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Airborne particulate matter and a western style diet as potential environmental factors in the pathogenesis of Inflammatory Bowel Disease

by

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To my family and my love, for all your support and most of all, for always believing in me

Abstract

Inflammatory bowel disease is believed to occure in response to environmental factors resulting in immune dysregulation in genetically predisposed individuals. Such environmental factors include diet, (specifically a "western diet"rich in refined sugars, and fat) and airborne particulate matter (PM) pollution. Normally the gastrointestinal tract maintains tolerance to luminal antigens and the enteric flora, however, it's proposed that in genetically predisposed individuals, presence of such antigens acts to trigger or exacerbate intestinal inflammation resulting in IBD development. In this thesis, I demonstrate the ability for short-term, oral exposure of PM to initiate an acute intestinal inflammatory response in wild type mice. Next, long term oral PM exposure alters the expression of various Th1/Th2 mediated cytokines microbial composition in WT and IL10$^{-/-}$ mice. Finally, I demonstrate that exposure to a western diet, with or without, PM results in immunosuppression in the colon and significant inflammation in the small intestine of IL10$^{-/-}$ mice.

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List of Abbreviations

αCD3	Anti-CD3 antibody
Ag	Antigen
AP-1	Activator Protein 1
APC	Antigen Presenting Cells
B2m	Beta 2 microglobulin
BCFA	Branched Chain Fatty Acid
BSA	Bovine serum albumin
CD	Crohn's disease
CD	Cluster of differentiation
cDNA	complementary DNA
CCL	Chemokine Ligand
CCR	Chemokine receptor
CHO	Carbohydrates
CSF	Colony stimulating factor
CTLA	Cytotoxic T-Lymphocyte Antigen
CXCL	Chemokine (CXC motif) ligand
DC	Dendritic cells
ddH20	Distilled water
DEP	Diesel exhaust particulate
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EHC-93	Ambient air particulate matter
ELISA	Enzyme linked immunosorbent assay

EPA	Eicosapentaenoic acid
H&E	Haematoxylin and eosin
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
ICOS	Inducible T-cell co-stimulator
INFγ	Interferon gamma
IL	Interleukin
IPA	Ingenuity Pathway Analysis
GI	Gastrointestinal
HCl	Hydrochloric acid
MHC	Major Histocompatibility complex
MICA	Microbial Community Analysis
mRNA	Messenger Ribonucleic acid
MUFA	Monounsaturated fatty acids
NFκB	Nuclear Factor kappa B
NK	Natural Killer
NO2	Nitrous Oxide
NOS	Nitric oxide synthesis
PAH	Poycyclic aromatic hydrocarbons
PBS	Phosphate buffered solution
PCA	Principle component analysis
PCR	Polymerase Chain Reaction
PPARγ	Peroxisome proliferator active receptor γ
PUFA	Polyunsaturated fatty acids
PM	Particulate matter

RIPA	Radioimmunoprecipitation assay
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute media
rRNA	Ribosomal Ribonuclei acid
ROS	Reactive oxygen species
SCFA	Short Chain Fatty Acid
SELE	Selectin E
SOCS	Suppressor of cytokine signalling
SI	Small Intestine
Sig	significant
STAT	Signal Transducer and Activator of Transcription
TBX	T-box
TH	T helper type
TLDA	TaqMan Low Density Array
TNFα	Tumor Necrosis Factor alpha
T-RFLP	Terminal Restriction Fragment Length Polymorphism
UC	Ulcerative Colitis
VCAM	Vascular cell adhesion protein
WT	Wild Type
ZO-1	Zonula occludens 1

