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### Title

Identifying and Validating Novel Genes Associated with Host-Microbe Interaction in Response to Deficiency of an Ibd-Associated gene, Ptpn2

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IDENTIFYING AND VALIDATING NOVEL GENES ASSOCIATED WITH HOST-  
MICROBE INTERACTION IN RESPONSE TO DEFICIENCY OF AN IBD-ASSOCIATED  
GENE, *PTPN2*

By

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A capstone project submitted for Graduation with University Honors

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## Abstract

Inflammatory bowel disease (IBD) is a term for illnesses such as ulcerative colitis and Crohn's disease that are caused by chronic inflammation in the gastrointestinal tract. There are over 200 candidate genes that have been associated with increasing the risk of developing IBD, and one of these genes is protein tyrosine phosphatase non-receptor type 2 (*PTPN2*). *PTPN2*, which encodes for the protein T-cell protein tyrosine phosphatase (TCPTP), plays an important role in maintaining intestinal homeostasis. Studies have shown that loss of *Ptpn2* in immune cells in mice and single-nucleotide polymorphisms (SNPs) in *PTPN2* in humans result in an altered microbiome, specifically with an increase in pathobiont bacteria such as Adherent-Invasive *Escherichia coli* (AIEC). Recently, we identified in a constitutive *Ptpn2*-deficient mouse, expansion of a novel mouse AIEC (*mAIEC*) that significantly overlaps with the IBD-associated AIEC. My role will consist of identifying novel genes involved in the binding and colonization of pathobiont bacteria using *PTPN2*-deficient human intestinal cells and mice. We hope to identify novel mechanisms of host-microbe interaction in an IBD setting in an effort to improve therapeutic targets against IBD.

## **Acknowledgments**

I would like to thank my faculty mentor, Dr. Declan F. McCole, for his help and support in completing this capstone project with me. I would like to thank him for his guidance and willingness to make accommodations for my project during this time of the COVID-19 pandemic. I would also like to thank my postdoctoral mentor, Ali Shawki, for training me since I first joined the McCole Lab and for helping me draft the initial abstract and prospectus for this project. Also, I would like to thank the members of the McCole Lab, notably Vinicius Canale, Pritha Chatterjee, Alina Santos, and Anica Sayoc-Becerra, for their time in mentoring me in the lab and providing me with the supplemental knowledge and skills needed to complete this project.

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## Introduction

Inflammatory bowel disease (IBD), composed of ulcerative colitis (UC) and Crohn's disease (CD), is a major health issue affecting about ten million people around the world ("[About IBD](#)"). IBD is mainly prevalent in North America, Europe, and Oceania with about 50-200 per 100,000 persons for CD and 120-200 per 100,000 persons for UC, however, the incidence of IBD is also rising in developing countries in Asia, Africa, and South America (Siew et al., 2017). Direct and indirect costs of treating IBD patients in 2014 were estimated at around \$14.6 to \$31.6 billion, and much of the costs were increased by inappropriate treatment and lack of adherence to therapeutic regimens for IBD (Mehta, 2016). Therefore, IBD not only affects the health of global populations but also carries a significant economic burden on healthcare systems worldwide.

Although the exact cause of IBD is unknown, genetic and environmental factors are known to play a role in disease pathogenesis. Recent biomedical research has identified over 200 candidate genes associated with IBD and one of these genes is protein tyrosine phosphatase non-receptor type 2 (*PTPN2*), which encodes for the protein T-cell protein tyrosine phosphatase (TCPTP) (Jostins et al., 2012; Liu et al., 2015). *PTPN2* plays an important role in maintaining intestinal homeostasis where it has been shown to regulate epithelial barrier function and pro-inflammatory signaling (McCole, 2012; Scharl et al., 2009; Scharl et al., 2010; Scharl et al., 2011). We identified that deficiency of *Ptpn2* in mice causes increased expression of pro-inflammatory cytokines, disrupted barrier function, and an altered intestinal microbiome, specifically increased abundance of barrier modulating pathobiont bacteria (Shawki et al., 2020). Therefore, we aim to identify novel genes involved in the host-microbe interaction using *PTPN2*-

deficient human intestinal cells and mice to better understand the role that *PTPN2* plays in modulating the microbiome and maintaining intestinal homeostasis.

## **Background**

Inflammation in the gut primarily occurs when the function of the intestinal epithelial barrier is compromised. Intestinal epithelial cells (IECs) lining the gastrointestinal tract that form the epithelial barrier are critical for the regulated absorption of nutrients, as well as prevention of invading pathogens (Shanshan et al., 2018). When the barrier is no longer intact, it increases permeability and allows pathobiont bacteria to colonize in the intestine, causing significant alterations in the intestinal microbiome in human IBD patients.

The pathobiont Adherent-Invasive *E. coli* (AIEC) and its reference strain, LF82, have been reported to have increased abundance in IBD patients. Although LF82 was first identified in a CD patient, it is prevalent in both UC and CD (Darfeuille-Michaud et al., 1998). LF82 alters the intestinal microbiome by adhering to and invading IECs, surviving and replicating in macrophages, and inducing increased pro-inflammatory response (Demarre et al., 2019). Specifically, LF82 and other AIEC strains bind and adhere to the receptor of carcinoembryonic antigen-related cell-adhesion molecule 6 (CEACAM6) on IECs. CEACAMs are proteins in the intestinal epithelium that are involved in cell and bacteria adhesion and proliferation. Some CEACAMs are regulated by commensal bacteria such as segmented filamentous bacteria (SFB) that help maintain intestinal homeostasis (Kelleher et al., 2019). Thus, when AIEC strains bind to CEACAMs, they can inhibit SFB function and alter CEACAM regulation, disrupting the intestinal microbiome and contributing to human IBD pathogenesis. CEACAM6 has been

identified as the receptor for AIEC adhesion because it is abnormally expressed by ileal epithelial cells in CD patients (Barnich et al., 2007). AIEC strains were able to adhere to CEACAM6 receptors and cause upregulated expression in CD ileal enterocyte biopsies by using a type 1 pili-mediated mechanism and performing glycosylation of the receptor's mannose residues to bind to the receptors on the brush border of the enterocytes, increasing CEACAM6 expression on the apical side (Barnich et al., 2007).

