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Disruption of Immunoregulatory Functions of Intestinal Mesenchymal Stromal Cells and their Progenitors in Inflammatory Bowel Disease

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Disruption of Immunoregulatory Functions of Intestinal Mesenchymal Stromal Cells and their Progenitors in Inflammatory Bowel Disease

by

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Dedication

This work is first and foremost dedicated to my wife, Margaret Becker, who has walked with me every step of the way and endured so much in her own right during my graduate studies. To be able to walk this harsh world with her unconditional love to aid me is a constantly renewed blessing. This would not have been possible without you.

This dissertation is also dedicated to my son, Timothy Barack Grim, who has come to me only recently but already given so much to my life. I hope that this achievement on my part motivates you, but also gives you something to shoot for and eclipse.

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I would first like to acknowledge my wife, who has given the support and love I needed all these years to keep going with my dissertation research, and the writing of this document itself.

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To my graduate mentor, Iryna Pinchuk, who as a new PI, also took a chance on me. Thank you for allowing me to be your first graduate student. This an amazing yet unbelievably grueling process for me, but despite everything, you always pushed me forward, towards this dissertation.

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A special acknowledgment is reserved for Don W. Powell, M.D., my co-mentor and committee member. Your help and guidance, especially in this last month, have been vital to my success, and I will not forget it.

Disruption of Immunoregulatory Functions of Intestinal Mesenchymal Stromal and their Progenitors in Inflammatory Bowel Disease

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Abstract: Crohn's Disease (CD) and Ulcerative Colitis (UC), the two major forms of Inflammatory Bowel Disease (IBD), are multifactorial autoimmune diseases caused by an abnormal immune response to gut microbiota in genetically susceptible individuals resulting in dysregulation of type 1/2/17 immune responses. $CD90^{+}$ (myo)fibroblasts (CMFs) are abundant innate immune cells in normal colonic mucosa that express Programmed Death-Ligands 1 & 2 (PD-L1/2) to promote immune tolerance. However, PD-L1 signaling is altered in IBD for as yet unknown reasons. Gremlin 1^{+} (Grem1) Mesenchymal Stromal progenitor Cells (MSCs) are progenitors of CMFs under homeostasis, but their fate during IBD immunopathogenesis is unknown. I **hypothesize** that PD-L1 expressed by CMFs is a critical driver of immune imbalance in IBD and that CMFs developed altered PD-L1 expression due to the sensitivity of their progenitor MSCs to the IBD inflammatory milieu. This dissertation utilizes various *in vitro* and *in situ* experiments to determine the role of altered PD-L1 signaling by CMFs in driving altered type 1/2/17 immune responses and investigating the role of MSCs in the development of altered CMFs. This dissertation research has found that differentially expressed PD-L1, but not PD-L2, alters T cell proliferation and type 1 immune responses. IBD-derived colonic MSCs have altered stemness properties compared to multipotent cells isolated from the normal colon and human bone marrow. Furthermore, MSCs are sensitive to the IBD inflammatory milieu, and I observed that this sensitivity resulted in the altered expression on stemness potency factors, and differential expression of PD-L1 similar to that of IBD-CMFs. The data in this dissertation strongly suggests that altered PD-L1 expression by IBD-CMFs is likely to contribute to the inflammatory immune responses in IBD, and that alteration of MSCs by the IBD inflammatory milieu may contribute to the differential expression of PD-L1 in CMFs. Further, the changes in IBD-MSCs and of normal MSCs exposed to the IBD inflammatory milieu demonstrated here have significant implications for the use of normal MSC in stem cell therapy for IBD patients.

TABLE OF CONTENTS

96

List of Figures

List of Abbreviations

ALDH	Aldehyde Dehydrogenase
ALDH1A2	Aldehyde Dehydrogenase 1A2
ANOVA	Analysis of variance
APCs	Antigen Presenting Cells
α-SMA	alpha-smooth muscle actin
BM-MSCs	Bone Marrow-derived Mesenchymal Stem Cells
BMP	Bone Morphogenenetic Protein
CD	Crohn's Disease
CD105	Cluster of Differentiation 105
CD11b	Cluster of Differentiation 11b
CD14	Cluster of Differentiation 14
CD1d	Cluster of Differentiation 1d
CD34	Cluster of Differentiation 34
CD40	Cluster of Differentiation 40
CD45	Cluster of Differentiation 45
CD73	Cluster of Differentiation 73
CD80	Cluster of Differentiation 80
CD86	Cluster of Differentiation 86
CD90	Cluster of Differentiation 90
CMFs	$CD90^+$ Colonic (Myo)Fibroblasts
CO2	Carbon Dioxide
Cox-2	Cyclo-oxygenase 2
CSCs	Cancer Stem-like Cells
CTCF	Corrected Total Cell Fluorescence
DAN	Deadenylating nuclease/Cerberus protein family
DAPI	4′, 6-diamidino-2-phenylindole
ECM	Extracellular Matrix
EMT	Epithelial-to-Mesenchymal Transition
Foxl1	Forkhead box protein l1
Gata3	GATA-binding factor 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Grem1	Gremlin 1
IBD	Inflammatory Bowel Disease
IDO	Indolamine
IFNγ	Interferon gamma
IgG1	Immunoglobulin type 1
IKKα	IκB kinase
IL-13	Interleukin 13
IL-17a	Interleukin 17a
IL-17f	Interleukin 17f
IL-22	Interleukin 22
IL-23	Interleukin 23
IL-33	Interleukin 33
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
ILCs	Innate lymphoid cells

Ki67	Kappa light chain 67
MHC	Major Histocompatibility Complex
MSCs	Mesenchymal Stromal Progenitor Cells
MStCs	Chemotaxic Bone-Marrow derived Mesenchymal Stem Cells
MyD88	Myeloid Differentiation primary Response 88
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKTs	Natural Killer T Cells
Oct4	Octamer Binding Protein 4
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PD-L1Fc	Programmed Death-Ligand 1 functional chimera
PD-L2	Programmed Death-Ligand 2
PGE2	Prostaglandin E2
PLZF	Promyelocytic leukemia zinc finer protein
POU5F1	*See Oct4*
qRT-qPCR	Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
RORγ	Retinoic Acid Receptor-related Orphan Nuclear Receptor gamma
RSPO2	R-spondin 2
RSPO3	R-spondin 3
SEM	Standard Error of the Mean
SMAD	Mothers against decapentaplegic homologs
STAT3	Signal Transducer and Activator of Transcription 3
STAT4	Signal Transducer and Activator of Transcription 4
STAT6	Signal Transducer and Activator of Transcription 6
T-bet	T-cell specific T-Box 21 Transcription Factor
TGFβ	Transforming Growth Factor Beta
Th1	T helper 1 cells
Th17	T helper 17 cells
Th2	T helper 2 cells
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
tMSCs	tissue resident Mesenchymal Stromal progenitor Cells
TNFs	Tumor Necrosis Factors
TNFα	Tumor Necrosis Factor alpha
UC	Ulcerative Colitis
Wnt	Wingless/Integrated proteins

CHAPTER 1. GENERAL INTRODUCTION

Objectives of the dissertation

My dissertation deals with the role of intestinal stromal cells, specifically Mesenchymal Stromal progenitor Cells (MSCs) and $CD90^{+}$ Myofibroblasts and Fibroblasts (CMFs) in the immunological health of the healthy intestine and how they are altered in inflammatory bowel disease (IBD). Chapter 1 defines IBD and its putative causes and outlines the histology and morphology of the mesenchymal cells in the lamina propria layer of the colonic mucosa. This chapter explains what is known about the primary functions of the CMFs that reside in the lamina propria, notably their role in mucosal tolerance that is mediated by expression of the immune checkpoint inhibitors Programmed Death Ligands 1 and 2 (PD-L1/2). Chapter 2 explicates how variations of PD-L1 by subepithelial CMFs in the two primary disease forms of IBD, Crohn's Disease (CD) and Ulcerative Colitis (UC), cause an imbalance of Type 1 (Th1) and Type 2 (Th2) helper T cell responses and increases the presence of Th17 cells in both diseases. Chapter 3 describes how similar MSCs are to CMFs and gives evidence that we can isolate MSCs from healthy or diseased intestine are MSCs. I also describe how the stemness properties of the isolated MSCs differ significantly in the two forms of IBD, which are also different from MSCs isolated from normal colonic tissue and from commercially obtained bone marrow-derived MSCs. Chapter 4 affixes the conclusion that these multipotent mesenchymal progenitors are detrimentally aberrant in the IBD colonic mucosa. This has importance in both the immunopathology of these diseases and the potential use of MSCs as therapeutic targets for the treatment of IBD.

Inflammatory Bowel Disease Epidemiology, Diagnosis, and Therapy

Crohn's Disease (CD) and Ulcerative Colitis (UC) are the two most commonly diagnosed forms of IBD. CD can occur as acute and chronic inflammation in any portion of the gastrointestinal tract (GI) tract from the mouth to the anus, although it is most prevalent in the terminal ileum and colon, with Crohn's colitis occurring in approximately 40% of CD patients [1]. CD inflammation can be submucosal or transmural, resulting in deep ulcers and fistula, granulomas, and transmural fibrosis. UC is confined to the colon, and limited to the mucosal layer, and results in surface ulcerations, loss of goblet cells, and inflammation, abscesses, and distortion of the colonic crypt structures [2].

CD and UC have a prevalence of 26-199 and 37-246 cases per 100,000 people, respectively; and the prevalence of both has been increasing since 1960, with the disease currently diagnosed in 1.6 million Americans [3]. Until the late 20th century, IBD was primarily isolated to North American and European populations [4]. More recently, populations that were thought to be at reduced risk of IBD, such as South and East Asians, have had a startling increase in IBD prevalence [4]. Interestingly, the increasing incidence of Crohn's disease in these areas remained stagnant for nearly a decade after the observed increase in UC incidence before an alarming increase that placed CD diagnosis of similar incidence in these nations, but currently, CD remains less prevalent than that of Western nations [5]. IBD is not mainly associated with increased mortality, however CD patients are more likely to suffer premature death, and all IBD patients have an increased risk of dying from gastrointestinal causes [6]. Both of these reported increases in mortality remained in patients who received therapeutic interventions [6]. A significant detriment of IBD is the poor quality of life and expense. The primary methods of diagnosis for IBD consist of endoscopic and radiologic imaging and endoscopic-

obtained biopsy with histopathologic definition [7]. Treatments for IBD include aminosalicylates, corticosteroids, cyclosporine, immunosuppressants, biological therapies including monoclonal antibodies and fusion proteins especially against TNF-alpha, and partial or total surgical resection of the intestine [8, 9]. Despite decades of intense IBD research, there remain a significant gap in our knowledge that prevents the development of effective IBD therapies due to the lack of understanding of the immunoregulatory mechanisms that are disrupted in IBD [1].

Th1/2/17 cell responses

The etiology of IBD is unknown, and there is no curative treatment. However, it has been postulated that IBD may be due to an aggressive autoimmune response to the intestinal microbiota in a genetically predisposed host [7, 10-12]. However, while probiotic therapy has been demonstrated to contribute to symptom relief, its therapeutic success is minimal [13]. Other suspected environmental initiators of disease onset that have been suggested include antibiotic exposure, smoking, general stress, diet, and lifestyle, though no factor has emerged as a direct contributor with full scientific consensus [4]. While the exact etiology the IBD remains debatable, it has been well established that the $CD4^+$ T helper (Th) cells, NKT, and innate lymphoid cells are principal effectors cells involved in the imbalance of type 1, 2 and 17 immune responses during the immunopathogenesis of IBD [14]. In healthy adults, Th1 cells produce Interferon gamma (IFN-γ) and Tumor Necrosis Factors (TNFs) that activate macrophages and $CD8^+$ T cells which target and eliminate viruses, pathogenic bacteria, and other intracellular pathogens [15]. Th2 cells respond to inflammatory cues caused by helminths or damaged tissue, and in turn release several type 2 cytokines that activate B cells, M2 macrophages, mast cells and eosinophils [16]. Th17 cells are a relatively recently identified group of Th cells that have vastly transformed our understanding of adaptive

immunity [17]. Th17 differentiation is driven primarily by Interleukin 1β and 6 (IL1 β and IL-6) and Transforming Growth Factor Beta (TGFβ), and primarily express Interleukins 17A and F (IL-17A and IL-17F) after maturation, maintaining the commensal microbial population on the skin and in the gut [17, 18].

Although both CD and UC involve several innate and professional immune cells, their implication in the immunopathogenesis of IBD is vary (Figure 1). Despite some contradictory reports in the field, the majority of our and other data show that in CD, the Type 1 response is predominant compared to Type 2 leading to an increased presence of interferon gamma resulting in direct and indirect tissue damage [19, 20]. In UC, the Type 2 response is elevated in proportion to Type 1 causing increased levels of IL-5 and -13, again leading to direct and indirect tissue damage [21]. Il-5 is implicated in tissue damage through the activation of eosinophils [22]. IL-13 produced by Cytotoxic Natural Kill T (NKT) cells [23] contributes to IBD-associated fibrosis [24]. In both forms of IBD, increases in Interleukin (IL) 1β, IL-6 and Il-23 lead to high Th17 responses that increases IL-17A causing a further increase in Th17 proliferation and causing tissue damage [21, 25]. However, a growing body of work have demonstrated that the role of the Th17 response in IBD may be much more complicated, with Th17 possibly prompting a simultaneous protective effect during IBD immunopathogenesis utilizing the relatively novel IL-22 cytokine cascade, although the overall mechanisms of the beneficial versus inflammatory effects of this cytokine remain unclear [26, 27]. Natural Killer T cells (NKT) have also been demonstrated to play a role in both innate protective immunity and IBD pathogenesis [28]. After responding to CD1d expression on the surface of APCs, which detect infiltration of microbes via lipid detection, NKT cells in IBD release pro-inflammatory cytokines and contribute the granuloma formation that occurs in CD [28].

Recently, innate lymphoid cells have also been observed to contribute to IBD immunopathogenesis via similar proliferation and inflammatory proteins seen as integral to Type 1, 2, and 17 responses [29, 30]. Despite the wealth of knowledge that exists in the field regarding Type 1/2/17 immune responses, a **gap** currently remains regarding the mechanisms that trigger these immune cells to switch from promoting immune tolerance to facilitating an inflammatory milieu.

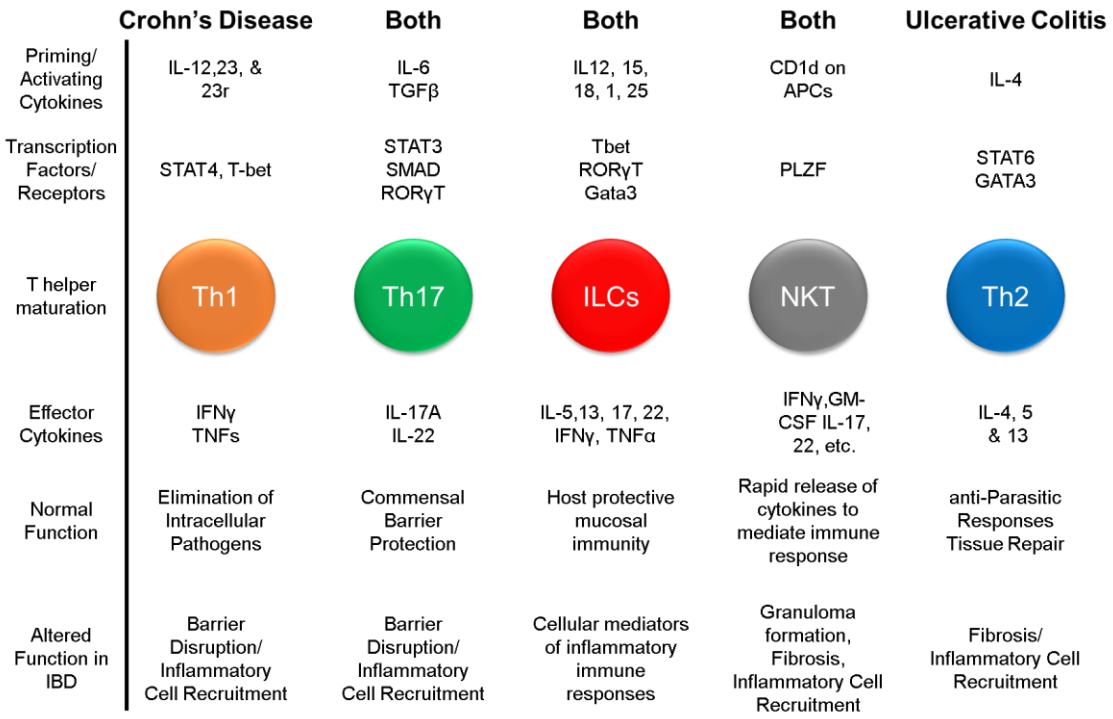

Figure 1. The Critical Factors of Effector Th Cells in Inflammatory Bowel Disease. Inducer transcription factors effector cytokines and functions of Th1/2/17 cells in IBD. Type 1 and 17 responses are elevated in CD, while Type 2 and 17 responses are high in UC. Figure Adapted from Imam, et al., and Fuss, et al. [21, 31].

Neither treatments generally aimed at inflammation (corticosteroids of immunomodulator) nor targeting abnormal Th/Treg cells or the cytokines they produce, does not always result in optimal therapeutic efficacy [32, 33]. An important new avenue of treatments are biologics, including monoclonal antibody treatments such as anti-TNFα

antibodies such as Infliximab and Integrin receptor antagonists such as Vedolizumab. Despite the improved treatment provided by such medicines, non-response or decreased efficacy over time, and severe, but rare, risk profiles including increased risk for cancer result in constraints to current IBD treatments [6, 34]. Therefore, identifying the cellular and molecular regulators of imbalanced Th1/2/17 responses may lead to novel IBD treatments.

Despite this advance in the knowledge, the mechanisms responsible for dysregulation of Type 1, 2 and 17 immune responses in both CD and UC, remain poorly understood. My dissertation aims to identify the contribution of a novel group of nonprofessional antigen-presenting cells, intestinal stromal cells, to these imbalanced immune responses.

Intestinal Stromal Cells

COLONIC TISSUE ARCHITECTURE

The composition of the gastrointestinal tract has the same four tissue layers from the stomach to the anus; the mucosa, submucosa, muscularis, and the serosa [35]. The mucosa, in turn, has three layers; the epithelium, the lamina propria, and the muscularis mucosae, the last one separate mucosa from submucosal area[35]. It is in the lamina propria of the colonic mucosa where cells of mesenchymal lineage reside in large quantities (Figure 2). While the mesenchymal cells of the colonic mucosa lamina propria also include pericytes the focus of this dissertation is on myofibroblasts, fibroblasts, and stromal mesenchymal progenitor (stem) cells [36]. These cells are numerous, making up approximately 30% of the cells in the colonic mucosa. CD90 also referred to as Thy-,1 is a 25-37kDa extracellular glycosylphosphatidylinositol–linked glycoprotein that is expressed (fairly specifically in humans) by cells of mesenchymal lineages, including

mesenchymal progenitors and myo-/fibroblasts (MF) [37]. Intracellular antibodies such as ER-TR7, which mark thymic stromal cells, have also been identified as possible markers of colonic MFs (CMFs) for both human and murine tissue *in situ* [38, 39]. Activated myofibroblasts in colon can be distinguished by their expression of the intracellular cytoskeleton microfilament protein alpha-smooth muscle actin (α-SMA)(Figure 2) [36]. Type 3 intermediate filament proteins such as vimentin and desmin have also been utilized as markers for differentiation of certain subtypes of mesenchymal cells [40]. The stable expression of CD90 by both fibroblasts and myofibroblasts in the human colonic mucosa lamina propria have led to the descriptive term $CD90^{+}$ Colonic (Myo)Fibroblasts (CMFs).

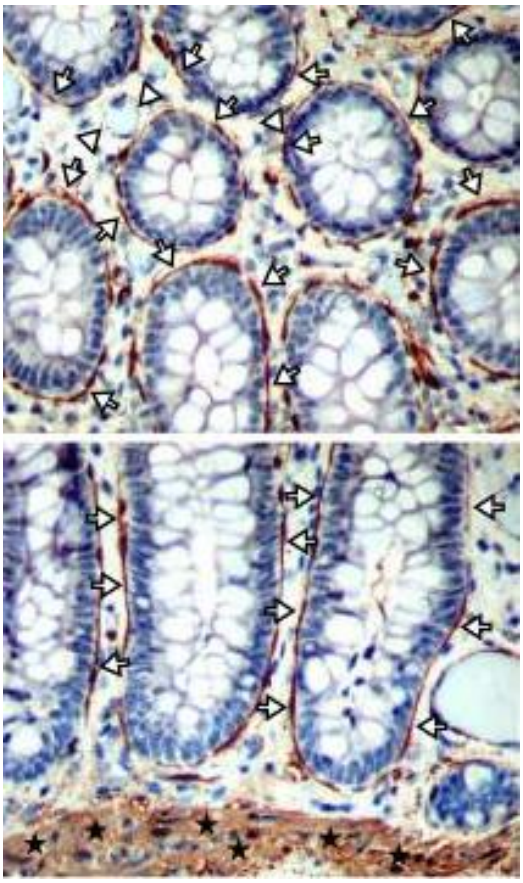

Figure 2. Histology of the human colonic mucosa.

Cross-section and longitudinal sections of the human colonic mucosa display epithelial layer forming the colonic crypts, the lamina propria is under the epithelium, between the crypts, and the muscularis mucosae (stars) is a strip of smooth muscle below the crypts. Brown staining indicates α-SMA positivity, arrows denote myofibroblasts, and the stars identify smooth muscle cells of the muscularis mucosa [41].

CD90^{+} COLONIC (MYO)FIBROBLASTS (CMFS) AS INNATE IMMUNE CELLS.

MFs have been observed in the human embryo as early as 21 weeks, with neural crest cells believed initially to be the progenitor of these cells. However, there is strong evidence to suggest that CMFs originate from the serosa via epithelial to mesenchymal transition (EMT) from the serosal epithelium (mesothelium) [40]. CMFs (stromal cells) are non-professional, innate immune cells of mesenchymal origin that reside in the large intestine lamina propria, located beneath the epithelium. As stated previously, CMFs

constitute approximately 30% of mononuclear cells in the intestinal mucosa, the connective layer between the basement membrane of the surface epithelial layer of the colonic crypt and the smooth muscle membrane of the muscularis mucosa [42]. When activated, CMFs express α-Smooth Muscle Actin (α-SMA) [41], a marker of a mesenchymal lineage that has been observed to be upregulated in areas of fibrosis in CD [1].

IMMUNE FUNCTION OF COLONIC MYOFIBROBLASTS

Our team has shown that CMFs support a state of mucosal tolerance in the normal colon [10, 36, 43]. By contrast, CMFs phenotype is altered in IBD, switching these cells activity to pro-inflammatory by yet unknown mechanisms. CMFs are among the primary regulators of T cell responses in colonic mucosa. Thus, abnormality within CMFs regulatory function and its progenitors is likely among critical events contributing to the IBD progression, and, thus, is an attractive therapeutic target [43, 44]. It has been firmly established that CMFs play a critical role in acute and chronic inflammation; in the Normal (N) colonic mucosa, N-CMFs support homeostatic (a.k.a. tolerogenic) responses via mechanisms involving B7 inhibitory molecules PD-1 ligand 1 (PD-L1) [45]. While our group previously show that CMFs may potentially present antigen through MHC class 2 to T cells, the bulk of data from our and other laboratory support the idea that CMFs have a tolerogenic function in the colon and are known to sustain Th cell proliferation and cytokine production via PD-L1-mediated signaling. Our group has determined that expression of PD-L1 by CMFs determines their capacity to suppress IFNγ producing Th1 cells [46]. Stimulation of TLR4 reinforces these tolerogenic properties via a mechanism involving the innate immunity adaptor MyD88 [10]. Therefore, CMFs are essential regulators of T-cells in the colonic mucosa. Therapeutic approaches directly targeting CMFs have not been reported. In CD, the PD-L1 expression of CMFs is decreased

compared to expression in N-CMFs, while the PD-L1 expression of UC-CMFs is increased compared to N-CMFs (Figure 3 A-D) [45]. In some cases, such as colon cancer, CMFs produce high amounts of IL-6 and TGF-β, which may contribute to Th17 cells induction [2]. However, the mechanisms contributing to a pro-inflammatory CMF phenotype and activity in IBD are unknown.

