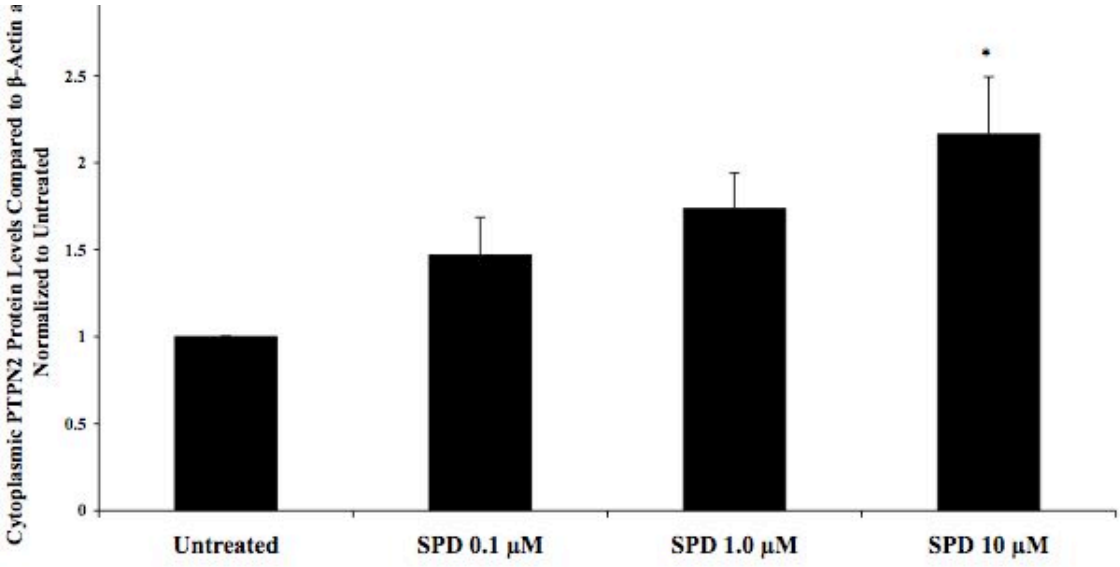
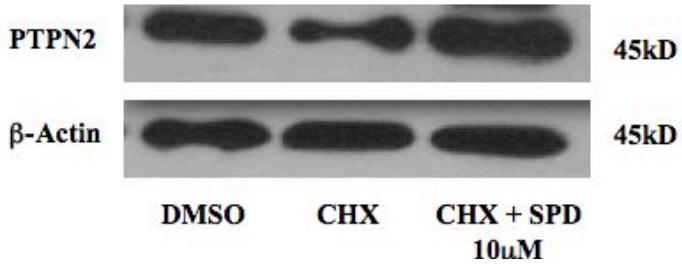

Cytoplasmic PTPN2 Protein Levels Compared to β-Actin a
Normalized to Untreated
2.5
2
1.5
1
0.5
0
Untreated
SPD 0.1 μM
SPD 1.0 μM
SPD 10 μM

Figure 5 | The Effect of Spermidine on PTPN2 Does not Require Protein Synthesis

Following translational inhibition by the ribosomal inhibitor, cycloheximide (CHX), PTPN2 protein levels were decreased. Co-administration of spermidine (SPD) restored PTPN2 protein levels to those of the vehicle control. Cytoplasmic PTPN2 protein levels in CHX and SPD-treated T_{84} cells are shown by a representative Western Blot in the upper panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 4). Data are expressed as a percentage of the vehicle control ± SEM. (**P* < 0.05 compared to control, ##*P* < 0.01 compared to CHX, using Anova and the Student-Newman-Keuls post-hoc test).