Also, single nucleotide polymorphisms (SNPs) in the *PTPN2* gene locus in humans as well as the loss of *Ptpn2* in immune cells in mice result in chronic inflammation and alterations of the intestinal microbiome (Knights et al., 2014; Yilmaz et al., 2018). SNPs are variations of a single base pair in an individual's DNA sequence that can confer risks of several diseases ("[Single Nucleotide Polymorphisms](#)"). These changes in the human genome can create mutations that alter the expression and function of essential proteins required for normal cellular processes. Although *PTPN2* is highly expressed in immune cells and IECs, some SNPs in the *PTPN2* gene locus have been shown to create loss of function mutations that impair PTPN2 protein (TCPTP) function and reduce enzymatic activity or efficacy (Scharl et al., 2012; Spalinger et al., 2016). As a result, loss of *PTPN2* expression and TCPTP function hinders the ability of IECs to form an effective barrier, making *PTPN2*-deficient cells more susceptible to adherence and invasion of AIEC LF82 and the development of IBD (Shawki et al., 2020). Our lab confirmed this by validating the role of CEACAM6 in the AIEC invasion of *PTPN2*-deficient cells. Previous studies identified increased gene and protein expression of CEACAM6 on the apical surface of the primary ileal enterocytes of CD patients through RNAseq and western blot analyses (Barnich et al., 2007). We used similar techniques to analyze CEACAM6 expression in response to AIEC invasion of *PTPN2*-deficient human IECs. We found that loss of *PTPN2* in human IECs



promotes increased expression of CEACAM6 proteins that significantly elevate host susceptibility to AIEC invasion and IBD (Shawki et al., 2018). We then validated the role of CEACAM6 by using an antibody to block the binding activity of CEACAM6 in *PTPN2*-deficient cells. Our results showed that the antibody partially prevents AIEC LF82 from adhering to IECs (Shawki et al., 2018). Thus, we confirmed the role of CEACAM6 in the binding of AIEC in IECs and the mechanism by which *PTPN2* loss of function mutations contribute to microbiome alteration and IBD pathogenesis.

In our mouse model of IBD, we identified an altered microbiome, specifically an increased abundance of AIEC that had a 100% sequence match of a 250 base pair sequence to human AIEC LF82 (Shawki et al., 2020). We have confirmed that this novel mouse *E. coli*, *mAIEC*, adheres to and invades IECs, survives in macrophages, and causes disease in mice (Shawki et al., 2020). To continue to support this research, we aim to use *PTPN2*-deficient cells and mice to identify and validate other genes involved in the binding and colonization of AIEC so that we can better understand how *PTPN2* restricts AIEC colonization and contributes to developing future therapeutic targets against IBD.

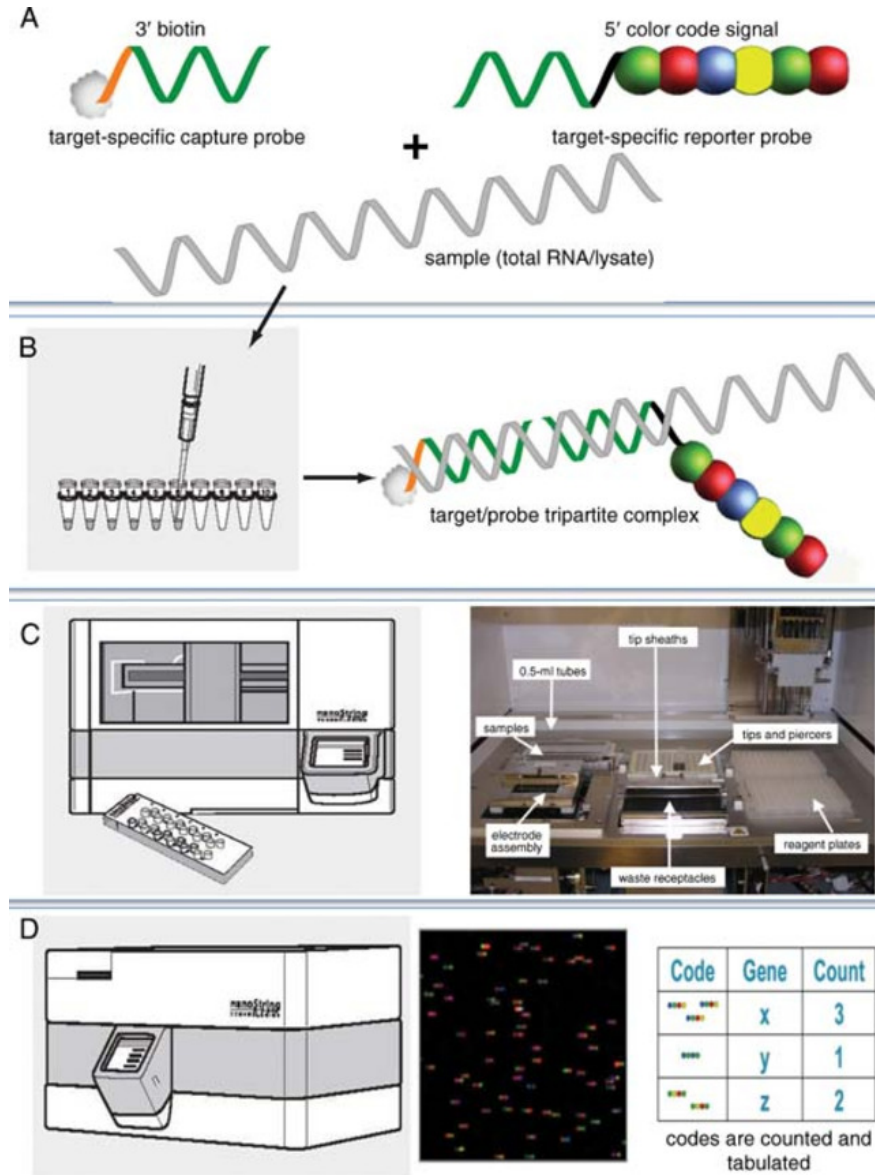
## Methods

We will use data available from bioinformatics techniques such as RNAseq and Nanostring to selectively identify highly significantly altered novel genes associated with host-microbe interactions similar to CEACAMs using *PTPN2*-deficient human IECs and *Ptpn2*-deficient mouse intestinal tissue. RNAseq is a commonly used technique that determines which genes are turned on in a cell, what their level of expression is, and at what times they are

activated or shut off (MacKenzie, 2018). However, for this experiment, we will use the Nanostring nCounter analysis system to identify genes that have significantly altered expression in *PTPN2*-deficient cells. The Nanostring nCounter platform performs a multiplex analysis of up to 800 RNA, DNA, or protein targets using molecular barcode technology (“[Nanostring Overview](#)”). Through this technique, RNA is directly tagged with capture and reporter probes that are specific to the target gene of interest, creating a unique target-probe complex (see fig. 1). The complex undergoes hybridization and excess probes are removed to leave only purified target-probe complexes. The purified complexes are then immobilized and aligned on an imaging surface and scanned by an automated fluorescence microscope. Finally, barcodes are labeled on the sample and are directly counted for data analysis. This technique obtains reproducible results faster than RNAseq because it generates a highly multiplexed single reaction without requiring amplification, cDNA conversion, or library preparation (“[Direct Digital Detection](#)”). Performing cDNA conversion and amplification through the RNAseq technique is more time-consuming and can introduce variability, leading to biased data and an inability to replicate results.

In this study, we will collect samples of IECs isolated from the ileum of constitutive *Ptpn2* knockout (KO) mice and *Ptpn2* heterozygous (HET) mice to perform the differential gene expression analysis. We will then use pre-formatted Nanostring panels to examine which genes in which pathways are turned on or off and detect altered gene expression in the mouse intestinal tissue. We will use the Inflammation Panel to measure the effects of immune response and *PTPN2* deficiency on the expression of novel candidate genes associated with IBD (“[Inflammation Panels](#)”). We will also use the Autoimmune Profiling Panel to evaluate the

pathways, processes, and cell types that these novel genes are involved in to identify specific therapeutic targets against IBD (“[Autoimmune Panel](#)”).