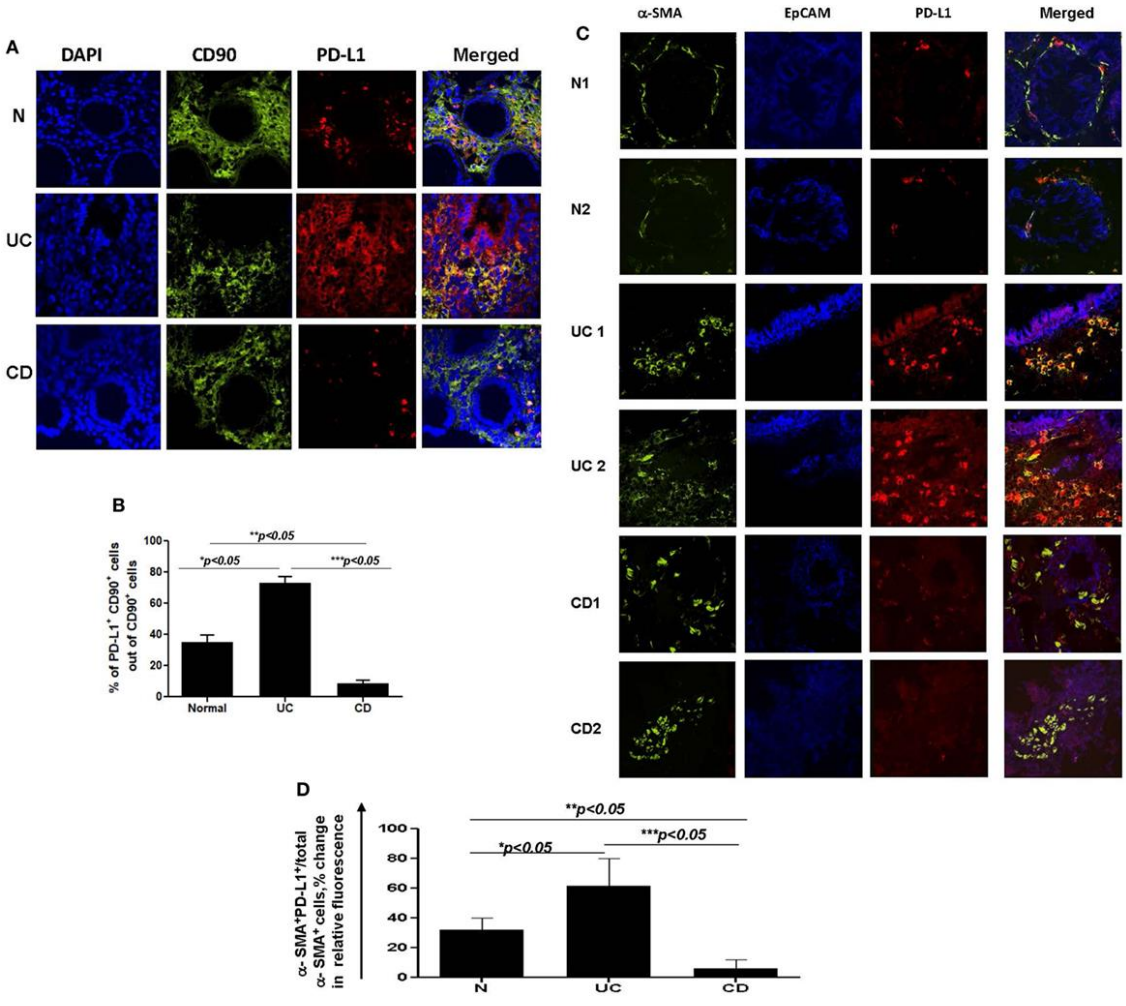

Figure 3. Programmed death-ligand 1 (PD-L1) expressing CD90$^+$ mesenchymal cells are increased in ulcerative colitis (UC) and decreased in Crohn's disease (CD) colonic mucosa.

(A) Confocal analysis of representative cross-sections of Normal, CD, and UC (n = 10 per group) were performed. Cell nuclei were stained with DAPI (blue). CMFs may be identified by morphology, location in the lamina propria, and staining with anti-human CD90 monoclonal antibodies (mAbs) conjugated to AF488 (clone 5E10, green). PD-L1 staining was performed using AF633-labeled anti-PD-L1 human mAbs (clone MIH1,

red). Co-localization of PD-L1 and $CD90^+$ CMFs is yellow-orange (arrow) on the merged images. (B) Summary of changes in PD-L1 Expression by CMFs in situ. Image J software was utilized for the quantification analysis. Data were expressed as means ± SEM of percentage of the changes in the count of corrected total cell fluorescence (CTCF) of PD-L1 positive $CD90^+$ cells (yellow-orange color formation) over total $CD90^+$ CMFs (both, green and yellow-orange stained cells), n = 10 per group, *p < 0.05. (C) Changes in PD-L1 expression in IBD-mesenchymal stromal cells is strongly associated with α-smooth muscle actin (α-SMA^+) CMFs. Confocal microscopy images of two representative cross sections from UC, CD, and normal human colonic mucosa (n = 7 per group) are shown. Myofibroblasts were detected by anti-α-SMA mAb (green), and epithelial cells were identified with anti-EpCAM mAb (in blue). Co-localization of PD-L1 with epithelial cells and α-SMA^+ myofibroblasts results in the formation of magenta/yellow-orange color on merged images. (D) Summary of the changes in the PD-L1 expression by α-SMA^+ CMFs *in situ*. Image J software was used for the quantification analysis. Data were expressed as means ± SEM of percentage changes in the CTCF of PD-L1 positive α-SMA^+ cells (yellow-orange color formation) over total α-SMA^+ myofibroblasts (both, green and yellow-orange stained cells), n = 7 per group, *p < 0.05 [47].

Mesenchymal Progenitors as the origin of CMFs in Adults

Until the mid-2000s, data seemed to support that the gut mesenchyme, including CMFs, originated from the neural crest, but recent studies suggest that the embryological mesenchyme is also the candidate for the origin of CMFs and other mesenchymal cells such as myofibroblasts, pericytes, and smooth muscle cells [35]. Thus, Mesenchymal Stromal progenitor Cells (MSCs) have emerged as major progenitors CMFs [48-50] and are believed to be derived from Mesenchymal Stem Cells (MStCs) also known as adult, tissue mesenchymal stem cells. MStCs are nonhematopoietic, rare, heterogeneous, and migratory cells that were first identified in bone marrow and have roles in most biological functions [12, 51, 52]. MStCs have further been observed in the umbilical cord, amniotic fluid, placenta, joint synovium, synovial fluid, dental pulp, endosteum periosteum and adipose tissue [53] and have been shown to migrate to injured tissue to differentiate into osteocytes, chondrocytes, adipocytes, and fibroblasts [47]. Intestinal MStCs have been proposed, but not isolated from the adult intestine.

MStCs are generally classified as being negative for the cell surface markers CD11b, 14, 34, and 45, while positive for CD73, 90, and 105, and have been regarded as both nonimmunogenic and immunosuppressive due to their poor expression of MHCI and II^{-}, and co-stimulatory molecules CD40^{-}/80^{-}/86 [51, 52]. They are converted from immunosuppressive to pro-immune function when exposed to IFNγ produced by innate and adaptive immune cells. While IFNγ can activate the immune functions of MStCs alone, TNFα, IL-1α, and IL-1β have also been determined to act in concert with the gamma interferon in many tissues [54, 55]. Activation of inducible nitric oxide synthase in MStCs has also been observed to decrease T cell proliferation in murine experiments [56]. The immunosuppression of T cells by MStCs occurs via both contact and soluble mediators by a variety of mechanisms, including cell cycle arrest, decreased production of IFNγ, IL-2, and TNFα, and have also been observed to increase IL-4 secretion of professional and non-professional immune cells [48, 52, 57].

However, there is significant controversy in the field of MStC research, specifically in regards to whether these cells are genuine stem cells or undifferentiated semi-potent cells that remain capable of differentiation into various other cell types as stated previously. The classic stem cell community requires that stem cells be cells that can both regress and differentiate into specific lineages, be able to undergo serial transplantations and not have their ability to differentiate and de-differentiate be altered, be capable of rapid proliferation even at small seeding densities, and must be capable of immortalization in their most potent state [58, 59]. While MStCs have been demonstrated in some cases to achieve these standards, there is evidence demonstrating that other cells, such as pericytes, can give rise to cells that are remarkably similar to MStCs [35, 60-62]. A separate but equally important issue regarding MStCs research is the lack of uniformity by researchers to utilize similar methodology to isolate MStCs from different tissue and to use various names to describe MStCs [59]. This dissertation does not seek

to wade into the controversy regarding the validity of membership of multipotent Mesenchymal Stromal Progenitor Cells in the stem cell community. Thus, the progenitor cells isolated from intestinal tissue will be notated as stromal progenitor cells and not stem cells. However, by convention, we will notate progenitor cells isolated form human bone marrow as BM-MStCs.

As with many lineages of stem/stromal cells, MSCs have been observed to have high expression of Pituitary-specific Octamer transcription factor Unc-86 transcription factor or class5, transcription factor 1 (POU5F1), commonly known as Octamer-binding transcription factor 4 (Oct4) [16, 17]. While crucial for embryogenesis [63], Oct 4 has been inextricably linked with inducing and maintain pluripotency in adult human fibroblasts [4, 64, 65]. Oct4 has also been observed to be increased in peripheral blood samples of patients with Crohn's Disease, indicating a possible presence of stem cells in the circulatory system [5, 63].

Aldehydes have been shown to play a crucial role in tumor initiation and maintenance of cancer stem-like cells (CSCs)[38]. CSCs are believed to be a driving force of tumor maintenance and evasion of oncotherapies in colon cancer [26, 39, 64]. Aldehyde dehydrogenase 1A2 (ALDH1A2) has been observed to be upregulated in carcinoma cancer stem cells (CSCs). Although CSCs synthesize tumor-arresting Retinoic acid (RA), they have also been implicated in poorer prognosis in colorectal and pancreatic cancers for reasons that are still unclear [26, 38]. However, the presence of and increase expression of Oct4 and ALDH1A2 in blood and tissue in normal and IBD colonic tissue remains a preliminary observation and has not been confirmed in IBD tissues in situ or *in vitro*.

Tissue-based progenitor cells of CMFs

In the adult, CMFs are thought to derive from either tissue-resident stationary stem cells that reside in the colonic mucosa (tMSCs), or migratory stem cells that arrive in the intestine by chemotaxis from the bone marrow (BM-MStCs) [66-69]. tMSCs are present in the gut mucosa, can remain stationary or home to pericyptal locations via TLR-dependent Cox-2 signaling, and may be important in the regeneration of CMFs during homeostasis [61, 62]. Much of the difficulty in characterizing MSCs that either reside in or home to the gut is the determination of a lineage marker specific to both the MSCs and their progeny in the gut. Recently, it has been observed that Gremlin 1 (Grem1) may identify possible progenitors of CMFs in the murine small intestine [68]. Grem1 is a highly conserved glycoprotein in the Deadenylating nuclease (DAN)/Cereberus protein family discovered in Xenopus as a Bone Morphogenetic Proteins (BMP) antagonist. It was identified by inhibiting their association with BMP receptors on cell membranes and shown to localize to the Extracellular Matrix (ECM) and endoplasmic reticulum [47]. Furthermore, Gremlin 1 has been demonstrated to identify a single cell in the base of the murine small intestine epithelium that regenerates the entirety of ileal mucosal CMF syncytium in approximately 12 months and persists for the life of the mouse [68]. Gremlin 1 suppression has also been demonstrated to contribute to osteogenic capacity of MSCs [74]. Grem 1 is disruptive to normal intestinal function when overexpressed and is an identifying protein of highly clonogenic bone marrow stromal cultures [68, 70-73]. As noted above, during normal murine postneonatal development, $Grem1^+$ MSCs have been shown to populate the small intestinal mucosa within 11 months and to preserve their progenitor capacity. It has been reported in lineage tracing experiments that $Grem1^+$MSC progeny may acquire expression of α-SMA^+ suggesting they differentiation to CMFs. Further, they may persist for up to two years or more , which suggests that

this CMF syncytium is a very slowly renewing organelle during homeostasis, in contrast to the rapid turnover of intestinal epithelial cells which may take on 4-7 days from epithelial stem cell division to death and extrusion of the mature epithelial cell into the gut lumen[68] Grem1 has further been observed to be upregulated in colorectal cancer tumors when compared to normal colonic mucosa. However, there has been no characterization of Grem1^{+} population of cells and function of this protein in the IBD-inflamed colonic mucosa [27]. While tMSCs have been identified in intestinal wounding and murine DSS colitis models by their extremely robust expression of COX-2, tMSCs presence/activity in IBD is unknown [62].

Interestingly, the epithelial barrier may also rely heavily on MSCs presence in the lamina propria to facilitate growth and wound repair via R-Spondin-2 and 3 (RSPO2 and RSPO3) mediation of Wnt signaling, in addition to TLR-dependent Cox-2 signaling. RSPO2 expression has been linked to the susceptibility of colitis, and RSPO3 has been determined to be a gene the belays susceptibility to CD in humans. [75] RSPO2 floxed mice were determined to have retarded limb bud formation, along with decreased weight and bone mass after birth due to decreased Wnt stimulation of osteocytes during development [76]. Further studies by Kang, et al. at McGill University determined that RSPO2/3 were expressed exclusively by CD45^{-} (non-hematopoietic) cells residing in the lamina propria [75]. RSPO2 and RSPO3 have also been observed to mediate Wnt signaling via orphan receptors LGR4 and LGR5, the major proliferating receptors of intestinal epithelial stem cells, and murine crypt organoids from cells harvested from LGR4$^{-/-}$ mice failed to grow *in vitro* [77]. While CD34 has long been thought of to be a negative marker for MSCs, recent work has shown that CD34^{+} multipotent cells are positioned near the epithelial crypts, express Wnt2b, Grem 1, and RSPO1, and are sufficient in maintaining LGR5^{+} intestinal epithelial stem cells[78]. Similar work has also been shown with a second, known Wnt modulator cell, Forkhead Box l1 (Foxl1)$^{+}$

mesenchymal cells. Foxl1^{+} cells appear to prime LGR5+ epithelial cell division, thus making a significant contribution to the epithelial stem cell niche [79]. Taken together, this data suggests strong evidence for the presence of tMSCs in the colonic mucosa lamina propria and that these cells are important precursors of CMFs and are critical for maintenance of the epithelial barrier.

Bone-marrow multipotent cells may also be progenitors of CMFs

Another possible source for CMFs is chemotaxis of Mesenchymal stromal progenitor cells from the bone marrow. Elegant experiments utilizing Y chromosome-tagging of male mesenchymal stem cells injected into irradiated, TNBS colitis-induced female mice have demonstrated that up to 40% of CMFs weeks post-injury are derived from the injected Bone Marrow Mesenchymal Stem Cells (BM-MSCs) [69]. In other studies, cyclooxygenase (COX)-2 expressing cells have been shown to reposition and facilitate repair near the basal crypt of the colon [67]. Despite this sophisticated study with bone marrow stem cell infusions in a colitis model, little work has been done to determine the exact role of BM-MSCs in the human colon during homeostasis or after damage (colitis), and if they are altered by the colonic environment after migration to the lamina propria. However, this data does illustrate that BM-MSCs migrating from bone marrow may play a significant role in wound healing of the murine and perhaps human colon. However, the data and observations regarding chemotaxic bone marrow progenitors in the gut is sparse, and there is almost no data from human tissue.

Immune Tolerance in the Human Colonic Mucosa

CMFs are innate immune cells that interact with both commensal gut bacteria and professional immune cells to promote tolerogenic immune responses in the healthy human colon [10, 45, 47]. Although MStCs have only been recently identified as progenitors in the adult human colonic mucosa lamina propria, MSC in other tissues have been identified and known for their immunosuppressive function. Thus, tissue MSCs have many biological properties in common with CMFs in terms of promoting immune tolerance, making them a potential target for new therapeutic avenues in the treatment of autoimmune diseases such as IBD [61, 62, 74].

In humans, CMFs have been demonstrated to be among the major cell population present in the colonic *lamina propria* and are capable, when primed with IFN-γ, of presenting antigens via Major Histocompatibility Complex II (MHC II), molecules traditionally associated with professional Antigen Presenting Cells (APCs) and have been shown to initiate immune responses to extracellular pathogens [80]. Interestingly, CMFs were also observed to uptake and phagocytose specific *Salmonella* antigens, further indicating their role as APCs in the gut mucosa [81]. Despite the presence of MHC II, the low basal expression of CD86, the positive co-stimulatory molecule necessary for antigen presentation, by CMFs indicates that they do not play a major role in the activation and proliferation of naïve $CD4^+$ T cells. This, taken together with the strong expression of B7 immunosuppressive molecules PD-L1 and PD-L2, indicate that the primary role for CMFs in the normal colonic mucosa is tolerogenic [44, 82]. Importantly, MStCs share with CMFs the properties of lowered CD86 and higher PD-L1/2 expression, most likely contributing to their already elucidated immunosuppressive functions [83].

The GI tract is home to populations of resident and transitory microbes [84]. Toll-Like Receptors on innate immune cells recognize patterns present on the surface of microbes and have been identified as the classic source of innate immune responses triggered by the associated ligands produced by microbes throughout the body, including the gut [85]. CMFs have also been demonstrated to have enhanced PD-L1 mediated immunosuppressive qualities via stimulation of TLRs 2, 4, and 5 [10]. This is accomplished via the adaptor protein Myeloid Differentiation factor 88 (MyD88), which is required to initiate the intracellular signaling required for response to the recognition of microbial ligands of TLRs (except TLR 3) [86]. TLR 4 has been observed to upregulate expression of PD-L1 on the surface of CMFs, resulting in increased suppression of (1) $CD4^+$ T cell proliferation and (2) the suppression of production of Type 1 immune response cytokine, IFNγ. PD-L1 has also been demonstrated to play a role in Type 1 immune responses. Thus, the regulation of PD-L1 expression by TLRs may serve as a serve to enhance tolerogenic functions. Interestingly, the reports on the outcome of the TLR stimulation on MSCs remain contradictory. Chen et al (ref 88) reported that stimulation of TLR3 and 4 affect only the capacity of MSCs to differentiate to adipocytes and osteoblasts, but did not change their immunogenic simmunosuppresve function. By contrast Liotta et al (ref 89) observed a decrease in immunosuppresive capacity of MSC via mechanism involving Notch ligand Jagged-1[87-89]. Therefore, the effects of TLRs on the potency, immunogenicity and tolerogenicity of MStCs requires further investigation.

MSCs and CMFs also share in common the secretion of soluble mediators of intestinal immune tolerance and commensal homeostasis, specifically Prostaglandin E2 (PGE_2), Indolamine (IDO), and Transforming Growth Factor Beta (TGF-β), all of which have been determined to play a vital role in effector T cell and Regulatory T cells (Treg) balance [40, 90]. Murine experiments have also demonstrated that PGE_2 is upregulated by pro-

inflammatory effector cytokines IFNγ and TNFα, indicating that secretion of PGE2 and the contact immunosuppressive molecules such as PD-L1/2 with PD-1 are critical immunosuppressive mechanisms of MStCs [61]. CMFs have also been demonstrated to produce immunosuppressive cytokines Interleukins 10 and 21 (IL-10/21), which are also crucial in the instilling equilibrium between Th and regulatory T (Treg) cells [40, 90]. Tregs have been identified as critical for gut mucosal tolerance, and our group has demonstrated that PGE_2 is crucial to CMF-mediated peripheral induction of Tregs from naïve $CD4^+$ T cells, indicating further importance of soluble mediators such a PGE2 in gut homeostasis, with the contact ligand PD-L1 only responsible for approximately 10% of Treg induction [91, 92] . These data illustrate that CMFs and MSCs are very similar potent immunosuppressive mesenchymal cell populations whose disruption may be critical to IBD immunopathogenesis.

MSC Therapy May Be a Potential Treatment for IBD

Since MSCs are most likely the progenitors of CMFs in adults and possess several crucial immunomodulatory effects (Figure 4), MSCs have been identified as a therapeutic target for treating IBD [41]. While MSC therapy resulted in a reduction of the inflammatory response, long-term remission in comparison to IBD patients undergoing standard treatments remains in question [93-95]. It does not appear that infused MSCs will permanently engraft in the diseased intestine, so immunosuppression appears to be the main therapeutic mechanism of this form of experimental therapy. The proper route of administration, the source of MSCs, and methods to enhance the density of cells required at the site(s) of inflamed tissue must be determined [95]. Optimization of MSC therapy is also cited as the main area for concern in improving results [96]. The **lack of knowledge** in how MSCs are altered during IBD development is a significant obstacle

preventing the development of effective MSC therapy for prolonged remission/cure of IBD.

Despite the advances and controversies in the field, MSCs/MStCs therapy has been considered for numerous diseases such as pancreatitis, sclerosis, radiation injury, various autoimmune diseases, and myocardial infarctions [80-82, 85, 86]. Despite several preclinical animal studies that demonstrate the immunosuppressive effects of MSC therapy, it remains unclear whether MSCs exert their immunomodulatory function via paracrine signaling, mitochondrial or vesicle transfer, or cell contact and adhesion [88, 89, 97, 98]. While the number of clinical trials attempting to utilize MStCs as a therapeutic agent has ballooned, there have been relatively few successes that justify the immense expense of moving the therapy to market [80, 98, 99].

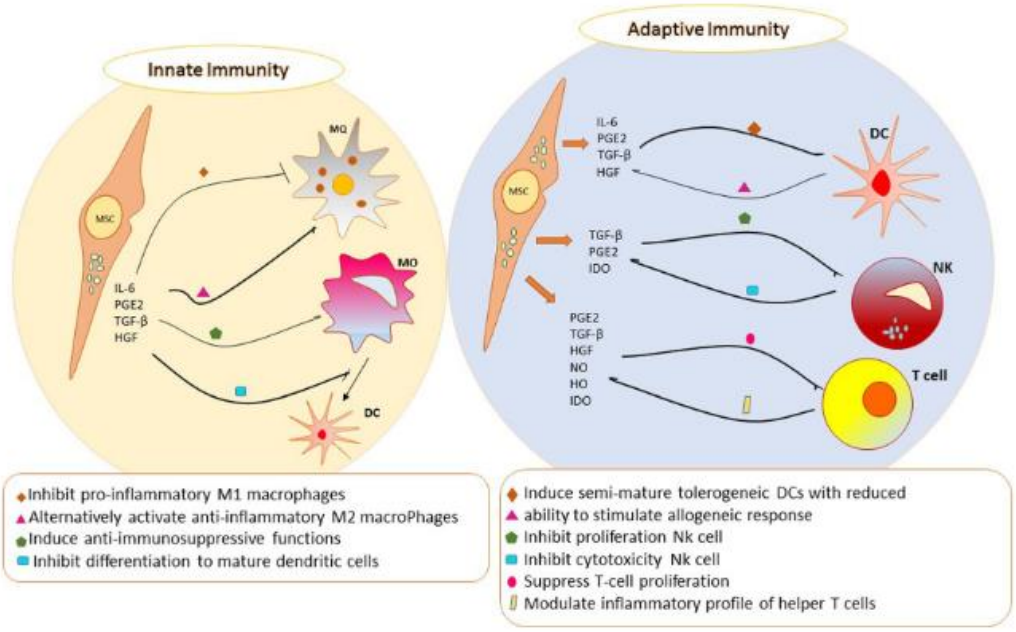

Figure 4. Immunomodulatory properties of MSCs.

MSCs have been demonstrated to interact with innate and adaptive immunity lymphocytes. In both innate and acquired immune responses, MSCs have immunosuppressive properties that inhibit the activation and proliferation of inflammatory macrophages and monocytes and prevent the maturation of monocytes into dendritic

cells. MSCs further reduce the toxicity of natural killer cells and have been shown to suppress the proliferation of T cells [97].

