INVESTIGATION OF CD36 SCAVENGER TYPE B RECEPTOR EXPRESSING MACROPHAGES IN INTESTINAL INFLAMMATION

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A Thesis
Submitted to the School of Graduate Studies
In Partial Fulfillment of the Requirements for the Master Degree of Science

McMaster University

MASTER OF SCIENCE (2012): McMaster University
(Medical Sciences, Infection & Immunity) Hamilton, Ontario

TITLE: THE ROLE OF CD36 SCAVENGER TYPE B RECEPTOR EXPRESSING MACROPHAGES IN INTESTINAL INFLAMMATION

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NUMBER OF PAGES: 167

ABSTRACT:

Currently, there is no effective cure for Inflammatory Bowel Disease (IBD), medications are aimed solely at alleviating symptoms and not curative. Great scientific efforts have been aimed at elucidating the mechanisms underlying the pathogenesis of IBD. Macrophages—antigen presenting cells—play a chief role in the pathophysiology of IBD. It has been proposed that CD36 receptor present on the surface of macrophages, may play a role in the inflamed intestine. CD36-expressing macrophages have been implicated in a variety of human diseases; however the role of CD36+ macrophages in the intestine has been limited. The aim of this study was to decipher whether or not CD36+ F4/80+ macrophages are inflammatory in the colonic intestine. Our study discovered the proportion of CD36+ F4/80+ macrophages were markedly upregulated in active IBD patients and TNBS-induced colitis mice. CD36+ macrophages isolated from the LPMC of the small and large intestine of Balb/c treatment groups(a) 12TNBS, (b) MHS+12TNBS, and (c) MHS+PA mice confirmed these macrophages expressed some level of proinflammatory cytokine TNF-α. Two macrophage ligands, LCFA: Palmitic Acid and PGN, in conjunction or separately, appeared to be two culprits which induced MHS macrophages to produce TNF-α both *in vitro* and *in vivo*. It is possible these two ligands may work in concert, however the mouse model has yet to be examined. The precise role(s) of these CD36+ F4/80+ macrophages requires further scientific inquiry and elucidation in the context of intestinal inflammation. It is quite possible understanding the mechanisms and roles of these macrophages will greatly advance our knowledge in the pathophysiology of IBD and potential therapeutic treatments or targets.

ACKNOWLEDGEMENTS

First, and foremost I would like to Thank Dr. PingChang Yang for taking me on as a Master's Student. Dr. Yang has been a great mentor: he is supportive, encouraging, challenging, and always pushes you to excel and do your best. He has been a great supervisor and I have learned so much. I would also like to acknowledge my Advisory committee, Dr. Paul Forsythe and Dr. Damu Tang, for their great insights, advice and guidance in completion of this Master's degree. I want to thank my lab members: Dr. Zhiqiang, Xiao Chen, Nala, Natasha, Hong, and Tahrin, for your great laughs, support and advice; I couldn't have asked for a better lab. I want to thank my lovely sisters Kathryn and Victoria Garside and dear friend Kayla Driver for always being there, pushing me, being strong, and being such great and everlasting supporters of me. I want to thank Nala, just for being herself: a happy, kind, brilliant, positive spirit that has keep me going ever since the day we started research together. I would also like to thank the man I love, Eric Wadsworth, if it had not been for you, your great support, loving nature, great perspective on life, I don't think I'd have come this far. Most importantly, I want to thank my parents: Dr. Brian Garside and Alida Garside. Ever since I was little, you never knew why but I didn't believe in myself, but you never gave up on me; you always told me "just follow your nose kid". You knew me as that clever little girl who saw the world with open eyes and questioned everything. You encouraged me, nurtured me, and inspired me in countless ways. Now, I have become the strong woman that, deep down, you knew I always was. There are no words to describe the amount of love I have for both of you. Because of your support, I was able to realize my dreams of becoming a

scientist, and I will continue to aspire to greatness as I continue to excel in my academic career. Lastly, I would like to quote my Grandmother, Edith Garside, my kind and kindred spirit, whom I loved very dearly: "Let Those who have eyes to see, to see."

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LIST OF ABBREVIATIONS:

LCFA: Long Chain Fatty Acid
PA: Palmitic Acid
VLCFA: Very Long Chain Fatty Acids
FA: Fatty Acid
TNBS: Trinitrobenzene Sulfonate
TNF-α Tumor Necrosis Factor Alpha
NMHS: Normal unstimulated NMHS
MHS+PA: MHS treated with 24 hour high dose Palmitic acid
MHS+12tnbs: Unstimulated MHS and 12 tnbs colits
12TNBS: Half dose tnbs colitis treated mice
LPMC: Lamina Propria Mononuclear Cells
CD36: Scavenger Type B receptor
F4/80: Intestinal Marcrophage marker
CD11b: Intestinal Macrophage marker
UC: Ulcerative colitis
CD: Crohn's Disease
RPM: Revolutions per minute
RPMI: Roswell Park Memorial Institute medium
RT-PCR: Reverse Transcription-Polymerase Chain Reaction
SDS: Sodium Dodecyl Sulphate
PGN: Peptidoglycan
NF-κB: Nuclear factor kappa B
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
MPO: Myeloperoxidase
LP: Lamina Propria
APC: Antigen Presenting Cell
ANOVA: Analysis of Variance
BSA: Bovine Serum Albumin
DNA: Deoxyribonucleic acid
RNA: Ribonucleic Acid
EC: Epithelial Cell
EL: Epithelial Lumen
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-Linked Immunosorbent Assay
FBS: Fetal Bovine Serum
HE: Hematoxylin and eosin stain
IBD: Inflammatory Bowel Disease
IHC: Immunohistochemistry
GI: Gastrointestinal Tract
FITC: Fluorescein isothiocyanate
BMM: Bone Marrow Derived Macrophages

INTRODUCTION:

1.1 Innate& Adaptive Immunity

The immune system is subdivided into two major arms : innate and adaptive immunity. The innate immune system is the body's first defence against pathogens and takes place within minutes to hours[1-5,7-9,11,12,14,16,28].The innate immune system is a hard wired and immediate immune response that responds first to a pathogen when it exceeds an infectious load[1-5,7-9,11-12]. This innate immune response is essential to the function and integrity of mucosal tissues, such as the intestine, wherein innate immune cells constantly sample and monitor the components of the epithelial lumen and microbiota [1-5,7-14,19,28].One of the major players involved in mediating and initiating the innate immune responses in the intestine are primarily macrophages[1-5,7-9,14,28]. Innate immune cells respond to PAMPs—pathogen-associated molecular patterns—which are conserved repetitive structural elements composed of distinct patterns of lipid and carbohydrates present on the surface of pathogens, viruses, and protozoa[1-5,7-9,11,12,28.] PAMPs are recognized and bound by germ-line encoded pattern recognition receptors (PRRs) which are found on antigen presenting cells (APCs): macrophages, DCs and B cells[1-5,7-10,12].When these recognition receptors, PRRs, are activated this initiates downstream signalling cascades that produce proinflammatory cytokines, chemokines, which induce inflammation and attract more inflammatory mediators amplying the immune response. Upon stimulation, macrophages will phagocytose, process, and present antigen to adaptive lymphocytes (T and B cells) which proliferate and differentiate into professional effector cells; thus bridging the innate to adaptive immune response[1-5,7-9,11,12,14,16,28].

Adaptive immunity is a more focused immune response to pathogens which occurs within days to weeks and has the unique ability to retain memory[1-7,9-10,14,16]. Once cytokines are released this polarizes B and T lymphocytes towards their differentiated fates—TH1, TH2, TH17, Treg and plasma cells, thus polarizing different adaptive immune responses[1-3,5,8-10].

Macrophages are the chief immune cells in bridging innate and adaptive immune responses, and are among the first inflammatory mediators recruited to the site of inflammation and infection[1-3,5,8,17,26-28.] After activation of both arms of the immune system, lymphocytes and leukocytes will effectively destroy pathogen(s) through several layers of immune responses in a matter of minutes, hours, days, or weeks[1-7,9-10,14,16]. In collaborative efforts and extensive scientific investigation into the underlying pathogenic mechanisms in IBD, it was discovered that dysregulation of the innate immunity to commensal microbiota appeared to be the main factor in driving the in early pathogenesis of this disease. A crucial inflammatory cell that induces these abnormal intestinal immune responses to microbiota are macrophages[4-5,8-10,16-19,21]

1.2 Breakdown of Immune tolerance & Intestinal barrier

In the normal human condition, the immune system can decipher 'self' from 'non-self' and ascertain which microorganisms are tolerated in the body and which are pathogenic; the host normally only perments mutalistic bacteria to live harmoniously in the intesine [1-5,7-10,12]. There is approximately 10^{14} bacterial cells in the intestine and nearly10^{13} of all human cells comprise our entire body and organ systems[1]. Commensal microbiota is essential to sustain life, via inducing metabolism, energy, and digestion in

the human body [1,11-12,14,16-17]. During the development of IBD, a crucial error occurs wherein the immune system begins to target host microorganisms as if it were a pathogen, and thus initiates an inflammatory response to destroy microbiota, destroying surrounding intestinal tissues[1-2,4,6,8-13,16,19,21].The majority of this damage is mediated by macrophages [1,4,7-10,16-17,19,28,43,44].

IBD collectively destroys immune tolerance via dysregulated innate immunity mediated via macrophages[1-10,17, 28]. Macrophages induce intestinal damage via the release of proinflammatory cytokines, inducing damage to the intestinal epithelial barrier, inducing lesions, and permitting bacteria leaking into the lamina propria which further amplifies inflammation and damage to the intestine[1,11-12,14].Over time IBD destroys immune tolerance to beneficial commensal microbiota in a uncontrollable manner and thus activates the adaptive immune system, and at this stage the symptoms of IBD are devlop or a relapse of the disease is observed [1,11-14].

In brief, the intestine is comprised of three major tissue layers: epithelial lumen, epithelial barrier, and lamina propria[1,11-12]. All three layers are very important for maintaining the health and integrity of the intestine, each layer has a critical functional role within the intestine. In the outermost layer of the intestine is the epithelial lumen were mucosa and resident commensal microbiota resides in[11]. The lamina propria is the most internal layer of the intestine, where the innate and adaptive immune cells are found. Normally, macrophages and dendritic cells continuously sample the bacteria in the intestinal lumen for surveillance for potential invaders[1,11]. The epithelial barrier is a specialized single cell epithelial layer that protects the delicate balance of these two

internal and external worlds, keeping separate under normal conditions[1,11-12, 14,16]. This barrier of single cell layer of epithelial cells contains several other types of cells such as Goblet cells, Paneth cells, M cells, Enteroendocrine cells and columnar epithelial cells, these cells originiate and differentiate from the stems cells of intestinal crypts[1,11,15]. Epithelial cells role in the intestine is to absorb nutrients, produce digestive enzymes, detect and sample antigens in the epithlial lumen, and secrete antimicrobial molecules to maintain a balanced mucosa environment and mucosal immunity[11].These intestinal epithelial cells comprise both a protective and physical barrier against invading pathogens and a beneficial niche for commensal microflora[1,11-12,14].

To maintain a healthy intestinal environment DCs, macrophages, neutrophils and epithelial cells, continuously monitor and sample to environment to decipher 'self' from non-self, in the normal condition and this tightly regulated to sustain the homeostasis of the intestine[1-5,7-9,11-12.14]. However, when a pathogen is presented, the immune cells in the barrier change allowing immune cells to extravagate through the tissue and APCs such as macrophages to phagocytose, process, and present antigen to adaptive immune cells in the lamina propria. This will induce a potent inflammatory response that will collectively destroy the invading pathogen. [1-5,7-10,12].

The health, integrity and homeostasis of the intestine cannot be well maintained during active IBD and is a major reason why individuals with this disease chronically relapse[1-5,7,11-12,14,16,19,25]. Treatment for IBD is challenging because this condition is chronic[1, 11,16]. In the first stage of IBD, macrophages and dentritic cells are activated; they subsequently phagocytose, attack, and degrade host microbiota. After engulfment,

macrophages release proinflammatory cytokines such as,TNFα, which elicits damage to the intestinal epithelium by punching holes, forming lesions in the intestinal barrier[1,11,12,14].

These lesions permit bacterial leaking into the lamina propria and amplifies the immune response of both arms of the immune system: innate and adaptive immunity[1-5,7-9,11-12,14]. As second role, macrophages are APCs and present antigen to activate and recruit other adaptive immune cell such as effector T and B cells [1-5,7-9,11-12,14,16].

The focused, potent, and overactive inflammatory response generated by the adaptive immunity leads to the destructive and uncontrollable stage of IBD where symptoms and relapse take place [1]**.** Taken together, IBD will manifest in a genetically susceptible host, wherein these genetic differences affect the ability of the host to properly regulate the innate and adaptive immune system, the physiology and function of the intestinal epithelial barrier, and potentially predispose this barrier to inappropriate sampling of microbiota antigens to the mucosal immune system[1,11.]

1.3 Inflammatory Bowel Disease

Inflammatory Bowel Disease, IBD, is defined as a dysregulated mucosal immune response to indigenous commensal microbiota[1-4,7-10,16]. IBD is well-characterized through its chronic relapsing and remitting nature and clinically manifests itself in two forms: CD—Crohn's disease and UC—Ulcerative Colitis[1-10,16-21]. These two subtypes of IBD can be differentiated by the location and the nature of the inflammation[1,11,16]. Crohn's Disease is multifocal and can affect the gastrointestinal tract anywhere from mouth to anus—frequently attacking the ileum and colon, whereas UC produces continuous

inflammation restricted to the large intestine: the lining of the rectum, colon and caecum[1,16,18,20].

These two inflammatory diseases have skewed cytokine profiles, TH1—T-helper 1 is primarily observed in Crohn's Disease and TH2—T-helper two for Ulcerative colitis; however IBD can also exhibit mixed phenotypes [1,11,16-18,19]. At the histological level, intestinal biopsies from patients with active IBD reveal large proportions of leukocytes—polymorphonuclear leukocytes—monocytes, macrophages, neutrophils, lymphocytes in the intestinal and colon interstitium [1,3,16-20]. In addition to a high mononuclear and polymorphonuclear cell infiltrate, the histology of IBD induces widespread intestinal damage by inducing edema (swelling between tissues), crypt destruction and hyperplasia, loss of goblet cells, decreased mucus synthesis, and lesions [1,3,16,18.20].

The exact cause of IBD or the mechanism(s) underlying the pathogenesis of this inflammatory disease remain unknown and requires much further characterization and scientific inquiry[1-5,7-10,12,16,18-20]. However, there have been some recent connections in the dysfunction in the mucosal immunity and the manifestation of IBD[1,16-19].Although the innate and adaptive immunity are involved in the early onset of IBD, the precise mechanisms are still being investigated[1-10,12,16-20]. The use of multiple IBD colitis mouse models has made it clear that chronic inflammation in the intestine is resultant from a dysregulated innate immunity to commensal microbiota, and macrophages are a key inflammatory mediator in the pathogenesis of IBD [1,11,16-20, 27,41,49].

1.4 Epidemiology of IBD

Globally, IBD significantly lowers the quality of human life and there is no effective cure to date[1-5,7-11,16,27,41,49]. Currently there are over four million reported patients that suffer from this disease; not all of these individuals can afford proper care and treatment [1,16,23-24,25]. The highest prevalence and reported incidences of Crohn's disease and Ulcerative Colitis is within the demographic area of Northern Europe, North America and the United kingdom [24]. IBD results in significantly higher health care costs, and morbidity rates worldwide[1, 23-24,25]. One of the largest challenges in IBD, is simply the diagnosis of this disease, as the symptoms can develop gradually, suddenly, and differ from patient to patient; there is 'no gold standard' or specific list for diagnosis [24]. Inflammatory bowel disease appears to be highest in Westernized worlds, and a most concerning aspect is that the prevalence of IBD is steadily growing[1,16,23-24,25]. Canada alone has one of the highest incidences of IBD and nearly 1 into 180 Canadians currently suffer from this inflammatory disease [24]. Annually Canada spends nearly two billion dollars on treatment and medications for IBD patients, all of which are solely aimed at trying to control inflammation and relapse [23-25]. Currently a higher incidence of CD is reported in Canada relative to UC; the reason for this remains relatively unknown[1,11]. Based on cohort studies, both types of IBD generally manifest between the ages of 15- 25 with similar proportions in both males and females—generally IBD does not appear to affect either younger or older age groups and this disease is usually a life long diagnosis[1,16,23-25]. There are a multitude of different factors that contribute to the

pathogenesis of IBD the two main contributing factors are: genetic predisposition and environmental factors[1,16,23-25].

Although the pathogenesis of IBD have strong genetic predispositions, it is highly influenced by multiple environmental factors as well[1,11,23-25]. Some potential risk factors that have been identified: smoking, antibiotic use, stress, poor diet, infectious agents, alcohol consumption, steroids, appendectomy, birth control pill, and bacterial infections[1,11,23-25]. Interestingly, the highest risk factors in manifesting IBD have confirmed to be smoking and appendectomy[1,16,23-25].

As previously aforementioned the exact cause(s) of IBD remain unclear it is believed that genetic factors exhibit a strong influence in this inflammatory disease[1,23-25]. The natural interaction between host and commensal microbiota is carefully regulated in the body and there some essential genes involved which maintainance this homeostatic relationship[23-24,27,30-35]. Although some genetic and risk factors have been identified, one crucial gene discovery was NOD2 and its direct link to the susceptibility to Chron's Disease[1,11,16,27].NOD2 is an intracellular pattern recognition receptor—present in macrophages—which normally bind to muramyl dipeptide in peptidoglycan a component of bacterial cell walls [27]. The NOD2/CARD15 gene plays a role in the innate immune system, and this gene encodes a pathogen recognition molecule which is altered in Crohn's Disease patients[16,19,27]. This gene affects cross talk between the innate and adaptive immunity[16,19,27]. NOD2 is found on the IBD1 susceptibility locus on chromosome 16 and is disrupted via either a insertion mutation, frameshift mutation, or missense mutation. It was recently discovered in human IBD that nearly 30% of patients

with small intestinal CD have a polymorphism in NOD-2 demonstrating the importnace of this gene; the polymorphism in this gene is one of the largest genetic predispositions identified in IBD [27].

In addition, autophagy plays a pivitoal role in clearing up cellular waste and pathogens in order control of bacteria, viruses, parasites, and recently one autophagy-associated gene was associated with susceptibility to Chron's disease, the Atg16L1 gene [27,30-33].This gene is highly expressed by normal intestinal epithelial cells and is normally functional in intestinal macrophages and becomes dysregulated in a number of IBD patients[27,30-33]. It is believed this malfunction in this gene contributes to the pathogenesis of this disease via an insufficiency in the clearance of waste and pathogens [27-28,30-33]. Lastly a protein involved in innate immune defence, IRGM, Autophagy Immunity-Related GTPase family M, a polymorphism in this gene was also identified in susceptibility to Crohn's Disease and is associated with more surgical procedures[27-28, 33-35]. Extensive investigative research continues to identify more IBD susceptibilty genes, in attempts to better understand the onset and development of IBD, and precisely what components of the immune system are dysregulated; it is possible once genes are identified genentic engineering may be another possibilty to aid in prevention or treatment of this chronic inflammatory disease [27-28,30-35].

1.5 Symptoms, Complications & Treatment of IBD

IBD patients experience a wide array of clinical symptoms: diarrhea, anemia, rectal bleeding, bloating, malnutrition, abdominal cramps & swelling, ulcers, fever, and weight loss[1-5,7-13,16-24,26,39]. One of the biggest challenges in IBD—and the reason for such

intensive study—is that current treatments for IBD are solely aimed at alleviating symptoms, and there has been no successful cure to this chronically relapsing and reemitting nature[1,-5,7-13,16-24,39].

Success in therapeutic treatments for IBD has been limited[1,11,16-24]. Conventional treatment for patients suffering from IBD in the past were typically corticosteroids, prednisolone, anti- inflammatory agents such as 5-aminosalicylates—mesalamine, olsalazine, sulphasalazine—and immunosuppressant's such as 'Azathioprine' that dramatically suppress inflammation in the intestine; however these medications put patients in an immunocompromised state [16,26,29,38]. Long term use of these medications not only caused steroid dependence and illnesses, but also predisposes these individuals to opportunistic pathogens and cancer; in addition the efficacy of these drugs also varies widely from patient to patient[16,29]. It is unfourunate that the individuals being treated with these medications will still experience relapses of CD and UC, be subjected to a wide array of potential side effects, and in some cases still require surgical intervention[16,26,29,38].

One of the inflammatory mediators responsible for the intestinal damage observed in IBD is exhibited via a potent proinflammatory cytokine, tumour necrosis factor alpha, TNFα; as mentioned before this cytokine induces lesions in the epithelial barrier and is a chief player in the pathogenesis of IBD[1,5,8,11,16-18,21,39,40]. After this discovery TNFα, it became a major target for therapeutic treatments in IBD for some length of time[16,39,40]. Through extensive research it was discovered that neutralizing antibodies directed against TNFα was an effective treatment for many patients which

appeared to work very well in some patients reducing hospital visits, surgery, and inflammatory relapses[16,39,40]. TNFα inhibitors such as Infliximab—an IgG1 monoclonal antibody, Adalimumab—a recombinant human IgG1 monoclonal antibody and Certolizumad pegol—a pegylated humanized Fab fragment unit monoclonal antibody—demonstrated some efficacy in treating severe CD patients in reducing relapse of disease[16, 39]. Moreover, Infliximab in particular was most capable in promoting remission in patients that could not respond to previous conventional treatments [16,39,40]. However one of the largest pitfalls of these treatments was the unexpected toxic and dangerous side effects[16,39,40]. However useful these TNFα inhibitors were in reducing inflammation, relapse and surigcal intervention, the exact molecular mechanisms involved were poorly characterized, one mechanism in reducing inflammation was mediated by inducing T cell apoptosis, however the secondary effects of this was unsafe and not beneficial to the patient overtime[16].

TNFα is intimately involved in a multitude of different cellular processes such as epithelial cell growth and apoptosis, matrix metalloproteinases, inflammation, and intestinal permeability[1,16,39,40]. Long term use of neutralizing or blocking TNFα therapeutics raised some considerable concerns for increasing infections, malignancy and reducing immunity[39]. Another problem with TNFα inhibitors was that even after some success in controlling intestinal inflammation, the patient often lost responsiveness to TNFα treatments[16,39,40]. Immunogenicity was also a problem as a small subset of patients may experienced allergic reactions from cutaneous rashes, eruptions, anaphylatic shock, urticaria, and angioneurotic edema[39]. It was repeatidly confirmed these treatments are too

danergous for long term use, and clearly demonstrated the dire need for new therapeutic approaches in IBD[11,16,39,40]. Great scientific efforsts are being made to elucidate the mechanisms underlying the pathogenesis of this disease, downstream signalling events, in order to produce an effective target and better hope for future treatment of IBD patients[1-5,7-9,11-12].

Two other cytokines have been implicated as inflammatory cytokines in IBD—IL-12 and IL-23—which are involved in T cell activation and differentiation [16]. Activated innate immune cells release IL-12 and IL-12 which is composed of two subunits, p35 and p40; these cytokines are important for producing TH1 IFN-γ cells [16]. Antibodies directed against IL-12 inhibited apoptosis in lamina properia mononuclear cells from CD patients, and a monoclonal antibody, IL-12p40, demonstrated high efficiency for reducing remission rates [16]. However, further investigation demonstrated that the neutralization of IL-12, was actually due to the expression of IL-23, which shared the equivalent p40 subunit as IL-12, and it was in fact IL-23 that enabled the therapeutic effects[16]. IL-23 has two subunits: p19 and p40 and promotes the growth and survival of Th17 cells, a distinct T cell lineage implicated in inflammatory conditions of IBD. Normally Naive CD4 T cells will differentiate into TH17 T cells in the presence of TGFβ and IL-6[1,5,16]. Th17 cells express the transcription factor RORγt and stimulates other macrophages to produce proinflammatory cytokines: TNF-a and IL-6 [1,5,16]. Experiments on Mice deficient in IL-23p19 subunit, were protected against initial intestinal inflammation and IL-17 receptor knockout mice were also protected from TNBS colitis[16]. Furthermore IBD patients demonstrate increased expression of intestinal IL-23 MRNA and IL-17 protein expresion

in serum and inflammaed mucosa [16]. It remains relatively clear that the activation of TH17 cells via IL-23 plays an important role in the pathogenesis of IBD and thus presents another potential therapeutic target[16]. In addition, this finding also suggests other TH17 cytokines such as IL-17a and IL-17f could also be beneficial therapeutic targets[16]. The antibodies targeting IL-23, and IL-17 cytokines, will likely prevent or downregulate the expansion of TH17 cells and induce apoptosis of IL-17 T cells or potentially reduce inflammation in the intestine of IBD patients. However these cytokines are involved in many different cells, tissues, and functional roles so one has to be careful in designing these targets[1,11,16].

1.6 Macrophages in the Intestine

It has been made clear that macrophages play a crucial role in driving the inflammatory pathogenesis of IBD, but first it is important to understand the difference between natural resident macrophages and inflammatory macrophages; resident macrophages do not orchestrate inflammatory disease, they are homeostatic in nature.[1,11,22,25,27,28, 41,49]. In normal non-inflammatory conditions, intestinal macrophages are relatively sentinel phagocytic cells, continuously sampling and surveying commensal microbiota; the inflammatory tone in the intestine is greatly dampened via a multitude of mechanisms to maintain homeostatic balance and inhibit autoimmunity, or over-reactivity[1,11,17, 22,25,27, 28,41].

At first monocytes are first differentiated from a common myeloid progenitor, these monocytes are then released from the bone marrow and into the periphery[17,28,44,47-48]. These blood circulating monocytes then migrate out to host tissues and are influenced

and shaped via its target microenvironmental niche[17,27,28,44,47-49]. Innate and adaptive cues are what essentially shape macrophage physiology and function[17, 27,28,44,47-48]. Macrophages can play a variety of different roles in the body; the primary functions of these phagocytic APCs are wound repair, tissue remodelling, and immune defense[1,11 17, 21,28,41, 44,47-49]. Macrophages can be protective or destructive in the human condition[1-5,7-9,11,12,17-19, 21,28,41,43-48]. These antigen presenting cells protect the host against infection by induction of inflammatory mediators and cytokines to induce inflammatory macrophages to elicit potent pathogen killing, once pathogen is eradicated, then resident macrophages resume their natural role. However in IBD these inflammatory macrophages stay activated in the intestinal tissues, are continuously recruited and damage the intestine. Inflammatory macrophages can be destructive to the host when they turn the on immune system targetting self as 'other' inducing autoimmunity, hypersensitivity and allergy[1,5,8, 17, 21,28,41-48]. Macrophages are distinctively plastic; these cells are able to change in response to environmental cues and can change their functional roles accordingly[1, 17,28,41,44,47,48].

Macrophages can be categorized into two simple subgroups, M1 and M2 macrophages[28,44]. These macrophage subgroups have been characterized as 'Classical' M1 and 'Alternative' M2 macrophages that contribute to a skewed Th1 and Th2 immune response respectively [28,44]. M1 is defined by high IL-12 and low IL-10 and M2 is characterized as decreased levels of IL-12 and high IL-10 expression [28,44] .M1 macrophages are activated in cell-mediated immune responses that are dependent upon IFN-γ and TNF family cytokines and are primarily the macrophages activated in

inflammatory IBD [17,28,41,44,49]. M1 macrophages mediate host immune response via the production of IL-1, IL-6, IL-23 and IL-12[28,44]. This subtype of macrophage can be identified via the expression of a few markers: nitric oxide synthase, C-C motif chemokine 15 (CCL15) and CCL20, CXCCL9 and CXCL10 and these macrophages aid in killing intracellular bacteria[28,44].

Alternatively activated macrophages, M2, are a separate group of macrophages which are comprised of a variety of different macrophage phenotypes[44]. These macrophages have both regulatory and wound healing functions [28, 44]. When an injury occurs, there is a marked production of IL-4 and IL-13 which cause a conversion of these sentinel macrophages into macrophages that will induce wound repair via production of the Extracellular matrix, ECM [28,44]. M2 regulatory macrophages are increased during an acquired or innate immune response and are influenced via IL-10 and TGF-β which suppress immune response and decrease inflammation [28,44]. When cytokines TGF-β, IL-10 and PGE2, Prostaglandin E2, are produced and IL-12 are downregulated, this is characteristic of M2 macrophages [28,44].

The large and small intestine contain the highest proportions of macrophages in the human body; and intestinal macrophages are crucial in the fine balance and interactions with commensal microflora with host immunity [1,11,17-19,28, 41,44,47-48]. In the intestine there are different and distinct subgroups of resident and inflammatory macrophages [17,28,41-42,44-45]. Under normal conditions, resident macrophages are always present in the intestine; these immune cells are inert, phagocytic, exhibit decreased proliferation, chemotactic ability, utilize TLRs to dampen immune response, interact

with microbiota, release chemokines and ligands; however these macrophages do not produce inflammatory cytokines[17,28,41,44]. Resident mucosal macrophages typically exhibit features of normal macrophages with a special mononuclear size and shape, granular cytoplasmic-like appearance, and are immune tolerant and phagocytic [27].

Interestingly, these inert macrophages still retain the ability to kill microbes; they still remain bactericidal, and naturally produce anti-inflammatory cytokines—TGF-β and IL-10—which inhibits or downregulate NFkb and Trem1 activation[17,27,41]. Resident macrophages are also important in the regeneration of damaged epithelial cells as reported by colitis mice models[41]. The resident non-inflammatory intestinal macrophages remain mostly inactive because the local microenvironment relays signals or 'cues' to keep the macrophage response suppressed; an entirely opposite behavior is observed in inflammatory macrophages[1, 17-18,28,41,44,47-48]. Inflammatory macrophages are highly bactericidal, phagocytic, and active, these macrophages over react to microflora, TLR ligands, and produce an entire array of proinflammatory cytokines and chemokines[1,27,28,41,44].Inflammatory macrophages are defined by their distinctive patterns and interactions with specific ligands[17,28,41,44]. In addition, the two subtypes of intestinal macrophages can be defined by different precursors; resident macrophages develop from monocytes expressing $CX3CR1^{hi}GR^{-}$ and inflammatory macrophages develop from $CX3CR1^{lo}GR1^{+}$ monocytes [17,28,44,47]. It is largely believed that normal lamina propria macrophages differ in phenotype, function, and phylogeny from inflammatory intestinal macrophages and it is believed that resident macrophages are different[17,28,44,47].

Primarily the highest proportion of macrophages in the intestine are located in the Lamina Propria; however some macrophages are found in the mucosa, the underlying submucosa and muscularis mucosae area [28,41]. The presence of mucosal macrophages appears to be highly correlated with bacterial load and naturally the distal colon contains the highest level of microbiota, and is a site wherein the majority of the commensal microbiota are present[1,11,17,18,28]. It is at this location that IBD inflammatory macrophages are most destructive and the intestinal section of interest in this study[1,17,28,41].

1.7 Role of Macrophages in IBD

Macrophages are among one of the first inflammatory mediators recruited to the site of an infection, and collectively they form one of the largest populations of mononuclear phagocytes in the entire body[1,28, 43,49]. Macrophages have been implicated as the chief innate immune cells that induce the inflammation and destructive phase of IBD [1,11,17,27, 28,41,44]. One of the major inflammatory mechanisms underlying the pathogenesis of IBD is primarily induced by inflammatory macrophages which promote inappropriate inflammatory responses via release of cytokines and chemokines, and recruting of other effector cells [1-5,7-9,17,28,41,49].

These inflammatory macrophages become inflammatory due to the release of pro-flammatory cytokines that collectively destroy host tissue and attack indigeous microbiota [17, 27 28,41,44,49]. More specifically, the release of these proinflammatory cytokines induce epithelial cell apoptosis, lesions in the barrier, compromise the integrity and permeability of the barrier, and promote necrosis, fibrosis, and the formation of granulomas [1,11,17,19,27-28,41,49].

In IBD and colitis mouse models it has been observed that inflamed mucosal tissues exhibit a markedly higher infiltrate of monocytes and macrophages relative to healthy patients and controls[17,27-28,41,49]. In experimental studies of colitis, the importance of macrophages was further established by a observed depletion and knockdown of lamina propria macrophages [17,27,28,41,49]. When these intestinal macrophages were decreased or depleted, this reduced the levels of intestinal inflammation—measured via increased loss of body weight, higher DAI (daily activity indexes), colon length, decreased histological scores and reduction of inflammatory cytokines—across four different mic of colitis strains[17, 28]. In support of murine evidence, lamina propria macrophages isolated from IBD patients have demonstrated that these inflammatory macrophages upregulated the expression of T cell co-stimulatory molecules—CD80 and CD86, CD14,TREM, NFkβ activation, and release of proinflammatory cytokines: TNfα, IFN-γ, IL-6, IL-1, IL-23, IL-17 ,and neutrophil chemoattractant, IL-8[17,28,41,50,52,53]. The Triggering receptor expressed on myeloid cells-1, TREM1, augmented the inflammatory process via enhancing proinflammatory cytokine secretion from these macrophages [53]. Under the normal condition resident intestinal macrophages lack the TREM1 receptor; however in IBD patients and colitis mice models inflammatory macrophages markedly expressed this receptor, and it is believed this receptor is upregulated due to a deleterious change in macrophages [19,53].

Another phenotypic change in macrophages is believed to be a lack of production of anit-inflammatory IL-10; normal intestinal macrophages secrete this cytokine and it is believed to suppress immune response to commensal microbiota[16,17, 27,28,41,49]. Also

inflammatory macrophages and IL-10 knockout mice, exhibit a marked expression of IL-23 and this cytokine is believed to play a role in the pathogeneis of IBD via inducing IL-17 cytokines and polarizing TH17 T cell proinflammtory response and neutrophil and macrophage recruitment[1,16,19,127]. Many cytokines have been confirmed to play an inflammatory role in IBD, however, above all, TNFα appears to be the most potent inducer of intestinal inflammation and relapse.[1,11,17-19,27,28, 39,40-41,49,55]. Thus production of TNFα is good pro-inflammatory indicator of IBD, and is secreted primarily from activated inflammatory macrophages in IBD[17,27,28,39-41,55].

1.8 The role of Tumor Necrosis Factor Alpha in IBD

Tumor necrosis factor, TNF, is a 19 member cytokine superfamily which are structurally and functionally similar based on genetic sequence simillarties[55,57,58]. TNF superfamily is generally involved in response to injury or inflammation, and elicits a multitude of different protective and proinflammatory roles [55,57,58,113].

The TNF receptor exists in two forms: membrane bound and a soluble form[55,57,58,113]. TNF is first sythesized as its membrane bound 26-kDa mTNF receptor, and when it is activated it releases its soluble 17-kDa sTNF form[55,57,58,113]. The soluble form of TNF can signal through the two TNF receptors (a)TNFR1(p55) and (b) TNFR2(p75) [59,113,114]. The TNFR1 receptor is found across a multitude of different tissues, and TNFR2 is found solely on immune cells [59,113,114]. These receptors can uniquely act as a ligand or signalling molecules.The soluble form of TNF generally binds to the TNFR1 receptor(p55), and this receptor transduces signals to induce caspase activated apoptosis [59,112,113].

Membrane-bound TNF stimulates TNFR2 and both soluble and membrane TNF can activate the TNFR1 receptor [59,113,114]. In membrane bound, mTNF, preferentially binds to TNFR2(p75) with a higher affinity [59,113,114]. After the TNF receptor has been activated this inhibits SODD to dissociate from the intracellular death domain, permiting Tumor Necrosis Factor Receptor Type 1-Associated DEATH domain(TRADD), to dock and bind to a platform, this recruits RIP and TRAF2, and TRAF2 recruites IKK, the IkB kinase, enabling serine theonine kinase RIP to become activated, causing IKK complex to phosphorylate IkB, a protein that noramally sequesters NFkB in the cytosol. After the phosphorylation and release of IkB this enables translocation of NFkB to the nucleus, and activating NF-kβ to transcribe downstream proinflammatory targets such as TNFα [59,113,114].

Tumor necrosis factor alpha plays a regulatory role and the purpose of this cytokine is to regulate immune cells of innate and adaptive immunity, when this cytokine becomes dysregulated that is when disease will manifest [1-5,7-9,11,12,17,27,114]. TNFα cytokine is the first member of the TNF superfamily [55,57-58,113,114]. TNFα, is an endogenous pyrogen, it is a cytotoxin, which plays a dual role in first inducing septic shock, acute phase reaction, systemic inflammation, cachexia, cell activation and proliferation and secondly it acts as inhibitor in tumorigenesis and viral replication [58]. TNFα, is an inflammatory mediator across many autoimmune diseases and past studies have revealed this cytokine, is highly upregulated in IBD patients[1,5,8, 11,16,17,19,39,40,49,50,55-58,114].The over expression of this particular cytokine has been identified as a key feature of Inflammatory Bowel Disease, as TNFα is markedly upregulated in lamina propria macrophages of IBD

patients[1,5, 8,16,17,27,28, 41, 49]. The strong correlation between increased TNFα production and IBD development has been made clear via numerous studies on murine colits models, TNBS, DSS-induced colitis, TNFα neutralizing monoclonal antibodies, and gene knockouts that demonstrated marked reductions in inflammation and inflammatory relapses[1,16,39,40,55,56].

The important role of TNFα in the pathogenesis of IBD is well supported[1,5, 8,16,17,27,28, 39, 40,54-56.] Past studies have reported serum and mucosal extracts from the intestine in active CD patients discovered TNFα was markedly upregulated in comparison to healthy patients [16,27,28, 55,56]. In addition, a proceeding study examined CD14+(adaptive component for TLR2 receptor) intestinal macrophages from Crohn's disease patients revealed that TLR2 activated macrophages to induced high levels of TNFα, thus suggesting TLRs and TNFα may play a synergistic role in inducing the pathogenesis of IBD [17].

1.9 TLRS and IBD

The recognition of pathogens is essential for maintaining homeostatic conditions in the intestine and for initiating appropriate immune responses [1-5,7-9,11,12,16]. In the innate immune system, recognition of pathogens is mediated via PRRs—Pathogen recognition Receptors which are present on APCs— Macrophages, DCs, and B cells[1-5,7-9,11,12,16,-18]. These receptors bind to, and recognize, conserved molecular structures expressed via different microbes, fungi, protozoa and bacteria[1-5,7-9,11,12,16]. One such PRR are TLRs, which belong to a conserved group of an 11 member family of Toll-Like Receptors[1-5,7-9,17,28,60-70,108].These evolutionarily conserved germline encoded receptors play a vital role

in the innate immune system and immune surveillence via recognizing specific pathogen associated molecular patterns, PAMP's presented via APCs [1-5,7-9,17,28,60-70,108]. TLRs are type I transmembrane glycoproteins that are a part of the IL-1 receptor family [60-70,108]. There are two main components: the cytoplasmic domain consists of a signalling intracellular domain: Toll/IL-1R TIR which initiates downstream signalling, and a extracellular domain mediates specific ligand binding, and this binding initates TLR ligation[60-70,108]. Once ligand activates TLR receptors, receptors dimerise inducing a conformational change,which permits downstream signalling via the TIR domain [60-70,108]. Toll-like receptors are unique in their ability to be either cell surface or intracellular receptors; these receptors distinguish types of pathogenic components and based on ligands and effectively direct innate immune responses[60,61,63,108]. TLR's 3, 7, 9 are intracellular homodimers that bind to double stranded RNA, single stranded RNA and CpG DNA respectively[60,61,63,108]. Toll like receptors 4, 5 are also homodimers on the cell surface which recognize LPS and Flagellin [60,61,63,108]. The last group of TLR's are heterodimers which dimerize with different TLRs: TLR-1/2 and TLR 2/6, they bind to Lipoteichoic acid (LTA) and Lipoproteins of gram positive and gram negative bacteria respectively[3,7,9,3460-63,108]. The majority of signalling from these Toll-like receptors is dependent on an adapter protein, Myeloid Differentiation Primary Response Protein, MyD88, that leads to the activation of NF-kB gene trancription which releases proinflammatory cytokines, TNFα[108]. Only TLR3, which is stimulated via dsDNA, signals entirely independant of Myd88 and signals through TRIF—TIr-domain containing adapter— and TLR4 is unique as it can signal through both pathways[60-70,108, 111].

TLR receptors trigger the response of antigen presenting cells, such as macrophages, to initiate cells to phagocytose, internalize, and degrade the pathogen, and induce secretion of proinflammatory cytokines [3, 16, 25,29,3.] It is well known that TLRs induce proinflammatory cytokine responses during infection and dramatically shape the immune response according to encountered pathogen[108,109].

At basal levels, normal intestinal mucosa and epithelial cells constitutively activate apical TLRs to maintain the homeostasis in a hyporeactive state to reduce the inflammatory tone to indigenous microbiota[1,6,11-12,22,25,28,42]. In a healthy host, TLRs induce distinct immunoregulatory mechanisms for barrier protection and integrity, detecting and eliminating foreign invaders whilst maintaining tolerance to self antigens and native microflora[1-5,7-9,17,28,60-70,108-109]. However in IBD, TLRs become over activated activation on APCs, such as macrophages inducing inappropriate inflammatory immune responses which are destructive to the intestine [1-5,7-9,11,16,17,60,61,108-109]. Over-reactive TLRs on inflammatory macrophages induce pronounced activation of NFkB and release of proinflammatory cytokines:TNFα, IL-17, IFNγ,Il-4,IL-1β. TLRs have been widely supported to play an essential role in the pathogenesis of IBD[1 ,5,11,16-19,25,27,28,41,49,60-70].

