# THE POTENTIAL PROTECTIVE ROLE OF CAVEOLIN-1 IN INTESTINAL INFLAMMATION IN EXPERIMENTAL COLITIS

BY

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# Abstract

**Background:** Caveolin-1 (Cav-1), the major component of caveolae, is a multifunctional scaffolding protein that serves as a platform for the cell's signal-transduction and plays a role in inflammation. However, its role in inflammatory bowel disease (IBD), a chronic inflammatory condition in the gastrointestinal tract, is not clear. A recent study shows that Cav-1 mediates angiogenesis in dextran sodium sulphate (DSS)-induced colitis. These results contradict our data, in which Cav-1 levels decreased significantly in 2,4,6-trinitrobenzene sulphonic acid (TNBS)–induced colitis.

**Methods:** To test whether Cav-1 is involved in IBD pathogenesis, various models representing different dominant Th subtype responses and mimicking the immune pathologic mechanisms of different clinical IBD setting were employed: acute colitis was induced by intra-rectal administration of a single dose of TNBS in BALB/c and C57BL/6J mice, or by drinking 3% DSS water for 6 days in C57BL/6J mice. Chronic colitis was induced by administration of TNBS once a week for 7 weeks in BALB/c mice. To assess the effects of complete loss of Cav-1, Cav-1 knock-out (Cav-$1^{-/-}$) and control wild-type C57BL/6J mice received a single TNBS administration. To further test the possible role of Cav-1, one of two peptides (that either mimicked (Caveolin scaffolding domain; CSD) or

antagonized (Caveolin-1 binding domain; CBD1) Cav-1)) was administered intraperitoneally to mice receiving TNBS. Body weight and clinical scores were monitored. Colon Cav-1 and pro-inflammatory cytokine levels were quantified by ELISA. Inflammation was evaluated through histological analysis.

**Results:** Cav-1 levels in mouse colon tissue were significantly decreased in TNBS-induced colitis mice when compared to normal mice and also inversely correlated with colon inflammation and cytokine levels. Furthermore, a loss of Cav-1 (Cav-$1^{-/-}$) showed increased clinical and inflammatory scores and increased body weight loss. Mice receiving peptides to alter Cav-1 levels, showed surprising effects. The mimicking peptide (CSD) showed decreased Cav-1 levels, while the antagonizing peptide (CBD1) showed increased Cav-l levels. These changes in levels were associated with clinical and inflammatory scores and body weight loss that supported the TNBS-induced data. DSS-induced colitis mice showed increased disease activity index, however no significant difference in Cav-1 levels was found between colitis and normal mice.

**Conclusions:** Cav-1 plays an important role in the protection of TNBS-induced colitis, but not in DSS-induced colitis, an entirely different result from a previous report, suggesting that enhancement of Cav-1 expression and functions may be beneficial to IBD treatment in some specific clinical settings. Further studies are warranted.

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# Table of Contents

# Table of Figures

# Table of Tables

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# List of Abbreviations

| | |
|---|---|
| Cav-1: | caveolin-1 |
| CD: | Crohn's disease |
| ELISA: | enzyme-linked immunoabsorbant assay |
| eNOS: | endothelial nitric oxide synthase |
| IBD: | inflammatory bowel disease |
| mAbs: | monoclonal antibodies |
| mg: | milligram |
| TGF-β1: | transforming growth factor-beta 1 |
| µl: | microliter |
| UC: | ulcerative colitis |

# Introduction

## I. Immune disorders

The term ‘immunity’ is derived from the Latin immunitas meaning exempt. Classical immunology studies the relationship among the body systems, pathogens, and immunity. The earliest written mention of immunity can be traced back to the plague of Athens in 430 BCE. The historian Thucydides noted that people who had recovered from a previous bout of the disease could nurse the sick without contracting the illness a second time.[6] By the 19th and 20th centuries this theory had developed into scientific practice. In developed countries, new treatments and vaccines have caused many infectious diseases to enter remission or become almost completely eradicated. In 2000, mumps, measles, and tuberculosis had an extremely decreased incidence of infection compared to nearly 100 percent in 1950. Yet in that same time period, immune disorders have shown an opposite trend (Fig. 1). In the past 50 years, epidemiological data have provided strong evidence of a steady increase in allergic and autoimmune diseases in developed countries. Asthma, diabetes, and inflammatory bowel disease have increased exponentially. Today Crohn's disease has more than tripled in Europe since the 1950s.[3]

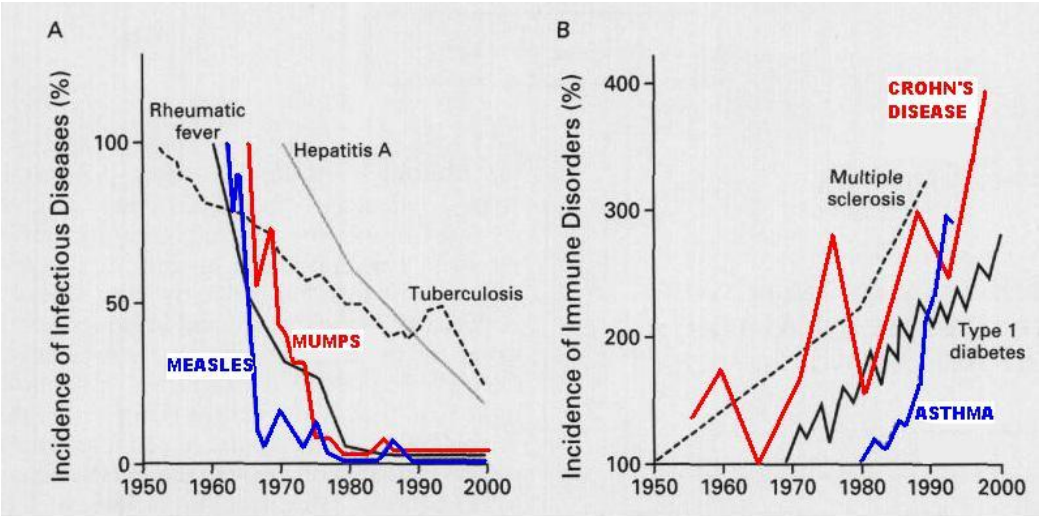


**Figure 1**: Inverse relation between the incidence of prototypical infectious diseases (A) and incidence of immune disorders (B) from 1950 to 2000.[3]

Protective immunity against microbes is mediated by the early reactions of innate immunity and the later responses of adaptive immunity. The immune system possesses several properties which are of fundamental importance for normal function: specificity for different antigens, a diverse repertoire of responses capable of recognizing a wide variety of antigens, antigen-specific memory, specialized responses to different microbes, self-limitation, and the ability to discriminate between foreign and self-antigens. Without all these qualities, the immune system would be unable to function effectively.[7]

Under normal conditions, the immune system recognizes foreign antigens and produces specific immune responses that protect the body from these substances, all the while remaining tolerant to self-antigens. An autoimmune

disorder is a condition that occurs when the immune system mistakenly attacks and destroys healthy body tissue. Under certain conditions, the immune system cannot tell the difference between healthy body tissue and foreign antigens, and thus the body mounts an abnormal immune attack with antibodies against a person's own self-tissue antigens. The result is an immune response, called hypersensitivity, which destroys normal body tissues. This occurs in autoimmune diseases, including inflammatory bowel disease (IBD).[7, 8]

### *a. Adaptive immune system dysregulation*

Dysregulation of the innate immune system causes functional abnormalities of the adaptive immune system, which reveals many characteristics of chronic inflammatory processes in IBD.[9]

$CD4^+$ T helper (Th) cells play a critical role in the orchestrating adaptive immune responses to various infectious microbes.[10, 11] They are also involved in the pathogenesis of autoimmune and allergic diseases. Upon activation by T-cell receptor complex, naïve $CD4^+$ T cells may differentiate into four major types of Th cells in the presence of different cytokines, including Th1, Th2, Th17 and inducible T-regulatory (iTreg) cells. They can be characterized by their special cytokine production profiles, transcription factors and their functions.[12] Under the stimulation of IL-12, naïve $CD4^+$ T cells differentiate into Th1 cells, mainly

producing IFN-γ and are vital for protective immunity against intracellular viral and bacterial infections.[12] Under the stimulation of IL-4, naïve $CD4^+$ T cells differentiate into Th2 cells, producing IL-4, IL-5, IL-9, IL-13, and IL-25, and are critical for eliminating extracellular parasites such as helminths.[12] TGF-β and IL-6 induce naïve $CD4^+$ T cells to differentiate into Th17 cells, producing IL-17, IL-17F, IL-21 and IL-22, and are important for controlling extracellular bacterial and fungi infections.[12] In the presence of TGF-β without IL-6, naïve $CD4^+$ T cells differentiate into iTreg cells. iTreg together with naturally occurring T-regulatory (nTreg) cells, are vital for maintenance of immune tolerance, and regulation of lymphocyte homeostasis, activation, and function.[12] Transcription factors also play important roles in the differentiation of Th cells and production of cytokines. The vital transcription factors of Th lineage are T-bet/Stat4 for Th1, GATA-3/Stat5 for Th2, RORγt/Stat3 for Th17, and Foxp3/Stat5 for iTreg.[12]

## II. Inflammatory bowel disease

The digestive system is a set of organs that convert the foods we eat into nutrients and facilitates the absorption of these nutrients into the bloodstream to fuel our bodies. We seldom notice its function unless something goes wrong, such as what happens in inflammatory bowel disease.[13] IBD is a chronic inflammatory disease of the gastrointestinal tract. It includes many chronic inflammatory conditions, most significantly ulcerative colitis (UC) and Crohn's disease (CD). Inflammation of the intestinal mucosa in IBD is characterized by episodes of diarrhoea, bloody stools, weight loss, abdominal pain, fever, and fatigue.[14]

### *a. Epidemiology*

The incidence and prevalence of IBD has markedly increased in recent years, and now IBD is considered to be one of the most prevalent gastrointestinal diseases. There are over 1.3 million IBD patients in the United States.[13] Recent estimates from a population-based, multiple province-wide study in Canada indicate that approximately 0.5% of Canadians have IBD, and incidence rates across Canada are among the highest in the world.[15]

These chronic diseases usually begin in young adulthood and last throughout life. The nature and gravity of the symptoms patients endure cause it to be a very difficult disease to live with. Current therapy focuses on symptom

relief by prescribing anti-inflammatory drugs or by surgical resection of the affected intestine, rather than a true cure.[16, 17] It places a heavy burden on populations as it reduces quality of life and capacity, and has a high direct annual medicare cost.[18] Thus, the importance of determining disease pathogenesis and a more effective way of treating it becomes apparent.

Over the years, many factors have been suggested as the cause of IBD, however it appears to be an extremely complex disorder with a combination of genetic and environmental factors, enteric flora, and immunological abnormalities contributing.[19, 20] Nevertheless, through the study of patients and mouse models, it has become apparent that in both Crohn's disease and ulcerative colitis, tissue damage likely results from an inappropriate or exaggerated immune response to antigens of the gut microflora. Targeting these exaggerated responses has emerged as a biological therapy in IBD. [21, 22]

### *b. Clinical manifestations*

Crohn's disease is a chronic, progressive, granulomatous disorder that can affect the gastrointestinal tract from mouth to anus, although it commonly affects the ileum, cecum, and peri-anal area (Fig 2). This disease is a chronic inflammatory process involving the submucosa, the layer of dense irregular connective tissue that supports the mucosa (the lining involved in absorption and secretion). Transverse fissuring and crypt abscesses are common.[23] Studies

have shown that inflammation may be due to an increased Th1 immune response, and to the more recently described excessive IL-23/Th17 pathway activation in response to bacterial antigens in genetically predisposed individuals. [24,25] Th1 cytokines (such as IFN-γ) are most likely critical in generating and perpetuating the chronic inflammation, resulting in infiltration by granulocytes and macrophages, leading to the release of enzymes, reactive oxygen intermediates, and pro-inflammatory cytokines. All of these factors contributed to the characteristic discontinuous ulceration and inflammation of CD that extends through the full thickness of the bowel wall.[26, 27]

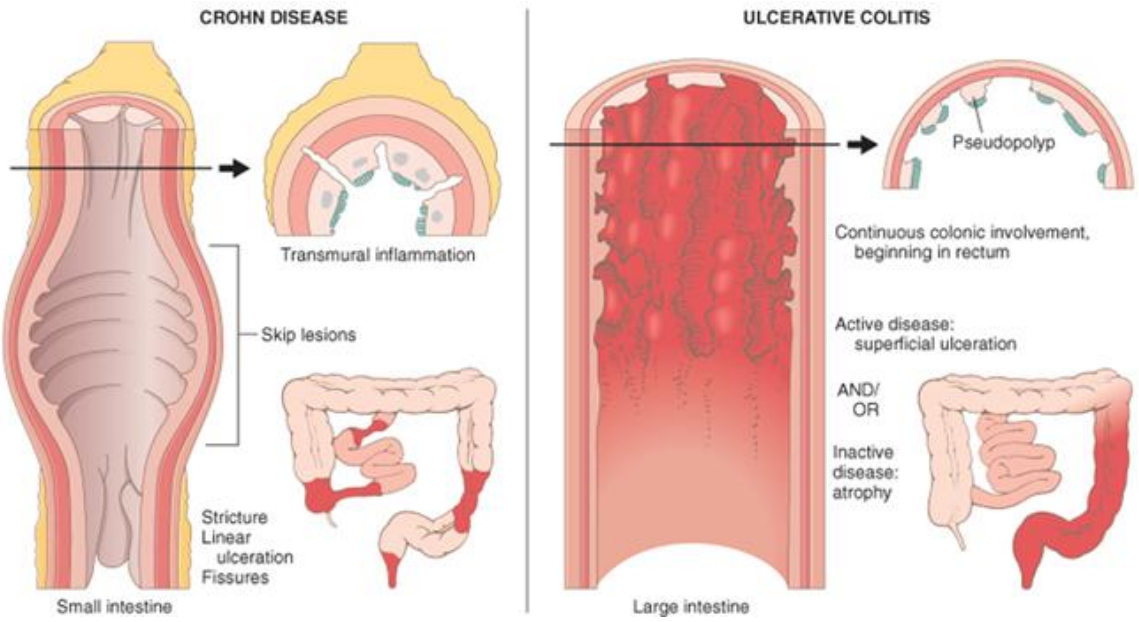


**Figure 2:** The various afflicted locations of IBD.[1]

Ulcerative colitis is characterized by continuous inflammation and ulceration, but involves only the innermost lining, or mucosa, and affects only the colon and the rectum (Fig 2). Pathological findings show that UC has an increase in certain inflammatory mediators (such as IL-13 and IL-5), signs of oxidative stress, and an unbalanced colonic microflora.[13, 20, 28] Histologically, UC shows cryptitis and crypt abscesse, a collection of dead neutrophils that has accumulated in a cavity due to damaged tissue releasing cytokines.[14] There is evidence that UC is characterized by a T-helper 2 (Th2 humoral) response (enhanced cytokines IL-5, IL-6, IL-8), because of high levels of immune globulins (especially IgG1 isotype) and autoantibodies. Researchers have found that patients with UC also had one or more type of invasive or adhesive fecal coliform bacteria and higher counts of certain pathogens (Escherichia coli, Costridum difficile, Shigella sp).[13]

### *c. Pathogenesis of IBD*

#### i. Genetic factors

Genome-wide association studies (GWAS) have identified 99 non-overlapping genetic risk loci, including 71 identified in Crohn's disease and 47 in ulcerative colitis.[29-31] Of these genetic risk loci, 28 are shared between Crohn's disease and ulcerative colitis. The analysis of the genes and genetic loci

identified in IBD indicates that several pathways play important roles in maintaining intestinal homeostasis, such as epithelial barrier function, innate mucosal defence, immune regulation, cell migration, autophagy, adaptive immunity, and metabolic pathways associated with cellular homeostasis.[29] The permeability of epithelial barrier enables microbial incursion, which is recognized by the innate immune system, which then launches appropriate tolerogenic, inflammatory, and restitutive responses partially by secreting extracelluar mediators that recruit other cells, including adaptive immune cells.[29]

Nucleotide-binding oligomerization domain 2 (NOD2) is the first gene found to be associated with Crohn's disease, which is frequently mutated in patients with Crohn's disease, occurring in around one third the patients.[32, 33] For instance, Crohn's disease patients associated with the 1007fs mutation in the NOD2 gene show a much more severe disease phenotype than other Crohn's disease patients; while the R702W and G908R mutations lead to increased inflammatory cytokine responses.[34] NOD2, a member of the cytosolic Nod-like receptor (NLR) family based on their triggers and the signaling pathways that they control, is one of the two important and distinct detection systems to sense microbial invaders.[34] NLR proteins are found in the cytoplasmic compartment, and the other detection system is membrane-bound receptors, termed toll-like

receptors (TLRs). NOD2 can recognize the minimal bioactive fragment of peptidoglycan found in the cell wall of both Gram-negative and Gram-positive bacterial, called muramyl dipeptide (MDP). Thus NOD2 is thought to be important as an intracellular sensor of bacterial components.[34-36] Upon binding to its ligand-MDP, a conformational change of NOD2 occurs that allows it to bind the caspase recruitment domain of the adaptor protein RIP2. RIP2 then induces the polyubiquitination of nuclear factor kappa B (NF-κB), an essential modulator, which is the key scaffolding protein of NF-κB and thus activates NF-κB, leading to secreting some proinflammatory cytokines, such as IL-12. It can also activate MAPK signaling pathway.[34, 35]