As discussed above, the role and origin of MSCs in the gut remain poorly understood. Bone Marrow Mesenchymal Stem Cells (BM-MtSCs) have been shown to migrate to the sites of colonic damage [69]. Novel data have demonstrated that there may also be a population of tissue based adult MSCs that persist solely in gut tissue [66, 68, 100]. MSCs have also been observed be an immunosuppressive population of cells in the gut, and other tissues are making them candidates of therapeutic targeting to treat IBD [12, 16-18, 41]. While MSC therapy has proved promising in regards to reduction of the inflammatory response, long-term remission in comparison to currently approved IBD treatments remains questionable [18, 34, 93-95].

Programmed Death Ligands 1&2 is critical to the CMF-mediated suppression of inflammatory responses during colonic homeostasis.

Our laboratory has published that normal CMFs play a suppressive role in the colonic mucosa via induction of Treg and expression of immunosuppressive B7 molecules, Programmed Death Ligands (PD-L) 1 and PD-L2, and their receptor PD-1 (Figure 3 above, reprinted below), which is present on lymphocytes, endothelial and some cancer cells [47]. PD-1, initially isolated from apoptotic T cells *in vitro*, is a 55-kDa transmembrane protein that contains one extracellular IgV-like domain and a 97-amino acid cytoplasmic tail [101]. PD-1 is expressed on the surfaces of T cells, B cells, and myeloid cells, indicating that PD-1 and its ligands have a vast spectrum of immunoregulatory functions [102]. PD-L1 and PD-L2 expression have been observed in immune and stromal cells in a broad range of normal human and murine tissues such

as the heart, placenta, spleen, pancreas, and thymus [103-106]. Expression of these ligands, especially PD-L1, has also been observed in aberrant tissues such as solid tumors and both resident lymphocytes and the cancer cells may present PD-1.Further, these negative co-stimulatory molecules have been demonstrated to aid in tumor development through the mechanism of immune evasion [107-109]. This has made the PD-1/PD-L1 immune response checkpoint therapy a prime therapeutic modality for cancer treatment, with ant-PD-L1 antibodies on the market which have become standard of care treatment for many cancers and with clinical studies to ascertain their efficacy for treating other cancers underway [58-60]. Interestingly, the expression of PD-1, PD-L1 and PD-L2 have also been observed in tissues responding to bacterial infection and autoimmune inflammation with data indicating that it plays a role in regulating T cell responses [110].

Our lab and others determined that signaling via PD-L1 was critical for the suppression of the activated Th cell and cytokine production, and the induction of Treg cell [40, 45, 111]. We observed that depending on levels of expression of PD-L1 by CMFs, there is a switch from immunosuppressive to an inflammatory phenotype in IBD and this was associated with the abnormal Th1/Th2 balance and increase in Th17 promoting IL-6 cytokine production. Our recent *in situ* data has demonstrated that PD-L1 expression is altered in human IBD tissue (Figure 3). In Crohn's Disease, PD-L1 on CMFs is low when compared to normal tissue, whereas in UC tissue there is increased expression of PD-L1 on CMFs [46]. These data suggest that changes in PD-L1 is instrumental to the dysregulated T cell responses in the human IBD colonic mucosa.

CHAPTER 2. THE ROLE OF PD-L1 EXPRESSION BY CMFS IN TYPE 1/2/17 IMMUNE RESPONSES IMBALANCE

Modified in part from:

Expression of Programmed Death-Ligand 1 by Human Colonic CD90^{+} Stromal Cells Differs Between Ulcerative Colitis and Crohn's Disease and Determines Their Capacity to Suppress Th1 Cells

Beswick, E. J., Grim, C., Singh, A., Aguirre, J. E., Tafoya, M., Qiu, S., Rogler, G., McKee, R., Samedi, V., Ma, T. Y., Reyes, V. E., Powell, D. W. & Pinchuk, I. V. (2018). Frontiers in Immunology, 9, 1125. doi:10.3389/fimmu.2018.01125

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Preliminary data, aim and specific hypothesis

Several laboratories have determined that Type1/2/17 responses are dysregulated in IBD [24, 31]. It has also been observed that PD-L1 and PD-L2 may be expressed by nonprofessional immune cells such as CMFs, providing crucial signaling that alters T cell proliferation and responses [10, 45]. Interestingly, our published results demonstrated that PD-L1, but not PD-L2 mRNA expression is altered in IBD (Figure 5A-D) [46].

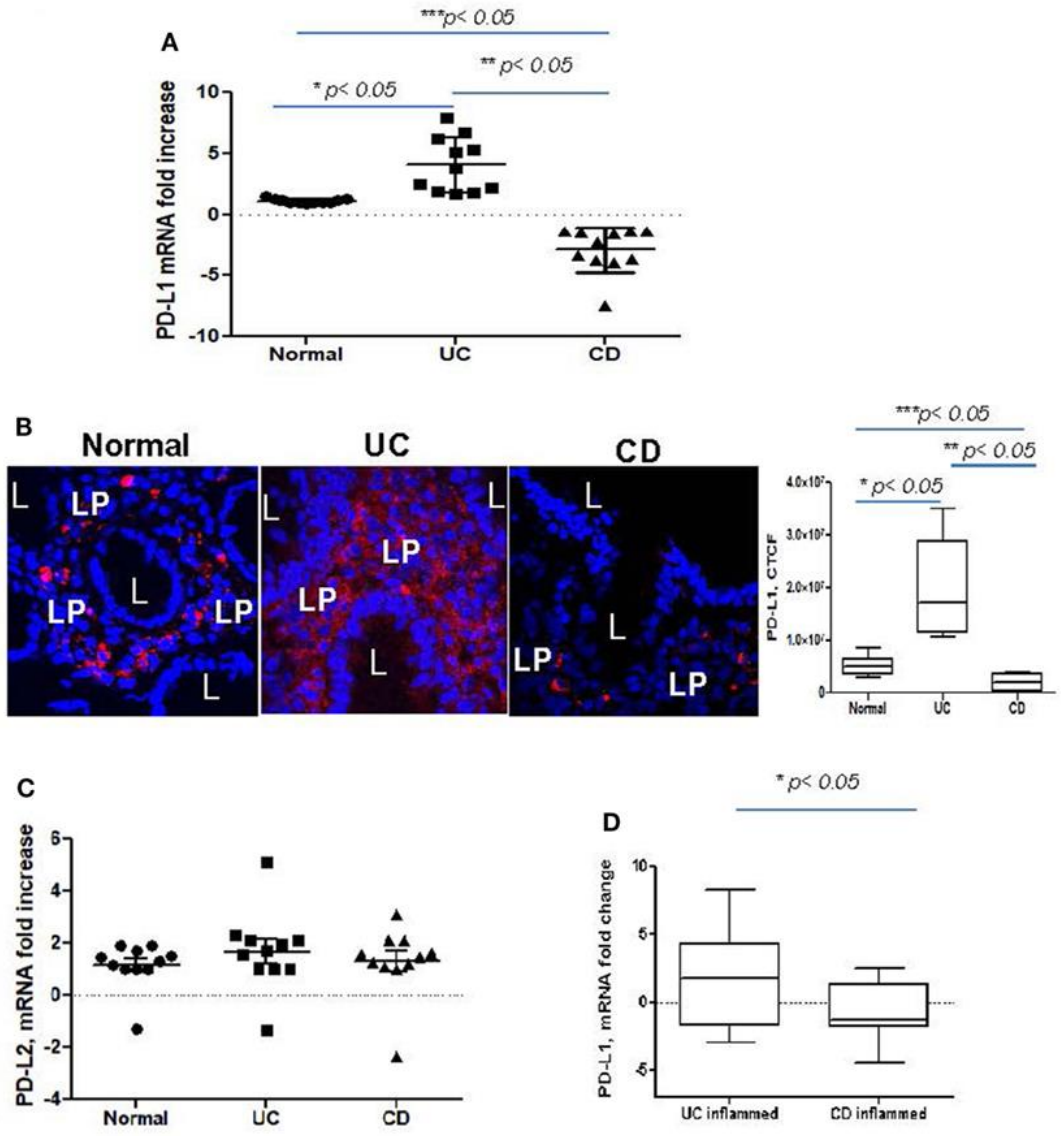


Figure 5. Programmed Death-ligand 1 is increased in the UC colonic mucosa but decreased in the CD colonic mucosa.

(A) PD-L1 mRNA levels in UC and CD colonic mucosa were compared to normal tissue controls obtained from healthy individuals (real-time RT-PCR analysis). The means ± SEM are shown as the results of duplicates of each tissue sample, n = 11 per group. (B) PD-L1 protein expression in situ was increased in UC and somewhat decreased in CD. In these experiments, frozen colonic tissue sections were stained with DAPI to identify cell nuclei (blue) and anti-PD-L1 monoclonal antibodies (clone MIH, in red) and analyzed by confocal microscopy (63×). A representative cross sections and summary of fold changes in the corrected total cell fluorescence (CTCF) of PD-L1 expression in UC, CD, and normal human colonic mucosa (the means ± SEM are shown, n = 10 per group) are shown, L, lumen; LP, lamina propria, *p < 0.05. (C) PD-L2 mRNA levels in UC and CD colonic mucosa were compared to normal tissue controls obtained (real-time RT-PCR analysis). The means ± SEM shown are the results of duplicates of each tissue sample, n = 11 per group. (D) PD-L1 mRNA levels in UC and CD inflamed

colonic mucosa was normalized to its matched, non-involved tissue controls (real-time RT-PCR analysis). The means ± SEM shown are the results of duplicates of each tissue sample, n = 20 per group. [47].

In order to determine if the above–noted increased tissue expression of PD-L1 was due to CMFs, and not by other cells present in the lamina propria, we confirmed our initial confocal microscopy observations via flow cytometry analysis on significantly higher number of tissue derived from IBD patients. We observed that the total number of PD-L1- expressing fibroblasts, as determined by CD90 positivity of the cells, as well as expression by activated fibroblasts, marked by alpha-smooth muscle actin (myofibroblasts), is increased in UC, but decreased in CD colonic mucosa *in situ* (Figure 6A-C, Figure 3D) *[46]*.

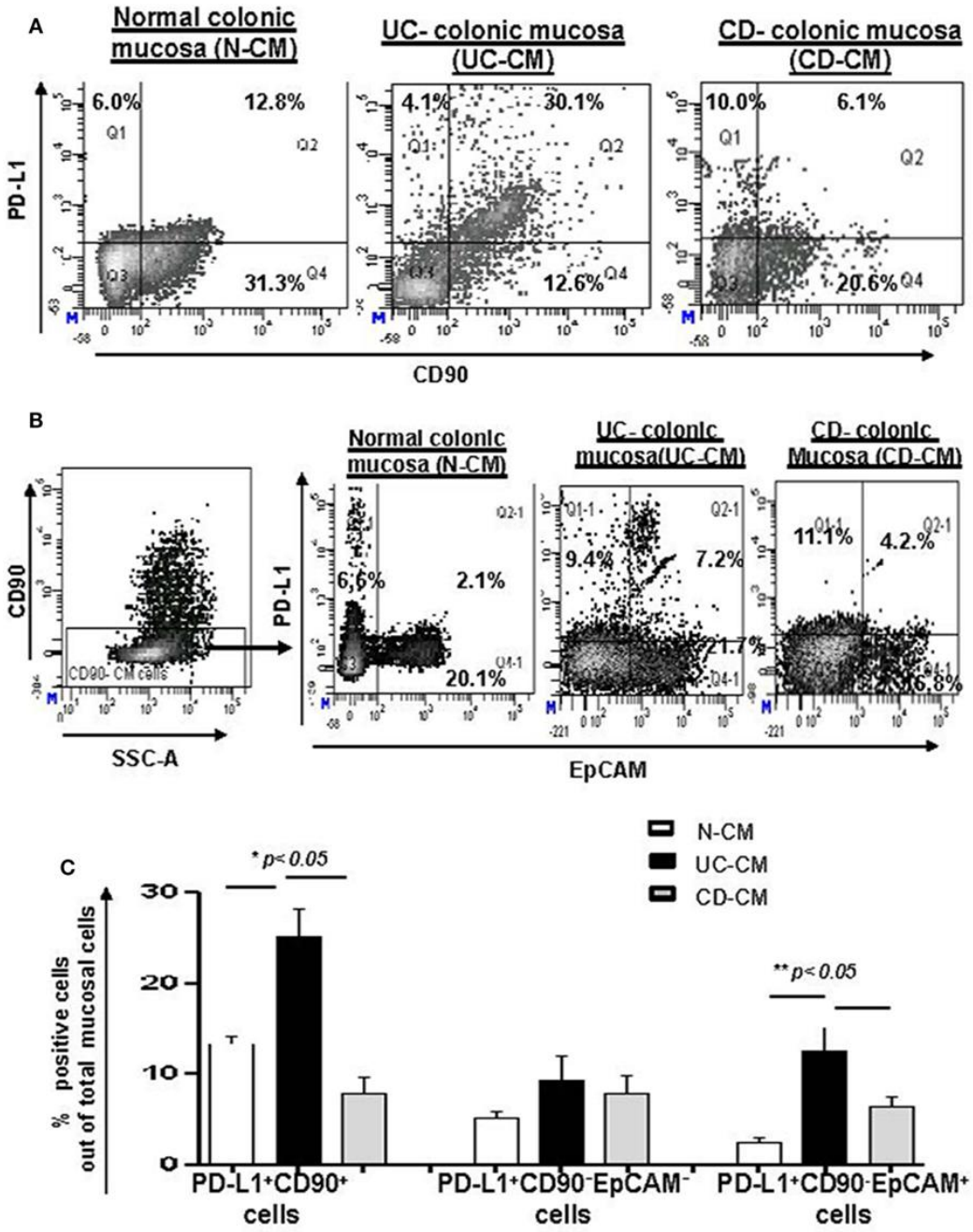


Figure 6. CMFs have increased expression of PD-L1 in UC but decreased expression in CD.

The number of programmed death-ligand 1 (PD-L1) expressing CD90+ colonic stromal cells (CMFs) is increased in ulcerative colitis (UC), but decreased in Crohn's disease (CD) colitis. Freshly digested Normal (N), UC, and CD colonic mucosal single cell suspensions (CM) were prepared and immunostained and analyzed by multi-color flow cytometry. Live events were gated and mucosal cells (CMs) were analyzed for the PD-L1 expression. (A) A representative density plot for the flow cytometry analysis for the expression of PD-L1 by CD90+ CMFs. (B) A representative density plot for the flow cytometry analysis for the expression of PD-L1 by EPCAM+ cells out of CD90− colonic

mucosal (CD90− CM) cells. (C) Summary of the PD-L1 expression by CD90+ EpCAM− cells (CMFs) CD90− EpCAM+ cells (epithelial cells) and CD90−EpCAM− professional immune cells in normal, UC-CM, and CD-CM preparations (flow cytometry analysis). Values are expressed as a mean of percentage positive cells ± SD, n = 9 per group. [47].

These data confirms a strong association between the expression of PD-L1 by CMFs and the specific type of IBD. Therefore, the objective of the investigation described in this chapter was to test the hypothesis **that abnormal expression of PD-L1 on IBD-derived CMFs contributes to the dysregulation of T helper cell responses in Inflammatory Bowel Disease.**

Results & Discussion

Changes in PD-L1 Expression Modify IBD-CMF-Mediated Suppression of $CD4^+$ T Cell Proliferation

To begin testing this hypothesis, allogenic CMFs derived from normal or IBD tissue were co-cultured with pre-activated $CD4^+$ T cells. All appropriate controls were included in these experiments, and CMF co-culture were maintained up to five days. CMFs have also been determined to maintain their PD-L1 phenotype and activity during extended passages *in situ [45, 80]*. In certain experimental subgroups, anti-PD-L1 blocking antibody or murine IgG1 isotype control was also added to the co-culture. Because Il-2 is a cytokine required for T cell proliferation, we then measured the IL-2 cytokine production as a surrogate for T cell proliferation in the co-cultures. We first confirmed that CMF cultures shared similar PD-L1 expression profiles with our previous data and confirmed that PD-L1 mRNA expression by CMFs was significantly increased in UC-CMFs and decreased in CD-CMFs (Figure 7A-B). This finding was then confirmed via

immunostaining and flow cytometry analysis. We also confirmed that PD-L2 showed no significant differential expression between Normal and IBD samples (Figure 7C). To determine if the changes in PD-L1 expression in IBD-CMFs could alter the proliferation of $CD4^+$ T cells, the singleplex IL-2 cytokine analysis was carried out to quantify levels of IL-2, a potent T cell growth factor, secretion by CMFs in the presence of anti-PD-L1 blocking antibody or controls [112]. It was observed that when PD-L1 was blocked by anti-PD-L1 antibodies in N-CMFs and UC-CMFs, there was a significant increase in IL-2 secretion. Thus, Normal and UC-CMFs exert suppression of IL-2 production in PD-L1 dependent manner.

In contrast, use of PD-L1 blocking antibodies did not affect the IL-2 secretion observed when activated $CD4^+$ T cells were co-cultured with CD- CMFs; Thus CD-CMFs failed to significantly suppress production of IL-2 by activated T cells (Figure 7D) and this is likely due to the already low level of PD-L1 present on CD-CMFs.

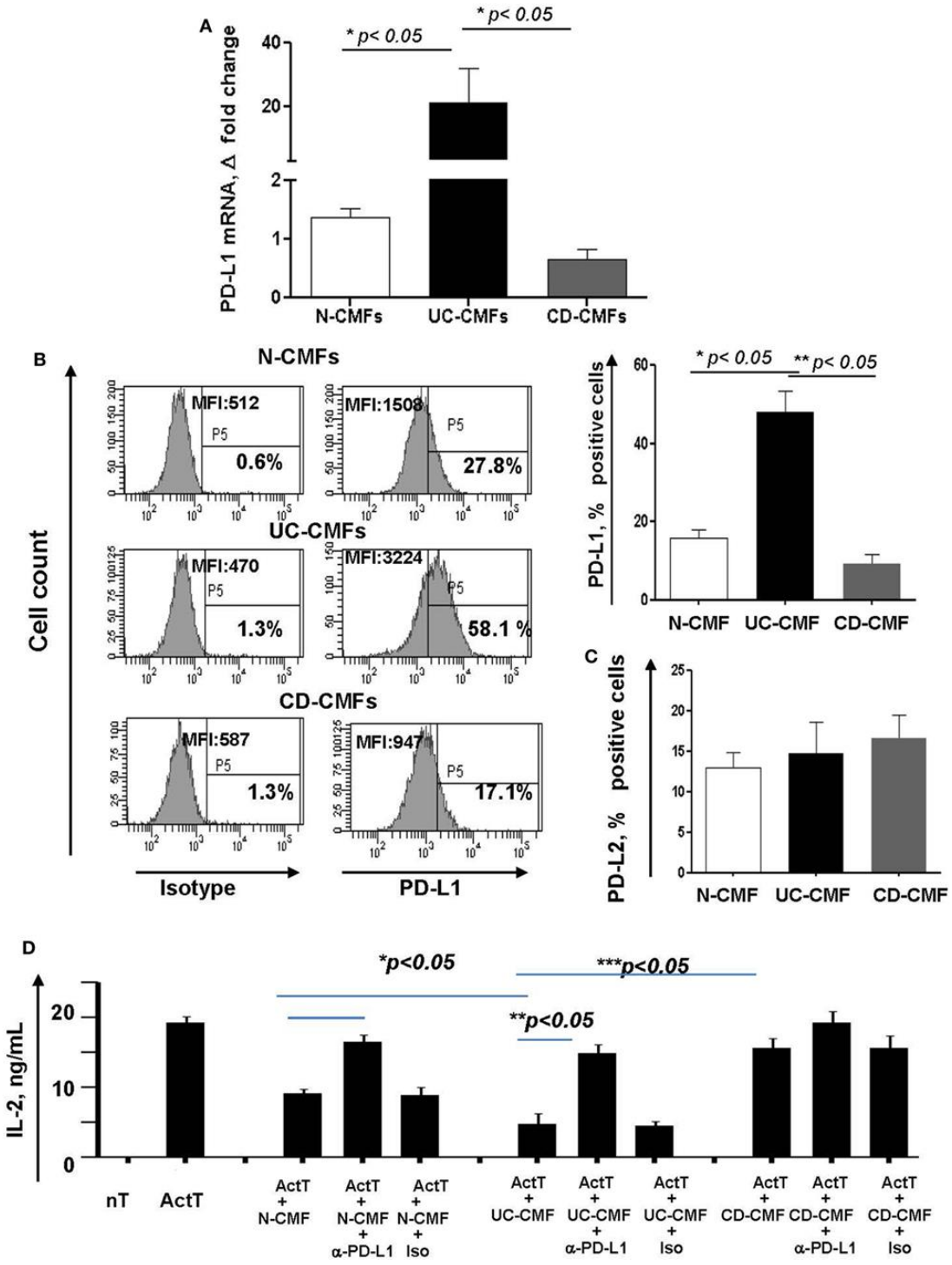


Figure 7. PD-L1 contributes to the immunosuppression of T cells in the Normal and UC colonic mucosa.

An increase in surface programmed death-ligand 1 (PD-L1) by UC-CMFs and a decrease by CD-CMFs modify cell-mediated suppression of IL-2 production byCD3-/CD28-activated CD4+ T cell IL-2 production. (A) PD-L1 mRNA (real-time RT-PCR analysis) and (B) surface protein (flow cytometry analysis) expression was increased in

6–7 days, 90–100% confluent primary cultures of UC-CMFs, but decreased in CD-CMFs when compared to N-CMFs. Representative flow cytometry histogram and summary of the surface PD-L1 expression shown on panel (B). The means ± SEM are shown, n = 8 per group. (C) PD-L2 surface expression on primary UC-, CD-, and N-CMFs (flow cytometry analysis); the means ± SD are shown, n = 8 per group. (D) CMFs were cocultured with allogeneic CD3-/CD28-preactivated naive CD4+ T cells (Act T cells) at a ratio of 1:2.5 for 72 h in 24-well plates. PD-L1 blocking mAbs (clone MIH1) or isotype controls were added in the concentration 1 μg/ml. IL-2 production was analyzed using singleplex cytokine analysis. The means ± SD are shown, n = 5 allogeneic donor pairs per group, two experimental replicates each [47].

INTERFERON-GAMMA IS DIFFERENTIALLY EXPRESSED IN CD AND UC COLONS

As stated in Chapter 1 and notated in Fig 1, IFNγ is a major effector cytokine of the Type immune response, of which PD-L1 is a negative modulator. Therefore, it was vital to determine if the changes in PD-L1 expression being observed could be effecting IFNγ expression in the IBD colonic mucosa. Utilizing frozen tissue sections and confocal microscopy, we observed that IFNγ response was significantly decreased in UC tissue, indicative a suppressed Type 1 immune response (Figure 8). In contrast, IFNγ expression was significantly increased in CD tissue as compared to both normal and UC tissue [47]. Taken together, this data indicates that PD-L1 expression by CMFs plays a crucial role in regulating Type 1 immune responses in the human colonic mucosa.