The role and influence of TLRs in the inflammatory pathogenesis of IBD have become increasingly evident and widely acknowledged [1,11,16-19, 27,28, 41,49,60-70]. Although there is some uncertainty in which TLRs are involved in IBD, TLR 2,4, 5,and 9 have been implicated [17,27,63]. Murine colitis models, TNBS, DSS, have demonstrated that mice deficient in TLR2,4, or 9 exhibit increased disease susceptibility and severity of the colitis[17]. TLR4 has shown compelling evidence of its involvement in IBD [17, 61,63,65]. In

TLR4 KO and MyD88 Knockout mice, this significantly impaired barrier function and mucosal healing in DSS (Dextran Sulfate Sodium) induced colitis [63]. Some very convincing evidence of the involvement of TLR4 in the pathogenesis of IBD was demonstrated by TLR4 expression was markedly upregulated in both CD and UC patients, and a higher level of TLR4 mRNA is observed in inflamed colon mucosa relative to healthy patients [63]. In addition, Human genomic studies has also identified there is an association between a TLR4 polymorphism and the susceptibility to IBD [63].

Despite some conflicting evidence, TLR5 is generally believed to contribute to the pathogenesis of IBD [64,63]. In one reported study, intrarectal administration of flagellin directly into DSS treated mice greatly enhanced colitis symptoms realtive to control mice; thus suggesting TLR5 is involved in inflammation[64]. In support of this, TLR5 knockout mice develop spontaneous colitis and markedly higher proinflammatory cytokine production [63]. Furthermore the role of TLR5 in IBD was discovered by the extraction of serum from active Crohn's Disease patients, which exhibitd marked elevation of antiflagellin antibodies realtive to healthy patients[17]. However, at present, there has been no discovery of a genetic linkage between Crohn's Disease and TLR 5 in active disease patients [17,63].

Another TLR, that has been extensively studied is TLR9 for its unique immunosuppressive role in IBD[17,63,66]. Some studies have demonstrated subcutaneous and intragastric injections of CpG oligodeoxynucleotides decreased the severity of TNBS and DSS induced acute murine colitis and chronic colitis in IL-10 KO mice [63]. Another study revealed one of the successes of treating colitis mice with bacterial probiotics was,

in part, facilitated via CpG activated TLR 9 signalling[63,66]. Lastly, in attempts to study the relationship of TLR9 in IBD, the effect of CpG oligodeoxynucleotides used to stimulate TLR9 was investigated via utilizing *ex vivo* colon mucosal biopsies from active UC and healthy patients [63]. When these human mucosal tissues were stimulated with CpG this significantly reduced colonic expression of IL-1β and TNFα; this expression was TLR9 dependant in UC patients but was not observed in control patients [63]. In one study murine Intraperitoneal injections of CpG-ODN was observed to increase DSS induced colitis and TLR9-/- deficient mice induction of DSS colitis these mice were largely reduced in terms of intestinal inflammation and proinflammatory cytokine production [63]. However some controversial data has also been reported in which TLR9 appeared to induce an inflammatory role, thus demonstrating TLR9 must play a dual role in the intestine, perhaps dependant on the context or inflammatory stimulus [17,63].

Interestingly, TLR2 also appears to play an essential inflammatory role in the pathogenesis of IBD [17,27,60-63,65,67-68]. One of the first lines of evidence was established by Hausmann et al, whereby they he discovered a large increase in TLR2 antigen expression in IBD macrophages derived from submucosal and inflammed intestinal mucosa, in comparison to normal healthy patients which exhibited little to no expression of TLR2 [62]. In addition, Hausmann et al identified the expression of TLR2 was localized to inflammatory intestinal CD68+ macrophages in the Lamina Propria [62]. Futhermore, TLR2's role in IBD was also confirmed via TLR2 knockout mice, as these mice elicited an increased susceptibility to DSS-induced murine colitis [63]. Another study conducted by Szebeni and colleaugues investigated TLR2 expression in colonic biopsies taken from

children in newly diagnosed IBD, and relapsing IBD, together they found a marked increase in the mRNA expression of TLR2,4 in both types of IBD relative to control patients [65]. Also a soluble form of TLR2, sTLR2, has been implicated in UC and CD patients, by Candia and fellows study that showed sTLR2 and TLR2 were largely evelvated in mucosa explants, LPMC—lamina propria mononuclear cells—taken from Ulcerative Colitis and Crohn's patients [69].

Interestingly, a novel study reported by Canto and colleagues, demonstrated that a large proportion of monocytes isolated from colonic lamina propria mononuclear cells, LPMC, in patients with active IBD exhibited high levels of TLR2 on the cell surface, and secondly that TLR2 activated monocytes induced markedly high levels of TNFα production in comparison to inactive IBD patients and healthy patients [63,68]. Limited epidemiological evidence is available on the precise role that TLR2 has in the development and pathogenesis of IBD, and as of yet there is no direct evidence of a genetic linkage for TLR2 in IBD [63,67]. However, it is the study reported via Canto and his colleages which initially sparked our interest in the further investigation of TLR2's role in intestinal macrophages of the Lamina propria in the intestine[68] .

1.10 CD36 Type B Scavenger Receptor

The Cluster of Differentiation 36, CD36, receptor was first isolated nearly thirty years ago and named 'Glycoprotein IV' as this receptor appeared as the fourth major band observed on an SDS page gel of proteins extracted from platelet membranes[72]. Structurally, CD36 is an 88kd integral membrane glycoprotein that is situated on the cell surface; it is also synonymously known as FAT [73,75-76,79,90-94]. CD36 is the primary

member of a gene family which accounts for two other members in vertebrates: LIMP-2— Lysosomal integral membrane protein-2 and CLA-1—CD36 and LIMP-2 Analogous, also known as SR-B1[73,75-76,79,90-94]. CD36 is believed to be a Serum Amyloid A (S.A.A) acute phase protein which is involved in a multitude of physiological and pathological processes [71]. This receptor is believed to function as a binding site for S.A.A and mediate downstream proinflammatory targets [71]. The SAA are acute phase proteins that belong to a family of apolipoproteins associated with HDL-high density lipoproteins [71]. These proteins are expressed in response to inflammatory stimuli and are generally released in an acute phase of inflammation[71]. Acute phase SAA have been implicated in inflammatory diseases such as Atherosclerosis, Rheumatoid Arthritis and Inflammatory Bowel Disease [71]. SAA can be utilized as a marker for systemic inflammation [71,100].

The CD36 receptor is best characterized as a type B Scavenger receptor and functions as a pathogen recognition receptor (PRR) on innate immune cells[75,76,90-94]. A hallmark of these receptors is their primitive ability to recognize specific types of molecular patterns present on pathogens[75,76,90-94,96]. These PRRs bind to specific ligands derived from bacterial, fungi and protozoa, however CD36, which is a type of PRR, binds to lipids, lipoproteins, and β-glycans in fungi and can these trigger an innate immune responses[75,76,90-94]. However, CD36 is best characterized as a scavenger receptor phagocytosing, clearing lipids, LCFA and apoptotic cells, in the body in order to maintain homeostatic conditions,[75,76,79,90].

CD36 type B Scavenger receptor are receptors that mediate the removal of foreign substances and waste in the body through the binding of specific ligands[75,76,90-94]. CD36

binds to a variety of different ligands: OxLDL, AcLDL, oxidized phospholipids, lipoprotein, Fatty acids, apoptotic cells, thrombosponin, collagen, and fatty acids[73,75,76,78,79,88,90-94,96,97]. In addition, this receptor is expressed on a wide variety of cell types & tissues, which include monocytes/macrophages and dentritic cells, hepatocytes, cardiac and skeletal myocytes, erythrocytes, platelets, amyloidogenic peptides, microvascular endothelial cells, and adipose tissue[75,76, 88,90-94,96]. CD36 receptor is considered to be a highly dynamic receptor as a result of its involvement in a wide variety of biological processes: lipid & glucose metabolism, clearance of apoptotic cells, antigen presentation, inflammation and pathogenesis[76,90-94]. This receptor also binds to LCFA—long chain fatty acids—and aids in their transport into cells, facilitates lipid metabolism and absorption and energy storage [73,75,76,79,90]. In the event of dysregulated CD36 receptor-ligand interactions, especially in lipid metabolism and absorbtion, it is believed to be a factor promoting pathogenesis across a number of inflammatory diseases[74,76,96,97].

CD36 has been implicated in the following human diseases: Arthritis, Alzheimer's, Atherosclerosis, and Angiogenesis, Diabetes, Heart Disease and Ischemic Brain injury[72,75,76,78,79, 88,90-94,88]. One central theme of this receptor which is consistent across cell & tissues types is its universal involvment in pathogenesis and lipid metabolism[76, 88,90-94,96,97.]

1.11 CD36 Expressing Macrophages & Human Disease

CD36 plays a chief role in fatty acid metabolism via binding to, internalizing and degrading lipids in the body[73,75,76,79,90-94]. However there has been a recent connection

between the overexpression of CD36+ macrophages, fatty acid accumulation, and the early pathogenesis of several human diseases [73,76-78,80,85,86,89,90-94,97,98].

Recently, despite some conflicting evidence, CD36 is generally considered to be an early indicator of insulin resistance, which is a well-known early indicator of diabetes type II [77-79,80-82,84]. In Diabetes type II the membrane-bound plasma CD36 in macrophages and monocytes are upregulated via OxLDL ligand [80,82-84]. In Type II diabetic patients, the expression of CD36 and OxLDL appears to be markedly upregulated in monocytes and macrophages, resulting in an increase of up to 34% compared to non-diabetic patients[77].

Also glucose intolerant and pre-diabetic patients exhibited high circulating levels of oxLDL, and CD36+ macrophages in serum[77,83,84]. Furthermore two diabetic risk factor conditions, namely hyperglycemia and Insulin resistance, also induce a higher expression of CD36+ monocytes [83]. In Diabetes type II these monocytes have an impaired fatty acid metabolism and inflammatory foam cells also develop inducing inflammation[83.]

A study conducted by Handberg and colleagues discovered that Diabetic patients and in prediabetic conditions—insulin resistance, glucose intolerance, and polycystic ovary syndrome—exhibited high expression of a soluble form of CD36, sCD36, from plasma derived monocytes and macrophages; this finding was not observed in healthy patients[77]. This soluble form of the scavenger receptor reflects CD36 expression in activated and infiltrating macrophages, and higher CD36+ macrophages and monocytes appeared to be a strong indicator in early insulin resistance and manifestion of Diabetes[77].

Furthermore, rodent studies conducted by Pravenec and colleges supported CD36's role in fatty acid metabolism via studying spontaneous hypertensive rats—

SHR/NIH [81]. This mouse strain has a deletion variant in CD36 fatty acid transporter and developed human-like insulin resistance, dysregulated fatty acid metabolism and hypertension[81]. When Pravenec introduced transgenic expression of CD36 this ameliorated insulin resistance and decreased fatty acids in serum[81]. Therefore, CD36 expressing macrophages and monocytes play a fundamental role in the pathophysiology of diabetes type II and Insulin resistance[77-81,83,84].

CD36 expressing macrophages also appear to play a pathogenic role in Alzheimer's disease [85,86,87]. This disease is driven by an accumulation of inflammatory microglia in plaques; the plaque accumulation is believed to be driven from an innate immune response to β-amyloid fibrils, which causes progressive neurodegeneration[85-87]. CD36 is expressed on both microglia and macrophage cells which bind to β-amyloid fibrils in normal and diseased brain tissue[85-87]. Past studies reveal there was a significantly higher expression of CD36 in diseased tissue[85-87]. El Khoury and colleages conducted a study with CD36 null mice and isolated both microglial and macrophage cells from diseased tissue; they discovered a marked reduction of cytokines, chemokines, and reactive oxygen species[86]. Therefore, the CD36 receptors are implicated in the induction of an inappropriate innate immune response to β-amyloid fibrils via macrophage and microglial cells; thus strongly suggests that CD36 plays a part in the early pathogenesis of Alzheimer's disease [85,86,87].

In a state of Hyperlipidemia—high lipids and/or lipoproteins in the blood—is a major risk factor for developing atherosclerosis, thrombosis, coronary heart disease and stroke[72,76,79,88,90,92-94,96-98]. In such inflammatory diseases particularly Atheroslerosis, one

established pathogenic mechanism is mediated via lipid laden macrophages called 'Foam cells' wherein excess fats accumulate and macrophages exhibit a dysregulated ability to degrade these fats and drives the inflammatory pathogenesis of this disease [76-78,80,88,90,93,94,96,97]. Accumulation of foam cells predisposes blood vessels and arteries to forming atherosclerotic plaques and chronic inflammation via proinflammatory cytokines through the activation of NFkβ; which when activating release of TNFα.[88,90-94,98].

Atherosclerosis is a chronic inflammatory disorder of the blood vessel wall and CD36 has been confirmed as a major contributing factor to the inflammatory state of this disease[76,89,90-94]. In the blood vessel, due to free radicals, low density lipoprotein (LDL), becomes oxidized to form, OxLDL, and this ligand loses the affinity to bind LDL receptors and gains a new affinity for scavenger receptors CD36 present on macrophages/monocytes[89,90-94]. Once LDL is converted into OxLDL this is then considered an atherogenic ligand[80,88,89,90-94,95,98].

Nicholson and colleages discovered the interaction of CD36-OxLDL, in a murine sarcoma derived macrophage ATCC cell line, J774, induced foam cell development and contributed to atherosclerotic lesions *in-vivo*[94]. To further elucidate CD36's role in Atherosclerosis, Maria Febbario and colleagues conducted a study investigating an ApoE null mice strain crossed with CD36 deficient mice, and proatherogenic ApoE mice. She examined fat accumulation, foam cell development, and atherosclerotic lesions in mice fed on normal and Western diets[93]. Febbario et al discovered that CD36-Apo E null mice fed a high fat diet had 77% less aortic lesion development, relative to normal proatherogenic Apo E mice which displayed marked lesion formation and advanced

stages of atherosclerosis[93]. Absence of CD36 almost completely abolished lesion formation in normal and western high fat diets[93].

Moreover, CD36 ApoE deficient mice also exhibited significantly less fat accumulation and foam cell development relative to ApoE null mice[93]. Thus CD36 expressing macrophages and its cognate ligand, OxLDL, have been widely accepted to play a fundamental role in the pathogenesis of Atherosclerosis[77,88,89,90-96,98].

Past studies reported by Silverstein and colleagues demonstrated that CD36 ligands were generated during injury to carotid arteries and the results were consistent with their observations that CD36 null mice were less sensitive to arterial injury showing and had longer onsets to thrombosis[91-92]. Of interest, an additional study by Eunhee and colleages identified CD36+ peripheral macrophages as inflammatory mediators in ischemic brain injuries in mice[98]. In hyperlipidemia, CD36+ macrophages exacerbated brain injury through promoting CD36-induced inflammation via the release of proinflammatory cytokines[98]. It is believed that the lipid saturated macrophages are a major contributor to the swelling of the hyperlipidemic brain; in support of this, the absence of CD36 receptor on macrophages reduced swelling and lesion size[98]. It is evident CD36 plays a an inflammatory role in pathogenesis of many human diseases and that this scavenger receptor does so via macrophages/monocytes[77,80,82-85,88-96,98].

CD36 scavenger receptor is highly dynamic receptor and appears to play a dual role as either proinflammatory or protective entity; however in most human pathologies, primarily when the innate immune system becomes dysregulated, it appears CD36 plays a dominant pathogenic inflammatory role[75-76,90-94,98].

1.12 Role of CD36 in the intestine

Interestingly, a study conducted by Oz and colleagues suggested that CD36 plays a protective role in the intestine. Researchers discovered mice dually deficient in CD36 and SRA type B scavenger receptors appeared to exacerbate colitis in DSS treated mice groups[100]. These doubly deficient scavenger receptor mice magnified colitis through increased anemia, decreased colon length, increased Serum amyloid A (an indicator of systemic inflammation), dysregulated proinflammatory cytokines of TNFα, IL-6, and pronounced lesions in comparison to Wildtype or singly deficient mice[100].

However, CD36-/- alone did not induce more severe colitis in comparison to DSS treated wildtype, and singly deficent mice significantly decreased in weight, which suggested this receptor is essential to the health of these mice [100]. Taken together the Oz et al study suggested the two scavenger receptors work in concert, to induce an synergistic protective role in the intestine[100]. When both scavenger receptors were present, SAA was largely reduced, mice exhibited decreased intestinal pathology, and increased proinflammatory cytokines production[100]. It is clear these two receptors have an additive role in association; however it is not entirely clear what the seperate roles that CD36 and SRA receptors play in the intestine. Interestingly, LPMC macrophages extratced from CD36 deficient mice significantly decreased the expression of proinflammatory cytokine, TNFα, which also implied that perhaps CD36 may play a inflammatory or regulatory role in the intestine[100].

As previously aforementioned, CD36 plays a major role in lipid metabolism via binding to, internalizing and degrading fatty acids, in order to maintain intestinal

homeostasis[1-5,8,11,17,27,28]. However, in several human diseases, this scavenger receptor plays a pathogenic role via inducing inflammation across a variety of different tissue types. One chief mechanism involved in inducing inflammation is mediated by CD36 receptor, lipid accumulation, and a dysregulated proinflammatory response via release of proinflammatory cytokines[88,90-94,98]. Although much research has been undertaken in lipoproteins, such as oxLDL, there is limited scientific investigation into the effects of long chain fatty acids (LCFA) in the intestine. Based on previously characterized mechanisms, it is possible that LCFA accumulation in conjunction with inflammatory stimulus may play a significant role in the inflammatory pathogenesis of IBD [88,90-94,98,101,105-106].

A recent study conducted by Drover and colleagues investigated long chain fatty acids absorption by CD36 receptor-mediated endocytosis and discovered that the longer the Fatty acid chain, the higher the absorption of the fatty acid and this was specific for saturated fatty acids and that contained very long acyl chains[101]. Interestingly, Drover and colleagues discovered that the absorption of very long chain fatty acids, VLCFA, in the intestine of CD36-null mice was completely nullified in mice fed on a high fat diet, which demonstrated CD36 had a dominant role in fatty acid absorption in the intestine[101].

Although the Drover's study demonstrates the importance of CD36 in VLCFA uptake,it also reveals that CD36 has an important role in Fatty acid absorption, and since the most common fatty acid in normal diet is LCFA: Palmitic Acid, we would first like to examine, the effects of this simple LCFA, and its interaction with CD36 for its potential inflammatory role in the intestine[101,102,105,106].

Palmitic acid—hexadecanoic acid—(16:0) is a hydrocarbon chain and is one of the first synthesized and abundant long chain fatty acid in humans and in mice[101,102]. Palmitic acid can be found in foods such as meats cheese, butter and dairy products; it is also found in coconut oil, palm oil and palm kernel oil. After consumption of carbohydrates, excess carbohydrates will first be converted to Palmitic acid; this fatty acid is the first LCFA produced during fatty acid synthesis[101,102].

A recent study by Seimon et al. demonstrated that the CD36 receptor directly interacted with Palmitic acid in ER stressed peritoneal macrophages; this interaction markedly induced apoptosis of macrophages in wildtype cells[102]. When cells were deficient in CD36, this significantly decreased the proportion of apoptotic macrophages, indicating Palmitic acid and CD36 significantly contributed to apoptosis of macrophages[102]. In addition, they also found TLR2-/- macrophages decreased macrophage apoptosis; it appears this process was also TLR2 dependent [102]. Although our focus is not on apoptotic cells and necrosis—although this would make for another interesting project—our main interest is in the interaction of LCFA Palmitic acid, TLR 2, of CD36 expressing macrophages in the intestine[101,102].

A paper by Nassir et al determined that absorption of fatty acids in intestinal enterocytes was CD36 dependant, and that this receptor was particularly important in fatty acid absorption in the proximal, not distal intestinal segments in a gradient-dependant manner[103]. CD36 expression in enterocytes largely decreased towards the distal parts of the colon [103]. This study also demonstrated that CD36-/- mice reduced fatty acid uptake by up to 50% , and that this receptor was crucial in the intestine for the uptake

of fatty acids and cholesterol[103]. In the intestine, it is clear that a large proportion of CD36 was upregulated by enterocytes orginating from the epithelial lumen, however it is interesting to ponder about the role of intestinal lamina propria CD36+ macrophages and their with fatty acids in the intestine [103].

With the knowledge of consumed fats and the digestion of these fatty acids in the intestine, coupled with the fact that Palmitic acid is one of the most abundant and first synthesized fatty acids in the intestine, leads to a conjecture of whether the interaction of CD36 receptor and high dose of Palmitic acid could potentially induce production of proinflammatory cytokines in the intestine. In addition it is interesting to examine the effects of Palmitic acid and TLR2 stimulated macrophages in the intestine to determine whether these conditioned macrophages could in fact induce inflammation in the intestine. We believe it is possible that LFCA stimulation, in conjunction with PGN stimulated TLR2, in CD36+ MHS Macrophages can produce proinflammatory cytokine expression and this may potentially elucidate a potential inflammatory mechanism in the intestine.

CHAPTER TWO:

2.1 Hypothesis & Study Aims.

It is a well-known fact that inflammatory macrophages are intimately involved in the inflammatory pathogenesis of IBD, and it is likely that there are multiple receptor-ligand interactions that mediate this inflammatory process. One receptor present on the surface on these macrophages is CD36 which binds to, internalizes, and degrades fatty acids in order to maintain intestinal homeostasis. It is possible when this interaction becomes dysregulated, or over stimulated, in conjunction with other inflammatory stimuli such as TLR2, that this may be a mechanism involved in the induction of inflammation in the intestine.

CD36-expressing macrophages have been implicated in the pathogenesis across many inflammatory diseases in human condition. Recently, a strong correlation has been identified between CD36+ macrophages and the induction of inflammatory disease. One established inflammatory mechanism in atherosclerosis is induced by CD36+ macrophages, an accumulation of oxLDL, and proinflammatory cytokine production. CD36 positive macrophages also play a role in the pathogenesis of other human diseases such as: Alzheimer's disease, Atherosclerosis, Arthritis, Angiogenesis, Rhematoid Arthritis, Insulin resistance, Thrombosis, and Ischemic brain injury. These diseases implicate the over-expression of CD36+ macrophages in inflammatory pathogenesis. There are many potential ligands that bind to, and activate CD36 macrophages. A multitude of evidence implicates two ligands that are involved in stimulating these CD36+ Macrophages, TLR2 and LCFA: Palmitic acid[68,69,101,102]. I believe that CD36

expressing macrophages may induce an inflammatory response when treated with a high dose of LCFA, perhaps coupled with a inflammatory stimulus, such as TLR2, would induce inflammation in the intestine.

I hypothesize that CD36 macrophages stimulated by both Palmitic acid and TLR 2, will induce inflammation in the intestine. To test this hypothesis, I have designed three research aims:

(1) Identify CD36-expressing macrophages in IBD patients and TNBS colitis mice.

(2) Examine *In vitro* stimulation of peripheral lung derived MHS macrophages with high dose Palmitic acid and TLR ligand 2 both individually and together to assess proinflammatory cytokine production.

(3) To induce intestinal inflammation by adoptive transfer of conditioned CD36+MHS macrophages.

CHAPTER 3: MATERIALS AND METHODS:

3.1. List of Antibodies & Regents

The following reagents were purchased from Sigma Aldrich, Oakville, Ontario: Ammonium Chloride, Blenzyme, DTT,DAPI, DPX Mountant for histology, Ethidium Bromide, ETDA, GenElute Plasmid Miniprep kit, Tween 20, Trition X-100, DMSO, Crystal violet, Dimethyl Sulfoxide, DNase, Eosin, Ly6C, Ly6G, Hematocylin, Percoll, Protease inhibitor cocktail, PGN-Peptidoglycan, LPS-Lipopolysaccride, PolyIC, Dipotassium hydrogen orthophosphate (K_2HPO_4), Disodium Hydrogen Orthophosphate (Na_2HPO_4), Sodium Dihydrogen Orthophophate (NaH_2PO_4), Palmititc acid and fatty acid free BSA. Bioshop Burlington ON purchased products were: Agarose, BSA-Bovine Serum Albumin, Glycine, TEMED, Tris, Sodium Citrate, Sodium chloride, Sodium Dodecyl Sulphate(SDS), OCT medium. Invitrogen Inc, Carlsbad, Canada purchased supplies were as follows: DEPC water, DMEM— Dulbecco's Modified Eagle's Medium, GeneAmp® dNTP, TNBS—Trinitrobenzene Sulphonate, Trypsin, FBS— Fetal Bovine Serum, Penicillin-Streptomycin Solution, and cell culture RMPI-1640, Roswell Park Memorial Institute media, BamHI, EcoRI, and T4 DNA ligase kit. Products from Bio-Rad Mississauga, Ontario: iScript™ cDNA Synthesis Kit, 20% SDS solution and 30% Acrylamide/Bis Solution. From Caledon Laboratories, Georgetown, Ontario: Chloroform, Glycerol, Isopropyl alcohol, and Methanol. From Fisher Scientific, Ottawa, Ontario: Acetone, Trizol, Xylene. Ethanol was purphased from the Scientific Store, with permission from McMaster University. St. Joesph's Hospital, Hamilton, Ontario kindly

supplied formalin reagent. CD36 SHRNA primers were designed by myself and Oligomers were synthesized at MOBIX lab at McMaster University.

FACS antibodies were purchased from eBioscience, Inc Ontario: F4/80-APC conjugated, F4/80-PE conjugated, F4/80-APC, CD36-PE, CD11b-FITC, IFN-g-APC, IFN-g-PE, IL-17A-PE, TNFa-FITC. Western blot antibodies were purchased from Santa Cruz Biotechnology, Inc, U.S.A: Polyclonal Goat Anti mouse CD36, and Polyclonal rabbit anti mouse CD36, TNFa polyclonal goat antibody, Beta actin mouse monoclonal IgG, Anti mouse HRP, anti-goat HRP, and goat anti rabbit HRP. CD36-FITC monoclonal antibody for IHC was purchased from Abcam. T25 culture flasks, 5, 10 and 25ml serological pipettes, 15 and 50ml tubes were purchased from Sarsted company. White, yellow and blue small and large tips, 1.5ml microcentrifuge tubes were ordered from McMaster Health Sciences Science store and purchased from Diamed.

3.2 Animal Models

3.2.1 *Origins of Animals & Treatment*.

All Scientific investigations were undertaken with Balb/c Mice purchased from Charles River Laboratories International. Mice were housed in CAF facility in a pathogen-free room at St. Joseph's hospital according to AUP protocol. Experiments were granted approval from AUP, BUP, permission from the CAF facility and McMaster University. When animals arrive, it is mandatory to give a minimum of one week to adapt and settle to the new environment before any experiments are permitted. Animals 10-12 weeks of age were weighed prior to the beginning of any experiments, and only animals 22g or more were considered to be healthy.Five mice were assigned per group.

3.2.2. *Naive Mouse Model*

Mice were fasted 24 hours prior to treatment, but allowed to keep hydrated. On the experimental day, control mice were injected on day 1 with 50% ethanol with a soft tube inserted intrarectally, 0.1mm was injected directly and slowly into the colon 4 cm deep. Mice were anesthetized with oxygen set and isofluoranefor induction on anaesthesia equipment in the CAF facility, and placed on a heating pad whilst under anaesthesia. Glycerol was used to ensure tube is inserted softy and slowly, and to stimulate rectal opening. After injection, mice are then wiped and cleaned to ensure no blockage of fecal route, then these mice are placed back into their cage and behaviour of mice are monitored every 2-3 minutes over 30 minutes to ensure the mice recovered. When conducting this procedure, there is a small chance you can perforate the intestine or cause blockage, mice must be observed periodically over several hours after operation. Each calendar day the mice weights were recorded and DAI is assessed. If there was any error in the procedure, or mice did not recover well, i.e. greyish color of fur, blue-to grey tone to feet, closed or swollen orbits, fir standing up, lack of activity and/or hiding behaviours over a sustained period of time this mouse was euthanized. The mice protocol we follow is a seven day treatment. Mice are sacrificed on day 7 by $C0_2$ followed by cervical dislocation. For all experimental procedures the small and large intestine were dissected and used for experiments.

3.2.3 *TNBS Colitis Mice*

Mice were fasted 24 hours prior to treatment, and allowed to keep hydrated. On the experimental day, control mice were injected on day 1 with 5mg TNBS diluted in

50% ethanol, per mouse. Each mice is as described for the naive mouse model in 3.2.2. The TNBS mixture is was made in the BSCII hood following aseptic techniques. A mixture of 500ul 50% ethanol is mixed with 500ul of TNBS (Picrysulfonic acid solution). Tubes are inverted up and down 10-15 times to ensure mixture prior to injection. Glycerol is utilized to ensure tube is inserted softy and slowly, and 0.1mm was injected per mouse. After the procedure, behaviour of mice are monitored every several minutes over 30 minutes to ensure recovery. Mice were euthanized if any wanring signs of illness or lack of recovery presented. The mice were scarified on day seven, or earlier for mice that reached a low body weight (<19kg) or endpoint. The small and large intestine are dissected and used for experiments.

3.2.4 *TNBS half-dose Model*

After analyzing full dose TNBS, we were interested in examining the response of a half dose of TNBS to examine whether or not half dose developed inflammation in the intestine and its effects on CD36+ Macrophages. In a similar manner as mentioned previously in 3.2.2 and 3.2.3, a dose of 1.25mg TNBS was diluted in 50% ethanol per mouse and 0.1mm was injected intrarectally on day one. Mice were also fasted for 24 hours prior to treatment and were sacrificed on day seven; if mice reached an endpoint mice were sacrificed immediately. The small and large intestine are dissected and used for experiments.

3.2.5. *MHS & TNBS half-dose Model*

After a high expression of CD36 receptor was confirmed in MHS macrophage cell line via FACS analysis, this cell line was utilized to explore the effects of conditioned

CD36+Macrophages, and their role in the murine intestine. CD36+ MHS macrophages were grown and cultured in RMPI media until 80% confluency, these cells were harvested using trypsin, centrifuged at 1500 rpm for 5minutes, and resuspended in 1ml of RMPI 1640. cells were slowly pipetted up and down until suspension was uniform; a milky pink consistency of cells should be observed. The cell suspension was measured via the haemocytometer and adjusted to 10^8 cells per ml. Cells were then put on ice and prepared for tail vein injection. 0.1mm MHS macrophages were injected into mice.

The tail vein procedure is conducted by first using a container filled with warm water, using the tail vein trap for mice and submersing their tails into warm water until the vein is visible. After waiting 30 seconds to one minute the vein should be fully visible and at the surface. Then holding mice tail parallel to the arm and needle, the needle should neatly scoop into the vein and should not be resistent, the needle must go in straight, and the suspension can be visibly observed following down the central vein if done correctly. Injections should first start at middle of the tail and work upwards when experiencing difficulty. After each injection you must put pressure on the wound to stop the small bleed, and ensure clotting and minimize infection. After wiping off the tails of mice with ethanol wipes, tails were re-labelled and mice were put back into appropriate cages. For this particular mouse model, half dose TNBS injections were completed the next day to avoid unnecessary stress to the animals. On the second day 1/2 TNBS was injected intrarectally as previously described above in 3.2.4. Mice are sacrificed on day 7 and the small and large intestine are dissected and used for experiments.

3.2.6 *MHS Stimulated Palmitic Acid Mice*

MHS macrophages were cultured in RMPI 1640 until 80% confluency and then stimulated with high dose (600ul) of Palmitic acid for a 24 hour time course. Afterward the cells were trypsinized, harvested, and centrifuged at 1500rpm for 5 minutes. Macrophages were resuspended with 1ml of fresh RMPI 1640 media and pipetted up and down until the cells are uniformly distributed. These cells were measured via the haemocytometer to be 10^8 cells per ml and diluted down to this concentration. MHS+PA macrophages were then put on ice and prepared for tail vein injection. Mice were injected with 0.1mm of MHS+PA solution via tail vein procedure outlined above 3.2.5. Mice were fasted for 24 hours prior to treatment, and injected on day one follwong sacrificed on day seven. If mice reached an endpoint as described above mice were sacrificed immediately. Mice are sacrificed on day 7 via CO_2 and cervical dislocation. The small and large intestine are dissected and used for experiments.

3.2.7 *Normal MHS Control Mice*

MHS macrophages were cultured in RMPI 1640 until 80% confluentcy and then were trypsinized, harvested, and centrifuged at 1500rpm for five minutes and resuspended with 1ml of fresh RMPI 1640 media. This suspension was pipetted up and down until the cells were uniformly distributed. The cell suspension was then put on ice and prepared for tail vein injection. These cells were measured via the haemocytometer and 10^8 cells per ml, were used, 0.1mm of MHS macrophages were injected via tail vein as described in 3.2.5. Mice were also fasted for 24 hours prior to treatment and were injected on day one and sacrificed on day seven. If mice reached an endpoint as described above mice

were sacrificed immediately. Mice are sacrificed via cervical dislocation and CO_2. The small and large intestine are extracted and used for experiments.

3.3 Origin of Cells & Tissues

3.3.1 Human Tissue Samples

Human samples were obtained from active Crohn`s Disease and Ulcerative Colitis patients from China, little information is found on patient origins. Different samples were marked on an indexed paper and appropriately labelled per slide to ensure samples were taken from different patients. Human samples were fixed in formalin and immediately preserved in paraffin wax and stored in 4°C. Human controls samples were also fixed in paraffin and were dissected from colon cancer patients, and normalized tissue sections were utilized as non-IBD patient controls. Immunohistochemistry was undertaken to examine the expression of CD36 receptor and F480 in colonic sections of the intestine.

3.3.2 Cell Culture & Cell Lines

MHS murine derived lung macrophages originally purchased from ATCC were a generous gift from Dr. Zhou Xing and was cultured with RPMI 1640 according to the outlined conditions advised from ATCC. MHS cells were grown in RPMI 1640 culture media with 10% FBS and 1% penicillin-streptomycin in 37^{o}C placed in the 5% CO_2 in the cell culture incubator. Cells were only passaged three to four times in culture.

3.4 LMPC Extraction and Culture:

Immediately upon sacrifice of mice the small and large intestine were dissected. Tissues were cleaned in ice cold phosphate buffered saline and then transferred to an additional clean petri dish containing 20ml PBS. These petri dishes are then transferred

into the biological safety cabinet to carry out the remaining procedures. The intestines were cut open with sterile autoclaved scissors and stools were removed. Subsequently the intestines were cut into 1cm pieces and put into a 15ml conical tubes containing 10 ml of EDTA and DTT solution to wash the intestine and sterilize the tissues. The tubes were then placed in the shaker for 15 minutes at room temperature and shook at 60rpm, resulting in the solution turning opaque. IN BSCII hood, the intestines were then cut into smaller pieces of approximately 0.5cm and are then put into a 15ml conical sterile tube filled with 10ml of RMPI 1640 media, then 5ul of Blenzyme and 5ul of Dnase was added to digest the tissues and release LPMC cells. These tubes are again put into the thermo shaker for ten minutes at 37°C and shook at 60rpm speed for 10 minutes.

The solution was then pipetted through a 45µm strainer into a new clean 15 ml conical tube, and centrifuged for 10 minutes at 1500 rpm. Pellets were washed again with 10ml media under sterile conditions and centrifuged at 1500rpm again for five minutes. The supernatants were discarded and cells are cultured in 20ml RMPI 1640 media in T25 flasks and put into the CO_2 incubator.

The cells are harvested after a week and put into labelled tubes and centrifuged for 6 minutes at 1500rpm. Pellets were washed with media and cells were re-suspended in 10ml of 40% Percol solution by pipetting up and down. The cells in Percol were slowly overlayed with a transfer pipette onto 5ml of 80% Percol solution in a 15ml conical tube, forming a gradient. During this procedure careful not to agitate the layers as cells will be lost in solution. The Percol tubes are then centrifuged at 2400rpm for 20 minutes. After centrifugation, an opaque white layer was observed in the centre of the suspension,

containing the LPMC cells. Using sterile pipettes the top layer was pipetted off and disposed of, then the LPMC layer was carefully removed with a new pipette in a circular motion and pippetted into a new sterile 15ml tube, washed again with RMPI 1640 media and centrifuged at 1500rpm for 5 minutes. The supernatant was removed and cells were resuspended in RMPI 1640 media. Cells were recultured with 20ml RMPI media in a T25 flask and ready for FACs analysis.

3.5 Immunohistochemistry

3.5.1. Preservation of Tissues

There were two ways to preserve tissue samples (a) cryostat and OCT or (b) paraffin preservation. Immediately after mice tissues were sacrificed, dessected and cleaned in sterile PBS the colon tissues were immediately preserved in formalin or in OCT compound. For cryostat sections, colons were dropped lengthwise into a aluminium foil container labelled with specimen name and then quickly dropped with forecpts into a sealable container with liquid nitrogen, and immediately be put into -80°C storage until use. For paraffin sectioning, microcentrifuge tubes containing 1ml 10% Formalin were labelled and prepared for each mice per group. After using sterile forceps the tissues are inserted into formalin until all mice are sacrificed and all tissues are collected. Then microcentrifuge tubes were brought into a clean sterile BSC II cabinet and tissue cassettes were labelled in pencil per mice, per group and two pieces of 1-2cm colon tissues were placed upright. Subsequently, these tissues cassettes were placed in a 500ml 10% formalin container and brought to the second level of St. Joseph's Hospital to the pathology room for paraffin embedding and H&E staining.

3.5.2 *Cutting of Tissue samples, Cryostat & Microtome*

Tissue samples preserved in OCT(VMR Clear Frozen Section Compound) were cut in 6μm sections via Jane Foster's BBI cryostat machine in T-3330 and fixed on coated blood smear slides. The cryostat blade was provided by the Yang lab. Briefly, the blade fixed to the machine, and allowed to incubate in the apparatus for 20-30 minutes to ensure the blade was cooled to -37°C for proper cutting. Samples were removed from -80°C and placed in the OCT machine. The OCT block was fixed to the apparatus and the tissue was affixed to the metal block via OCT compound. Blocks were then adjusted to the desired angle in order to begin cutting. Tissue sections were placed on slides, with 3-5 tissue sections per slide, multiple slides per mouse. Slides were allowed to dry for 24 hours at 24°C and residual samples were placed in 4°C fridge.

For paraffin embedded tissues, the paraffin block is first fixed on the BBI microtome with the safety on and then, the blade is carefully placed and locked in. Samples are fixed with the bottom of the cassette facing left, the sample is clipped in. Samples are cut using the microtome at 3μm in diameter and 3-5 sections were cut per slide, and multiple slides were cut per each mouse. When cutting the sample, the microtome is rolled in a forward clockwise manner, and the machine is rolled backwards from sample in a counter-clockwise manner. Using chilled forceps, the sections were transferred to a sterile 40°C water bath, to flatten wax samples, and air bubbles must be avoided for proper mounting of slides. Paraffin tissues sections were gently picked up via forcepts and slid onto pre-treated blood smear slides. Finished slides are placed in a case and allowed to dry for 24 hours at room temperature, then placed into 4°C fridge.

3.5.3 *Preparing samples for Staining*

Cryostat OCT preserved samples required fixation by adding acetone drop wise to each sample and incubation for 20 minutes at room temperature. Alternatively, 4% Formalin may also be utilized with an 1 hour incubation time. Following fixation, samples were washed 3-4 times in PBS for five minutes at room temperature in IHC chamber. Washing at this stage is crucial as clearing excess fixative from the tissue is essential for proper antibody binding to its epitope[107].

For Paraffin-embedded samples, the tissues are first dipped into the xylene container to de-wax for 3-5minutes, generally 4 minutes is best. The longer in xylene the more likely it is to strip tissue from the slide. Tissues are rehydrated of tissues with 100% ethanol, followed by 90% ethanol and then 70% ethanol for an incubation time of five minutes each. Subsequently, these samples are then incubated in ddH_20 followed by PBS for five minutes. Each slide is transferred to PBS-filled slide holders and steamed (high humidity, 100°C in PBS) for 4-5 minutes for antigen retrieval[107]. Then blood smear slides are fixed with acetone for 20minutes, then washed three times for five minutes in ddH_20; then slides are ready to be stained.

3.5.4 *Immunohistochemistry Staining Protocol*

Tissue sections were blocked with 1% BSA for thirty minutes at room temperature in a humidity chamber. The majority of BSA was removed, and prepared 1% BSA 1:100 primary antibody was incubated for 1 hour at room temperature, then incubated at 4°C overnight; our lab has demonstrated this increases antibody binding efficiency(Xiao Chen). The following day, slides were washed with PBS 3 times, for five minutes, and

secondary antibodies were added for1 hour at room temperature. Secondary antibodies were washed 3-4 times with PBS for 5 minutes. Slides were kept covered in order to minimize exposure to light. Slides are permitted to dry and anti-fade mounting media is overlayed followed by a cover slip. Twenty pictures from each mice group, five mice per group, were taken with the BBI Zeiss fluorescent microscope. In order to minimize bias, randomized numerical tables were followed and sample numbers were picked at random to choose the odd or even samples for cell counting; to explain further a slide may contain 5 samples from same mouse, if the random numbers table first selected odd numbers, samples 1,3,5 would be utilized for staining and competent cell counting. Competent cells were counted per mice groups and sorted via expression of the following markers (a) CD36 only, (b) F4/80 only or (c) Double Positive.