NOD2 has also been implicated in the initiation of autophagy.[29, 37] Autophagy is a highly conserved recycling process involving the degradation of cytosolic contents and organelles. It also plays a role in resistance against infection by the removal of intracellular microbes.[29, 37] MDP stimulation can activate the autophagy process leading to confinement of intracellular bacteria within autophagosomes and subsequent control of infection.[38] Following bacterial recognition, NOD2 serve as molecular scaffolds for the nucleation of the autophagy machinery by physically interacting with ATG16L1. ATG16L1 is essential for all forms of autophagy. Interestingly, ATG16L1 polymorphisms are also linked to Crohn's disease like NOD2.[38]

ii. Microbial Factors

IBD can also result from an abnormal response to intestinal microbiota. The human gastrointestinal tract is colonized at birth by a wide range of microorganisms. With many patients, tolerance is maintained and coexistence occurs. However, some pathogens can also be detrimental to the host. [39] Bacteria are detected by pattern-recognition receptors (PRRs) which activate the innate immune system, triggering the NF-κB signaling pathway, ultimately stimulating the production of pro-inflammatory cytokines and chemokines. Since the pathogen-associated molecular patterns (PAMPs) recognized by PRRs are small and conserved within many species of pathogenic and non-pathogenic microbes, they may recognize commensal organisms and cause an imbalance in gut homeostasis, contributing to the development of intestinal inflammation.[40] Unfortunately, this system is very complex and, as a result, the specific roles of intestinal flora in inflammatory conditions, which can initiate IBD, have not been determined. However, certain intestinal microbiota clearly promotes the development of IBD.[39, 41] For example, Clostridium difficile counts are increased in both Crohn's and ulcerative colitis patients.[42]

Researchers have found that patients with UC also had one or more type of invasive or adhesive fecal coliform bacteria and higher counts of certain pathogens (Escherichia coli, Clostridium difficile, Shigella sp).[13] There is

considerable data on the role bacteria play in colitis: 1) highest bacterial concentrations result in higher occurrence of bowel lesions; 2) normal enteric bacteria are needed in animals models of colitis; and 3) using anti- or probiotics to manipulate gut flora can result in symptom improvement.[13]

iii. Other environmental factors

Other environmental factors are also important in the pathogenesis of IBD. The frequency of IBD has increased in developed countries over the past 50 years and there is recent recognition of a correlation with industrialization in developing countries.[43, 44] Diet is another dynamic player, with studies showing that foods high in fat and sugar may exacerbate the development of existing Crohn's disease.[45] Smoking affects cellular and humoral immune responses, promoting colonic mucus production.[43] Evidence also suggests that smoking impairs autophagy.[46] Nicotine could modulate the immune balance to Th1-dominant via nAChR in the intestine, to improve Th2-type enteritis.[47] These studies propose that smoking is a disease-specific modifier that seems to aggravate Crohn's disease, while being protective against ulcerative colitis.

iv. Immunological Abnormalities

IBD is characterized by epithelial damage, infiltration of cells into the lamina propria, and by a failure to regulate the inflammatory immune response.

The activated lamina propria cells then produce high levels of TNF, IL-1β, IFN-γ, and cytokines of the IL-23/TH17 pathway.[21, 48]

The immune system is classically divided into the innate and adaptive responses, although interplay occurs commonly between the two arms. Intestinal innate immunity includes the barrier function of intestinal mucosa, antibacterial proteins, acidic pH of the gastric juices (to limit microbial growth), innate immune cells (neutrophils, macrophages, dendritic cells, natural killer T cells), and innate cytokines (IL-1, TNF). Proinflamamatory cytokines secreted during intestinal inflammation, such as TNF or IFN-γ, can increase epithelial permeability. Mice that have defective epithelial barrier functions develop colitis.[32] Intestinal macrophages maintain homeostasis by capturing and clearing bacteria that breach the epithelial layer without initiating an inflammatory response.[49] However, IBD patients have an increased number of macrophages and also express high levels of co-stimulatory molecules, such as CD40, CD80, and CD86.[50] Aberrant CD14-expressing macrophages from the mucosa of IBD patients produce high levels of IL-12 and IL-23 *in vitro*.[51]

The intestinal adaptive immune system is much more pathogen-specific and is usually initiated when innate responses cannot clear the invading pathogen. Adaptive immunity, which results in the activation of pathogen-

specific T and B cells, takes 4-14 days to become fully active after the initial insult.[52]

The balanced response to intestinal flora is very highly regulated, determining whether a tolerant or a defensive response should occur. Deviations from this equilibrium can cause IBD.[14]

$CD4^+$ T cells are major players in the adaptive immune response to infections. Upon activation of T-cell receptor complexes, CD4+ T cells differentiate into one of the Th subtypes important in IBD pathogenesis (Th1, Th2, Th17, and T-regulatory cells).[10, 12]

It has been widely accepted that Crohn's disease is caused by an overly aggressive Th1 immune response.[20, 53] The resulting infiltration of granulocytes and macrophages in the bowel leads to a release of enzymes, reactive oxygen intermediates, and proinflammatory cytokines. This causes discontinuous ulceration, full thickness bowel wall inflammation and is characterized by an excessive IL-23/Th17 pathway activation.[19,24] Ulcerative colitis, on the other hand, is more of a Th-2 like disease, with increasing amounts of IL-5 and IL-13.[54, 55]

**Th1 cells**: A number of observations indicate Th1 cells are involved in the pathogenesis of Crohn's disease.[36, 56] T cells in the colonic lamina propria of Crohn's disease patients produce large amounts of IFN-γ, and increase the

expression of IL-12Rβ2, T-bet and STAT4.[56] IFN-γ producing lamina propria lymphocytes are accumulated in the mucosa of patients. Macrophages in Crohn's disease patient produce high levels of IL-12.[56] At the initial phase of Crohn's disease, mucosal T cells mount a typical Th1 response that resembles an acute infectious process, and gradually disappear with progression to late Crohn's disease.[57] In addition, clinical responses are induced in a subcohort of patients with Crohn's disease treated with anti-IFN-γ antibody. In animal colitis model, abrogation of IFN-γ in the $CD4^+$ $CD45RB^{hi}/Rag^{-/-}$ transfer model potently prevents the development of colitis; T-bet-deficient $CD4^+CD45RB^{hi}$ cell cannot induce the colitis in $Rag^{-/-}$ recipients. These results indicate that Th1 play a role in the pathogenesis of Crohn's disease.[58]

**Th17 cells:** The recent findings of the IL-23/Th17 pathway highlights the role it plays in the pathogenesis of IBD.[58] Studies have shown that large amounts of IL-17-producing cells are mainly accumulated in the lamina propria of ulcerative colitis patients, and in the submucosa and muscularis propria of Crohn's disease patients.[59] Flow cytometry analysis of mucosal cells also show that the number of IL-17 producing T cells is increased in Crohn's disease patient than in normal controls, but some of these cells co-express IFN-γ.[60] Gut biopsies grown *ex vivo* and LPMC cultured *in vitro* also produce higher levels of

IL-17 in IBD patients than in controls.[61] Other Th17 cytokines, such as IL-21, IL-22 and IL-23 are also increased in the inflamed tissue of IBD patients.[62]

**Treg cells**: The gut-associated lymphoid tissue (GALT) is believed to be the primary site where naïve conventional $CD4^+$ T cells convert to iTregs after exposure to oral antigens in a lymphopenic environment.[63, 64] This conversion is dependent on TGF-β and retinoic acid producing $CD103^+$ DCs in the GALT. It has been supposed that nTregs mainly protect against autoimmunity in location, but iTregs primarily inhibit immune responses against environmental and food antigens in the gut.[65]

The dysfunction of Tregs in IBD is usually believed to be due to the defective numbers of Tregs or their suppressive function, and thus cannot control intestinal inflammation.[64] For instance, patients with a *FOXP3* gene mutation have defective Tregs and always suffer from intestinal inflammation.[66] When compared with healthy controls, the numbers of Treg are decreased in peripheral blood, but are increased in inflamed colons of patients with IBD. [64] Also, the ratio of Tregs to Th17 in peripheral blood is reduced in IBD patients when compared with controls.[67] However, the increased number of Tregs in the colon lamina propria of IBD patients is still lower than that of patients with infectious enteritis or diverticulitis.[68] Tregs isolated from inflamed colon or peripheral blood maintained normal cell-contact-dependent, cytokine-

independent suppressive capacity in vitro.[68-70] However, effector T cells from IBD patients display relative resistance to Treg-mediated suppression because effector T cells express high levels of Smad7, an inhibitor of the TGF-β signalling pathway.[71] These data indicate that Treg dysfunction might be due to an extrinsic milieu of activated cells that are resistant to suppression.[64]

More recently, a new type of iTregs, called iTR35, have been identified which mainly produce the suppressive cytokine IL-35 (not IL-10 or TGF-β).[72, 73] Adoptive transfer of IL-35-deficient Tregs cannot cure $CD4^{+}CD45RB^{hi}$-induced murine colitis,[73] whereas adoptive transfer of iTR35 generated *in vitro* can significantly improve intestinal inflammation.[72] IL-35 also shows strong ability in the control of intestinal inflammation. Administration of recombinant IL-35 significantly reduces the development of several forms of experimental colitis and reduces levels of cytokines of Th1 and Th17 cells.[74]

Both iTreg and Th17 differentiation require TGF-β which induces Foxp3 and RORγt, so there is a fine balance existed between these two types of cells under the control of many factors.[75] For instance, low concentrations of TGF-β together with IL-6 and IL-21 induce the expression of IL-23R and promote the differentiation of Th17 cells. On the contrast, high concentrations of TGF-β inhibit the expression of IL-23R and promote the development of iTregs. Foxp3

directly interacts with RORγt to suppress its function, but IL-6, IL-21 and IL-23 down-regulate the Foxp3-mediated suppression of RORγt.[76]

On the other hand, there is a close relationship between Treg and Th17 cells. Recent data have documented that memory Tregs can convert into Th17 cells under inflammatory conditions, and IL-1 is the key molecule in promoting this conversion.[77, 78] A hybrid subpopulation of memory Tregs co-expresses Foxp3 and RORγt which, in turn, exert suppressive functions but concomitantly secret IL-17 ex vivo.[79-81] In the presence of IL-1, IL-2, IL-23 and TGF-β, human Th17 cells preferentially differentiate from natural naïve regulatory cells, rather than from conventional $CD4^{+}CD25^{-}$ naïve T cells.[82]

Endothelial nitric oxide synthase (eNOS) has been shown to play a role in IBD as well. Using a DSS experimental colitis model, Sasaki *et. al*, demonstrated that disease activity was dramatically increased in $eNOS^{-/-}$ mice compared to wild types. A loss of eNOS resulted in greater leukocyte infiltration and gut injury, exhibiting that eNOS plays a role in limited injury to the intestine during experimental colitis.[83]

Taken together, the pathogenesis of IBD is associated with genetic susceptibility of the host, intestinal microbiota, other environmental factors, and immunological abnormalities.

### *d. Animal Models*

The clinical manifestation of human IBD is heterogeneous, a characteristic that is also reflected by the steadily increasing number of transgenic or gene-targeted mouse strains displaying IBD-like intestinal alterations. When chosen appropriately, these models can be used to investigate pathophysiological mechanisms and they are valuable tools to test emerging therapeutic strategies in the preclinical phase. As the onset of inflammation is immediate and the procedure is relatively straightforward, chemically induced models of intestinal inflammation are some of the most commonly used IBD animal models. Although they have limitations, like other models, they bare important immunological and histopathological aspects of human IBD.[84] Much of what we know about IBD mechanisms is gathered from our use of animal models that, although having similar features of IBD, also have limitations.

The two most common chemical models of IBD are dextran sodium sulfate (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS).[85] Both models show an inflammatory mediator cascade (such as cytokine dysregulation) that is similar to human IBD.

Hapten-induced colonic inflammation is a widely used animal model of human Crohn's disease.[86-88] Intrarectal delivery of 2,4,6-trinitrobenzene sulphonic acid (TNBS) resolved in ethanol induces colitis by haptenation of

colonic proteins, leading to a delayed-type hypersensitivity reaction by causing a Th1 reaction. This reaction leads to rapid onset of inflammation in the distal part of the colon, acting as a colitis similar to Crohn's disease, with transmural mononuclear cell infiltrate, abnormal crypt architecture, ulcerations, and occasional granulomas.

DSS-induced murine colitis is another widely used colitis model. In this model, it is believed that DSS is directly toxic to epithelial cells of the basal crypts and affects the integrity of the mucosal barrier.[89]

There are at least 66 other animal models that are used to study IBD, which are classified primarily into chemical induced, cell-transfer, congenial mutant, and genetically engineered models. The most popular include IL-10 knockout, Oxazolone-induced, and IL-17 altered expression models.[89]

IL-10 Knockout mice are genetically engineered to lack the IL-10 gene, and spontaneously develop colitis after three months. Enteric microbiota plays the major role in this colitis.[89]

Oxazolone (4-ethoxylmethylene-2-phenyloxazol-5-one) is also a haptenating agent that causes onset of inflammation in the distal part of the colon. Characterized by severe submucosal edema and hemorrhagic inflammation, it is mediated by IL-4 and IL-13 natural killer cells.[89]

The role of Th17 cells in the pathogenesis of IBD has also been evaluated in animal models. IL-17 is shown to be elevated in the IL-10 knockout and RAG1 knockout mouse models of IBD.[90, 91] Anti-IL-17 Ab ameliorates the severity of intestinal inflammation in RAG1 knockout mice reconstituted with IL-10 knockout $CD4^+$ T cells.[91] Deficiency of IL-17R (receptor) prevents the development of TNBS-induced murine colitis, including improving body weight loss, decreasing productions of IL-6 and local macrophage inflammatory protein-2, ameliorating colonic inflammation, and reducing tissue myeloperoxidase activity.[92] IL-17F-deficiency improves the development of DSS-induced murine colitis, whereas IL-17-deficiency exaggerates the development of DSS-induced murine colitis, indicating that IL-17F rather than IL-17A is important in sustaining DSS colitis.[93]

Enhanced production of IL-17 in the gut is also found in the C3H/HeSnJ SCID transfer colitis model, and adoptive transfer of IL-17-producing T cells to SCID recipients leads to severe colitis.[94] The model of $CD8^+$ T cell-dependent colitis shows that a single adoptive transfer of naïve $CD8^+$ T cells into syngeneic RAG-deficient mice results in severe colitis, with rapid spontaneous proliferation of these $CD8^+$ T cells in MLN.[95] These $CD8^+$ T cells in the MLN co-express IL-17 and IFN-γ. Adoptive transfer of naïve $CD8^+$ T cells isolated from either IL-17 or

IFN-γ deficient mice induced a remarkably less severe colitis, suggesting IL-17 and IFN-γ can cooperate to cause colitis in this model.[95]

The role of IL-21 in murine colitis is also indicated.[96] DSS colitis and TNBS-relapsing colitis are significantly decreased in IL-21-deficient mice, which is associated with reduced expression of Th17 cell-related genes (IL-17, IL-17F and RORγt) in the colon tissue. Furthermore, blockade of IL-21 using a specific IL-21R-fusion protein improves intestinal inflammation and down-regulates Th17 responses during the course of DSS colitis.[96] Taken together, these data indicate that the Th17 pathway play important roles in the pathogenesis of IBD.

Animal models of colitis also demonstrate the role of Tregs in the control of intestinal inflammation.[64] Adoptive transfer of naïve T effector cells in the absence of Tregs into SCID mice leads to colitis, whereas co-transfer of T effector cells and Tregs does not induce colitis.[64] Furthermore, adoptive transfer of $CD4^+CD25^+$ Tregs cures established $CD4^+CD45RB^{hi}$ transfer colitis.[97] In this model, Tregs are capable of suppressing colonic inflammation by down-regulating Th1 and Th17 responses depending on the presence of IL-10 and TGF-β.[98, 99]

The cytokine network is a complex and dynamic system in which cytokines, chemokines, and growth factors regulate the initiation and perpetuation of inflammation. In CD we see a Th1 disorder, while UC is primarily associated with

Th2 type responses.[84] Given the role that Cav-1 plays in a number of cytokine signaling pathways, it seems possible that varying models might produce various results.

## III. Current Biological Therapies for IBD

There are several treatment models for IBD including medication and surgery. Most commonly, doctors use drugs such as corticosteroids.[100] However, only around 50% of patients maintain remission using these drugs, resulting in researchers focusing on biological therapies which have shown promising results. Current biological therapies include monoclonal antibodies (mAbs) against cytokines, blockade of leukocyte migration, anti-T cell activation, small molecules, hematopoietic stem-cell transplantation, and growth factors.[101]

Administration of mAbs against TNF (such as infliximab) are effective in the treatment of Crohn's disease,[16, 102-105] as well as ulcerative colitis.[106, 107] Although this therapy provides a novel treatment, the agents all act as passively administered antagonists with short half-lives. For example, the half-life of infliximab is 9.5 days,[108] and thus, repeated injections are required to maintain the effects. Side effects to mAb treatment include acute infusion reactions and the development of antibodies to the infused mAb.[102, 103] Another highly relevant concern is the extremely high cost ($35,000-45,000/year per patient) associated with such passively administered therapeutics. These disadvantages limit the usefulness of this approach, as IBD is chronic and requires long-term treatment.