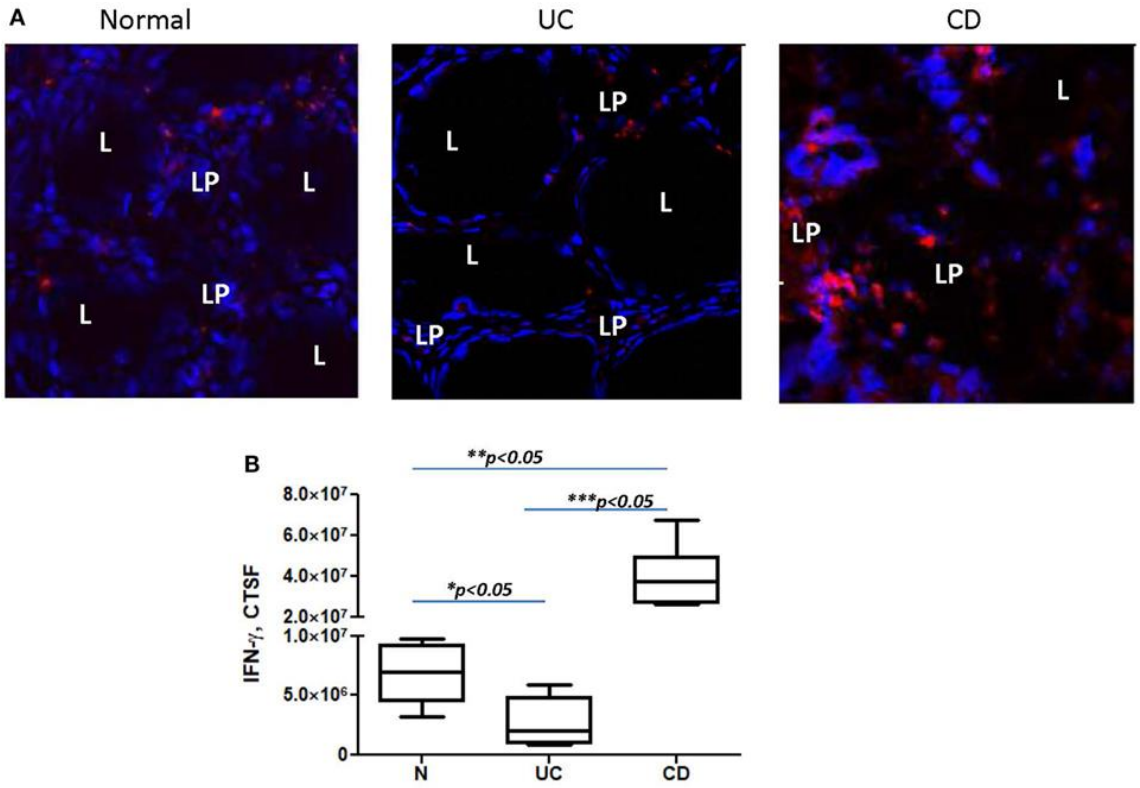


Figure 8. IFN-γ expression is decreased in the colonic mucosa of ulcerative colitis (UC) but increased in Crohn's disease (CD) when compared to normal controls. In these experiments, frozen colonic tissue sections were stained with DAPI to identify cell nuclei (blue) and anti-IFN-γ monoclonal antibodies (clone 4S.B3, in red) and analyzed by confocal microscopy, objective used is 63×. (A) Representative cross sections of UC, CD, and normal human colonic mucosa and (B) summary of fold changes in the corrected total cell fluorescence (CTCF) of PD-L1 expression in UC, CD, and normal human colonic mucosa (the means ± SEM are shown, n = 5 per group), L, lumen; LP, lamina propria. [47].

PD-L1 SUPPRESSES THE PRIMING OF TYPE 1 IMMUNE RESPONSES

We next sought to determine if PD-L1 is critical to the alteration of T cell responses by CMFs in the human IBD colonic mucosa. Normal or IBD-derived CMFs were co-cultured with pre-activated $CD4^+$ T cells for five days. The T cells were separated from co-cultures, and real-time RT-PCR and flow cytometry analysis were performed, and conditioned media was harvested for cytokine analysis. We observed that priming of

activated T cells with normal or UC, but not CD-derived CMFs suppressed mRNA expression of Th1 transcriptional factor Tbet and the cytokine IFNγ in these T cells (Figure 9A).

Similar data were obtained on protein level (flow cytometry and single plex cytokine analysis): T cells had decreased expression of Tbet (Figure 9B), and decreased co-expression with Type 1 effector cytokine IFNγ when co-cultured with UC-CMFs (Figure 9C). Alternatively, both expression profiles are increased when the activated T cells are co-cultured with CD-CMFs. [46]. These data suggest that CMFs in UC tissue contributes to the overt with suppression of Th1 responses as previously reported. This is in contrast to CD-CMFs, which failed to suppress Th1 responses.

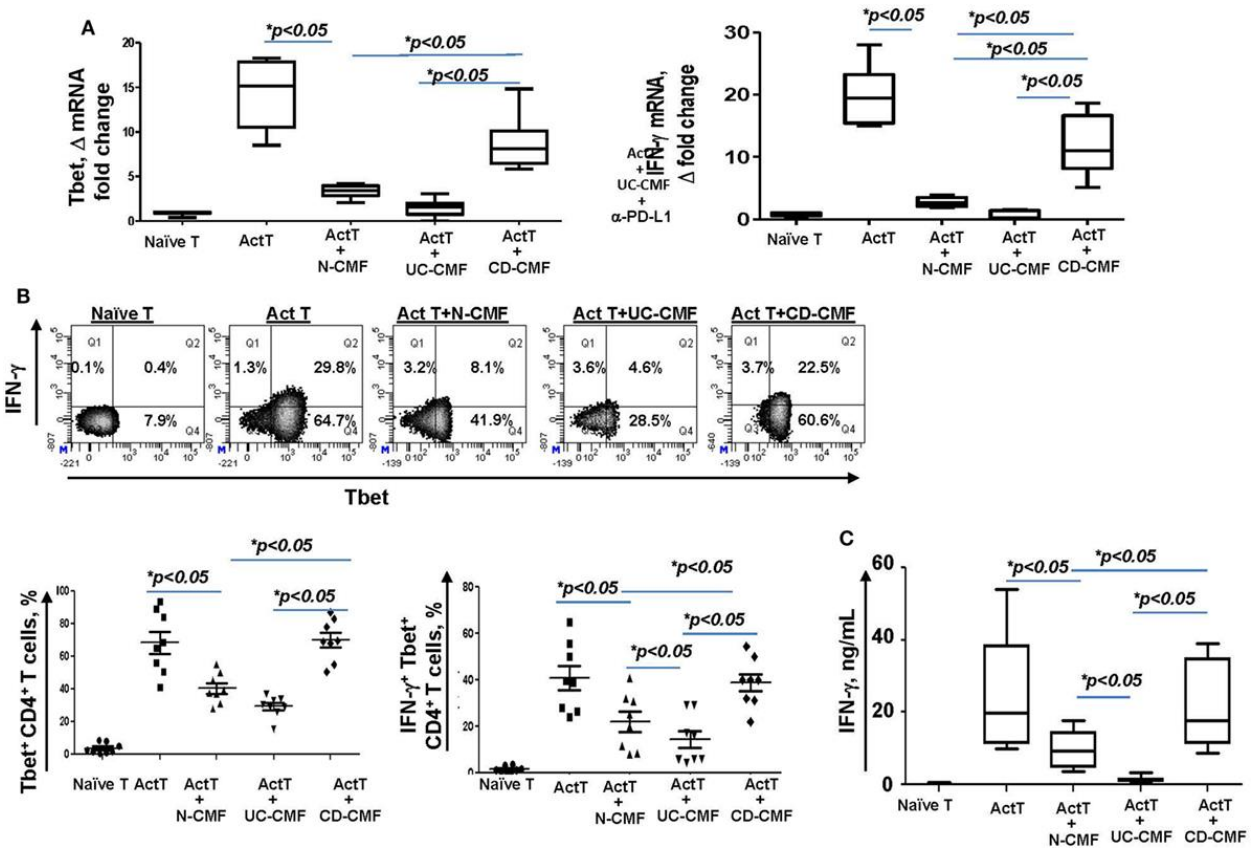


Figure 9. Priming of Th1 cells and Type 1 immune responses are significantly suppressed by PD-L1^{normal} N-CMFs and PD-L1^{high} UC-CMFs [11, 46].

UC-CMFs demonstrate strong suppression of Th1 type responses in the CD3/CD28-activated CD4+ T cells, while CD-CMF-mediated suppression was significantly reduced. (A) Tbet and IFN-γ mRNA expression by the T cells was analyzed using real-time RT-PCR analysis. The means ± SEM are shown, n = 4 allogeneic donor pairs per group, two experimental replicates each. (B) The percentage of the T cells expressing Th1 transcription factor Tbet and the Th1 cytokine IFN-γ was analyzed using intracellular immunostaining for flow cytometry. The means ± SED are shown, n = 8 allogeneic donor pairs per group. (C) IFN-γ production was analyzed using singleplex cytokine analysis. The means ± SD are shown, n = 8 allogeneic donor pairs per group, two experimental replicates each.

PD-L1, BUT NOT PD-L2, IS RESPONSIBLE FOR DECREASED TYPE 1 IMMUNE RESPONSE WHEN EXPRESSED BY CMFS.

Since activated T cells are capable of expressing low levels of PD-L1, we then confirmed the primary role of CMFs using PD-L1 specific siRNA (Figure 10). A contribution of PD-

L2 to the suppression of the Th1 type responses was previously observed by others in the animal models of airway hyper reactivity and in infectious diseases. Because both Normal and IBD-derived CMFs expressed basal levels of PD-L2 and PD-L1, and to determine the relative contribution of either ligand to the CMF-mediated suppression of Th1 immune response, experiments were included in which PD-L2 was silenced using specific siRNA. Since silencing of PD-L2 in CMFs did not significantly change the CMF-mediated suppression of the IFN-γ^{+}Tbet^{+} cells (Figure 10 A-B), we concluded that CMF-mediated suppression of the Th1 type responses was specific to PD-L1, but not PD-L2. [46]. This data demonstrates that PD-L1 expression by CMFs is key molecule in the suppression of Th1 cell proliferation and type 1 immune response in the normal and UC colonic mucosa, and its decrease in CD likely contributes to the increase in type 1 immune responses.

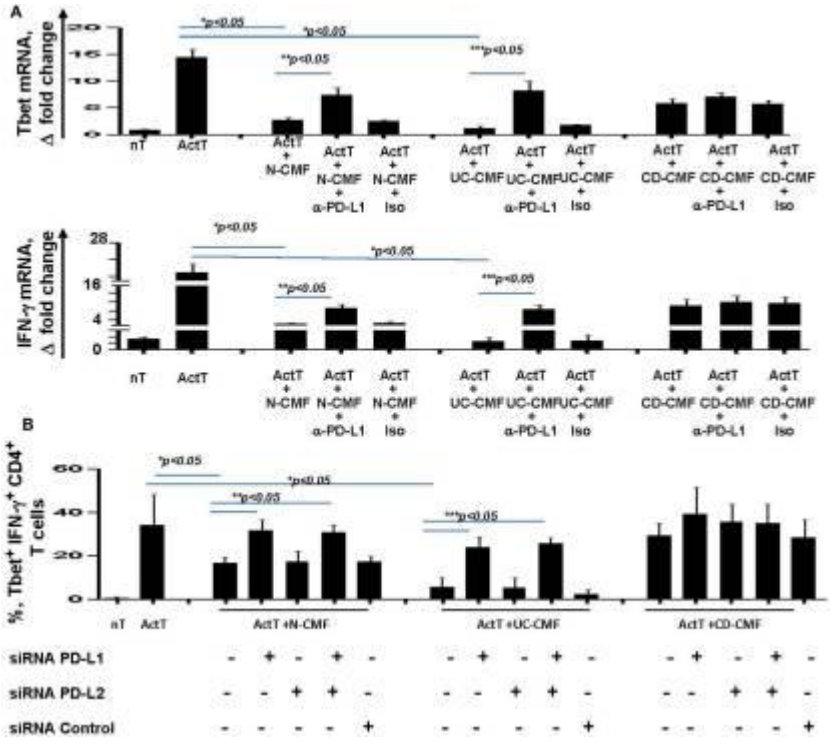


Figure 10. PD-L1, but not PD-L2, expression by CMFs suppresses Th1 cell proliferation and type 1 immune response in Normal and UC-CMFs.

CMF-mediated suppression of Th1 type responses depend on the level of expression of the programmed death-ligand 1 (PD-L1), but not PD-L2. (A) N-, UC-, and CD-CMFs were cocultured with allogeneic CD3/CD28-activated naive CD4+ T cells in presence/absence of PD-L1 blocking monoclonal antibodies (clone MIHI) or isotype control. Tbet and IFN-γ mRNA expression by the T cells was analyzed using real-time RT-PCR analysis. The means ± SEM are shown, n = 4 allogeneic donor pairs per group, two experimental replicates each. (B) N-, UC-, and CD-CMFs transfected or not with small interfering RNA (siRNA) specific to PD-L1, PD-L2, or siRNA control were cocultured with allogeneic CD3/CD28-preactivated naive CD4+ T cells. The percentage of the T cells expressing Tbet and IFN-γ was analyzed using intracellular immunostaining followed by flow cytometry. The means ± SD are shown, n = 4 allogeneic donor pairs per group, two experimental replicates each.

RESTORING PD-L1 ON CD-CMFS PARTIALLY RESTORES SUPPRESSION OF TYPE 1 IMMUNE RESPONSE

As pointed out in Chapter 1 and above, Crohn's Disease is exemplified by high and imbalance Type 1 immune response. I have also described how CMFs modulate Type 1

immune responses via PD-L1 expression, which is significantly blunted on the surface of CD-CMFs. Due to the low expression of PD-L1 observed on the surface of CD-CMFs, it remained unclear if CD-CMFs could suppress Type 1 immune responses if PD-L1 expression was restored. Since PD-L1 hIgG1 chimera protein (PD-L1Fc) may be able to bind to free Fc receptors on the surface of CD-CMFs, we investigated if the attachment of this PD-L1 chimera could produce a rescue effect on the ability for CD-CMFs to suppress Type 1 immune response. IgGFc antibody was utilized as a control. Treatment of CD-CMFs with the chimera partially restored their ability to suppress Type 1 immune response via decreased Tbet expression and IFNγ production when compared to controls (Figure 11A). These data indicate that the lack of PD-L1 expression by CD-CMFs plays a critical role in the failure of these cells to be able to effectively suppress Type 1 immune responses.

Finally, we examined the effect of CMFs on T cells polarized toward a Th1 phenotype. In these experiments, levels of the Th1 transcriptional factor Tbet and the Th2 transcriptional factor GATA2 was used to assess the efficiency of the naïve $CD4^+$ T cells polarization toward Th1 or Th2. When compared to N-CMFs, UC-CMFs strongly suppressed the number of $Tbet^+GATA2^-$ Th1 cells. In contrast, CD-CMFs did not suppress Th1 cells (Figure 11B). The Normal- and UC- CMFs-mediated suppression of Tbet expression in Th1 cells was partially reversed when anti-PD-L1 blocking mAbs were added to the cocultures (Figure 11B).

Taken together, these data suggest that increased PD-L1 expression by CMFs in UC very likely contributes to the overt suppression of a Th1 type response. In contrast, downregulation of PD-L1 in CD-CMFs is likely to be an important contributor to the pathological increase in the inflammatory Th1 type responses observed in CD colonic mucosa.

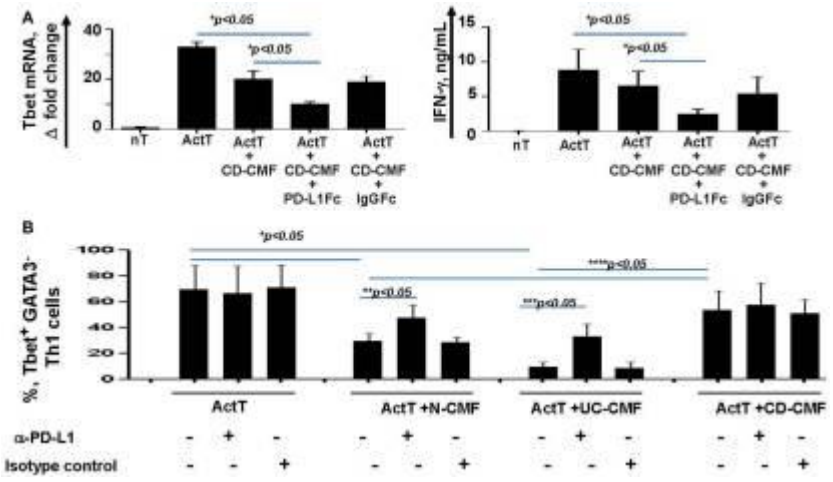


Figure 11. CD-CMFs treated with PD-L1 hIgG1 fusion protein were able to partially suppress Type 1 immune responses [47].

(A) CD-CMFs were cocultured with allogeneic CD3/CD28-preactivated naive $CD4^+$ T cells in presence/absence of the PD-L1Fc or IgG Fc control. IFN-γ expression (real-time RT-PCR) and secretion (singleplex cytokine analysis) were analyzed. The means ± SEM are shown, $n = 3$ allogeneic donor pairs per group, two experimental replicates each. **(B)** Th1 cells were differentiated from $CD4^+$ naïve T cells and cocultured with N-, UC-, and CD-CMFs in presence/absence of the PD-L1 blocking monoclonal antibodies or isotype control. Percentage of the T cells expressing the Th1 transcription factor Tbet was analyzed by flow cytometry. The means ± SED are shown, $n = 7$ allogeneic donor pairs per group.

PD-L1 EXPRESSION SUPPRESSES TYPE 17 IMMUNE RESPONSE

PD-L1 has also been demonstrated to contribute to Th17 responses in the intestinal stroma [75]. To investigate the role of PD-L1 in Th17 responses, we conducted T cell co-culture experiments with normal and CD-CMFs and treated the co-cultures with anti-PD-L1 blocking antibody or isotype control. Results were analyzed via flow cytometry analysis. We observed that N-CMFs decreased Th17 cells activity when co-cultured with activated T cells. CD-CMFs did not suppress Th17 activity. Blocking with anti-PD-L1, but

not isotype control, abrogated Th17 suppression in the N-CMF co-culture and caused no significant alteration in the CD-CMF co-culture (Figure 12). These data indicate that PD-L1 may also play a critical role in regulating Th17 responses in conjunction with Th1 responses.

Experiments with UC-CMFs co-culture are underway. Current data from our lab suggests that this altered Type 17 immune response may be due to cleavage of PD-L1 on CD-CMFs by CD-CMF-overexpressed Matrix Metalloproteinases- (MMPs) 7, 9 and 10. Expression of these MMPs are not observed to be upregulated in UC (Aguirre, et al. 2018 [under review]), therefore IL-17 responses should be decreased in UC, but they are elevated. It remains unclear why increased PD-L1 expression in UC does not contribute to a decreased Th17 response, especially since such has been demonstrated in other autoimmune disorders such as lupus [70]. Inflammatory responses can be highly complicated, and perhaps it is inducers of NFκB such as IL-6 that primes Th17 responses in UC. Regulation of Th17 transcription factor RORγτ has been shown to occur via both dependent and independent phosphorylation of IKKα [71].

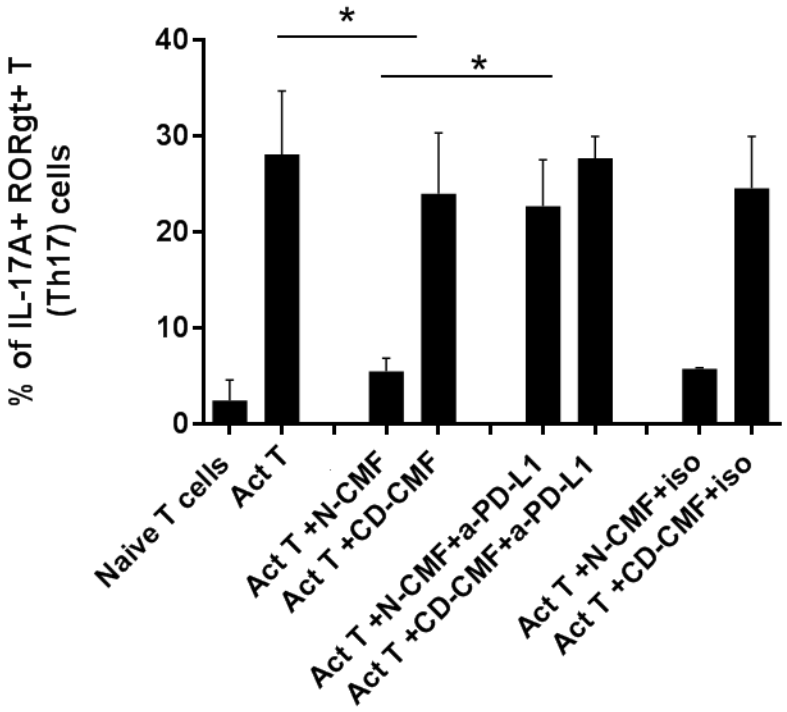


Figure 12. Low PD-L1 expression on CD-CMFs fails to suppress Th17 immune response in the normal human colonic mucosa.

CMFs were isolated from normal and CD patients and then co-cultured with Activated T cells and treated with an anti-PD-L1 antibody or isotype control. Naïve T cells we cultured alone as controls. Normal CMFs had increased production of Th17 effector cytokine IL-17a and transcription factor RORγT. When PD-L1 was blocked. PD-L1 blocking antibody did not affect the Th17 population in CD co-cultures. Data are expressed as mean ± SEM, $n = 4$ allogeneic pair per group, $^{*}p < 0.05$.

SUMMARY

The work in Chapter 2 highlights the importance of PD-L1 and its capability to suppress Type 1 immune responses. PD-L1, but not PD-L2 mRNA is upregulated in IBD, suggesting that PD-L1 is the primary PD-1 ligand that plays a role in the immune response in IBD. The increased PD-L1 expression in UC is associated with decreased Type 1 effector cytokine IFNγ in the colonic mucosa *in situ*. Conversely, IFNγ expression is increased in CD tissue while PD-L1 is decreased. This PD-L1 expression is explicitly

localized in CMFs, identifying these abundant subepithelial cells as a significant source of PD-L1 in the colonic mucosa in both health and disease. T cells are identified as the effector cells of IBD immunopathogenesis, and it has been considered by most investigators in the field that T cells and other professional immune cells, but not CMFs, are the significant effector cells in IBD. Our research has demonstrated through PD-L1-signaling CMFs contribute to the regulation of T cell responses via suppression of IL-2 and the Type 1 cytokine IFNɣ in the Normal and UC colonic mucosa . Since PD-L1 is decreased in CD, it is not surprising that PD-L1 blocking antibodies had no significant effect on IL-2 expression and fail to suppress type 1 immune responses. PD-L2 did not facilitate any significant alterations in Type 1 cell response, allowing for the conclusion that PD-L1, and not PD-L2 is the primary PD ligand that alters Type 1responses in both normal and IBD colon. While PD-L1 function in the regulation of Th1 responses is more explored here, our data has shown that PD-L1 also suppresses Th17 responses in the normal human colonic mucosa, and its low level in CD may contribute to the increase of Th17 cell activity.

Taken together, our current work regarding the role of PD-L1 expression by CMFs **has demonstrated** that PD-L1, but not PD-L2, expression was upregulated in the inflamed mucosa from UC and downregulated in CD when compared to the non-involved matched mucosa and healthy control tissue. Increased PD-L1 expression likely contributes to the persistent Type 2 inflammatory responses in the UC colonic mucosa at least by overt suppression of type 1 immune responses while low PD-L1 expression contributes to Type 1 inflammatory responses in CD colitis. $CD90^{+}$ mesenchymal cells (CMFs), and not epithelial or other lamina propria innate immune cells, are the dominant cell phenotype associated with differential expression of PD-L1 in the two major forms of

IBD. Furthermore, change in PD-L1 expression contribute to the alteration in BD-CMF-mediated regulation of Th1 and Th17 responses.

CHAPTER 3: THE CONTRIBUTION OF MESENCHYMAL STROMAL PROGENITOR CELLS TO THE GENERATION OF ALTERED CMFS IN INFLAMMATORY BOWEL DISEASE

Preliminary data, aims, and hypothesis

The data from Chapter 2 has elucidated the changes in CMF-PD-L1 in IBD patients, and the crucial role played by PD-L1 expression by CMFs in altering the colonic inflammatory milieu in these diseases. However, there has to date been sparse investigation into the mechanism whereby the CMF PD-L1 phenotype and activity are altered in IBD. As described in the introduction, MSCs and multipotent progenitors have been demonstrated to be highly clonogenic and capable of differentiating into multiple cell lineages in other tissues [113-116]. MSCs are the progenitors of CMFs and have been identified in both small intestine and colonic lamina propria in both human and murine tissue [66, 68, 78]. However, as stated in Chapter 1, a **gap of knowledge** remains regarding how CMFs develop aberrant PD-L1 expression in diseases such as IBD. Consequentially, the contribution of MSCs that are precursor cells to CMFs to the generation of pathogenic IBD-CMFs with altered PD-L1 expression is unknown.