For CD36 antibody staining a dilution of 1:100 in 1% BSA was utilized for the primary antibody, and a 1:200 for anti-goat FITC or anti-rabbit-FITC, dependent upon which CD36 antibody was selected. For F4/80-PE this antibody was already conjugated and used in the secondary antibody stage as it was fluorescently labelled; also 1:100 dilution in 1% BSA was used. In the first step, primary antibody only CD36 rabbit or goat was added, in the second step CD36 goat or rabbit-FITC, F4/80-PE 1:100 dilution and ≤0.5ul of DAPI was mixed into this 1:100 ratio. Isotype IgG was employed in the same fashion as mention depending on which host was used: rabbit or goat.

3.6 Fluorescence-Activated Cell Sorting

Cells were cultured in RMPI-1640 until 80% confluency, and harvested via one of two methods (a) trypsinization, for MHS, or (b) flask tapping and cell dislodging for

LPMC, dependent upon the nature of cells being used for FACS analysis. When using trypsin method, 1%trypsin was added to cell culture flasks that had been previously washed with 10ml sterile PBS to remove serum. T25 cell culture flasks were then placed back into the $C0_2$ incubated maintained at 37°C for 5-10minutes, until culture microscope displayed ≥80% of cells dislodged. Once cells were fully dislodged, in the BSCII hood 10ml solution was pipetted into a sterile 50ml tube and these tubes are then centrifuged at 1500 rpm for 5minutes at room temperature. Under sterile conditions the supernatant is discarded, and 10ml of sterile PBS is used to wash the cell pellet. After pipetting solution well with media, the cells were centrifuged again at 1500rpm for 5 minutes at 4°C and supernatant was discared; this is repeated twice to ensure no trypsin was present. Cell pellets were resuspended in sterile PBS, with the volume adjusted depending on the nature of the experiment. Cell suspensions were aliquoted, 1 ml per microcentrifuge tube, and centrifuged at 1500 rpm for 5minutes at 4 °C in the mini centrifuge. Pellets were resuspended in 400ul of FA buffer (1% FBS serum in PBS) and incubated for 20 minutes, immediately followed by another centrifugation; FA buffer was carefully removed and discarded. Cell pellets were blocked in 200ul of 1% BSA for 20 minutes. Antibodies were added at a 1:100 ratio in a total volume of 200ul per tube, consisting of 2ul of antibody to 198ul BSA, and incubated on ice and avoided light for 1 hour. The cells were then pipetted and centrifuged as previously described, and washed twice with sterile PBS in the BSC hood. If primary antibody was not conjugated, the cells were resuspended in 1%BSA 200ul into each microcentrifuge tube, following a dilution of 1:100 2ul of fluorescently-conjugated secondary antibody into 198ul of blocking solution (1%BSA)

and incubated for 1 hour. Then cells were washed twice in sterile PBS and centrifuged at 1500rpm for 5minutes at 4°C. Either fluorescently-labelled primary or secondary-treated cells were resuspended in 400ul of FA buffer for FACS analysis, to permit an equal and well distributed flow through the FACs BBI Canto machine. Note: LPMC cells were first filtered via 0.45um filter into the FACS tubes to ensure minimal debris and epithelial cells in cell suspension. Cells are then sorted via their wavelength and fluorescent labels and FACS Flow Joe was employed to examine data analysis.

3.7 Real-time & RT-PCR

3.7.1 RNA extraction

Immediately upon sacrifice, 1-2cm of murine colon tissues were cleaned and placed into microcentrifuge tubes containing 1ml of sterilized PBS on ice. RNA extraction was carried out on the day of dissection for optimal extraction. However, samples may be preserved at -20°C for up to 1 week. Samples were put on ice, and 1ml of TRIzol was added to100-150mg per each sample. The solution and tissues were put into glass tubes and homogenized on speed 6 of the Yang`s lab homogenizer for five minutes until the solution is uniform and an opaque, pink colour. The solution is then incubated on ice for 15 minutes, inverting tubes every 5 minutes, after time was up 200ul of chloroform was added to suspension and incubated for 15minutes; tubes were inverted similarly. Samples were then centrifuged at 12000rpm for 15minutes at 4°C. After centrifugation was complete three phases were visible: (a) a clear layer, which contains RNA (b) an opaque layer and (c) and a pink layer. The clear layer was carefully extracted and transferred into a newly labelled microcentrifuge tube; note the clear layer cannot

touch the opaque or pink layers, otherwise RNA is contaminated. Once clear layers were extracted and placed in labelled microcentrifuge tubes, 500ul Isopropanol was added and tubes are inverted several times. RNA is allowed to precipitate at room temperature for approximately 10 minutes, afterward samples were centrifuged at 12,000rpm for 10 minutes at 4°C. The pellet was washed twice, and mixed with 200ul sterile 70% ethanol and centrifuged at 12,000rpm for 10minutes, to purify the RNA samples. The microcentrifuge tubes are then placed in the BSCII cabinet tops opened for 4 minutes to allow residual ethanol to evaporate. Dried pellets were resuspended in 20-40ul DEPC water dependent upon pellet size. Rna samples were stored in -20°C and labelled appropriately for storage.

3.7.2 cDNA Synthesis

RNA concentration was measured with Nano drop Spectrophotometer in Jane Foster's lab in St. Joseph's Hospital BBI centre. Concentration was recorded in mg/ml, in addition to $A_{260/280}$ values, and tubes are stored at -20°C for later use. Reverse transcription using 0.5-2µl of RNA, dependent upon DNA product length, template was prepared using the Iscript reverse transcriptase enzyme system (BioRad) as directed and prepared in DNA clear tubes (Diamed). Tubes were pipetted and centrifuged briefly to ensure sample collection and removal of bubbles, and placed in a thermocycler (BioRad) for cDNA synthesis; please note NTPs were already present in the iscript mix (Table 1). Resultant cDNA strands were stored at -20°C prior to use in PCR reactions, samples should be aliquoted as multiple freeze, thaw times of cDNA is not advisable.

Table 1. Thermocycler conditions for reverse transcription of RNA templates

25°C	42°C	85°C	4°C
5 minutes	30 seconds	5 minutes	Hold

3.7.3 Standard PCR

cDNA strands were removed from -20°C and used to set up PCR to amplify CD36, Beta actin and TNFα, using primers designed Liz Garside, Xiao Chen and Natasha Leung respectively; See primer sequences as summarized in Table 2.

Table 2. Primer sets used in PCR, RT-PCR, and Real-time PCR Experiments

Primer Set		Sequence (5' to 3')
B-Actin	Fwd	AGG AAT TCA TGG TGT GTC TG
	Rev	AAG GAT CCT CAG CTC AGG AAT
CD36	Fwd	ACA GGG CTG GAG ATA TCA GGG ACT C
	Rev	CTC CTC GTG CAG CAG AAT CAA GGG
TNFα	Fwd	AGG AGG GAG AAC AGA AAC TC
	Rev	AAT GAG AAG AGG CTG AGA CA

Note: B-actin primers were provided by Xiao Chen; TNFα by Natasha Leung

The PCR reaction was set up using 0.5µl of each forward and reverse primer, 0.5µl template cDNA, 10µl Master Mix and 8µl of sterile DEPC water, to a total reaction volume of 20µl. Reaction tubes were pipetted, mixed, and centrifuged to ensure mixture collected at the bottom of the tube, and all air bubbles were removed. PCR was carried out using the BIO RAD PCR machine according to the conditions outlined in Table 3 subject to different annealing temperatures for each primer; each primer required a

separate PCR**.** The optimal annealing temperature for CD36 56°C was determined via a gradient PCR (data not shown). In addition the annealing temperatures for TNFα and β-actin primers were determined to be 52.5°C and 54°C as determined via gradient PCRs as confirmed previously via Hong Song and Natasha Leung; fellow Master`s students. CD36 should yield an amplicon of approximately 296 kb, β-actin 240kb and TNFα 150kb. After PCR amplified samples were completed, samples were then placed on ice and prepared for agarose gel electrophoresis by dilution in a 1:9 ratio of DNA to loading buffer.

Table 3. Thermocycler conditions for PCR from cDNA strands.

	35 Cycles			
98°C	98°C	56°C	72°C	4°C
1 minute	10 seconds	30 seconds	1 minute	Hold

3.7.4 Real Time PCR

All cDNA used for real time PCR was first quantified via an ND-1000 NanoDrop spectrophotometer from Jane Foster`s Lab for all test groups. The mean cDNA concentration per tube in each reaction was calculated and this was designated as the starting concentration for the standard curve. For each reaction 1µl of cDNA was mixed with 0.5µl of forward and reverse primer, 1µl cDNA,10µl of SYBR-Green (BioRad), and 8µl DEPC yielding a final reaction volume of 20µl. The first tube will utilize 2µl of 20µl reaction mixed and dilute serially 1µl into 1ul DEPC, mixing slowly and very carefully. The standard curve of 7 other concentrations was prepared by this initial serial dilution; the conditions for Real time PCR is as outlined in Table 4. BIORAD white sealed flat top tubes were used for all samples and tubes to minimize light exposure to SYBR-green, as

exposure to light will degrade fluorescence. The standard curve is set up in the first row and all remaining samples afterwards, and the reaction was set up and followed by the designed protocol Opticon RTPCR machine using the conditions outlined in Table 4. The PCR machine can then calculate the efficiency and amount of CD36, TNFα and β-actin expression in each sample (in ng). Efficiencies generally varied between 50-100% of gene expression (dependant on mouse sample and individual differences observed in mice) and was calculated accurately by determining the line of best fit from the standard curve.

Table 4. Thermocycler conditions for Real-time PCR

		35 Cycles		
98°C	98°C	56°C	72°C	4°C
1 minute	10 seconds	30 seconds	Hold	

3.8 Western Blotting

3.8.1 Protein Extraction

Frozen mouse colon samples were thawed gently on ice. The tissue was homogenized in 360µl lysis buffer with 40µl of Protein inhibitor cocktail (Sigma) with Yang labs homogenizer for 5 minutes on speed 6, spinning BD Falcon glass tubes to ensure equal homogenizing of tissues. Tissues are then incubated for 30 minutes on ice, transferred back to 1.5ml microcentrifuge tubes and centrifuged at 14,000rpm for 20 minutes at 4°C, yielding a transparent supernatant containing the protein fraction**.** If the supernatant was opaque, it was transferred to a new microcentrifuge tube for a second centrifugation, or centrifuged again until fully clear protein containing supernatant was observed. The

supernatant was then transferred to a fresh microcentrifuge tube and protein concentration in mg/ml was measured using the BBI Nano Drop Spectrophotometer in Jane Foster`s lab.

3.8.2 Preparing Sample, Running Gel & Transfer

As a standard, 7.5µl of sample buffer and 3µl of reducing agent was added to each microcentrifuge tube for preparation of protein samples. Then, protein samples were diluted to contain 50mg in a final volume of 30ul for gel loading. Protein samples were mixed thoroughly and heated up to 100°C for 5 minutes, and centrifuged briefly at low speed prior to gel loading. Then 15-20µl of tissue or cell sample was loaded into each well, along with one lane containing 8µl of protein page ladder marker (McMaster scientific store) can be added to each gel band location and size comparison. Yang lab followed laboratory protocol to prepare gel(s) as seen in SOP binder under laboratory techniques.

SDS-PAGE is run at 90volts BioRad Western blotting machine until samples have passed through the top stacking gel, approximately 30 minutes were required to pass this layer, and then voltage is scaled upward to 142 volts for 1 hour through separating gel. Gels were removed when the ladder lanes were clearly separated and had reached the bottom of the gel, and then transfer could be prepared. Transfer to nitrocellulose membranes was completed using a semi-dry sandwich layering method with BioRad transfer equipment, in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol). Please note it is mandatory that membranes are pre-treated with 1% reagent grade methanol before transfer of protein from gel to membrane; when everything is

prepared ensure equipment is set right to ensure protein travels downward on the gel. For one blot using the BioRad apparatus it is set at 22volts for 1 hour, and two blots require transfer for a total of 1.5 hours.

3.8.3 Western Blotting, Antibody incubation

Once transfer is complete, the membrane is extracted from the transfer apparatus with forceps into a container and membrane(s) were blocked for 1 hour at room temperature with agitation using 5% non-fat dry skim milk powder in TBST, 1gram skim milk into 20ml TBST (Tris Buffered Saline and 0.1% Tween 20). Primary antibodies are incubated at a concentration of 1:1000 in 5% skim milk TBST solution as described above for 1 hour on the shaker. Alternatively, some antibodies, including Santa Cruz rabbit-anti CD36 and goat-anti TNFα were incubated for different membranes and put into labelled containers; the membranes incubated at 4°C with agitation overnight.

Following primary antibody incubation, blots were washed 3 times with 1% TBST for 15 minutes on the shaker on minimum speed. Then blots were incubated with secondary antibodies were added in 0.05% w/v BSA/1%TBST (1:5000 Mouse anti-rabbit-HRP and donkey anti-goat-HRP Santa Cruz Biotechnology). Three more washes in 1%TBST were implemented and blots were analyzed by autoradiography by Western Lightning Enhanced Chemiluminescence (ECL) reagents (Perkin-Elmer, Boston, MA). Following visualization of the band of interest, blots were washed 3 times with TBST and re-probed with loading controls (i.e.β-Actin) as described. However if bands were in the same location, it was required to strip the gels first. Western Blot Bands can be analyzed and

quantified via Image J and were graphed graph pad prism 5 software program and Microsoft excel 2007.

3.9 Histological Score & Mononuclear cell counting.

3.9.1 Histological Scoring

To determine the inflammatory score of mice colon samples, histological ranking was carried out according to the standards set by Cooney et. al (Table 5)[112]. H&E stained tissue sections were obtained from the STH pathology laboratory and mounted as described in subchapter 3.5. Twenty pictures were taken via BBI Zeiss microscope at 20X magnification and histological score were calculated per picture, per mouse, ranked and averaged. Briefly, to access the severity of inflammation in the intestine three basic parameters were investigated: (a) Crypts & Glands, (b) Edema, and (c) Mononuclear cell infiltration; which are scored 0-3 based on whether there is, focal, zonal, or throughout the tissue layers. The sections on each slide were chosen by random number tables. Statistical analysis is described for all data sets in 3.14.

Table 5. Histological parameters and assessment in IBD[(112)].

Parameter	Score	Condition
Epithelium and Glands	0	Normal
	1	Focal disruption of epithelial surface and/or glands
	2	Zonal disruption of epithelial surface and/or zonal crypt loss
	3	Diffuse mucosal alteration involving sub-mucosa and/or diffuse crypt loss
Inflammatory Cell Infiltration	0	Absence of infiltrate
	1	Sub-epithelial and in the Lamina Propria
	2	Infiltrate reaches the muscular mucosa
	3	Severe and diffuse infiltrate reaching the sub-mucosa and/or involving the Muscolaris Propria
Edema	0	Absent
	1	Focal
	2	Zonal and/or moderately diffuse
	3	Extensive and severe

Note: Image adapted by from Cooney et. al. (2007)[112] and Natasha NurtanioThesis (2012).

3.9.2 Mononuclear Cell Counts

Tissue sections were chosen at random as described in 3.9.1. Pictures were taken at 20X magnification for mononuclear cell counting. Mononuclear cells are visualized by their small, rounded, significantly dark purple staining. Twenty cell counts were calculated per mice (n=5) and averaged over each mouse group yielding an avergae score. The diameter of the slide was accounted for in order to calculate the number of mononuclear cells/mm^2

from the BBI fluorescent Zeiss microscope. Cells were counted utilizing the cell counter and grid functions of Image J software and statistically analyzed as outlined in 3.14.

3.10 MPO: Myeloperoxidase Activity Assay

First reagents must be prepared before experiment begins it is best to use freshly made buffer and reagent for optimal results. First solution A is prepared via adding 3.4grams of 1M KH_2P0_4 into 25ml ddh_20 and then solution A must be mixed well. Then Solution B is prepared 4.5grams of K_2HP0_4 into 25ml ddh20 and mixture is shaken well before use. In order to prepare Potassium Phosphate buffer, Add 1ml of Solution B into all of solution A and mix until all components are dissolved and ph muste be adjusted from 4 to 6. In addition, the MPO sample solution was prepared via adding 0.0175g of HTAB to the above buffer, and mixed well until fully dissolved, this buffer should be made separately for samples only.

Frozen MPO samples are thawed gently on ice and then placed alternately in a 100°C water bath and liquid nitrogen, for three freeze thaw cycles of one minute each at each temperature. After freeze thaw and samples are brought to room temperature, PBS is decanted and 400ul HTAB buffer solution is added to each ependorf tube, then colonic tissue sample is placed into homogenizer tubes and homogenized mechanically as described in 3.8.1. Each different mice group of colon tissues required sterlization with 70% prior to homogenizing. Samples are then ready for MPO Elisa Sarssted plate and use. To prepare the next step, another potassium phosphate buffer is prepared with HTAB, Hexadecyltrimethyl, as previously outlined with the addition of 0.167mg/ml of O-Dianisidine Dihydrochloride into solution, this solution is used to start reaction when

H_20_2 is added. When the plate is set up the very last step is to add hydrogen peroxide, dependent upon how many samples you use, for every 10ml of solution 20ul of peroxide is used. perioxide is addes only when plate is ready to be read. First,50ul of Evans blue is added to 1ml ddHh$_2$o and mixed well, this solution starts off the standard curve, 200ul of this solution is added to the plate for the standard curve into the first top well, and concentration is diluted serially downwards by adding 100ul of standard, into 100ul ddh$_2$0.Once reaction was set up and plate was ready, 40ul of H202 was added to the HTAB potassium phosphate buffer containing Dianisidine Dihydrochloride. Plate was swirled quickly to ensure all hydrogen peroxide was starting the reaction equally. Immediately 100ul of the reaction solution was pipetted into each lane via a serial Elisa pipette, this process is time sensitive as the reaction started right away. Then plate was immediately read by Gene5 program previously set up and ready to read plate and plates were read at 450nm. Differences between groups were analyzed via subtracted differences between 450nm from plate 2-7 readings, and this presented the overall change in myeloperoxidase per group. Results were plotted using Graph pad prism 5 and Statistical analysis is carried out at described in 3.14.

3.11 Palmitic Acid Preparation

For LCFA stimulation, a 5mM Palmitic Acid in BSA stock solution was prepared, and 20ul was added per 1ml media per T25 flask.. For LCFA, Palmitic acid, this preparation generally yields 20ul of stock solution per 1ml of media; since 20ml of RMPI 1640 cell culture media is added to T25 cells, then a regular dose of Palmitic acid would be 400ul per 20ml of media, since this project was interested in examining high dose

Palmitic acid, we utilized a high dose of Palmitic Acid, 600ul to stimulated MHS macrophages for a time course of 24hours. The first step was to warm up water bath to 70°C, whilst waiting prepare 20% FAF:BSA/PBS solution. For this solution one gram of Sigma Low Endotoxin FAF:BSA into 5ml of 1xPBS and dissolve solution at room temperature inverting tubes and shaking every 5minutes. This procedure may take 30 minutes to dissolve under sterile conditions. To ensure no contamination, the Palmitc Acid stock solution needs to be filtered through 0.45um filter and stock solutions can be stored at 4°C for up to one week. After solution was dissolved, 50ul of 1N NaOH was added into 5ml of cell grade Sterile H_20 in a 15ml conical tube and heated in 70°C water bath. Then 28mg of Palmitic Acid(Sigma) was added into heated solution whilst stirring to the H_20/NaOH solution. The suspension was incubated for 25 minutes, inverting tube(s) 2-3 times every 3 minutes. After incubation was complete, the tubes were inverted 2-3 times and 50µl of 1N NaOH solution was added while continuously swirling the tube. The NaOH solution was continuously added 25µl at a time until solution was clear, this can take 1-4 additions of NaOH before the solution will turn. When solution had gone clear, it was incubated for five more minutes in 70°C water bath.

In the next step, the FAF:BSA/PBS solution was thawed on ice, and 1.5ml of the solution was pipetted into 15ml conical tubes and this tube was heated in the hot water bath for twenty minutes at 37°C. After incubation is up, tubes were taken out of water bath, then the tube containing Palmitic Acid was swirled vigorously, and the slow addition of 500ul of the warm FAF BSA/PNS solution was added drop wise; note do not add the solution too fast or it will not dissolve properly, and do not allow solution to drop

to room temperature as this will cause the FA to separate out. It is important to add the FAF:BSA slow enough to allow the Palmitic acid to complex to the FAF:BSA solution. After solutions have been successfully mixed 2ml of Palmitic acid solution was added to 18ml of RMPI 1640 cell culture media and sterile filtered through 0.45µm filters and 600ul of solution was aliquoted out into sterile ependorf tubes and labelled appropriately. Sterile Palmitic Acid solution was stored in -20 C fridge, and thawed upon PA stimulation of MHS macrophages following sterile procedures in the BSCII hood.

3.12 *In vitro* stimulation of MHS macrophages

MHS, peripheral lung-derived murine macrophages purchased from the ATCC, were cultured in RMPI 1640 until 80% confluentcy, and stimulated by either (a)TLR ligands PGN (Sigma), (b) high FA dose of Palmitic acid, (c) both PGN and high dose Palmitic acid. PGN stimulated MHS macrophages at a concentration of 100ng/ml for a 24 hour time course. In addition, to test the response of Long chain fatty acids on MHS macrophages, these macrophages were treated with a high dose of Palmitic acid, 600ul, prepared as described in 3.11. TLR ligand, PGN, was purchased from Sigma and first diluted into a 1mg/ml working stock solution then diluted to a working concentration of 100ng/ml for stimulation of cells. After cells had been stimulated for the majority of their 24 hour time course, three hours before time course was completed the macrophages were pretreated with the intracellular cytokine stain GolgiStop^{TM} protein transport inhibitor (BD Bioscience). For every 3ml of media 6µl of Golgi Stop was added and incubated for 3 hours at 37°C in the CO_2 incubator. Upon time course completion, the cells were harvested and collected for flow cytometry, protein extraction, and rna extraction. The

following techniques were utilized to study stimulated macrophages: FACS, Western Blot, ELISA, RT-PCR, IHC which the expression of CD36, macrophage markers: Cd11b, and F4/80 and expression of proinflammatory cytokines: TNFα, IFN-γ, IL-4, and IL-17a.

3.13 Enzyme-Linked Immunosorbent Assay

ELISA 96-well plates (Corning Costar 9018) were coated with 100 µl per well of working concentration of capture Antibody from DuoSet Elisa kit (R&D Systems).Then plates were sealed and incubated overnight at 4°C. The following day, each well was aspirated and tapped to ensure all liquid and bubbles were removed; then each well was washed three times with 100ul of wash buffer(0.05% Tween20 in 1xPBS); each time wash buffer must be completely removed. Subsequently, each well was blocked by adding 100ul of Reagent Dilutent(1%BSA in filtered PBS), to each well and incubated and sealed at room temperature for an hour. After incubation wells were aspirated, and tapped, and washed again three times with wash buffer to ensure complete removal of liquid and no bubbles in the wells. After plate preparation was complete 100ul of standard or sample was added (prepared in reagent dilutent) the plate was covered with an adhesive strip and incubated for two hours at room temperature. Please note standard curve started at the initial concentration of 280ng/ml and serially diluted across eight wells as outlined in R&D protocol. After time course was up, the plate was washed three more times with wash buffer. The next step was to 100ul of biotin-tagged detection antibody solution to each well, and cover the plate with another adhesive strip, avoiding light, for an additional 2 hours. Plates were washed 3 more times. After ensuring no bubbles and all solution was aspirated, 100ul of Strepavidin-HRP was added to each well

and plate was incubated for 20 minutes in the absence of light. The plate was washed three times again. Then substrate solution was prepared via addition of 1:1 ratio, 5ml and 5ml, of solution A &B: Color Reagent A, H_20_2, and Color Reagent B, Tetramethyl-benzidine; solution must be mixed well. 100ul of substrate solution was added to each well for a 20 minutes at room temperature and avoided light. Once the ELISA plate reading protocol was set up via Gene 5 program, and the yang lab ELISA machine was set up, 50ul of stop solution (2N H_2S0_4) was added to each well and tapped to mix. The plates were immediately read using ELISA machine at 450nm and analyzed by the Gen5 program. For our experiment we examined the concentration of TNFα protein (pg/ ul) and was analyzed via standard curve and plotted via graph pad prism program 5. Statistics were analyzed as outlined in section 3.14.

3.14 Statistical Analysis

All data are represented as mean ± standard error (SE). Statistical analysis was preformed via GraphPad Prism version 5.04 and Microsoft excel 2007. Two-tailed unpaired T-Test's were utilized to analyze data differences between experimental and control groups. In addition, when comparing more than two groups, a one-way ANOVA with Tukey's test was preformed to examine differences between and among groups.

RESULTS:

4.1 Higher frequency of CD36-expressing macrophages in Crohn's Disease and Ulcerative colitis Patients

Macrophages play an instrumental role in the pathogenesis of IBD, as demonstrated by UC and CD patients and colitis mice which exhibit markedly higher infiltrate of inflammatory macrophages in the intestine[1,11,27-28,41,49]. Recently, receptors that activate inflammatory macrophages have become a extensive research area in IBD, one receptor, is the CD36 scavenger type B receptor. The purpose of a scavenger receptor is to bind to, internalize, process, metabolize or degrade fatty acids or waste, in order to maintain the homeostasis of the intestine. It is possible the dysregulated mucosal immune responses seen in IBD, could be mediated in part, by abberant CD36 scavenger receptor activation on macrophages with accumulated lipids. Although CD36 has been previously investigated for its role in enterocytes and IBD, this receptor is a major receptor found on the cell surface of macrophages. In my study, I first wanted to assess whether CD36+ macrophages were involved in the inflammatory pathogenesis of IBD and TNBS colitis mice.

To determine whether CD36 expressing macrophages are upregulated in UC, and CD, I utilized colonic biopsies from three patients of both types of IBD, and normalized sections of colon biopsies taken cancer patients as non-IBD patient controls. I employed Immunohistochemistry in attempts to examine whether or not these CD36+ macrophages were (a) present in the colon (b) exhibited higher expression in IBD patients, (c) localization of CD36+macrophages. A macrophage marker, F4/80, that is expressed by intestinal macrophages was utilized to confirm the presence of macrophages.

Immunohistochemical analysis revealed that CD36+F4/80+ macrophages were present in colons of three patients with active Chron's Disease (CD) and Ulcerative colitis (UC) (Figures 1,3).The majority of CD36+expressing macrophages appeared to localize to the Lamina Properia of the intestine, and some macrophages were observed in the epithelial lumen (Figures 1). Control patients had markedly lower frequencies of CD36+ F480+ macrophages, and these macrophages were also observed in the Lamina Propria (Figure 1). As a staining control, Isotype IgG was employed and showedno flase positives, and thus confirmed the validity of antibody staining. Of note, the strong green fluorescence exhibited in isotype staining, is in part, due to the intestines natural autofluorescence (Figure 2). Interestingly, The frequency of Cd36+ F4/80+ macrophages were higher in UC and CD patients relative to control patients (Figure 3, P=0.0001) In addition, both CD and UC patients exhibited significantly higher levels of F4/80+ macrophages in comparison to control patients (Figure 3, P=0.0001). Furthermore the proportion of CD36 scavenger receptor competent cells was largely upregulated in IBD patients relative to the low expression observed in non-IBD controls (Figure3, P=0.0001); expression of CD36+ receptor was highest in UC patients relative to CD (Figure3, P=0.0001).

In control patients, there was a lower frequency of F4/80+ macrophages, although this is not surprising as resident macrophages are always present in the Lamina Propria sampling antigens in the epithelial lumen; this is part of the normal immune mucosal surveillance[1,11,27,28]. Of special interest to my study is the fact that the level of CD36+ F4/80+ macrophages in IBD patients was markedly upregulated relative to control patients (Figure 3, P=0.0001). This data provides evidence that CD36+ F4/80+ macrophages play a

role in active CD and UC disease, and indicates their potential proinflammatory role in the pathogenesis of IBD.

4.2 The number of CD36-expressing macrophages were higher in TNBS-colitis mice

Since the number of CD36+ macrophages were higher in IBD patients, my next step was to investigate whether TNBS colitis mice, a novel mouse model of IBD, also exhibited increased numbers of CD36+F4/80+ macrophages. I wished to determine whether or not these colitis mice presented a higher infiltrate of CD36+ expressing macrophages in intestine. First Balb/c mice were induced via the intrarectal injection of 2,4,6-Trinitrobenzenesulfonic Acid (TNBS) which formed lesions in the intestine and sucessfully induced severe inflammation as determined by histology[110]. The TNBS model requires that 50% ethanol mixed in the solution as this will degrade the mucosa of the intestine allowing this acid and haptens to form lesions in the intestine, as a result of this we implemented a naive 50% ethanol control [110].

To examine the presence of CD36+ F4/80+ macrophages, Immunohistochemistry (IHC), was performed on colonic tissue sections extratced from TNBS and Naive mice; this technique revealed that there was a markedly higher frequency of CD36 expressing macrophages in the LP in the colon of TNBS mice (Figure 4). Although there was low to moderate CD36 receptor expression in naive mice, only a small proportion of these cells were double CD36 expressing macrophages. As a control for IHC, isotype IgG staining was undertaken and confirmed that the antibody staining for TNBS and naive mice were specific, as no positive cells were observed (Figure 5).

After observing CD36+ F4/80+ macrophages were higher in the LP of TNBS mice we then proceeded to count the frequency of macrophages per mice in each group(n=5).Twenty pictures were taken via the BBI Zeiss fluouresent microscope per mice per group and 100 pictures were averaged and analyzed. Naive and TNBS colon samples were analyzed seperately for three parameters(a) CD36, (b) F4/80 and (c) double positive (CD36+F4/80+) to identify the proportion of competent cells. TNBS induced colitis mice demonstrated a noticeably higher level of CD36-expressing macrophages relative to Naive mice; this pattern was also observed in the expression of the CD36 receptor. Due to limited sample size and variation in the data, this result was not signifcantly different; however the increased number of CD36 positive macrophages approached significance and it is likely more samples and replicates would reveal a difference (Figure 6, P= 0.054). In addition, as the Yang lab has observed, there is some variability between mice and their ability or inability to compensate for intestinal inflammation, it is likely these differences influence results. This data supports that CD36-expressing macrophages are recruited to the Lamina Propria of the intestine in response to intestinal inflammation as both human IBD patients and TNBS colitis mice show markedly higher proportions of CD36+ F4/80+ positive macrophages. This suggests that the CD36 receptor is somehow involved in the pathogenesis of colonic inflammation, this is also an area where microbiota is highest[1,11, 22,25,27]. It is currently unclear what the role of the these CD36 expressing macrophages have in the inflamed intestine, however it has now been made clear these macrophages are highly upregulated in inflammatory conditions.

To further confirm the CD36 expressing macrophages were higher in the inflamed intestine, we further employed other techniques to establish this relationship: we utilized Real time q-PCR, Western Blotting and Flow cytometry. Colonic samples of TNBS mice displayed significantly higher Cd36 protein expression relative to naive mice (Figure 7, P=0.00095). In addition the same pattern was observed in CD36 gene expression, wherein CD36 expression was significantly higher in TNBS mice (Figure 8, P=0.049). Although IHC demonstrated that the numbers of CD36+ F4/80+ positive cells were not significantly higher in TNBS mice, the levels of CD36 protein and gene expression was significantly higher in TNBS mice (Figures 7,8; P=0.00095, P=0.049 respectively).

After confirming high expression of CD36 in protein and DNA, and observing an increased pattern of CD36+F480+ macrophages in TNBS mice, we decided it would be better to examine the exact numbers of CD36 expressing macrophages from the Lamina properia mononuclear cells (LPMC) from the colonsof both Naive and TNBS mice. LPMC was isolated and extracted from the colon and small intestine and large intestine of five TNBS and Naive mice. FACs analysis was undertaken four times from four different LPMC extractions from naive and TNBS mice and compiled. Each group of TNBS and Naive mice consisted of five mice each, and data was pooled and averaged over the four repetitions. Collectively, the expression of the CD36 receptor (Figure 9, P=0.0018), CD36 and F480 (Figure 9, P=0.0044) and CD36 and a Monocyte marker(LY6C)(Figure 9, P= 0.0098), was statistically higher in TNBS colitis; thus providing solid evidence of this association (Figure 9).

Collectively data confirms the role of CD36+ F4/80+ macrophages are largely upregulated in the inflamed intestine from a multitude of different experiments. The expression of CD36 receptor is markedly higher in TNBS colitis mice and that these macrophages appear to be somehow involved in the inflammatory pathogenesis of IBD. The exact role of the CD36 Scavenger type B receptor and its expression in macrophages requires further characterization.

4.3 *In vitro* stimulation of MHS macrophages with Palmitic Acid, PGN, and PA +PGN and proinflammatory cytokine production.

To further examine the role of CD36 positive macrophages, my next step was to use a simple cell line, MHS, derived from murine lung macrophages (ATCC) that I found largely expressed the CD36 receptor (Figure 10).

To verify that the MHS cell line expressed the CD36 scavenger receptor, I utilized flow cytometry. I confirmed that CD36 expression was present in MHS macrophages wherein nearly 65% of the cells were CD36 and F4/80 positive demonstrating the majority of MHS macrophages highly expressed CD36 (Figure 10). Additionally, to further confirm the validity and purity of cell line, macrophage markers CD11b and CD11c were employed and approximately 96.9% of MHS cells expressed these markers confirming cell line (Figure 10).

Next, to test whether an inflammatory response could be induced by *in vitro* MHS macrophages I stimulated CD36 expressing MHS macrophages with two ligands together or independantly. The ligands chosen to induce a proinflammatory response in MHS cells were high dose long chain fatty acid, Palmitic acid, and the TLR2 ligand PGN[68, 102]. The

point of this study was to examine whether these stimuli could promote, separately or together, CD36+ MHS macrophages to become inflammatory in the intestine.

Three proinflammatory cytokines were examined: TNFα, IL-17 and IFNγ. The reason I chose to examine TNFα, IL-17 and IFNγ cytokines was due to the significant evidence implicating these cytokines in the pathogenesis of IBD [1,5,11,17]. In particular, I wanted to test toll-like receptor 2 (TLR2) activation following stimulation with PGN, as a few studies in the intestine have implicated TLR2 activation in inflammatory macrophages in IBD [60-62,65,68,69,102].Interestingly, TLRs are well known to induce inflammatory cytokines from stimulated macrophages, such as TNFα in the intestine [17,27,60,61,68,69].

Additionally, given that macrophages involved in Atherosclerosis are stimulated by lipoproteins such as OxLDL, (a ligand of CD36) and that these lipid laden foam cells induce dysregulated fat accumulation and release of proinflammatory cytokines dependent on CD36 receptor [80,90-94,98,115-118]. Based on all evidence I quieried whether a simillar mechanism existed in the intestine. I hypothesized that LCFA, Long chain Fatty Acids, which are intimately linked in lipid metabolism, and degradation in the intestine, if in access,could potentially evoke a similar proinflammatory response in conjunction with TLR2.

To determine if LCFA could induce an inflammatory response(s), CD36 expressing MHS cells were stimulated with (a) high dose Palmitic Acid, 600ul as described in methods, (b) 100ng/ul of PGN, a TLR2 ligand, and (c) a combination of high dose PA and PGN and incubated for a 24 hour time course. Following stimulation, MHS

cells were assessed for their production of proinflammatory cytokines: TNFα, IFN-γ , and IL-17a via FACs analysis. However first an ELISA test was used to examine whether TNFα expression was upregulated in MHS macrophages first. RMPI 1640 supernatants from 24 hour stimulated MHS were collected and a half-sandwhich ELISA technique performed to examine expression of TNFα via DuoSet (R&D systems) Elisa kit (Figure 11).

The supernatants from MHS macrophages treated with PA, PGN and PA+PGN revealed a significantly higher level of TNFα relative to NMHS (Figure 11; P=0.0179, P=0.0198,P=0.028 respectively). Interestingly, high dose Palmitic acid on its own stimulated macrophages to induced a small amount of TNFα production relative to control NMHS cells (Figure 11, p=0.0179). The highest production of TNFα was exhibited in macrophages stimulated with both PA and PGN (Figure 11, p=0.028), whereas a moderate production of TNFα was shown in PGN stimulated macrophages (Figure11, p =0.0198). This data demonstrates that PGN was sufficient to drive MHS macrophages to induce a proinflammatory response *in vitro* and as a result of this PGN was chosen as the TLR ligand of interest and used throughout subsequent experiments. It is not surprising, that Palmitic acid and PGN stimulated macrophages induced a higher expression of TNFα as we predicted high dose Long chain fatty acids in conjunction with TLR2 stimulation would induce these macrophages to secrete proinflammatory cytokines. Results suggest both factors may work synergisticallyor have an additive role in TNFα production; however production was relatively low. This may be attributed to the fact that a PGN dose of 100ng/ul may have been too low to efficiently stimulate MHS a high

cell concentration of 10^8 macrophages, or perhaps a longer time course of 48 hours was more appropriate.

After determining CD36+ MHS macrophages could be driven via high LCFA concentration and TLR2 stimulation to induce TNFα, I sought to investigate other proinflammatory cytokines: IFN-γ and IL-17a evidenced in IBD and to further confirm TNFα production via Flow cytometry.

The next step was to investigate these cytokines in CD36+ MHS macrophages after stimulation with PA, PGN, and PA and PGN, in flow cytometry (Figure 11). Three repetitions of FACs was performed and samples were average and pooled. The frequency of CD36 receptor in macrophages remained relatively high throughout all treatments (Figure 12). There was a small detectable difference between NMHS and MHS+PA treated macrophages, wherein MHS+PA exhibited a slightly higher proportion of CD36 positive macrophages (Figure 12, P=0.0022). In addition, NMHS and MHS+PA+PGN exhibited a a small difference in the number of macrophages that express the CD36 receptor (Figure 12, P=0.0019). However, there was no significant difference in number of CD36 positive cells between NMHS and PGN stimulated macrophages (Figure 12, P=0.2578). Interestingly, there was also no difference observed between MHS+PA and PA+PGN stimulated CD36 positive macrophages (Figure 12, P=0.9631) and there was also no difference in the number of CD36-expressing macrophages between MHS+PA and PGN treatments (Figure 12, P=0.1444,).

Secondly, I wanted to confirm macrophage markers, F4/80 and CD11b, expression in MHS macrophages, after stimulation with PA, PGN, or both, to examine if

there were differences in the overall numbers of macrophages (Figure13,14). As expected, there were no significant differences observed in number of cells positive for either macrophage markers, F4/80 or CD11b (Figure 13,14). This measure was solely to confirm the purity of the MHS cell line throughout the time course experiments (Figure 13, 14).

The next step was to examine the differences in the induction of proinflammatory cytokines: IL-17a, IFN-γ, and TNFα in MHS macrophages following stimulation with PA, PGN, or both, flow cytometry was performed.

The number of IL-17a positive cells was relatively low in NMHS as expected , but significantly higher when compared to PA treated MHS macrophages (Figure 15, P=0.0255) . In addition, PGN stimulated macrophages exhibited increased numbers of IL-17a positive cells relative to NMHS control (Figure 15, P=0.032) which was similar to the numbers of IL-17a positive cells in the PA-treated macrophages. This data suggest that both Palmitic acid and TLR2 ligand, PGN, can induce IL-17a expression in MHS macrophage in *vitro*; although the addition of both does not appear to have an additive effect (Figure 15).

Next, IFN-γ was examined in control and stimulated MHS macrophages and no significant differences in positive cell numbers were observed in any of the treatments, PA, PA+PGN, or PGN only (Figure 16; P=0.9719, P=0.4904, P=0.2486 respectively). Overall, intracellular expression of IFN-γ was low and does not appear to change, although a slight trend towards an increase in the number of IFN-γ positive cells was observed in PGN stimulated MHS but was not significant (Figure 16, P=0.2486).

Additionally, there were no differences in the number of IFN-γ positive cells between the MHS+PA treated macrophages and PA+PGN (P=0.3694) or PGN (P=0.2442) treated macrophages (Figure 16). MHS lung derived perpherial macrophages stimulated with Palmitic Acid, PGN or both, also did not have significant changes in the number of IFN-γ competent cells and therefore does not appear to be a important proinflammatory cytokine when conditioned with Palmitic Acid, PGN or both; this was also observed in standard pcr with no expression of IFN-γ demonstrated (data not shown).