## IV. Caveolae and Cav-1

### *a. Caveolae structure*

Caveolae were first morphologically identified in 1953, and described as 'little caves' due to the fact that they appeared as 50 – 100nm vesicular invaginations of the plasma membrane (Fig 3A).[109] They are enriched in sphingolipids (lipids that contain a set of aliphatic amino alcohols that includes sphingosine and play a role in signal transmission and metabolism), cholesterol, and caveolin proteins, and are distinct from other lipid rafts due to this composition. A family of three proteins have been identified (Caveolin- 1, 2, 3) with Caveolin 1 (Cav-1) as a structural component and marker for caveolae and trans-Golgi derived transport vesicles.[109] All three caveolins have a constant 'FEDVIAEP' stretch (in the single-letter amino-acid code) within their hydrophilic amino-terminal domains. The functional importance of this sequence has yet to be determined, however.[109] The protein adopts a hairpin-loop conformation in the lipid bilayer, thereby exposing both the N and C termini to the cytoplasmic surface. A stretch of amino acids referred to as the caveolin scaffolding domain interacts with many signaling proteins (Fig 3B).[5] Cav-1 is a necessary requirement for caveolae formation as Cav-1-null cells have no caveolae and the majority of caveolae in cells and tissues require only Cav-1 for their proper formation (Fig 3C).[2] After Caveolin-1 synthesis in the endoplasmic

reticulum, caveolae biogenesis continues with the assembly of caveolin-1 oligomers. Cholesterol is essential for oligomerization, and oligomerization is critical for Cav-1 passage through the Golgi apparatus (where caveolae form) and delivery to the plasma membrane as stable units.[110, 111]

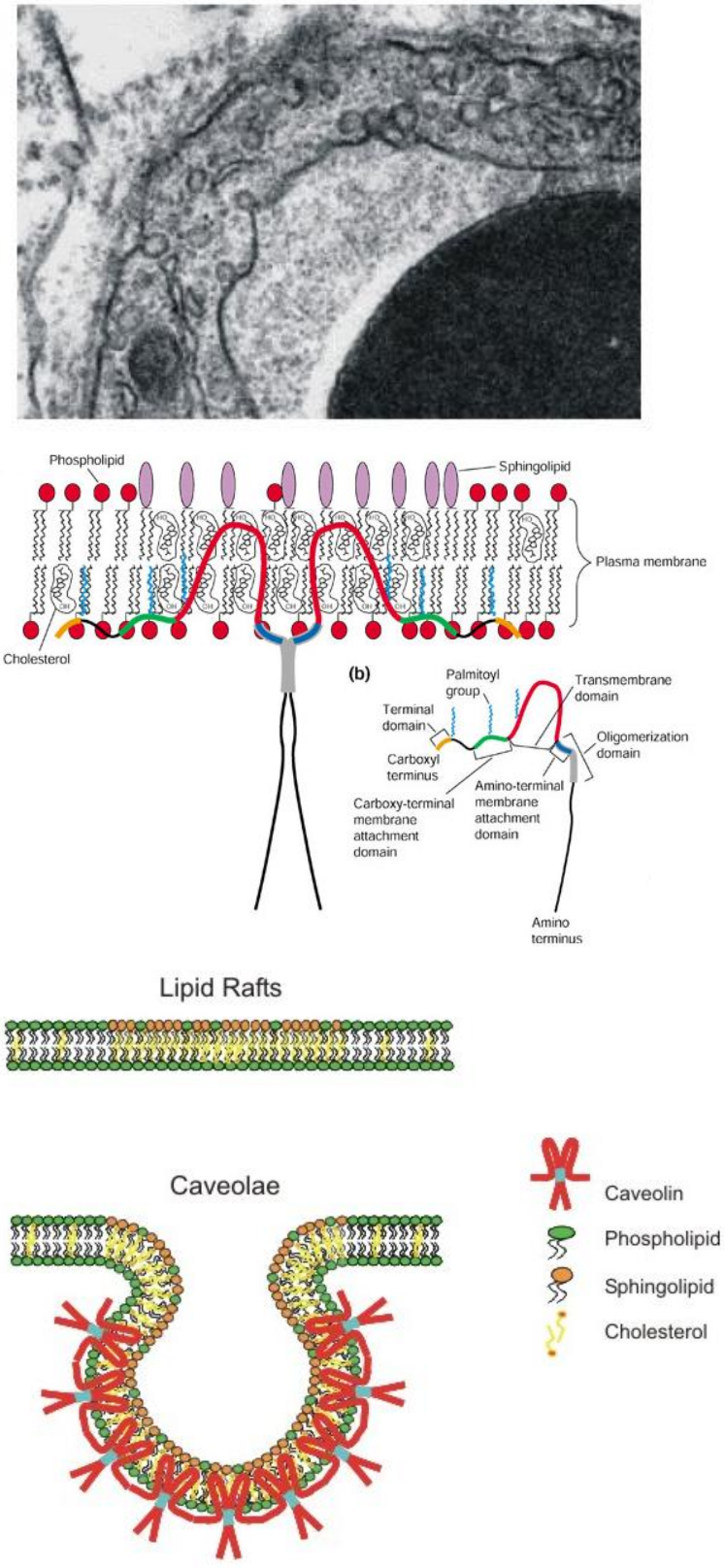


**Figure 3**: Caveolae structure. A) Electron micrograph of an endothelial cell showing caveolae. B) Primary structure and topology of Cav-1. C) Diagram comparing the biochemical composition of lipid rafts and caveolae. Oligomers of caveolin cause the formation of invaginations of the plasma membrane.[5]

Cav-1 is expressed in most cell types but is enriched in adipocytes, endothelial cells, myocytes, smooth muscle cells, fibroblasts, and macrophages.[109] These specialized lipid rafts can function as cell signalling platforms. The microdomain created by caveolae is ideal for amplification of cell signalling both through the clustering of many types of receptors and enhanced bioavailability of signalling molecules and through the direct actions of the caveolin proteins.[109, 112] It is believed that this scaffolding ability is due to oligomerization of Cav-1.

Cav-1 has an unusual structure, with both its C-terminus and N-terminus facing the cytosol. In between is a membrane-embedded hydrophobic domain. There are two isoforms of Cav-1 – α and β. [2, 113].The two forms are translated from different mRNA with Cav-1 β form starting from methionine at position 32 and being truncated by 31 residues, while α is 178 residues starting from position 1 of methionine.[114] The two isoforms have in common a hydrophobic stretch, the scaffolding domain, and the C-terminal.[5] The two isoforms have different potentials in caveolae formation, giving the difference in molecular composition (deep or shallow caveolae).[114]

Cav-2 is co-expressed with Cav-1 and, although not required for caveolae formation, results in the development of deeper, more uniform and more abundant caveolae. Cav-2 is retained mainly at the level of the Golgi complex. It

requires Cav-1 to form stable high molecular mass oligomers.[114] It is thought that Cav-2 plays a modulatory role in caveolae biogenesis. Analysis has shown that the phosphorylation of Cav-2 is necessary to modulate Cav-1 dependent caveolae assembly.[5]

Cav-3 is a muscle specific isoform of caveolin, and the major caveolar protein of differentiated skeletal, cardiac, and smooth muscle cells.[114] It is similar to Cav-1 in structure and function.[115] Cav-3 also interacts with proteins, including nitric oxide synthase and type I myostatin receptor (a muscle-specific TGF-β superfamily member controlling negative regulation of skeletal muscle volume).[116]

### *b. Caveolae functions*

#### i. Caveolae endocytosis

Cells use endocytosis to control the presence of receptors at the cell surface. This allows regulation of cell signalling, receptor turnover, and the magnitude or duration of events (Fig 4A). [2]

Caveolae contain the molecular machinery for vesicular transport and can undergo GTP-dependent, dynamin-mediated internalization from the plasma membrane. [117] Because of this, caveolae has been thought to participate in the transportation of macromolecules across endothelial cells through a process

called transcytosis. As detailed knowledge on internalization, shuttling through the cell, and release at the surface of the endothelium is limited, caveolae trafficking is the object of intense study.[2]

Caveolae internalization requires local actin polymerization, the presence of cholesterol and GM1 ganglisoside (the prototype ganglioside that contains one sialic acid residue and affects repair mechanisms and the release of neurotrophins in the brain), and the involvement of kinases. Src has shown to play a major role in caveolae endocytosis. In fact, silencing Src causes caveolar structures to build up at the cell surface and reduce the movement of caveolae.[2]

Microtubules serve as tracks for long range transport between donor and acceptor compartments. Actin acts coordinately with this to control membrane trafficking in the caveolar system. As caveolae mediate the movement of molecules across cells, motile vesicles and tubules serve as transport intermediates between caveolae and caveosome. Experiments by Mundy *et. al* show that the distribution of caveosomes substantiates the existence of an extensive caveolar membrane system. This system may be equivalent to the endosomal membrane system. [118]

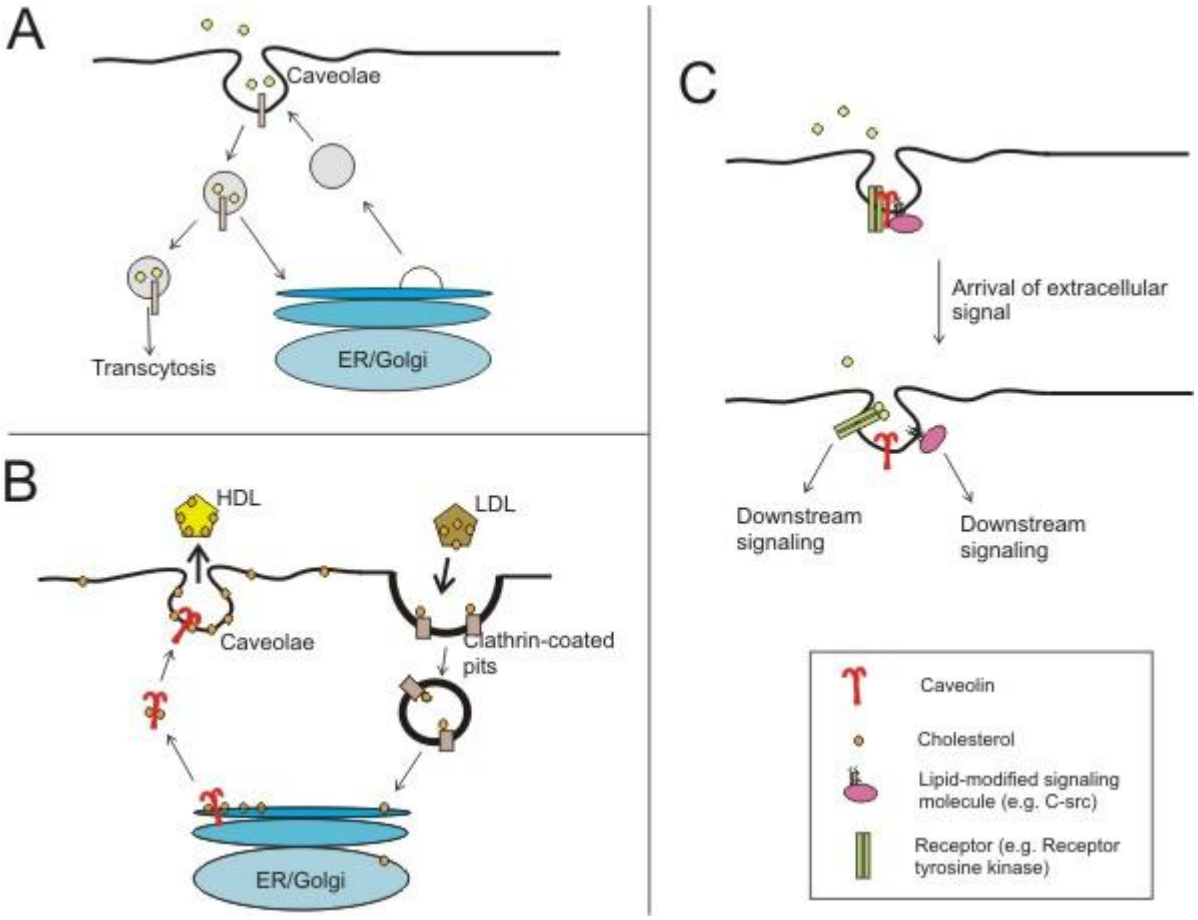


**Figure 4**: Proposed functions of caveolae and the caveolins. A) Certain molecules have been shown to be endocytosed via caveolae. B) Intracellular cholesterol is thought to be transported to plasma membrane caveolae via a golgi-independent caveolin-mediated route. C) Caveolae are now thought to act as entities in which signal transduction events can take place efficiently.[2]

ii. Caveolae signalling

As mentioned above, Cav-1 has the ability to bind and regulate the activity of signalling proteins (Fig 3C). This interaction has been mapped to amino acids 82 – 101, and is known as the caveolin scaffolding domain (CSD). Interaction between the CSD and the signalling protein generally inhibit signalling activity of the bound protein.[119] Binding of the CSD to the transient receptor potential channel-1 in endothelial cells, allows for Cav-1 to administrate the calcium-ion influx.[120]

Also, evidence suggests that interactions can occur based on cholesterol depletion, cofraction, and coimmunoprecipitation. Despite the variability of the protein binding, there is evidence that the CSD peptide can regulate signalling of known Cav-1 interacting partners.[119, 121]

Cav-1 is able to facilitate signalling because of its ability to provide functional platforms where multiple sets of signalling molecules are preorganized and sequestered. This provides a specific spatial and temporal compartmentalization, as the signalling machinery is carried to different locations in cells by caveolae relocation.[122] Combining this compartmentalization with the ability of Cav-1 to directly interact with and regulate, results in an elegant regulation. eNOS is a prime example of this: compartmentalization of eNOS and other involved signalling players enhances the process leading to

eNOS activation. However, direct interaction with Cav-1 maintains an inactive state of eNOS until stimulation occurs.[123]

Cav-1 is a very complex protein, as its role in the cell extends beyond that of caveolae. It has been identified on intracellular membranes, such as Golgi structures,[124] the endoplasmic reticulum[125], and in certain mitochondria[126]. It is also found in an intracellular soluble form known as cytoplasmic Cav-1. Here Cav-1 interacts with chaperone proteins to form a soluble complex with cholesterol to assist in cholesterol transport. The complex relationship between caveolae and cholesterol is well documented. Caveolae are enriched with cholesterol, yet free cholesterol is required for the proper formation of caveolae, as depletion or oxidation of cholesterol results in the loss of Cav-1 architecture. Cav-1 is one of the few proteins that binds cholesterol. Cholesterol also stabilizes caveolae oligomers.[127]

Cav-1 can also be an extracellular secreted protein, as it was identified in the lumen of serous secretory vesicles of exocrine cells. There is a lot of data which links Cav-1 secretion to prostate cancer.[128, 129] Increased expression of Cav-1 was identified in metastatic prostate cancer cells. Secreted Cav-1 seems to be biologically active and can protect the prostate cancer cells from apoptosis.[130]

### *c. Caveolin Scaffolding Domain & Cav-1 Binding Domain*

The ability of Cav-1 to bind to a variety of kinases, thereby inhibiting their activity, is now being explored as a therapeutic approach to reduce microvascular hyperpermeability and tumor progression in mice,[131] to inhibit collagen and alpha smooth muscle actin (α-SMA) expression *in vitro,* and to inhibit the progression of lung fibrosis *in vivo*.[132] This ability has been mapped to a sequence known as the caveolin scaffolding domain (CSD). The CSD consists of amino acids 82-101 (DGIWKASFTTFTVTKYWFYR) on Cav-1 (Fig 5).[131, 133] A peptide equivalent to the CSD, has been used to mimic the kinase-inhibiting activity. It can cross the plasma membrane when synthesized as a fusion peptide on the C terminus of the antennapedia internalization sequence (RQUIKIWFQNRRMKWKK).[131] Like the intact molecule, this CSD peptide binds to and inhibits the activity of various kinases. It is particularly useful because it is functional when delivered *in vivo*.[132, 134]

The CSD is the region that interacts with various proteins containing one of the caveolin-1 binding motifs.[135] The Cav-1 binding domain (CBD1) is a 16 amino acid long peptide from the HIV gp41 envelope. One of the distinct feature of the CBD1 peptide is its capacity to bind specifically caveolin-1.[136] These peptides can penetrate into cell membranes and bind to CSD, thus antagonizing Cav-1 activity directly.[137] This will inhibit the interaction with Cav-1, thus antagonizing the

activity of the CSD. This may, in turn, decrease the amount of binding to the CSD and thus, lower the inhibition of it.