Chapter 1. Introduction

1-1. Background

Inflammatory bowel disease (IBD) is a group of chronic, relapsing and remitting inflammatory conditions affecting the gastrointestinal (GI) tract (1-3). The most common manifestations of IBD occur as Ulcerative colitis (UC) and Crohn's disease (CD), two disorders which differ from each other in severity and location of inflammation. UC is restricted to the colon and rectum, occurs in a continuous fashion, and lesions are limited to the mucosal layer of the intestinal wall (2, 4). CD on the other hand, is characterized by the formation of transmural lesions that can occur anywhere along the GI tract (i.e. from mouth to anus), usually in a discontinuous ("skip lesion") fashion. This intestinal injury often results in fibrosis and fistula formation in CD patients, and is not something typically observed in UC (1). Despite these differences, there are however, some overlapping pathological and clinical characteristics between these disorders, which suggest similar causes of disease (2). The exact etiology for this group of inflammatory disorders remains unknown; however, there is wide belief that IBD is the result of the combination of genetic predisposition, immune dysregulation against enteric flora, and environmental factors (5-9).

There has been a rapid increase in the prevalence, incidence, and severity of IBD over the last 50 years (10, 11), which is not due to improvements in diagnosis and disease surveillance alone (12). This increase is not only observed

in North America and Europe, which have always reported higher incidences of disease, but also in countries which have classically reported low incidence of disease such as Japan, China, and India (13, 14). Studies have also demonstrated increased incidence of disease in migrants who have moved from regions with low disease prevalence to westernized nations (13). The worldwide increase in IBD over such a short period of time can't be explained by genetic predisposition or immune dysregulation alone, and along with the observation that not all individuals with IBD associated genes go on to develop disease (2), strongly suggests a role for environmental factors in the development of IBD.

Smoking, appendectomy, microbiota, stress, geographical location, diet and airborne pollutants (1, 15) are examples of environmental factors believed to be involved in the pathogenesis of IBD. Some factors, such as smoking, gut microflora, and appendectomy have been extensively studied and consistently shown to play a role in the etiology of IBD. For instance, smoking is a known risk factor for CD, but plays a protective role in UC (16), and appendectomy, if performed in individuals younger than 23, is also shown to be protective in UC (17). Other factors have been studied only to a small extent or results have remained inconclusive, so therefore have yet to be proven as causative factors in IBD. The role of microbiota, dietary factors, and airborne pollutants, which are factors associated with susceptibility to IBD are discussed in greater detail below.

1-2. Microflora and IBD

There are approximately 10^{15} microorganisms, consisting of between 300

and 1000 different species of bacteria (18-21), within the human body, with the

highest concentration of bacteria present in the distal ileum and colon (22). This

is 10 times greater than the amount of cells in the body and accounts for more

than 100 times the amount of genes present in the human genome (23). Because

of this and the interrelationships observed between enteric bacteria and the

human host, the intestinal microbiota is often regarded as another organ in the

human body which has evolved with us over time (20).

Microbes are necessary for the maintenance of intestinal health and are

required for proper gut development and function. They are also needed for the

development of the mucosal immune system, provide key nutrients, are involved

in fermentation of substrates that the body can't break down on its own, and

prevent growth of pathogenic bacteria (2, 24, 25). However, because of this close

association to intestinal function, it appears that any dysregulation in the host

response towards the bacteria, or imbalance in bacterial function can result in

disease. There is significant epidemiological and experimental evidence to

suggest that enteric microflora and their products are key to the development of

disordered immune function and initiation of inflammation within the GI tract

(26-29), and in the pathogenesis of IBD in particular. Microbial factors are

believed to act as an environmental trigger to initiate IBD in genetically

predisposed individuals, instigate flare-ups, cause the systemic complications

often observed in CD patients, and contribute to the development of many of the extra-intestinal components associated with IBD (26).