Lastly, FACs analysis for intracellular expression of TNFα in MHS stimulated with PA,PGN, or both, revealed macrophages stimulated with PA induced the highest TNFα production in comparison to NMHS control and other groups (Figure 17, P=0.0086). Surprisingly, MHS macrophages treated with both PA and PGN induced a lower number of TNFα positive cells compared to PA treated macrophages (Figure 17, P=0.0086). There were no detectable differences observed between NMHS and PA+PGN and PGN (Figure 17, P=0.9987, P=0.5288). Finally, no differences were detected in the number of TNFα positive cells between MHS+PA and PGN stimulated macrophages (Figure 17, P=0.7273). Overall, it seems clear that a high dose of PA induces a TNFα response, whereas TNFα production was lower in PA+PGN and PGN treated macrophages. This suggests that high long chain fatty acid stimulus could elicit a proinflammatory cytokine response in macrophages. Despite the discrepancies observed between the TNFα ELISA and FACs analysis, both results support high dose of Palmitic acid stimulated MHS macrophages were capable to produce some proinflammatory response, in addition it appears possible that PGN stimulated TLR2, may amplify this

proinflammatory cytokine response. After examining the relationship between high dose Palmitic acid, PGN, and inflammatory cytokine production, I wanted further explore PGN and PA conditioned macrophages *in vivo* in Balb/c mice.

4.4 Adoptive transfer of conditioned CD36+MHS macrophages in BALB/c mice

In the next stage of my experiments, I wanted to examine the effects of conditioned macrophages on the intestine of Balb/c, to do this I conducted adoptive transfer of stimulated and unstimulated MHS macrophages (dependant upon mouse model). In MHS+PA mice, MHS macrophages were stimulated with a 24 hour time course of high dose Palmitic acid, and these macrophages were injected via tail vein. In addition MHS+1/2TNBS and NMHS mice 10^8cells/ml of unstimulated MHS macrophages were injected via tail vein, and in 1/2TNBS injections mice were injected intrarectally on the second day under anethesia. MHS+1/2TNBS were treated with both, MHS 10^8cells/ml were injected via tail vein on day one and 1/2TNBS was intrarectally injected on day 2, mice were sacrificed on day 7.

To determine if there were any changes in our conditioned mice, the first measure of change was to examined in the mice behavior (DAI), Daily Activity Index and mice weights. DAI is assessed via a variety of measures, the acivity of mice, hiding behaviours, health of fur, orbits, body size, stool consistency, and group behaviours. Weights are particularly important to assess in TNBS induced colitis as mice weights typically will drop with colitis; mice will exhibit swelling, discomfort, diarrhea , constipation, lesions, and lack of nutrient absorption; this needs to be watched carefully as a weight $\leq$ 19kg must be sacrificed immediately.

As previously mentioned weight measurement of mice provides an indication of health, illness, or disorder in an animal following treatment and this measurements must be taken every morning and before and after injections (Figure 18). The weights of control mice (NMHS), and MHS+PA presented fairly normal and stable weights which indicated the mice were healthy. Surpringly, the weight of Naive mice were a little lower than expected and one possibilty was that these mice were treated with 50% ethanol as a control for TNBS and it is possible this may have lead to some weight loss; in addition although we waited adaptation time of a week mice arrived to the CAF facility at lower than average weights (Figure 18).

NMHS exhibited the highest weights and mice were very healthy as expected for control. In full dose TNBS mice the weights dropped rapidly after day one post-injection until day three where the TNBS mice weight only stabilized; mice did not gain sufficient weight (Figure 18). TNBS animals exhibited signs of colitis such as discomfort, watery stool, diarrhea, decreased appetite, exhibited hiding behaviours, less active, and displayed blood in the stool (Figure 20).

MHS+1/2 TNBS and 1/2TNBS mice had similar weight loss patterns where weights decreased day2 post-injection till day five and then began to recover to a more normalized weight (Figure 18). Interestingly, 1/2TNBS mice weights dopped slightly lower than MHS+1/2 TNBS mice and did not recover as well as MHS+12tnbs (Figure 18). Also, 1/2tnbs mice displayed discomfort, watery stool, stool inconsistency, some inactivity, and exhibited a mild colitis. In comparison to MHS+1/2 TNBS mice no signs of colitis was present and mice appeared healthy suggests perhaps unstimulated

macrophages, could potentially play a protective role, perhaps via endogenous macrophage cross-talk elicited by MHS macrophages. Perhaps unstimulated MHS macrophages may play a role in the recovery of the intestinal tissues, and that these macrophages require stimulation in order to generate inflammatory macrophages, or indirectly aid recovery by recruiting other lymphocytes and leukocytes to the site of inflammation. This result was unexpected, as we postulated that 12TNBS may induce these macrophages to become inflammatory, it is possible after day seven the mice intestine may have recovered it is difficult to say whether exogenous MHS macrophages, resident macrophages aided in recovery; perhaps CD36+ MHS macrophages play a dual role and are protective via apoptotic cell clearance in the intestine, masking the potential inflammatory role (Figure 18, 20).

From the same experiment detailed above, colonic tissue was extracted from control and experimental mice, fixed in parafin and stained with hemotoxylin and eosin, performed by a histology lab at SJH (Figure 19).The colon of naive mice appeared healthy, exhibited no crypt destruction, or edema, and displayed very little infilitrate of mononuclear cells (Figure19). NMHS colons were mostly normal but did demonstrate a some edema, mild some focal crypt destruction or crypt hyperplasia, and moderate mononuclear cell infiltration (Figure 19). Despite the normal weights of MHS+PA mice, MHS+PA colons exhibited focal and zonal edema, some crypt destruction and a high mononuclear infiltrate throughout the intestinal tissues (Figure 19). This was surprising as the mice weights and behaviours appeared completely normal and did not suggest any discomfort or inactivity no did dissected colons show inflammation (Figure

18-10). However, it is possible that the mononuclear infiltrate could attributed to macrophage scavenging of long chain fatty acids (PA) and this potentially recruited further macrophages (Figure 19).

Full dose TNBS induced colitis induced widespread crypt destruction, systemic edema throughout all three layers of intestine, and a high level of inflammatory infiltrate of mononuclear cells throughout intestinal tissues (Figure 19). MHS+1/2 TNBS exhibited a low to moderate level of inflammatory infiltrate, with little edema, focal crypt damage and some hyperplasia (Figure 19). Interestingly, the colons with 1/2TNBS colitis exhibited more damage to the intestine than the MHS +1/2 TNBS colons which again suggested macrophages somehow may have induced a protective role or recruited other macrophages to dampen inflammatory insult (Figure 19, 20).

To get an idea of what the entire colon and intestinal morphology looked like intetsines were dissected from NMHS, Naive, TNBS, 12TNBS, MHS+12TNBS, MHS+PA mice and macroscopic images were undertaken (Figure 20). Naive and NMHS mice colons and intestine looked healthy, elongated, solid stool consistency, no swelling was observed as expected (Figure20). As expected, full-dose TNBS colon and intestine, induced severe colitis, displaying bloody spots in the large and small intestine, swelling of the colon, watery stool, diarrhea, evidence of ulceration, and decreased colon length[110] (Figure 20). The intestines of the MHS+PA group looked relatively healthy and similar to controls (Naive and NMHS);(Figure 20). In the MHS+PA intestine there was no evidence of inflammation and tissues looked markedly healthy despite H&E results exhibited inflamatory features; perhaps response is localized (Figure19,20). 1/2TNBS colon and

small intestine exhibited minor bloody spots in the small intestine, some swelling and redness, and soft/watery stool showing a mild colitis.Colon length was mostly normal in MHS+1/2 TNBS and 1/2 TNBS (Figure 20).

To determine the severity of inflammation in the intestine between the mouse treatment groups; three inflammatory measures where employed: Histological score, Myleoperoxidase (MPO), and Mononuclear cell counts. For each of the five mice groups: NMHS, Naive, full does TNBS, half dose TNBS, MHS+PA, and MHS+1/2TNBS colon samples were scored and averaged based on the histological parameters as previously described. For each mouse, 20 images were scored and averaged, and a total 100 images were taken per each group(n=5), and averaged to obtain a total histological score for treament and control groups (Figure 21).

Full dose TNBS colitis mice were determined to have the highest histological score of 8 (Figure 21). As one would expect, Naive mice scored much lower relative to TNBS mice, yielding an average score of two (Figure 21).The histological score of TNBS colitis mice was significantly higher than Naive mice (Figure 21, P=0.0001). Half dose TNBS exhibited an average histological score of five and was significantly higher in comparison to naive mice (Figure 21, P=0.0001). Similarly, MHS+PA mice scored 5.5 when compared to NMHS which demonstrated a histological score of 3 (Figure 21, P=0.0001).Surprisingly NMHS scored a slightly higher histological score in comparison to naive, however, this may be attributed to effects of exogenous cross-talk to endogenous macrophages that may have affected the intestinal tissues; in addition injected macrophages will increase mononuclear cell count and infiltrate. The MHS+1/2 TNBS

had an average score of 3.8 which was similar to the control NMHS mice, however MHS+12TNBS was still slightly higher than NMHS control mice (Figure 21, P=0.0340). Furthermore, MHS 1/2TNBS and 1/2TNBS mice were significantly different wherein 1/2TNBS exhibited a markedly higher histological score (Figure 21, P=0.0001). Interestingly, 1/2TNBS and MHS+PA exhibited similar histological scores, 5 and 5.5; however MHS+PA mice displayed a significantly higher histological score than 1/2TNBS (Figure 21, P=0.0001).

To further strengthen and support my findings that inflammation was present in the colonic intestine, I examined another inflammatory measures, such as the infiltration of mononuclear cells (Figure22). The infiltrate of mononuclear cells are comprised of inflammatory mediators such as lymphocytes and monocytes[112]. Mononuclear cell counts were undertaken according to cooney et al protcol and we counted based on size, morphology, dark purple color, and location. Cells were counted in the same way as previously mentioned and counted with a slide scale to determine cells/mm^2[2,112]. Full dose TNBS colitis mice demonstrated the highest proportion of mononuclear cell infiltrate (180cells/mm^2) among all mouse groups was significantly higher in comparison to Naive control mice (Figure 22, P= 0.0004, unpaired T-test). Secondly MHS+PA exhibited the second highest rank exhibiting a moderate mononuclear cell infiltrate (130cells/mm^2) and was significantly higher in comparison to NMHS mice (Figure22, P=0.0002). Half dose TNBS(75cells/mm2) and MHS1/2TNBS (60cells/mm2), were both higher than NMHS control; however only 1/2tnbs exhibited significantly higher mononuclear infiltrate (Figure22, P=0.8764, P=0.007). In addition, the MHS+PA mice displayed

significantly higher mononuclear cells relative to MHS+1/2 TNBS and 1/2TNBS mice and was statistically different from the two groups (Figure 22, P=0.0004, P=0.0041 respectively). Both contols exhibited lower mononuclear cells in the intestine as expected Naive (50cells/mm^2) and NMHS(58cells/mm^2); controls were not statistically different (Figure22, P=0.0026). In addition 1/2TNBS exhibited a significantly higher mononuclear cell count in contrast to MHS+12TNBS; although this was a relatively small difference (Figure 22, P=0.0116). As expected Half dose TNBS demonstrated a lower mononuclear cell count in comparison to full dose TNBS (Figure 22. P=0.0052). There was no statistical difference in mononuclear cell infiltrate between full dose TNBS and MHS+PA (Figure 22, P=0.1293). High mononuclear cell infiltrates strongly suggest that there is inflammation in TNBS colitis mice and perhaps a localized inflammatory response in MHS+PA mice.

As an additional method to confirm inflammation was present in the intestine, I utilized a technique called myeloperoxidase (MPO)[119,120]. This technique is a unique method which measures the infiltration of activated inflammatory neutrophils based on MPO expression[119,120]. MPO is a hemoprotein which is highly expressed by polymorphonuclear leukocytes, and in particular MPO is expressed by activated neutrophils[119,120].

Myeloperoxidase (MPO) activity was significantly higher in the colons of TNBS Colitis among all groups and significantly higher in comparison to Naive mice (Figure 23, P=0.0057; unpaired T-test). The level of MPO was lower than expected in the half dose TNBS colitis mice and was not significantly different from naive control (Figure

23, P=0.4513). MHS+12TNBS exhibited a low level of MPO which which was slightly higher than NMHS control mice (Figure 23, P=0.0146). In second rank, MHS+PA induced a moderate expression of MPO, and was significantly different from MHS+1/2TNBS, 1/2TNBS and NMHS (Figure 23, P=0.0092, P=0.0014, and P=0.0015, respectively). In both experimental groups, full dose TNBS and MHS+PA, exhibited a higher expression of MPO relative to other mice groups; TNBS displayed a significantly higher mpo activity (Figure 23, P=0.0333). Neutrophils are the first inflammatory mediators recruited to the site of inflammation and these results indicate there was a high infiltration of activated neutrophils in TNBS colitis mice and MHS+PA mice which strongly suggests that PA and TNBS induce significant inflammation in the intestine (Figure 23).

Since we confirmed three measures of inflammation were present in the colonic intestine of TNBS and MHS+PA mice, in our next experiments I wanted to examine if there was expression of pro-inflammatory cytokines in LPMC macrophages in mice. I investigated lamina propria macrophages isolated from the colons and small intestines of NMHS, MHS+PA, 12TNBS and MHS+1/2 TNBS. We first needed to confirm the proportion of CD36+ macrophages in LPMC via sorting by macrophage markers: Cd11b and F4/80, and subsequently gating these macrophages for the expression of proinflammatory cytokines: IL-17a, IFN-y, TNFα and IL-4.

To obtain the macrophage population from the colon and intestines of treated mice, I used FACs analysis sorting with two macrophages cell markers, CD11b and F4/80.The number of CD36 receptor positive cells in the LPMC population was the

highest in 1/2TNBS and moderately upregulated in MHS+PA relative to NMHS mice (Figure 24, P=0.0001, P=0.0033,unpaired T-test) . There was no significant differences between NMHS and MHS+1/2tnbs in CD36 positive cell numbers (Figure 24, P=0.6925). However MHS+1/2tnbs and 12TNBS mice groups were different, 1/2TNBS exhibited significanly higher porportion of CD36 competent cells (Figure 24, P=0.0001). MHS+1/2tnbs and MHS+PA were also different; MHS+PA exhibited a moderately higher CD36 positive cells (Figure 24, P=0.0112). Lastly 1/2TNBS CD36 positive cells were significantly higher relative to MHS+PA; these two groups were also significantly different (Figure 24, P=0.0001).

To determine and sort the number of macrophages extracted from LPMC, I first utilized FACs to sort for the macrophage marker, CD11b. The number of CD11b positive cells was highest in 1/2TNBS colitis mice and there were moderate numbers of CD11b positive cells observed in both NMHS and MHS+PA mice groups (Figure 25). The amount of Cd11b positive cells in MHS+1/2tnbs were significantly lower in comparison to NMHS mice (Figure 25, P=0.0006); which was surprising as MHS macrophages were injected into these mice; it is possible that LPMC extraction was low, or perhaps these macrophages migrated to other tissues. 1/2TNBSdemonstrated high numbers of CD11b+ macrophages relative to control mice (Figure 25 P=0.0047). No significant differences were observed between MHS+PA and NMHS and MHS+PA and MHS+1/2 TNBS (Figure 25, P=0.2435; P=0.1146). MHS+1/2tnbs and 1/2TNBS were significantly different with a higher expression of CD11b positive macrophages in 1/2TNBS mice (Figure 25, P=0.0001). In addition 1/2TNBS mice exhibited a significantly higher

proportion of Cd11b+ macrophages in comparison to MHS+PA(Figure 25, P=0.0015); demonstrating a higher macrophage infiltrate in the mild-to moderately inflammed intestine.

Simillarily, F4/80, an additional intestinal macrophage marker demonstrated a simillar pattern with moderate expression in NMHS and high expression in 1/2TNBS, in the other two groups exhibited a lower expression of F4/80+ markers (Figure 26). Half dose colitis mice displayed a moderately higher F4/80+ macrophage positive cells relative to NMHS control (Figure 26, P=0.0155). In addition 1/2TNBS expressed statistically higher proportion of F4/80 macrophages in comparison to both MHS1/2tnbs and MHS+PA treatment groups (Figure 26, P=0.0001, P=0.0001). In addition F4/80 positive cells in NMHS and MHS1/2tnbs and NMHS and MHS+PA mice were not statistically different (Figure 26, P=0.2147, P=0.1592). No statistical difference was observed between MHS1/2tnbs and MHS+PA (Figure 26, P=0.4267).

To identify double positive CD36+F4/80+ intestinal macrophages, cells were double gated using CD36 and F4/80 markers via FACs analysis to isolate out CD36+ expressing intestinal macrophages. Interestingly, CD36+F4/80+ LPMC macrophages were highest in the 1/2TNBS mice and a moderate cell number was exhibited by MHS+PA mice relative to other groups (Figure 27). A high number of CD36+ F4/80+ macrophages were present in 1/2TNBS colitis in comparison to NMHS control (Figure 27, P=0.0001). MHS+PA exhibited a moderate proportion of CD36+F4/80+ LPMC macrophages and these cells were significantly higher relative to NMHS control (Figure 27, P=0.0272). Additionally, 1/2TNBS demonstrated a significantly higher number of

double positive cells in comparison to MHS+PA (Figure 27, P=0.0011). Additionally 1/2 TNBS exhibited a higher proportion of double positive macrophages relative to MHS+1/2 TNBS group, confirming patterns of inflammatory scoring; 1/2tnbs exhibited higher proportion of CD36+ inflammatory macrophages (Figure 27, P=0.0001). There was a small but detectable difference between NMHS and MHS+1/2TNBS with a slightly higher number of double positive cells in the latter group (Figure 27, P=0.0018). No significant differences were found between MHS+ 1/2TNBS and MHS+PA (Figure 27, P=0.5110). With the high proportion of CD36 expressing macrophages in intestinal inflammation, such as half dose TNBS colitis, and high LCFA, the data suggests that CD36+F4/80+ macrophages are recruited to the inflamed intestine.

After confirming that CD36+F4/80+ macrophages were signifcantly higher in half dose TNBS, MHS+PA and MHS+ 1/2TNBS mice relative to control mice (Figure 27, P=0.0001, P=0.0272, P=0.0018), as a next step, I wanted to confirm that the expression of CD36 at the protein level in these mice. Western Blots were performed on colonic protein samples from each mice group utilizing antibodies specific for CD36 (Santa cruz, 1:1000) and Santa cruz 1:100 TNFα and as a loading control Santa cruz β-actin was used (Figure 32). CD36 protein was highly expressed in MHS+PA group relative to NMHS control mice (Figure 32, P=0.0009). Also, 1/2 TNBS colitis mice exhibited significantly high levels of CD36 protein in contrast to control mice (Figure 32, P=0.0001). There were no significant differences observed in CD36 protein levels between MHS+PA and 1/2TNBS mice (Figure 32, P=0.3955). Despite a small visible difference, NMHS and MHS+1/2tnbs were not significantly different in CD36 protein expression (Figure 32,

P=0.0551). However, there was a significant difference between MHS+1/2TNBS and 1/2TNBS mice whereby 12TNBS mice exhibited higher CD36 expression (Figure 32, P=0.0001). In addition, there was a small difference in the expression of CD36 between MHS+1/2TNBS and MHS+PA; expression was higher in MHS+PA (Figure 32, P=0.027). Therefore CD36 protein expression was largely upregulated in 1/2TNBS and MHS+PA mice confirming trends of Flow results.

The next parameter was to confirm the production of proinflammatory cytokines from LPMC intestinal macropages across the four mouse groups: NMHS, MHS+12TNBS, 12TNBS and MHS+PA. The production of proinflammatory cytokines: TNFα, IL-17a, IFN-γ, and IL-4 were investigated and LPMC was extracted from the small and large intestine and examined via flow cytometry.

The first cytokine I examined was the TNFα which revealed a simillar trend in to the Elisa and FACs *in vitro* data (Figures 11,17, 28). However, *in vivo* Palmitic acid conditioned MHS macrophages induced a small detectable difference in TNFα production when compared to the NMHS control (Figure28, P=0.0021). Although the production of this proinflammatory cytokine was relatively low in MHS+PA, this data provides support that high dose LCFA: Palmitic acid does indeed induce a small proinflammatory response in the intestine (Figure 28). It was not surprising, based on inflammatory scores, that half dose TNBS colitis LPMC macrophages demonstrated the highest production of TNFα, and this was significantly different from control mice (Figure 28, P=0.0001). MHS+1/2 TNBS demonstrated a significantly higher level of TNFα relative to control mice; however this difference however was quite minor (Figure 28, P=0.0005). There

was a large difference in TNFα between 1/2TNBS and MHS+1/2TNBS LPMC macrophages whereby a higher production of this cytokine was elicited by 1/2TNBS mice (Figure 28, P=0.0013). Lastly, MHS+PA production of TNFα was significantly lower in comparison to both MHS+12TNBS and 1/2TNBS (Figure 28, P=0.0071, P=0.0001), this was not as expected however reasons for this finding was previously discussed.

Next, I investigated IL-17a proinflammatory cytokine as this cytokines has been implicated in IBD and skews the response of T cells toward a inflammatory TH-17 phenotype[1,11,127] . MHS+PA treatment group displayed a slight increase in IL-17a, however due a high level of variability in the data there was no statistical difference when mice were compared to NMHS(Figure29, P=0.0518).There were no statistical differences in the amount of IL-17a positive cells between MHS+PA and MHS+1/2TNBS, and 1/2TNBS mice groups (Figure29, P=0.1279, P=0.4076). Following a simillar trend in the expression of proinflammatory cyokines, 1/2TNBS colitis macrophages induced the highest level of IL-17a production among all groups and this was significantly different from control mice and MHS+1/2 TNBS (Figure 29, P=0.0001, P=0.0001). A small dectable difference in IL-17a positive cells was observed in MHS+12TNBS and proportion of IL-17 positive macrophages was significantly higher relative to NMHS mice (Figure 29, P=0.0005).

The third pro-inflammatory cytokine investigated across the four different mice groups was IFNγ, a cytokines known to promote TH1 responses in IBD[1-5,11,127]. Flow cytometry demonstrated the level of IFNγ remained relatively low in LPMC macrophages

from NMHS, MHS+1/2 TNBS and MHS+PA mice, however was drammatically upregulated in 1/2TNB mice (Figure 30). A very minor difference in IFN-γ was detected in MHS+PA relative to NMHS control, however looking at proportion of IFN-y positive cells it appears these levels are quite simillar (Figure 30, P=0.0444). The levels of IFN-γ produced by the 1/2 TNBS was strikingly high when compared across all the other mice treatment groups and was significant relative to control mice (Figure30, P=0.0001). Additionally, 1/2 TNBS and MHS+PA were significantly different from each other in the level of IFN-γ production, 1/2TNBS production of this cytokine was markedly higher in comparison (Figure 30, P=0.0001). Lastly a very small difference in IFN-γ production was observed between MHS+PA and MHS12tnbs with a slightly higher production of this cytokine in MHS+PA (Figure 30, P=0.0479). The production of IFNγ in NMHS and MHS+ 1/2TNBS, and MHS+PA groups exhibited very low levels of positive cells and the production of IFN-γ in these mice does not appear to be an influential proinflammatory cyokine in the intestine of these mice and macrohages (Figure 30).

The last cytokine investigated via flow cytometry was proinflammatory IL-4, a TH2 polarizing cytokine evidenced in IBD, moreover in UC [1]. Similar to what I observed earlier with the other inflammatory cytokines, 1/2TNBS produced high levels of IL-4 competent cells, whereas MHS+12TNBS, MHS+PA, and NMHS produced little to low levels of IL-4 positive macrophages (Figure 31). Of note, MHS+PA exhibited a much lower IL-4 in comparison to both MHS + 1/2TNBS and 1/2TNBS groups (Figure31, P=0.0003, P=0.0001).There were no statistical differences between MHS+PA and NMHS in the production level of IL-4 (Figure 31, P=0.8990). 1/2 TNBS displayed a high level of

IL-4 and was significantly different from NMHS control and MHS 1/2TNBS (Figure 31, P=0.0001, P=0.0001). Lastly, MHS 1/2 TNBS macrophages elicited a higher production of IL-4 in comparison to NMHS(Figure 31, P=0.0003). This data suggested that 1/2TNBS colitis and some small amount of MHS+ 1/2TNBS LMPC macrophages induce IL-4, and this suggests a TH2 response is present in the intestine of these mice; however is most significant in 1/2TNBS colits mice. 1/2TNBS colitis mice exhibited a mixed TH1/TH2/TH17 ratio which is observed in the pathology of IBD[1,127].

To further confirm that TNFα expression was in fact higher in the intestine of the 1/2TNBS, and MHS+PA mice, I also examined the TNFα protein expression level. Interestingly, only a small difference in TNFα production was previously detected by flow cytometry, in MHS+PA, however the TNFα protein expression in these mice was highly expressed in comparison to NMHS control mice (Figure 33, P=0.0001) . Consistent with my previous results there was also a significantly higher TNFα production in the 1/2TNBS colitis mice relative to control mice (Figure 33, P=0.0001). There were simillar expression profiles for TNFα in both MHS+PA and 1/2TNBS mice and no significant differences were observed (Figure 33, P=0.2862). MHS+1/2TNBS demonstrated a moderate expression of TNFα in relative to NMHS control (Figure 33, P=0.0001). In addition, MHS+1/2TNBS revealed significantly lower TNFα expression when compared back to MHS+PA and 1/2TNBS mice groups (Figure 33, P=0.0004, P=0.0004). This result confirms a simillar trend as compared to the previous FACs data demonstrating that 1/2TNBS mice and MHS+PA induce the production of TNFα in the intestine. Taken together Western blotting has confirmed that the expression of CD36

scavenger receptor and TNFα proinflammatory cytokine expression was highest in MHS+PA and 1/2 TNBS colitis mice, suggesting that these two treatment groups induce an inflammatory response in the intestine.

To establish direct evidence that CD36+ macrophages were highly expressed in the lamina propria of the MHS+PA and 1/2TNBS intestines and to determine the frequency of CD36+ macrophages in the inflamed intestine, immunohistochemistry was again undertaken.

As a first step, the expression of CD36 scavenger receptor was examined in the colons of NMHS, MHS+PA, MHS + 1/2TNBS and 1/2TNBS (Figures 34). As predicted 1/2 TNBS mice colonic sections demonstrated the highest frequencies of CD36+ compentent cells in the lamina propria of the intestine and was significantly higher relative to MHS control mice (Figure 34, P=0.0001). Based on FACs analysis and CD36 protein expression levels, it was expected and confirmed that MHS+PA exhibited the second highest proportion of CD36 positive cells in the LP of colon in comparison to control mice (Figure 34, P=0.0001).There were no significant differences observed between MHS+PA and 1/2 TNBS although this was not surprising as the amount of CD36+ cells appeared similar in number (Figure 34, P=0.0677). MHS+1/2TNBS displayed a moderately higher proprtion of CD36+ cells relative to control mice (Figure 34, P=0.0001). In additon, MHS+PA demonstrated significantly higher CD36 positive cells in colon in comparison to the lower amount positive cells observed in MHS+1/2tnbs (Figure 34, P=0.0009). MHS+1/2tnbs also demonstrated significantly lower CD36 competent cells in comparison to 1/2tnbs colitis mice(Figure 34, P=0.0001).

To determine the proprotion of macrophages infiltrating the intestine of all four mice groups intestinal macrophages marker, F4/80, was utilized for IHC to visualize positive cells in lamina propria of the colonic intestine samples (Figure 35). Half dose TNBS mice displayed the highest amount of F4/80+ macrophages in the colon and among all groups and was significantly higher than control mice (Figure 35, P=0.0001). MHS+PA demonstrated a high percentage of F4/80+ macrophages relative to NMHS control (Figure 35, P=0.0001). Similar to the number of CD36 positive cells, MHS+1/2TNBS displayed a moderate frequency of F4/80+ positive macrophages in the colon in contrast to NMHS mice (Figure 35, P=0.0006). As expected1/2TNBS exhibited higher F4/80+ postive cells in the intestine in comparison to MHS+ 1/2 TNBS (Figure 35, P=0.0079). No significant differences were observed between MHS+1/2tnbs and MHS+PA in the proportion of F4/80+ positive cells (Figure35, P=0.4343). However, a small difference was observed between MHS+PA and 1/2tnbs, wherein 1/2tnbs revealed a moderately higher proportion of F4/80+ competent cells in the intestine (Figure 35, P=0.0327).

The next step was to confirm the expression of CD36+F4/80+ macrophages in the colonic intestine of NMHS, MHS+1/2TNBS, and 1/2TNBS via examining double positive staining. MHS+PA and 1/2 TNBS colitis exhibited markedly higher proportions of CD36+F4/80+ cells and were significantly different from NMHS control mice (Figure 36, P=0.0001, P=0.0001). MHS+1/2TNBS demonstrated a moderate proportion of CD36+F4/80+ double positive cells in comparison to NMHS (Figure 36, P=0.0004).

However a slightly higher but significant proportion of double positive cells were found in 1/2TNBS colitis mice in contrast to MHS+PA mice (Figure 36, P=0.0126).

Given the considerable amount of evidence, it does appear evident that CD36 expressing macrophages are upregulated in active IBD patients and TNBS colitis mice. Some significant confirmation has been presented to indicate these CD36+F4/80+ macrophages are inflammatory in intestine via the upregulation of proinflammatory cytokines, in particular TNFα. In addtion it appears high LCFA Cd36+ F4/80+ stimulated macrophages also induce TNFα both *in vitro* and *in vivo*. In addition three inflammatory methods were utilized: Histological scoring, Mononuclear cell counts and MPO, all of which confirmed inflammation was present in the intestine in colitis mice and high LFCA conditions. In addition Lamina Propria Mononuclear cells gated intestinal macrophages directly confirmed CD36+ F4/80+ macrophages induced proinflammatory cytokines: TNFa, IFN-y, IL-17a, IL-4 depandant upon the mouse model.

4.1 Higher frequency of CD36-expressing macrophages in both Crohn's Disease and Ulcerative colitis Patients.

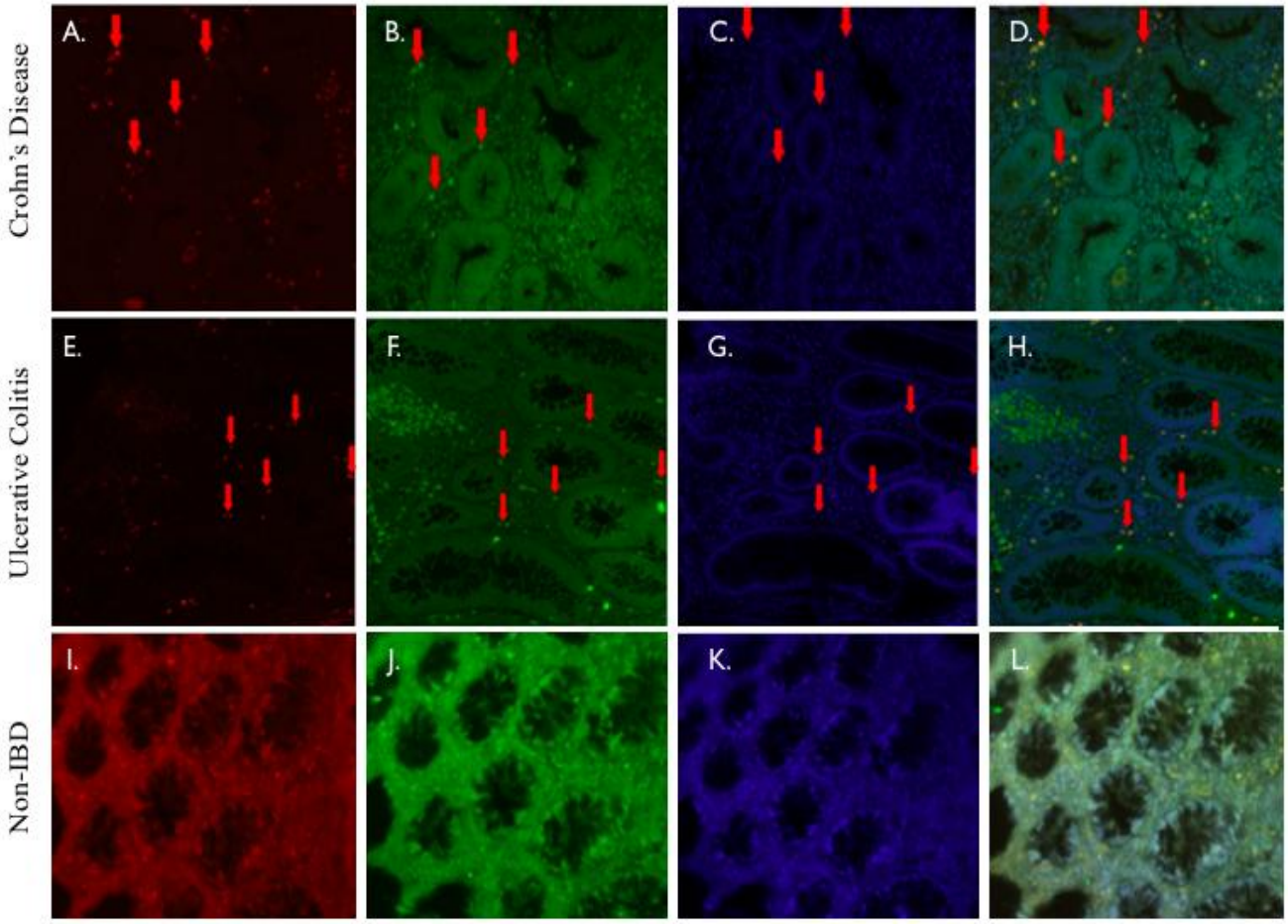

Figure 1.CD36 expressing macrophages are higher in the Lamina Propria of IBD patients. Immunohistochemistry staining on colonic biopsies taken from active CD and UC, and control Chinese patients (n=3). Please notes Non-IBD patients colons were obtained from non-diseased portions of colon cancer biopsies. A single representation of the proportion of infiltrating CD36+ macrophages were selected and taken from the BBI zeiss microscope in CD, UC and control patients. Representative IHC panel is shown for one patient biopsy at 40X magnification for FITC-CD36 and IF480-PE. Top Panels A-D demonstrate FITC-CD36, F4/80-PE, DAPI, and merged panels for Crohn's Disease; panels E-H for Ulcerative Colitis and panels I-L for non-IBD patients. Note: Cells that are double positive for CD36 and F4/80-PE are denoted by red arrows. The expression of CD36 expressing macrophages is higher in IBD and appears to localize to the LP of the intestine.

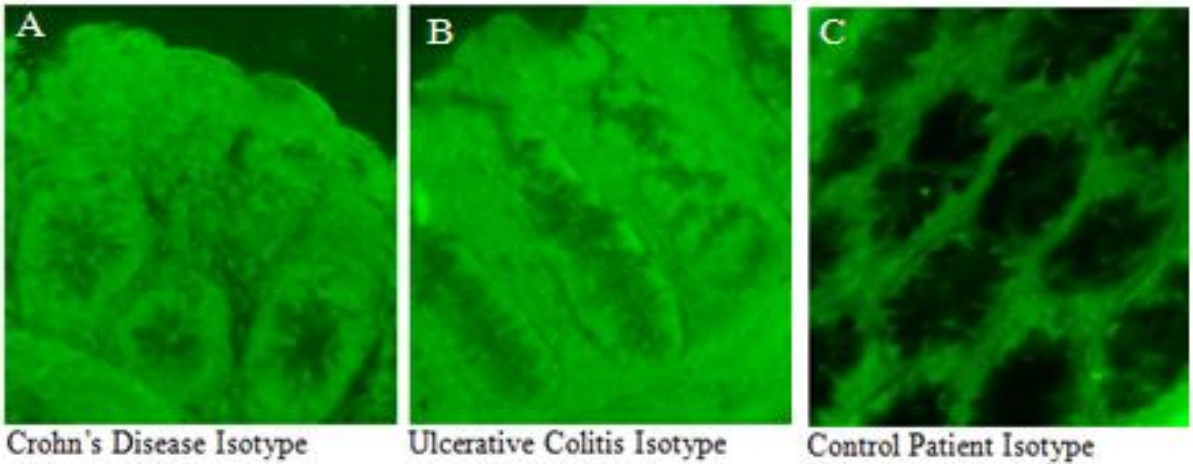

Figure 2. Goat Isotype IgG-FITC staining control for CD36 antibody validates positive stainging in disease states. Immunohistochemistry staining of colon biopsy of (A) Crohn's Disease, (B) Ulcerative Colitis, and (C) non-IBD (n=3) for Goat Isotype IgG. Representative panels at 40X magnification for each disease state are shown. Negative staining observed in IgG isotype verifies the specificity of the CD36 antibody in all human patients.

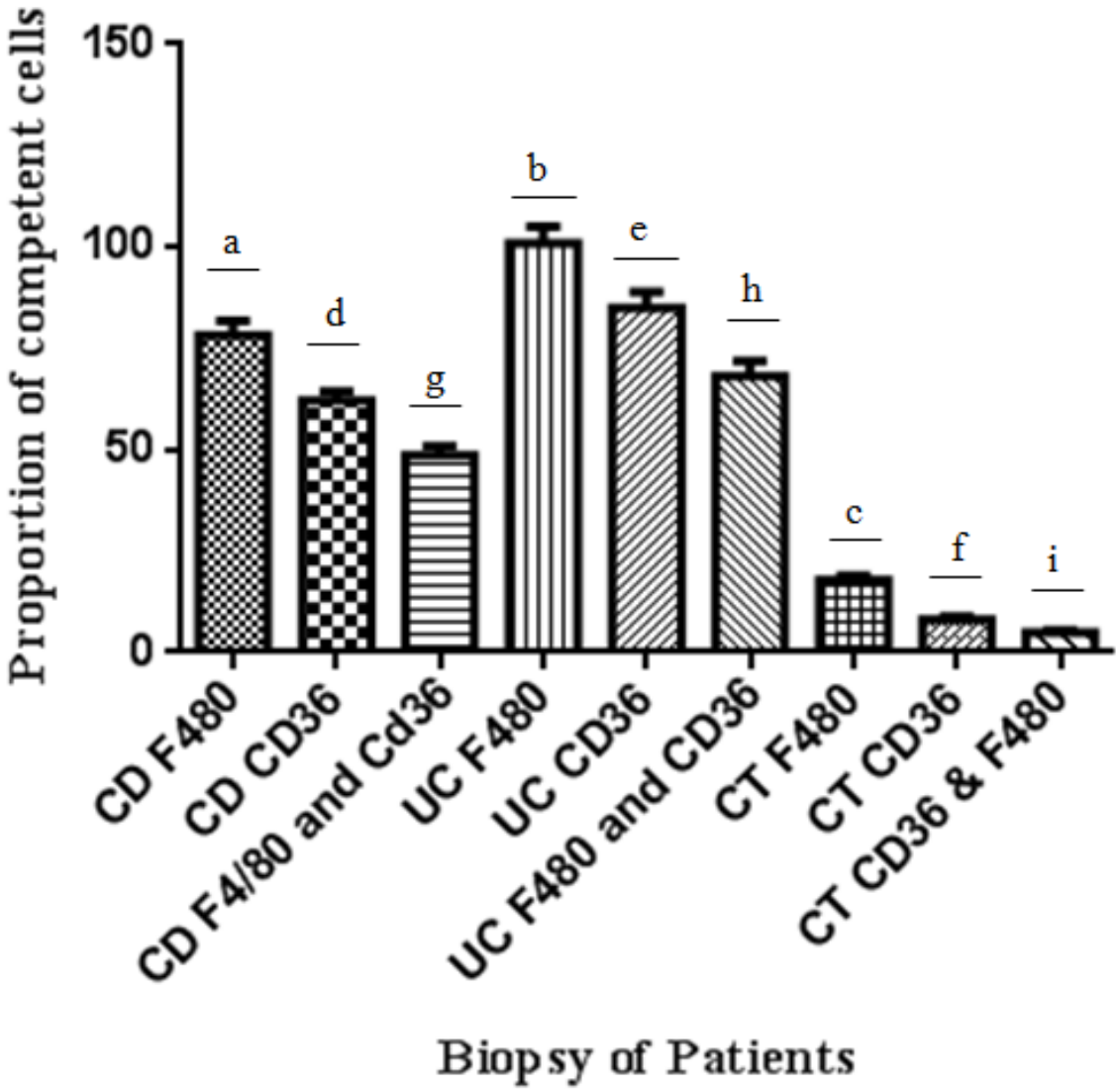

Figure 3. Immunohistochemical analysis reveals CD36 expressing macrophages is markedly expressed in IBD patients with a higher frequency in UC. Immunohistochemistry was utilized to examine competent cell counts in colonic biopsies from CD, UC and non-IBD patients (n=3) were calculated at 40X magnification for CD36, F4/80 and CD36+F4/80 and twenty pictures were averaged per patient per group. Samples were pooled for results and averaged for statistical analysis. CD36 expression was significantly higher in CD and UC from non-IBD patients (a,b,c; P=0.0001; one-way ANOVA with Tukey's test). In addition, the proportion of F4/80 expressing macrophages was significantly higher in CD and UC patients compared to non-IBD patients (d,e,f; P=0.0001; one-wayANOVA with Tukey's test).Lastly double positive Cd36+F4/80+ macrrophages were markedly high in UC and CD relative to Control patients (g,h,i P=0.0001). Please note compared data reached statisitical significance $P \leq 0.05$.