PTPN2
45kD
β-Actin
45kD
DMSO
CHX
CHX + SPD
10μM

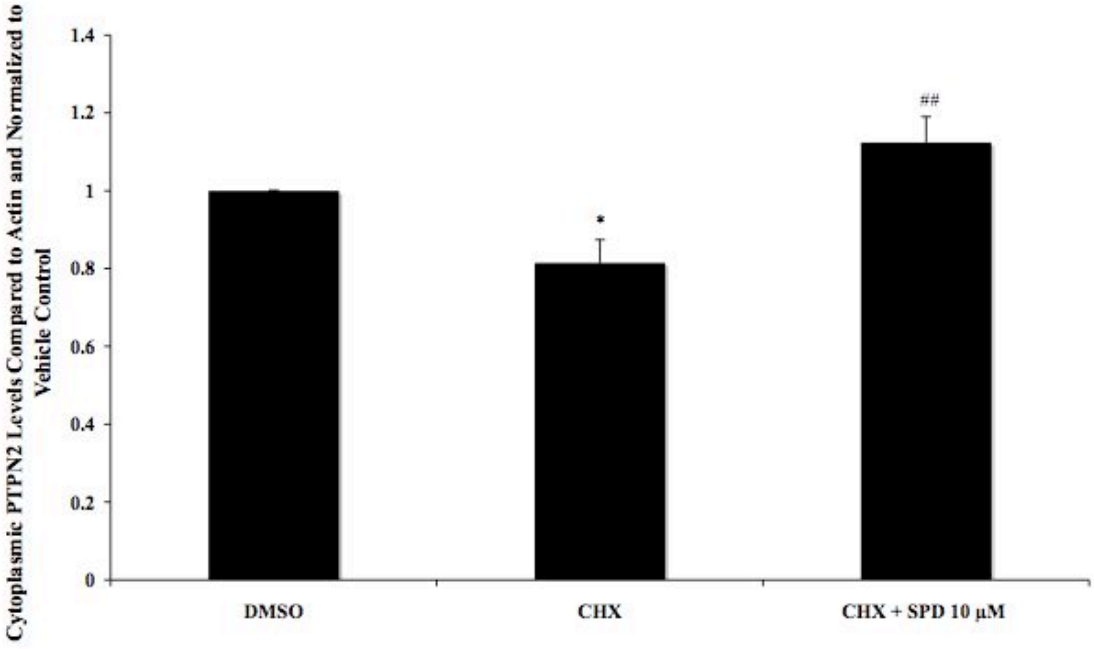

Cytoplasmic PTPN2 Levels Compared to Actin and Normalized to Vehicle Control
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
##
DMSO
CHX
CHX + SPD 10 μM

Figure 6 | The Effect of Spermidine on PTPN2 Enzymatic Activity

Following incubation +/- spermidine (SPD, 10μM) for 30 minutes, PTPN2 was immunoprecipitated from whole cell lysates and the activity assay was initiated by incubating immunoprecipitated PTPN2 with the fluorescent phosphatase substrate, DiFMUP. A sample from each immunoprecipitation was run on SDS-PAGE and probed for PTPN2 to account for equal protein loading. Fluorescence activity units were compared to PTPN2 protein levels to account for any differences in overall phosphatase amounts (n = 3). Error bars represent ± SEM. (*$P < 0.05$, **$P < 0.01$ compared to the respective untreated time point, using the Student's unpaired t-test).

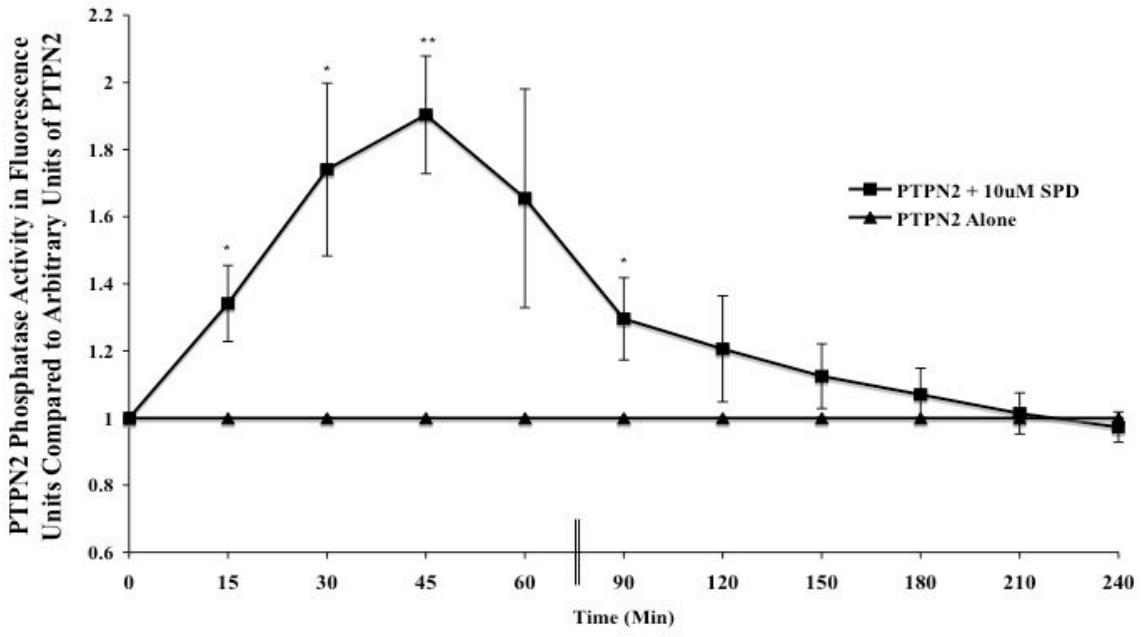
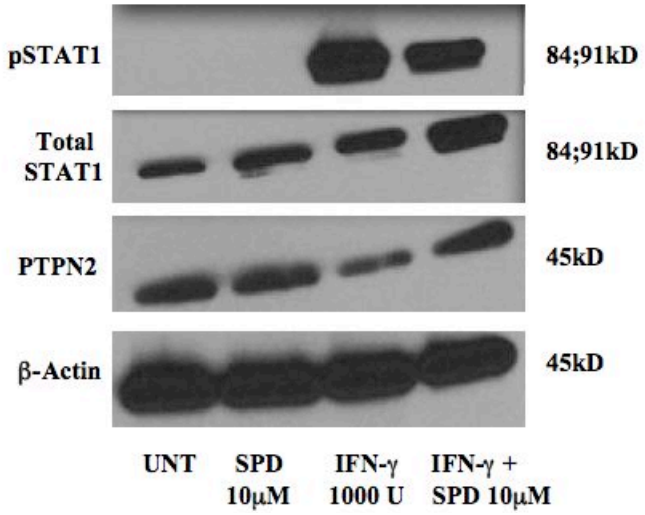

PTPN2 Phosphatase Activity in Fluorescence Units Compared to Arbitrary Units of PTPN2
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0
15
30
45
60
90
120
150
180
210
240
Time (Min)
PTPN2 + 10uM SPD
PTPN2 Alone
*
*
**
*