The evidence for linking enteric bacteria with IBD include: **(1)** intestinal lesions in IBD patients are most commonly found in areas with the highest concentration of bacteria (22); **(2)** studies have shown disease severity improves in CD patients with the diversion of the fecal stream, and relapse occurs in those patients after fecal restoration (30,31); **(3)** experimentally induced lesions can be induced in susceptible individuals by directly inoculating the non-inflamed bowel with fecal material (32,33); **(4)** alteration of the microflora with antibiotics and probiotics can have beneficial effects in UC and CD patients (34,35); and **(5)** studies using animal models show that presence of normal enteric bacteria is required for full expression of disease, regardless of the underlying genetic defect (22). This is perhaps the strongest experimental evidence for the involvement of enteric flora in the pathogenesis of IBD.

Many theories have been postulated to describe the role of bacteria in IBD, including: **(1)** IBD is due to a persistent intestinal infection with an unidentified pathogenic organism; **(2)** alterations in bacterial composition and function result in gut dysbiosis and initiate disease; **(3)** defective mucosal barrier function increases translocation of bacteria and bacterial products which increases antigen exposure to the mucosal immune system; and **(4)** IBD is the result of a dysfunctional host immune response, including a loss of tolerance,

towards the microbiota. These theories are not mutually exclusive; instead IBD is most likely the result of a combination of many host and microbial factors.

Role of Pathogens: Several pathogens have been suspected in causing CD and UC. *Mycobacterium paratuberculosis* and *Mycobacterium kansasii* are two examples of bacteria believed to be involved in the pathogenesis of CD (9). *M. paratuberculosis* is known to cause Johne's disease, an inflammatory disorder, with granuloma formation, within the colons of cattle (36), which is pathologically similar to CD and human idiopathic granulomatous enterocolitis (22). Both species of bacteria have also been isolated from IBD patients, but not controls (36-38). Mycobacteria are extracellular and intracellular bacteria which means they have the ability to stimulate both arms of the immune system (humoral and cellular immunity) (9), and along with the isolation from patients, makes them potential infectious agents involved in pathogenesis of CD. *Diplostreptococcus* and adherent *Escherichia coli* are two examples of other suspected infectious agents in UC, which have been found in higher incidences in patients with UC compared to controls (39,40). *Yersinia, Campylobacter, Clostridium, Pseudomonas, Shigella*, and *Fusobacterium necrophoum* are some of the other bacteria suspected in the pathogenesis of IBD (9, 22). Studies have demonstrated that the appearance of certain pathogens can precede onset of disease in some IBD patients, while others can exacerbate inflammation and initiate flare-ups (26). Unfortunately, it is not yet possible to determine if and what infectious microbe is responsible for the onset of IBD due to the lack of

prospective studies involving in-depth analysis of the gut microbiota in individuals prior to their developing IBD; therefore only basic associations can be suggested.

Gut Dysbiosis: Alterations in microbial function and composition, dysregulated immune function caused by defective sensing and clearance of bacteria, loss of immune tolerance towards enteric bacteria, and impaired barrier function are all suggested to play a role in the pathogenesis of IBD (41-43). Dysbiosis, which is the imbalance of microbial colonies within the intestinal tract, is a common characteristic in IBD; these patients tend to have an increase in aggressive, potentially harmful bacteria (eg adherent invasive *Escherichia coli*) and decrease in protective microbes (eg *Faecalibacterium prausnitzii*). In addition, IBD patients tend to exhibit a reduced overall microbial diversity within the intestinal tract (28, 44-47). These changes may increase the ability for pathogenic bacteria to invade intestinal epithelial cells resulting in activation of the immune system and release of pro-inflammatory cytokines. This could then cause damage to the intestinal epithelial barrier which increases tissue exposure to luminal bacteria and antigens; in a genetically susceptible individual this could result in an improper immune response towards enteric bacteria, causing uncontrolled inflammation and possible development of IBD.