4.2 The number of CD36-expressing macrophages were higher in TNBS-colitis mice

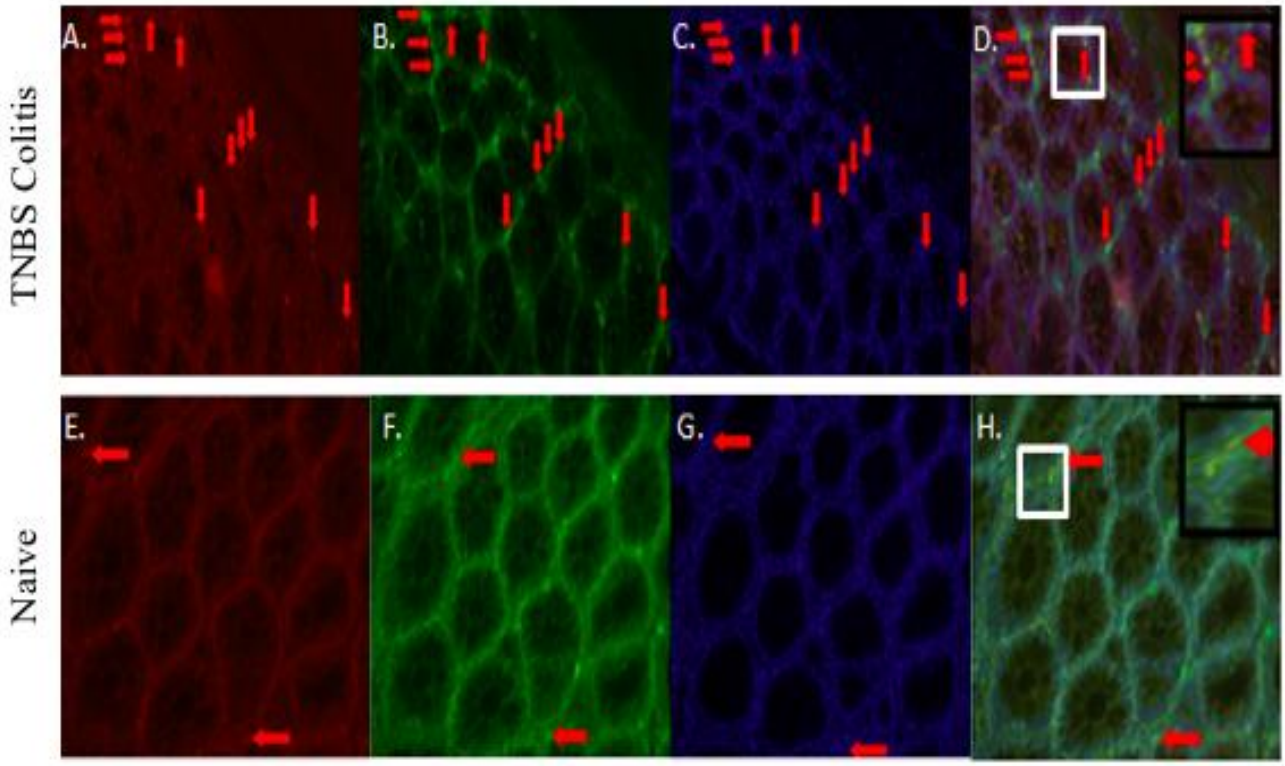

Figure 4. CD36 expressing Macrophages are higher in TNBS colitis realtive. Immunohistochemistry staining was undertaken on murine colonic tissues and competent cell counts were undertaken. Selected pictures of Naive and TNBS were taken as representative pictures for the expression of CD36 expressing macrophages. Representative IHC panel is shown for one colon at 40X magnification for FITC-CD36 and F4/80-PE.Top Panels A-D represent FITC-CD36, F4/80-PE, DAPI, and merged images for TNBS colitis mice, E-H for Naive mice. Note: Cells double positive for CD36 and F4/80-PE are denoted by red arrows. The frquency of Cd36 expressing macrophages in TNBS the was higher in comparison to Naive mice. CD36+ F4/80+ macrophages appear to localize in the lamina propria of the murine intestine.

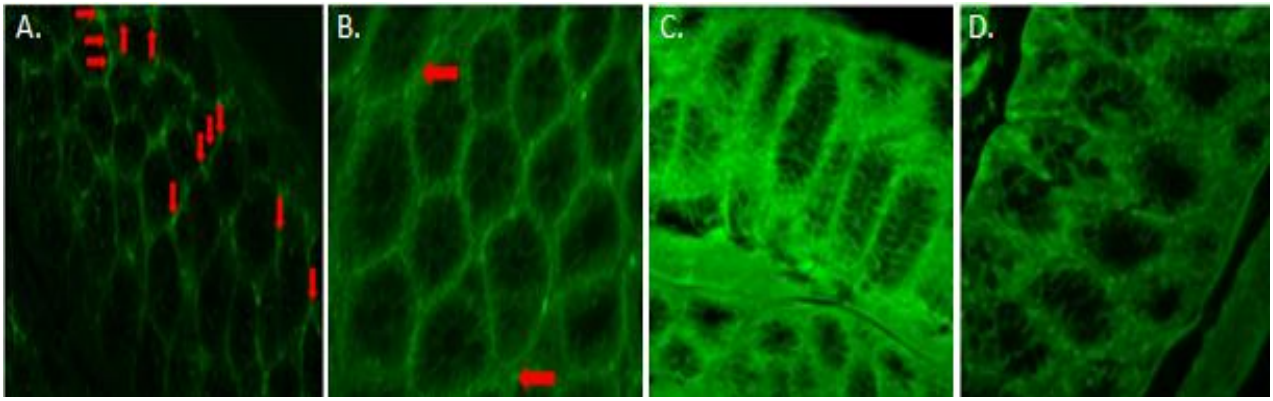

Figure 5. Goat Isotype IgG staining control for CD36 in Naive & TNBS Mice validates positive staining. Immunohistochemistry of mice colon sections of of Naive and TNBS Goat Isotype IgG exhibits no false positive staining, and thus validates true CD36 positive cells present in both Naive and TNBS mice (n=5). Representative panels at 40X magnification for each disease state are shown. Panels A and B represent FITC-CD36 staining for TNBS Colitis and Naive mice and Panels C and D represent Goat IgG Isotype staining for for TNBS Colitis and Naive mice. Please note some of the staining of naive mice shows a few false positive stains evident on the surface of the tissues; only a few true positive CD36 cells are shown. TNBS demonsrates a much higher frequency of true CD36 positive cells in the colon.

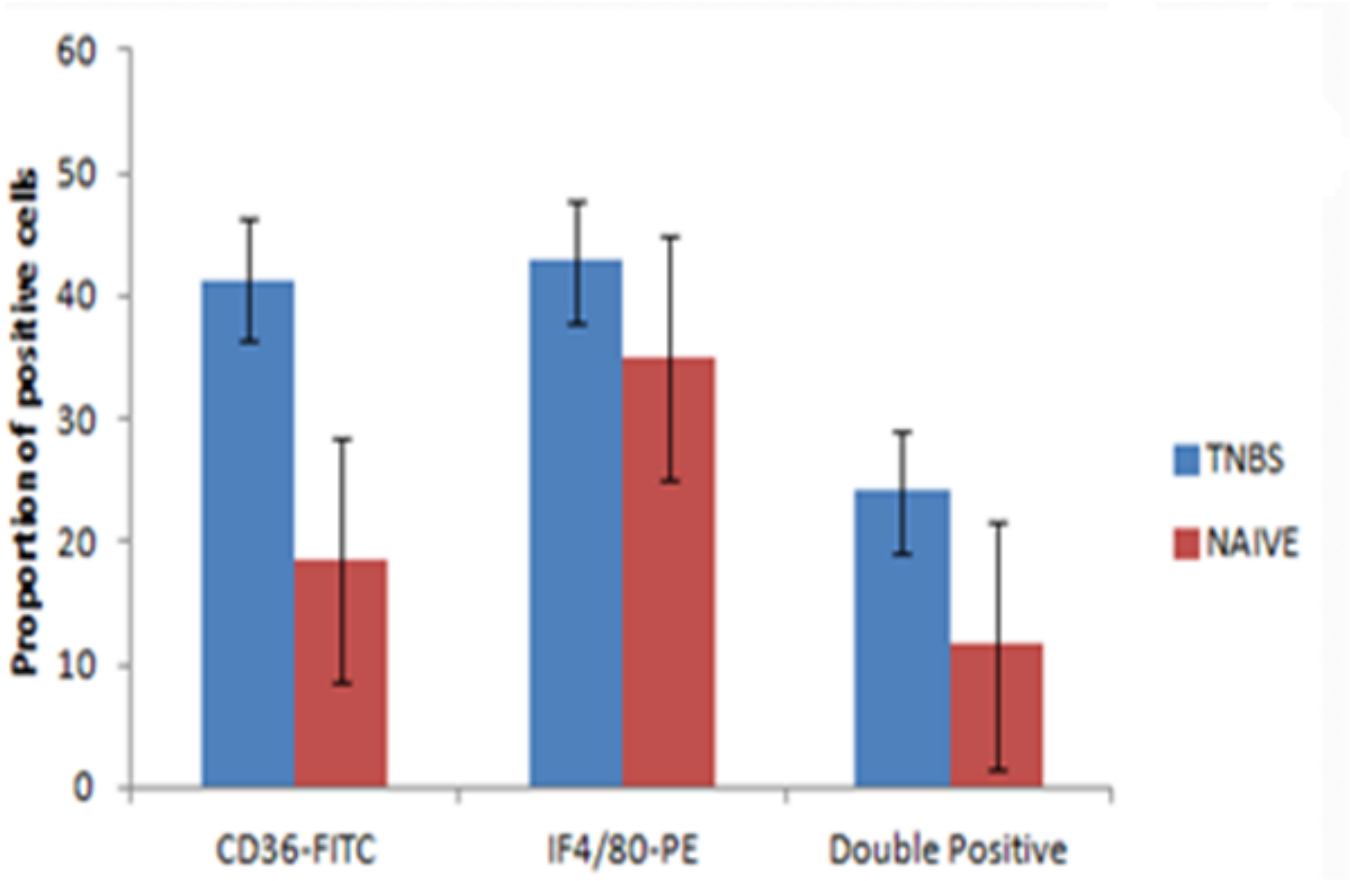

Figure 6. CD36+F4/80+ macrophages are higher in TNBS mice.
Immunohistochemistry was utilized to examine the competent cell staining of colonic samples of naive and tnbs mice; twenty pictures per mice per group were taken at 40X via the BBI Zeizz microscope, and counted and averaged over three parameters:(A) CD36, (B) F4/80 and (C) CD36+ F4/80+ per group(n=5). Although results were not statisically different the frequency of CD36 and double positive Cd36+F4/80+ macrophages was higher in TNBS colitis mice.

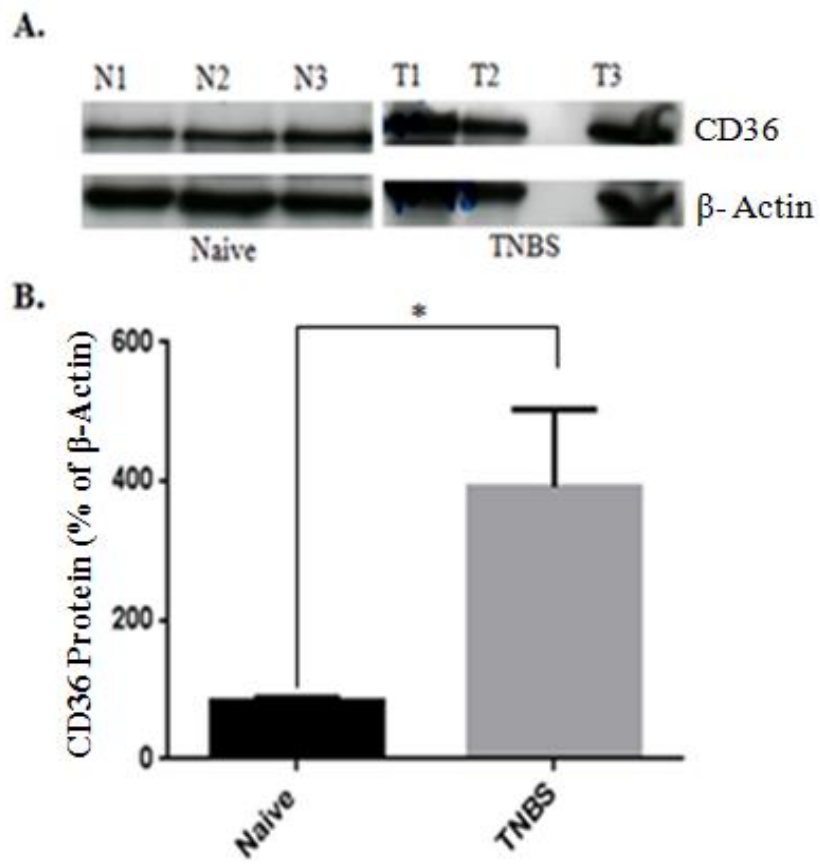

Figure 7. CD36 protein expression is higher in TNBS colitis mice. (A) Western blots depicting colonic CD36 expression in Naive and TNBS Colitis mice relative to β-Actin loading control; five protein samples from different mice per group were examined via integrated density differences between sample and β actin loading control. Colonic Protein was extracted immediately upon sacrifice from both Naive and TNBS following protein extraction protocol. (B) Quantification of CD36 protein expression relative to B-Actin shows significant elevation of CD36 protein expression in TNBS Colitis mice in comparison to naive mice (P= 0.0009, unpaired T-test). All data are represented as means ± SE

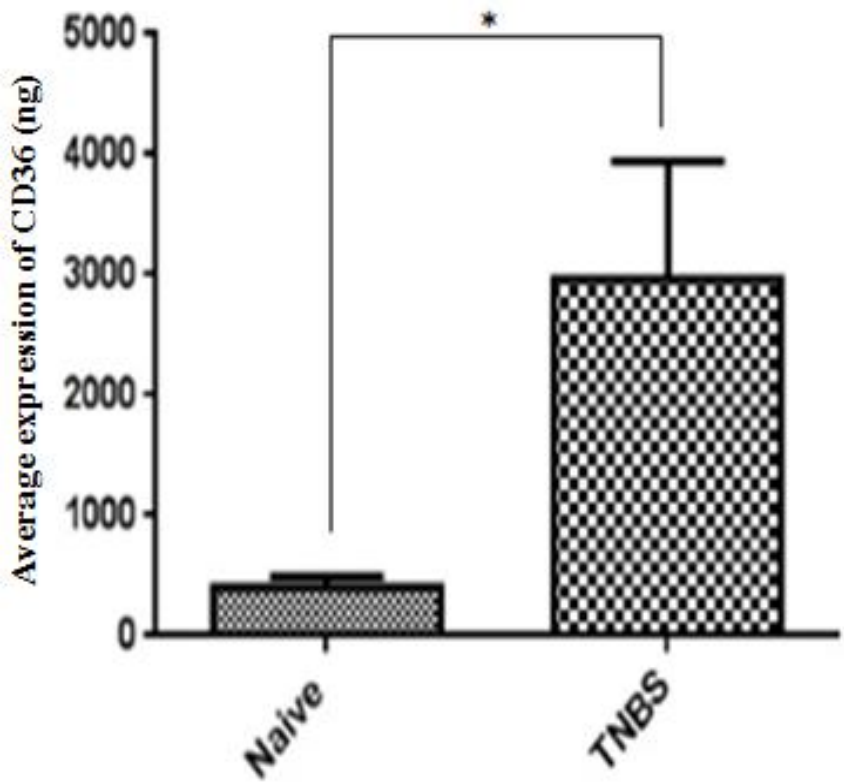

Figure 8. CD36 gene expression is higher in TNBS colitis. RNA was extracted from colon tissues extracted from Naive and TNBS Colitis mice(n=4) immediately after sacrifice as described and analyzed by Real time PCR. CD36 gene expression for each group is presented demonstrated CD36 gene expression is significantly higher in TNBS Colitis mice (P=0.049; unpaired T-test). All data is represented as a means ± SE.

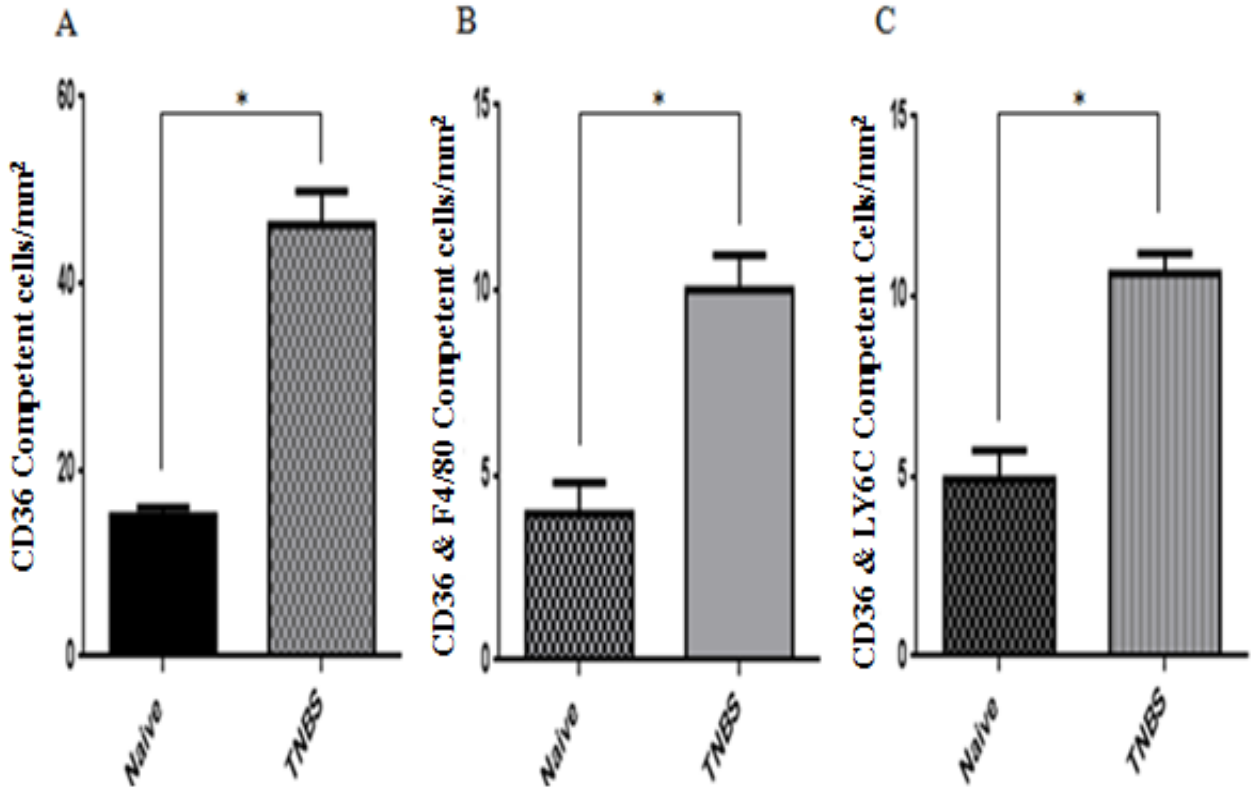

Figure 9. Flow Cytometry reveals TNBS colitis mice express higher levels of CD36, F4/80 and LY6C. LPMC isolated from the small and large intestine of Naive and TNBS Colitis mice were cultured with RMPI 1640 for several days, harvested, and cell sorted via BBI FACs CANTO machine to examine the following expression of markers (A) CD36, (B)CD36+F4/80 and (C) CD36+LY6C. F4/80 is an intestinal macrophage marker and Ly6C is represented as a monocyte marker. Graphs depict pooled results from multiple replicates of FACS analyses are statistically significant (n=4). Competent cell counts/mm^2 were significantly higher in TNBS Colitis mice for CD36 (A; P=0.0018, unpaired T-test), CD36+F480 (B; P=0.0044, unpaired T-test), and CD36+LY6C (C; P=0.0098, unpaired T-test). All data is represented as a means ± SE.

4.3 *In vitro* stimulation of MHS macrophages with Palmitic Acid ,TLR 2 Ligand PGN, and Palmitic acid +PGN and proinflammatory cytokine production

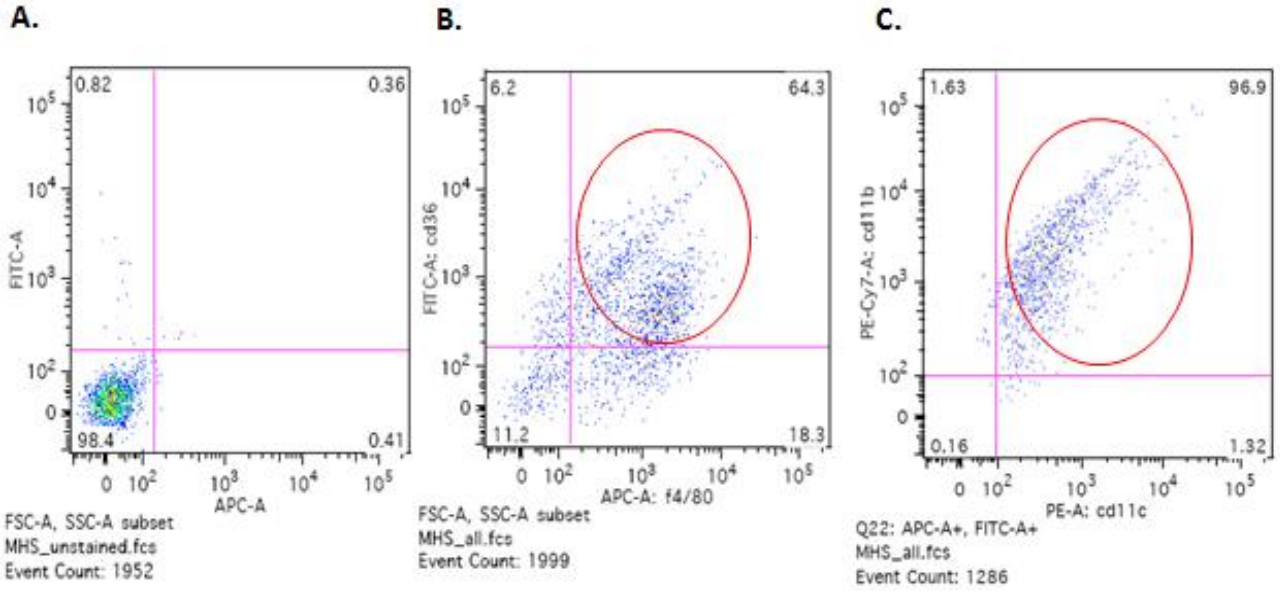

Figure 10. Flow cytometry demonstrates high expression of CD36+ Macrophages in MHS cell line. MHS cells were cultured in RMPI 1640 media, harvested, and cell sorted via BBI FACs CANTO machine. Dot plots represent (A) Unstained, and (B) CD36 & F480 and (C) CD11b & CD11c. Results reveal CD36+ MHS macrophage population is 64.3%, double positive CD36+F480+ and 70.5% CD36+ cells; majority of cells are confirmed macrophages. This result confirms MHS macrophage cell line and verifies MHS cell line highly express CD36 scavenger B receptor.

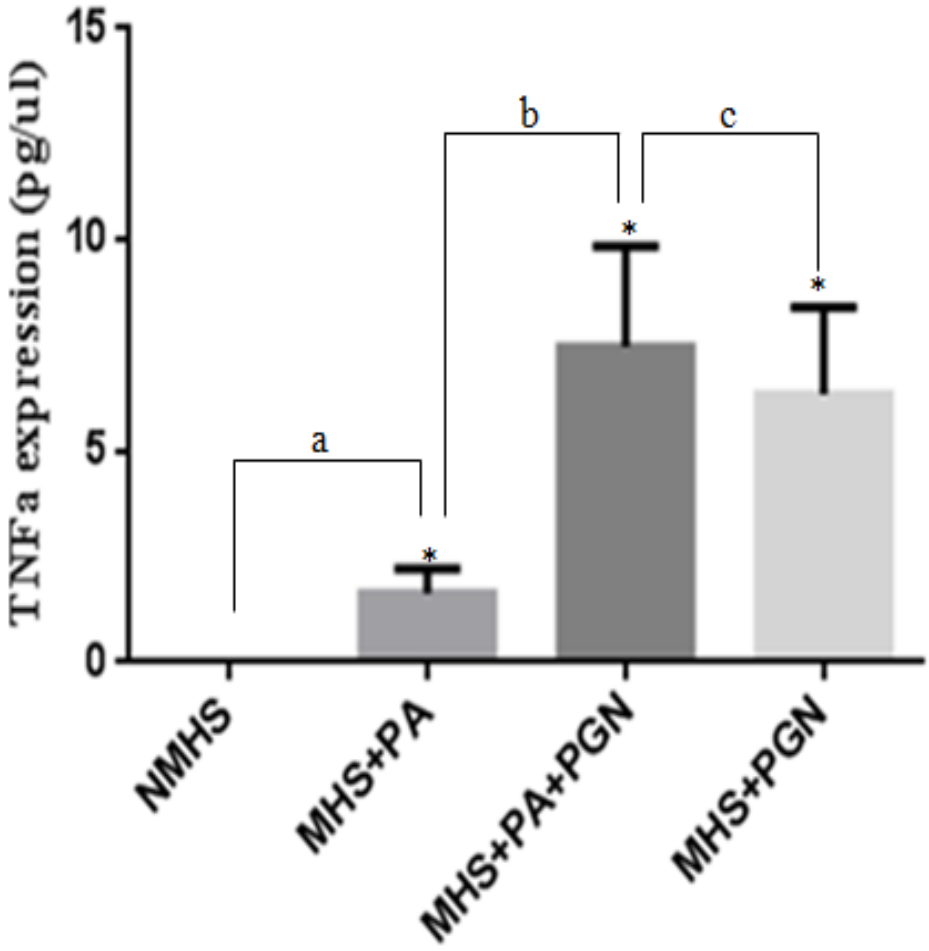

Figure 11. TNFα Expression in MHS Macrophages stimulated with PA, PGN, or PA+PGN.

Macrophages were stimulated with Palmitic Acid (600ul high dose), PGN (100ng/ul) or both for a time course of 24hours and cell supernatants were collected and analyzed via an TNFα ELISA duoset (R&D systems) kit. Standard curve, blank, and negative controls were utilized as plate controls. MHS macrophage supernantants revealed that there was significantly higher expression of TNFa in MHS+PA+PGN, PGN and some expression in MHS+PA relative to NMHS control (b, P=0.0198, unpaired T-test); (c, P=0.028, unpaired T-test) (a, P=0.0179, unpaired T-test) respectively. It was then determined PGN was sufficient to stimulate MHS macrophages and was used throughout proceeding experiments. Please note data is demonstrated as a means ± SE.

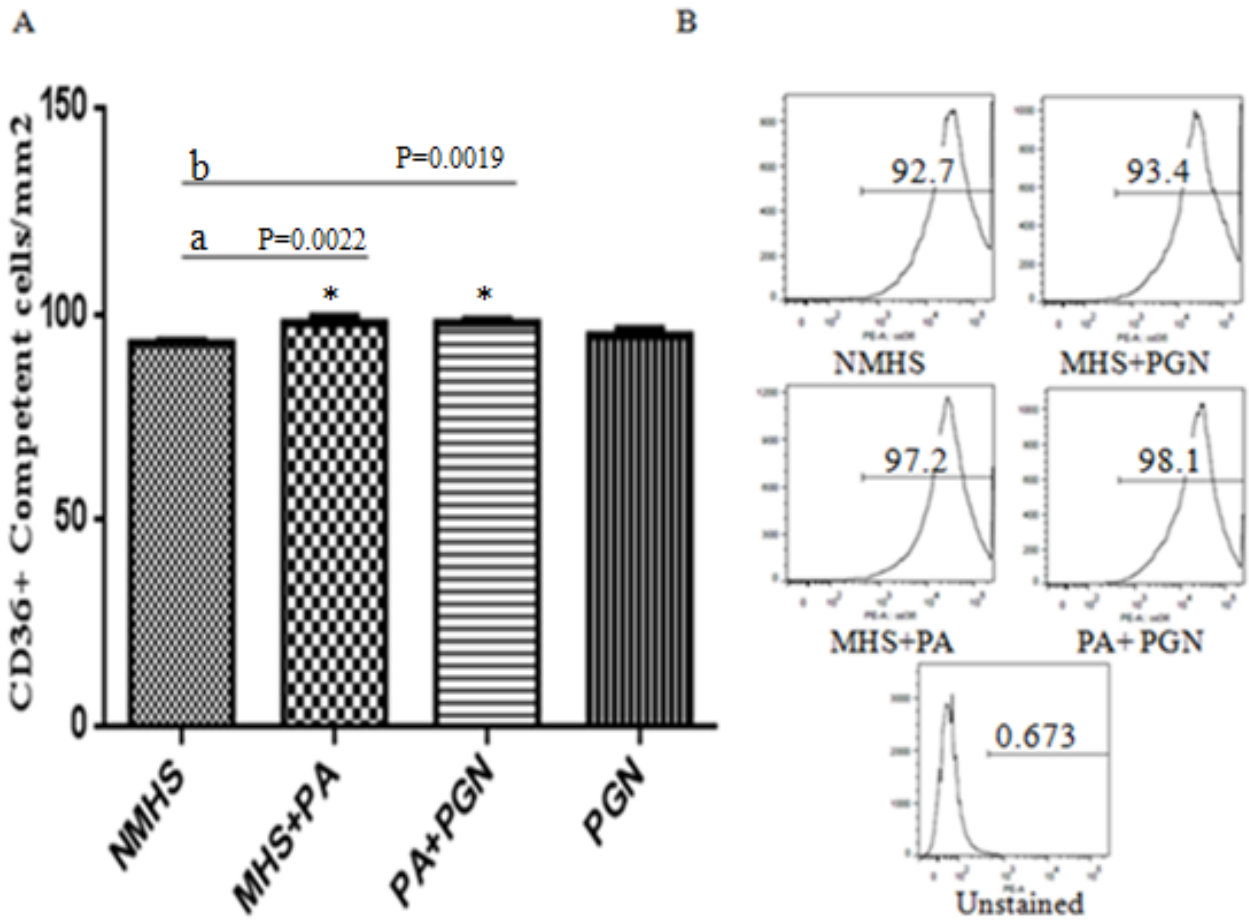

Figure 12. Flow cytometry demonstrates MHS highly express CD36 stimulated by Palmitic acid or Palmitic acid and TLR 2 Ligand, PGN. (A) MHS macrophages were incubated with a 24 hour time course of either high Palmitic acid concentration, high Palmitic acid and PGN or, PGN alone and analyzed via FACS and cell sorted via the BBI FACs CANTO machine. All MHS macrophages exhibited a high expression of CD36 receptor. CD36 expression is slightly elevated in PA-treated (a; P=0.0022; unpaired T-test) and PA+PGN-treated (b; P=0.0019, unpaired T-test) macrophages. PGN treated macrophages were not significantly different in comparison NMHs control (P=0.2578, unpaired T-test).There is also no significant difference between PA and PA+PGN-treated groups (P=0.9631). (B) Single CD36-PE histograms were utilized as a representation of quantitative CD36 upregulation in *in vitro* MHS macrophages.

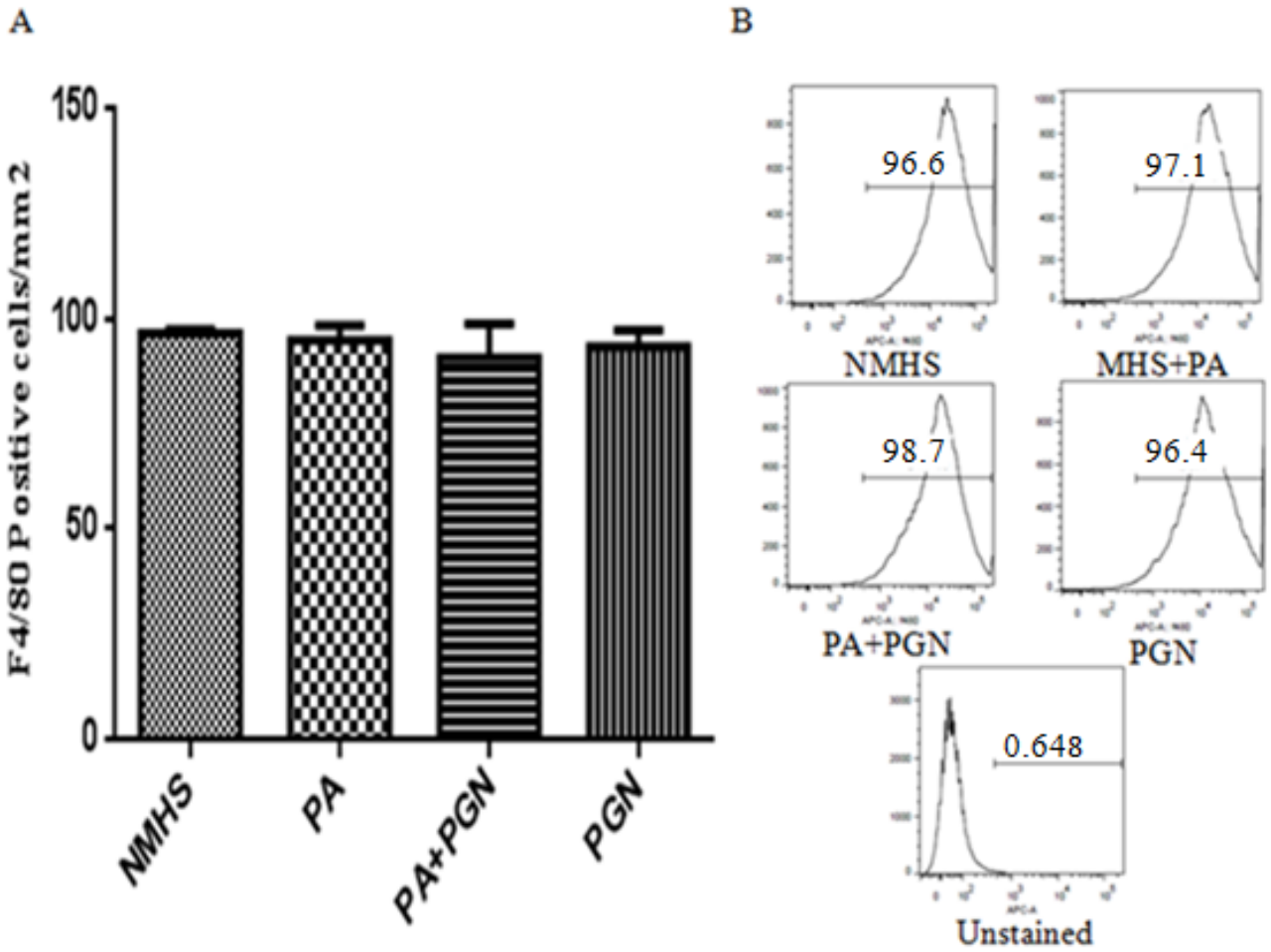

Figure 13. Flow cytometry demonstrates MHS highly express macrophage marker, F480, in all conditons. MHS macrophages were incubated with a 24 hour time course of either high Palmitic acid concentration, high Palmitic acid and PGN or, PGN alone and analyzed via FACS BBI CANTO machine. F4/80 expression is uniform, with no significant differences among treatment groups ($P>0.05$, unpaired T-test). (B) Single F4/80 histograms were selected to present an example of quantitative F4/80 upregulation in vitro MHS macrophage cell line. Data is demonstrated as means ± SE and analyzed via unpaired t-tests.

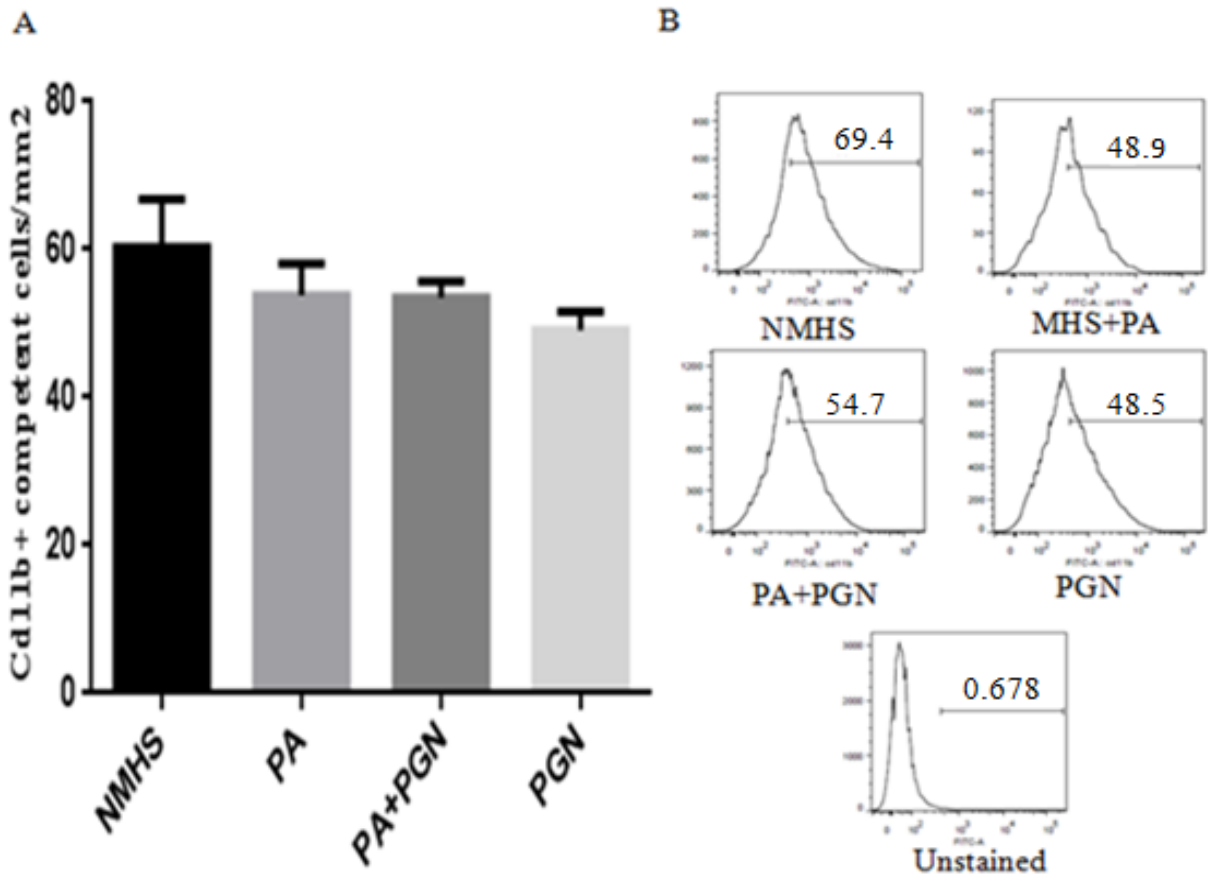

Figure 14. Flow cytometry demonstrates MHS highly express macrophage marker, CD11b, in allconditons. MHS macrophages were incubated with a 24 hour time course of either high Palmitic acid concentration, high Palmitic acid and PGN or, PGN alone and analyzed via FACs BBI CANTO machine. No significant differences in CD11b expression were found between NMHS, MHS+PA, PA+PGN and PGN macrophages (P>0.05, unpaired T-test), stimulated and unstimulated macrophages express this receptor similarly. (B) Single Cd11b-FITC histograms were utilized to present an example of the quantitative expression of CD11b in MHS macrophage groups. Data is demonstrated as means ± SE.