MARKER OF STEMNESS OCT4 IS INCREASED IN THE IBD COLONIC MUCOSA

The Oct4 expression of cells has been determined to be tightly regulated at all stages of cell life, is vital to induction and maintenance of stem cell potency, and PD-L1 has been observed to increase Oct4 in cancer tissue [28, 30, 117, 118]. Since the classical stem cell marker Oct4 was observed to be increased in the blood and whole colonic tissue of IBD patients, we first sought to confirm the presence of Oct4 in the human colonic mucosa [63]. We observed a significant increase in the mRNA expression of Oct 4 in tissue samples from both UC and CD patients when matched to non-involved control

tissue (Figure 13). Protein expression of the classical stem cell marker Oct4 in the colonic mucosa of UC but not CD patients were significantly increased when compared to normal controls (Figure 13, right).

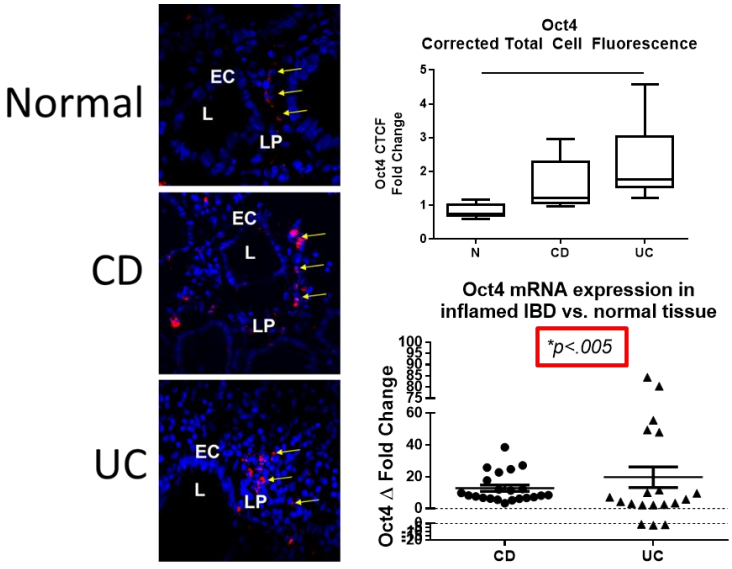


Figure 13. Stemness marker Oct4 is increased in the IBD colonic mucosa.

Normal and IBD tissue sections were stained with DAPI (blue) and Oct4 (red) and analyzed via confocal microscopy (A). Both CD and UC sections were determined to have significantly increased Oct 4 expression via Corrected Total Cell Fluorescence (CTCF) analysis (CTCF= Int. Density*Area-Background) (B). Human tissue from normal and IBD patients were analyzed from inflamed tissue of CD and UC patients and normalized to control tissue from those same patients. Oct4 was found to be significantly increased in both CD and UC patients (C). Data were expressed as means ± SEM of percentage of the changes in the count of CTCF of $Oct4^{+}$ cells, n =11 per group, $*p < 0.05$.

This observation, together with the fact the mesenchymal stem cells are major progenitor of CMFs, led me to the hypothesis that **Aberrant differentiation of MSCs in the IBD inflammatory milieu is the critical event in the generation of CMFs with abnormal expression of PD-L1.** Exploration of this hypothesis is the objective of this chapter and the data described in the Result section supports this hypothesis.

Results and Discussion.

IBD-MSCs HAVE DECREASED CLONOGENIC CAPACITY.

Clonogenicity, or the ability to proliferate even at lower seeding densities in *vitro* culture, is one of the major properties of MSCs when compared to other differentiated cells of mesenchymal origin. Because my laboratory has successfully isolated and cultured CMF progeny from human colonic tissue in the past, I isolated MSCs from normal and IBD tissue using proprietary stem cell culture media and compared their clonogenic capacity to commercially obtained bone marrow-derived MSCs, which constitute an accepted MSC control. As shown in Figure 14A, when compared to Bone Marrow-derived MStCs, normal colonic MSCs (N-MSCs) had less clonogenic capacity. UC-MSCs clonogenic capacity was further diminished when compared to BM-MSCs and N-MSCs. MSC-like cells isolated from CD tissue did not show any significant proliferative results in the clonogenic assay. My scoring system for this assay reflected these results, with CD-MSCs having impressively significantly less clonogenic potential than N- or UC-MSCs (Figure 14B)

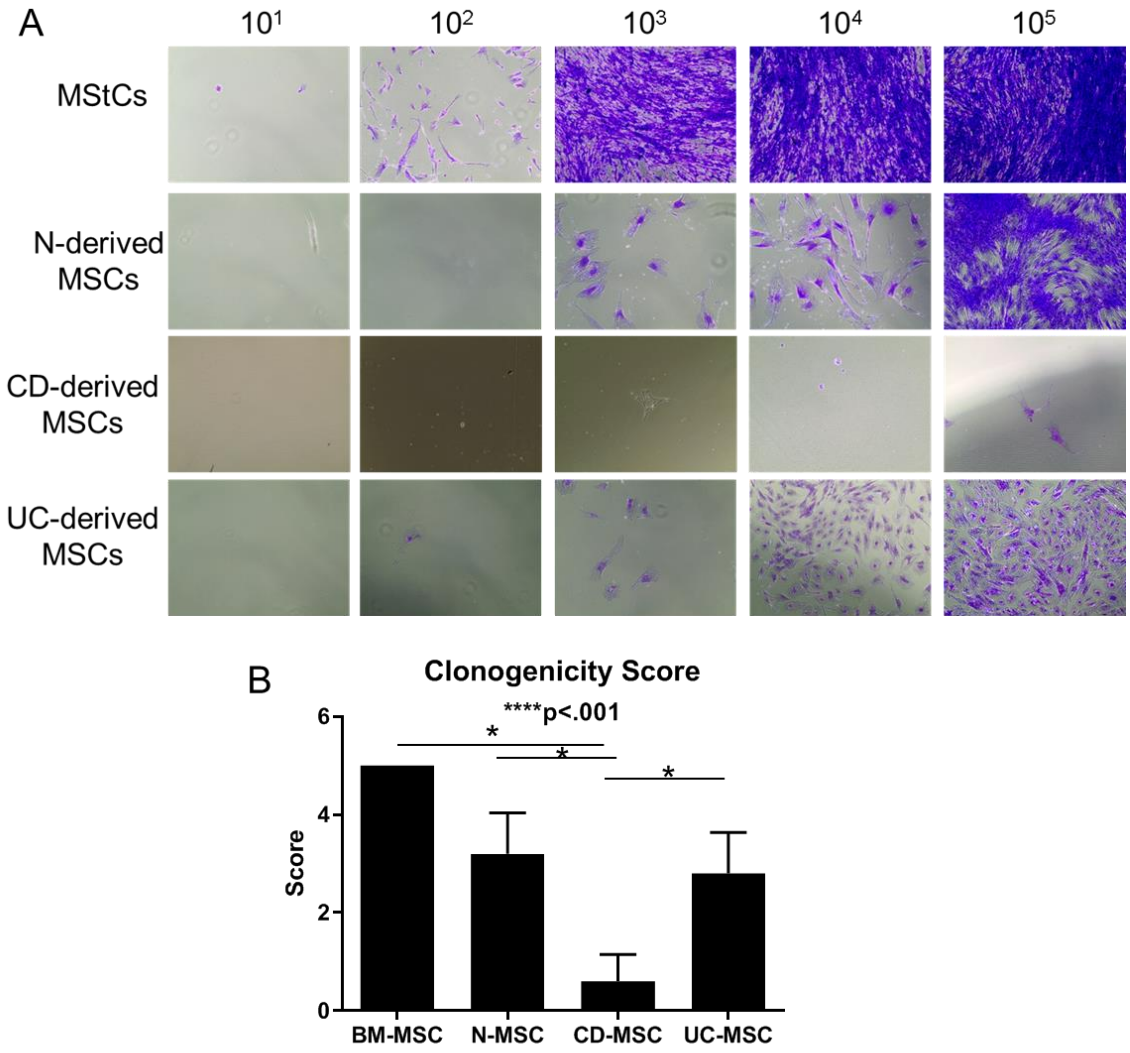


Figure 14. IBD-MSCs have decreased clonogenic capacity.

(A) MSCs isolated from normal, and IBD colons were plated in stem cell media at seeding densities 10^1-10^5 and stained with crystal violet for contrast. Bone Marrow-derived MStCs had the highest clonogenic capacity, with N-MSCs having slightly reduced capacity. UC-MSCs had no significant reduction in clonogenicity compared to N-MSCs, while CD-MSCs were not able to survive and colonize except at the highest seeding density. (B) Summary graph of the clonogenic scoring system. Wells were awarded 2 points for survival and confluency, 1 point for survival, and 0 points for no survival. MStCs had the highest clonogenic score. N- and UC-MSCs had similar clonogenic scores, and CD-MSCs had significantly lower clonogenic scores than all other MSCs. Data are shown as mean ±SEM; *n*=3 for MStCs and N-MSCs and 5 for CD- and UC-MSCs.

MSCS ISOLATED FROM THE COLONS OF IBD PATIENTS HAVE DECREASED DIFFERENTIATION CAPACITY.

Another unique property of stromal progenitor cells is the capacity to give rise to different mesenchymal cells, particularly adipocytes, chondrocytes, and osteoclasts. We, therefore, carried out differentiation experiments by plating normal-, UC- and CD-derived MSCs in special media designed to drive differentiation of the stem cells into bone, cartilage or fat cells. Results were compared to BM-MStCs (Figure 15A). We observed that when compared to the BM-MStCs, N-MSCs preserved their capacity to differentiate into osteoclasts and chondrocytes but to a lesser extent into adipocytes. IBD-derived CMFs completely lost their capacity to differentiate into adipocytes and osteoclasts. Furthermore, while UC-MSCs preserved their capacity to differentiate into chondrocytes, CD-MSCs capacity to differentiate into chondrocytes was reduced. Thus, the ability of CD-MSC s to differentiate was considerably less than both UC-MSCs and N-MSCs, while BM-MStCs exhibited significantly higher differentiation capacity than all the colonic derived MSCs. (Figure 15).

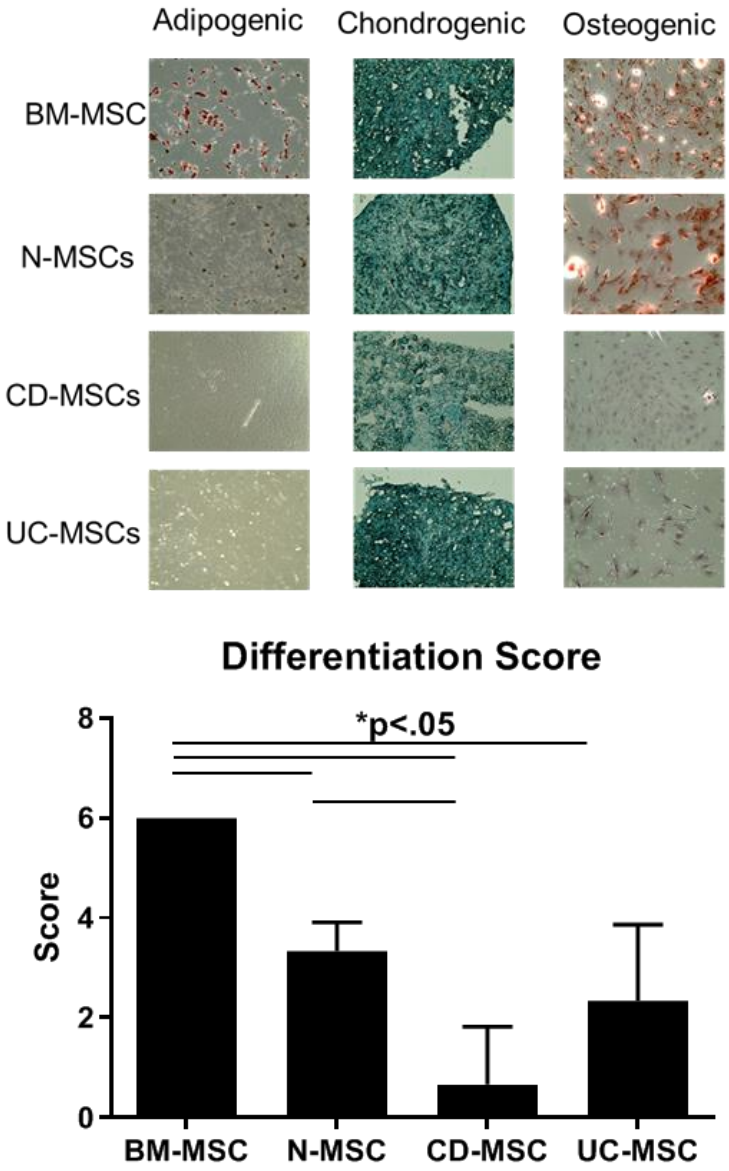


Figure 15. MSCs isolated from the colons of IBD patients have decreased differentiation capacity.

(A) MSCs isolated from normal, and IBD colons were plated in special differentiation media for adipocytes and osteocytes or pelleted in special media to grow chondrocytes. Wells were stained with Oil Red O to resolve adipocytes, Alizarin red to resolve osteocytes, or Alcian Blue to identify chondrogenesis. Bone Marrow-derived MStCs had the highest differentiation capacity, with N-MSCs having slightly reduced capacity. UC-MSCs had more reduction in differentiation capacity. CD-MSCs were unable to differentiate into osteocytes or adipocytes and had greatly decreased chondrogenesis. (B) Summary graph of the differentiation scoring system. Samples were awarded 2 points for survival and differentiation, 1 point for survival and limited or no differentiation,

and 0 points for no survival. MStCs had the highest clonogenic score. N- and UC-MSCs had similar clonogenic scores, and CD-MSCs had significantly lower clonogenic scores than MStCs and N-MSCs. Data are shown as mean ±SEM; *n*=3 for MStCs and N-MSCs and 5 for CD- and UC-MSCs.

EXPRESSION OF STEMNESS MARKER ALDH1A2 IS INCREASED IN THE IBD COLONIC MUCOSA

While the data from previous research indicates that both Bone Marrow-derived and tissue-resident MSCs contribute as CMF progenitors, the research in this area is sparse and contradictory [67-69]. Further, the few publications that investigate the presence of MSCs either focus solely on murine models of colitis or only utilize normal tissue samples. Due to the lack of foundational research on colonic MSCs, some characterization of the multipotent progenitors present in the normal and IBD colonic mucosa was required.

In addition to Oct 4, Aldehyde dehydrogenase 1A2 (ALDH1A2) has also been demonstrated to be a stem cell marker in the intestines of sheep, mice, and humans [73, 90-92]. We determined expression of ALDH1A2 by qRT-PCR and confocal microscopy as previously demonstrated with Oct4. Compared to normal colon, UC tissue had increased ALDH1A2 expression in confocal section, while CD did not (Figure 16A-B). However, both UC and CD tissue had increased mRNA expression when compared to matched controls from non-involved tissue (Figure 16C). Overall, we believe that this increase ALDH1A2 supports the concept that there is increased stemness of CMFs in UC and probably also in CD.

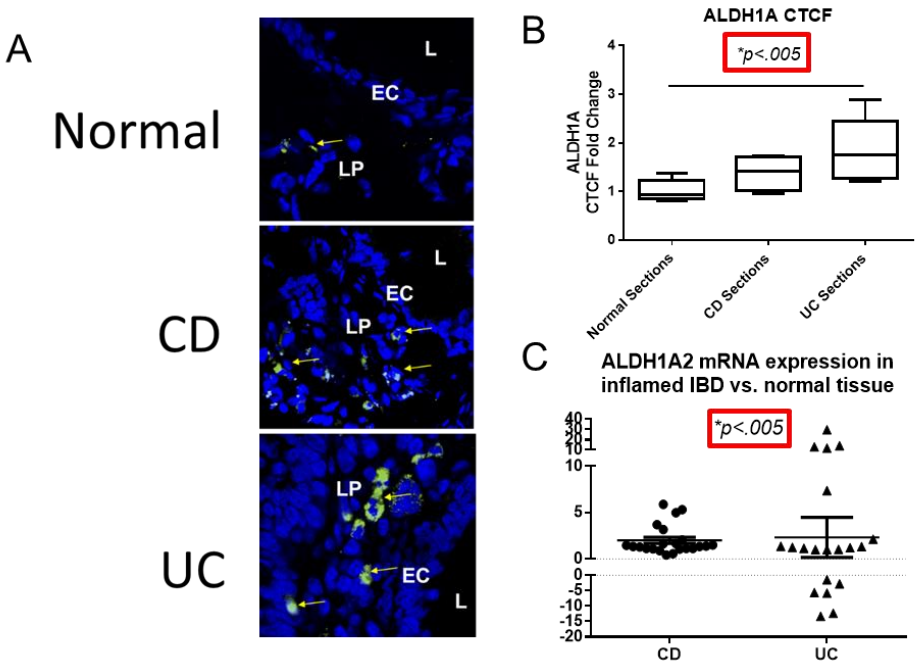


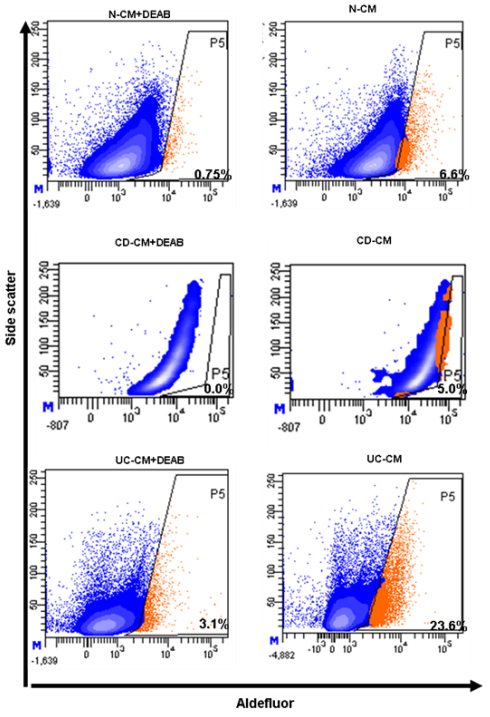
Figure 16. Stemness marker ALDH1A2 is increased in the IBD colonic mucosa.

(A) Normal and IBD tissue sections were stained with DAPI (blue) and ALDH1A2 (green) and analyzed via confocal microscopy (A). (B) Both CD and UC sections were determined to have significantly increased ALDH1A2 expression via Corrected Total Cell Fluorescence (CTCF) analysis (CTCF= Int. Density-Area*Background) (B). (C) Human tissue from normal and IBD patients were analyzed from inflamed tissue of CD and UC patients and normalized to control tissue from those same patients. ALDH1A2 was found to be significantly increased in both CD and UC patients. Data were expressed as mean ± SEM of CTCF of $ALDH1A2^{+}$ cells and ALDH1A2 mRNA expression; *n*= 6 per group for CTCF and 20 per group for mRNA expression.

ALDEHYDE DEHYDROGENASE ACTIVITY INCREASES IN UC BUT NOT CD OR NORMAL TISSUE.

Aldehyde dehydrogenases are enzymes that are vital to the synthesis of retinoic acid, which in turn has important immunomodulatory properties [119]. Dendritic cells have also been determined to utilize retinoic acid synthesis to imprint homing to T and B cells and promote the proliferation of tolerogenic Treg cells [120]. However, ALDH activity has also been identified as a marker of proliferation and differentiation regulation in non-immune tissues. Thus, ALDH enzymatic assay is a means to identify the activity of all

stem cell populations in a tissue [121]. However, the expression and roles of aldehyde dehydrogenase activity in MSCs of the normal and IBD human colonic mucosa remain sparsely investigated. In order to understand if ALDH activity was a viable identifier of MSCs and to determine if ALDH was differentially active in IBD, aldehyde dehydrogenase activity was quantified utilizing an enzymatic fluorescence assay and analyzed on dispersed whole tissue via flow cytometry (Figure 17). Our data demonstrate that ALDH expression is significantly increased in UC tissue, but not in Normal or CD tissue. Interestingly, these results vary from the activity of ALDH by professional immune cells present in the normal and IBD colon. Dendritic Cells from CD patients were determined to have similar ALDH activity as controls, while UC activity is decreased [122]. However, the role of professional immune cells and immunosuppressive cells such as MSCs vary significantly. Although not shown, a significant percentage of ALDH positive cells were determined to be CD90 positive.



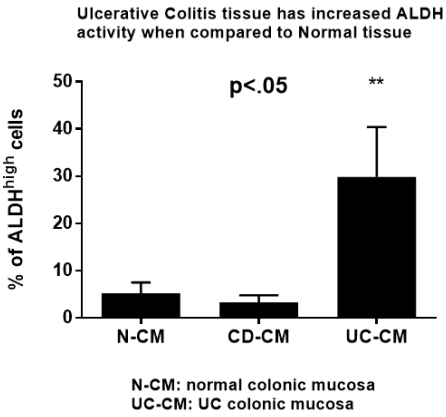


Figure 17. Aldehyde Dehydrogenase enzymatic activity is increased in UC but not CD or normal tissue.

Whole sections of colonic mucosa were isolated from healthy and IBD patients digested and placed in a cell suspension. The cells were then treated with the commercial Aldefluor assay to investigate ALDH activity via an enzymatic reaction. Flow cytometry and quantitative analysis determined that ALDH activity was increased in UC, but not CD or normal tissue. Data were provided as mean ± SEM of $ALDH^+$ cells; *n*=3 for N- and CD-CM and 5 for UC-CM.

MESENCHYMAL LINEAGE MARKER GREM1 IS UPREGULATED IN UC COLONIC MUCOSA

As mentioned previously, Gremlin 1 is a mesenchymal cell lineage marker that was determined to identify multipotent progenitors in the murine small intestine mucosa [68]. Therefore, we investigated the expression of Gremlin 1 in the IBD colonic mucosa. We observed a significant increase in Grem1 mRNA and protein level in IBD tissue as determined by qRT-PCR and confocal microscopy (Figure 18A-C). Unlike stemness markers, Oct4 and ALDH1A2, Grem1 protein and mRNA expression were upregulated in both CD and UC tissues *in situ* (Figure 18B), suggesting while cells of mesenchymal lineage may increase in both form of disease, the preservation of stemness properties of these cells occurs in UC, but not in CD colonic mucosa.

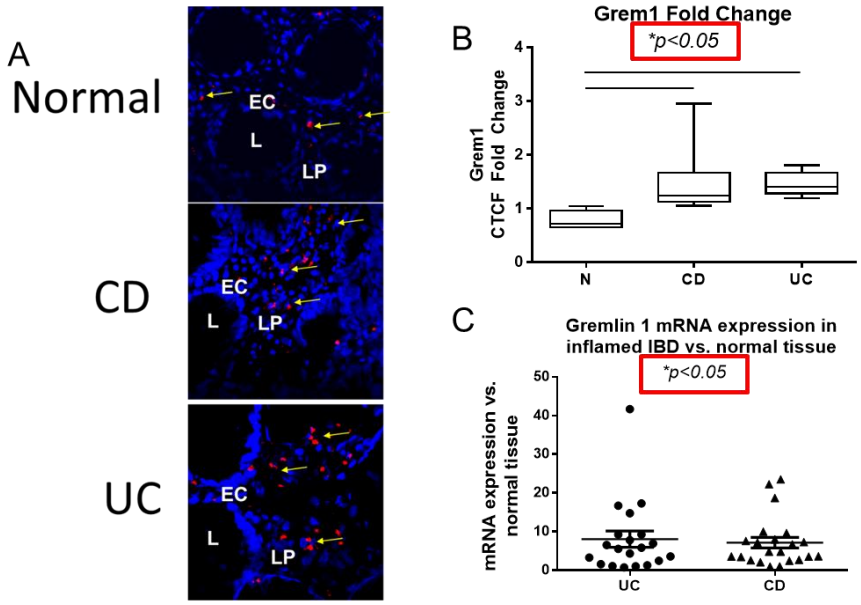


Figure 18. Stem cell lineage marker Gremlin 1 is increased in the IBD colonic mucosa.