**Figure 1.** Overview of nCounter Gene Expression Assay from Kulkarni, Meghana. “Digital Multiplexed Gene Expression Analysis Using the NanoString nCounter System.” *Current Protocols in Molecular Biology*, vol. 94, no. 1, April 2011, pp. 25B.10.1-25B.10.17. Wiley Online Library, doi:10.1002/0471142727.mb25b10s94.

### *Inducible Ptpn2 Deletion in Intestinal Epithelial Cells in vivo*

We will use *Ptpn2*-VilCre/Ert2 (*Ptpn2*<sup>ΔIEC</sup>) mice as our *Ptpn2*-deficient mouse model to validate novel genes involved in the host-microbe interaction in the gut during *PTPN2* deficiency. For this study, inducible tissue-specific *Ptpn2*-KO mice were generated by crossing a mouse line where critical *Ptpn2* exons were flanked by LoxP sites (*Ptpn2*<sup>tm1a(EUCOMM)Wtsi</sup>), with a transgenic mouse expressing Cre recombinase enzyme under the villin-1 promoter [Tg(Vil-cre/ERT2)23Syr]. Induction of recombinase activity was achieved by Tamoxifen administration intraperitoneally (50mg/kg body weight) for five consecutive days. Mice with *Ptpn2* exons flanked by LoxP sites without expressing the recombinase enzyme in IECs, (*Ptpn2*<sup>fl/fl</sup>) were used as controls, whereas mice expressing the recombinase enzyme in IECs (*Ptpn2*<sup>ΔIEC</sup>) had deletion of PTPN2 only in IECs but expression is retained in other intestinal cell types.

## **Preliminary Results**

### *Identification of Novel Genes Associated with IBD by Nanostring Analysis*

Gene expression analysis was performed using the Nanostring technique to evaluate changes in the expression of genes located in the IECs of the ileum in our constitutive *Ptpn2*-deficient mouse model. We tested for levels of gene expression in the ileum IECs of wild-type mice (WT) in comparison to constitutive *Ptpn2* heterozygous mice (HET) and knockout or *Ptpn2*-deficient mice (KO). For this study, we specifically looked at the Nanostring results from the KO vs. WT mice to identify novel genes that had significant changes in gene expression in response to *Ptpn2* deficiency. Based on the results, 25 out of 188 genes identified in the ileum IECs had the most significant changes in gene expression in the KO mice and were highlighted

in green (see table 1). The results denoted changes in gene expression as Log<sub>2</sub> fold change ranging from -1.76 to 6.63. Genes with a Log<sub>2</sub> fold change highlighted in red were significantly upregulated, and genes with a Log<sub>2</sub> fold change highlighted in blue were significantly downregulated in the KO mice compared to the WT mice. The Nanostring results also identified various gene sets such as Type I Interferon Signaling, Lymphocyte Trafficking, and MHC Class II Antigen Presentation to categorize which cellular pathways the novel genes were involved in. Based on this information, additional literature research was conducted to determine which of the 25 genes could be involved in the binding and colonization of pathobiont bacteria in our *Ptpn2*-deficient mice.

**Table 1.** Ileum IECs Differential Expression Nanostring Results – KO vs. WT

	Log2 fold change	BY p-value	P-value	Gene sets
Maf-mRNA	-0.734	0.0109	9.95E-06	Th2 Differentiation
H2-Aa-mRNA	5.39	0.0109	1.07E-05	Lymphocyte Trafficking, MHC Class II Antigen Presentation, T-cell Checkpoint Signaling, T-cell Receptor Signaling, Type II Interferon Signaling
Ly6c1-mRNA	6.17	0.0109	1.18E-05	Lymphocyte Trafficking
H2-Eb1-mRNA	4.8	0.0109	1.43E-05	Lymphocyte Trafficking, MHC Class II Antigen Presentation, T-cell Checkpoint Signaling, T-cell Receptor Signaling, Type II Interferon Signaling
Cd74-mRNA	5.01	0.0109	1.74E-05	MHC Class II Antigen Presentation
Psmb9-mRNA	2.85	0.0118	2.26E-05	Apoptosis, B-cell Receptor Signaling, Fc Receptors and Phagocytosis, Immunometabolism, MHC Class I Antigen Presentation, NF-kB Signaling, T-cell Receptor Signaling
Ifitm3-mRNA	3.87	0.0231	5.64E-05	Type I Interferon Signaling
Socs1-mRNA	3.8	0.0231	5.91E-05	MHC Class I Antigen Presentation, Toll Like Receptor Signaling, Type I Interferon Signaling, Type II Interferon Signaling
Cd63-mRNA	-1.1	0.0246	7.99E-05	
Casp3-mRNA	3.06	0.0246	8.78E-05	Apoptosis, Oxidative Stress, TNF Family Signaling
H2-DMa-mRNA	3.51	0.0246	9.14E-05	Lymphocyte Trafficking, MHC Class II Antigen Presentation
H2-DMb1-mRNA	4.89	0.0246	0.000101	Lymphocyte Trafficking, MHC Class II Antigen Presentation
CUBN-mRNA	-1.76	0.0246	0.000121	
Psmb8-mRNA	2.92	0.0246	0.000122	Apoptosis, B-cell Receptor Signaling, Fc Receptors and Phagocytosis, Immunometabolism, MHC Class I Antigen Presentation, NF-kB Signaling, T-cell Receptor Signaling, Type I Interferon Signaling
P2rx4-mRNA	-1.16	0.0246	0.000126	Endothelial Activation
H2-Ab1-mRNA	3.93	0.0246	0.000126	Lymphocyte Trafficking, MHC Class II Antigen Presentation, T-cell Checkpoint Signaling, T-cell Receptor Signaling, Type II Interferon Signaling
Idd1-mRNA	3.41	0.0246	0.000136	Immunometabolism
Sp100-mRNA	2.92	0.0246	0.000142	Autoantigens, Type II Interferon Signaling
Stat1-mRNA	1.91	0.025	0.000152	Chemokine Signaling, Interleukin-12 Signaling, NLR Signaling, Other Interleukin Signaling, Th1 Differentiation, Toll Like Receptor Signaling, Type I Interferon Signaling, Type II Interferon Signaling
Zfp1-mRNA	4.88	0.025	0.00016	Cytosolic DNA Sensing
Ifi44-mRNA	6.63	0.037	0.000249	Type I Interferon Signaling
Cd274-mRNA	3.76	0.0382	0.000269	Lymphocyte Trafficking, T-cell Checkpoint Signaling
Elavl1-mRNA	-1.33	0.0425	0.000313	Th17 Mediated Biology
Oasl1-mRNA	1.78	0.0468	0.000359	Type I Interferon Signaling, Type II Interferon Signaling
H2-DI-mRNA	1.42	0.0496	0.000397	Lymphocyte Trafficking, MHC Class I Antigen Presentation, Type I Interferon Signaling, Type II Interferon Signaling
Ripk3-mRNA	1.72	0.068	0.000566	Cytosolic DNA Sensing, NLR Signaling, TNF Family Signaling, Toll Like Receptor Signaling
Oas2-mRNA	3	0.068	0.000605	NLR Signaling, Type I Interferon Signaling, Type II Interferon Signaling
Cd24a-mRNA	-1.47	0.068	0.000609	T-cell Checkpoint Signaling
Maml3-mRNA	-2.98	0.0751	0.000697	Th1 Differentiation
Lamp1-mRNA	-0.919	0.0773	0.000742	
Stat5b-mRNA	-0.929	0.0877	0.000887	Chemokine Signaling, Other Interleukin Signaling, Th2 Differentiation
Bst2-mRNA	3.69	0.089	0.000936	Type I Interferon Signaling
Ifi27-mRNA	-1.71	0.089	0.00094	Type I Interferon Signaling
Trex1-mRNA	1.9	0.089	0.000969	Cytosolic DNA Sensing
Irf1-mRNA	1.64	0.0909	0.00102	Type I Interferon Signaling, Type II Interferon Signaling
Ccl5-mRNA	2.3	0.0946	0.00109	Chemokine Signaling, Cytosolic DNA Sensing, NLR Signaling, Oxidative Stress, TNF Family Signaling, Toll Like Receptor Signaling
Ifi13-mRNA	3.12	0.0965	0.00123	Type I Interferon Signaling
Dlx1-mRNA	-1.73	0.0965	0.00126	Chemokine Signaling, Growth Factor Signaling, Immunometabolism, Interleukin-12 Signaling, NLR Signaling