Role of the epithelial barrier: The condition of the epithelia barrier is important because it separates the luminal contents such as bacteria and food antigens

from the mucosal immune system and allows for the maintenance of immune

tolerance towards the enteric microbes. Any disruption within the intestinal

epithelium can result in inflammation and loss of tolerance (48). Alterations in

the mucosal barrier can be the direct result of damage from pathogenic bacteria

and microbial products on the intestinal epithelium, caused by intrinsic defects in

the epithelium, or occur as a result of secondary effects caused by inflammation

within the intestine (48). Impaired barrier function increases intestinal

permeability and results in the stimulation of the mucosal immune system by

enteric bacteria and food antigens normally present in the lumen. Experimental

studies in mice have confirmed the importance of an intact epithelial barrier in

IBD pathogenesis, showing that altered barrier function results in the

development of colitis (48). Therefore, breakdown of the epithelial barrier

results in the inappropriate exposure of the mucosal immune system to

commensal microorganisms leading to inflammation and possibly IBD.

Overall, there is significant evidence to link the role of bacteria to the

pathogenesis of IBD. Microbes appear to induce or exacerbate intestinal

inflammation by initiating an abnormal host immune response towards enteric

bacteria and their products or toxins, and other luminal antigens in genetically

predisposed individuals, resulting in uncontrolled intestinal inflammation and

disease development. Improving our understanding of how intestinal microbes

function, how they are influenced by environmental factors and how this affects

intestinal health will perhaps one day allow us to fully understand the

pathogenesis of IBD and possibly provide new therapeutic targets for better

treatment of IBD.

1-3. Particulate matter and IBD:

Particulate matter (PM) is a key component of air pollution that is

responsible for an increasing number of adverse health conditions in urban

centers around the world. PM comes from a variety of sources, including, vehicle

exhaust, road dust, forest fires, and industrial emissions (49). It can be composed

of a complex mixture of metals (aluminum, copper, iron, zinc, vanadium, and

lead), sulfate and hydrogen ions, water, and organic components (50). There is

also a polycyclic aromatic hydrocarbons (PAH) component, which are

combustion derived by-products that have attracted a lot of attention because of

their ability to generate reactive oxygen species (ROS) in target cells, and in

many cases are considered carcinogenic (51,52). PM in ambient air is normally

classified as PM_{10} (particles with an aerodynamic diameter less than 10uM),

which means it is made up of coarse and fine particles. Fine particulate is

referred to as $PM_{2.5}$ (particles with an aerodynamic diameter between 0.1uM

and 2.5uM) and represents the combustion derived particulate component in air

pollution (50, 53). There is also ultrafine PM (aerodynamic diameter less than

0.01uM), which most often is referred to as diesel exhaust particulate (DEP),

because it is almost exclusively derived from the combustion of fossil fuel and is

a major component of vehicle exhaust (54). These particles have the largest

surface area and highest concentration of toxic PAH out of all PM sources, and due to their small size, have the ability to penetrate the deepest into lung tissue. The exact negative health effect generated by PM exposure will vary depending on the exact chemical composition, surface area, and size of the particulate.

An estimated 500,000 deaths each year are related to PM exposure (52), and epidemiological studies show a strong association between pollution and various adverse health conditions, including an increased risk of stroke, myocardial infarction, arrhythmia, cardiac arrest, venous thrombosis, and lung cancer (55-61). There is also a direct effect on pulmonary diseases such as pneumonia and asthma, and PM exposure exacerbates obstructive lung disease (62, 65). Recent evidence also demonstrates an association between PM exposure and increased risk of various intestinal inflammatory disorders including, the development of gastroenteritis in children, appendicitis, colorectal cancer, and CD in individuals younger than 23 who are exposed to traffic related pollutants (15, 66-69).

The possible effect of PM on the GI tract should not be surprising considering that, the intestinal system is constantly exposed to a high concentration of pollutants similar to that seen in the lung on a daily basis. The majority of inhaled fine and coarse particles are removed from the airways via the mucociliary clearance system which transports the particles to the pharynx where they are swallowed and carried through the GI tract (70, 71). Intestinal

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exposure also occurs with the ingestion of PM contaminated food such as fruits and vegetables (71). A significant proportion of PAH exposure occurs via this route; for instance, one study estimated that in non-smoking individuals, up to 18% of all PAH exposure occurred via the ingestion of PM contaminated vegetables (72). Therefore, the GI tract can be exposed to high levels of PM, making it an important environmental factor to consider in the initiation or exacerbation of intestinal inflammatory disorders.