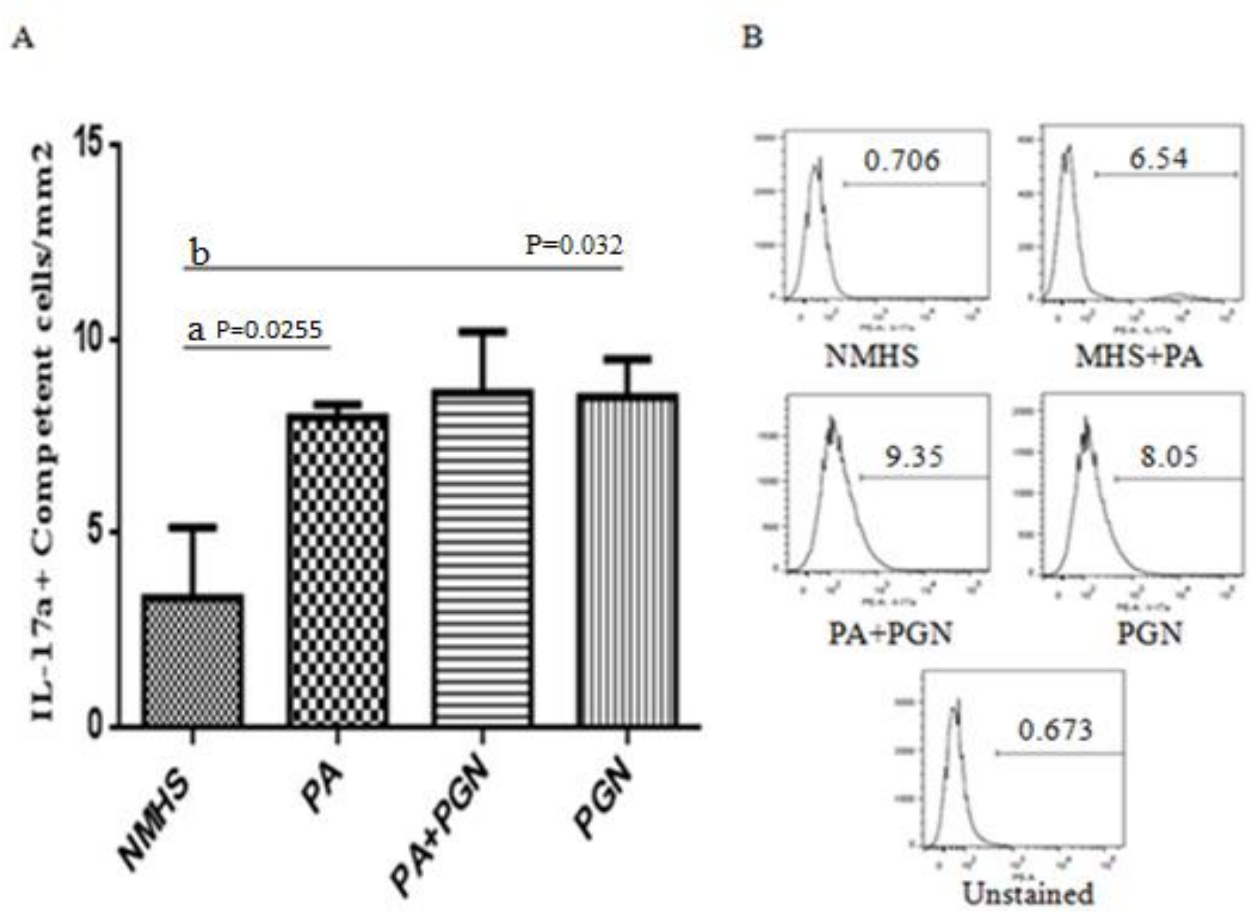

Figure 15. Flow cytometry shows significantly higher IL-17a expression in MHS+PA and MHS+PGN stimulated macrophages. (A) MHS macrophages were incubated with a 24 hour time course of either high Palmitic acid concentration, high Palmitic acid and PGN or, PGN alone and analyzed via BBI FACs CANTO. IL-17a expression was significantly higher in MHS+PA treated macrophages (a, P=0.0255; unpaired T-test) and in PGN-treated macrophages (b, P=0.032; unpaired T-test) relative to control NMHS. No significant differences were observed between NMHS and PA+PGN (P=0.063;unpaired T-test). No significant differences were observed between MHS+PA, PA+PGN, and PGN stimulated macrophages (P=0.7001;unpairedT-test);(P=0.6248; unpaired T-test) correspondingly. (B) Single IL17a-PE histograms were selected to represent quantitative IL-17a upregulation per each MHS macrophage in *vitro* group. Data is represented as means ± SE.

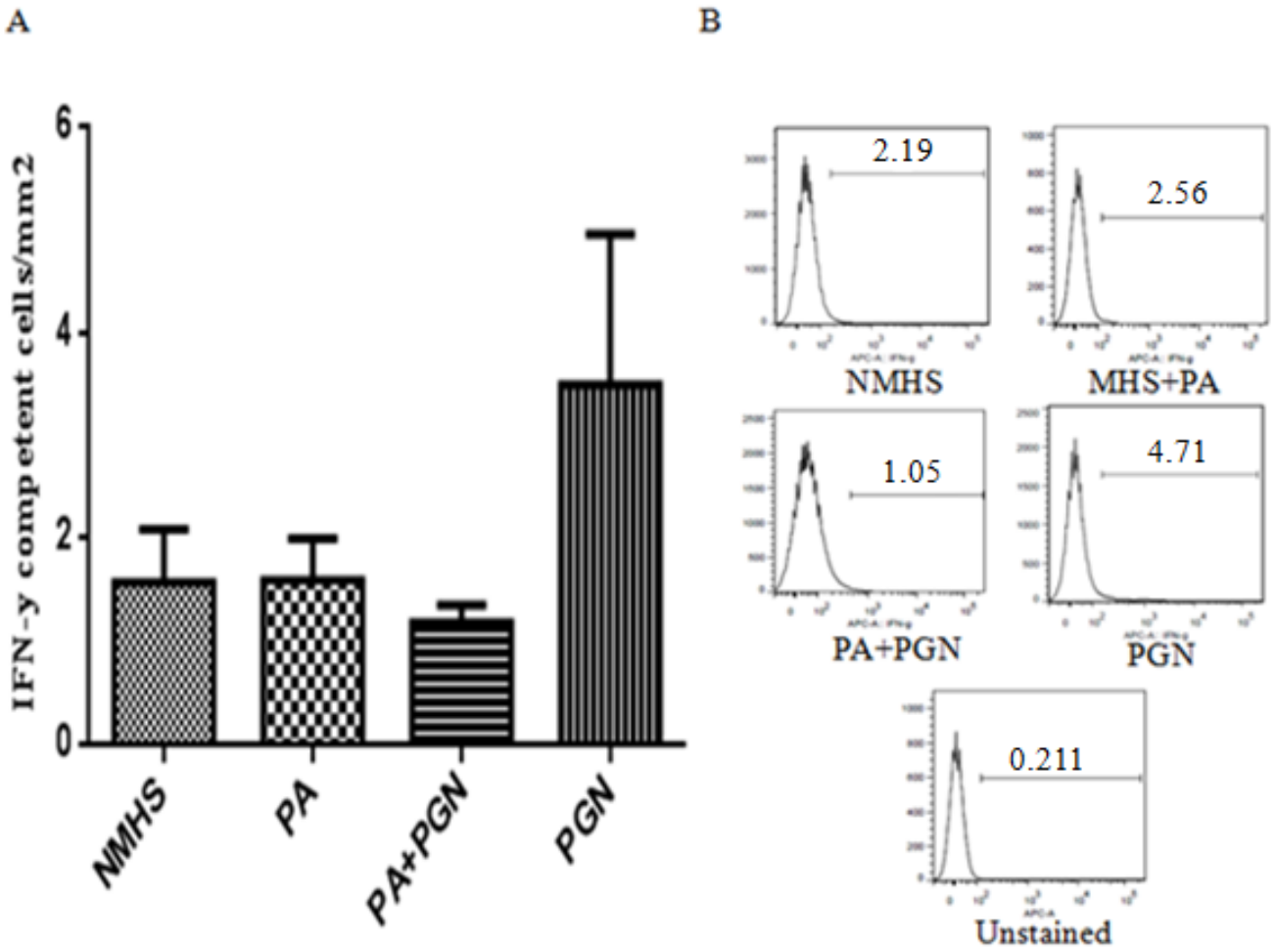

Figure 16 . Flow cytometry reveals little to no expression of IFN-γ in MHS macrophages. **(A)** MHS macrophages were incubated with a 24 hour time course of either high Palmitic acid concentration, High Palmitic acid and PGN or, PGN alone and analyzed via FACs BBI CANTO machine. No significant differences were observed between all treated groups and control NMHS ($P>0.05$, unpaired T-test). No significant differences were observed between NMHS and MHS+PA, PA+PGN and/or PGN ($P=0.9719$);($P=0.4904$);($P=0.2486$) respectively. MHS+PA treated macrophages expression of IFN-γ was not significantly different from either PA+PGN ($P=0.3694$) or PGN($P=0.2442$) treated macrophages.**(B)** Single IFN-γ histograms were utilized to present an example of average IFN-γ expression per MHS macrophage group.

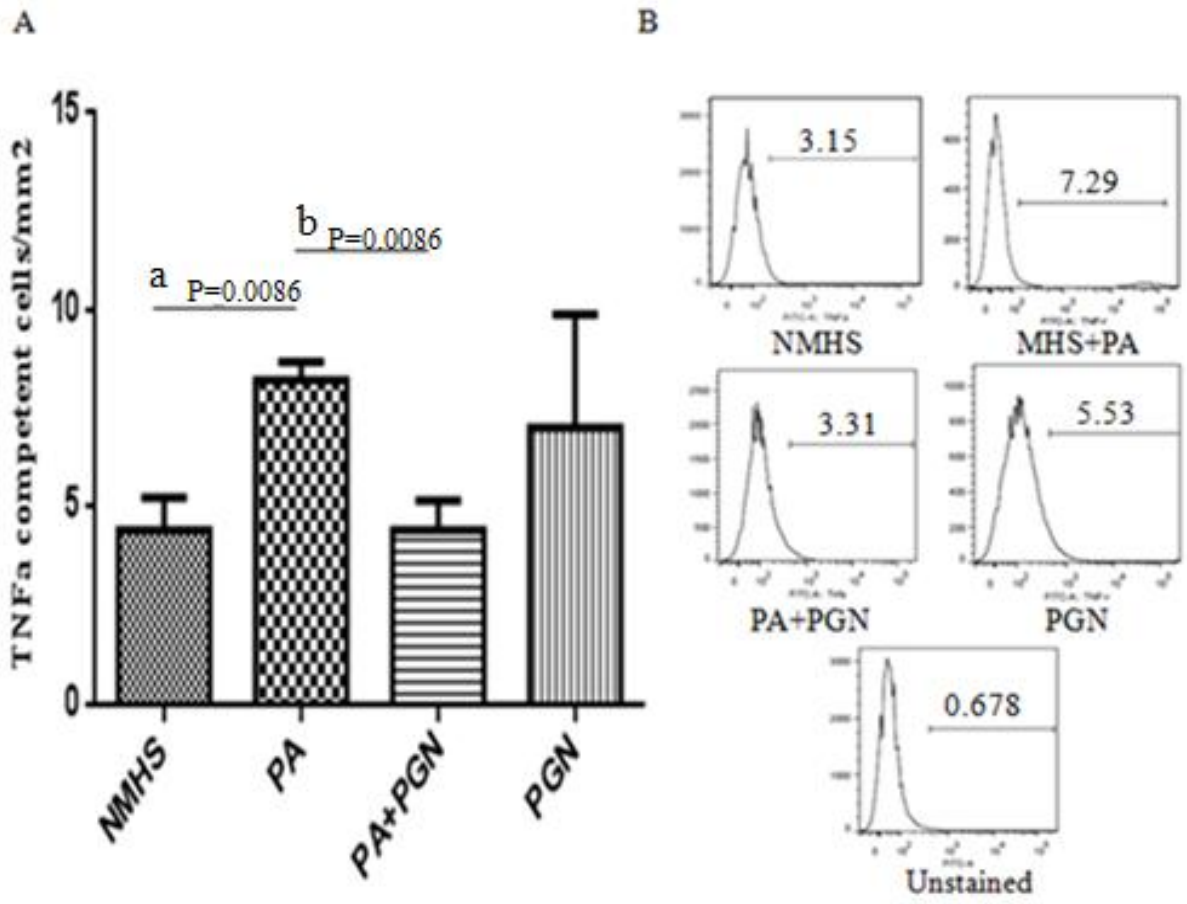

Figure 17. Flow cytometry reveals high dose Palmitic Acid induces a higher expression of TNFα in MHS macrophages.(A) MHS macrophages were incubated with a 24 hour time course of either high Palmitic acid concentration, High Palmitic acid and PGN or, PGN alone and analyzed via FACs BBI CANTO machine. TNFα expression was significantly higher in PA-treated MHS cells compared to control (a, P=0.0086, unpaired T-test). Alternatively, TNFα expression was significantly lower in PA+PGN-treated cells relative to PA treatment alone (P=0.0086, unpaired T-test). There was no significant difference among NMHS and PA+PGN or PGN-treated cells (P>0.05). **(B)** Single TNFα histograms were selected to represent an example of average TNFα expression per each stimulated and unstimulated MHS macrophage group.

4.4Adoptive transfer of conditioned CD36+MHS macrophages in BALB/c mice

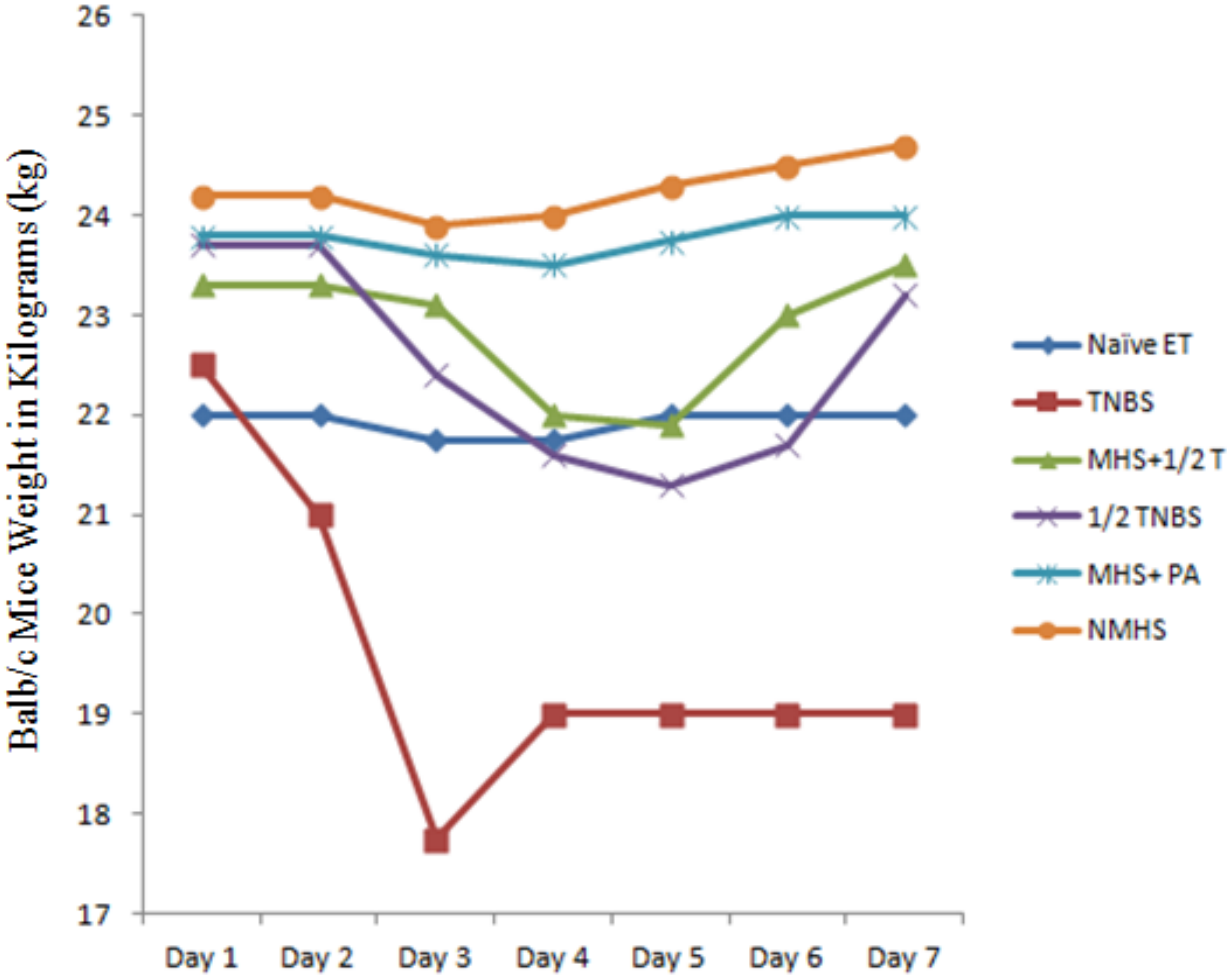

Figure 18. Seven day Mice weight chart for treatment and control mice . Balb/c Mice Group weight chart. Mice were treated in the same condition injected on day 1 and sacrificed on day 7 (n=5 for each cohort) and weighed daily at 10:00am by the CS2000 compact CE scale and measured before and after injection to ensure recovery of animals. At any point an animal was determined sick, poor health, distress, or fails to recover, these mice were sacrificed immediately. Five mice were assigned per each group, and replications of mice groups were conducted.

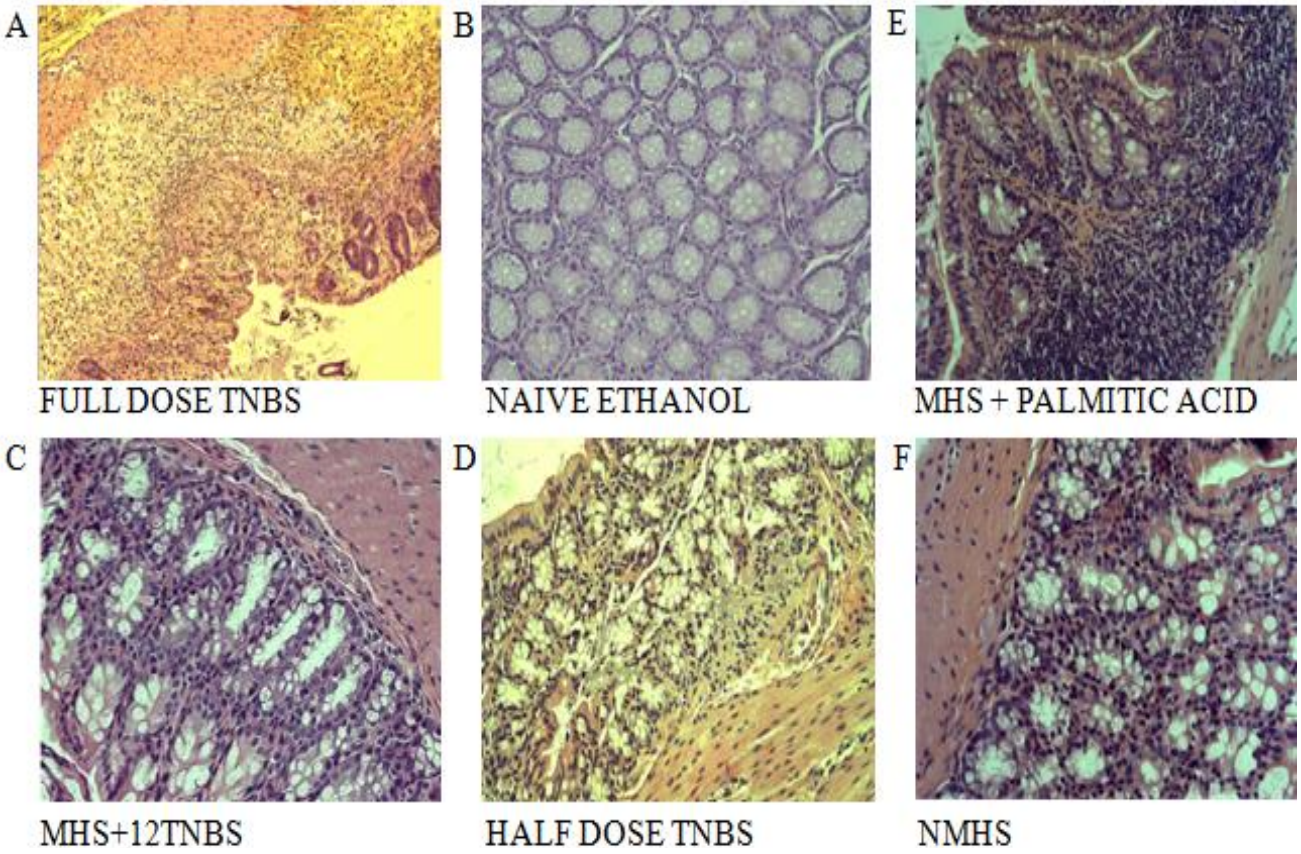

Figure 19. Colonic sections were stained via H&E, Hematoxylin and Eosin stain, and one representative histological picture is selected to demonstrate the average Histology of each mice group(n=5). Representative colon tissues were sectioned and underwent H&E staining as to determine histology, each picture is selected as a representation per mice group (n=5). Both Naive and NMHS exhibited little pathology with some mononuclear cell infiltration in the lamina propria, little zonal edema and crypt hyperplasia. NMHS exhibited a slightly higher histological scoring but is still considered in normal range. MHS+12ntbs and half dose tnbs displayed simillar histological features with mononuclear infiltration in muscle and LP, and some edema; 12tnbs exhibited higher histological scoring due to focal and zone crypt destruction. Most severe pathogology was observed in the full dose TNBS group wherein there was systemic mononuclear infiltration throughout all tissues, zonal or complete crypt destruction and severe edema, indicating full dose TNBS induced severe colitis; half dose induces a milder colitis. In addition MHS+PA displayed an unexpected higher monnuclear infiltrate, and histological score than anticipated, this group also exhibited some crypt destruction and mild to moderate edema.

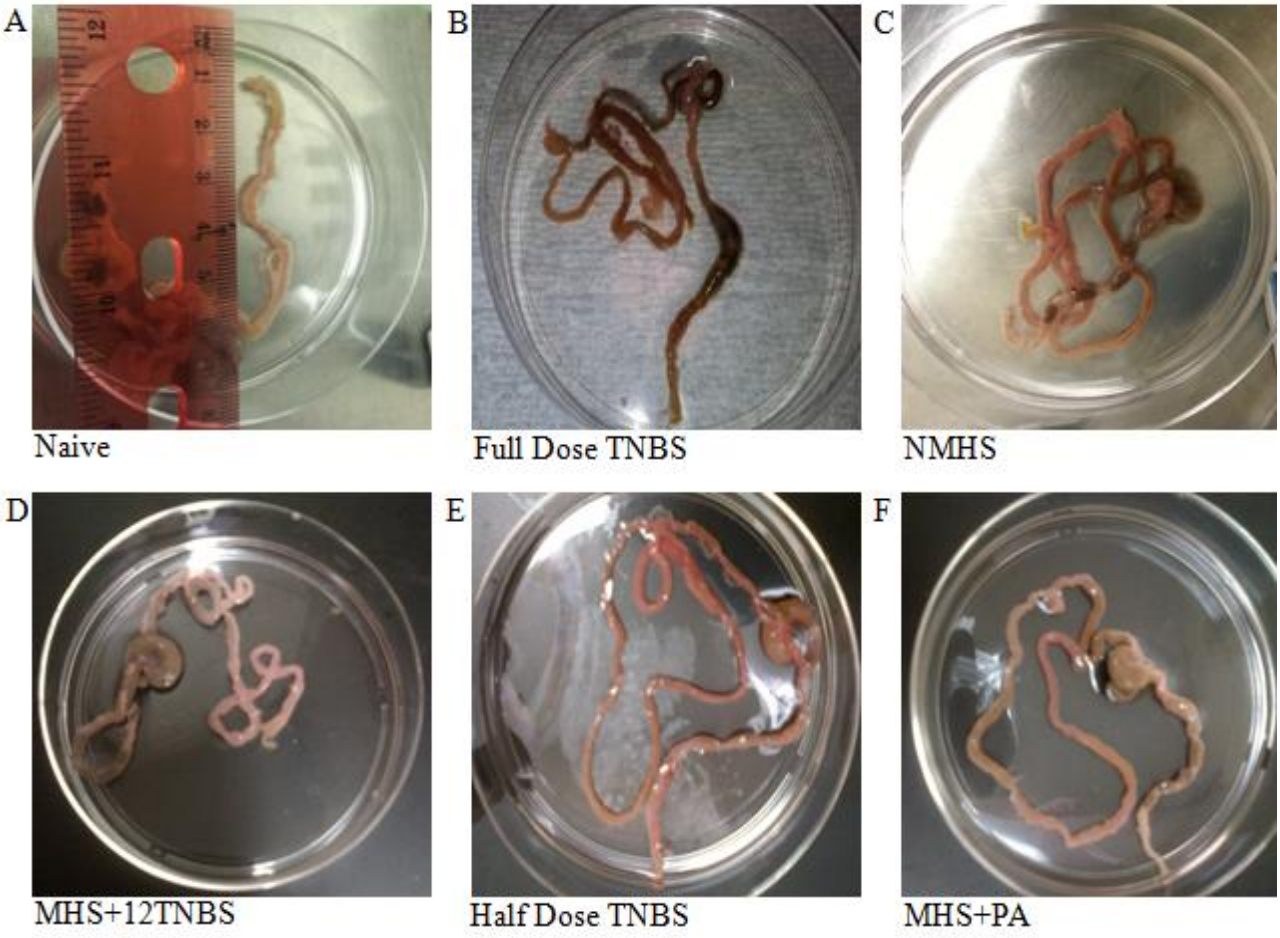

Figure 20. Representative Colon dissections per each mouse group. Macroscope pictures where taken to examine inflammation in colon, colons were dissected and one picture was selected to represent each mice group.Colons extracted from (A) Naive, (C) NMHS, (D)MHS+12tnbs and (F) and surprisingly MHS+PA remained unremarkabely healthy. These colons were elongated, elastic, and bared no signs of constipation, diarrhea, blood spots, inflammation, and no swelling of the intestine was visible; stools had a healthy consistency.Some inflammation was expected in MHS+12tnbs as half dose tnbs should have induced some mild irritation or swelling, however mouse colons from thsi group appeared simillar to controls. Full dose TNBS induced marked inflammation and swelling in the colon, decreased colon length, blood spots, watery stool, some constipation or perhaps bowel obstructions; the integrity of colon was compromised as the tissue was fragile and tore easily. Additionally many small small blood spots and sweeling in the colon was observed and also in the small intestine as well, establishing TNBS has caused inflammation in the intestine. Lastly, half dose TNBS induced minor swelling in the colon, with a few blood spots, loose to watery stool, irriatation and redness of the colon; no colonic shortening was observed. Half dosTNBS colitis did not induce pronounced damage or inflammation to the intestine as seen in full dose TNBS.

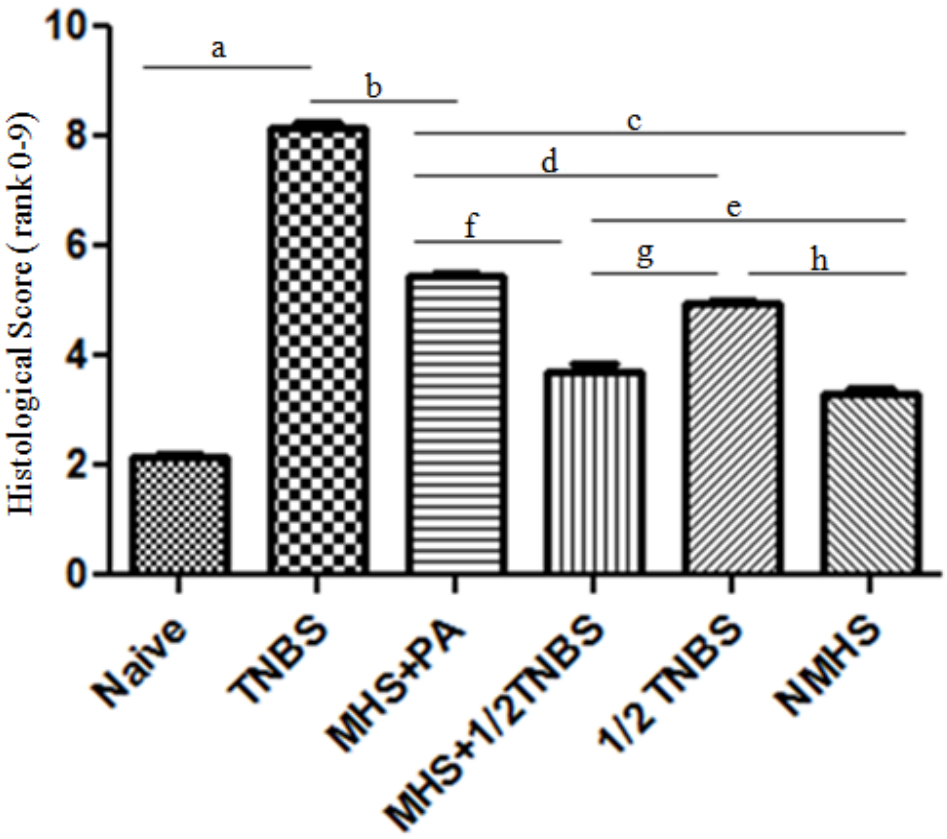

Figure 21: Histological scoring of mice groups. Histological scoring is a method of grading inflammation in the intestine via three parameters: edema, crypt destruction and mononuclear infiltrate and scored 0-3 in terms of severity according to Cooney et. al[112]. Using BBI Zeiss microscope twenty pictures per mice per group were taken, yielding 100 pictures per group (n=5); data was pooled and averaged per group. TNBS colitis mice yielded the highest inflammatory score and was significantly different from control mice (a, P=0.0001; unpaired T-test). In addition although 1/2TNBS exhibited a lower histological score, this value was also significantly higher than naive ethanol control (P=0.0001). MHS+PA scored a higher inflammatory score than both MHS+1/2TNBS and 1/2TNBS and was statically significant from both groups (f, P=0.0006 and d, P=0.0001). Experimental groups TNBS, and MHS+PA, exhibited either a severe and moderate histological score; TNBS exhibited a significantly higher histological score (b, P=0.0001). Full dose TNBS exhibited a markedly higher histological score than 1/2TNBS (P=0.0001). All three groups MHS+PA, MHS+1/2TNBS and 1/2TNBS were significantly different and exhibited a higher in comparison to NMHS control mice(c, P=0.0001, e, P=0.034, and h, P=0.0001, respectively). TNBS mice reveal that there is extensive inflammation and damage to the intestine. MHS+PA and 1/2 TNBS exhibited similar histological scores; mice demonstrate moderate inflammation and scores in the intestine. All data are represented as means ± SE

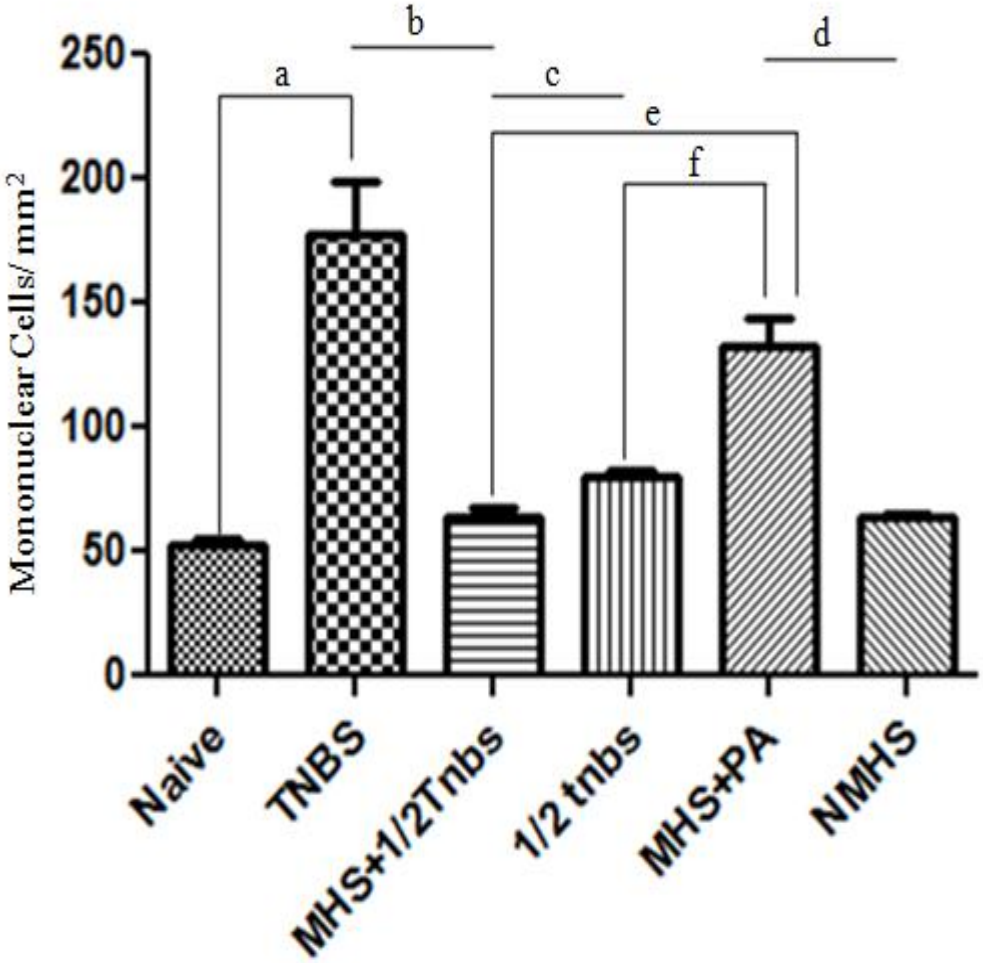

Figure 22. Mononuclear cell counts are significantly higher in TNBS, MHS+PA and 12tnbs. Tissues were fixed via formalin and stained via H&E protocol. Using the Zeiss BBI microscope twenty pictures were taken per mice in each group (n=5); cell counts per group were pooled and averaged for statistical differences. Each bar graph represents the mice groups average mononuclear cell count. Naive and TNBS were statically significant (a, P=0.0004; unpaired T-test) full dose TNBS exhibited the highest mononuclear infiltrate. MHS+PA and MHS+1/2TNBS and 1/2TNBS were also statistically significant (e, P=0.0004 and f, P=0.0041) with MHS+PA exhibiting a moderately higher mononuclear cell infiltrate. MHS+PA was significantly higher then NMHS macrophage control (d, P=0.0002). MHS+1/2TNBS and 1/2tnbs mononuclear infiltrate were statistically significant (c, P=0.0116); 12tnbs exhibited a moderately higher mononuclear cell count. In addition, full dose TNBS treatment was markedly higher than half dose tnbs (P=0.0052). 1/2TNBS mice groups exhibited a significantly higher infiltrate in comparison to NMHS(P=0.007). There was no statistical difference between TNBS and MHS+PA and MHS+1/2tnbs and NMHS (P=0.1293 and P=0.8764). Collectively, all data are presented as means ± SE.

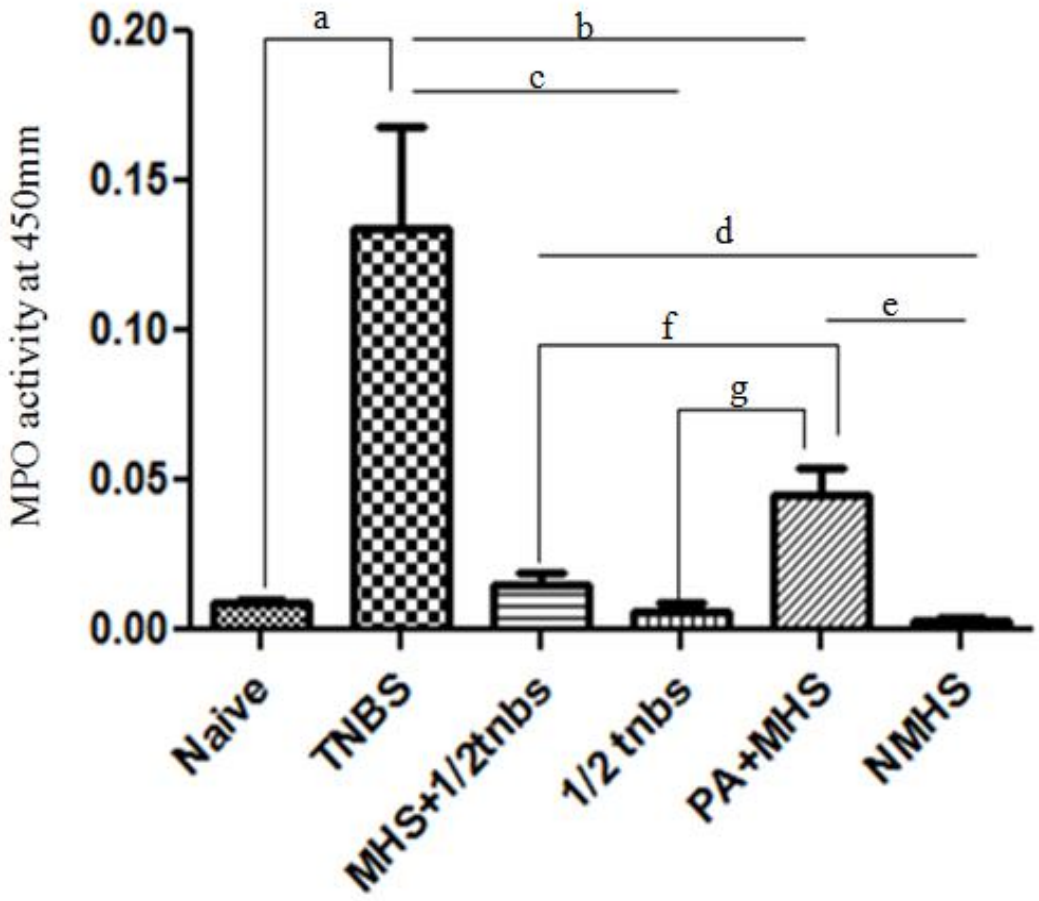

Figure 23. Myeloperoxidase activity is significantly higher in TNBS and MHS+PA. Myeloperoxidase activity was determined as described, by MPO protocol. Myeloperoxidase activity was significantly higher in the colons of TNBS mice compared to Naive mice (a, P=0.0057; unpaired T-test). In second rank, MHS+PA induced a moderate expression of MPO, and was significantly different from MHS+1/2TNBS, 1/2TNBS and NMHS (f, P=0.0092, g, P=0.0014, and e, P=0.0015). MHS+1/2TNBS displayed a small MPO expression and was significantly different from NMHS control mice (d, P=0.0146). In addition full dose TNBS mice showed a marked difference in MPO production in comaprison to NMHS control (P=0.0023); however half dose tnbs mice did not show any MPO expression. In both experimental groups, TNBS and MHS+PA, exhibited a higher expression of MPO relative to other mice groups; TNBS displayed a significantly higher mpo in comparison to MHS+PA(b, P=0.0333). Collectively this demonstrates that TNBS and MHS+PA mice are recruiting neutrophils and there is inflammation in the intestine.All above data all data are presented as means ± SE.

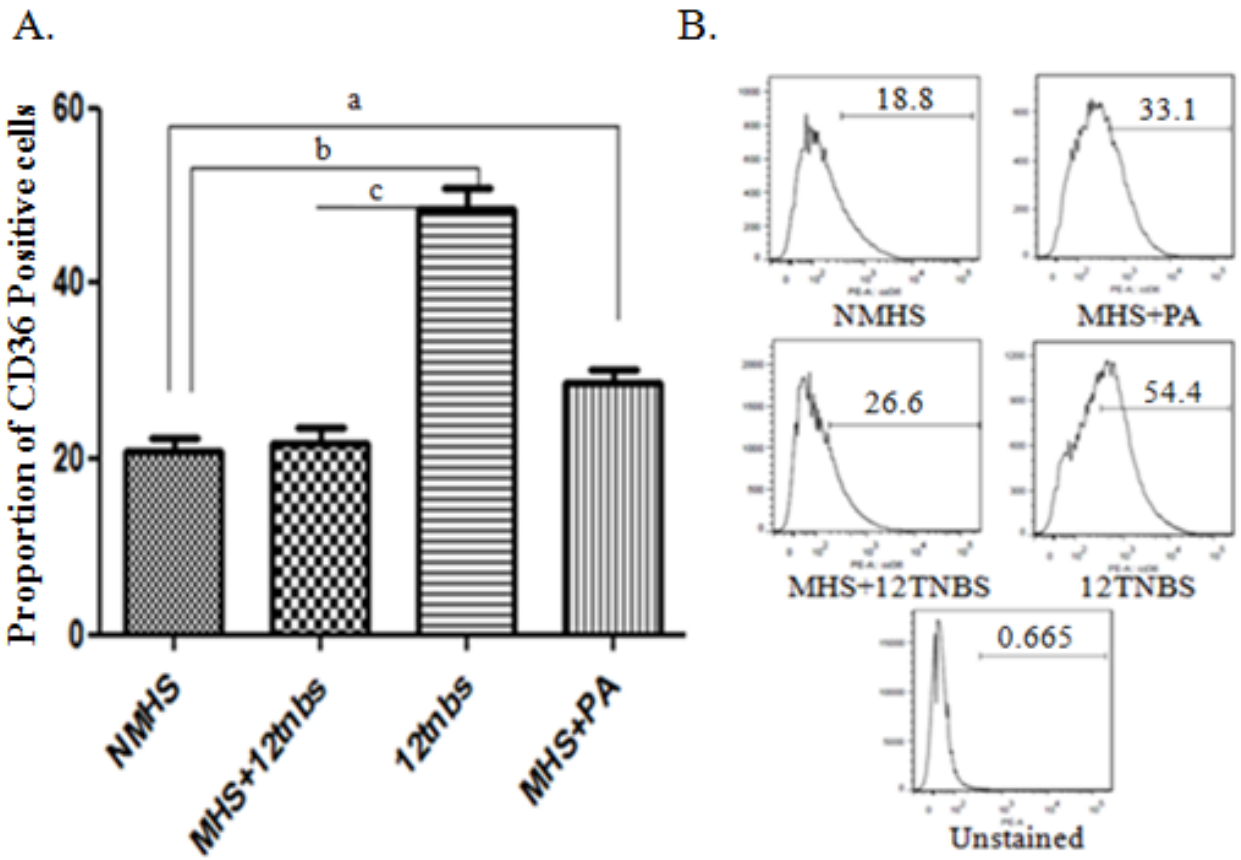

Figure 24. Flow cytometry reveals CD36 expression is highest in 12tnbs colitis and moderate in Palmitic acid mice. Each mice group was assigned a minimum of five mice per group (n=5). Mice were treated with tail vein injections of (a) normal unstimulated MHS, MHS+PA, macrophages stimulated with a 24 hour high dose of Palmitic acid and injected via tail vein, and/or (c) half dose intrarectal tnbs injections ; this applies to MHS+12tnbs and 12tnbs mice groups only. Normal MHS mice group were used as a control group. LPMC was extracted from the colon and small intestine in sterile PBS and cultured in RMPI 1640 for a week; cells were harvested, stained, and cell sorted via BBI FACs CANTO machine. Both 12tnbs and MHS+PA were significantly different from NMHS control group and upregulated higher CD36 expression (b,P=0.0001, unpaired T-test);(a,P=0.0033, unpaired T-test) respectively. There was no significant difference between NMHS and MHS+12tnbs (P=0.6925, unpaired T-test). However MHS+12tnbs and 12tnbs mice groups were significantly different (c, P=0.0001, unpaired T-test). MHS 12tnbs and MHS+PA were also different; MHS+PA expression of CD36 was moderately higher (P=0.0112, unpaired T-test). Lastly 12tnbs CD36 expression was higher relative to MHS+PA; these two groups were also significantly different (P=0.0001, unpaired T-test). (B) Single CD36-PE histograms were utilized as a representation of quantitative CD36 upregulation per each mice group. All represented data is demonstrated as a means ± SE.