Figure 7 | The Effect of Spermidine on STAT1 Phosphorylation

IFN-γ (1000 U/mL) induced STAT1 phosphorylation following 30 minutes of treatment. Co-administration of spermidine (10 μM) for this time attenuated IFN-γ-induced phosphorylation of STAT1 on average by 26 ± 7% as shown by a representative Western Blot in the upper panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 5). Data are expressed as a percentage of the control ± SEM. (***$P < 0.001$ compared to untreated control, ##$P < 0.01$ compared to IFN-γ-treated cells, using Anova and the Student-Newman-Keuhls post-hoc test).


pSTAT1
84;91kD
Total STAT1
84;91kD
PTPN2
45kD
β-Actin
45kD
UNT
SPD 10μM
IFN-γ 1000 U
IFN-γ + SPD 10μM

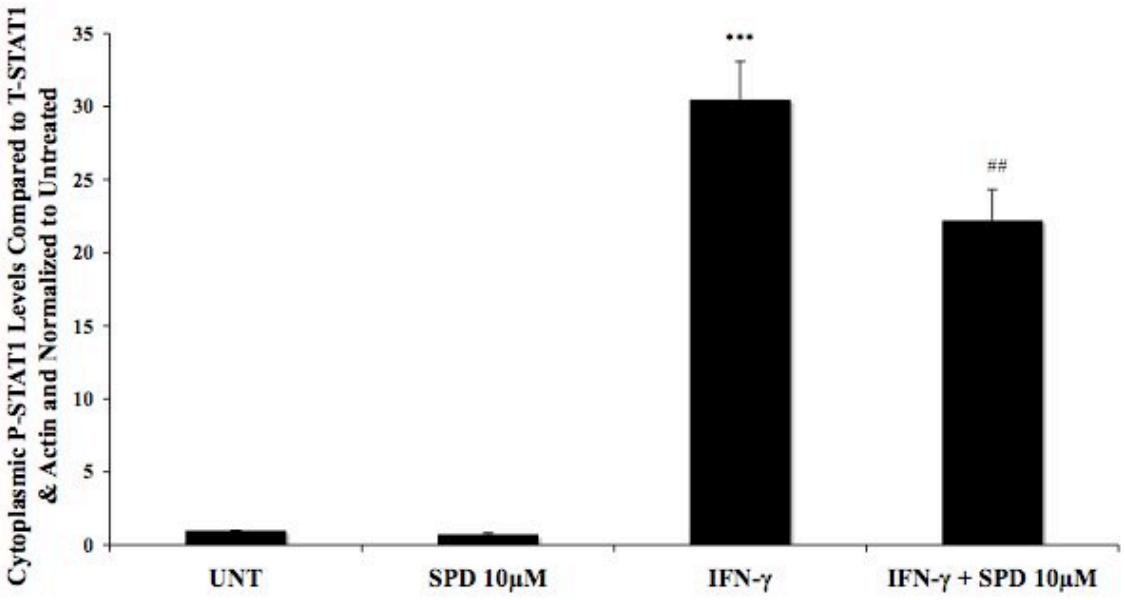
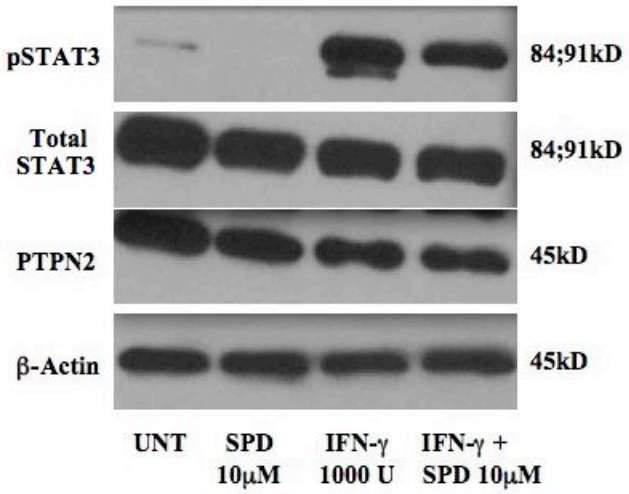

Cytoplasmic P-STAT1 Levels Compared to T-STAT1 & Actin and Normalized to Untreated
35
30
25
20
15
10
5
0
***
##
UNT
SPD 10μM
IFN-γ
IFN-γ + SPD 10μM

Figure 8 | The Effect of Spermidine on STAT3 Phosphorylation

IFN-γ (1000 U/mL) induced STAT3 phosphorylation following 30 minutes of treatment. Co-administration of spermidine (10 μM) for this time attenuated IFN-γ-induced phosphorylation of STAT3 on average by 26 ± 7% as shown by a representative Western Blot in the upper panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 4). Data are expressed as a percentage of the control ± SEM. (**$P < 0.01$, *$P < 0.05$ compared to untreated control, using Anova and the Student-Newman-Keuhls post-hoc test).


pSTAT3
84;91kD
Total STAT3
84;91kD
PTPN2
45kD
β-Actin
45kD
UNT
SPD 10μM
IFN-γ 1000 U
IFN-γ + SPD 10μM