The PAH and transition metal component of PM is thought to be the major culprit in the production of airway inflammation, which act to induce reactive oxygen species (ROS) and oxidative stress in bronchial epithelial cells (73-76). This has been confirmed in several animal models and human exposure studies, which have demonstrated that exposure to DEP, caused pronounced airway inflammation through the up regulation of vascular endothelial adhesion molecules and pro-inflammatory cytokines, and recruitment of neutrophils, mast cells and T cells (77-81). PM_{10} has also been shown to generate free radicals, which are known to activate inflammatory associated transcription factors such as NFκB and AP-1 (82, 83).

However, the exact biological mechanisms underlying air pollution associated intestinal disease is still poorly understood. It is believed, in a similar fashion as the respiratory tract, PM can initiate intestinal inflammation by inducing cellular oxidative stress in the epithelium, with subsequent cytokine

release and disruption in the mucosal barrier (71). There has been an effort to describe in detail the immune-pathologic effects of PM in the intestines. For instance, several studies have demonstrated an ability to induce ROS formation and NFκB activation in intestinal epithelial cell lines (71,84), and Dybdahli *et al.* (85) has shown dietary exposure to DEP in rats, results in the formation of DNA adducts and strand breaks in colonic epithelia. These features can go on to activate intracellular signaling pathways resulting in mucosal inflammation (86).

Mutlu *et al.* (71) have further shown that intestinal permeability increases in mice after oral exposure to PM, and this is due to reduced expression of the tight junction protein ZO-1. This was confirmed *in-vitro*, which also demonstrated discontinuous distribution of ZO-1 around the junctions of the intestinal epithelial cells. PM exposure also increased apoptosis in colonocytes and IL6 mRNA expression. Thus, this would suggest a possible role for dietary PM in the ability to initiate inflammation and increase intestinal permeability.

Interestingly, PM exposure has also been shown to interfere with the host's ability to clear bacteria from the respiratory tract, resulting in increased susceptibility to bacterial and viral lung infections (87, 88). If PM has similar effects on the intestinal immune system, then increased oral exposure to pollutants may increase the risk of bacterial invasion and intestinal inflammation.

Overall, it appears as though dietary exposure to PM has the ability to induce intestinal inflammation and alter mucosal barrier function; this could then increase the translocation of particles and enteric bacteria from the lumen, perpetuating the inflammatory response. If PM also has the ability to decrease intestinal host defence mechanisms, the cycle of mucosal inflammation would continue and be exacerbated, this in a genetically susceptible individual, could act as a trigger initiating IBD.

1-4. Role of Diet in IBD

Diet is an environmental factor that has long been suggested to play a role in IBD pathogenesis, mainly because of the location of this disease. Dietary antigens are present in abundance within the intestinal lumen and have the ability to directly interact with the epithelium and mucosal immune system, alter microbial composition, and impair barrier function (89-93). Therefore, it is possible that in a genetically predisposed individual, the presence or absence of specific dietary factors in association with enteric bacteria, act to initiate or exacerbate intestinal inflammation, leading to the development of IBD.

Increased consumption of refined sugar, starch, and processed fats are examples of dietary components believed to increase the risk of developing IBD, and CD in particular, whereas consumption of fruits, vegetables and dietary fibre appear to decrease this risk (94-98). A large number of case-control and prospective studies have been published examining the possible role of these

dietary factors in IBD, which have been summarized in Table 1 and are discussed in more detail below.