(A) Normal and IBD tissue sections were stained with DAPI (blue) and Grem1 (red) and analyzed via confocal microscopy. (B) Both CD and UC sections were determined to have significantly increased ALDH1A2 expression via Corrected Total Cell Fluorescence (CTCF) analysis (CTCF= Int. Density-Area*Background). (C) Human tissue from normal and IBD patients were analyzed from inflamed tissue of CD and UC patients and normalized to control tissue from those same patients. Grem1 expression was found to be significantly increased in both CD and UC patients. Data were expressed as mean ± SEM of CTCF of $Grem1^+$ cells and Grem1 mRNA expression; *n*= 6 per group for CTCF and 20 per group for mRNA expression.

EXPRESSION OF STEMNESS MARKERS OCT4 AND ALDH1A2 ARE SIGNIFICANTLY CORRELATED WITH MESENCHYMAL POTENCY MARKER GREM1 IN UC, BUT NOT CD

Despite the illuminating findings regarding mRNA and protein expression of Oct4, ALDH1A2, and Grem1 in the human colonic mucosa, how these expression profiles fit together in the same tissue remained unclear. In order to determine the relationship between Oct4 and ALDH1A2 expression *in situ*, Pearson correlation analysis was performed. The analyses demonstrated that there is a significant positive association between the expression of ALDH1A2 and Oct4 in both CD and UC (Figure 19).

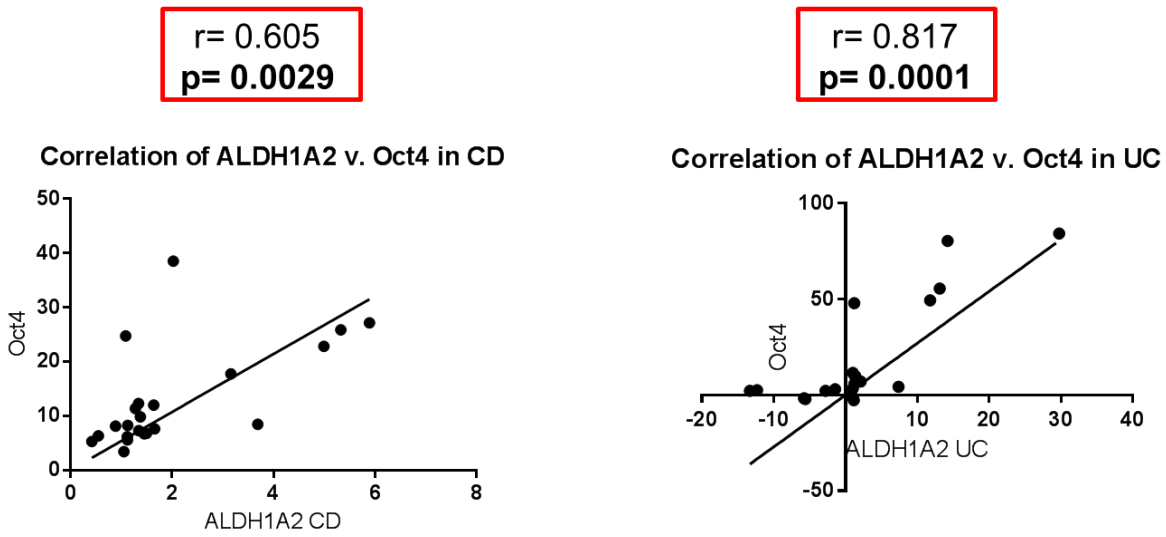


Figure 19. mRNA expression ALDH1A2 and Oct4 have a significant positive correlation in both UC and CD tissue.

Human tissue acquired from areas of active inflammation and non-involved control areas were attained from patients with CD and UC. mRNA expression of ALDH1A2 and Oct4 were then compared via Pearson correlation analysis. Correlation of ALDH1A2 and Oct4 from CD patients was significant and had a goodness of fit of .605. Correlation of ALDH1A2 and Oct4 from UC patients was significant and had a goodness of fit of .817. The linear regression trend line has been constrained to X=0, Y=0, *n*=20 for CD and 17 for UC.

Pearson correlation analysis demonstrated that there is a significant positive association between expression of stem cell marker ALDH1A2 and mesenchymal lineage marker Grem1 in UC, but not in CD, suggesting that at least in UC, there is an increase in $ALDH1A2^{+}$ MSCs population within the colonic mucosa (Figure 20).

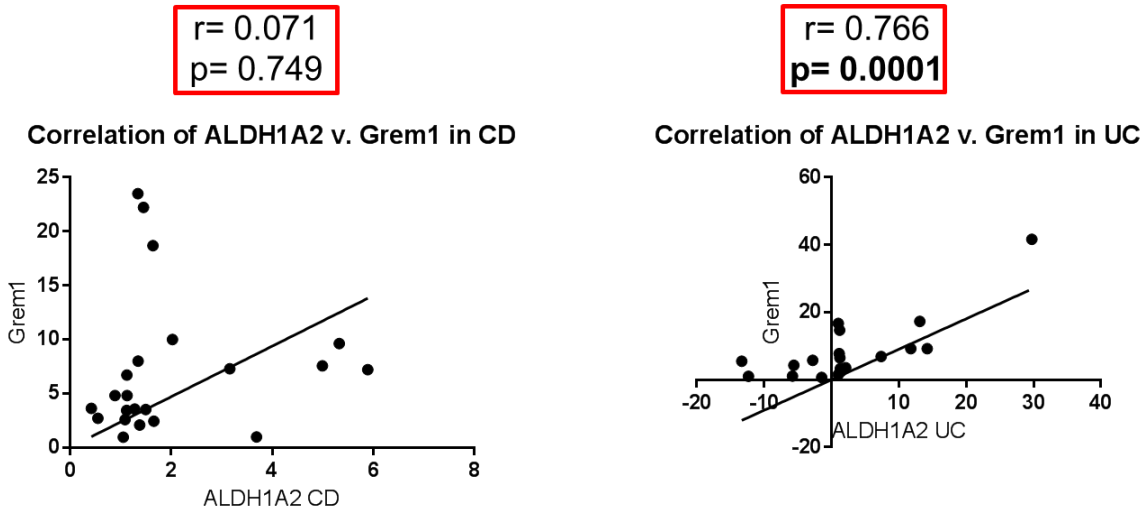


Figure 20. mRNA expression ALDH1A2 and Grem1 have a significant positive correlation in UC but not CD tissue.

Human tissue acquired from areas of active inflammation and non-involved control areas were attained from patients with CD and UC. mRNA expression of ALDH1A2 and Grem1 were then compared via Pearson correlation analysis. Correlation of ALDH1A2 and Grem1 from CD patients was not significant and had a goodness of fit of .071. Correlation of ALDH1A2 and Grem1 from UC patients was significant and had a goodness of fit of .766. The linear regression trend line has been constrained to X=0, Y=0, *n*=22 for CD and 18 for UC.

Surprisingly, despite the strong correlation between Oct4 and ALDH1A2 in both UC and CD as well as a significant correlation observed between Oct4 and Grem1 in both CD and UC (Figure 21), the correlation between ALDH1A2 and Grem 1 was not significant. This may indicate that there may be other processes that MSCs utilize ALDHIA2 for (e.g., retinoic acid metabolism) and these may be more altered in CD-MSCs than in UC-MSCs.

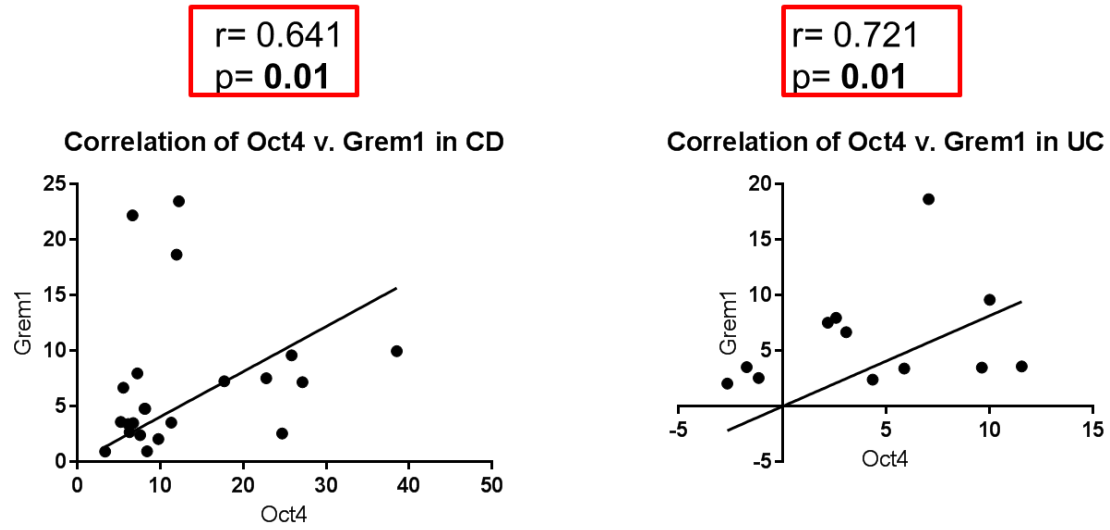


Figure 21. mRNA expression of Oct4 and Grem1 have a significant positive correlation in both UC and CD tissue.

Human tissue acquired from areas of active inflammation and non-involved control areas were attained from patients with CD and UC. mRNA expression of Oct4 and Grem1 were then compared via Pearson correlation analysis. Correlation of Oct4 and Grem1 from CD patients was significant and had goodness of fit of .641. Correlation of Oct4 and Grem1 from UC patients was significant and had goodness of fit of .721. The linear regression trend line has been constrained to X=0, Y=0, *n*=20 for CD and 12 for UC.

OCT4+/GREM1+ MSC LIKE CELLS ARE MORE PREVALENT IN UC AND LESS IN CD AND NORMAL TISSUE

Due to the sparse data regarding stromal progenitors in the human colonic mucosa, descriptive studies are necessary to accurately characterize MSCs in the IBD colonic mucosa. Therefore, we next performed co-immunostaining followed by confocal microscopy for the classical stem cell marker Oct4 and the mesenchymal lineage marker Gremlin 1. As shown in this representative section below in Figure 22, we observed a substantial increase in colocalization between Oct4 and Grem1 in UC and to a much lesser extent in CD. This data suggests that at least in the UC colonic mucosa, there is a substantial increase in the Mesenchymal Stem Cell-like cell population.

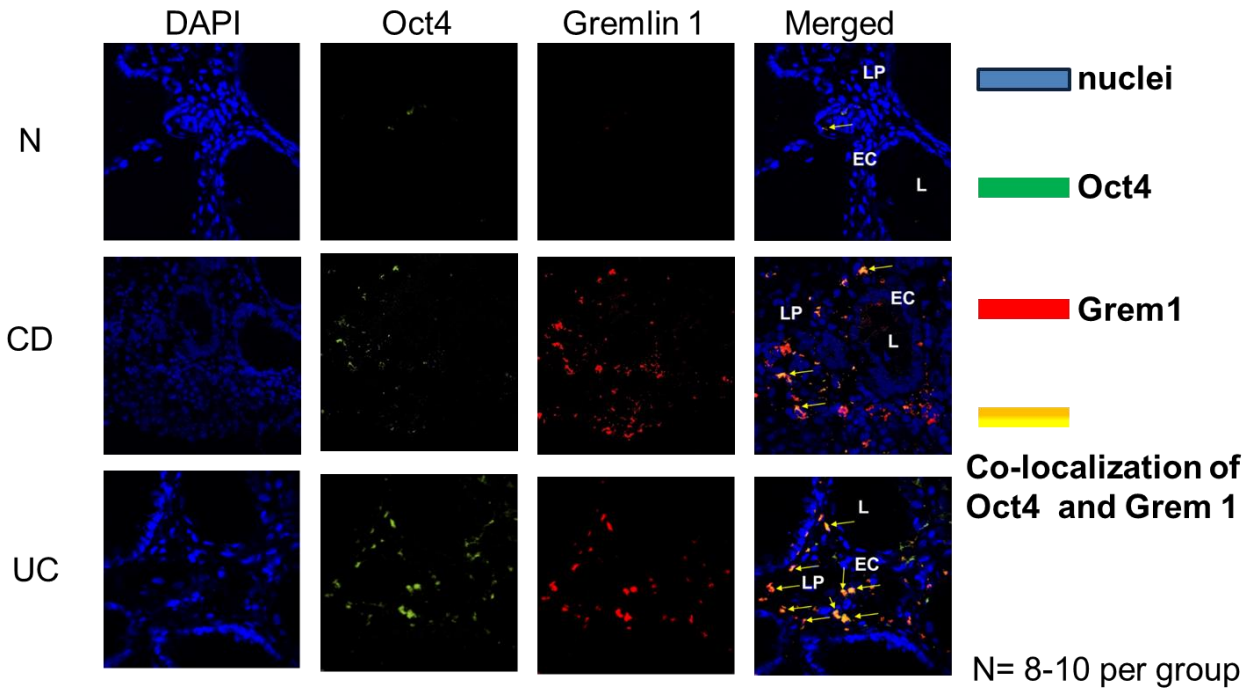


Figure 22. Oct4+/Grem1+ MSC like cells are more prevalent in UC and CD than normal tissue, and colocalization is decreased in CD compared to UC.

Normal and IBD tissue sections were stained with Oct4 (green), Grem1 (red), and DAPI (blue) (*n*=8-10 per group). Co-localization of Oct4 and Grem1 appear as a yellow/orange color. Increased expression of both Oct4 and Grem1 were observed in CD and UC tissue. However, there was less co-localization in CD tissue.

PROLIFERATION AND ABERRANT DIFFERENTIATION OF OCT4+GREM1+ MSC-LIKE CELLS IS STRONGLY INCREASED IN THE UC, BUT NOT IN CD COLONIC MUCOSA

Ki67 is a classical marker for the analysis of proliferation of cells in situ. Alpha Smooth muscle actin is a marker of differentiated fibroblasts (myofibroblasts) and, in the classical Mesenchymal Stromal progenitor Cells field, is used to differentiate activated fibroblasts from MSCs. It then follows that analysis of these markers would aid in further understanding the impacts of IBD on MSCs in the human colonic mucosa lamina propria. As shown on Figure 23 below, we observed that there was an increase in the proliferation of MSC-like cells in the UC colonic mucosa, when compared to the normal and CD colonic mucosa, as shown by the corrected total cell fluorescence graph shown

below the representative confocal sections. We also observed an increase of co-localization between stem cell markers Oct 4 and lineage marker Grem1 and the differentiation marker alpha smooth muscle actin in UC when compared to the normal and CD colonic mucosa (Figure 24). This data suggests that at least in UC, MSC-like cells undergo proliferation and differentiation to CMFs.

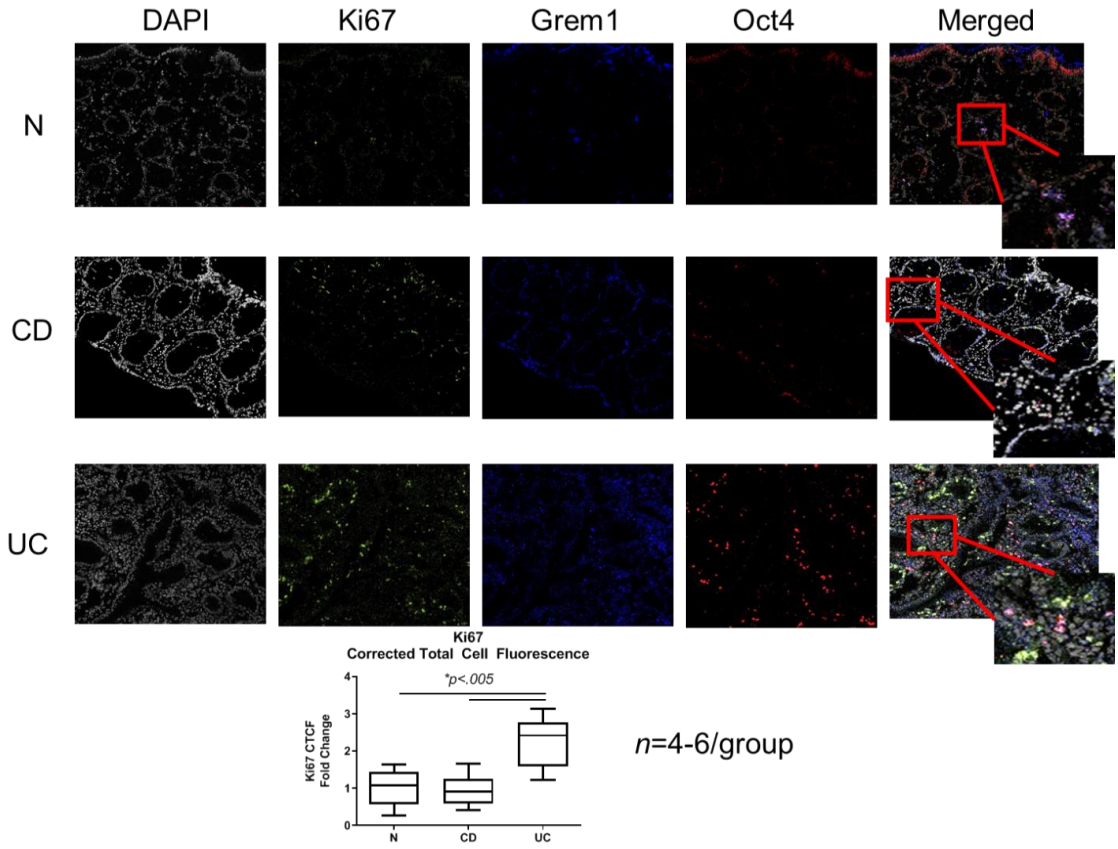


Figure 23. The proliferation of Oct4^{+} Grem1^{+} multipotent cells in increased in UC tissue, and decreased in CD tissue.

Human colonic samples were mounted section and stained for proliferation marker Ki67 (green), mesenchymal marker Grem1 (blue), and stemness marker Oct4 (red). The sections were then analyzed via confocal microscopy, with triple colocalization indicated by bright pink/white coloration. UC samples demonstrated increased proliferation of multipotent cells versus normal controls, while CD samples had decreased triple colocalization. Ki67 expression alone was quantified using Corrected Total Cell Fluorescence, CTSF. Summary graph is shown, data express as a means ± SEM of CTCF, *n*=4-6 per group, **p< 0.005*.

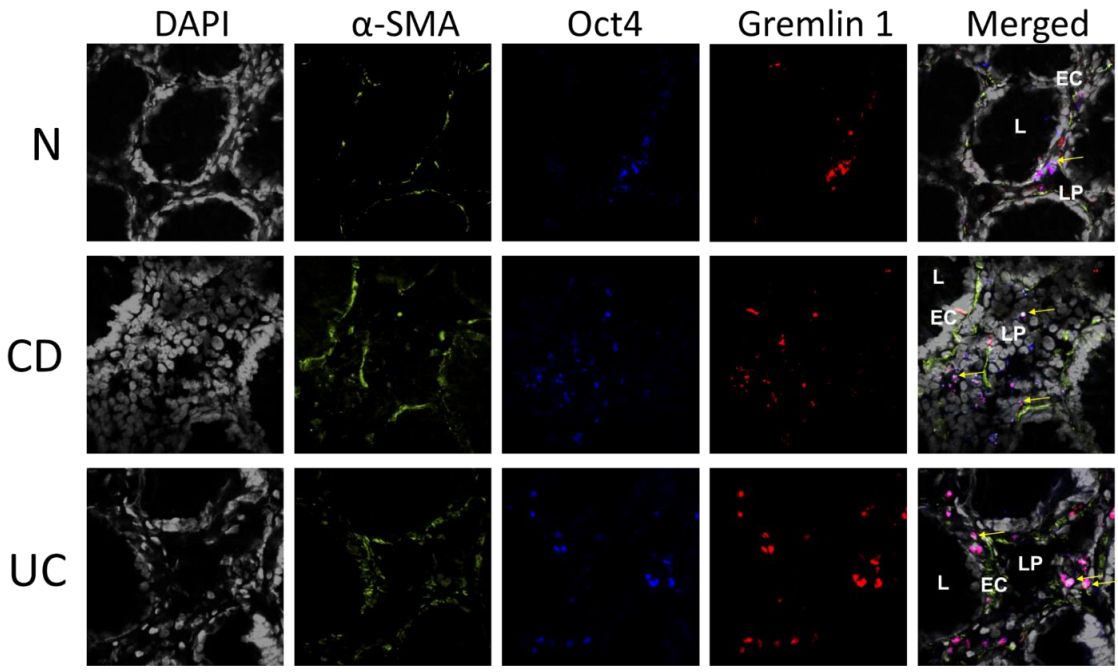


Figure 24. Multipotent cells in the UC colonic mucosa express differentiation maker α-SMA.

Human colonic mucosa tissue from Normal and IBD patients were stained for a marker of fibroblast activation and differentiation α-SMA (green), Oct4 (blue), and Grem1 (red). UC samples displayed the highest levels of triple colocalization (pink/white). CD and Normal both had several small areas of double colocalization, but few areas of triple colocalization were observed; *n*=4-7/group.

EXPRESSION OF IMMUNOSUPPRESSIVE MOLECULE PD-L1 IN UC, BUT NOT CD IS POSITIVELY ASSOCIATED WITH STEMNESS MARKERS OCT4 & ALDH1A2, AND LINEAGE MARKER GREM1 IN IBD

Thus far, it had been demonstrated that stem cell markers Oct4 and ALDH1A2, as well the mesenchymal lineage marker Gremlin 1are increased in the IBD colonic mucosa,. There is an also increase in the MSC-like cells that co-express Oct4 and Gremlin1. However, MSCs isolated from the colonic mucosa of IBD patients have reduced

clonogenicity and differentiation capacity compared to MSCs isolated from Normal colonic mucosa and bone marrow. Finally, it was determined that there is an increase in the proliferation of MSC-like cells in the UC colonic mucosa that are may be subject to aberrant differentiation. This data now allowed the investigation to begin to determine if CMF phenotype in IBD is the result of aberrant differentiation of MSC function as the result of exposure to the inflamed IBD mucosa microenvironment.

As shown in Chapter 1 and 2, the expression and Type 1 inflammatory activity of PD-L1 is increased in CD and decreased in UC, and this was mostly associated with fibroblast-like mesenchymal stromal cells. In our next set of experiments, we analyzed whether the increase in the total PD-L1 mRNA is correlated with the increase of Oct4 in IBD colonic mucosa (Figure 25). We observed that UC had significant positive correlations between PD-L1 and Oct4/ALDH1A2/Grem1, indicating that the increased PD-L1 expression may be associated with MSCs in the colon. However, there was no significant positive correlation with the expression of these proteins in CD, with the correlation being slightly negative and not approaching significance. This suggests that MSCs present in the CD milieu may have concomitantly blunted PD-L1 expression regardless of their levels of expression of Oct4, ALDH1A2, or Grem1.

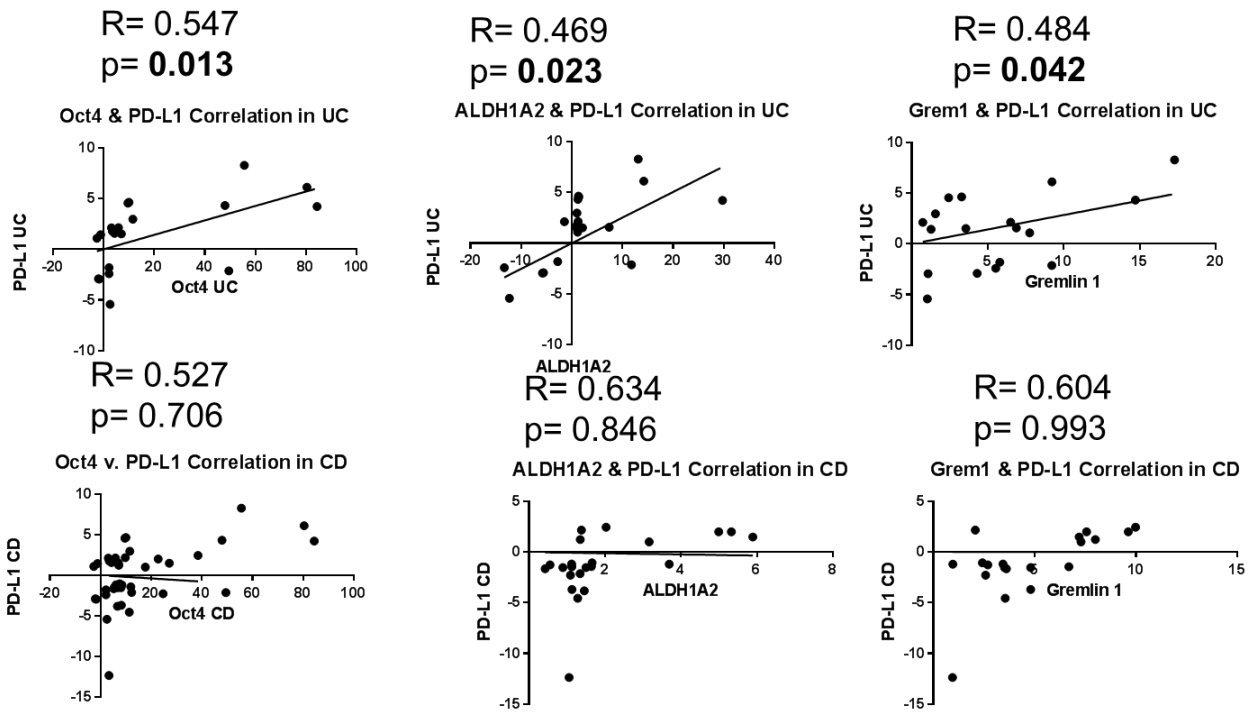


Figure 25. PD-L1 positively correlates with stemness markers Oct4 and ALDH1A2, and lineage marker Gremlin 1 in UC but not CD.