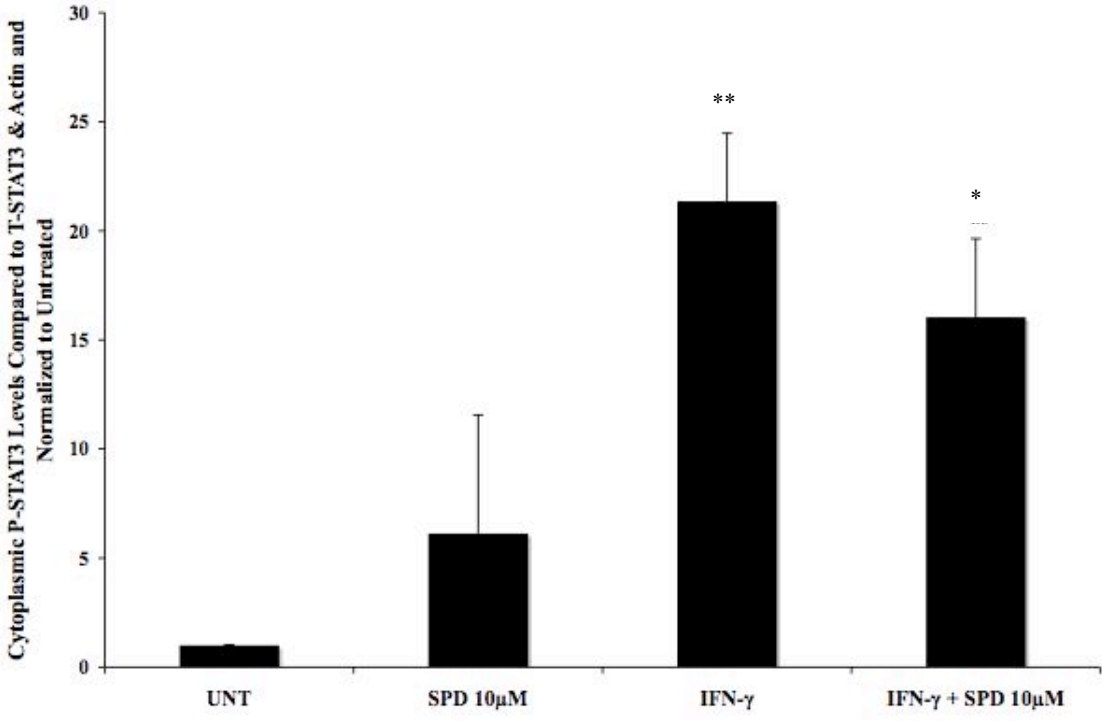
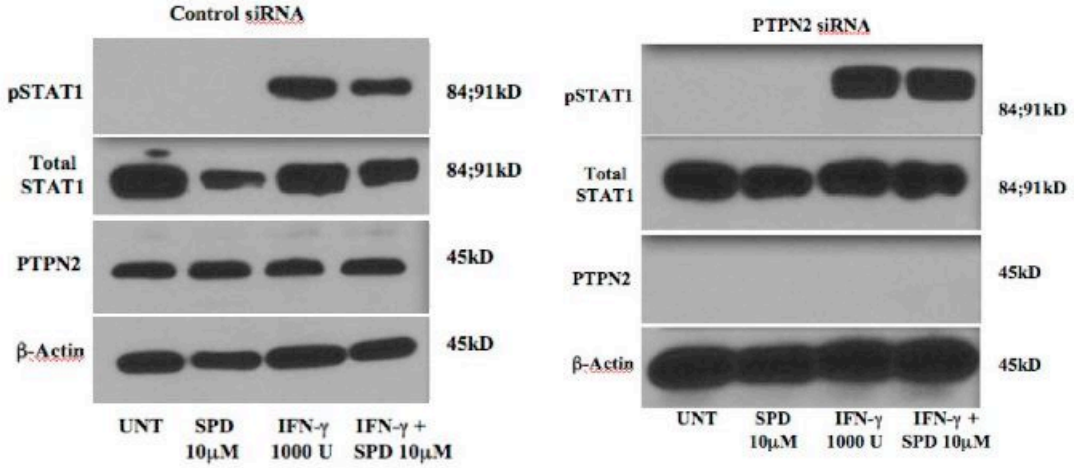

Cytoplasmic P-STAT3 Levels Compared to T-STAT3 & Actin and Normalized to Untreated
30
25
20
15
10
5
0
**
*
UNT
SPD 10μM
IFN-γ
IFN-γ + SPD 10μM

Figure 9 | Spermidine Attenuates IFN-γ Signaling in a PTPN2 Dependent Manner

T_{84} cells were transfected with either PTPN2-specific siRNA or nonspecific control siRNA. IFN-γ (1000 U/ml) induced STAT1 phosphorylation following 30 minutes of treatment and this level was increased in the PTPN2 knockdown samples. A decrease in STAT1 phosphorylation was observed in control siRNA transfected cells co-administered spermidine (10μM) with IFN-γ. The decrease in STAT1 phosphorylation by spermidine was not realized in co-administration studies in PTPN2 knockdown cells as shown by a representative Western Blot in the upper right panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 4). Data are expressed as a percentage of the control ± SEM.