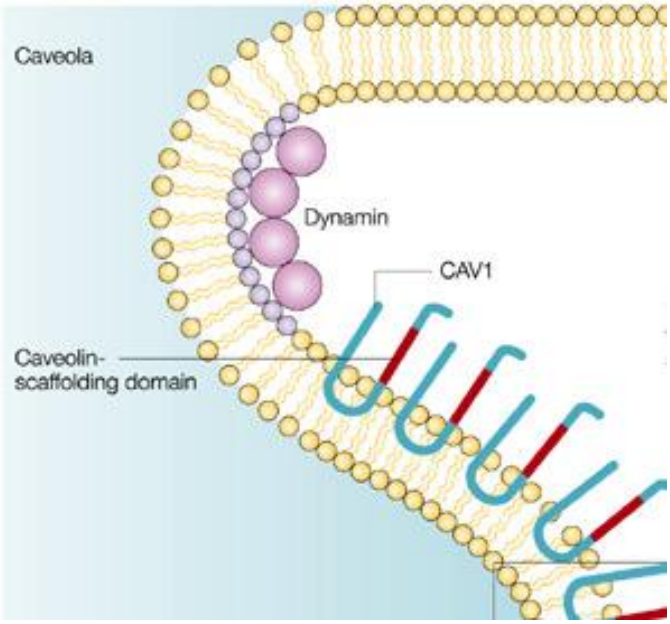


**Figure 5**: Select signalling molecules concentrate in caveolae, where they are bound to the scaffolding domain (red) of caveolin-1 (CAV1).[4]

### *d. Cav-1 & Disease*

Cav-1 has shown importance in a number of biological functions. For example, in the context of pulmonary hypertension, short-term administration of a cell-permeable Cav-1 peptide prevents hyperactivation of the STAT3 signaling cascade and upregulation of cyclin D1 and D3 protein levels. This, in turn, prevents the development of pulmonary artery medial hypertrophy.[133] It

has also been shown to be involved with diabetes-associated inflammation,[138, 139] atherosclerosis,[140] and cardiovascular diseases.[141, 142]

There is conflicting evidence for the role of Cav-1 in cancer, as it can act as both a tumor suppressor and as a tumor promoter. Its expression has been seen to increase or decrease depending on the tissue. In breast cancer cells, Cav-1 expression is significantly lowered when compared with normal breast tissue and Cav-1 inhibits oncogenic signalling pathways,[143] In addition to that, when Cav-1 cDNA is transfected to cancer cells, the overexpression of Cav-1 results in substantial inhibition of growth.[144] A decrease in Cav-1 expression (mRNA and protein level) is also seen in ovarian, gastric and colon cancer cell lines.[145-147]

On the other hand, in carcinoma of the thyroid Cav-1 expression is elevated, [148] while Cav-1 levels are associated with tumor dedifferentiation in bladder cancer. [149] Another report showed that Cav-1 enhances the invasive capability of lung cancer cells lines. [150]

Cav-1 also plays an important role in normal lung function, as well as pathology. It regulates acute inflammation and capillary leakage during lung injury, [151] as well as bleomycin-induced lung injury and fibrosis. [152]

The role of Cav-1 in inflammation is controversial. Literature has supported the role of Cav-1 in inflammatory functions. Cav-1 expression in peripheral mouse polymorphonuclear neutrophils promotes the infiltration of

neutrophils to injury sites, thereby increasing the adhesion and migration responses and signaling for cytokines.[153] As well, Cav-1 suppresses transforming growth factor-beta 1 (TGF-ß1) signalling. As TGF-ß1 represses the differentiation and activity of antigen presenting cells (such as macrophages, dendritic cells and neutrophils), a decrease in TGF-ß1 signalling, mediated by Cav-1, could result in an increase in inflammatory responses.[154]

However, other research supports anti-inflammatory functions of Cav-1. The expression of Cav-1 in alveolar macrophages inhibited the expression of inflammatory mediators and promotes the expression of anti-inflammatory cytokines such as IL-10.[26] It can act as a protective modulator in animal sepsis.[155] As well, Cav-1 negatively regulates endothelial nitric oxide synthase (eNOS), which is involved with the hyperemia and permeability changes associated with acute inflammation.[83]

### *e. Cav-1 and Inflammatory Bowel Disease*

The intestinal immune system is finely balanced, where pro-inflammatory and anti-inflammatory cells and molecules are carefully regulated. Since caveolae, and more specifically Cav-1, play a role in the pathogenesis of many diseases and inflammatory pathways, they have been considered as potential therapeutic targets.

Currently, there is one published study on Cav-1 and inflammatory bowel disease. Those suffering from IBD have shown to have increased vascular density correlating with disease activity. This suggests that pathological angiogenesis influences the progression of IBD.[156] Angiogenesis refers to the formation of new capillaries from pre-existing blood vessels. Pathological angiogenesis has been shown to lead to chronic inflammation of IBD. [157] There are many mediators of this angiogenic response and caveolae are the site of regulation for many of these mediators.[158] As a result, Chidlow *et. al* believed that if endothelial Cav-1 is lost, the signaling components are no longer organized correctly and endothelial cell signaling is perturbed. Using a 3% DSS Cav-1 knock out model, as well as injection of the CSD peptide, they discovered that disruption of Cav-1 resulted in attenuation of disease, suggesting that endothelial caveolae are crucial points for microvascular regulation of experimental colitis. [159]

# Research Rationale

Inflammatory bowel disease (IBD) is a lifelong disease occurring early in life in both males and females. The incidence and prevalence of IBD has markedly increased over the last 50 years. As the quality of life improves, especially in developed countries such as Canada, the hygiene hypothesis suggests that the incidence of IBD will continue to rise.[75]

A population-based, multiple province-wide Canadian study by Bernstein et al. indicates that approximately 0.5% of Canadians have IBD (about 170,000 or approximately 1 in 180); and incidence rates across the country are among the highest in the world. Unfortunately, the etiology and pathogenesis of IBD has not yet been clarified, and the current management of the disease is not efficient in controlling IBD, as there is a relatively high relapse rate.[15]

Roughly 30% of those afflicted with IBD are children and young adults.[33] Children generally suffer from a more severe form and are susceptible to more complications from the disease. Due to the lack of effective treatment, these patients are burdened with frequent flare-ups of the disease their entire lives, and as a result, their quality of life becomes lower than the healthy population.[160]

Deregulation of mucosal immunity in the gut, causes an overproduction of inflammatory mediators. It has been suggested that phosphorylation of Caveolin-1 increases vacscular hyperpermeability through inflammatory

mediators[156, 158, 161] and thus may be involved in this mucosal regulation. Yet, the role of Cav-1 in IBD remains unclear. Therefore the goal of this study was to investigate the roles of Cav-1 in IBD. Determining how it regulates inflammatory mediators in various species and disease models, as well as knowledge of the interplay between Cav-1 and the immune system will help in the search for ways to block disease progression associated with fibrosis and inflammatory cytokines. Cav-1 can therefore be considered a potential therapeutic target for the treatment of IBD.

# Hypothesis

Cav-1 plays an important role in the development of IBD by exacerbating intestinal inflammation. Those with higher Cav-1 levels will experience more severe DSS- & TNBS-induced murine colitis model symptoms, such as body weight loss, clinical scores, and inflammation. Genetic loss of Cav-1 will alleviate experimental murine colitis symptoms. Increasing or decreasing Cav-1 function through the use of two Cav-1 related peptides (CSD and CBD1), will alter inflammatory responses in the mouse colon. This will hopefully provide a direction towards a potential therapeutic approach for intervening IBD progression.

# Study Plan and Endeavours

As outlined, caveolae play a role in the inflammatory signaling process of a number of diseases. Cav-1 inhibits oncogenic signalling pathways,[143] markedly ameliorates pulmonary fibrosis,[152] and acts as a protective modulator in animal sepsis.[155] It has also been shown to be involved with diabetes-associated inflammation,[138],[139] atherosclerosis,[140] and cardiovascular diseases.[141, 142]

Until now, only one study has reported the role of Cav-1 in experimental colitis.[159] Childlow *et al*. observed that endothelial Cav-1 was up-regulated during DSS-induced murine colitis and a loss of Cav-1 significantly protected against inflammatory tissue damage. The authors concluded that endothelial Cav-1 mediates angiogenesis in experimental colitis and suggested that Cav-1 might be a novel therapeutic target for IBD.[159]

Our laboratory has developed vaccines against IL-12, IL-23 and TGF-β for the treatment of IBD and has evaluated their effects in TNBS-induced experimental murine colitis.[162] In the present study, I looked to not only assess the role of Cav-1 in TNBS-induced colitis, but to also use various mouse species, including Cav-1 knock-out mice, two different disease models (TNBS and DSS), and two peptides (CSD and CBD1, which can either antagonize or mimic Cav-1 to study the role of Cav-1) in intestinal inflammation. The results of this study

could lead to the development of a therapeutic strategy, thus alleviating colitis symptoms.

# Specific Aims

1. To determine whether Cav-1 expression is altered in colon tissue in acute and chronic models of TNBS- and DSS-induced murine colitis, in two species of mice (Balb/c and C57BL/6J mice).
2. To assess the relation of Cav-1 levels with colon inflammation, by quantitatively determining Cav-1 levels through developing a sandwich ELISA technique
3. To assess the effects of complete loss of Cav-1 (Cav-1 knockout mice) on the induction and resolution of TNBS-induced colitis, and thus clarify the possible roles of Cav-1 in IBD development.
4. To explore whether administration of two Cav-1 related peptides affects the intensity and duration of intestinal inflammatory responses, how it alters clinical scores of mice with induced disease, and to understand their underlying immune mechanisms in models of murine colitis.

# Methods

## I. Animals

Female Balb/c mice (7-8 weeks old), and female and male C57BL/6J mice (10-12 weeks) were purchased from Charles River Laboratories (Saint-Constant, Quebec, Canada). Female C57BL/6J Cav-1 knock-out mice (Cav1$^{tm1Mls}$/J, 7-8 weeks old) and control C57BL/6J (7-8 weeks) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). They were maintained at the Central Animal Care Services, University of Manitoba. All experiments were performed in accordance with the standards set by the Canadian Council for Animal Care.

## II. Protocols for Induction of Colitis

Experimental colitis was induced by both TNBS and DSS (Sigma-Aldrich, St. Louis. MO). In two TNBS-induced acute colitis experiments: Balb/c mice received 1 mg of TNBS (n=12 (normal), n=16 (TNBS)); C57BL/6J mice received 2.5 mg of TNBS at day 0 (n= 9 (Cav-1 -/- TNBS), n=12 (wild-type TNBS), n=3 (Cav-1 -/- normal), n=7 (wild-type normal)). In one chronic colitis experiment, Balb/c mice were induced by weekly administration of increasing doses of TNBS eight times (1.0 - 2.3 mg in 45% ethanol) as previously described.[162, 163]

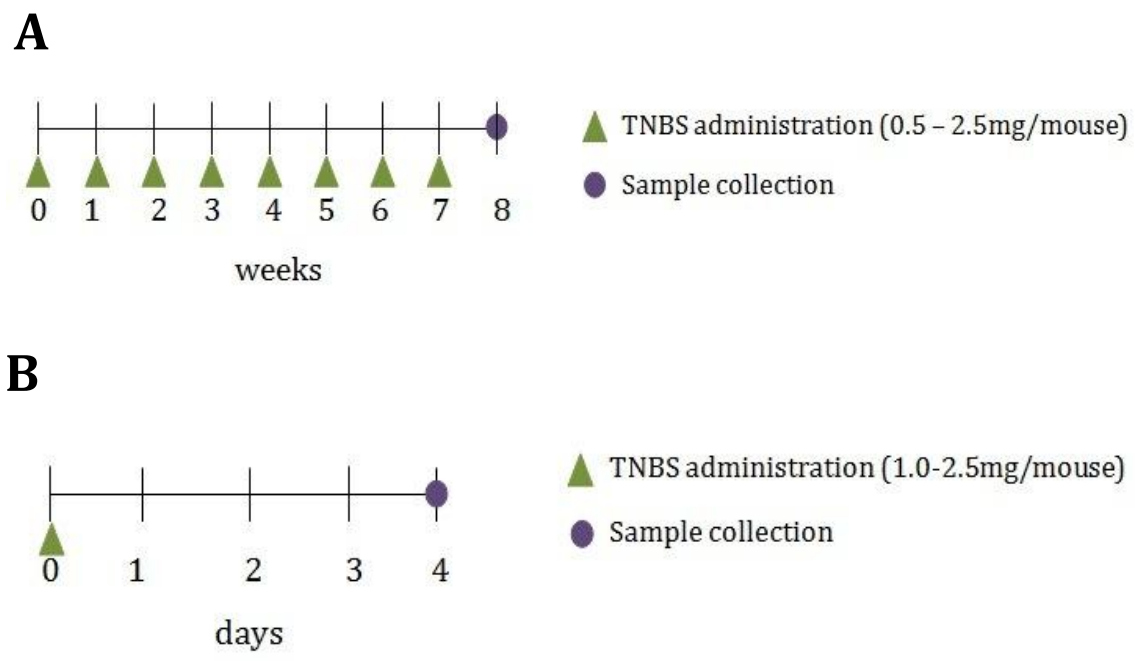


**Figure 6**: Protocol of TNBS administration. (A) Acute. (B) Chronic.

Mice were lightly anesthetized with isoflurane, and then intrarectally administered with TNBS in 45% ethanol via a 3.5 F catheter affixed to a 1-mL syringe. The catheter was advanced into the rectum to a point 4 cm proximal to the anal verge (Fig 7), and TNBS was injected to a total volume of 100 µl. To ensure distribution of TNBS within the entire colon and cecum, mice were held in a vertical head-down position for 50 seconds after the injection. Four days (acute) and ten days (chronic) *after* the final TNBS administration, mice were sacrificed for tissue collection (Fig. 6).

In DSS-induced colitis, female and male C57BL/6J mice received 3% (w/v) DSS through drinking water for six days (n=10). Approximate 40-50ml of water per cage was drunk (four mice/cage, ~10-12ml/mouse). Mice were sacrificed on day 6. In the above experiments, normal mice served as controls (n=7).

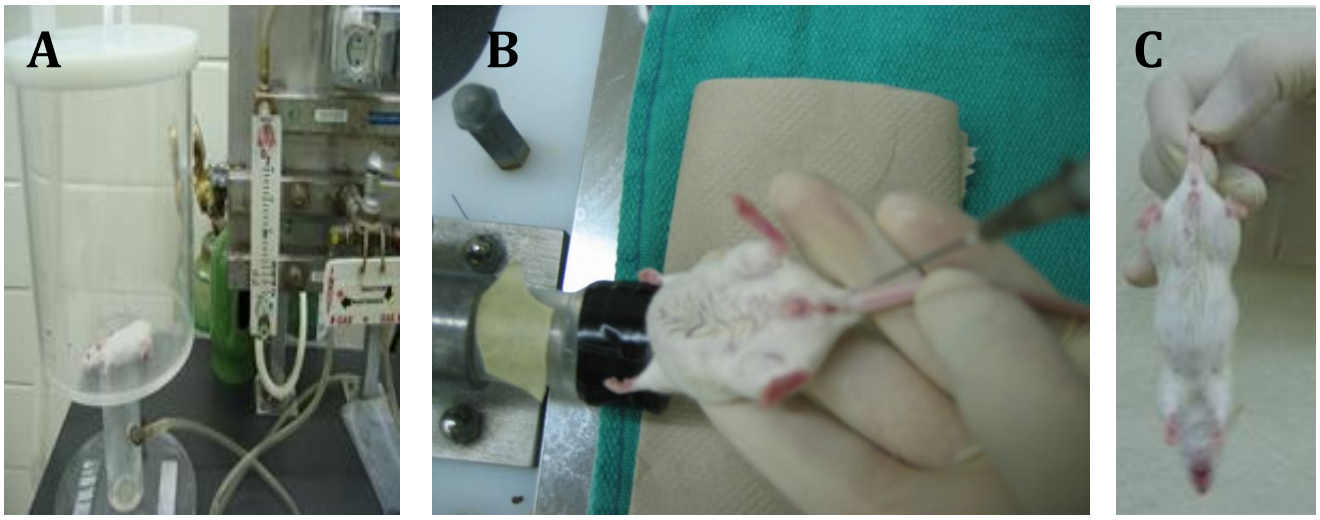


**Figure 7:** Protocol of TNBS-induction. (A) Anesthesia (B) Delivery of TNBS (C) Vertical holding (head down) to ensure complete entry of TNBS.

## III. Cav-1 Peptides

Peptides, corresponding to the full-length of the Caveolin scaffolding domain (CSD; amino acids 82-101; DGIWKASFTTFTVTKYWFYR) and of the Cav-1 binding domain (CBD1; amino acids 618-633; SLEQIWNNMTWMQWDK based on the sequence from the HIV gp41 envelope[136]) were synthesized as a fusion peptide to the C terminus of the *Antennapedia* internalization sequence (RQIKJWFQNRRMKWKK) by GenScript USA Inc (Piscataway, NJ). Before each experiment, desiccated peptides were weighed and dissolved in 10% dimethyl sulfoxide (DMSO) to 1mg/ml.