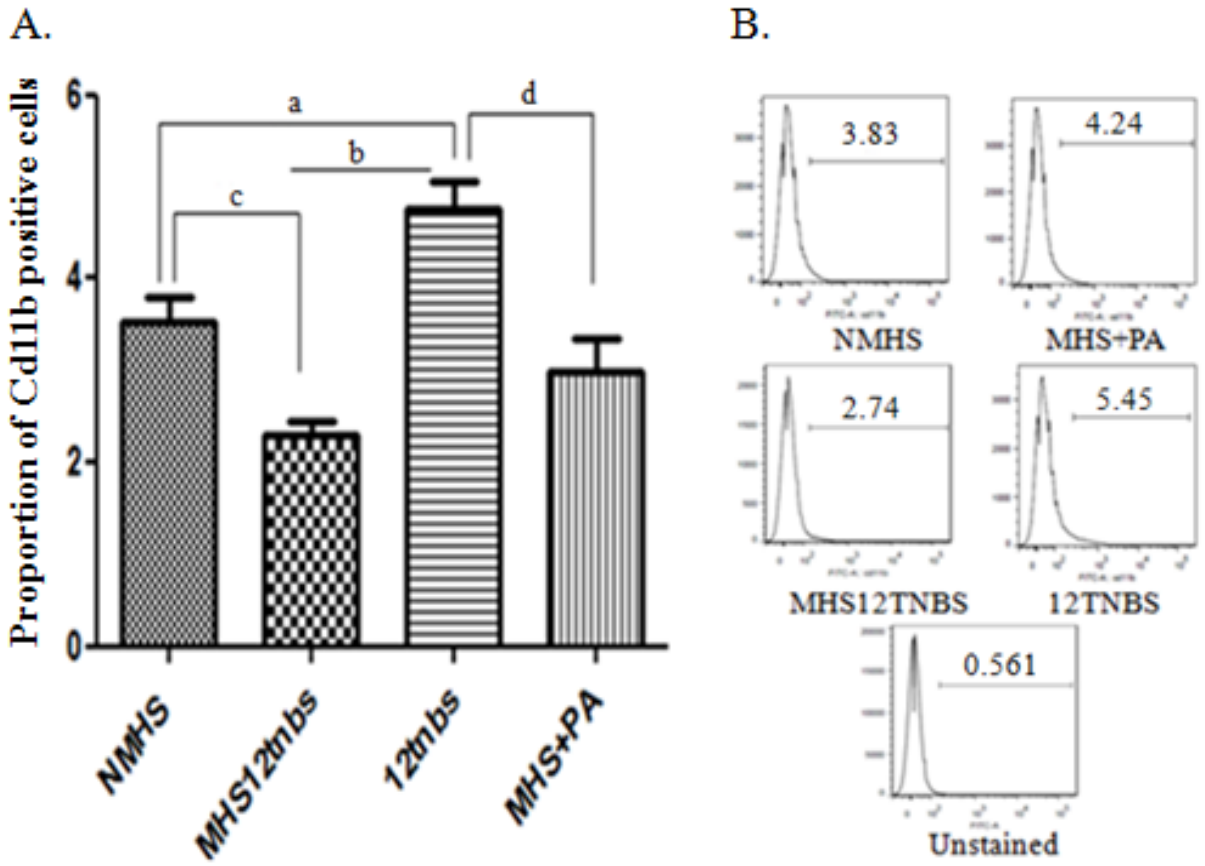

Figure 25. Flow cytometry reveal CD11b macrophages are higher in half dose TNBS mice. (A) Each mice group was assigned a minimum of five mice per group (n=5). Mice were treated with tail vein injections of (a) normal unstimulated MHS, MHS+PA, macrophages stimulated with a 24 hour high dose of Palmitic acid and injected via tail vein, and/or (c) half dose intrarectal tnbs injections ; this applies to MHS+12tnbs and 12tnbs mice groups only. Normal MHS mice group were used as a control group. LPMC was extracted from the colon and small intestine in sterile PBS and cultured for a week; cells were harvested, stained, and sorted via BBI FACs CANTO machine. No significant differences were observed between NMHS and MHS+PA and MHS+PA and MHS 12tnbs (P=0.2435, unpaired T-test);(P=0.1146, unpaired T-test) respectively. NMHS and MHS12tnbs were significantly different, with a slightly lesser proportion of Cd11b+ macrophages in MHS+12tnbs (c, P=0.0006, unpaired T-test). CD11b+ macrophages were significantly higher in 12TNBS relative to control NMHS (a, P=0.0047, unpaired T-test). MHS12tnbs and 12tnbs were significantly different with a moderately higher expression of infiltrating macrophages in half dose tnbs mice (b, P=0.0001, unpaired T-test). In addition MHS+PA and 12tnbs, there were significantly different was also a higher percentage of CD11b+ macrophages in 12tnbs (d, P=0.0015,unpaired T-test). **(B)** Single Cd11b-FITC histograms were utilized to present an example of the quantitative expression of CD11b per each mice. All represented data is demonstrated as a means ± SE.

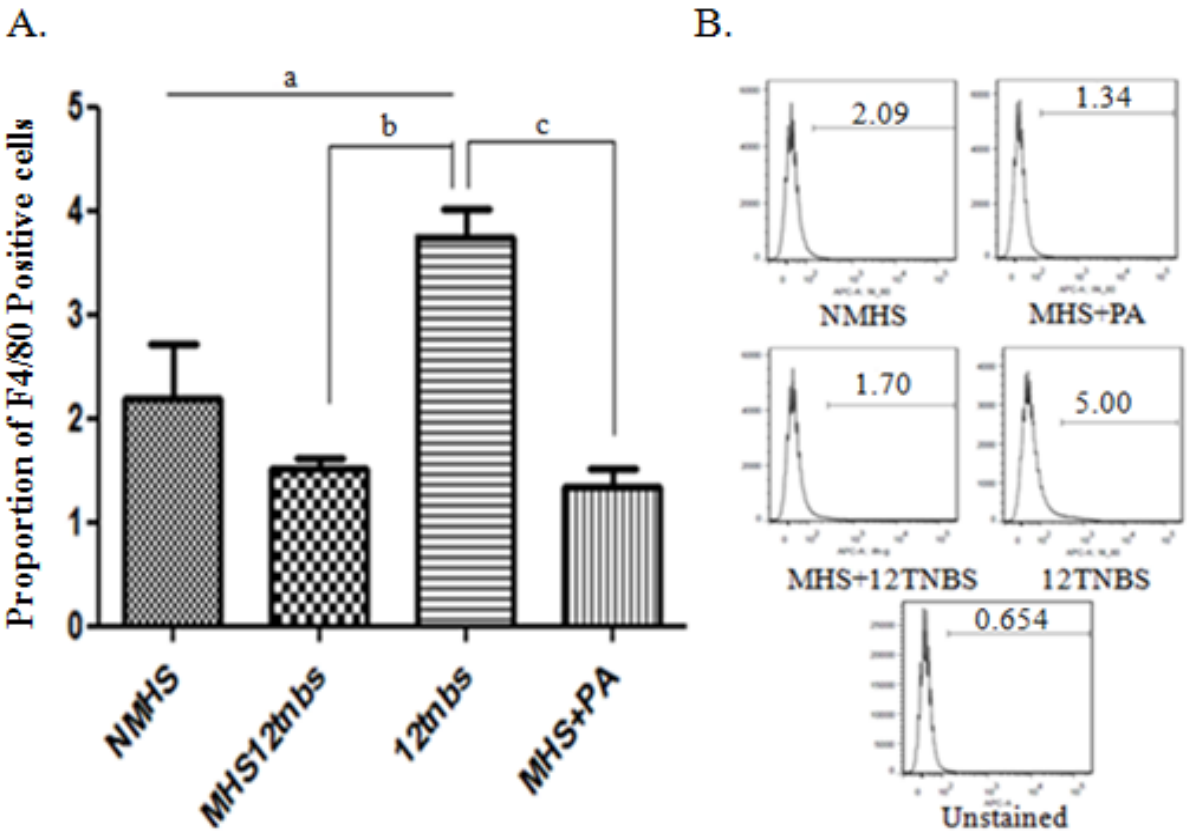

Figure 26. Flow cytometry reveal F4/80+ macrophages are higher in half dose TNBS mice.(A) Each mice group was assigned a minimum of five mice per group (n=5). Mice were treated with tail vein injections of (a) normal unstimulated MHS, MHS+PA, macrophages stimulated with a 24 hour high dose of Palmitic acid and injected via tail vein, and/or (c) half dose intrarectal tnbs injections ; this applies to MHS+12tnbs and 12tnbs mice groups only. Normal MHS mice group were used as a control group. LPMC was extracted from the colon and small intestine in sterile PBS and cultured for a week; cells were harvested, stained, and sorted via BBI FACs CANTO machine. NMHS and MHS12tnbs and NMHS and MHS+PA mice groups were not statistically different in there expression of F4/80+ macrophages (P=0.2147; P=0.1592, unpaired T-test) correspondingly. Half dose TNBS had a moderately higher F4/80+ macrophage infiltrate relative to NMHS control (a, P=0.0155, unpaired T-test). In addition half dose TNBS expressed statically higher F4/80+ macrophages in comparison to MHS12tnbs and MHS+PA, mice groups (b, P=0.0001; c, P=0.0001, unpaired T-test). No statistical difference was found between MHS12tnbs and MHS+PA (P=0.4267). **(B)** Single F4/80 histograms were selected to present an example of quantitative F4/80 upregulation per each mice group. All data is represented as means ± SE.

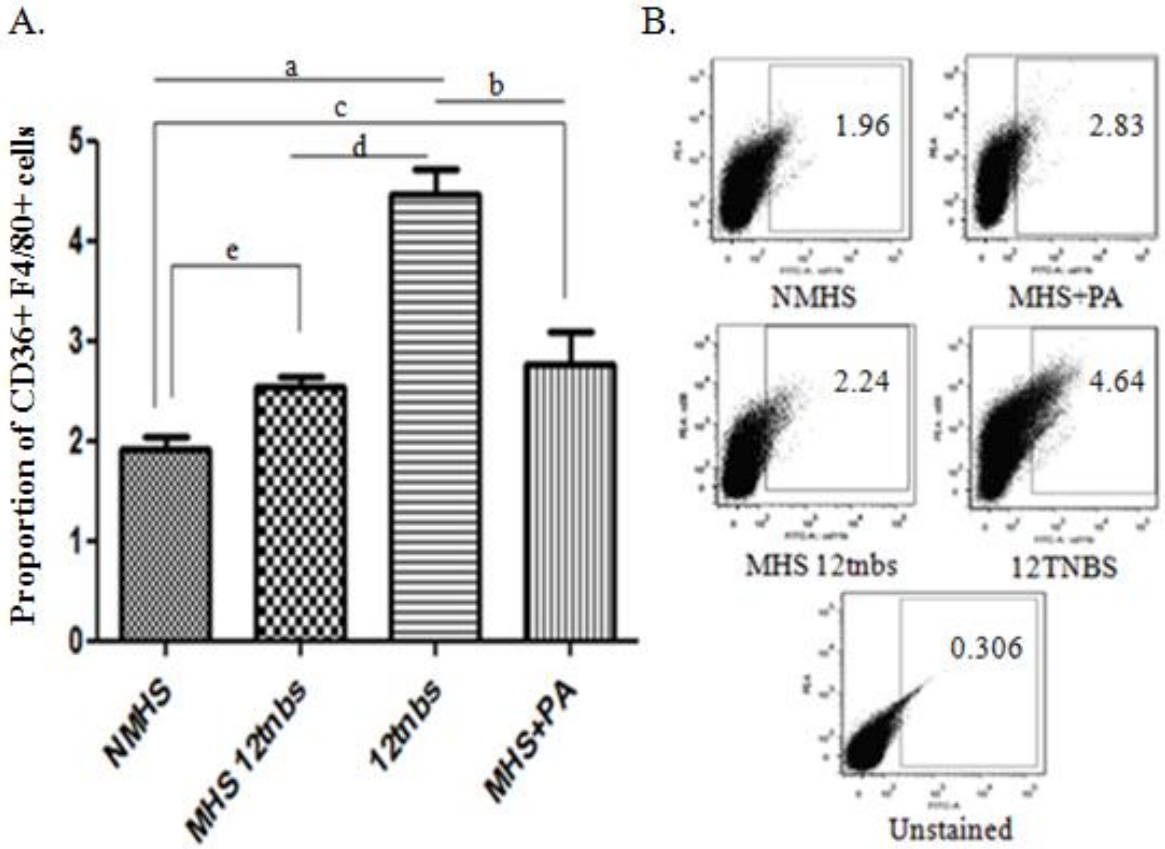

Figure 27. Flow cytometry reveals CD36+ F4/80+ macrophages are highest in half dose tnbs colitis and moderate in MHS+PA. Each mice group was assigned a minimum of five mice per group and a maximum of ten. Mice injections and procedures is as previously aforementioned. After sacrifice, LPMC was extracted from the colon and small intestine in sterile PBS and immediately extracted and cultured following laboratory protocol; cells were cultured for one week in RMPI 1640, after 80% confluency cells were harvested, and stained and cell sorted via BBI FACs CANTO machine. There was a small difference between NMHS and MHS+PA wherein a slightly higher expression of CD36+F4/80+ macrophages was exhibited in MHS+PA (c, P=0.0272 unpaired T-test). Similarly there was also a small difference between NMHS and MHS12tnbs with a slightly higher expression of CD36 expressing macrophages in MHS12tnbs (e, P=0.0018, unpaired T-test). There was a markedly higher expression of CD36+ macrophages in half dose TNBS colitis relative to NMHS control (a, P=0.0001, unpaired T-test). In addition MHS12tnbs and 12tnbs were also significantly different with a larger expression of CD36+ macrophages in 12tnbs (d, P=0.0001, unpaired T-test). There was no significant difference between MHS12tnbs and MHS+PA (P=0.5110). Half dose tnbs displayed moderately higher expression of CD36+ macrophages in comparison to MHS+PA (b, P=0.0011, unpaired T-test). (**B**)Double Positive Dot plots were selected to represent the avergae CD36 F4/80+ macrophages per four mice groups; one representative dot plot is displayed for an example of quantitative differences. All data is demonstrated as means ± SE.

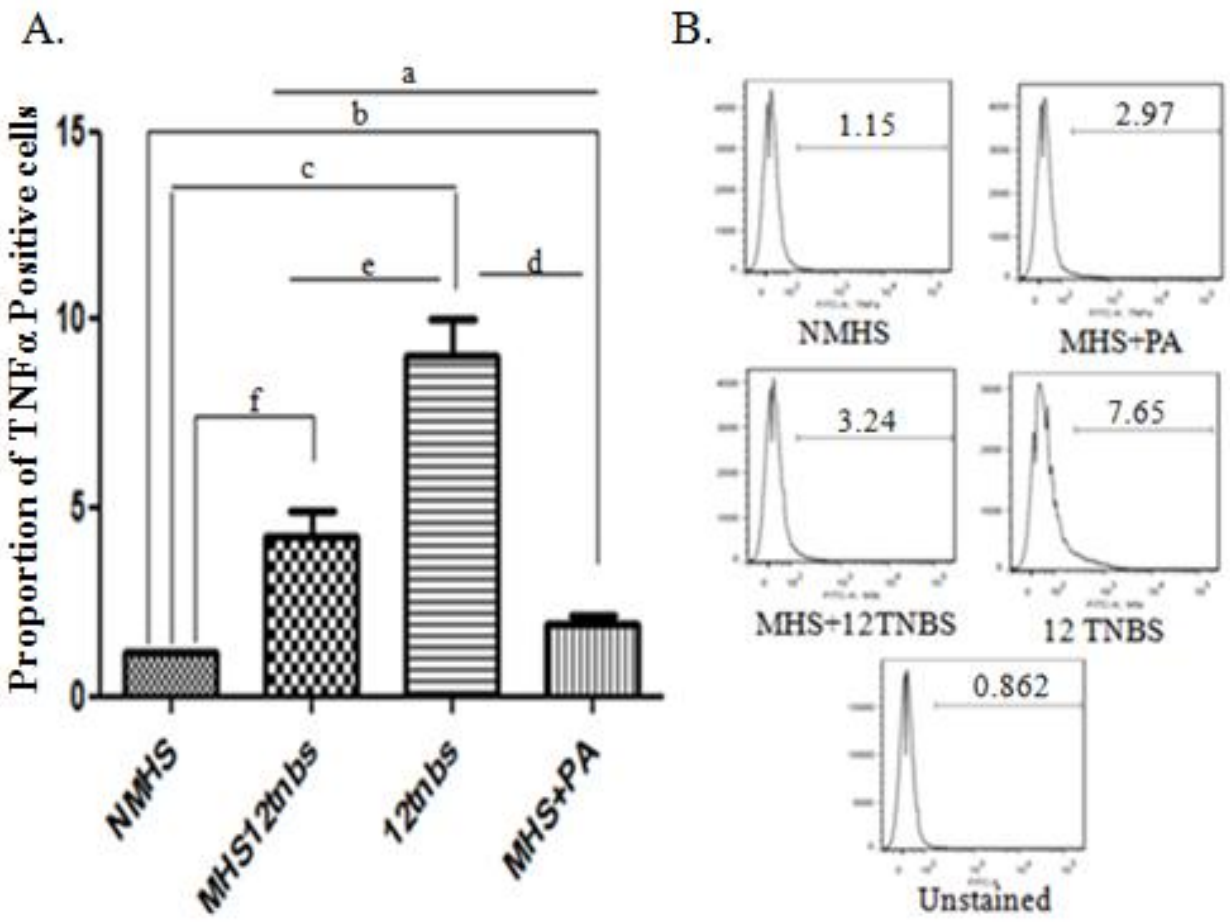

Figure 28. Flow cytometry reveals high expression of TNFα in 12tnbs, moderate expression in MHS+12tnbs and a slight elevation in MHS+PA mice group. Five mice were assigned per each mice group. **(A)** Mice injections and procedures are as previously mentioned. LPMC cells were extracted from the colon, cultured for one week, harvested, and analyzed by BBI FACs CANTO. MHS12tnbs exhibited a significantly higher expression of TNFα relative to NMHS control mice (f, P=0.0005, unpaired T-test). In addition half dose tnbs displayed the highest expression of TNFα in comparison to control (c, P=0.0001, unpaired T-test). A slight difference was observed between MHS+PA and NMHS, wherein MHS+PA induced a small production of TNFα (b, P=0.0021, unpaired T-test).There was also a significant difference between MHS12tnbs and 12tnbs; with a larger TNFα in 12tnbs mice (e, P=0.0013, unpaired T-test). Lastly MHS+PA was also significantly less TNFα in comparison to MHS12tnbs and 12tnbs (a, P=0.0071; d, P=0.0001, unpaired T-tests) respectively.**(B)** Single TNFα histograms were selected to demonstrated the average TNFα expression per each mice group. All data is analyzed using two-tailed unpaired t-tests and data represented as means ± SE.

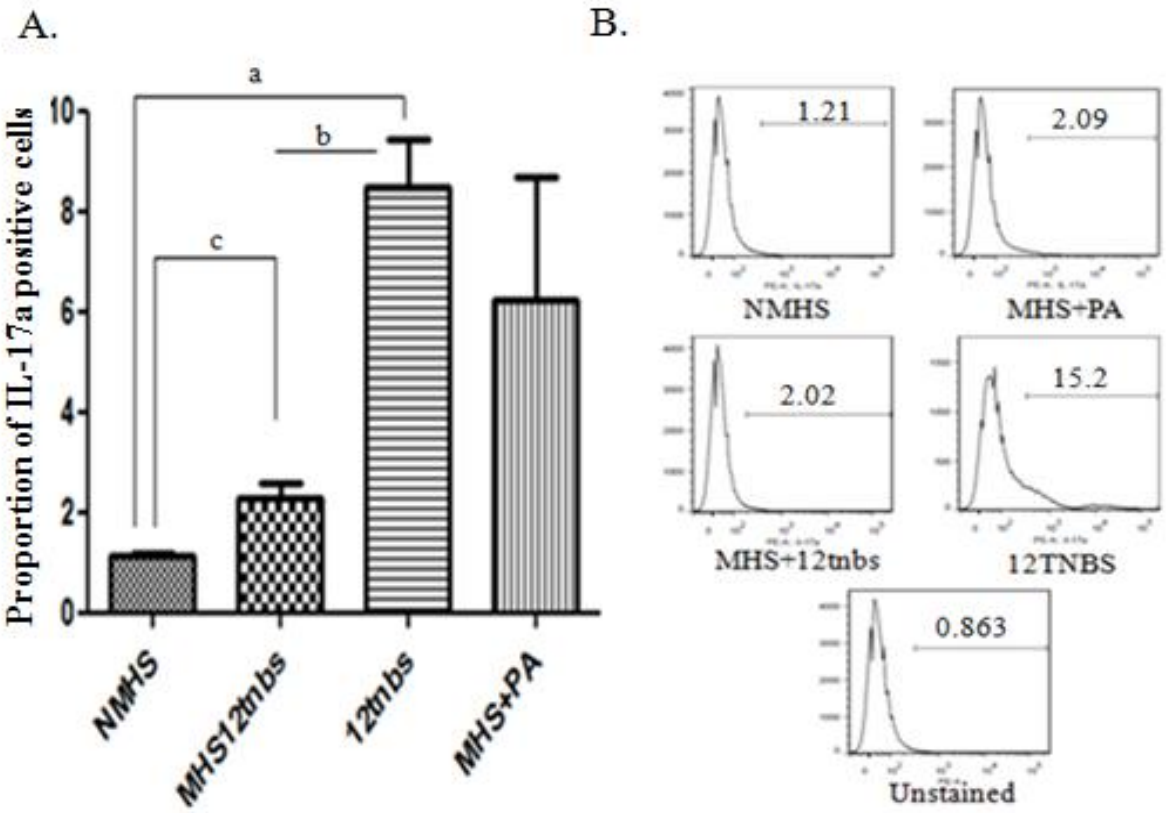

Figure 29. Flow cytometry reveals IL-17a is highly expressed in 12TNBS colitis and elevated in MHS+PA mice groups. Five mice were assigned per each mice group. **(A)** Mice injections and procedures are as previously mentioned. LPMC cells were extracted from the colon, cultured for one week, harvested, and analyzed by BBI FACs CANTO.NMHS and MHS12ntbs, 12TNBS were statistically different exhibiting higher IL-17a expression in both groups, particularly the latter (c, P= 0.0005; a, P=0.0001; unpaired T-test). There was no statistical difference between NMHS and MHS+PA, although MHS+PA does appear to have an increased in IL-17a expression (P=0.0518, unpaired T-test). In addition there was no statistical difference between MHS+PA between either MHS12tnbs and 12tnbs mice groups, although there appears to be a higher IL-17a expression in MHS+PA relative to MHS12tnbs, the data was not significantly different (P=0.1279; P=0.4076, unpaired T-test) respectively. However, MHS12tnbs and 12tnbs were statistically significant with a markedly higher IL-17a expression in half does tnbs colitis group (b, P=0.0001, unpaired T-test). **(B)** Single IL-17a histograms were selected to demonstrate average TNFα production per each mice group. All data is represented as means ± SE.

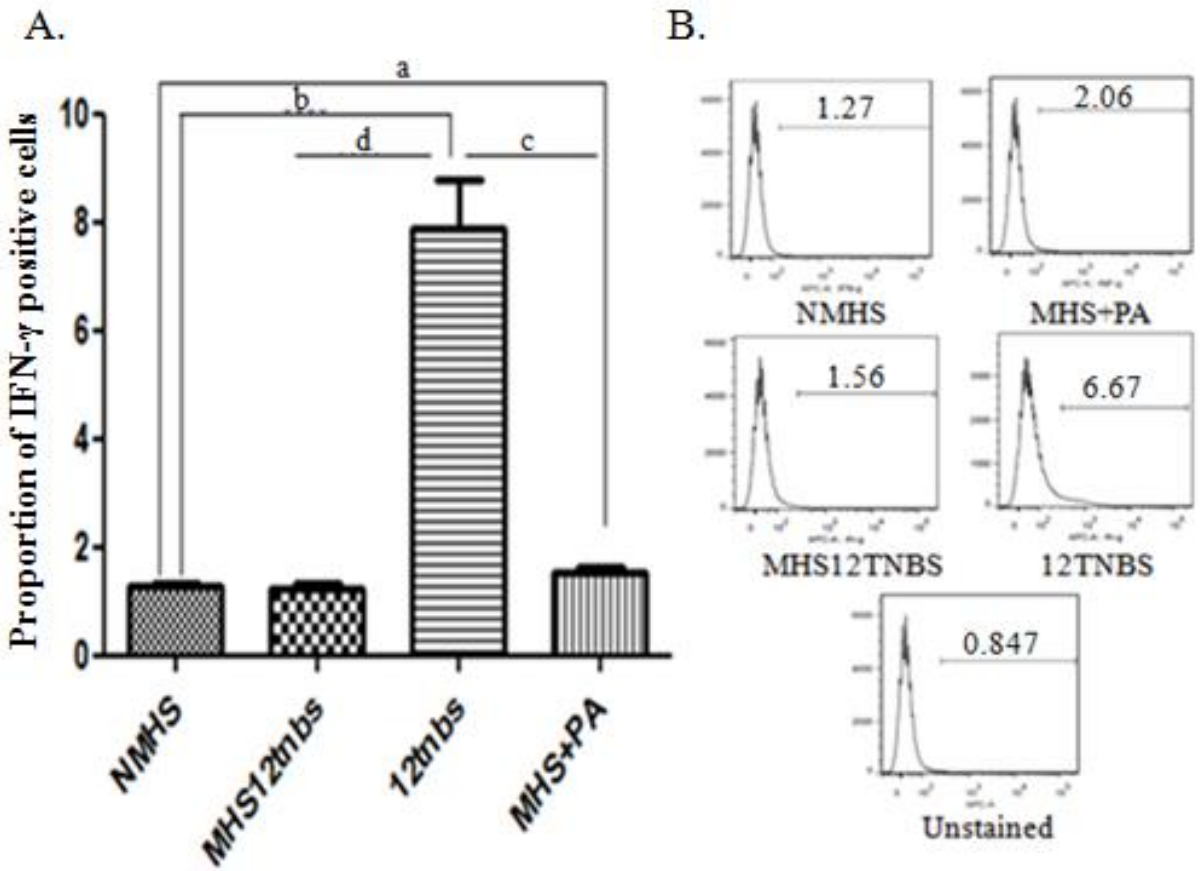

Figure 30. Flow cytometry confirms high expression of TH1 IFN-γ expression in TNBS colitis mice. Mice injections and procedures are as previously mentioned. LPMC cells were extracted from the colon, cultured for one week, harvested, and analyzed by BBI FACs CANTO.NMHS and MHS12tnbs groups were not statically different in IFN-γ expression, expression of this cytokine remained at basal levels (P=0.618, unpaired T-test). However IFN-γ expression between NMHS and 12TNBS were markedly different; 12tnbs highly elevated IFN- γ production (b, P=0.0001, unpaired T-test). There was a small difference between NMHS and MHS+PA wherein a slightly elevated IFN-γ was observed in the latter group (a, P=0.0444, unpaired T-test); however this result suggested MHS+PA is not producing IFN-γ as an inflammatory cytokine. There was a large difference between MHS12tnbs and 12tnbs expression of IFN-γ with a marked TH1 cytokine production in 12tnbs (d, P=0.0001, unpaired T-tests). In addition 12tnbs and MHS+PA were also significantly different with a marked increase of IFN-γ in 12tnbs colitis mice relative to Palmitic acid treated mice (c, P=0.0001, unpaired T-tests). In addition, a small difference between MHS+PA and MHS12tnbs in IFN-γ production was observed, with a very minor increased of IFN-y in MHS+PA (P=0.0479); however results indicate IFN-γ is not a proinflammatory cytokine in MHS+PA or MHS+12TNBS mice. (**B**) Single IFN- **γ** histograms were utilized to display an average representaton of IFN-γ production a per each group. All data is analyzed and represented as means ± SE.

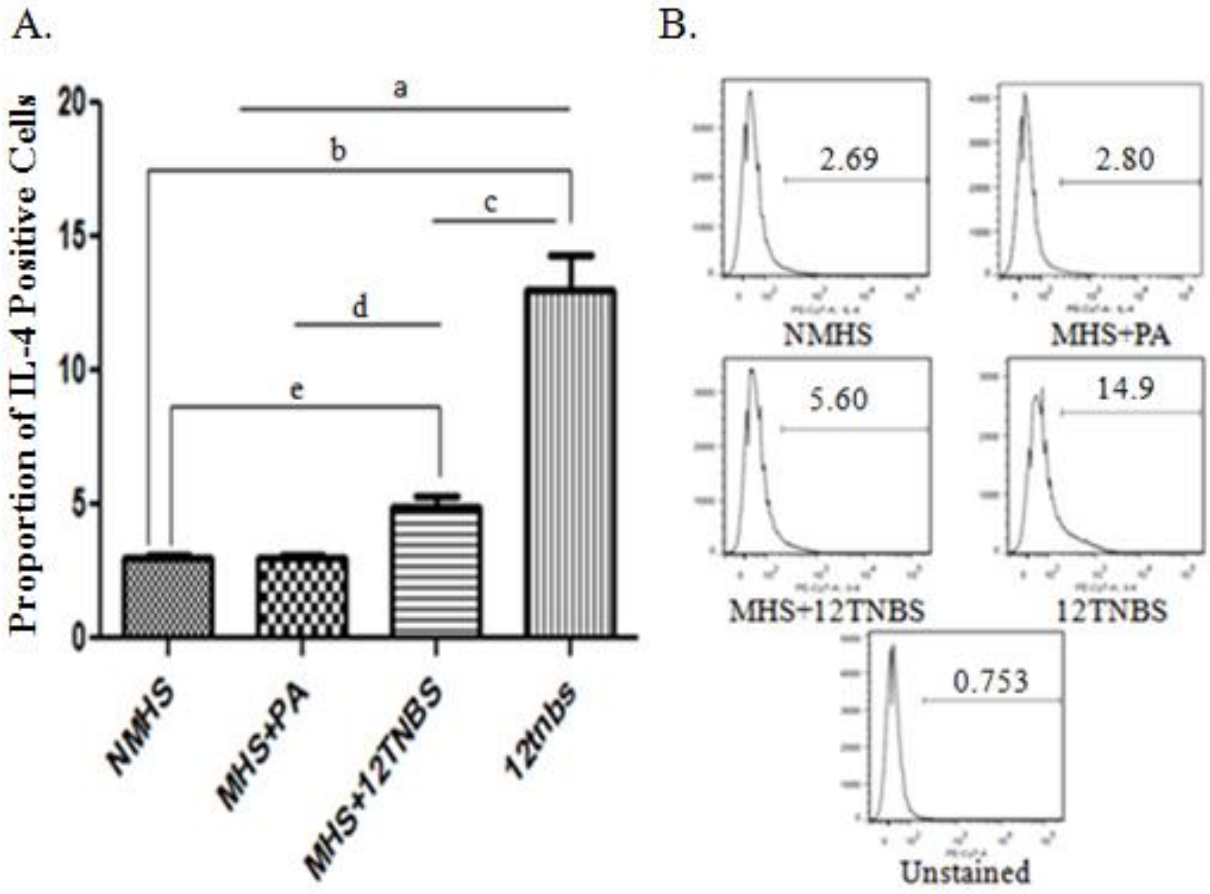

Figure 31. Flow cytometry reveals IL-4 expression is highly expressed in 12TNBS colitis mice. Balb/c mice injections and procedures are as previously mentioned. LPMC cells were extracted from the colon, cultured for one week, harvested, and analyzed by BBI FACs CANTO.There was no statistical difference between NMHS control group and MHS+PA in IL-4 expression (P=0.8990, unpaired T-test). NMHS and MHS12tnbs were moderately different, with a higher production of IL-4 in MHS12tnbs group(e, P=0.0003, unpaired T-test). 12TNBS laregely upregulated the TH2 cytokine, IL-4, in comparison to control NMHS mice (b, P=0.0001, unpaired T-test). 12TNBS and MHS12tnbs were also significantly different with a moderately higher IL-4 in the half dose TNBS colitis mice (c, P=0.0001, unpaired T-test). MHS+PA exhibited significantly lower IL-4 expression in comparison to both MHS12tnbs and 12tnbs groups (d, P=0.0003; a, P=0.000, unpaired T-tests). **(B)** Single IL-4 histograms were selected as a representation of quantitative IL-4 production per each mice group. All data is represented as means ± SE.

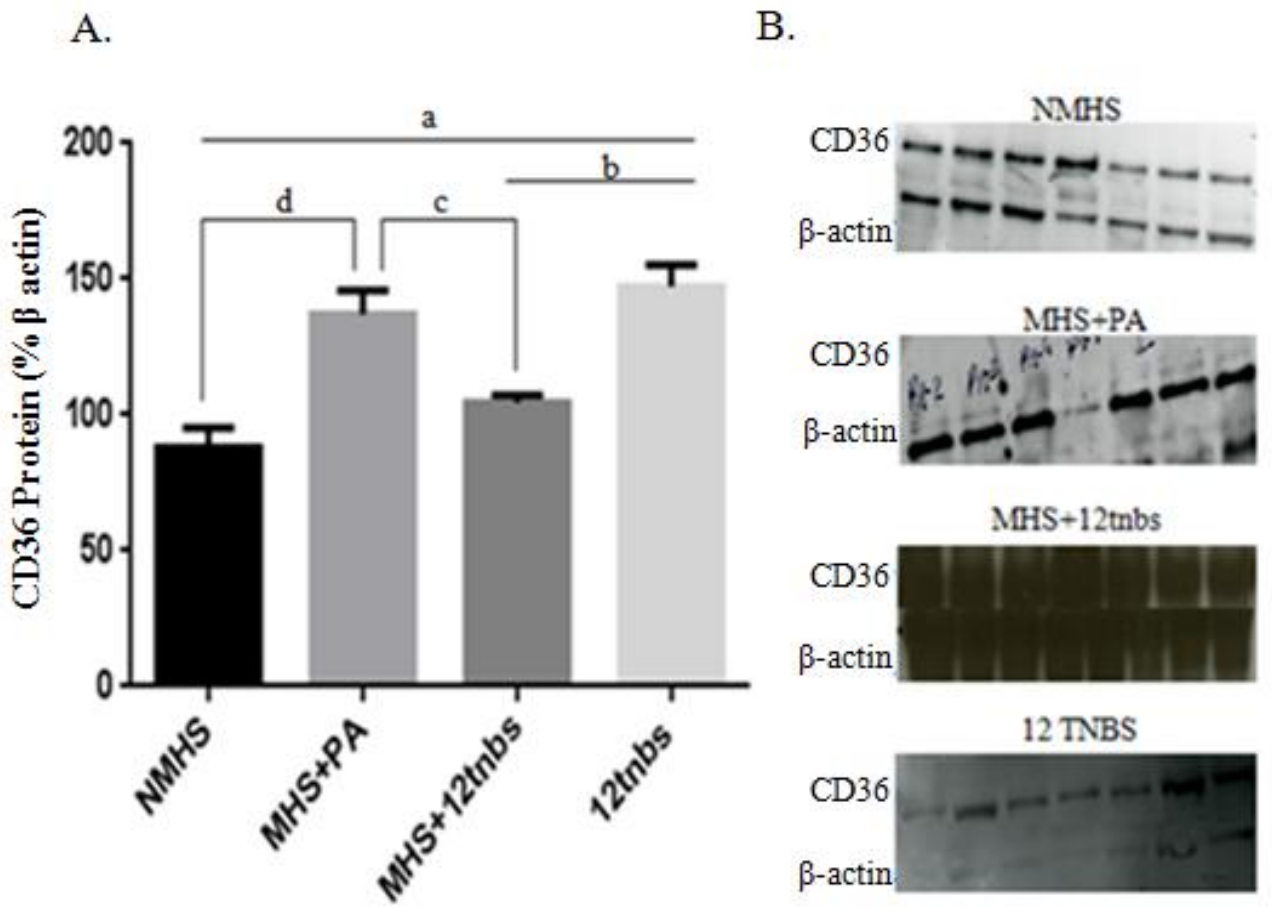

Figure 32. CD36 Protein Expression is higher in half dose colitis and in MHS+PA. (A) Upon sacrifice of mice, protein was extracted from 8 samples of 2-3cm colonic tissues preserved in sterile PBS from NMHS, MHS+PA, MHS+12tnbs and 12tnbs mice groups(n=5) following protein extraction protocol. The blot was analyzed via Image J, the expression of CD36 was determined by the difference between the integrated density of CD36 and β-actin, and expressed as a percentage of beta actin.MHS+PA upregulated a markedly higher expression of CD36 in comparison to NMHS control mice (d, P=0.0009, unpaired T-test). NMHS and MHS+12tnbs were not significantly different in the expression of the CD36 receptor (P=0.0551, unpaired T-test). 12TNBS exhibited a higher expression of the CD36 receptor in comparison to control(a, P=0.0001, unpaired T-test), andCD36 expression was similar to that of MHS+PA (P=0.3955, unpaired T-test). MHS+12tnbs and 12tnbs were also significantly different the expression of CD36; more CD36 expression was exhibited by 12TNBS mice (b, P=0.0001, unpaired T-test). A small difference was observed between MHS+12tnbs and MHS+PA, wherein a higher expression of CD36 was observed in MHS+PA mice (c, P=0.027, unpaired T-test). **(B)** Single immunoblot representation of bands in CD36 88kDa and Beta actin 42kDa location demonstrated per each mice group**.** The CD36 receptor appears to be markedly uprgulated in the inflammated intestine and high fatty acid stimulated macrophages. Data is showna as means ± SE.