Control siRNA
pSTAT1
Total STAT1
PTPN2
β-Actin
84;91kD
84;91kD
45kD
45kD
UNT
SPD 10μM
IFN-γ 1000 U
IFN-γ + SPD 10μM
PTPN2 siRNA
pSTAT1
Total STAT1
PTPN2
β-Actin
84;91kD
84;91kD
45kD
45kD
UNT
SPD 10μM
IFN-γ 1000 U
IFN-γ + SPD 10μM

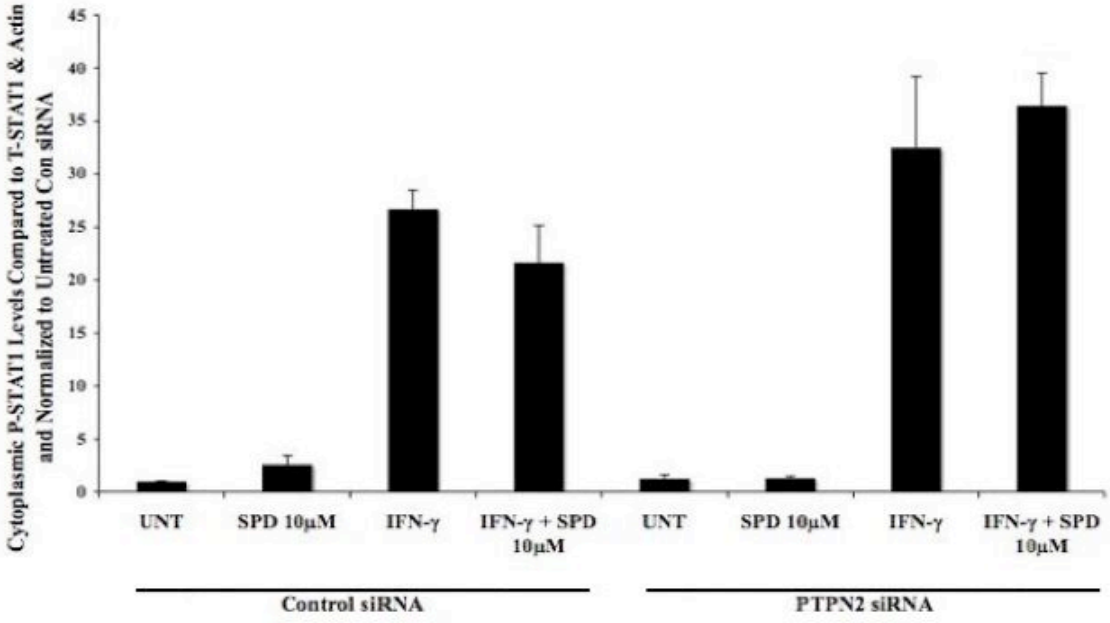

Cytoplasmic P-STAT1 Levels Compared to T-STAT1 & Actin and Normalized to Untreated Con siRNA
0
5
10
15
20
25
30
35
40
45
UNT
SPD 10μM
IFN-γ
IFN-γ + SPD 10μM
UNT
SPD 10μM
IFN-γ
IFN-γ + SPD 10μM
Control siRNA
PTPN2 siRNA

Figure 10 | The Structural Formula of Spermine

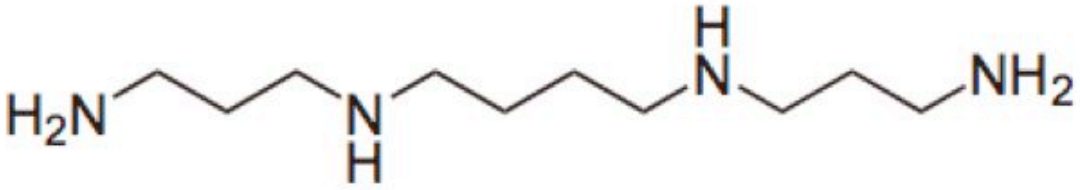
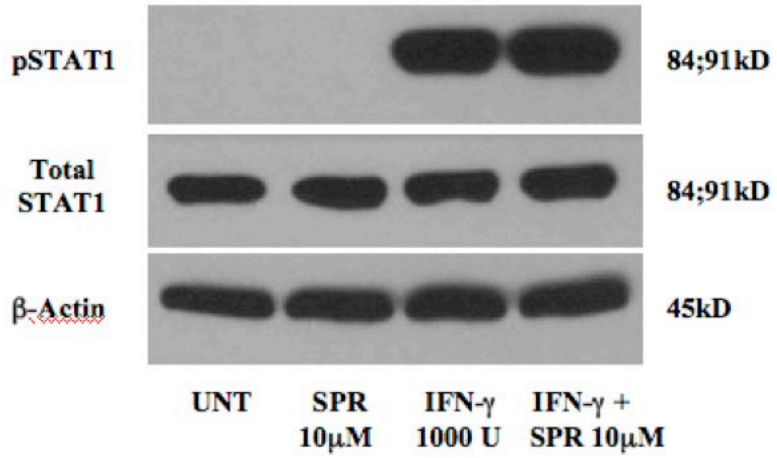

H2N
N
H
H
N
NH2

Figure 11 | Activation of PTPN2 is Specific for the Chemical Nature of Spermidine

IFN-γ (1000 U/mL) induced STAT1 phosphorylation following 30 minutes of treatment. Co-administration of spermine (SPR, 10 μM) for this time showed no significant decrease in IFN-γ-induced phosphorylation of STAT1 as shown by a representative Western Blot in the upper panel. β-Actin was used throughout as a loading control. The relative protein level was assessed by densitometry (n = 4). Data are expressed as a percentage of the untreated control ± SEM. (**$P < 0.01$ compared to untreated cells, using Anova and the Student-Newman-Keuhls post-hoc test).