1-4-1. Fat

There has been numerous studies published that have attempted to determine what role fat consumption, and in particular fatty acid intake, has in the development of IBD. For instance, a study by Belluzzi *et al.* (99) tested the effect of omega (n) 3 fatty acids in the form of enteric-coated fish oil in 39 patients with CD, while another 39 patients were given placebo. They found that there was a marked decrease in the relapse rate among the patients receiving the enteric coated preparation, and this was not confounded by other variables such as smoking, sex, age, or duration of disease. This study showed the potential anti-inflammatory effects of n-3 fatty acids which are generally considered to have beneficial health effects. A later study found that supplementation with fish oils containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) significantly inhibited the production of interferon gamma (IFNγ) in peripheral mononuclear cells in patients with CD (100).

In another study, however, it was found that n-3 fatty acids and n-6 polyunsaturated fatty acids (PUFA) correlated strongly with the development of CD in a Japanese population. This study also found strong correlation with the ratio of n-6 to n-3 PUFA, milk protein, animal fat, and total fat to the

development of CD (101). The ratio of n-3: n-6 PUFA appears to be an important consideration regarding the risk factor for IBD (89,102,103).

Similarly, consumption of n-6 PUFA has been associated with the development of UC. One Japanese case-control study found a significant relation between the consumption of western foods and UC. In particular margarine, which is high in n-6 polyunsaturated fats, was independently associated with UC (104). A later European prospective cohort study found that there was a significant positive association between the consumption of total polyunsaturated fats and the development of UC (105). A follow-up study found that, among 126 patients with UC, there was an increased risk for the development of disease among those who were in the highest quartile of linoleic acid consumption (106). Other studies have also found that increased intake of total fat, monounsaturated fatty acids (MUFA) and PUFA are associated with increased risk of developing UC (107), and CD (102).

Overall, there is epidemiological evidence to suggest a link between increased fat intake, dependent on individual fatty acids, and IBD. The mechanism that has been suggested is through the direct effect of the fatty acids on the intestinal inflammatory response (1).

1-4-2. Carbohydrates

Carbohydrates can be divided into 3 groups: **(1)** monosaccharides and disaccharides which are simple sugars, **(2)** oligosaccharides, and **(3)** polysaccharides which include starch and fibre. Fibre can be further classified as soluble or insoluble. Soluble fibre, which includes pectins, beta-glucans, inulins, and dextrins, is readily fermented in the colon by enteric bacteria (1). This process results in gas, lactate, and short chain fatty acids (butyrate, acetate, and propionate) production. Insoluble fibre on the other hand, is not fermentable; instead it absorbs water as it passes through the intestinal tract, increased bulking activity and softening of stool (108). Examples of nonfermentable fibre include cellulose, hemicelluloses, lignins, waxes, and resistant starch (1).

Several studies have tried to examine the link between carbohydrate intake and disease susceptibility, but results remain inconclusive. There are many studies which suggest an association between increased sugar consumption along with decreased fibre are involved in IBD development. For instance Thorton *et al.* (109) found newly diagnosed CD patients consumed higher amounts of refined sugar and total carbohydrates, and lower amounts of dietary fibre, fruit, and vegetables then matched controls. Another study which compared intake of refined carbohydrates and fibre in 250 patients with CD and UC, also found increased sugar intake to be correlated to risk of disease development and fibre to be protective (1). Similar results were observed by

Reif and colleagues (110), which looked at the dietary histories from 54 patients

with UC and 33 with CD and compared to 144 controls. They found that there

was an increased risk for IBD in individuals who consumed high levels of sucrose.

Some studies however, have suggested that no relationship exists between fibre

consumption and IBD development (111,112).