Normal and IBD tissue samples were acquired from biopsy, digested, and processed for mRNA extraction. The samples were then analyzed for PD-L1, Oct4, ALDH1A2, and Grem1 via qRT-PCR. The analysis was performed via GraphPad linear regression with the regression line being constrained through X=0, Y=0; *n*=16 per group. UC tissue has a significant positive correlation between PD-L1 and all other proteins of interest, while CD had no significant positive correlation.

These data demonstrated that Mesenchymal Stromal progenitor Cells are present in the normal and IBD colonic mucosa and that the expression of Oct4 and Grem1 are increased in IBD. However, the contribution of MSCs to the generation of pathogenic IBD-CMFs remained poorly understood**.** We **hypothesized** that aberrant differentiation of MSCs in the IBD inflammatory milieu might be the key event in the generation of CMFs with abnormal expression of PD-L1.

In order to investigate the presence of PD-L1 within cells that may be undergoing aberrant differentiation *in situ*, we next performed confocal microscopy analysis of normal and diseased colonic tissue staining for alpha-smooth muscle actin, Oct4, and

PD-L1 (Figure 26). We observed that PD-L1 expression in the normal and IBD tissue reflect our results from mRNA analysis. Further, we observed an increase in triple location between these markers, especially in UC, indicating that PD-L1^{high} MSCs may have developed because of aberrant differentiation in UC.

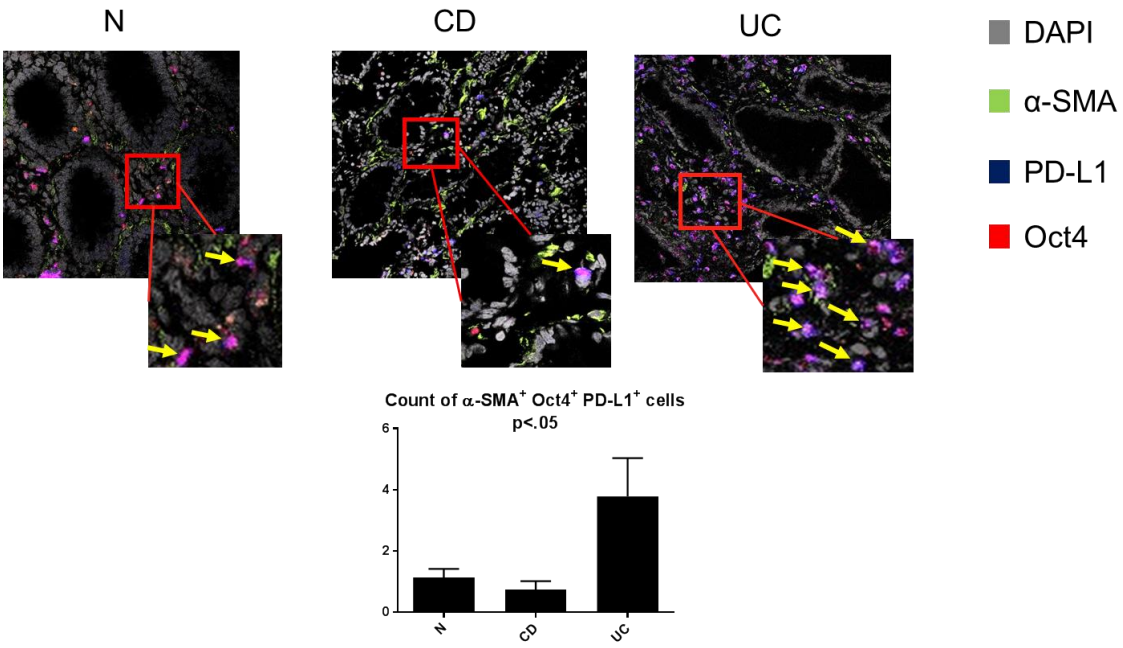


Figure 26. PD-L1^{+} Oct4^{+} cells undergo increased differentiation in the human colonic mucosa.

Human colonic mucosa tissue from Normal and IBD patients were stained for a marker of fibroblast activation and differentiation α-SMA (green), PD-L1 (blue), and Oct4 (red). UC samples displayed the highest levels of triple colocalization (pink/white), indicating that these cells may be undergoing differentiation. CD and Normal both had several small areas of double colocalization, but few areas of triple colocalization were observed; *n*=5-7/group.

CYTOKINES ALTER STEMNESS, DIFFERENTIATION, AND IL-6 IN CD, BUT NOT UC AND NORMAL TISSUE-DERIVED MESENCHYMAL CELLS MSCS.

Having observed an increase in mesenchymal progenitor cells in ex vivo and situ analyses, we next sought to investigate if Mesenchymal progenitor cells could be altered by classically identified inflammatory cytokines present in the IBD inflammatory milieu. This was approached by treating freshly isolated progenitor cells from normal, CD and UC patients with inflammatory effector cytokines. Our initial results demonstrate that TNFα upregulated expression of Oct4 in MSCs isolated from CD tissue, but not UC tissue, nor in MSCs isolated from normal tissue (Figure 27). We next observed that both IFNγ and IL-17a increased expression of alpha-smooth muscle actin in CD-MSC, but not UC-MSC or N-MSCs. We then observed that treatment with a combination of TNFα and IL-17a, a combination of IFNγ/TNFα/ and IL-17a, and combination treatment with IL- 1β, 6, and 17a increased expression of IL-6 in CD and normal tissue, but not UC. Treatment of UC tissues with the same cytokines did not initiate reproducible responses (data not shown). This may be indicative of unique mechanisms of MSC alteration of UC compared to CD, but more experiments with additional UC tissues will be need to confirm this idea. However, clearly, CD-MSCs had increased expression of Oct4 when treated with TNFα, increased expression of α-SMA when treated with IFNγ and IL-17a separately, and increased expression of Il-17a when treated with multiple cytokines.

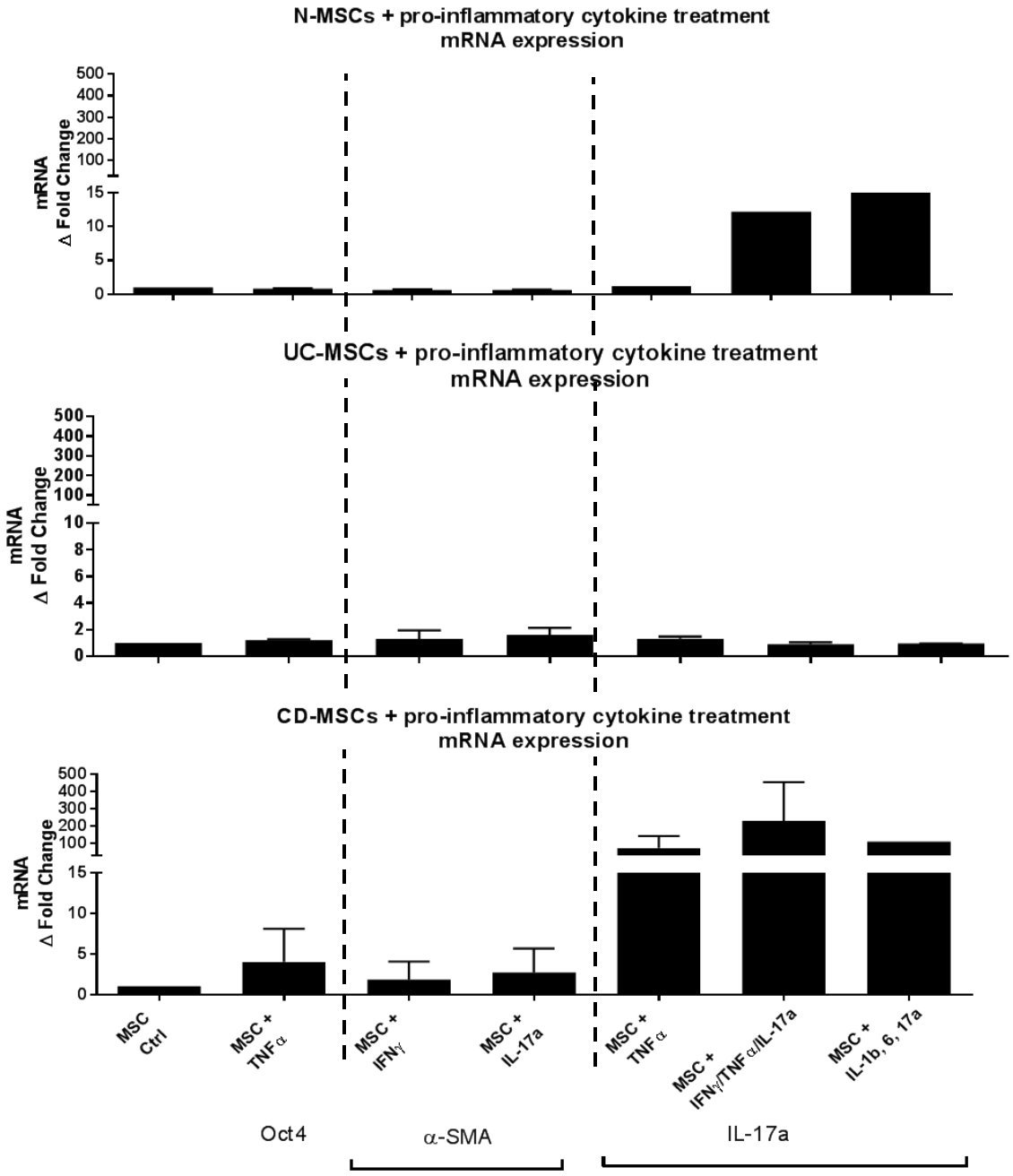


Figure 27. MSCs isolated from CD, but not UC tissue have increased sensitivity to inflammatory cytokines leading to increased mRNA expression of stemness marker Oct4, CMF differentiation marker α-SMA, and inflammatory cytokine IL-17A

MSCs were isolated from digested normal, UC and CD tissue and plated for 72 hours before cytokine treatment. Cells were then treated with TNFα, IFNγ, IL-17a, a combination of TNFα/IFNγ/IL-17a, or IL-1, 6, and 17a. CD-MSCs were then lysed and analyzed via qRT-PCR. CD-MSCs had increased expression of Oct4 when treated with

TNFα, increased expression of α-SMA when treated with IFNγ and IL-17a separately, and increased expression of Il-17a when treated with both multi-cytokine treatments. UC showed no differential expression between cytokines. Data trended toward but did not achieve statistical significance. Data are expressed as mean ± SEM, *n*=2 for N and CD, 3 for UC.

CONDITIONED MEDIA FROM UC BUT NOT CD INCREASES PD-L1 MRNA IN BM-MSCS

To investigate the possibility of that the IBD inflammatory microenvironment might modify MSCs function, we developed *in vitro* conditioned media experiments in which bone marrow-derived MSCs were cultured in MEM and conditioned media derived from incubated UC-CMFs and CD-CMFs using equal portions of media. Some cultures were chronically pre-exposed to TLR4 agonist lipopolysaccharide, which is implicated in IBD immunopathogenesis. Cells were then lysed, mRNA extracted and analyzed via qRT-PCR analysis. We observed that exposure of BM-MStCs to the conditioned media derived from CD- and UC-CMFs increased expression of stemness marker Oct4 mRNA, but only in the presence of LPS (Figure 28), indicating that Oct4 expression of MSCs may be sensitive to the inflammatory TLR agonists that may be derived from the dysbiosis present in the IBD colon.

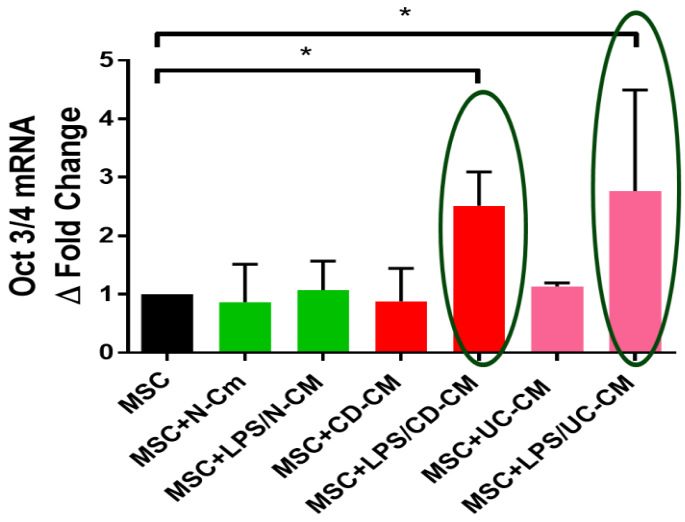


Figure 28. Conditioned media (CM) from IBD-CMFs exposed to the TLR4 agonist LPS increased expression of Oct4 in BM-MStCs.

MStCs isolated from human bone marrow were cultured with conditioned media from cultured of CD- or UC-CMFs with or without the addition of TLR4 agonist LPS and Oct4 mRNA expression was measured via qRT-PCR. MStCs were treated with media derived from N-CMFs or kept in untreated media as controls. Samples treated with both CM from IBD-CMFs and LPS had significantly upregulated Oct4 expression versus treatment with CM alone, treatment with media derived from N-CMFs, and untreated controls. Data are shown as mean ±SEM; *n*=3 for untreated and N-CM, 5 for CD-CM, and 6 for UC-CM.

It was also observed that MSCs treated with CD-CMF conditioned media had a significant decrease in PD-L1, while MSCs treated with UC-CMF conditioned media had a significant increase in PD-L1 expression, regardless of whether LPS was present or not (Figure 29). These data suggest that MSCs, which are crucial immunomodulators in the mucosa, may have their PD-L1 as well as their progeny's (CMFs) expression of PD-L1 altered by the IBD inflammatory milieu. Such changes in PD-L1 expression on IBD-MSC or IBD-CMFs could then facilitate altered suppression of Type 1 immune responses and Th cell imbalance.

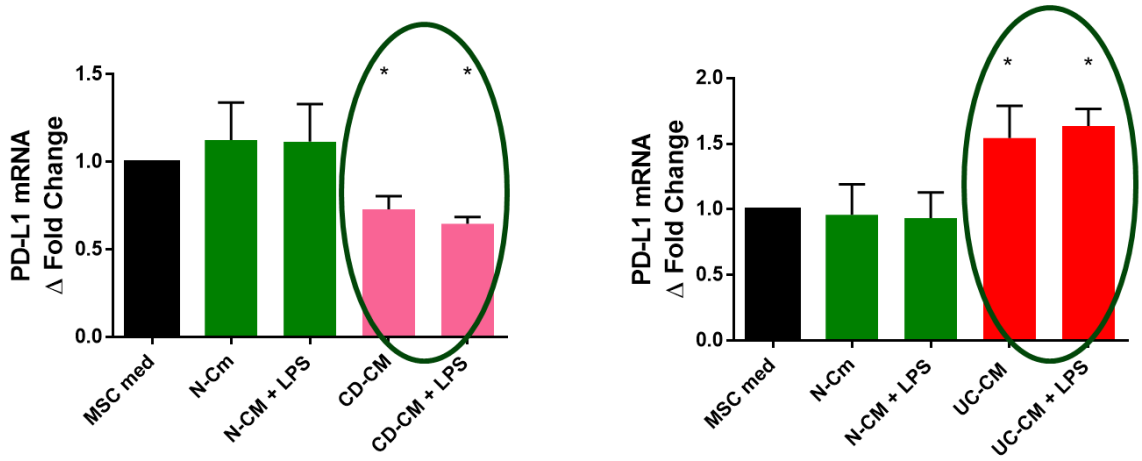


Figure 29. Treatment with conditioned media derived from CD-CMFs decreased PD-L1 expression while treatment with media derived from UC-CMFs increased PD-L1 expression of BM-MStCs with and without exposure to the TLR4 agonist LPS.

MStCs isolated from human bone marrow were cultured with conditioned media from cultured of CD- or UC-CMFs with or without the addition of TLR4 agonist LPS and Oct4 mRNA expression was measured via qRT-PCR. MStCs were treated with media derived from N-CMFs or kept in untreated media as controls. Samples treated with both CD- or UC-CM and CD- or UC-CM + LPS had significantly decreased expression of PD-L1 in the case of CD treatment and increased PD-L1 expression in the case of UC treatment versus treatment with CM alone, treatment with media derived from N-CMFs, and untreated controls. Data are shown as mean ±SEM; *n*=3 for untreated and N-CM, 5 for CD-CM, and 6 for UC-CM.

Summary

Although our previous research identified the importance of the CMF-PD-L1 phenotype in IBD immunopathogenesis, it remained unclear how the alteration in phenotype between UC and CD could occur. I hypothesized that the PD-L1 expression in IBD was due to the sensitivity of Mesenchymal Stromal progenitor Cells to cytokines of the inflamed gut, resulting in subsequent alteration by the inflammatory milieu. The data from this chapter demonstrated that MSCs isolated from both UC and CD tissue show increased stemness markers (Oct4 and ADLH1A2), although it is even higher in UC. CD-MSCs also have decreased ALDH activity. However, other classic stem cell behaviors are deranged. Thus, normal and UC tissue showed slightly decreased

differentiation and clonogenicity capacity when compared to MStCs isolated from bone marrow, but CD-MSCs were severely impaired MSCs and do not regularly survive the differentiation or clonogenic assays. These early progenies that we have isolated from normal and UC colons, however, are still potent (expressing classical stemness markers), and retain that potency in the Normal and UC milieu. While the *in situ* confocal imaging data alone could be indicative of decreased recruitment of MSCs to sites of injury in CD, taken together with the decreased ALDH activity, differentiation, and clonogenicity, the data points to impaired progenitor cells in CD. Although ALDH1A2 and Oct4 correlate in both forms of IBD, and these two markers, in turn, correlate with Grem1 in CD and UC, the correlation with ALDH1A2 is not statistically significant in CD. Further, *in situ* analysis shows that the $Oct4^{+}$ and $Grem1^{+}$ cells are less likely to be co-expressed CD tissue. The proliferation marker Ki67 is also blunted in CD while being highly upregulated in UC tissue. This suggests that in spite of increased markers for stemness in both forms of IBD, the stem cells in UC are actively proliferating, while those in CD were not. Although evidence of MSC differentiation into CMFs (α-SMA expression) is present in both CD and UC sections, colocalization is higher in UC, and only in UC tissue do the cells triple co-stain for smooth muscle actin, Oct4, and PD-L1. This finding supports poorer clonogenicity and differentiation in CD- than in UC-derived MSCs Although UC cytokine treatment experiments were inconclusive (not shown), MSCs isolated from CD were demonstrated to be sensitive to various cytokine treatments. Although TNFα exposure did increase Oct4 expression in isolated CD MSCs, this is not observed *in situ*, suggesting lesser sensitivity of the confocal experiments, or possibly that cytokines not identified may be suppressing or altering Oct4 expression in CD tissues.

Culturing naïve Bone Marrow MStCs with conditioned media isolated from cultured IBD-CMFs demonstrated that PD-L1 could be significantly increased by treatment with UC

conditioned media and significantly decreased by treatment with CD conditioned media. Our lab has developed evidence that CD-CMF PD-L1 may be susceptible to autocrine cleavage by Matrix Metalloproteinases (MMPs) produced by CD-CMFs, but not UC-CMFs, and this may account for the decreased CD- PD-L1 expression.(Aguirre et al). However, our data suggest that cytokines and growth factors may also play a role in altering PD-L1 expression in both CD and UC. Thus, it is possible that the differences in PD-L1 expression in BM-MStCs exposed to conditioned media from UC-CMFs or CD-CMFs are due to presence or absence of cleavage of PD-L1 from the surface because MMPs are produced by CD-CMFs but not by UC-CMFs. However, it is also possible that there are other fundamental changes in MSC differentiation in the two forms of IBD that may significantly alter CMF-PD-L1 expression in the two forms of IBD.

CHAPTER 4: OVERALL DISCUSSION AND CONCLUSION

The focus of my dissertation research is to determined if PD-L1 expression by CMFs significantly contributes to Th1/2/17 imbalance the human IBD colonic mucosa and if this altered PD-L1 expression is influenced by aberrant MSC differentiation due to the inflammatory milieu.

My work in Chapter two was influenced by previous findings by my group and others who have shown that the expression of PD-L1 and PD-L2 are crucial to the tolerogenic function of CMFs [10, 82]. This dissertation advances that concept, demonstrating the increased PD-L1 expression significantly suppresses Type 1 immune responses, including Th1 cell activity, which we demonstrated in UC. Conversely, the loss of PD-L1 expression by CD-CMFs may be a crucial factor in the high Type 1 immune response in the CD inflammatory milieu. Taken together, this data advances the notion that the altered PD-L1 expression by CMFs in both CD and UC may be critical factors contributing to the imbalanced Type 1 immune responses observed in these diseases. While the mechanism responsible for the imbalanced Type 1 response of naïve T cells remains unclear, the data in Chapter 2 of this dissertation strongly demonstrates the role of PD-L1 expression by CMFs in this imbalance and, thus, in the immunopathogenesis of IBD. PD-1 and its ligands PD-L1 and PD-L2 have emerged as successful therapeutic targets for various cancers. However, a significant side effect of these treatments includes diarrhea and overt colitis [123-125]. The mechanisms of these serious side effects of PD checkpoint blockade therapy remain unclear. Is seems likely that PD-L1-mediated type 1responses may be implicated. As stated in Chapter 1, gastroenterologists are sometimes unable to confidently diagnose IBD colitis as either CD or UC. The data from Chapter 2 may aid in this endeavor since the data attained

supports the idea that the expression levels of PD-L1 may be a diagnostic tissue marker for the type of IBD. Further, this data also suggests that restoration of PD-L1 expression by IBD-CMFs to levels observed in N-CMFs may provide a novel therapy for IBD.
The experiments carried out in Chapter 3 were built upon work by other groups that identified MSCs as progenitors of CMFs, and further experiments that demonstrated that Gremlin 1 could be used as a successful marker for lineage-tracing experiments of MSCs in the murine small intestine mucosa [68, 69]. However, the function and role of multipotent cells in the human colonic mucosa remained poorly characterized and unclear. The data presented in Chapter 3 advanced the knowledge in this field, identifying that multipotent progenitors (MSCs) are present in both the Normal and IBD human colonic mucosa lamina propria. This is the first report of successful isolation of MSC progenitors from human colon.

My data also strongly suggests that immunopathogenesis of the two forms of IBD, Crohn's Disease and Ulcerative Colitis, differentially affect colonic MSCs. My work has shown that CD-MSCs have variable expression of vital stemness markers, and appear to significantly lose their stemness properties. The terminal differentiation daughter cells for MSCs in the colon appears to be fibroblasts, which secrete the structural, adhesive, and space-filling proteins vital to Extracellular Matrix production [126]. It has been noted by other investigators that, in CD, these proteins are constitutively secreted by mesenchymal cells such as fibroblasts, leading to scarring fibrosis and stenosis in the GI tract of CD patients [127, 128]. However, the exact mechanisms responsible for fibrosis in CD remain unclear.