Source: Vinicius Canale, McCole Lab, University of California, Riverside, January 2020.

**Table 2.** Nanostring Results Listed in Order of Log<sub>2</sub> Fold Change – KO vs. WT

	Log2 fold change	BY.p.value	P-value	Gene.sets
Ifi44-mRNA	6.63	0.037	0.0002	Type I Interferon Signaling
Ly6c1-mRNA	6.17	0.0109	1.18E-05	Lymphocyte Trafficking
H2-Aa-mRNA	5.39	0.0109	1.07E-05	Lymphocyte Trafficking, MHC Class II Antigen Presentation, T-cell Checkpoint Signaling, T-cell Receptor Signaling, Type II I
Cd74-mRNA	5.01	0.0109	1.74E-05	MHC Class II Antigen Presentation
H2-DMb1-mRNA	4.89	0.0246	0.0001	Lymphocyte Trafficking, MHC Class II Antigen Presentation
Zbp1-mRNA	4.88	0.025	0.0002	Cytosolic DNA Sensing
H2-Eb1-mRNA	4.8	0.0109	1.43E-05	Lymphocyte Trafficking, MHC Class II Antigen Presentation, T-cell Checkpoint Signaling, T-cell Receptor Signaling, Type II I
H2-Ab1-mRNA	3.93	0.0246	0.0001	Lymphocyte Trafficking, MHC Class II Antigen Presentation, T-cell Checkpoint Signaling, T-cell Receptor Signaling, Type II I
Ifitm3-mRNA	3.87	0.0231	5.64E-05	Type I Interferon Signaling
<b>Socs1-mRNA</b>	<b>3.8</b>	<b>0.0231</b>	<b>5.91E-05</b>	<b>MHC Class I Antigen Presentation, Toll Like Receptor Signaling, Type I Interferon Signaling, Type II Interferon Signaling</b>
Cd274-mRNA	3.76	0.0382	0.0003	Lymphocyte Trafficking, T-cell Checkpoint Signaling
H2-DMa-mRNA	3.51	0.0246	9.14E-05	Lymphocyte Trafficking, MHC Class II Antigen Presentation
Ido1-mRNA	3.41	0.0246	0.0001	Immunometabolism
Casp3-mRNA	3.06	0.0246	8.78E-05	Apoptosis, Oxidative Stress, TNF Family Signaling
Psmb8-mRNA	2.92	0.0246	0.0001	Apoptosis, B-cell Receptor Signaling, Fc Receptors and Phagocytosis, Immunometabolism, MHC Class I Antigen Presentati
Sp100-mRNA	2.92	0.0246	0.0001	Autoantigens, Type II Interferon Signaling
Psmb9-mRNA	2.85	0.0118	2.26E-05	Apoptosis, B-cell Receptor Signaling, Fc Receptors and Phagocytosis, Immunometabolism, MHC Class I Antigen Presentati
Stat1-mRNA	1.91	0.025	0.0002	Chemokine Signaling, Interleukin-12 Signaling, NLR Signaling, Other Interleukin Signaling, Th1 Differentiation, Toll Like R
Oasl1-mRNA	1.78	0.0468	0.0004	Type I Interferon Signaling, Type II Interferon Signaling
H2-D1-mRNA	1.42	0.0496	0.0004	Lymphocyte Trafficking, MHC Class I Antigen Presentation, Type I Interferon Signaling, Type II Interferon Signaling
Maf-mRNA	-0.734	0.0109	9.95E-06	Th2 Differentiation
Cd63-mRNA	-1.1	0.0246	7.99E-05	
P2rx4-mRNA	-1.16	0.0246	0.0001	Endothelial Activation
Elavl1-mRNA	-1.33	0.0425	0.0003	Th17 Mediated Biology
CUBN-mRNA	-1.76	0.0246	0.0001	

DE results - KO vs WT
DE\_KO - Low counts included
DE results - HET vs WT
DE\_HET - Low counts included

Source: Vinicius Canale, McCole Lab, University of California, Riverside, January 2020.

### *SOCS-1 Upregulation as a Compensatory Mechanism for PTPN2-Deficiency*

We identified one upregulated gene, suppressor of cytokine signaling-1 (*SOCS-1*), that has a clear overlap with signaling pathways modulated by *PTPN2* and could potentially be involved in controlling the host immune response to pathobiont bacteria during *PTPN2* deficiency. The Nanostring results revealed that *SOCS-1* had a Log<sub>2</sub> fold change of 3.8 and was categorized into gene sets of MHC Class I Antigen Presentation, Toll-Like Receptor Signaling, Type I Interferon Signaling, and Type II Interferon Signaling (see table 2). Studies have shown that SOCS proteins play an important role in the cytokine-induced Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway that controls the regulation of cellular proliferation, survival, and apoptosis (Weniger et al., 2006). The JAK-STAT signaling pathway plays a role in increasing intestinal barrier defects and must be deactivated by TCPTP to