pSTAT1
84;91kD
Total STAT1
84;91kD
β-Actin
45kD
UNT
SPR 10μM
IFN-γ 1000 U
IFN-γ + SPR 10μM

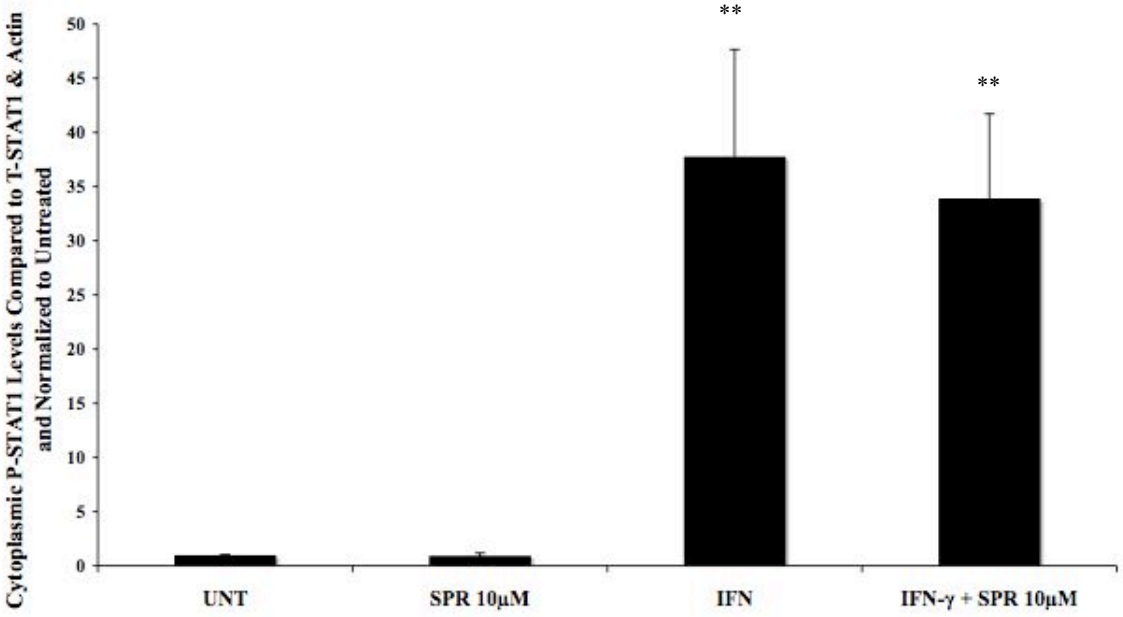

Cytoplasmic P-STAT1 Levels Compared to T-STAT1 & Actin and Normalized to Untreated
50
45
40
35
30
25
20
15
10
5
0
**
**
UNT
SPR 10μM
IFN
IFN-γ + SPR 10μM

Figure 12 | Spermidine Protects Intestinal Epithelial Barrier Function in the Setting of Inflammation

IFN-γ (1000 U/mL) was added basolaterally to T_{84} monolayers that had stable TER values of $\geq 1000\ \Omega \cdot cm^2$. Following 24 hours of treatment, TER values decreased by 35% of their initial baseline measurements. Co-administration of spermidine for this time reduced the effects of IFN-γ on barrier function. Error bars are representative of ± SEM. (***$P < 0.001$ compared to untreated cells; #$P < 0.05$ compared to IFN-γ-treated cells, using Anova and the Student-Newman-Keuhls post-hoc test).