## IV. Peptide Treatment

The ability of Cav-1 to bind to a variety of kinases and inhibit their activity makes it a new therapeutic target for hyperpermeability and tumor progression. The two peptides, known to mimic and antagonize Cav-1 action, will give the ability to experimentally alter the activity of Cav-1 and gain a greater understanding of the role Cav-1 plays in inflammation.

Mice received daily intraperitoneal injections of 100µl of a 0.15mM solution of CSD or CBD1 (in 10%DMSO), a 10% solution DMSO (control), or saline (normal) on day 0, two hours after TNBS injection (for CSD, CBD1 and DMSO groups), and at the same time thereafter on days 1, 2, and 3 (Fig. 8).

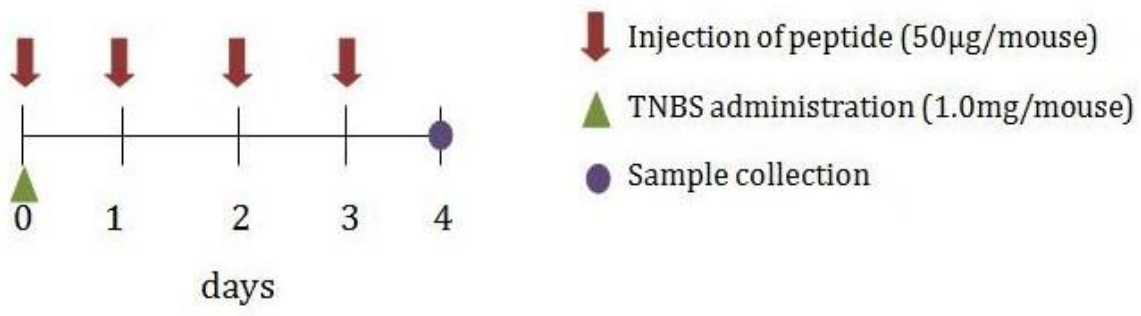


**Figure 8**: Protocol of peptide administration (n=12)

## V. Body Weight and Clinical Scores

In TNBS-induced colitis, starting body weight was measured prior to TNBS administration and weights were taken daily or weekly at the same time after that. Clinical scores were also determined at that time. Each mouse was observed and given a score (out of 20) based on appearance (0-4), behaviour (0-4), body condition (0-4), stool consistency (0-4), and rectal bleeding (0-4).

In DSS-induced colitis, DSS Disease Activity Index (DAI) was used to evaluate clinical scores according to the methods previously reported (Table 1).[164] Body weight was measured prior to DSS administration and monitored daily. Weight loss, stool consistency, and occult blood or gross bleeding scores were taken at the same time each day.

**Table 1.** Criteria for scoring Disease Activity Index*

| *Score* | *Weight loss (%)* | *Stool consistency °* | *Occult blood or gross bleeding* |
|---|---|---|---|
| 0 | None | Normal | Negative |
| 1 | 1-5 | Loose stool | Negative |
| 2 | 5-10 | Loose stool | Hemoccult positive |
| 3 | 10-15 | Diarrhea | Hemoccult postivie |
| 4 | >15 | Diarrhea | Gross bleeding |

*Disease acitivty index = (combined score of weight loss, stool consistency, bleeding)/3

°Normal stools = well-formed pellets; loose stools = pasty stool that does not stick to anus; diarrhea = liquid stools that stick to anus.

## VI. Histological examination

Colon tissue was fixed in 10% buffered formalin, for one week at room temperature and embedded in paraffin. Paraffin-embedded colon sections were cut sagittal plane(6-µm), stained with hematoxylin and eosin (H&E) and examined using an Olympus IX51 light microscope equipped with CCD camera under control of Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). The sections from each group of mice were assigned a random code to blind the examiners. Examiners were given three different parameters to estimate: severity of inflammation (based on polymorphonuclear neutrophil infiltration; 0–3: none, slight, moderate, severe), depth of injury (0–3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0–4: none, basal one-third damaged, basal two thirds damaged, only surface epithelium intact, entire crypt and epithelium lost). All values were added to a sum, in which the maximum possible score was 10.[165]

## VII. Preparation of colon tissue extracts

Colonic samples surgically removed from the mice were mechanically homogenized with a  in buffer containing 1M Tris-HCl, 3M NaCl, and 10% Triton supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis. MO) at a ratio of 1ml/100mg wet weight. Samples were then frozen (-70°C) and thawed

(37°C) three times over thirty minutes, followed by centrifugation at 14,000 *rpm* for 30 min at 4°C. Supernatants were frozen at -70°C until assay.[48]

## VIII. Measurement of cytokines by enzyme linked immunosorbent assays (ELISA)

Levels of TNF, IL-17, and IFN-γ in colon tissue extracts were measured by ELISA according to the manufacturer's instructions (BD Bioscience, Franklin Lakes, NJ). As colon tissue seizes varied, the cytokine amount in the extract was normalized to tissue weight, representing the amount per 100 milligrams.

## IX. Measurement of Cav-1 levels by ELISA

In order to quantitatively measure Cav-1 levels, a sandwich ELISA protocol was developed in which two commercial purified polyclonal anti-Cav-1 antibodies were chosen. The capture antibody was rabbit anti-human Cav-1, generated from recombinant human Cav-1 (#610060, BD Transduction Laboratories, Franklin Lakes, NJ). The detection antibody was an HRP-conjugated rabbit anti-human Cav-1 antibody, raised against a peptide mapping to the N-terminus of Cav-1 of human origin (#sc-894-HRP, Santa Cruz Biotechnology, Santa Cruz, California). To estimate relative amounts of Cav-1

and to minimize variations between assays, an in-house Cav-1 standard extract was prepared by pooling various samples of colon tissue extract from normal mice of both Balb/c and C57BL/6J species, known to have high levels of Cav-1 and defined as 1000 units/ml. The standard extract was aliquoted and stored at -85°C. Negative controls were taken from colon tissue extracts of C57BL/6J Cav-1 knock-out mice where no Cav-1 was detectable.

Costar microwell plates were coated with 1 µg/ml of capture antibody in 50 mM carbonate/bicarbonate buffer, pH 9.6 (50 µl/well) and incubated overnight at 4°C. After three washes with 0.02 M PBS containing 0.05% Tween 20 (pH 7.4), the wells were blocked with PBS containing 2.0% w/v BSA for 90 minutes at room temperature. Plates were washed once and samples (50 µl/well; 1:100 dilution with 0.2% BSA PBS) and standard extract dilutions (starting 1:20, 2-fold diluted for 11 dilutions) were added and incubated overnight at 4°C. Plates were washed three times and 50 µl of detection antibody, diluted 1:2000 in 0.2% BSA in PBS, was added. After incubation for 90 minutes at 37°C, plates were washed five times and 50 µl/well of 3,3',5,5' Tetramethylbenidine (TMB) liquid substrate system for ELISA (Sigma-Aldrich, St. Louis. MO; T 0440) was added. Plates were incubated at room temperature, in the dark, for 20 minutes. The absorbance was immediately read at 370nm. The

relative amount of Cav-1 was calculated by interpolation from the dilution curve of the standard extract.

## X. Detection of Cav-1 by Western blot

Colon tissue lysates were prepared in ice-cold SDS buffer (62.5 mM Tris-HCl, 2% SDS, 1mmPMSF, 1 mM protease inhibitor mix, and 1mM phosphatase inhibitor mix).

The proteins in colon samples were size fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on gels. Separating gel was 12% acrylamide and stacking gel was 5% acrylamide. Both upper and lower gels were prepared form a 30% acrylamide/bis-acrylamide (Fisher, Ottawa).

The Bio-Rad Protein assay kit determined total protein. Bovine serum albumin (BSA), Fraction V (Fisher, Ottawa) was used as a relative protein standard for all assays. Assay results were used to calculate the dilution required to bring all sample to a protein concentration of 3mg/ml.

Equal amounts of lysate (15µl/well) were subjected to electrophoresis. Proteins were fractioned by running the gels at constant voltage (200V) for 60-90 minutes. Fractionated proteins were transferred to nitrocellulose membranes overnight at 35V. Membranes were then blocked with a 5% w/v dried skim milk powder in Tris buffered saline containing 0.1% Tween-20 (TBST) for one hour

at room temperature. The membranes were then incubated overnight at 4°C with an HRP-conjugated rabbit anti-human Cav-1 antibody (#sc-894-HRP, Santa Cruz Biotechnology) diluted to by a factor of 1000 in TBST with 3% milk. Bands were visualized on film using enhanced chemiluminescence reagents (GE Healthcare Amersham ECL, Buckinghamshire, UK).

## XI. Statistical analyses

All experiments were completed in triplicate. Averages between the values were used. Experimental n values are reported in figure legends. Values were expressed as mean ± standard deviation. One-way analysis of varience (ANOVA) was used to compare differences between 3 or more experimental groups dependent on a single variable followed by Tukey multiple comparison test to determine if means were significantly different from one another. Unpaired t-test was used to compare two groups (GraphPad Prism, San Diego).

The results obtained were reproducible amoung replicates between dilutions and between assays.

Cytokine ELISAs were completed according to manufacture instructions and have been established in the lab for many years, thus sensitivity was well known. The sensitivity of the cav-1 ELISA was high (1.12 U/ml).

P values $< 0.05$ were considered statistically significant. In all figures, *represents $P<0.05$; **$P<0.01$; ***$P<0.001$.

# Results

## I. TNBS-induced colitis in Balb/c mice

### *a. Body weight loss, colon inflammation and cytokine levels are significantly increased in mice receiving TNBS*

As expected, mice receiving TNBS administration(s) showed significantly increased clinical scores (5.0 vs. 0, acute colitis) and displayed body weight loss (102.3 % normal vs. 98.8%, TNBS in chronic colitis at day 49; $P$ =0.02, Fig. 9). Mice receiving TNBS typically show a dip at the beginning. TNBS works by haptenation of colonic proteins, leading to a delayed-type hypersensitivity reaction by causing a Th1 reaction. This damages the colon wall and increases inflammation, resulting in severe diarrhea and loss of water, thus loss of weight.

Mice receiving TNBS also had significantly increased colon inflammation including distorted tissue architecture, inflammatory cell infiltrates and goblet cell reduction (Fig. 10 A & B). Semi-quantitative analysis showed the difference between colitis and normal mice was statistically significant ($P < 0.01$ in acute phase and $P < 0.001$ in chronic phase, Fig. 10C). After scarification, to explore possible mechanisms and anti-inflammatory effects, mouse colons were homogenized and assayed for levels of inflammatory cytokines IFN-γ, TNF and IL-17. For all three cytokines, mice that received TNBS showed increased levels

(*P's* < 0.01 for TNF and IL-17 levels in chronic phase) (Fig. 11) when compared with normal mice.

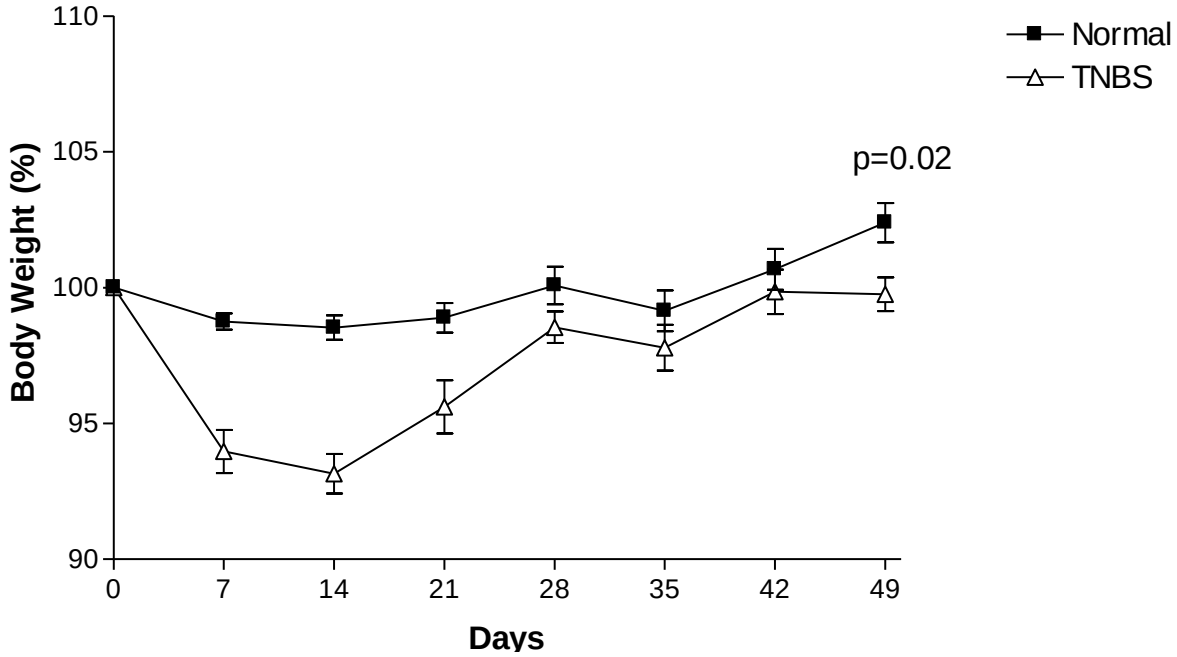


**Figure 9**: Body weight changes measured through to week 7 of TNBS-induced chronic colitis. Experimental colitis was induced by TNBS, Balb/c mice (n=12) received weekly administrations of increasing doses of TNBS eight times (1.0 – 2.3 mg in 45% ethanol). Comparison of body weight between Normal and TNBS on day 49, to determine ability to recover body weight, resulted in p=0.02.

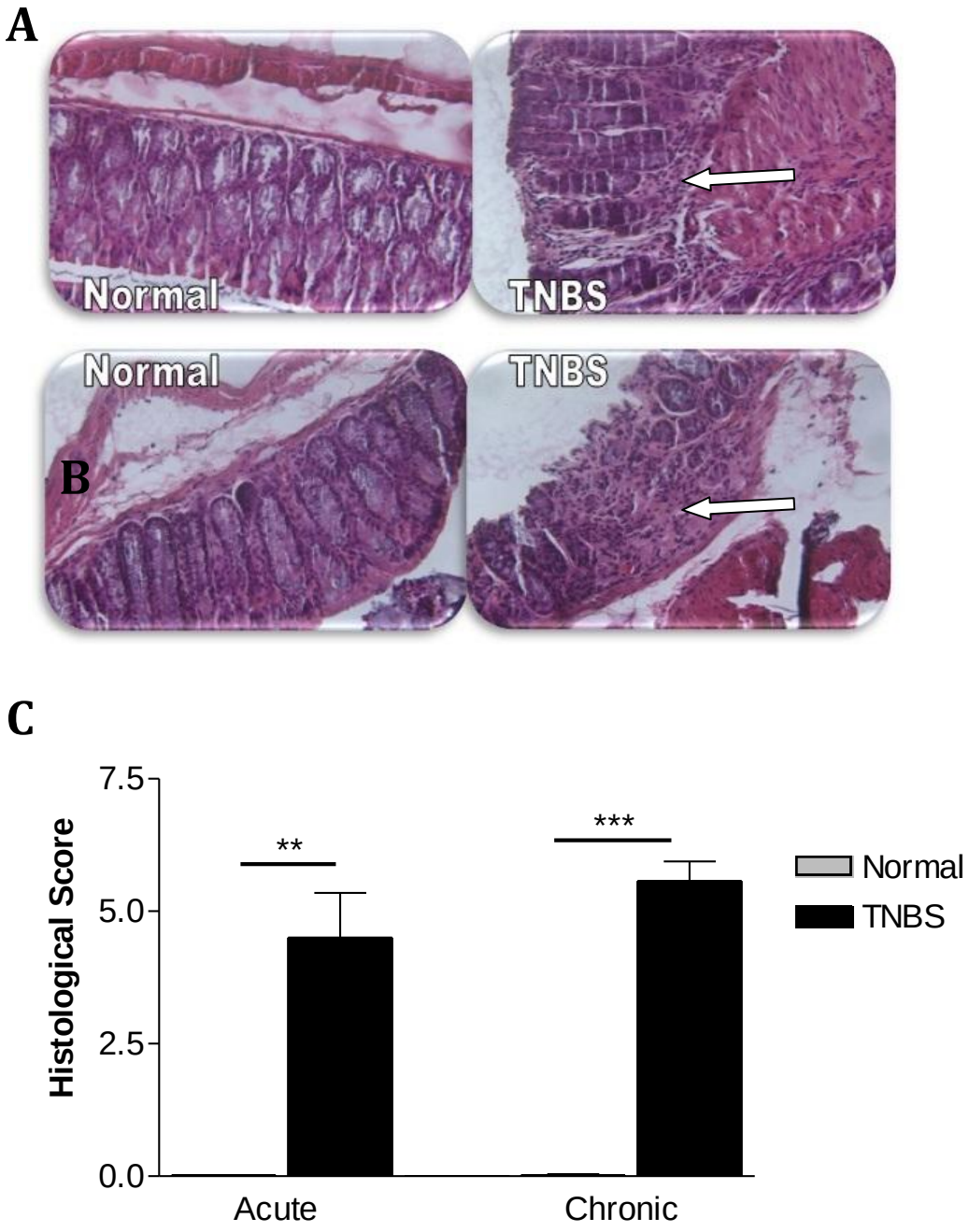


**Figure 10:** Histological analysis of colon sections stained with H&E in TNBS-induced colitis in Balb/c mice (n=12). (A) Representative pictures of inflammation in mouse colon tissue, acute. Mice receiving TNBS had distorted tissue architecture and inflammatory cell infiltrates (arrow) (B) Representative pictures of inflammation in mouse colon tissue, chronic IBD. Mice receiving TNBS had distorted tissue architecture and inflammatory cell infiltrates (arrow) (C) Semi-quantitative evaluation. Colon sections were semi-quantitatively evaluated double-blindly by a pathologist. Scores determined by severity of inflammation 0-3; depth of injury 0-3; and crypt damage 0-4. **P<0.01; ***P<0.001