regulate barrier function and correct these defects (Heinonen et al., 2004). Thus, deficiency of *PTPN2*, and consequently TCPTP, results in loss of barrier function and altered intestinal homeostasis. SOCS proteins have been found to play a similar role to TCPTP in regulating JAK-STAT signaling and repressing T cell proliferation because *SOCS-1* is a target gene of STATs, and *SOCS-1* gene expression rapidly increases upon cytokine-induced JAK-STAT signaling (Weniger et al., 2006). *SOCS-1* also directly inhibits the catalytic activity of JAK1, JAK2, and TYK2, and *SOCS-1* is a particularly potent inhibitor of JAK1 and JAK2 (Liau et al., 2018). Since JAK1 is a substrate of TCPTP and both *PTPN2* and *SOCS-1* negatively regulate JAK1 signaling, we hypothesize that *SOCS-1* may provide a compensatory mechanism to control elevated JAK1 signaling in *PTPN2*-deficient cells (Simoncic et al., 2002). Additional studies also found that *SOCS-1* is a potent inhibitor of the interferon-gamma (IFN- $\gamma$ ) pathway (Liau et al., 2018). IFN- $\gamma$  is a proinflammatory cytokine that is negatively regulated by *PTPN2* and plays an important role in the pathogenesis of CD and increased permeability of the intestinal epithelial barrier (McCole, 2012). Since *PTPN2*-deficient cells have an overactive JAK-STAT signaling pathway, the IFN- $\gamma$  in these cells exaggerates immune response because there is no restriction on signaling present. This also suggests that increased expression of *SOCS-1* in *PTPN2*-deficient cells could be a compensatory mechanism to reduce excessive immune response.

Based on these findings, we propose that *PTPN2*-deficiency may cause overexpression of *SOCS-1* to help control the strong immune response to pathobiont bacteria in the gastrointestinal tract. In addition to the role of *SOCS-1* in regulating cytokine signaling, studies have shown that *SOCS-1* is an essential negative regulator of responses to lipopolysaccharide (LPS), an integral cell wall component of Gram-negative bacteria, and can prevent LPS-induced microcirculatory dysfunction and inflammatory changes (Nakagawa et al., 2002). When bacterial flagellin is

present, *SOCS-1* is rapidly induced by LPS and negatively regulates LPS signaling by inhibiting T cell receptor (TCR)-mediated activation of T cells (Okugawa et al., 2006). This function of *SOCS-1* represents the host's attempt to limit inflammatory damage due to activation of the immune system by flagellated bacteria. In *Socs-1*-deficient mice, T cells also over-responded to flagellin stimulation through TCR activation, further suggesting that increasing *SOCS-1* expression has the potential to act as a compensatory mechanism to reduce the excessive immune response in *Ptpn2*-deficient mice. Therefore, we conclude that *PTPN2* deficiency may induce *SOCS-1* as a functional replacement to control cytokine and LPS signaling in the immune response to pathobiont bacteria. Since both *PTPN2* and *SOCS-1* control the JAK-STAT pathway, *PTPN2* may likely restrict *SOCS-1* expression through JAK1 signaling when *PTPN2* proteins remain functional but increase *SOCS-1* expression when their function is disrupted. Thus, we hypothesize that expression of *SOCS-1* would further increase in the presence of pathobiont bacteria, specifically AIEC, in *PTPN2*-deficient cells and mice to maintain intestinal homeostasis when symptoms of IBD become more severe. We will perform experiments to validate the role of *SOCS-1* upregulation and its effect on the binding and colonization of pathobiont bacteria, specifically *mAIEC*, in response to *PTPN2* deficiency.

### **Proposed Experiments**

Once we identify the panel of genes whose expression is altered in our models of *PTPN2* deficiency using the Nanostring nCounter analysis system, we will confirm these first by polymerase chain reaction (PCR) and quantitative PCR (qPCR), followed by protein expression by Western blot. For key validated genes of interest, we will visualize their cellular localization



by immunofluorescence. Then, we will validate the role that these novel genes/proteins play in host-microbe interaction by inhibiting or stimulating their expression, as described for CEACAM6, in *PTPN2*-deficient models in the presence of AIEC and determine their effects on AIEC colonization, binding, invasion, and survival.

To investigate the role of *SOCS-1*, we will start with a normal bacterial infection of *Ptpn2* knockdown (*Ptpn2*-KD) IECs from our *Ptpn2*<sup>AIEC</sup> mice with *mAIEC* vs. a control non-invasive *E. coli* bacterium, K12. We will first perform an invasion assay to measure the total number of *mAIEC* and K12 bacteria that adhere to and internalize in the IECs (Edwards et al., 2011; Shawki et al., 2020). Once we confirm the successful invasion of *mAIEC* in *Ptpn2*-deficient cells, we will use the Western blot technique to identify the effects of *mAIEC* invasion on *SOCS-1* protein expression. We will detect the level of *SOCS-1* protein expression in the infected *Ptpn2*-KD IECs to see if *SOCS-1* expression is significantly increased or decreased in response to *mAIEC*. If we identify significant alteration of *SOCS-1* expression in the Western blot, we will then confirm the results by using immunofluorescence techniques. To complement our studies of invasion of human IECs with a mouse-isolated bacterium, we will also examine the response to infection of *PTPN2*-KD human IECs with an equivalent human clinical AIEC isolate (LF82). We will also use the non-invasive human *E. coli*, K12, as a control condition for an *E. coli* without adherent-invasive properties. Using lysates from infected and uninfected IECs, we will perform Western blot analysis and immunofluorescence techniques to assess changes in *SOCS-1* expression in response to LF82 AIEC invasion (vs. K12) and compare them with the results found from the *mAIEC*-infected *Ptpn2*-deficient cells.

### *Inflammatory Marker Production in PTPN2-KD IECs and Macrophages*

To validate the role of *SOCS-1* in *PTPN2*-deficient cells and mice, we will first use *SOCS-1* small interfering RNA (siRNA) to reduce *SOCS-1* expression. RNA interference via siRNA helps precisely target genes that contribute to IBD by increasing the degradation of unwanted messenger RNA (mRNA) sequences (Chevalier, 2019). We will confirm knockdown by PCR and Western blotting of *PTPN2*-KD and *PTPN2*-Control IECs and macrophages, transfected with *SOCS-1* siRNA vs. control (scrambled) siRNA. We will then quantify inflammatory marker production in the *PTPN2*-KD vs. *PTPN2*-Control cells. Once we validate the role of *SOCS-1* in the host immune response to *PTPN2* deficiency, we will quantify the level of bacterial invasion in *PTPN2*-KD cells transfected with *SOCS-1* siRNA vs. control-siRNA (Shawki et al., 2020). Our lab has found that the levels of *mAIEC* and LF82 AIEC invasion in human IECs are similar, but *mAIEC* adheres to and invades mouse macrophages much more effectively than LF82 (Shawki et al., 2020). Therefore, we will use wild-type *mAIEC* to infect *Ptpn2*-KD IECs and macrophages in our *Ptpn2*<sup>AIEC</sup> mice and screen a panel of inflammatory markers to determine the effects of *Socs-1* knockdown and *mAIEC* invasion on the immune response in those cells.