My data concerning UC-MSCs strongly demonstrate that they retain factors vital to stemness and proliferation and thus the number of these multipotent cells are highly

increased in the UC colon. Specialized cancer stem cells are have emerged as an important area of investigation in the field of cancer research [129]. While specialized, $ALDH^+$ aberrant cancer stem cells have been identified in UC patients who have developed colorectal cancer, how these cells arise and what drives their proliferation remains unclear, although the increased activity of ALDH enzymes has been observed to play a role [64]. Perhaps the abnormally differentiated mesenchymal stem cells in the lamina propria of the colon provide an active stem cell niche for the cancer stem cells in these diseases.

Mesenchymal stem cell therapy has emerged as a potential treatment for IBD. However, the efficacy and treatment methodology remain unclear. The data I have attained in Chapter 3 demonstrates that the progenitor cells of IBD patients are negatively affected by their disease immunopathogenesis, which may lead to altered CMFs. Thus replacing diseased MSCs/CMFs of IBD patients with normal MSCs may be an important goal for mesenchymal stem cell therapy of IBD. However, methods must be developed to overcome the detrimental inflammatory milieu of the colon to prevent the alteration of therapeutic, normal mesenchymal stem cells, from undergoing rapid conversion to pathologic IBD mesenchymal stem cells.

APPENDIX A: METHODS

CMF Isolation and culture

For CMF isolation, full-thickness fresh human mucosal samples were obtained from discarded surgical resections of colons in compliance with protocols approved by the University of Texas Medical Branch Institutional Review Board. Areas of uninvolved colon tissue from patients undergoing colectomy for colon cancer or Inflammatory Bowel Disease were studied as controls. Total mucosal cell preparations were performed as described previously [44]. CMFs were isolated according to the protocol of Machida et al. [130], which is routinely used in our laboratory [44]. The purity of isolated CD90+ CMFs (98-99%) was confirmed by flow cytometry, as previously described [44]. Studies were performed with primary CMF isolates at passages 4-10. Cells were cultured as described previously [44]. Cells were cultured at 37°C in 5% CO2 atmosphere in complete MEM, which contains MEM base supplemented with nonessential amino acids, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin (Corning, NY, USA), 1 mM sodium pyruvate, and 10% heat-inactivated FBS (Sigma-Aldrich, MO, USA).

Isolation of MSCs from Normal and IBD tissue:

Whole tissue from normal and IBD patients were digested in a method similar to CMFs and placed in T25 flasks coated with commercial MSC attachment substrate and media with MSC potency supplement from StemCell Technologies. Media was exchanged every 48 hours, and MSCs were split at 80% confluency. Cells were then split to 6-well plates with adipogenic or osteogenic media, or pelleted in a 15ml conical tube for chondrogenic differentiation.

TLR4 stimulation of CMFs for production CMF-derived media

CMFs in T-75 cm^2 flasks were treated with a 1:1000 dilution of 1mg/ml LPS (Invivogen, CA, USA) for 72 hours or media control according to manufacturer instructions. The media was then collected for use in differentiation assays.

MSC culture

Mesenchymal Stem Cells (MSCs) purchased from StemCell Technologies (Vancouver, BC, Canada) were passaged according to manufacturer's instructions. Briefly, the purchased MSCs were thawed and plated in T-25cm^2 flasks at a density of 10 x 10^5 cells/flask. The MSCs were then plated in T-75cm^2 flasks at a density of 2.5 x 10^6 cells/flask, and in 6-well plates at a density of 3 x10^5 cells/well. Flasks and plates were pre-treated with StemCell Technologies Attachment Substrate. MSCs were cultured at 37°C in 5% CO2 atmosphere in complete Mesencult-XF medium (StemCell Technologies, Vancouver, BC, Canada), which contained Mesencult-XF base supplemented with Mesencult-XF supplement (StemCell Technologies, Vancouver, BC, Canada), nonessential amino acids, 2 mM l-glutamine, 100 U/ml penicillin/streptomycin (Corning, NY, USA), 1 mM sodium pyruvate, and 10% heat-inactivated FBS (Sigma-Aldrich, MO, USA).

MSC Differentiation Assays

Whole tissue from normal and IBD patients were digested and placed in T25 flasks coated with MSC attachment substrate and media with MSC potency supplement. Media was exchanged every 48 hours, and MSCs were split at 80% confluency. Cells were then split to 6-well plates with adipogenic or osteogenic media, or pelleted in a 15ml conical tube with special media for chondrogenic differentiation. Specialized

differentiation media was 50% exchanged every 48 hours for 21 days. Cells were then analyzed for differentiation via Oil Red O (adipogenic), Alizarin Red (osteogenic), or Alcian Blue (chondrogenic) staining. Bone Marrow-Derived MSCs had the highest level of differentiation, followed by N-MSCs. IBD-MSCs had significantly reduced differentiation capacity.

Quantifications of the scoring systems for differentiation and clonogenicity of Bone Marrow-derived, Normal, and IBD-MSCs. Confocal images from Normal and IBD human colonic sections were analyzed via Zeiss Zen software and points were assigned under the following criteria: for differentiation, each sample was awarded 2 points for differentiation and one point for survival. For clonogenicity experiments, each well for each sample that formed colonies was awarded 2 points, and each well that had surviving cells, but no colonies were awarded 1 point. If the well had not adhered cells at the end of the assay period, 0 points were awarded. In both assays, CD-MSCs had significantly decreased clonogenicity and differentiation compared to normal MSCs and stem cells isolated from human bone marrow. CD-MSCs also had significantly decreased clonogenicity compared to UC-MSCs. There was no significant difference between the differentiation and clonogenicity of N-MSCs and UC-MSCs.

Clonogenicity experiments

MSCs isolated from the mucosa of colonic biopsies or resections of normal or IBD colons were plated for 24 hours in a T75 flask with MSC attachment substrate and Mesencult media from StemCell Technologies. Cells were then detached from the plate and transferred to a 6-well plate with MSC attachment substrate and 1ml Mesencult media at seeding densities ranging from 10^{-1}-10^{5} cells/plate. Total media volume was then adjusted to 2ml for each well. Media was exchanged every 48 hours for seven days. Wells were then aspirated and stained with crystal violet. Briefly, media was

removed; cells were washed with PBS, and fixed with 70 EtOH for 20 minutes. Wells were then aspirated and allowed to dry. 2.5 ml of Crystal violet was added to each well for 30 minutes. Wells were then gently aspirated and washed three times with tap water, then allowed to dry.

Clonogenicity wells were then assigned a score utilizing the following scoring system; wells that contained cells that survived and proliferated were awarded 2 points, wells that had surviving cells but no proliferation was awarded 1 point, and wells with no surviving cells were awarded 0 points.

Reverse Transcriptase Real-Time PCR

Analysis of MSCs from differentiation assays was performed as previously described [45] according to the Applied Biosystems's two-step RT real-time PCR protocol (Applied Biosystems, Foster City, CA). Briefly, Fast Start polymerase was purchased from Roche (Switzerland), 96-well plates from Bio-Rad Laboratories (CA, USA), and all FAM probes from Applied Biosystems (CA, USA). The appropriate assays-on-demand™ gene expression assay mix (Applied Biosystems) for human 18S RNA and the gene of interest (a 20X mix of unlabeled PCR primers and TaqMan® MGB probe, FAM™ dye-labeled) and two µL of cDNA were added to the PCR reaction step. The reactions were carried out in a 20 µL final volume using a BioRad CFX96 real-time PCR machine with the protocol 10 min at 95°C, 10 sec at 95°C (1 cycle) and 30 sec at 60°C (50 cycles).

Luminex mRNA microarrays

mRNA from normal, inflamed and non-inflamed UC colonic tissue was analyzed via the QuantiGene® Plex Assay as per manufacturer’s instructions (Affymetrix, CA, USA). Briefly, mRNA was thawed and slightly vortexed. A working mix consisting of nuclease-free water, lysis mixture, blocking reagent, capture beads, and probe sets was

generated and dispensed into the hybridization plate. 20 µl of mRNA was added to each well, with nuclease-free water added to wells for background controls. The hybridization plate was pressure sealed and incubated for 22 hours in a shaking incubator at 54°C at 600 RPM. The hybridization plate was centrifuged for 240g for 1 minute, and the mRNA and bead solution was transferred to a magnetic separation plate. Magnetic beads were then separated to the bottom of the wells and the residual solution discarded. The wells were then washed with wash buffer three times and 100µl of pre-amplifier solution. The plate was sealed and shaken at 800 RPM for one minute at RT, followed by 600 RPM for one hour at 50°C. The pre-amplifier solution discarded and the plate was then washed as previously stated, followed by 100µl of amplifier solution is added to each well. The plate was then sealed and shaken as described above. The amplifier solution discarded and the plate was washed three times, followed by the addition of 100µl of label probe solution to each well and shaken as described previously. After wash, 100µl of SAPE working reagent was transferred to each well, and the plate was shaken as described above. The plate was then washed with SAPE wash buffer, and the plate was shaken at 800 RPM for three minutes at RT and read immediately on Luminex HTS 200 multiplex analyzer.

Data then inputted into GraphPad, with each sample being normalized to non-involved control tissue from the patient. Correlations were then determined via linear regression analysis, with trend lines constrained through X=0, Y=0. R-values were obtained by calculating the square root of r^2 values determined by the linear regression analysis in the software.

Confocal Microscopy

Frozen human colon tissue sections were fixed in 1% paraformaldehyde for 20 minutes at room temperature, blocked with normal mouse and rabbit serum (2.5% in PBS) for 15 min at room temperature, and then incubated with the following antibodies: AlexaFluor®594-conjugated or APC-conjugated anti-PD-L1 (clone MIH1, 29E.2A3; BioLegend and eBioscience; 0.5mg.ml or 100 µg/ml) mouse monoclonal alpha-smooth muscle actin mAbs (1:200, eBioscience or BioLegend, clone 29E.2A3) anti-human mouse Ki67 (clone 20Raj1; eBioscience), anti-human rat Oct4 (conjugated to AF-555 or 633, clone EM92; eBioscience), or APC-conjugated anti-PD-L1 (overnight at 4°C. The sections were then stained with AF®647-conjugated rabbit Gremlin 1 (0.5 mg/ml) IgG mAbs (1:200, BiOrbyt), eFluor®570 or 660 mouse anti-human Oct3/4 (0.2mg/ml) IgG mAbs (1:200, eBioscience clone EM92), and FITC-conjugated anti-α-SMA mAbs (1:200, Sigma clone 1A4), anti-human rabbit Gremlin 1 (conjugated to AF-555 or unconjugated, clone Orb10741; Biorbyt), anti-human CD90 (clone 5E10), α-SMA (clones 1A4 or 1A1; Sigma), or AF647 conjugated anti-human EpCAM, for 30 min at room temperature. Each staining step was followed by six washes with PBS with Ca++/Mg++. The sections were then mounted in SlowFade® Gold antifade reagent with DAPI (Invitrogen, CA, USA). Confocal microscopy was performed with a Zeiss LSM510 META laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY) as previously described (Pinchuk et al., 2008). To quantify Oct4 expression in stained human colon sections, several in-focus images from each tissue were opened in ImageJ (NIH, https://imagej.nih.gov/ij/), measured for integrated density, area, mean fluorescence along with background measurements from each image. The Corrected Total Cell Fluorescence (CTCF= Integrated Density – (area of selected image * mean background fluorescence) was then calculated [37]. The CTCFs from each tissue were averaged together, and the

generation of histograms and statistical analysis were performed using GraphPad Prism 6. The fold change in CTCF was calculated by comparing IBD samples to the averaged CTCF data from health normal colonic samples.

Flow cytometry, Immunostaining, and Aldefluor assay

Whole tissue was cut into small pieces aseptically and rinsed with 5% Dakin solution and PBS. The tissue was then digested with 100units/ml each of Collagenases I, II, and IV using a gentleMACS™ dissociator (Miltenyi Biotech; Germany). The cells were treated with DNAse I (Worthington Biochemical; Lakewood, NJ) and then filtered through a 40µm strainer and plated overnight with MEM complete media in one well of an ultra-low attachment 24 well plate. Cells were counted and assessed for viability. They were then washed and placed in a suspension at $1x10^6$ cells/ml in Aldefluor assay buffer. The Aldefluor assay was then carried out as per manufacturer's instructions with proper isotype controls. Briefly, 5ml of activated Aldefluor reagent was added per 10^6 cells. Half of the total cells were then immediately transferred to a separate polystyrene tube containing 10ml of DEAB enzymatic inhibitor per 10^6 cells. Both treatments were incubated for 60 minutes at 37C. Immunostaining was then carried out, with Aldefluor buffer used in place of generic FACS buffer. Fluorescent antibodies used for immunostaining include; α-SMA (clone 1A4; Sigma), CD90 (clone 5E10; BD Bioscience and eBioscience), CD4 (clone RPA-T4 and RM4-5; eBioscience), Tbet (clone eBio4B10; eBioscience), PD-L1 (clone MIH1, 29E.2A3; BioLegend and eBioscience), PD-L2 (clone MIH18; eBioscience), anti-human IFNγ (clone 45.B3; eBioscience). When necessary, conjugation to fluorophores was utilized via Zenon donkey anti-mouse or mouse anti-rabbit $IgG2_a$ or $IgG2_κ$ labeling kits for Alexa Fluor 488, 555, 633, and 647 purchased from Life Technologies (San Diego, CA). Functional grade anti-human IL-4 (clone B-S4),

along with recombinant human IL-12 and IL-2 were purchased from ThermoFisher Scientific [47].

T cell isolation, activation, and polarization [47]

Peripheral blood mononuclear cells were prepared from the blood of healthy donors using density gradient centrifugation over Ficoll-Plaque Plus (Amersham Bioscience, Piscataway, NJ, USA) according to the manufacturer's instructions. Naive human CD4+ T cells were then purified from these peripheral blood mononuclear cells by negative selection using a commercially available Naive $CD4^+$ T-cell isolation kit II (Miltenyi Biotec, Auburn, CA, USA) utilizing the manufacturer's instructions. Negative selection was the method chosen to avoid accidental activation of CD4+ T cells during purification. Purified human naive $CD4^+$ T cells were activated using Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation kit (ThermoFisher Scientific, Grand Island, NY, USA) according to the manufacturer's instructions. In some experiments, $CD45RA^+$ $CD4^+$ T cells were polarized toward Th1 prior the use in co-culture experiments. For this purpose 1×10^6 of CD45RA+ CD4+ T cell were cultured in standard MEM in the presence of the T cell -activator CD3/CD28 T Cell Expansion and Activation kit with a Th1 differentiation cocktail (rIL-12, 25 ng/ml + anti-hIL-4, five μg/ml). On day 4 (expansion step) cells were harvested, counted, and resuspended at the concentration 1×10^6 cell/ml and cultured in MEM media supplemented with the IL-2 (20 U/ml) and Th1 differentiation cocktail up to day 7. On day seven anti-CD3/CD28 beads were removed according to the T-Activator CD3/CD28 for T Cell Expansion and Activation kit instruction and described above procedure was repeated for a total of 14 days. The purity of isolated CD45RA+ CD4+ T cells (>98%) and efficiency of the Th1 polarization were controlled by flow cytometry prior to the use of cells in the CMF-T cell cocultures. In some experiments, T cells were labeled with Carboxyfluorescein Diacetate

Succinimidyl Ester (CFSE) using the cell trace™ CFSE cell proliferation kit (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's instructions.

Co-Culture of CMFs or MSCs with T cells [47]

Primary adherent CMFs or MSCs plated with attachment substrate and MSC Mesencult media from StemCell Technologies were grown until 80-90% confluency in 6- or 24-well plates (7-day culture) in proper media, and then used in the CMF: T cell co-culture experiments. Activated or unprimed $CD4^+$ T cells were co-cultured in 6- or 24-well plates in the presence or absence of CMFs in ration 2.5:1, respectively. mAbs against the studied co-stimulatory molecules, PD-L1 Fc human fusion protein or isotype controls were added to the cocultures (when necessary) at a final concentration one μg/ml. For the cytokine treatment co-cultures, IFNγ, TNFα, IL-17a, IL-1, and IL-6 were added to concentrations of 100ng-1ug/ml. Cocultures or control monocultures were then incubated for five days maximum at 37°C in 5% CO2. T cells were removed from the surface of the CMFs via brisk pipetting, and both CMFs and T cells were analyzed via flow cytometry.

Primary CMFs Transfection With Small Interfering RNA (siRNA)

Small interfering RNA technology was used to knockdown expression of PD-Ls molecules in human primary CMF isolates as described previously (16). Briefly, negative siRNA controls are included in each experiment. Stealth siRNAs Set of three siRNA probes to the conservative domains of PD-L1, PD-L2, or negative siRNA control were purchased from Thermo Fisher Scientific. Transfection of CMFs was performed using Human Dermal Fibroblast Nucleofector kit according to the manufacturer instruction (Lonza, Allendale, NJ, USA). The efficiency of the downregulation of the PD-L1 or PD-L2

expression by specific siRNA set was controlled by real-time RT-PCR and flow cytometry.

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Statistical Analysis

Results in all figures are expressed as mean ± SEM of data from a minimum of three experiments (except N and CD cytokine treatment data, where N and CD experiments only had two experiments). Duplicates were performed in all experiments. Unless otherwise specified, differences between means were analyzed by one-way ANOVA in GraphPad Prism 5 and 6. For this dissertation, p values <0.05 were considered statistically significant. You did not mention the Pearson correlation analyses.

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PREVIOUS EDUCATION/DEGREES GRANTED:

05/2014	Department of Biology San Francisco State University San Francisco, California B.S. (Cell and Molecular Biology)
05/2011	Southwestern Community College Chula Vista, California A.A. (Mathematics and Sciences)

PROFESSIONAL AND TEACHING EXPERIENCE:

2017:	Graduate Assistant: Advanced Academic Success Skills Course Parts 1 and 2
2015:	Secretary: Society of Cell Biology. University of Texas Medical Branch. Galveston, Texas.

2013-2014	President; Student Researchers in Bioscience. San Francisco State University. San Francisco, California
2013:	Teaching Assistant; NSF Research Experience for Undergraduates. San Francisco State University. San Francisco, California.
2013:	Assistant to the Director; Cell and Molecular Imaging Center. San Francisco State University. San Francisco, California.
2012-2013:	Vice President; Student Researchers in Bioscience. San Francisco State University. San Francisco, California.
2010-2011:	President; Student Veterans Organization. Southwestern Community College. Chula Vista, California.
2009-2010:	Vice President; Student Veterans Organization. Southwestern Community College. Chula Vista, California.

RESEARCH ACTIVITES:

2015-Present: "Alterations of Mesenchymal Stem Cell Functions by Inflammatory Bowel Disease." Pinchuk Laboratory: University of Texas Medical Branch.

2014- Present: "Regulatory role of CD90+ stromal cells in Th1/Th17 activity in Crohn's Disease." Pinchuk Laboratory: University of Texas Medical Branch.

Research interests in the Pinchuk Laboratory:
Determining the function of Mesenchymal multipotent Stromal Cells (MSCs and formally referred to as mesenchymal stem cells) in the Inflammatory Bowel Disease (IBD) colon, and determining if the inflammatory milieu contributes to the aberrant function and differentiation of MSCs.
Mechanisms responsible for the dysregulation of the gastro-intestinal mucosal immune responses in particularly those that involved in (IBD) and Colorectal Cancer.
Understanding of the immune function of the $CD90^{+}$ stromal cells (CMFs) in colonic mucosa and their immunosuppressive role contributing to the maintenance of the mucosal tolerance.
Development of new therapeutics and prognostic approaches for IBD and cancer

2012-2014: "Identifying the role of exogenous Wnt3a in the developing chick embryo." Undergraduate Research Assistant. Burrus Laboratory. San Francisco State University.
Duties: Cultured HH4-6 chick embryos in order to study the effects of overexpression of Wnt3a, and rescuing Wnt protein knockout embryos in order to identify the role of Wnt3a in spina bifida and its role in cell proliferation relating to certain cancers. Other duties included lab cleaning and upkeep, disposal of hazardous waste, performing and creating buffers and solutions for Western Blots, and embryo harvesting.

PUBLISHED MANUSCRIPTS:

Phinney, B. B., Ray, A. L., Peretti, A. S., Jerman, S. J., Grim, C., Pinchuk, I. V., & Beswick, E. J. (2018). MK2 Regulates Macrophage Chemokine Activity and Recruitment to Promote Colon Tumor Growth. Frontiers in immunology, 9, 1857. doi:10.3389/fimmu.2018.01857. Published 21 September 2018.

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SUBMITTED MANUSCRIPTS:

Carl Grim, Ellen J. Beswick, Jose Aguirre, Don W. Powell and Irina V. Pinchuk. Oct4-positive multipotent mesenchymal stromal cells have an increased presence but decreased potency and clonogenicity in the colonic mucosa of Inflammatory Bowel Disease patients.

Jose E. Aguirre, Ellen J. Beswick, **Carl Grim**, Marissa Tafoya, Gabriela C. Palma, Von Samedi, Rohini McKee, Gabriela Uribe, Romain Villeger, Yuriy Fofanov, Yingzi Cong, Don W. Powell and Irina V. Pinchuk. *Matrix Metalloproteinases cleave membrane bound PD-L1 on CD90+ (myo-)fibroblasts in Crohn's Disease and regulate Th1/Th17 cell responses*. Could list the journal and "Under Review"

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MEMBERSHIP IN SCIENTIFIC SOCIETIES/PROFESSIONAL ORGANIZATIONS

2016-Present:	Society of Mucosal Immunology
2015-Present:	American Gastroenterological Association
2012-2014:	American Society for Cell Biology

PRESENTATIONS AND HONORS:

2018:	Poster Presentation: Federation of Clinical Immunology Societies (FOCIS) 2018. "Mesenchymal Stem Cells have attenuated differentiation and clonogenic potential in Ulcerative Colitis."
2018:	Poster Presentation: UTMB Cell Biology Symposium. "Mesenchymal Stem Mesenchymal Stem Cells have attenuated differentiation and clonogenic potential in Inflammatory Bowel Disease."
2017:	Teaching Excellence Award, Cell Biology Graduate Program
2017:	Poster of Distinction: Digestive Disease Week 2017. "Mesenchymal Stem Cell Transition to Inflammatory (Myo)Fibroblasts in IBD colonic mucosa: Role in the Dysregulation of Th1/Th2/Th17 Responses."

2016:	Oral Presentation: Society for Mucosal Immunology Course and Symposium 2016. “Alteration of Mesenchymal Stem Cells during Crohn’s Disease Contributes to the Formation of the Inflammatory Colonic Fibroblast Phenotype.”
2016:	Poster Presentation: Society for Mucosal Immunology Course and Symposium 2016. “Alteration of Mesenchymal Stem Cells during Crohn’s Disease Contributes to the Formation of the Inflammatory Colonic Fibroblast Phenotype.”
2016:	First Place Oral Presentation: UTMB Cell Biology Symposium. “Alteration of Mesenchymal Stem Cells during Inflammatory Bowel Disease Contribute to the Formation of the Inflammatory Colonic Fibroblast Phenotypes.”
2014:	Honorable Mention; SFSU Personalized Medicine Conference. “Identifying Wnt Ligands Involved in Early Stage Chick Embryos During Neural Tube Closure.”
2014:	Second Place Poster; SFSU College of Science and Engineering Symposium. “Identifying Wnt Ligands Involved in Early Stage Chick Embryos During Neural Tube Closure.”
2011-2014:	Dean’s List; San Francisco State University
2013:	SFSU Instructionally-related Research Award; San Francisco State University
2012:	NSF Research Experience for Undergraduates Fellowship; San Francisco State University

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2008:	Honorably Discharged
2008:	Four month deployment; Operation Enduring Freedom
2007:	Seven month deployment; Operation Enduring Freedom
2005:	Six month deployment; Operation Enduring Freedom
2003:	Certified Naval Nuclear Operator by US Department of Energy
2002:	Enlisted; Recruit Training Command, Great Lakes, Illinois

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