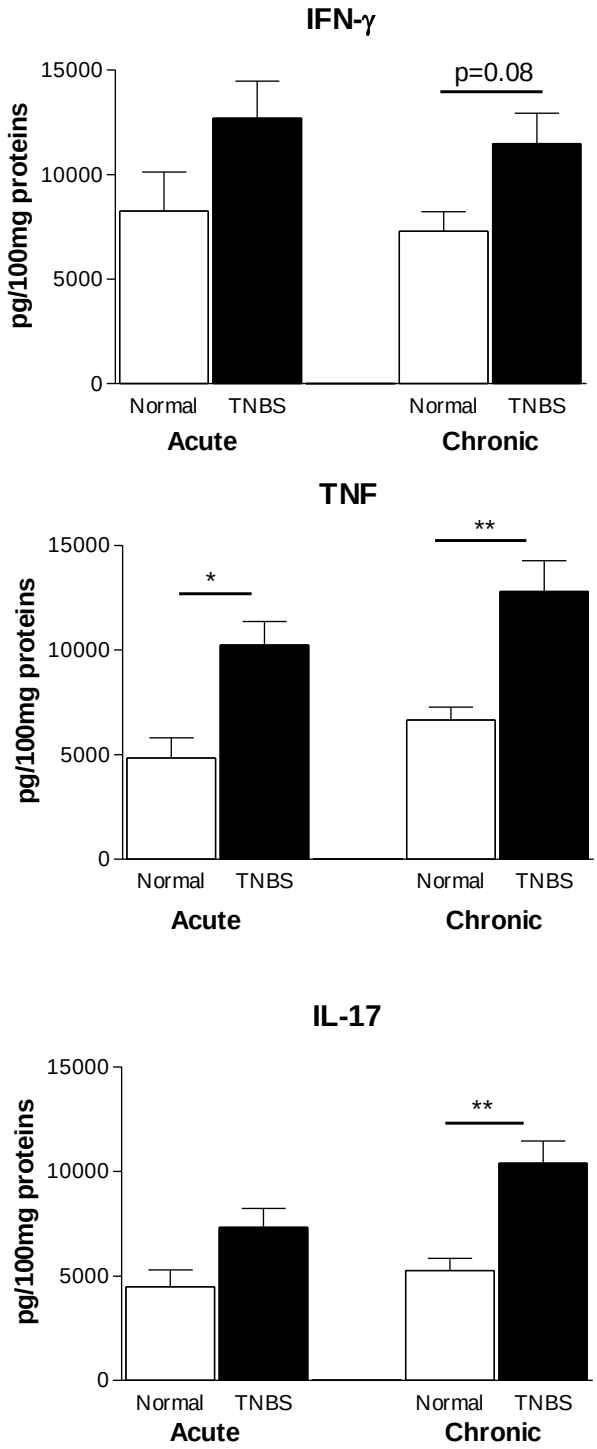


**Figure 11:** Cytokine levels in TNBS-induced colitis Balb/c mice (n=12) homogenized colon tissue after receving TNBS-induced colitis for five days (acute) or seven weeks (chronic) Measured by sandwich ELISA. Each experiment was duplicated and performed in triplicate. Cytokine amount in extract was normalized to tissue weight, representing the amount per 100 grams. *P<0.05; **P<0.01

### *b. Cav-1 levels were reduced and inversely correlated with inflammation and cytokine levels in TNBS-induced colitis*

We examined colonic Cav-1 levels in mice with acute and chronic TNBS-induced colitis, using two methods, ELISA and Western blot. Interestingly, mice receiving TNBS had lower levels of Cav-1 compared to normal mice as shown by both Western blot and ELISA results (Fig. 12 A&B). In fact, in the chronic study, this decrease was especially significant ($P < 0.001$). To determine the connection between Cav-1 and inflammation and cytokine levels, a correlation analysis was performed. Colonic Cav-1 levels strongly trended towards being inversely correlated with colon inflammatory scores in the chronic phase ($P = 0.056$, r=-0.679, Fig. 13 A&B). Cav-1 levels were also inversely correlated with cytokine levels significantly (*P's* < 0.005; r= -0.90 for IFN-γ; r= -0.91 for TNF; and r= -0.93 for IL-17 in chronic phase, Fig. 9 C).

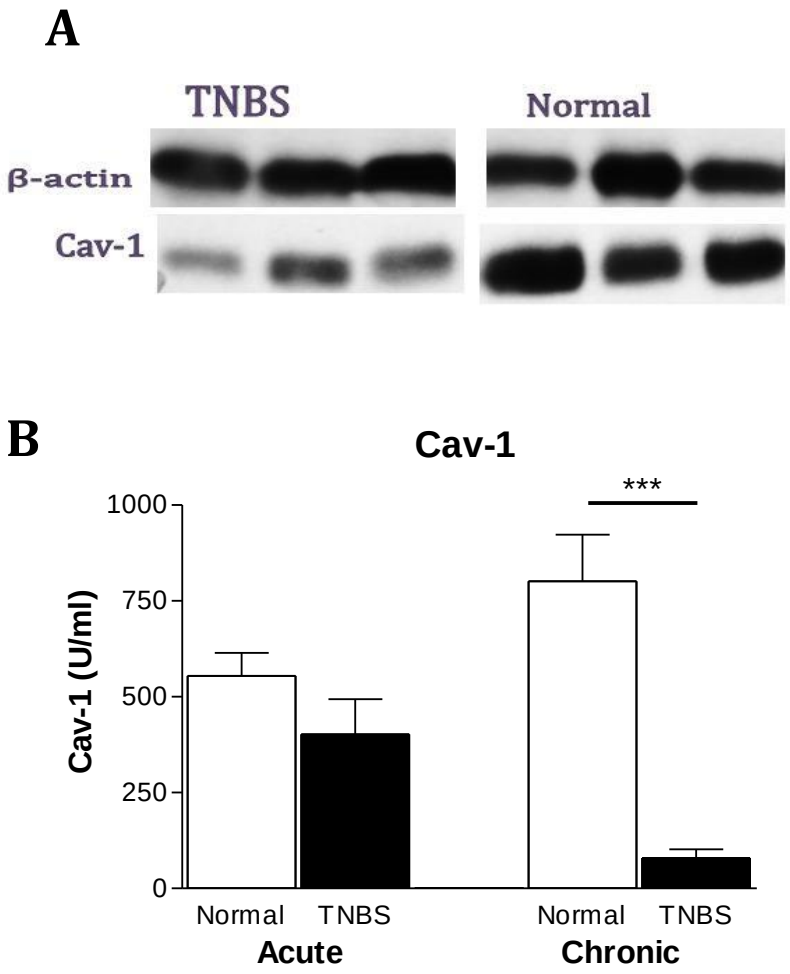


**Figure 12:** Detection of colon Cav-1 levels in TNBS-induced colitis in Balb/c mice (n=12) (A) Representative picture of Cav-1 levels detected by Westernblot. Three different mice for each group shown. Acute study. 3mg/ml loaded. (B) Cav-1 levels measured by ELISA. Acute and Chronic studies. ***P<0.001

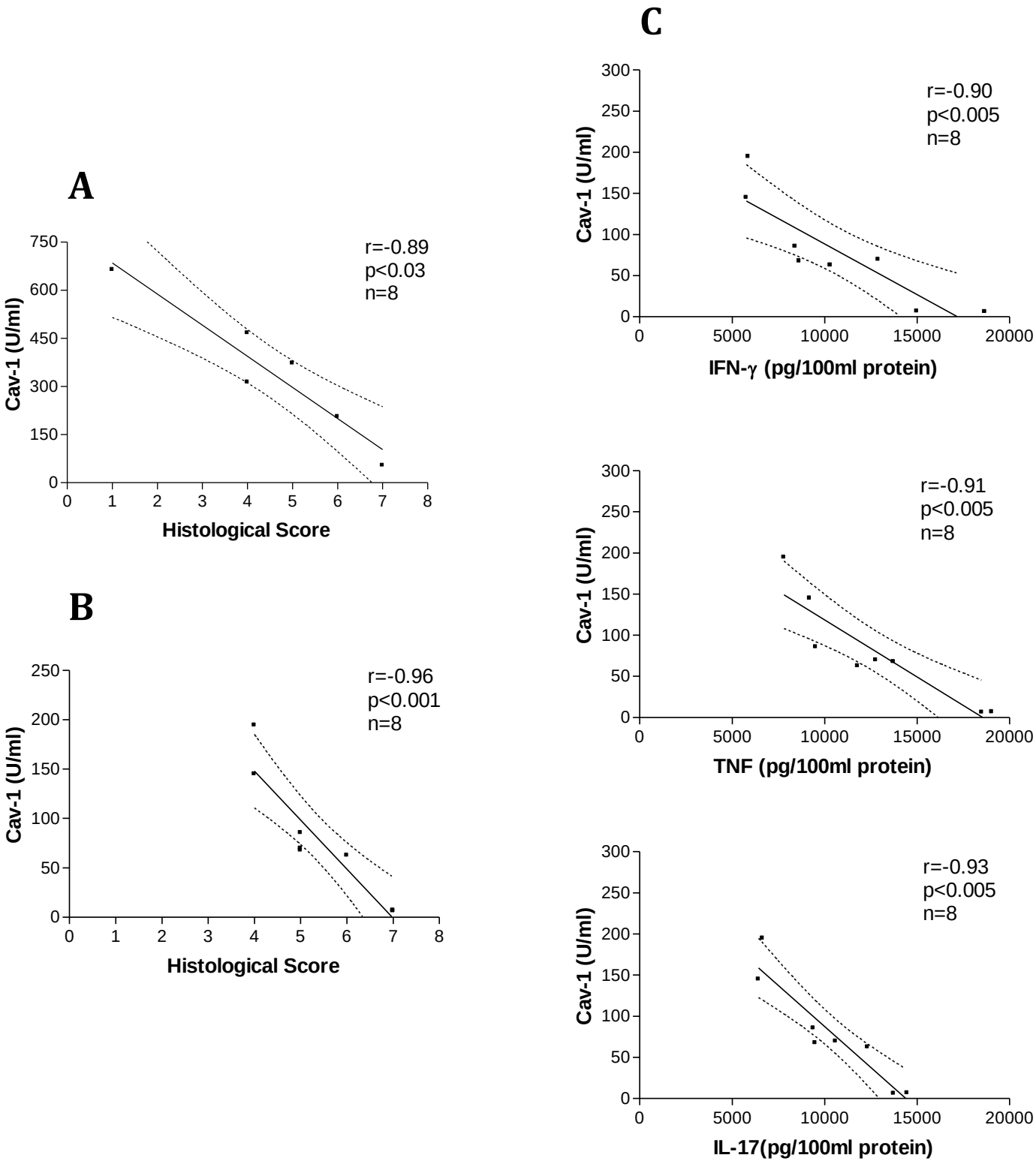


**Figure 13:** Correlation of Cav-1 levels of TNBS-induced colitis in Balb/c mice (n=12) with (A) inflammation, acute; (B) inflammation, chronic; (C) cytokine levels, chronic

## II. TNBS-induced colitis in C57BL/6J and Cav-1 knockout mice (Cav-1$^{-/-}$)

### *a. Genetic deletion of Cav-1 results in increased clinical symptoms*

To further confirm our findings that Cav-1 may play a protective role in TNBS-induced colitis, mice genetically deficient in Cav-1 underwent the acute TNBS-induced colitis protocol. Mice genetically knocked down of the Cav-1 gene were only of the C57BL/6J species. All experiments performed with Balb/c (previous stated) were redone with the control group of the C57BL/6J knock out species. As shown in Fig. 14, mice receiving TNBS have increased body weight loss and increased clinical scoring compared with controls. In agreement with our above findings, but not with those previously reported by others in which Cav-1$^{-/-}$ mice had significantly decreased colon inflammation in DSS-induced acute colitis,[159] mice lacking Cav-1 (Cav-1$^{-/-}$) and receiving TNBS showed significantly greater body weight loss and higher clinical scores than their wild-type counterparts (Cav-1$^{+/+}$) receiving TNBS. Also, Cav-1$^{-/-}$ mice showed signs of more severe inflammation ($P < 0.01$), including inflammatory cell infiltration and distorted tissue architecture, which was analyzed semi-quantitatively, compared to wild-type mice receiving TNBS (Fig. 15). As shown in Fig. 15, Cav-1$^{-/-}$ mice showed non-detectable Cav-1 levels.

Taking together, the above data indicate that Cav-1 may play a protective role in modulating disease activity and tissue inflammation in murine TNBS-induced colitis, as absence of Cav-1 results in an increase in inflammation and loss of body weight.

### *b. Cav-1 levels are decreased in TNBS-induced C57BL/6J mice*

To determine whether Cav-1 levels were different in a different mouse strain and to provide a control for the knockout species, wild-type C57BL/6J mice received one dose of TNBS administration. As in TNBS-induced colitis, Cav-1 levels were significantly decreased in mice receiving TNBS when compared to wild-type normal mice (Fig. 16).

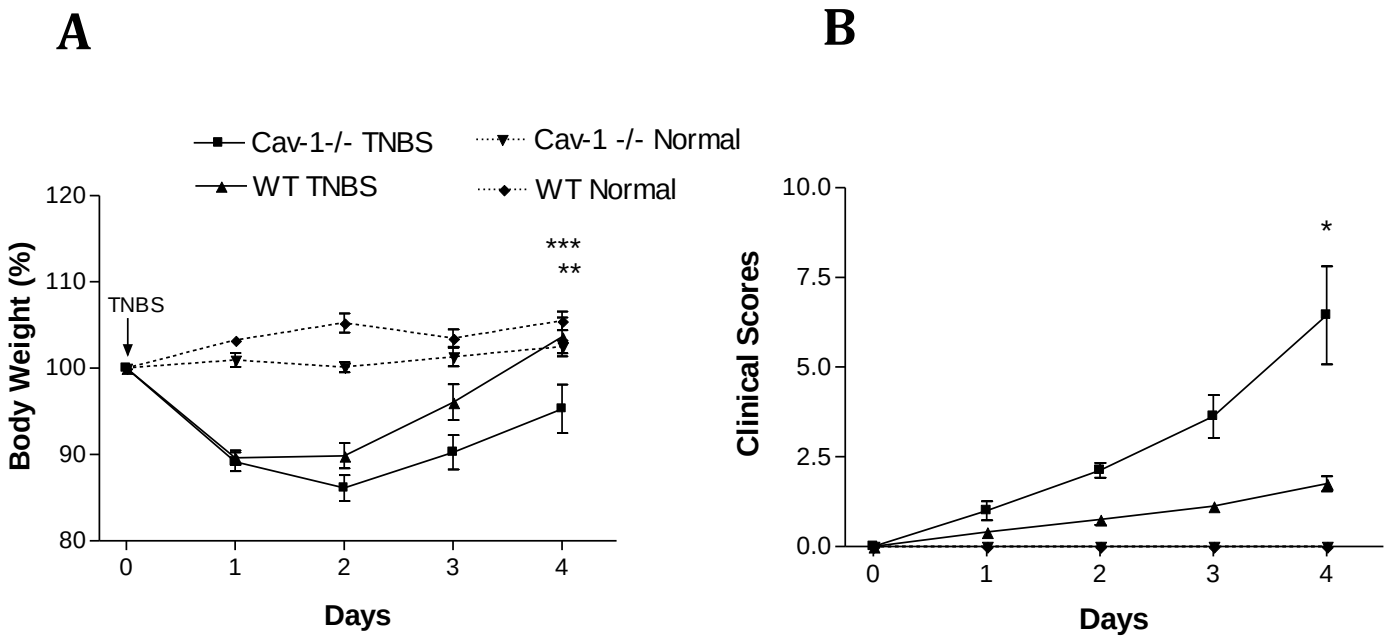


**Figure 14**: Acute TNBS-induced colitis for C57BL/6J Cav-1 knockout mice (n= 9 (Cav-1 -/- TNBS), n=12 (wild-type TNBS), n=3 (Cav-1 -/- normal), n=7 (wild-type normal)) (A) Body weight. **P<0.01, Cav-$1^{-/-}$ TNBS vs WT Normal; ***P<0.001, Cav-$1^{-/-}$ TNBS vo WT TNBS (B) Clinical Scores. *P<0.05, Cav-$1^{-/-}$ TNBS vo WT TNBS