### *SOCS-1 Expression in Response to mAIEC<sup>Red</sup> Infection*

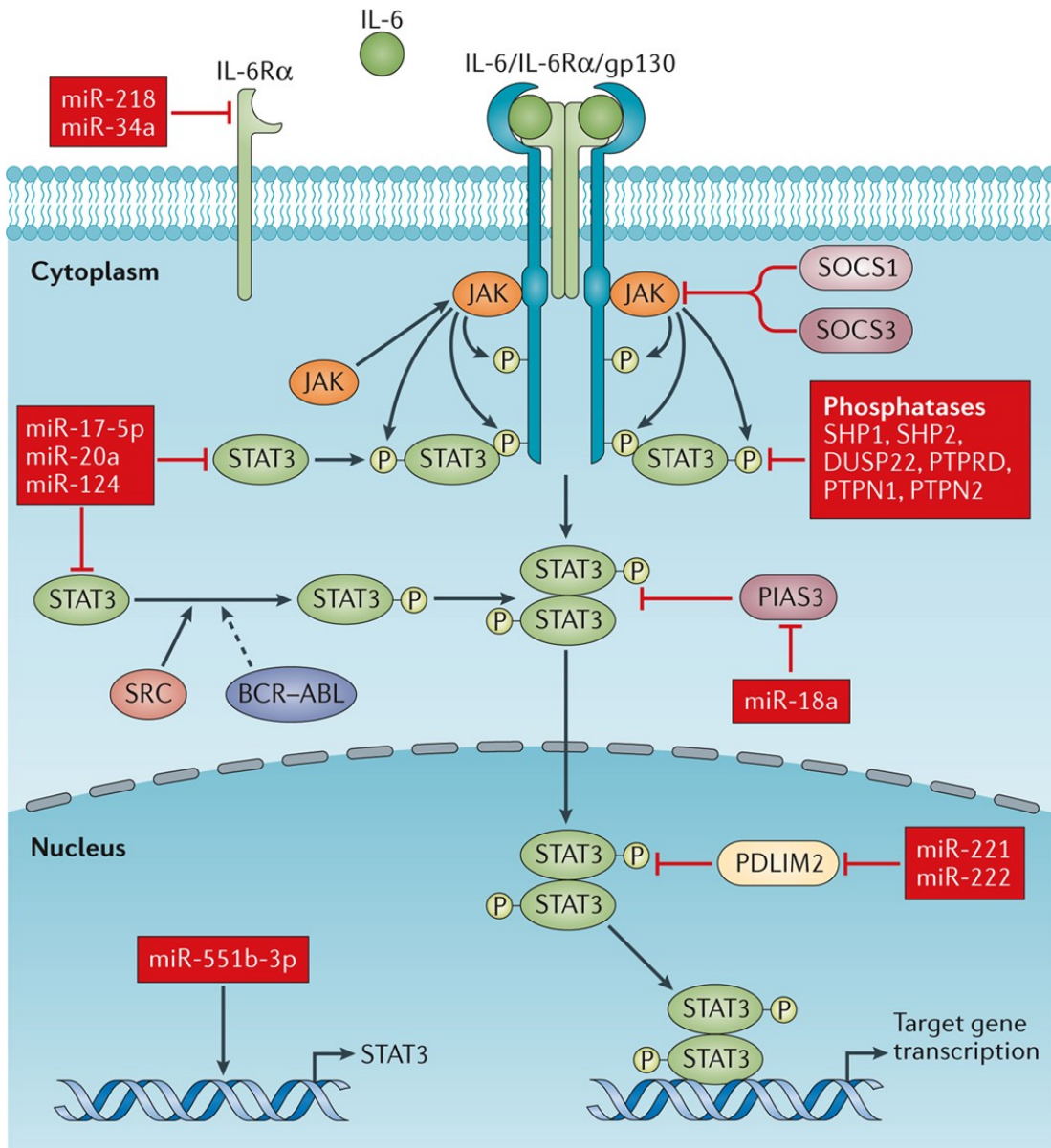
To confirm the role of *Socs-1* in *Ptpn2*-KD IECs and macrophages in response to wild-type *mAIEC* infection, we will evaluate *SOCS-1* protein function in host-microbe interaction by using *mAIEC*<sup>Red</sup>. We have generated a red fluorescence-tagged *mAIEC* called *mAIEC*<sup>Red</sup> that consists of a cassette containing red fluorescent protein (mCherry) and an antibiotic resistance

gene that was transformed into *mAIEC* (Shawki et al., 2020). We will use *mAIEC*<sup>Red</sup> to monitor the movement of the bacteria *in vivo* and determine its localization inside the cells (Shawki et al., 2020). We will then perform *in vitro* studies to identify the role of SOCS-1 proteins in mediating *mAIEC*<sup>Red</sup> entry and verify if it has a role in modulating bacterial binding and colonization in mice. We will calculate the number of *mAIEC*<sup>Red</sup> bacteria in *Socs-1*-KD IECs vs. control cells and co-stain the infected cells with staining markers for different cell compartments, such as 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining and lysosomal-associated membrane protein 1 (LAMP1) for lysosomal staining, to determine which compartments the bacteria localize in (Sayed et al., 2020). We will then use immunofluorescence techniques to visualize SOCS-1 expression and Western blotting of isolated IECs to confirm significant upregulation in the gastrointestinal tract of *mAIEC*-infected *Ptpn2*<sup>ΔIEC</sup> mice vs. wild-type mice. We will also identify possible direct interactions between SOCS-1 expression and invading *mAIEC* in the absence of *Ptpn2*.

#### *SOCS-1 Expression in Response to IL-6 Stimulated STAT3*

Finally, we will verify the role of *SOCS-1* upregulation in altering inflammatory cytokine signaling in *PTPN2*-deficient cells by characterizing SOCS-1 expression in response to IL-6 stimulated STAT3 (see fig. 2). Interleukin-6 (IL-6) is a pleiotropic cytokine produced by macrophages and an activator of the JAK-STAT signaling pathway that enables cell proliferation to generate an immune response against invading pathogens (Block et al., 2012). IL-6 is also a receptor for signal transducer and activator of transcription 3 (STAT3) that is important for host immune response but can contribute to IBD if it has abnormal prolonged activation (Wang et al., 2013). Studies have shown that SOCS proteins act as negative regulators of the IL-6/STAT3

pathway to prevent excessive activation of STAT3 (Wang et al., 2013). For this experiment, we will analyze SOCS-1 expression in response to IL-6/STAT3 signaling in *PTPN2*-KD vs. *PTPN2*-Control IECs and macrophages. Then, we will characterize SOCS-1 and IL-6 expression in *Ptpn2*-KD cells in our *Ptpn2*<sup>ΔIEC</sup> mice infected with *m*AIEC to verify that *SOCS-1* is upregulated in response to increased IL-6/STAT3 signaling. Likewise, we will characterize SOCS-1 expression in *PTPN2*-KD human IECs infected with LF82 AIEC to confirm *SOCS-1* as a functional substitute for *PTPN2*. Finally, we will use immunofluorescence techniques to visualize SOCS-1 and IL-6 expression in the *PTPN2*-KD IECs and macrophages and validate *SOCS-1* as an essential negative regulator of the IL-6/STAT3 pathway during *PTPN2* deficiency.