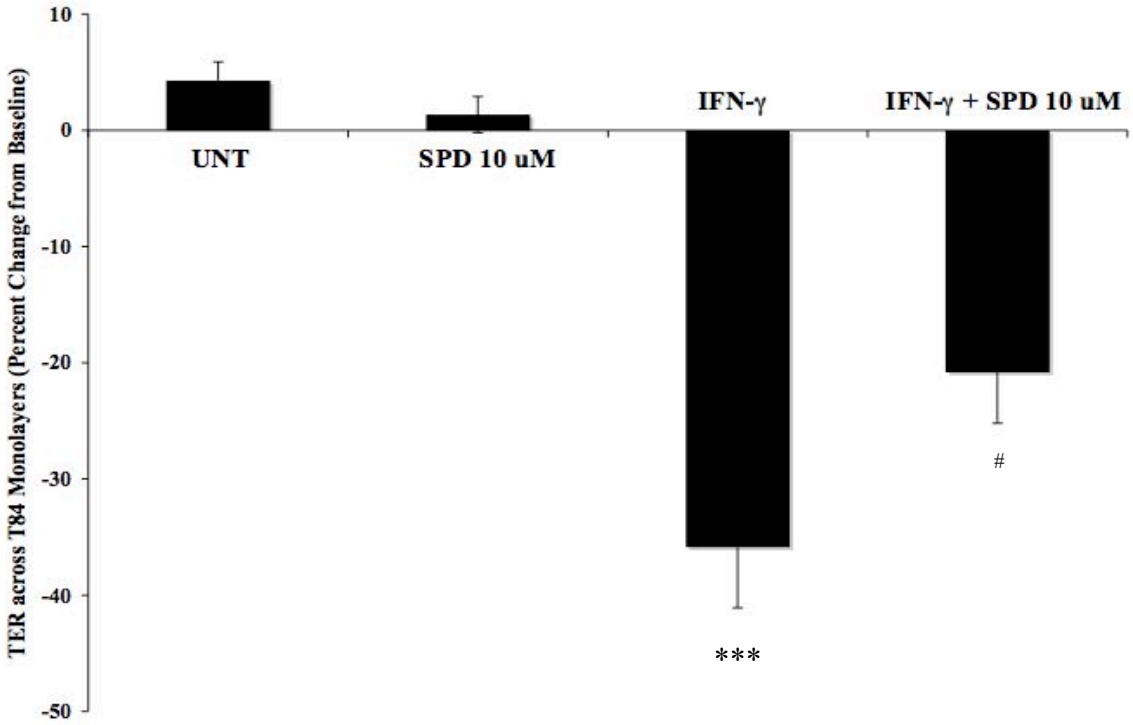
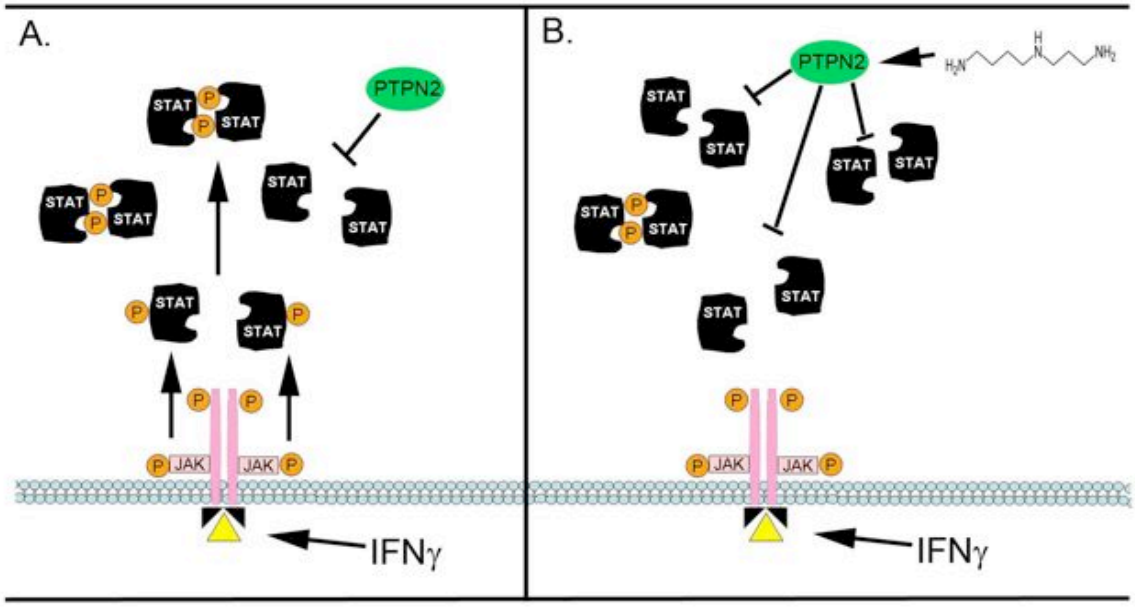

10
0
-10
-20
-30
-40
-50
TER across T84 Monolayers (Percent Change from Baseline)
UNT
SPD 10 uM
IFN-γ
IFN-γ + SPD 10 uM
***
#

Figure 13 | Spermidine's Proposed Mode of Action

A) PTPN2 protects epithelial barrier function during periods of inflammation by acting as a negative regulator of the pro-inflammatory cytokine, IFN-γ, through the dephosphorylation of STAT **B)** Spermidine increases PTPN2 protein levels and activity, thus amplifying PTPN2's protective effect during periods of inflammation


A.
PTPN2
STAT
P
JAK
IFNγ
B.
PTPN2
STAT
JAK
IFNγ

REFERENCES

1. Baumgart DC, Carding SR: Inflammatory bowel disease: cause and immunobiology. *Lancet* 2007, 369:1627-1640.

2. Robbins SL, Kumar V, Cotran RS: Robbins and Cotran pathologic basis of disease, 8th edn. Philadelphia, PA: Saunders/Elsevier; 2010.

3. Podolsky DK: Inflammatory bowel disease. *N Engl J Med* 2002, 347:417-429.

4. Wyatt J, Vogelsang H, Hubl W, Waldhoer T, Lochs H: Intestinal permeability and the prediction of relapse in Crohn's disease. *Lancet* 1993, 341:1437-1439.

5. Madsen KL, Malfair D, Gray D, Doyle JS, Jewell LD, Fedorak RN: Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. *Inflamm Bowel Dis* 1999, 5:262-270.

6. Schmitz H, Barmeyer C, Gitter AH, Wullstein F, Bentzel CJ, Fromm M, Riecken EO, Schulzke JD: Epithelial barrier and transport function of the colon in ulcerative colitis. *Ann N Y Acad Sci* 2000, 915:312-326.

7. Scharl M, Paul G, Weber A, Jung BC, Docherty MJ, Hausmann M, Rogler G, Barrett KE, McCole DF: Protection of epithelial barrier function by the Crohn's disease associated gene protein tyrosine phosphatase n2. *Gastroenterology* 2009, 137:2030-2040.

8. Fuss IJ, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W: Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *Journal of Immunology* 1996, 157:1261-1270.