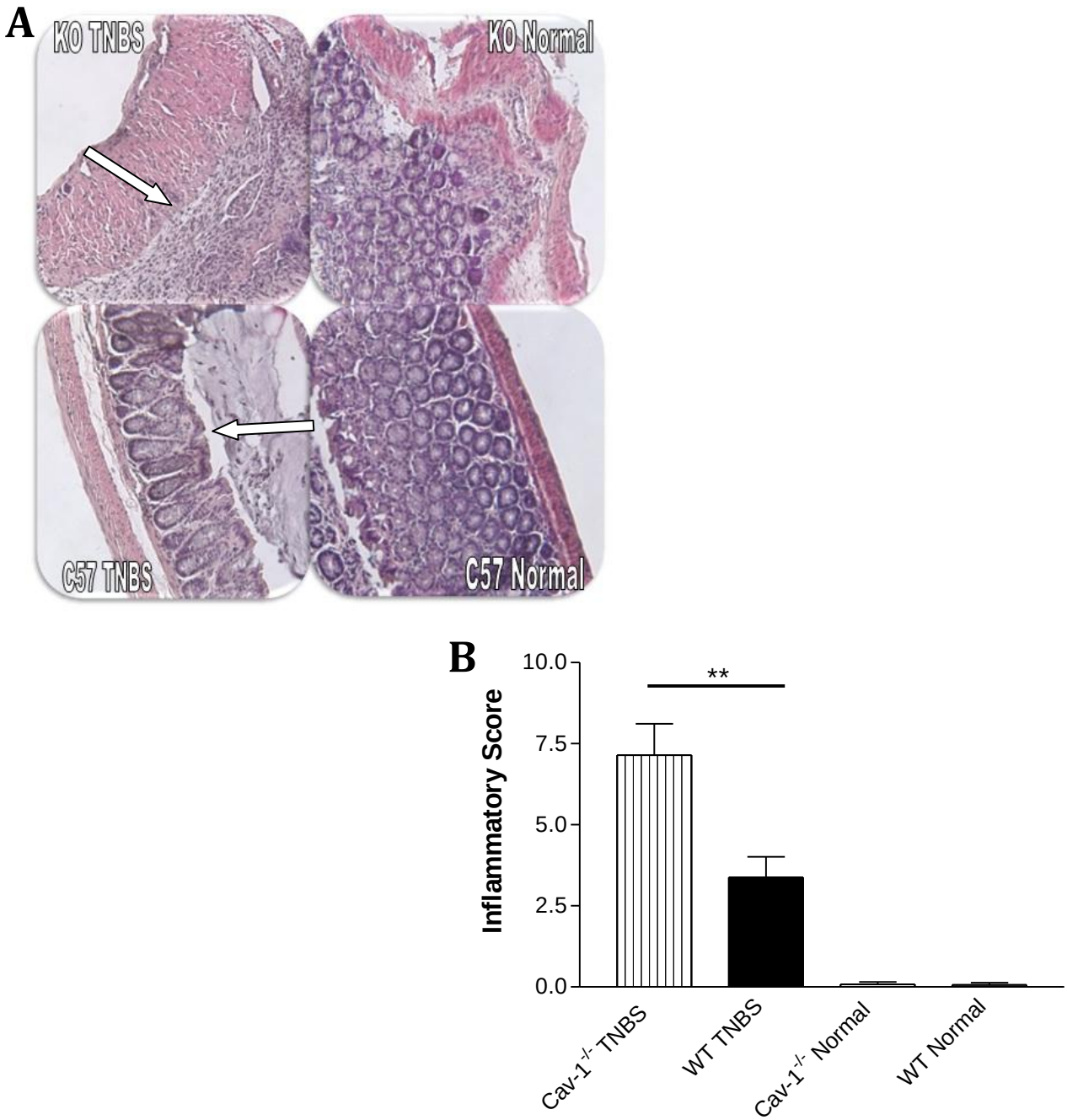


**Figure 15:** Histological analysis of colon sections stained with H&E in acute TNBS-induced colitis for Cav-1 knockout mice (n= 9 (Cav-1 $^{-/-}$ TNBS), n=12 (wild-type TNBS), n=3 (Cav-1 -/- normal), n=7 (wild-type normal)) (A) Colonic specimens were formalin-fixed and embedded in paraffin blocks, then 6-µm sections were stained with hematoxylin and eosin. Representative histological images of samples from normal control and TNBS-treated mice following treatment with vaccines, carrier or saline are shown (original magnification x 100). Mice receiving TNBS had distorted tissue architecture and inflammatory cell infiltrates, with Cav-1 $^{-/-}$ TNBS showing severe loss of tissue architecture (arrows). (B) Semi-quantitative evaluation. Colon sections were semi-quantitatively evaluated double-blindly by a pathologist. Scores determined by severity of inflammation 0-3; depth of injury 0-3; and crypt damage 0-4. **P<0.01

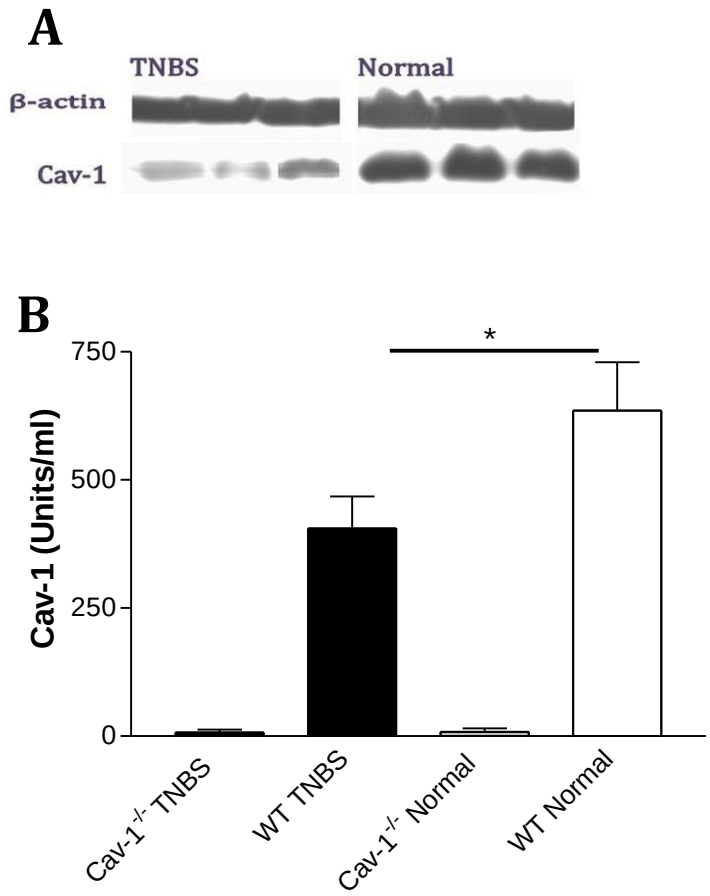


**Figure 16:** Detection of colon caveolin-1 levels in TNBS-induced colitis for C57BL/6J Cav-1 knockout mice (n= 9 (Cav-1 -/- TNBS), n=12 (wild-type TNBS), n=3 (Cav-1 -/- normal), n=7 (wild-type normal)) (A) Cav-1 levels detected by Westernblot, three representative mice from wild-type TNBS(n=12) and wild-type normal (n=7) Cav-$1^{-/-}$ lanes did not undergo luminescence, proving Cav-1 was not present (not shown)(B) Cav-1 levels measured by ELISA for all four groups. Cav-1 levels are non detectable in Cav-1 knockout mice. *P<0.05 WT TNBS vs WT Normal

## III. DSS–induced colitis in C57BL/6J mice

### *a. Cav-1 expression shows no significant difference between groups*

Since our findings were different from what was previously reported by Chidlow et al. 2009, in which colon Cav-1 levels were significantly increased in male C57BL/6J mice with DSS-induced acute colitis, we evaluated the levels of Cav-1 in 3% DSS-induced colitis in both female and male C57BL/6J mice. Both sexes of mice showed expected increase in Disease Activity Index (DAI) and significantly increased colon length shortening (Fig 17). However, neither group showed any significant change in Cav-1 levels in colon tissue (Fig. 18).

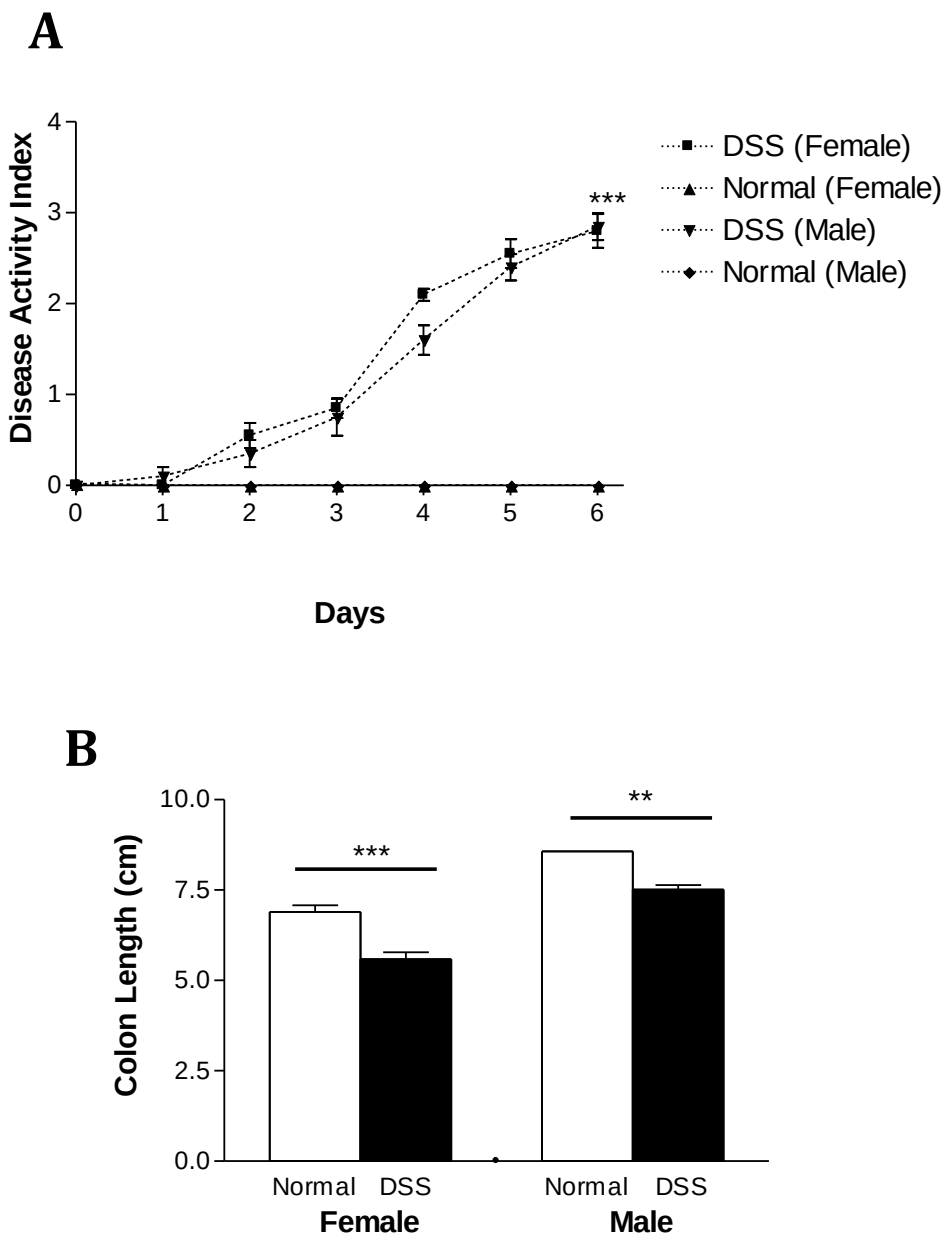


**Figure 17**: Acute DSS-induced colitis for C57BL/6J mice, male and female (n=10). (A) Disease activity index score. Normal mice show no DAI. ***P<0.001, DSS vs Normal (male and female). (B) Colon length. ***P<0.001, Normal vs DSS, Female. **P<0.01 Normal vs DSS, Male

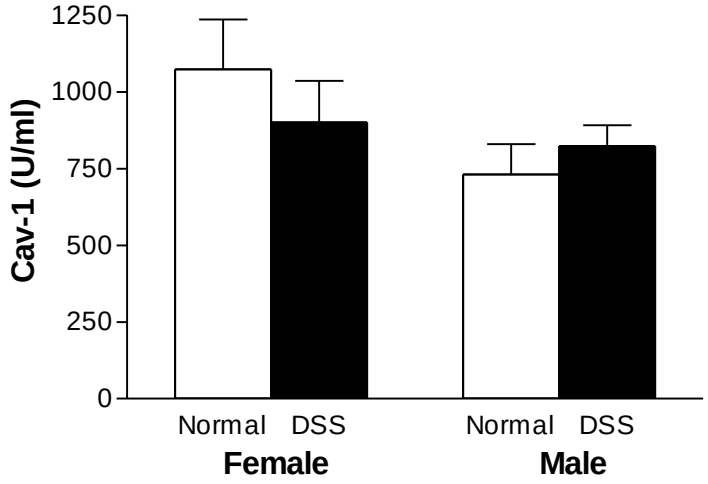


**Figure 18:** Colon Cav-1 levels for acute DSS-induced colitis (n=10), male and female, measured by ELISA. No statistical significance was ascertained.

## IV. Cell permeable peptides CSD and CBD1

### *a. Cav-1 peptides altered the clinical symptoms of TNBS-induced acute colitis in Balb/c mice*

Using the two peptides (CSD and CBD1), we evaluated how mimicking the ability of Cav-1 to bind or antagonizing the interaction of Cav-1 with binding partners would affect the duration and intensity of TNBS-induced murine colitis, as well as have an effect on the onset of the disease. Mice receiving the CSD peptide had significantly increased body weight loss and clinical scores. Whereas mice receiving CBD1 showed body weight recovery similar to normal and a decrease in clinical scores (Fig. 19). To further support our findings, mice receiving the CSD peptide had significantly higher inflammatory scores than the wild-type (WT) control, while mice receiving the CBD1 peptide showed decreased inflammation (Fig 20).

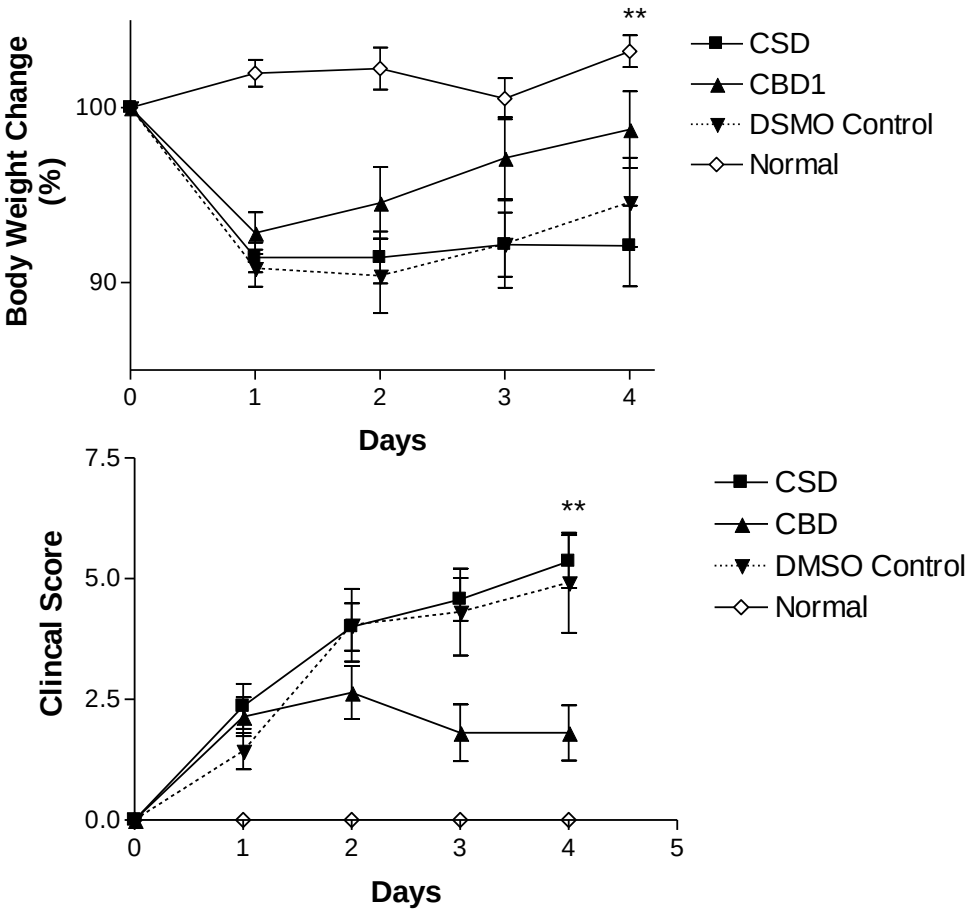


**Figure 19:** Peptide injection in TNBS-induced acute colitis for Balb/c mice (n=12). (A) Body weight change. Mice receiving TNBS (CSD, CBD1, DMSO) showed characteristic weight drop. **P<0.01 CSD vs Normal(B) Clinical scores. **P<0.01 CSD vs Normal; DMSO vs Normal

*b. Cav-1 levels are altered with cell permeable peptides; correlate with inflammatory scores.*

Using Western blot (not shown) and ELISA to measure colonic Cav-1 levels, we observed that mice receiving the CBD1 peptide had near normal levels of Cav-1 – significantly higher than the CSD group as well as the DMSO control group (Fig. 21). In support of our previous findings, mice which had higher Cav-1 levels (those receiving CBD1 or normal mice) had significantly lower inflammatory scores than those with lower Cav-1 levels (CSD1 and DMSO). In fact, mice receiving CSD showed a significant increase in inflammation compared to DMSO control (Fig. 20).