**Figure 2.** Signaling downstream of the IL-6 receptor from Johnson, Daniel, et al. “Targeting the IL-6/JAK/STAT3 signaling axis in cancer.” *Nature Reviews Clinical Oncology*, vol. 15, February 2018, pp. 234-248. Google Scholar, doi:10.1038/nrclinonc.2018.

## Expected Results

### *SOCS-1 Exhibits Increased Expression in AIEC-Infected PTPN2-Deficient Cells*

In our initial normal *m*AIEC infection of the *Ptpn2*<sup>ΔIEC</sup> mice, we expect that the total number of *m*AIEC bacteria in the *Ptpn2*-KD cells will be greater than the K12 bacteria, indicating that *m*AIEC invades *Ptpn2*-KD IECs and macrophages more successfully than the control K12 bacterium. Also, we expect to identify increased SOCS-1 protein expression in the Western blot analysis of the infected *Ptpn2*-KD cells and confirm these results using immunofluorescence techniques. We expect to identify similar results in the normal LF82-infected *PTPN2*-KD human IECs, where there is a higher number of LF82 AIEC in *PTPN2*-KD cells than K12 bacteria. We also expect to confirm SOCS-1 upregulation in these *PTPN2*-deficient cells through Western blot and immunofluorescence analyses.

### *SOCS-1 Functionally Replaces PTPN2 to Control Host Immune Response*

In the experiments using *PTPN2*-KD and *PTPN2*-Control IECs and macrophages transfected with *SOCS-1* siRNA and control siRNA, we intend to quantify inflammatory marker production in *PTPN2*-Control cells transfected with control siRNA, *PTPN2*-Control cells transfected with *SOCS-1* siRNA, *PTPN2*-KD cells transfected with control siRNA, and *PTPN2*-KD cells transfected with *SOCS-1* siRNA. In the *PTPN2*-Control cells transfected with control siRNA, we expect to detect no inflammatory marker production since the cells are under normal conditions where both *PTPN2* and *SOCS-1* are actively expressed and functioning. In the *PTPN2*-Control cells transfected with *SOCS-1* siRNA, we expect to quantify low inflammatory marker production because *PTPN2* still functions normally to control immune response despite

reduced *SOCS-1* expression. In the *PTPN2*-KD cells transfected with control siRNA, we also expect to detect reduced inflammatory marker production. Although *PTPN2* is deficient, we expect that *SOCS-1* expression remains active and becomes significantly upregulated to control the host immune response. Finally, in the *PTPN2*-KD cells transfected with *SOCS-1* siRNA, we expect to detect high inflammatory marker production because both *PTPN2* and *SOCS-1* are deficient and cannot negatively regulate the immune response. Therefore, we intend to confirm that RNA interference and the degradation of *SOCS-1* mRNA increases inflammation and IBD pathogenesis. This would verify that *SOCS-1* is an important negative regulator of the immune response during *PTPN2* deficiency and functionally replaces *PTPN2* to control inflammation in the gastrointestinal tract.

Furthermore, after performing bacterial invasion of the *Ptpn2*-KD IECs and macrophages in our *Ptpn2*<sup>ΔIEC</sup> mice, we expect to quantify even higher inflammatory marker production in the *Ptpn2*-KD cells transfected with *SOCS-1* siRNA. We expect AIEC infection to induce a stronger immune response and increased inflammation in the gastrointestinal tract since both *PTPN2* and *SOCS-1* are unable to regulate cytokine signaling. On the other hand, during *mAIEC* infection of the *Ptpn2*-KD IECs transfected with control siRNA, we expect to quantify even lower inflammatory marker production. This would indicate that *SOCS-1* expression is further upregulated in the presence of invading bacteria to help control immune response during *PTPN2* deficiency, thus confirming its role as a functional replacement for *PTPN2*.

### *SOCS-1 Upregulation Combats Invading AIEC in PTPN2-KD IECs*

To verify the role of *SOCS-1* in protecting hosts from pathobiont infection, we intend to use *mAIEC<sup>Red</sup>* to visualize interactions between *SOCS-1* proteins and invading *mAIEC* in *Ptpn2*-KD IECs in our *Ptpn2<sup>ΔIEC</sup>* mice. When we first evaluate the number of *mAIEC<sup>Red</sup>* bacteria in *Socs-1*-KD IECs vs. control cells, we expect to identify a lower number of *mAIEC<sup>Red</sup>* bacteria in the control IECs than the *Socs-1*-KD IECs. This would confirm that *SOCS-1* acts as an essential regulator of host immune response against bacterial infection. We also expect that the invading *mAIEC* and *SOCS-1* proteins localize at the same compartments in the IECs, indicating direct interactions between them. Then, when we evaluate *SOCS-1* expression in the *mAIEC<sup>Red</sup>*-infected *Ptpn2<sup>ΔIEC</sup>* mice vs. wild-type mice, we expect significantly increased *SOCS-1* expression in the *Ptpn2<sup>ΔIEC</sup>* mice compared to the wild-type mice. This would confirm that *PTPN2* likely restricts *SOCS-1* expression when it remains functional and induces *SOCS-1* upregulation when it becomes deficient. This would also verify that *SOCS-1* upregulation compensates for the loss of *PTPN2* by controlling immune response to prevent *mAIEC* infection.

### *SOCS-1 Functionally Replaces PTPN2 to Regulate IL-6/STAT3 Signaling*

When we characterize *SOCS-1* expression in response to IL-6/STAT3 signaling in *PTPN2*-KD vs. *PTPN2*-Control IECs, we expect to measure increased *SOCS-1* expression in the *PTPN2*-KD IECs compared to the *PTPN2*-Control IECs. Since *PTPN2* remains functional in the control cells, we expect that it negatively regulates IL-6/STAT3 signaling and restricts *SOCS-1* expression. However, in the *PTPN2*-KD IECs, loss of *PTPN2* induces increased IL-6/STAT3 signaling, so we expect to characterize *SOCS-1* upregulation and increased *SOCS-1* protein



expression in the PCR, Western blot, and immunofluorescence analyses. Additionally, we expect to characterize even higher SOCS-1 expression in the *Ptpn2*-KD IECs infected with *mAIEC* and the *PTPN2*-KD human IECs infected with LF82. This would indicate that increased IL-6/STAT3 signaling in infected *PTPN2*-deficient cells induces *SOCS-1* upregulation to limit inflammatory response against invading bacteria.

## Discussion

In this study, we intend to verify novel mechanisms that *PTPN2* uses to restrict AIEC binding and colonization in the gut when its function is disrupted. By infecting *PTPN2*-deficient cells and mice with AIEC, we intend to identify highly significantly altered expression of genes like *SOCS-1* in the *PTPN2*-regulated signaling network that provide functional compensation for *PTPN2* to control immune response against bacterial invasion. We expect to validate interactions between *PTPN2* and these novel genes so that we can better understand how they modulate the IL-6/JAK1/STAT3 pathway to prevent AIEC from binding and adhering to IECs. Therefore, by confirming the relationship between *PTPN2*, *SOCS-1*, and invading AIEC, we intend to identify alternative mechanisms in the host-microbe interaction to restore intestinal homeostasis and help improve future therapeutic targets against IBD.