9. Sartor RB: Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nat Clin Pract Gastroenterol Hepatol* 2006, 3:390-407.

10. Turner JR: Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 2009, 9:799-809.

11. Capaldo CT, Nusrat A: Cytokine regulation of tight junctions. *Biochim Biophys Acta* 2009, 1788:864-871.

12. Yuvaniyama J, Denu JM, Dixon JE, Saper MA: Crystal structure of the dual specificity protein phosphatase VHR. *Science* 1996, 272:1328-1331.

13. Tonks NK, Neel BG: Combinatorial control of the specificity of protein tyrosine phosphatases. *Curr Opin Cell Biol* 2001, 13:182-195.

14. Ibarra-Sanchez MJ, Simoncic PD, Nestel FR, Duplay P, Lapp WS, Tremblay ML: The T-cell protein tyrosine phosphatase. *Semin Immunol* 2000, 12:379-386.

15. Mattila E, Pellinen T, Nevo J, Vuoriluoto K, Arjonen A, Ivaska J: Negative regulation of EGFR signalling through integrin-alpha1beta1-mediated activation of protein tyrosine phosphatase TCPTP. *Nat Cell Biol* 2005, 7:78-85.

16. Galic S, Klingler-Hoffmann M, Fodero-Tavoletti MT, Puryer MA, Meng TC, Tonks NK, Tiganis T: Regulation of insulin receptor signaling by the protein tyrosine phosphatase TCPTP. *Mol Cell Biol* 2003, 23:2096-2108.

17. ten Hoeve J, de Jesus Ibarra-Sanchez M, Fu Y, Zhu W, Tremblay M, David M, Shuai K: Identification of a nuclear Stat1 protein tyrosine phosphatase. *Mol Cell Biol* 2002, 22:5662-5668.

18. Yamamoto T, Sekine Y, Kashima K, Kubota A, Sato N, Aoki N, Matsuda T: The nuclear isoform of protein-tyrosine phosphatase TC-PTP regulates interleukin-6-mediated signaling pathway through STAT3 dephosphorylation. *Biochem Biophys Res Commun* 2002, 297:811-817.

19. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW: Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene* 2002, 285:1-24.

20. Mudter J, Weigmann B, Bartsch B, Kiesslich R, Strand D, Galle PR, Lehr HA, Schmidt J, Neurath MF: Activation pattern of signal transducers and activators of transcription (STAT) factors in inflammatory bowel diseases. *Am J Gastroenterol* 2005, 100:64-72.

21. Franke A, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, Lees CW, Balschun T, Lee J, Roberts R *et al*: Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 2010, 42:1118-1125.

22. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 2007, 44:661-678.

23. Mattila E, Marttila H, Sahlberg N, Kohonen P, Tahtinen S, Halonen P, Perala M, Ivaska J: Inhibition of receptor tyrosine kinase signalling by small molecule agonist of T-cell protein tyrosine phosphatase. *BMC Cancer* 2010, 10:7.

24. Moinard C, Cynober L, de Bandt JP: Polyamines: metabolism and implications in human diseases. *Clin Nutr* 2005, 24:184-197.

25. Seiler N, Delcros JG, Moulinoux JP: Polyamine transport in mammalian cells. An update. *Int J Biochem Cell Biol* 1996, 28:843-861.

26. Hoet PH, Nemery B: Polyamines in the lung: polyamine uptake and polyamine-linked pathological or toxicological conditions. *Am J Physiol Lung Cell Mol Physiol* 2000, 278:L417-433.

27. Johnson LR, McCormack SA: Healing of Gastrointestinal Mucosa: Involvement of Polyamines. *News Physiol Sci* 1999, 14:12-17.

28. Hao L, Tiganis T, Tonks NK, Charbonneau H: The noncatalytic C-terminal segment of the T cell protein tyrosine phosphatase regulates activity via an intramolecular mechanism. *J Biol Chem* 1997, 272:29322-29329.

29. Dharmsathaphorn K, McRoberts JA, Mandel KG, Tisdale LD, Masui H: A human colonic tumor cell line that maintains vectorial electrolyte transport. *Am J Physiol* 1984, 246:G204-208.

30. Cafferata EG, Gonzalez-Guerrico AM, Giordano L, Pivetta OH, Santa-Coloma TA: Interleukin-1beta regulates CFTR expression in human intestinal T84 cells. *Biochim Biophys Acta* 2000, 1500:241-248.

31. Youakim A, Ahdieh M: Interferon-gamma decreases barrier function in T84 cells by reducing ZO-1 levels and disrupting apical actin. *Am J Physiol* 1999, 276:G1279-1288.

32. Heinonen KM, Nestel FP, Newell EW, Charette G, Seemayer TA, Tremblay ML, Lapp WS: T-cell protein tyrosine phosphatase deletion results in progressive systemic inflammatory disease. *Blood* 2004, 103:3457-3464.

33. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD: How cells respond to interferons. *Annu Rev Biochem* 1998, 67:227-264.

34. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr.: Stat3 as an oncogene. *Cell* 1999, 98:295-303.

35. Otsuka H: The toxic effect of spermidine on normal and transformed cells. *J Cell Sci* 1971, 9:71-84.

36. Guo X, Rao JN, Liu L, Zou T, Keledjian KM, Boneva D, Marasa BS, Wang JY: Polyamines are necessary for synthesis and stability of occludin protein in

intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 2005, 288:G1159-1169.