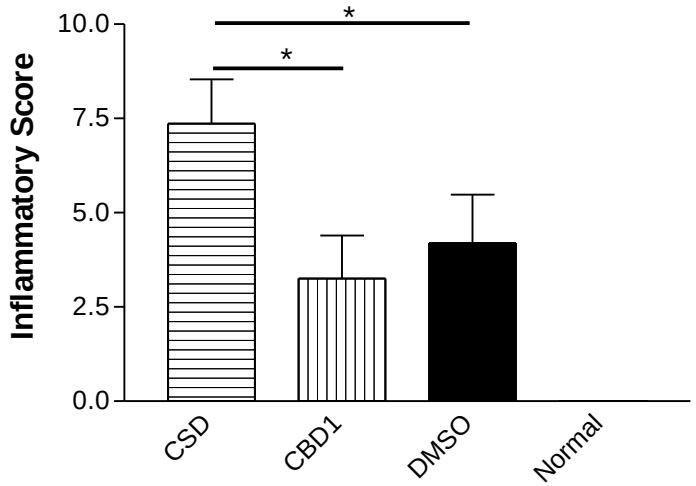


**Figure 20:** Histological analysis of colon sections stained with H&E in acute TNBS-induced colitis for peptide injected mice (n=12). Semi-quantitative evaluation. Colon sections were semi-quantitatively evaluated double-blindly by a pathologist. Scores determined by severity of inflammation 0-3; depth of injury 0-3; and crypt damage 0-4.*P<0.05

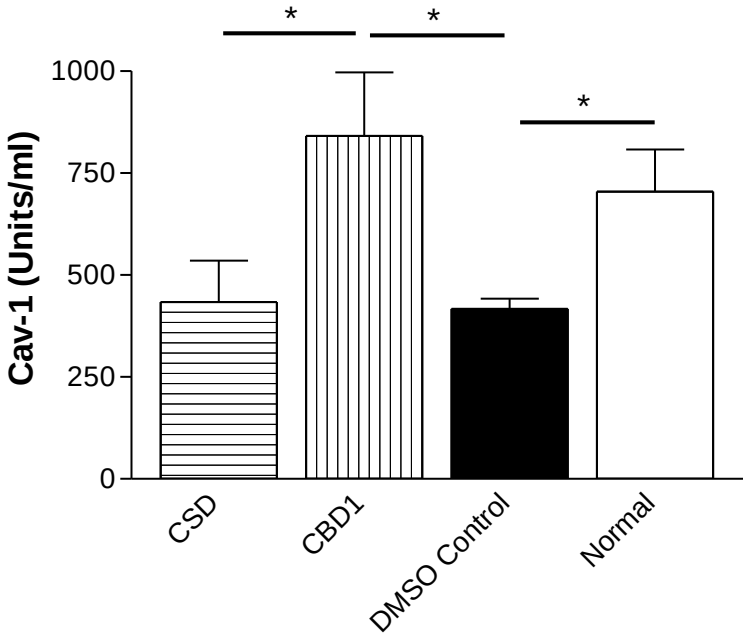


**Figure 21:** Colon Cav-1 levels for peptide injected acute TNBS-induced colitis, measured by ELISA (n=12).

# Discussion

Inflammatory bowel disease is a complicated, multi-factor disease. Studies are providing evidence that the pathogenesis of IBD is associated with genetic susceptibility, altered enteric flora, infectious agents and environmental triggers, and impaired immune regulation.[9, 159]

The resulting idiopathic, chronic, and relapsing inflammation is a key focus in research today. This abnormal inflammation may be a result of an imbalance between pro- and anti-inflammatory cytokines from an overly aggressive immune response.[166]

Crohn's disease and ulcerative colitis are characterized by inappropriate activation of the innate and adaptive arms of the immune system thought to result from many contributing factors (genetic and environmental factors, enteric flora, and immunological abnormalities), as well as a loss of tolerance to enteric commensal bacteria. Macrophages and dendritic cells are increased in absolute number and have an activated phenotype, and the production of local proinflammatory cytokines is enhanced in IBD.[84, 167, 168]

Recently there has been a discovery of 99 published loci/genes associated with in IBD. Amongst these are genes involved in multiple signalling pathways.[169-171] Various signaling pathways are involved in immune cell activation. [172, 173] Caveolae create an ideal domain for promoting cell signaling in

general. Not only do they localize many different types of receptors, they also directly interact with various signaling molecules. Cav-1 has been shown to play a role in a number of different inflammatory conditions. Most notably is the role of Cav-1 in endothelial nitric oxide synthase (eNOS) activation.[83, 174] eNOS and various downstream targets of the nitric oxide signaling cascade are concentrated in caveolae. eNOS interacts with the scaffolding domain of caveolin-1, causing eNOS to remain in an inactive state.[119] As a loss of eNOS results in dramatically increased disease activity in DSS experimental colitis, this further elaborates the possible role of Cav-1 in IBD.[83]

In 1994, Lisanti first described the role of Cav-1 in the lungs.[175] Since then, studies have shown the multiple roles Cav-1 plays in lung injury. Cav-1 regulates acute inflammation and capillary leakage during acute lung injury.[151] It also suppresses proliferative activity of airway smooth muscle cells, while the expression, distribution, and associations of receptors and signalling proteins contribute to contractile responses.[176] The ability of Cav-1 to alter signalling pathways or regulate membrane trafficking appears to directly influence cell response. For this reason, we believed that Cav-1 may alter signaling pathways and regulate inflammation in colon tissue.

Employing a TNBS-induced murine colitis model, we induced both acute and chronic colitis in Balb/c mice and acute colitis in Cav-1$^{-/-}$ and wild-type mice.

To quantitatively determine colonic Cav-1 levels, we developed a sandwich ELISA to measure Cav-1 levels in colon tissue lysates. We found that colon Cav-1 was significantly reduced in mice with TNBS-induced acute and chronic colitis in both Balb/c (Fig. 8) and C57BL/6J (Fig. 12B, right panel) mice. Western bloting was performed supporting the ELISA findings, as Cav-1 bands in TNBS-colitis mice appeared less dense than that in normal mice (Fig. 8A & 12A). Mice receiving TNBS had significantly decreased Cav-1 levels ($P < 0.001$ in chronic colitis) – a very different result than that reported in a prior study.[159] Further investigation showed that Cav-1 levels inversely correlated with colon inflammation and levels of inflammatory cytokines IL-17, IFN-γ and TNF (*P's* < 0.05*)* (Fig. 9) in colon tissues. In both the acute and chronic models, mice with low levels of Cav-1 had higher levels of colon inflammation, while high levels of Cav-1 showed low levels of inflammation (r=0.885, $P = 0.003$ in the acute phase). Thus, we believe that Cav-1 may play a protective role in TNBS-induced colitis.

To further understand the relevance of our findings in TNBS-induced colitis, Cav-1 knock-out mice were used to determine what effect the complete loss of caveolin-1 would have on inflammation in TNBS-induced experimental colitis. Mice lacking Cav-1 (Cav-$1^{-/-}$) and receiving TNBS showed decreased body weight recovery and increased clinical scores, compared to wild-type mice receiving TNBS (Fig. 10). After H&E staining, colon sections were semi-

quantitatively evaluated in a double-blinded protocol by a pathologist. Cav-1$^{-/-}$ mice receiving TNBS showed signs of more severe colon inflammation than wild-type mice receiving TNBS (Fig. 11). This supports my hypothesis that Cav-1 may play a protective role in regards to TNBS-induced intestinal inflammation.

A protective role for Cav-1 against inflammation could be due to the involvement of Cav-1 in cellular signalling. During caveolae associated signalling, Cav-1 acts as a scaffold protein – it binds and organizes various signalling complexes involved in diverse cell activities.[109] As a result, removing or mutating caveolin-1 may result in a number of disease and normal homeostatic processes being affected. Cav-1 has been shown to have a number of anti-inflammatory effects: through the regulation of eNOS, which is involved with the hyperaemia and permeability changes association with acute inflammation;[119] by inhibiting inflammatory mediators and promoting anti-inflammatory cytokines through alveolar macrophages;[177] or by suppressing airway smooth muscle cell proliferation and orchestrating receptor-mediated signal transduction that regulates phenotype expression of airway smooth muscle cells.[176] Studies have also reported that Cav-1 protects against the effects of sepsis by modulating inflammatory responses, alleviating bacterial burden, and suppressing thymocyte apoptosis.[155]

Cav-1 also has an inhibitory effect on cytokine production through several mechanisms.[152] It has been widely accepted that Crohn's disease is caused by an overly aggressive Th1 and Th17 immune response.[19] The IL-23/Th17 response is also critical for the development of chronic intestinal inflammation.[178] Recent studies demonstrate Cav-1 is involved with Toll-like receptor 4 (TLR4) in peritoneal macrophages, suggesting that regulation of TLR4 function may occur within caveolae or lipid raft microdomains.[179, 180] In fact, Wang *et al*, identified Cav-1 binding motifs within the amino acid sequence of murine TLR4.[179] Mutating this binding site abolished the interaction and reversed the inhibitory effect of Cav-1 on cytokine regulation (TNF, IL-6), indicating that Cav-1 is able to inhibit TNF production induced via TLR4. Thus, as we have seen in TNBS-induced experimental colitis, a decrease in Cav-1 is associated with higher tissue levels of TNF. The possibility that this may be due to a change in TLR4 regulation will require further study. Notably, experiments done by Tang *et. al*, showed that TLR4 activation is required for IL-17-induced tissue inflammation and wasting.[181] Our study has revealed an inverse correlation between Cav-1 and IL-17 levels in the colon. This begs speculation that reduced Cav-1 may be linked to reduced inhibitory effects through TLR4, thus leading to the increase of IL-17. Studies have also reported that Cav-1 can inhibit TGFβ signaling and reduced

Cav-1 expression is associated with the activation of TGFβ signaling.[182] Taken together, these studies, and my own, show an anti-inflammatory effect of Cav-1.

Due to the conflicting results between our study and a previously published report, we re-evaluated Cav-1 levels in DSS-induced C57BL/6J male and female mice following the experimental procedure previously reported.[159] Daily evaluation of both male and female mice showed increased disease activity index and increased colon length shortening. However, ELISA determination of Cav-1 levels showed no significant differences between treated and untreated mice. This may be possibly due to the higher levels of Cav-1. A better standard for Cav-1 may alleviate this problem.

There are a few possible explanations as to why there are opposing findings for the role of caveolin-1 in experimental mouse colitis in the two studies. First, the animal models used are different. IBD is a complex interaction of genes, environment, and intestinal flora. Much of what we know about IBD is gathered from our use of animal models that, although having many similar features to IBD, also have their differences. Intrarectal delivery of TNBS induces colitis by haptenation of colonic proteins, leading to a delayed-type hypersensitivity reaction by causing Th1 and Th17 responses, the colitis similar to human CD; while DSS-induced colitis more likely represents UC.[84] In the two mouse models, distinctive disease-specific cytokine profiles were identified.

TNBS-induced colitis exhibits heightened Th1/Th17 responses (increased IL-12 and IL-17) as the disease becomes chronic. In contrast, DSS-induced colitis switches from Th1/Th17-mediated acute inflammation to a predominant Th2-mediated inflammatory response in the chronic state.[19, 24] As Cav-1 is particularly abundant in endothelial cells, the two chemicals, TNBS and DSS, may affect endothelial cells differently.

Second, Cav-1 is a very complex membrane protein. Evidence indicates that Cav-1 function is cell context dependent resulting in different roles in diseases depending on stage or type, even being able to play opposite roles in the same disease. For example, Cav-1 can act as both a tumor suppressor and as a tumor promoter.[183] In breast cancer cells, Cav-1 expression is significantly lowered when compared with normal breast tissue.[184] This decrease in Cav-1 (mRNA and protein level) is also seen in gastric, colon, and ovarian cancer cell lines.[145-147] On the other hand, Cav-1 expression is elevated in carcinoma of the thyroid,[148] associated with tumor dedifferentiation in bladder cancer,[149] and enhanced the invasive capability of lung cancer cells lines.[150]

Cav-1 may positively or negatively influence the development of atherosclerosis, depending on the cell type and the metabolic pathways regulated by this protein. Endothelial-specific overexpression of Cav-1 accelerates atherosclerosis in apolipoprotein E-deficient mice,[140] whereas,

PPARgamma1-induced Cav-1 attenuates atherosclerosis in apolipoprotein E-deficient mice.[185] In the cardiovascular system, one study shows that Cav-1 induces cardio-protection through epigenetic regulation,[142] however, in biventricular damaged rodents, Cav-1 knock-out mice displayed decreased damage as well as decreased transcript levels of the proinflammatory marker plasminogen activator inhibitor-1.[186]

In lung injury, Cav-1 switches roles depending on the stage of the disease. In the initial state of acute lung injury, Cav-1 contributes to polymorphonuclear neutrophil-mediated inflammation, vascular injury, and non-cardiogenic pulmonary edema.

Yet in the late stage, Cav-1 may be beneficial as a potential antifibrotic protein.[151, 152] Given the role that Cav-1 plays in a number of cytokine signaling pathways and the fact that it can positively and negatively influence various diseases, it is not surprising that our finding of the protective role of Cav-1 is opposite to that previously reported.

To further investigate the effect of Cav-1 on IBD pathogenesis, one peptide that mimics the Cav-1 scaffolding domain (CSD), and another peptide that binds to the Cav-1 domain (CBD1), antagonizing the function of Cav-1, were synthesized and injected intraperitoneally into mice. Previous work has found that CSD mimics Cav-1 by inhibiting the functional activity of broad classes of

proteins in vitro (eNOS, G-protein, α-subunits, Ras, Src).[187] CBD1 has been thought to penetrate the cell membrane and interact with the cytofacial side of caveolin. This domain may be responsible for the translocation of extra-cellular caveolin-binding proteins allowing their interaction with Cav-1.[137]

As our data showed that Cav-1 played a protective role in intestinal inflammation, we thought CSD (that mimics Cav-1, thereby preventing association with endogenous Cav-1) would alleviate experimental colitis symptoms, while CBD1 (that antagonizes Cav-1 function) would exacerbate the symptoms.

Results showed that, mice receiving CSD showed similar low levels of Cav-1 to the wild-type mice, while mice receiving CBD1 showed increased Cav-1 levels. In fact, CBD1 group levels were near normal (those not receiving TNBS) and significantly higher than both CSD and wild-type groups ($P < 0.05$). Conversely, the Cav-1 levels did correlate with TNBS-induced colitis symptoms. Mice receiving the CSD peptide (low Cav-1 levels) showed increased body weight loss and clinical scores ($P <0.05$) compared to normal and wild-type mice (DMSO control). These mice also had increased inflammatory scores ($P<0.01$).

On the other hand, the CBD1 group (near normal Cav-1 levels) had less body weight loss ($P<0.01$) and significantly lower clinical scores ($P<0.01$) than the CSD or DMSO groups. The expected role of CBD1 in blocking downstream

Cav-1-dependent signaling pathways does not easily explain these effects since our data suggests Cav-1 signaling is actually clinically beneficial in colitis models. Thus, it is possible that the effects are rather due to a novel function for CBD1 in increasing Cav-1 levels through an unknown mechanism.

The question remains as to how much of the peptide actual travels to the colon tissue, and how much is dispersed throughout the body of the mouse. As results show changes in Cav-1 colon tissue levels, it is believed that peptides are arriving in the intestines. Further studies, involving labeling of the peptides to see amount, location, and action of peptides, is needed as the administration of each peptide did alter disease activity and demonstrate a correlation between Cav-1 levels and colitis development.

# Conclusion

We conclude that in TNBS-induced colitis, Cav-1 may play a protective role in experimental colitis inflammation. This suggests that enhancement of Cav-1 levels or function may be a beneficial IBD treatment. As the results are opposite to those previously reported in a DSS-induced colitis model, it raises an important issue regarding evaluations of molecule or treatment approaches in animals: varying models might produce varying results. To investigate the role of Cav-1 in inflammatory bowel disease, studies on human patients with CD or UC should be carried out to define whether the levels of Cav-1 are correlated with the severity of the disease and whether this correlation is positive or negative. It will also be necessary to look closely at the mechanistic insights derived from animal studies to determine what holds true in the human.

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