Based on our expected results, we intend to identify and validate *SOCS-1* as a novel gene involved in the binding and colonization of pathobiont bacteria in the gastrointestinal tract in response to *PTPN2* deficiency. Previous studies confirmed a robust genetic association with CD at the *SOCS-1* gene locus on chromosomal region 16p13 for SNP rs4780355, suggesting *SOCS-1* as a possible candidate gene of IBD (Ellinghaus et al., 2012). We expect that *SOCS-1*

upregulation provides a compensatory mechanism to reduce excessive host immune response in the absence of *PTPN2* since both negatively regulate JAK1 signaling. In our experiments studying inflammatory marker production in *PTPN2*-KD IECs transfected with *SOCS-1* siRNA vs. control siRNA, we intend to confirm that *PTPN2* restricts *SOCS-1* expression when it remains functional, but significantly increases *SOCS-1* expression when it becomes deficient. We also expect to validate the role of *SOCS-1* as a functional replacement for *PTPN2* when we verify *SOCS-1* upregulation in response to *mAIEC*<sup>Red</sup> infection of *Ptpn2*<sup>ΔIEC</sup> mice. We intend to confirm that *SOCS-1* proteins directly interact with *mAIEC* in the same cell compartments in IECs, indicating that *SOCS-1* plays an important role in protecting hosts from bacterial invasion. Furthermore, we intend to confirm that *SOCS-1* acts as a functional substitute for *PTPN2* by controlling the IL-6/STAT3 signaling pathway during *PTPN2* deficiency (see fig. 2). We intend to verify this through our characterization of *SOCS-1* upregulation in response to increased IL-6 expression in *PTPN2*-KD IECs, and even further upregulation during AIEC infection. Thus, we expect to validate *SOCS-1* as an important substitute for *PTPN2* in the IL-6/STAT3 signaling pathway to prevent excessive immune response and protect hosts from pathobiont infection.

In conclusion, we expect that *SOCS-1* proves to be a useful candidate gene for developing therapeutic targets against IBD. Since *SOCS-1* plays an important role in the host-microbe interaction and the regulation of cytokine signaling when *PTPN2* is deficient, we can develop therapeutic approaches to target *SOCS-1* upregulation in *PTPN2*-deficient cells to reduce inflammation in the gastrointestinal tract and relieve symptoms of patients suffering from IBD.

### *Experimental Limitations and Future Directions*

Although increased *SOCS-1* expression may prove to be a useful alternative in regulating host immune response to *PTPN2* deficiency and bacterial infection, there are still patients carrying a *PTPN2* loss-of-function variant that become sick and suffer from IBD. We may obtain similar results from our study if we identify an excessive immune response in our *Ptpn2*-KO mice after *SOCS-1* upregulation in IECs. One possible reason for this is that increased *SOCS-1* expression may not be strong enough to suppress the host immune response and reduce IBD symptoms. Additional regulators of the IL-6/JAK1/STAT3 pathway may be needed to help compensate for the loss of *PTPN2* to maintain intestinal homeostasis. This hypothesis would be correct if we detected no significant decrease in inflammatory marker production or low *SOCS-1* expression in response to increased IL-6/STAT3 signaling in *PTPN2*-deficient cells. On the other hand, *SOCS-1* upregulation could make a host more susceptible to bacterial binding and invasion if it suppresses cytokine signaling too strongly. In this case, the immune response would be insufficient to protect the host from bacterial infection. Previous studies have shown that the *Toxoplasma gondii* bacterium evades the host immune response by inducing *SOCS-1* upregulation to limit IFN- $\gamma$  signaling (Zimmermann et al., 2006). Thus, rapid *SOCS-1* upregulation may alter intestinal homeostasis by suppressing the immune response to both commensal and pathobiont bacterial binding, resulting in increased infection. This hypothesis would be correct if we examined decreased *SOCS-1* expression in response to *mAIEC*-infected *PTPN2*-deficient cells or if we calculated a high number of invading bacteria in the *PTPN2*-deficient cells after *SOCS-1* upregulation. These results would explain why *Ptpn2*-deficient mice and patients still suffer from IBD despite increased *SOCS-1* expression in IECs.

Therefore, we need to further investigate the relationship between *SOCS-1* and *PTPN2* and determine their interactions with each other and additional candidate genes for IBD that may be essential in regulating intestinal barrier function. We also need to further study the relationship between *SOCS-1* and AIEC to determine other signaling pathways bacteria may induce or repress to evade the host immune response and determine the impact of *SOCS-1* and AIEC interactions on cellular functions in different compartments of IECs. Future experiments can be performed using different *Ptpn2*-deficient mouse models in addition to *Ptpn2*<sup>AIEC</sup> mice, such as *Ptpn2*-LysMCre mice or whole body *Ptpn2*-Het mice, to validate the role of *SOCS-1* in host-microbe interaction and improve therapeutic targets against IBD.

## **Future Studies**

### *Identifying and Validating Novel Downregulated Genes in Response to PTPN2 Deficiency*

Moving forward, we would need to perform a broader literature search on additional genes with significantly altered expression to identify those from the group of 25 genes from the Nanostring results that could also serve as candidate genes for IBD or be part of a broader *PTPN2*-regulated signaling network. Since we focused on a novel gene that had increased expression in *PTPN2*-deficient cells and mice, we can perform additional research to identify genes with significantly decreased expression during *PTPN2* deficiency and validate their role in host-microbe interaction in the gut. For example, we identified one downregulated gene, cubilin (*CUBN*), that may be involved in disrupting the absorption of nutrients through the intestinal epithelial barrier. Out of the 25 genes that had the most significantly altered gene expression

from the Nanostring results, *CUBN* was the most significantly downregulated with a Log<sub>2</sub> fold change of -1.76 (see table 2).

*CUBN* is the intestinal receptor for the endocytosis of the intrinsic factor-cobalamin (IF-cobalamin) complex in the distal small intestine (Fyfe et al., 2004; Kozyraki et al., 1999). Mutations in *CUBN* have been shown to cause malabsorption of cobalamin, or Vitamin B12, resulting in cobalamin deficiency (Fyfe et al., 2004). Cobalamin deficiency is a common cause of macrocytic anemia and other extra-intestinal manifestations of IBD in CD and UC patients (Bager et al., 2010; Misora et al. 2017; Yakut et al., 2010). Therefore, downregulation of *CUBN* leads to decreased absorption of cobalamin in the intestine, which may disrupt the regulation of cytokine signaling, compromise intestinal iron homeostasis, and create increased host susceptibility to IBD. Similar experiments to analyze *CUBN* expression in *PTPN2*-deficient cells and identify how *CUBN* loss may enhance AIEC infection can be performed to better understand the role(s) of *CUBN* in the pathogenesis of IBD. Future studies to prevent *CUBN* downregulation or induce upregulation of *CUBN* can help develop additional approaches for improving therapeutic targets against IBD.

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