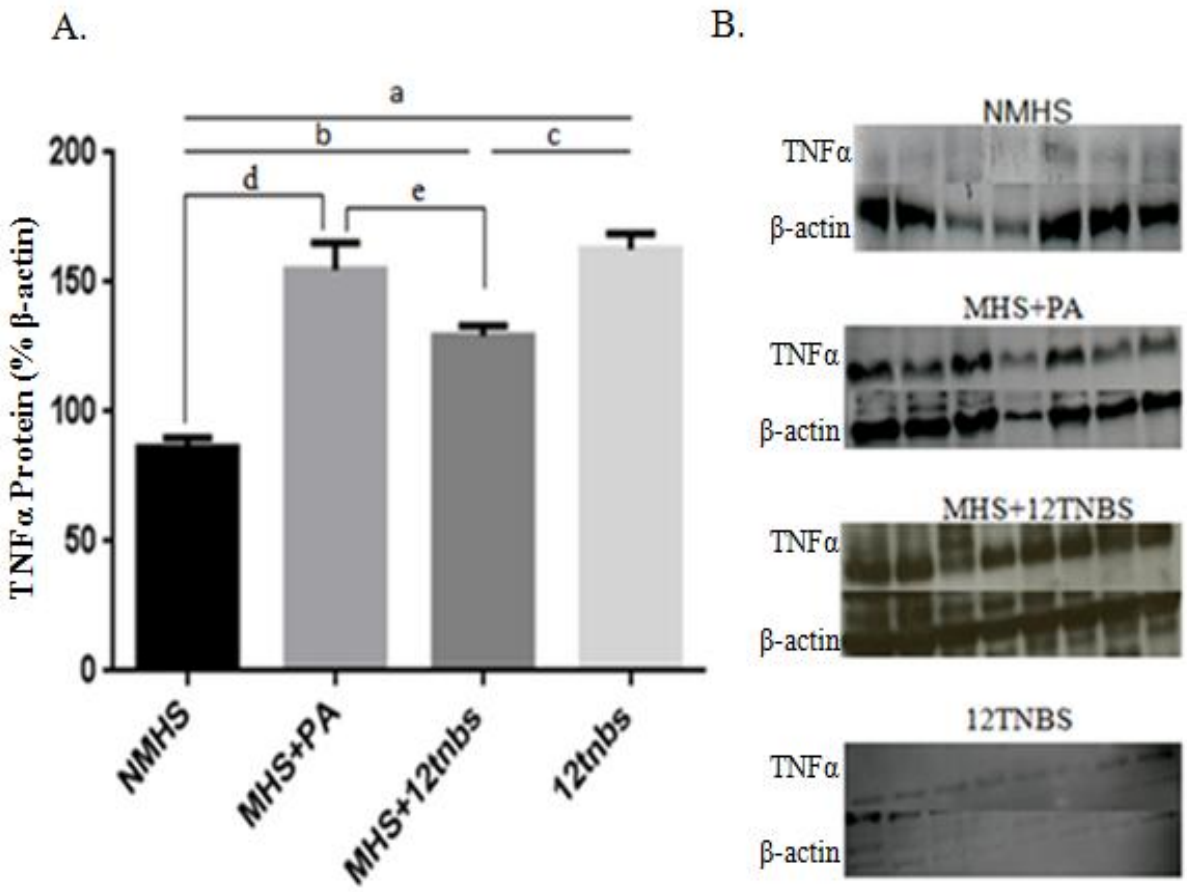

Figure 33. TNFα protein expression is elevated in MHS+PA & 12TNBS mice. Upon sacrifice of mice, protein was extracted from 8 samples of 2-3cm colonic tissues preserved in sterile PBS from NMHS, MHS+PA, MHS+12tnbs and 12tnbs mice groups(n=5) and subject to western blotting. **(A)** The blots were analyzed and examined via Image J, the expression of TNFα was determined via the differences in percent integrated density from loading control B-actin, TNFα is demonstrated as a percentage of Beta actin.MHS+PA exhibited significantly higher TNFa expression relative to NMHS control (d, P=0.0001, unpaired T-test). Similarly 12TNBS also revealed a higher TNFα production relative to NMHS (a, P=0.0001, unpaired T-test). No significant differences were observed between MHS+PA and 12tnbs (P=0.2862, unpaired T-test).MHS+12tnbs also displayed a moderate expression of TNFα in comparison to control NMHS mice (b, P=0.0001, unpaired T-test). MHS+12tnbs exhibited significantly lower TNFα expression in comparison to MHS+PA(e, P=0.0004, unpaired T-test) and 12tnbs(c, P=0.0004, unpaired T-test) mice groups.**(B)** Single immunoblot representation of bands in TNFα 19kDa and Beta actin 42kDa location demonstrated per each mice group**.** TNFα production appears to be markedly uprgulated in the inflammated intestine and high fatty acid conditions. Data is shown as means ± SE.

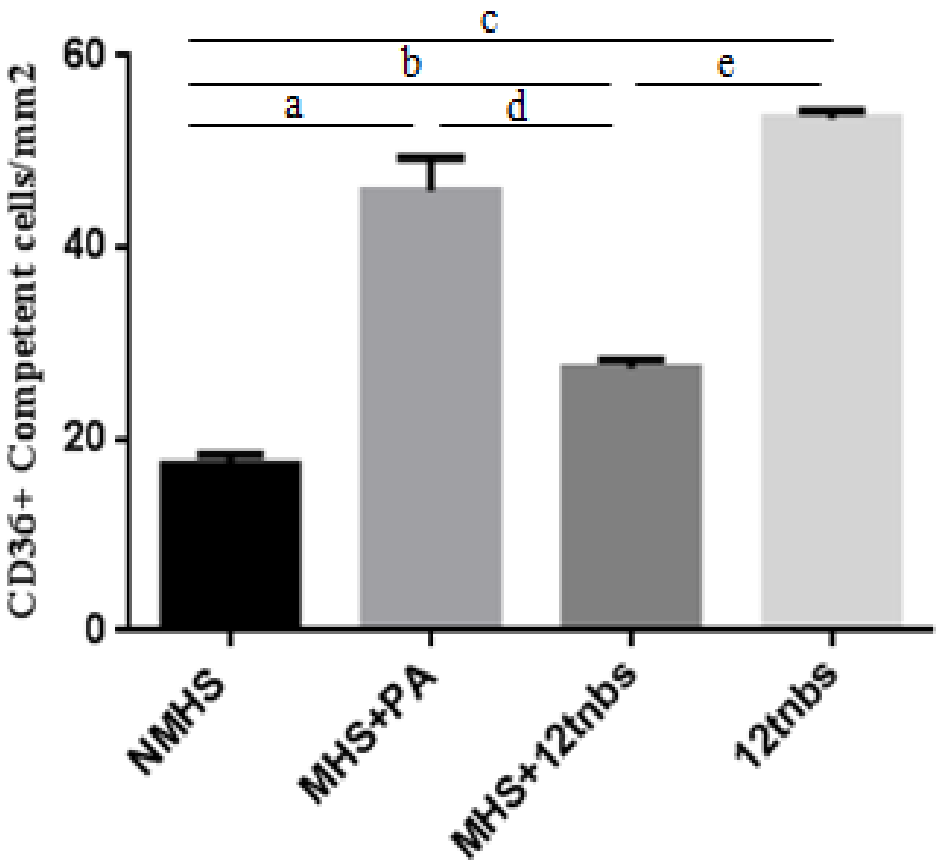

Figure 34. Frequency of CD36+ competent cells in the colonic tissue is higher in 12TNBS and MHS+PA mice (n=5). Colon tissue was immediately dissected after sacrifice and embedded in paraffin for immunohistochemistry, the following antibodies were utilized(a) Santa cruz CD36 polyclonal conjugated Fitc, (b) Ebioscience F4/80 monoclonal conjugated PE, and (c) double stained. Twenty pictures by the fluorescent Zeiss BBI microscope were taken per mice, per group of five mice, and totally 100 pictures were examined per group. Each twenty pictures were averaged per antibody and plotted via Graph pad prism; all mice groups were examined for all three antibody parameters. MHS+PA mice group exhibited a significantly higher proportion of CD36 positive cells in comparison to NMHS control mice (a, P=0.0001; unpaired T-test). MHS+1/2TNBS demonstrated a moderately higher expression of CD36-competent cells relative to control mice (b, P=0.0001,unpaired T-test). The highest expression of CD36 positive cells was exhibited via 1/2TNBS colitis mice, and competent cells remained highest among all groups, and significantly higher in contrast to control mice (c, P=0.0001, unpaired T-test). No significant difference was observed between MHS+PA and 1/2TNBS, as the amount of CD36+ competent cells appeared similar in frequency (P=0.0677, unpaired T-test). However, MHS+PA exhibited significantly higher CD36 positive cells in comparison to MHS+1/2TNBS which presented a lower frequency of CD36 positive cells (d, P=0.0009, unpaired T-test). In addition, MHS+1/2TNBS was also demonstrated significantly lower CD36 competent cells in comparison to 1/2TNBS colitis mice (e, P=0.0001, unpaired T-test). Data is represented as means ± SE.

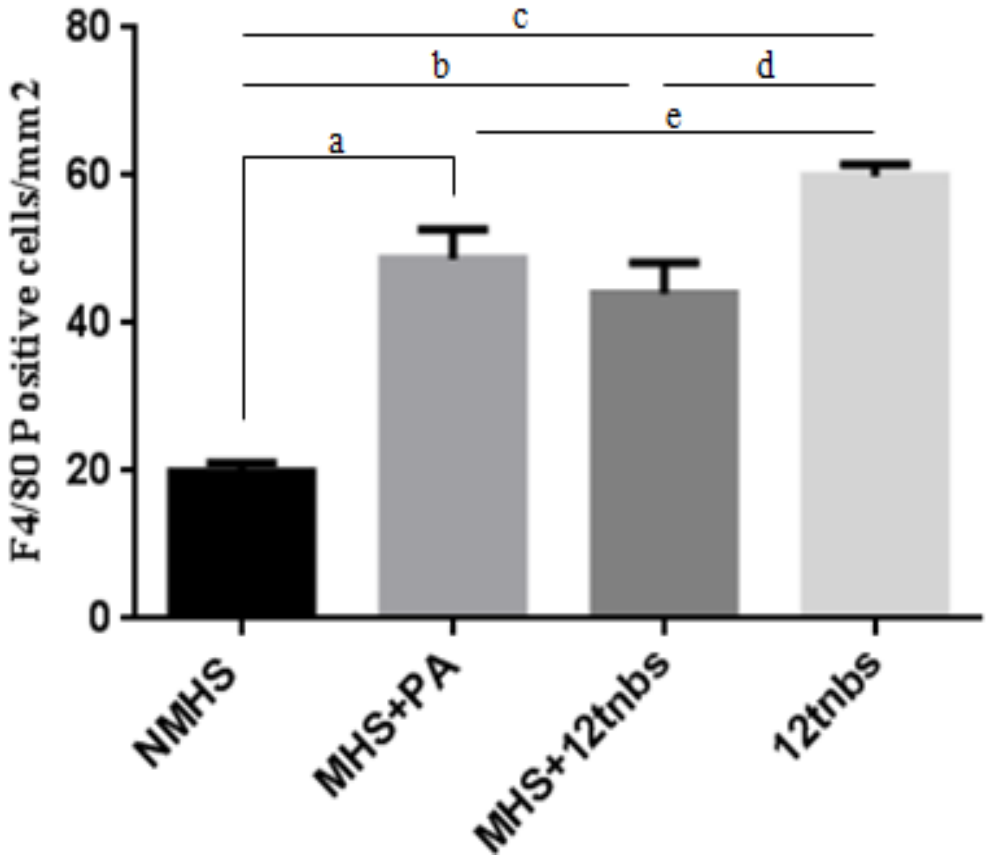

Figure 35. Frequency of F4/80 competent cells in the colonic tissue of MHS+PA, MHS+1/2TNBS, and 1/2TNBS mice. Colon tissue (n=5 for each group) was immediately dissected after sacrifice and embedded in paraffin for immunohistochemistry for (a) Santacruz CD36 polyclonal conjugated Fitc, (b) Ebioscience F4/80 monoclonal conjugated PE, and (c) double stained with both antibodies. Twenty pictures on the fluorescent Zeiss BBI microscope were taken per mice, per group of five mice, totally 100 pictures were examined per group. Each twenty pictures were averaged per antibody and plotted via Graph pad prism; each group was examined for all three antibody parameters. MHS+PA demonstrated a high frequency of F4/80+ macrophages relative to NMHS control (a, P=0.0001; unpaired T-test). Similar to CD36+ positive cells, MHS+1/2TNBS exhibited a moderate amount of F4/80+ positive cells in relative to control mice (b, P=0.0006, unpaired T-test). 1/2TNBS mice displayed the highest proportion of F4/80+ macrophages compared to control (c, P=0.0001, unpaired T-test). 1/2TNBS exhibited higher F4/80 positive cells in the intestine in comparison to MHS+1/2TNBS which expressed less of F4/80+ cells (d, P=0.0079, unpaired T-test). No significant differences were observed between MHS+1/2TNBS and MHS+PA in the expression of F4/80+ positive cells (P=0.4343, unpaired T-test). However, a significant difference was observed between MHS+PA and 1/2TNBS, wherein 1/2TNBS exhibited a slightly higher proportion of F4/80+ competent cells in the intestine (e,P= 0.0327, unpaired T-test). Data are presented as means ± SE.

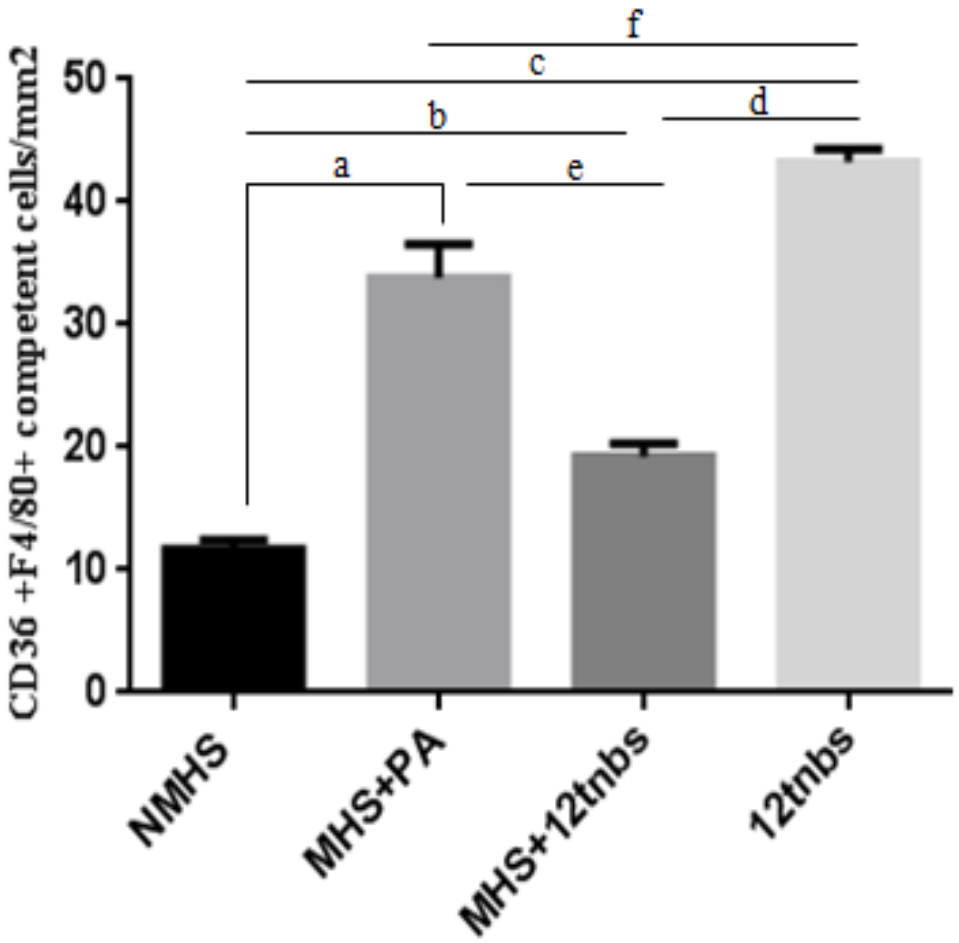

Figure 36. ImmunoHistochemistry reveals high proportion of CD36+ F4/80+ expressing macrophages in the colonic tissue of MHS+PA and 12tnbs mice. Colon tissue (n=5 for each group) was immediately dissected after sacrifice and embedded in paraffin for immunohistochemistry for (a) Santacruz CD36 polyclonal conjugated Fitc, (b) Ebioscience F4/80 monoclonal conjugated PE, and (c) double stained with both antibodies. Twenty pictures on the fluorescent Zeiss BBI microscope were taken per mice, per group of five mice, totally 100 pictures were examined per group. Each twenty pictures were averaged per antibody and plotted via Graph pad prism, and each group was examined for all three antibody parameters. MHS+PA and 1/2TNBS exhibited markedly higher CD36+F4/80+ competent cells relative to NMHS control mice (a, P=0.0001; c, P=0.0001 respectively; unpaired T-test). MHS+1/2TNBS demonstrated higher CD36+F4/80+ positive cells relative to control mice (b, P=0.0004, unpaired T-test), but significantly lower double positive competent cells in comparison to 1/2TNBS (d, P=0.0001, unpaired T-test) and MHS+PA (e, P= 0.0011, unpaired T-test). Lastly a significant difference was detected between MHS+PA and 1/2TNBS wherein a slightly higher proportion of double positive cells were exhibited by 1/2TNBS colitis mice (f, P=0.0126, unpaired T-test). Data are represented as means ± SE.

DISCUSSION

It is now a widely accepted scientific fact that a large proportion of inflammatory macrophages are recruited to the inflamed intestine in Crohn's Disease and Ulcerative colitis patients and colitis mice[1,17,27, 41, 43,49] . It is speculated that a variety of different receptors play a role generating and facilitating inflammatory macrophages in pathogenesis of IBD[1,11,17,18,27,28,41,49]. However, the mechanisms underlying the pathogenesis of IBD remain poorly characterized; in particular the role(s) of macrophages and their receptors[17,27,28,41,44,47,49]. It is likely that macrophages and a multitude of receptors-ligand interactions play a pivotal role in inducing inflammation in the intestine.[1-5,7,9,11,17,41,49].

Inflammatory Bowel Disease is, in part, driven by the dysregulation of innate mucosal immune response[1-12, 16,17,19-21,23,26,38]; this dysregulated response is likely mediated via pathogen recognition receptors (PRRs)[1-11]. These receptors are expressed on the surface of macrophages and are intimately tied to mounting innate immunity response(s) and maintaining intestinal homeostasis[1-11,12,16,17-19,20,21,23,26,38]. One Pathogen recognition receptor which is likely involved in the intestine is scavenger receptor Cluster of differentiation 36(CD36) [1-5,7,9,11].

A previous study observed that CD36+ macrophages isolated from the Lamina Propria mononuclear cells played a role in the inflamed intestine[100]. Oz and colleagues demonstrated that Lamina Propria macrophages from CD36 Knockout mice induced a protective role in conjunction with scavenger receptor, SRA, in the intestine of DSS treated colitis mice[100]. This study demonstrated that double deficient scavenger receptor

mice SRA-/-CD36-/- exhibited severe colitis relative to Wildtype DSS treated; this was demonstrated by increased anemia, decreased colon length, lesions, weight loss, expression of Serum Amyloid A, and a abnormal pro-inflammatory cytokine profile[100]. However one short coming in their study was that they did not directly investigate CD36 expressing macrophages for a potential proinflammatory role in the intestine [100]. Evidence to support an inflammatory role in the intestine was suggested by the observation that single deficient CD36-/- mice exhibited significantly decreased TNFα production in intestinal macrophages[100].

In my study, I directly identified, and confirmed a high upregulation of scavenger receptor CD36+ F4/80+ macrophages in the lamina propria of colonic biopsies from IBD patients. I also discovered significantly higher frequency of CD36+ F4/80+ macrophages in the inflammatory intestine of active CD, UC patients relative to non-IBD control patients. Of interest the proportion of CD36+F4/80+ macrophages was higher in UC patients.

One difficulty in assessing IBD patients is that the stage and phase of IBD may influence results, ie: whether it is newly diagnosed, relapsing, type and location of disease, geographical origins, environmental factors, and level of severity. Some short comings in drawing definitive conclusions from IHC data can be attributed to the variety of these confounding variables and the limitation of experimental tests from human samples[1-5,7-10,16, 19-21 ,23,24,26,27,38]. In my study, little information was known about patient biopsies originating from China; except that samples were taken from patients with active disease. In future, to fully confirm the relationship of the high frequency of CD36+

F4/80+ macrophages in UC relative to CD, a larger sample size is necessary. In addition, it is essential to investigate the expression of CD36+F4/80+ macrophages in inactive CD and UC patients to address and confirm whether these macrophages are largely elevated in the active stages of IBD. This would provide more solid evidence that these macrophages are indeed upregulated in active inflammatory stages of disease. It would also be interesting to compare active IBD patients colon samples from different geographic regions to examine whether CD36 expressing macrophages play a role in the inflammatory pathogenesis of IBD at a global level.

After confirming the frequency of CD36+ macrophages were largely increased in IBD patients, my study progressed to the next logical step: to examine this relationship in a murine colitis model. Results demonstrated the proportion of CD36+ F4/80+ macrophages were elevated in TNBS colitis mice in comparison to Naive; however, a larger sample size is required to determine if this relationshipis statistically significant. Unlike human control patients, naive mice presented a higher proportion of CD36+ F4/80+ positive cells than expected. It is likely the higher proportion of double positive cells in naive mice can be attributed to the 50% ethanol that was injected intrarectally, as this may have provoked a small level of inflammatory response in the intestine. In order to reduce this affect it would be beneficial for future studies to utilize a larger sample size, and additionally employ a naive saline mice group as a secondary control. Another limiting factor of my study was the varying response of Balb/c mice to TNBS colitis induction; some mice exhibit the natural ability to compensate for inflammation whereas others are more susceptible to develop severe colitis. This causes a large variability in

mice data. These mice can be identified by decreased amount of inflammation after TNBS Colitis treatment, more normal DAI, and weight gain after treatment, thus necessitating a much larger sample size is required for further studies or perhaps investigating a secondary colitis model such as DSS colitis.

Another intriguing point to consider in future work is illustrated by via Nassir and colleagues study whereby the researchers examined CD36 expression from proximal to distal healthy intestine sections, they found a steep decreasing gradient of CD36 expression towards the distal end of the intestine; thus suggesting higher CD36 expression is in the proximal, small intestine[103].

In retrospect, IHC of TNBS Colitis mice could have been much more powerful had I taken sections from both the proximal and distal intestine, rather than just focusing on the distal colon. It may very well be the same phenomenon that is witnessed in the colon and it is possible this relationship may be magnified in the small intestine consistent with the results from the aforementioned study[103]. It would also be interesting to obtain small intestine samples from active CD and UC patients, to examine whether the frequency of CD36+ F4/80+ macrophages was increased. In addition, despite the fact that Nassir et al study focused on CD36 expressing enterocytes, they also demonstrated that fatty acid absorption via CD36 was highest in the proximal which is important to consider in future work[103]. This may also account for the discrepancy between CD36+ F4/80+ competent cell counts from the IHC colon samples in comparison to flow cytometry results which used LPMC extracted from the small and large intestine; FACs displayed a repetitively higher proportion of CD36+ F4/80+ macrophages and monocytes.

In addition Immunoblotting and Real-time PCR, verified CD36 expression was significantly higher in TNBS colitis mice relative to Naive. However, one needs to be cautious in drawing conclusions from this data, as the expression of CD36 may be masked via a multitude of other cells in the colon, such as enterocytes, platelets, adipose tissue, and apoptotic cells[73,75,76,78,79,88,90-94,96,97,103].

Above all , results from flow cytometry revealed some persuasive evidence from Naive and TNBS mice. LPMC derived from the colon and small intestine were gated for macrophages and monocytes via: Cd11b, F4/80 and LY6G; and these macrophages exhibited significantly higher upregulation of the CD36 receptor in TNBS Colitis mice thus demonstrating the association was evidently clear.

Based on the knowledge of two ligands and their involvement in stimulating inflammatory responses, TLR ligand PGN and LCFA Palmitic acid, we stimulated MHS macrophage cell line to investigate whether these ligands could induce the expression of pro-inflammatory cytokines: TNFα, IL-17a and IFN-γ[68,69,80,90-94,98,102,115,116,117] .We were specifically interested in testing TLR2 based on previous findings by Canto and colleagues[68]. Their study showed that a large proportion of inflammatory monocytes/ macrophages in patients with active IBD exhibited high levels of TLR2 on the cell surface and induced a significantly high production of TNFα, thus sparking our interest to investigate this relationship in MHS macrophages both *in vitro* and *in vivo*[68]**.**

Several lines of evidence supported our interest in examining high LCFA Palmitic acid, firstly a study via Seimon et al identified that Palmitic acid directly binds CD36 receptor in ER stressed peritoneal macrophages and this process was TLR2-dependent.

Together this receptor-ligand interaction induced macrophage apoptosis, thus suggesting Palmitic acid and TLR2 are involved in a pathogenic process dependent on CD36+ macrophages [102].

Secondly, based on the knowledge that oxLDL, a lipoprotein, which is now considered a atherogenic ligand to CD36, is intimately involved fatty acid accumulation, and the release of pro-inflammatory cytokines; we queried whether a similar mechanism involving Long chain fatty acids played a role in the intestine[90-94,98,115,117,118].

A third study conducted via Nassir and colleagues demonstrated that CD36+ enterocytes in the epithelial lumen were largely involved in fatty acid absorption of Oleate Acid, a LCFA and that CD36-/- mice exhibited a 50% reduction of fat absorption in the proximal intestine[103]. We queried, due to the proximity of macrophages in the Lamina Propria to the epithelial lumen, whether macrophages were also highly important in fatty acid uptake, and whether these macrophages, given high dose of Palmitic Acid, could induce a inflammatory response in the intestine**.**

Taken together, we postulated that high dose Palmitic Acid and TLR2 stimulation of CD36 expressing MHS macrophages could induce a pro-inflammatory response in the intestine; and perhaps may stimulate CD36+ macrophages to induce inflammation in the intestine.

An Elisa containing supernatant from MHS macrophages stimulated with a 24 hour time course revealed MHS macrophages stimulated with high dose Palmitic acid in conjunction with PGN presented a significantly higher TNFα production in comparison to PGN or Palmitic acid stimulated macrophages. This result indicated that high dose

Palmitic acid and TLR2 may work in synchrony to elicit an pro-inflammatory TNF-α response [68,69,102].

After confirming PA and PGN stimulated MHS macrophages could produce inflammatory cytokine TNFα, FACs was undertaken to investigate other pro-inflammatory cytokines: TNFα, IFN-γ, and IL-17a. Surprisingly, all MHS macrophages conditioned and unstimulated exhibited a high level of CD36 expression. However, MHS+PA and MHS+PA+PGN induced a slightly higher CD36 expression in comparison to unstimulated macrophages. Interestingly, MHS+PGN and MHS+PA exhibited some expression of IL-17a suggesting TH-17 T cells may play a role in these CD36 expressing MHS macrophages. This worth some exploring in proceeding studies as IL-17 has been implicated in a proinflammatory role in IBD, and plays a role in neutrophil recruitment in inflamed tissues[1,5,11,16,27]. No significant expression of IFN-γ was observed in conditioned macrophages or control. However this does not rule out a role for this pro-inflammatory cytokine *in vivo*. Surprisingly, FACs analysis revealed that high dose Palmitic acid treated MHS could elicit TNFα production alone in contrast to MHS+PA+PGN and MHS+PGN, consequently suggesting high dose of long chain fatty acids could induce a TNFα response. Despite the discrepancy between the ELISA and FACs data, both *in vivo* experiments confirmed that TNFα is produced via high fatty acid stimulated macrophages and implicate a potential role for TLR2.

Due to our interest in further elucidating the role of CD36+ expressing macrophages in the intestine, and the role of the CD36 receptor and its ligands, we

proposed based on *in vitro* findings that perhaps a similar association may exist *in vivo* in Balb/c mice.

After confirming high CD36 expression in MHS macrophages, I utilized MHS macrophage cell line conditioned macrophages and adoptively transfer into mouse models in attempts to induce inflammation in the intestine.In my next study, Four mice models were examined (a) NMHS, unstimulated MHS, (b) MHS+PA, 24 hour stimulated high dose Palmitic acid, (c) half dose TNBS (1/2TNBS), and (d)MHS+1/2TNBS unstimulated MHS and half dose TNBS(MHS+12TNBS).

Mice weights demonstrated that full and half dose TNBS exhibited the most pronounced weight drops; however half dose TNBS mice were able to gain weight and in full dose the mice only stabilized. MHS+1/2TNBS exhibited an initial weight drop and then recovered quicker than 1/2TNBS. NMHS, Naive and MHS+PA exhibited normal healthy weights throughout the 7 day protocol. Colitis was evident in full dose TNBS by dissected colons exhibited swelling, lesions, blood spots, pronounced shortening of the colon , diarrhea, neucrotic tissue, stool inconsistency and bloating; establishing severe TNBS colitis mouse model [110]. In contrast half dose tnbs exhibited milder histology, suggesting a mild colitis or bowel irritation. The majority of dissected colons from Naive, MHS+PA, MHS+12tnbs and NMHS exhibited normal healthy, elongated colons, stool consistency, and good intestinal integrity. Surprisingly MHS+1/2tnbs looked remarkably healthy; reasons for this phenomenon have been explained in the results section.

However, looking deeper into colon histology at the microscopic level, H&E stained colon tissue from each mice group displayed a slightly different story.

As expected Full dose TNBS H &E stained colonic tissue revealed pronounced inflammation in all layers of the intestine: muscle layers, Lamina Propria, epithelial lumen, and mucosa. Mononuclear cells, edema and crypt destruction was throughout the tissues[110,112]. Half dose TNBS mice exhibited some focal to zonal edema, crypt destruction, and mild to moderate mononuclear cell infiltration. NMHS mice exhibited a moderate mononuclear cell infiltration with little to no edema and at times focal crypt destruction or hyperplasia. Naive mice displayed the most healthy morphology with little mononuclear infiltrate, no crypt destruction, little to no hyperplasia, and no edema. Lastly MHS+1/2TNBS exhibited some edema focal or zonal, little to no crypt destruction and moderate mononuclear cell infiltrate.

Unexpectedly, MHS+PA exhibited a markedly higher level of mononuclear cell infiltrate relative to other groups; the colons of these mice displayed focal to zonal crypt destruction and at times moderate edema. This result was surprising as the macroscopic pictures of colon tissue and mice weights did not indicate any visible inflammation in the intestine. Some possible reasons for the lack of morphological change could be that PA stimulated MHS macrophages likely internalized and digested the fatty acids within 24-48hours, or that intestinal epithelial cell turn over ocured after 2-5days sloughing off epithelial cell tissue, so it is likely by day seven the potential stimulatory effects were largely lost in the intestine[15, 101-103,105,106]. In addition, the effects of the natural inert environment in the intestine and inflammatory tone is maintained via a variety of immune suppression mechanisms, ie: Tregs and TGF-β and secretion of IL-10, anti-inflammatory cytokines, likely their suppressive roles mask pro-inflammatory cytokine production

[1,11,16,17,22,25,27,28]. It is possible that a more chronic model of high fatty acid stimulated macrophages with multiple doses over a few weeks time would better represent the inflammation in the intestine; if high fatty acid and stimulated macrophages could alone induce an inflammatory response. I suspect it is with high long chain fatty acids and TLR ligand(s), in particular TLR2, stimulated MHS macrophages and toegther these ligands could induce an inflammatory response in the intestine [68,69,102,104].

Intestinal inflammation was assessed via histological scoring and Myeloperoxidase (MPO). Histological scoring, was highest in full dose TNBS, half dose TNBS, and MHS+PA followed in rank, indicating that high to moderate inflammation was exhibited in TNBS and MHS+PA conditioned Balb/c mice. Furthermore myeloperoxidase was employed asa secondary confirmation for intestinal inflammation via infiltration of inflammatory neutrophils and monocytes that secrete Myeloperoxidase[119,120]. MPO confirmed inflammatory cells were present in the colon of full dose TNBS, MHS+PA, and moderately expressed in MHS+1/2TNBS. Unpredictably, half dose TNBS exhibited no expression of myeloperoxidase and was similar or less than control groups; this result was not expected. It is possible little neutrophils were recruited to the intestine of 1/2TNBS mice and potentially more macrophages were present. However, the low expression is likely due to a mechanical error, since neutrophils generally interact with macrophages and are intimately involved in inflammation[110, 122,123]. Taken together, inflammatory parameters confirm that inflammation is present in the intestine of full and half dose TNBS mice and moderate in MHS+PA.

Interestingly, despite some contradictory results it does appear, on some level, that high dose Palmitic acid stimulated MHS macrophages induce a localized inflammatory response in the intestine. This is particularly interesting, as past studies on Palmitic Acid in the intestine did not show a pro-inflammatory response; however reported studies focused on enterocytes not macrophages[101,103,105,106].

It is possible that MHS macrophages stimulated with Palmitic acid, an LCFA ligand that binds to CD36 receptor, that this interaction stimulates the production of TNFα. Furthemore it is probable this ligand may be involved in a mechanism whereby CD36+ macrophages, in a surplus of fatty acids, release pro-inflammatory cytokines to attract other macrophages and APCs to aid in the breakdown of lipids[101-102]. Also microbiota can influence the results, so it would be interesting to study PA stimulated CD36+ F4/80+ macrophages in the intestine of germ-free mice, to better evaluate whether these macrophages without commensal microbiota could induce an inflammatory response in the intestine[11,12,14,22,25,42].

LPMC was extracted on day 7 in the four mice groups and were gated for CD36, macrophage markers: Cd11b and F4/80, and pro-inflammatory cytokines: TNFα, IL-17a, IFN-γ and IL-4.

CD36 was largely upregulated in 1/2TNBS, moderately in MHS+PA, and slightly elevated in MHS+1/2TNBS mice. Macrophage markers Cd11b and F4/80 were highly expressed in LPMC from 1/2TNBS mice, moderately in MHS+PA, and minor increase in MHS+1/2TNBS. Results demonstrated that Cd11b+ F4/80+ macrophages were highly recruited to the intestine of MHS+PA and TNBS colitis mice. This suggests these CD36+

macrophages were being recruited to the intestine in high dose fatty acid and intestinal inflammation. However, overall proportion of gated macrophages from LPMC was relatively low. LPMC derived macrophage numbers often yield low numbers, and these cells are difficult to grow in culture. The variation in macrophages markers between groups can be attributed to the purity, cell number, and differences between cell populations.

A limitation of macrophages markers used in my study is that there is a significant overlap between DCs and macrophages ie: Cd11b and cd11c, and Eosinophils also express F4/80[124-126]. Thus future studies isolating LPMC macrophages should also stain for other monocyte/macrophage markers: MOMA2,LY6C, and LY6G to better confirm CD36 expression is highest in macrophages relative to other immune cells which express scavenger receptors[124-126].

Furthermore to confirm the high proportion of CD36+ F4/80+ macrophages in 1/2TNBS and MHS+PA mice, western blotting was conducted to examine CD36 expression in colonic tissues from NMHS, 1/2TNBS, MHS+PA, MHS+1/2TNBS mice. Results demonstrated CD36 expression was largely upregulated in 1/2TNBS and moderaltely increased in MHS+PA , again confirming CD36 expression in mild colitis and high LCFA stimulated macrophages.

To ensure this relationship was specific to macrophages in the intestine IHC was utilized to directly assess CD36 receptor in F4/80+ intestinal macrophages. The **e**xpression of CD36+ F4/80+ double positive cells exhibited the same pattern as seen previously, 1/2TNBS exhibited the highest frequency of competent cells, secondly

MHS+PA, and thirdly MHS+1/2TNBS. Results supported that macrophages upregulated CD36 receptor in mice treated with colitis and high dose Palmitic acid.

Collectively, *in vivo* experiments provide substantial evidence to support that CD36+ F4/80+ macrophages are markedly increased in response to inflammatory stimuli: high Palmitic acid (LCFA); and PGN stimulated TLR2, thus together these ligands may induce inflammatory macrophages in the intestine [1,11,17,18, 27, 41,49,68,69,102].

FACs analysis demonstrated CD36+ macrophages In 1/2TNBS and MHS+1/2TNBS exhibited a high to moderate expression of TNFα relative to NMHS; this suggested an inflammatory response present in the intestine. Unexpectedly, MHS+PA mice induced a relatively small, but significant expression of TNFα in comparison to control mice. This result was confirmed both *in vitro* and *in vivo*. Taken together, CD36+ MHS macrophages stimulated by a high dose Palmitic acid induced a mild pro-inflammatory response (TNFα) in the intestine.

However working against this finding, was that no morphological change was observed in colons of MHS+PA mice(n=5). However, as previously mentioned, this is likely due to the inert state of the intestine, tightly controlled immunosuppressive mechanisms, and that TNFα production was potentially downregulated or suppressed in the non-inflammatory intestine[1,11,14,22,23,25,27]. In addition, MHS macrophages were unstimulated so this may also explain why a low TNFα response was exhibited, as it is likely that PGN stimulated high Palmitic acid treated macrophages would induce a substantially higher TNFα response, this remains to be explored.

To further confirm TNFα was produced via MHS+PA ,1/2TNBS, and MHS+1/2TNBS mice, western blotting was undertaken to examine the protein expression of TNFα from colonic tissues. Results demonstrated a high expression of TNFα in the colons of 1/2TNBS and MHS+PA, and moderate expression in MHS+1/2TNBS mice. Although there are differences in the expression of TNFα in comparative experiments, results confirmed that MHS+PA and 1/2TNBS induced TNFα production.

It is possible the differences in TNFα between flow cytometry and immunoblotting could be attributed to the fact that isolated protein from the colon may demonstrate higher non-specific TNFα due to the influence from other immune cells, epithelial cells, platelets, apoptotic cells, enterocytes; likely flow data is most representative[73,75,76,78,79,88,90-94,96,97,103]. Nonetheless, results establish TNFα production is produced by 1/2TNBS colitis and MHS+PA mice, and IHC reveals that CD36+F4/80+ macrophages are recruited to the site of inflammation and these macrophages release TNFα. It is somewhat interesting that macrophages stimulated with fatty acid alone, unstimulated via TLR ligands, could induce a mild production of TNFα production at the cellular level. Lastly, the production of TNFα in these mice also suggests a skewed TH1 T cell phenotype is induced in the intestine[1,110, 127].

TH17, another polarized inflammatory T cell subtype, has been previously identified to play an inflammatory role in Inflammatory bowel disease. This subset of T cells, induce inflammation, tissue injury, recruite neutrophils, and release antimicrobial immunity at epithelial barriers of inflamed tissues[1-5,11,127]. Interestingly, the expression of

IL-17a, a cytokine involved in TH17 T cell activation, was significantly upregulated in 1/2TNBS mice and MHS+PA mice[1,11,127]. This result is not surprising in 1/2TNBS colitis mice as this cytokine has been evidenced to play a role in TNBS colitis mice models[1,5,11,127]. However, it is interesting that MHS+PA mice induced the production of IL-17a in the intestine, as this suggests CD36+ macrophages treated with high LCFA may in some way may activate TH17 pathway and TH17 T cell polarization. However due to large variations in data the results were not significant, and requires further study to validate results[1,127].

Pro-inflammatory cytokine IFN-γ, has been highly recognized as a TH1 inflammatory cytokine in IBD and colitis mice[1,11,110, 127]. Surprisingly, little to no IFN-γ was expressed in MHS+PA or MHS+1/2TNBS; however marked expression of IFN-γ was exhibited in 1/2TNBS colitis mice, suggesting a TH1 pro-inflammatory phenotype which has been previously observed [1,11, 110,127].

Lastly a TH2 inducing pro-inflammatory cytokine, IL-4, was investigated to elucidate whether any of the conditioned mice exhibited a TH2 skewed phenotype[1,5,127]. Interestingly, 1/2TNBS elicited IL-4 expression, suggesting these mice also exhibited a TH2 phenotype; and some expression was observed in MHS+1/2TNBS. Results suggest 1/2TNBS colitis induced a mixed T helper phenotype: TH1,TH2, TH17 cells as seen in IBD, whereas MHS+1/2TNBS and MHS+PA primarily induced a TH1 phenotype[1,5,110,127]. Based on the stimulus, this suggests that CD36+macrophages induce inflammatory responses through different arms of the T-helper pathway in intestinal inflammation.

Taken together, this study has discovered several novel findings: (a) CD36+F4/80+ macrophages are highly upregulated in Crohn's Disease, Ulcerative Colitis and TNBS colitis mice, (b) CD36 +F4/80+ macrophages induce the production of a pro-inflammatory cytokine, TNFα, dependent upon ligand stimulation or inflammatory stimulus, (c) that perhaps high Palmitic acid (LCFA), may be a potential pathogenic ligand in CD36+F4/80+ macrophages when fatty acids are in excess, or in conjunction with TLR2 and (d) CD36+F4/80+ macrophages upregulate other pro-inflammatory cytokines, and the role of these cytokines remains to be further elucidated.

FUTURE DIRECTIONS:

Future studies are required to further investigate PGN+PA conditioned macrophages in vivo, and whether these macrophages recruit a higher infiltration of CD36+ macrophages and production of proinflammatory cytokines such as TNFα. It is imperative to study these mice in future studies in order to draw more conclusive data from experiments. In addition it would be interesting to examine these parameters in conjunction with full dose and half dose TNBS, to observed whether or not CD36+ macrophages induced a higher magnitude inflammation in the intestine. Furthermore it is important that another macrophage cell line is utilized to validate previously observed results. Bone marrow-derived macrophages would be ideal, as it is blood derived monocytes that are stimulated to develop into inflammatory macrophages in IBD[17,43,44,47-49]. Examining the effects of other high dose long chain fatty acids would also be interesting to establish if high LCFA stimulation of CD36+ macrophages induced pro-inflammatory cytokines in the intestine. Investigating different intervals and doses of

Palmitic acid conditioned CD36+ macrophages in mice models would also prove helpful to examine if morphological changes may occur in a more chronic model, and also in conjunction with TLR 2. Future work could also investigate the effects of other pro-inflammatory cytokines elicited via CD36+ macrophages such as IL-17a, to investigate whether MHS+PA pr MHS+PA+PGN could polarize TH17 T cells, and if so investigate the role of this cytokine in CD36+ macrophages in the inflamed or high fatty acid intestine.

Another interesting study would be to investigate CD36-expressing macrophages and their role in apoptotic cell clearance as this may also be an alternate way that CD36+ macrophages attempt to regulate or control inflammation via clearing apoptotic cells and necrosis. It is possible this could result in release of pro-inflammatory cytokines in the intestine[76,80,89,90-94,96,98]. Studying this relationship appears to have some promise for future studies as the CD36 receptor has a central role in clearing apoptotic cells. It is would greatly advance the scientific field to elucidate the precise role(s) of CD36 expressing macrophages in IBD, and future aims should be at characterizing their inflammatory or potentially protective role in the intestine. It is also possible that dysregulated fatty acid metabolism could play a role in IBD via aberrant activation of CD36 receptor on macrophages. Although it remains relatively uncertain, our study highlights that these CD36 expressing macrophages in some fashion are unique and highly implicated in IBD.

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