Although no mechanism has been proposed to link high sugar and starch

consumption with IBD development, animal studies have shown increased intake

of sugar leads to insulin resistance and chronic inflammation (113,114). This

suggests this dietary factor in particular appears to have to ability to initiate

inflammation. Lack of fermentable fibre can also have important implications

regarding IBD susceptibility. Butyrate is involved in intestinal health as it is the

primary energy source for colonocytes, and has anti-inflammatory characteristics

due to its ability to inhibit NFκB activation, which in turn, prevents pro-

inflammatory cytokine production (2). It has also been shown to reduce colonic

permeability by increasing activation of peroxisome proliferator active receptor γ

(PPARγ) (42). Butyrate production is dependent on the type and amount of fibre

consumed and its transit time through the GI tract (41). Therefore, decreased

consumption of dietary fibre results in decreased butyrate production which

could result in increased permeability, which in genetically predisposed

individual, enhances the likely hood of disease initiation. Any possible positive

health effects associated with fibre consumption however, have yet to be seen

with IBD. Although an association appears to exist between carbohydrate

consumption and IBD development, there is still no direct epidemiological evidence to confirm this (1).

1-4-3. Protein

Increased consumption of dietary protein, whose major sources include meat, cheese, milk, fish, nuts, and eggs, is another factor that has been suggested in the pathogenesis of IBD. There have been a few studies published, which have attempted to confirm this association, including a paper by Tragnone and colleagues (111). They found that there was a correlation between increased protein consumption and development of UC, but not CD. A prospective cohort study performed in 2004 also examined the relationship between relapses in UC and dietary intake in 191 patients (115). They found 52% of the patients relapsed into active disease, and those that did, had a higher meat, protein, alcohol, and sulphur or sulphate consumption then those patients that didn't relapse. The authors postulated that the sulphur contained within these foods may be the trigger for relapse. Sulphur containing foods, such as milk, cheese, eggs, and meats may be metabolized into harmful by-products by the enteric flora leading to intestinal inflammation. (116). It has been shown that these by-products can inhibit butyrate oxidation and result in mucosal hyper-proliferation in UC patients (116).

Jantchew *et al.* (117) further evaluated the role of protein macronutrients in the pathogenesis of IBD in 2010. Among 67581 participants there were 77

incident cases of IBD and it was determined that high total protein intake was associated with a significant increased risk for developing IBD. Sources for such proteins that were associated with an increased risk included meat and fish but no eggs or dairy products (117).

Even though there is a smaller amount of published data examining the role of protein consumption in IBD development, what is there provides evidence to support the relationship, with the action of the microflora (1).

Various epidemiological studies have aimed at uncovering a link between specific dietary factors and the development of IBD, however their results are often inconclusive and in many cases contradictory. This can be due to the fact that much of the data used to make general associations often rely on case control studies, which are designed as retrospective based questionnaires (118). This allows for the possibility of recall bias and mistaken responses which can lead to confounding results (1,118). It is also hard to compare the results from several studies because there are different types of dietary questions asked from patients, and depending on the study, assessed at different time points in the disease (i.e. patients can be asked to recall their diet before diagnosis, at onset of symptoms, or after diagnosis) (1).

Another downside in many of these studies is their focus on single nutritional components as opposed to the entire dietary pattern of the individual. It seems more plausible that it is a combination of dietary factors

which, in association with the enteric flora, enhances the risk of developing IBD. For example, recent reviews by Cosnes (119) and Bernstein (120) have suggested that a western style diet (which is high in simple carbohydrates and fat, and low in fibre, fruits and vegetables) is associated with increased risk of IBD. This type of diet includes many of the components which on their own have been implicated in disease, and interestingly is also becoming more prevalent in countries such as Japan and China, which have reported increased rates of IBD in the past 50 years (121).

A few possible mechanisms that diet can have on intestinal inflammation include: **(1)** effect of insulin resistance due to increased sugar intake; **(2)** alteration of barrier function and enteric bacteria; and **(3)** initiating either a pro- or anti- inflammatory response, depending on which nutrients present in the diet (1).

Overall, there appears to be an association between diet, as an environmental factor, and the development of IBD. Whether this relationship is due to an individual dietary component or the result of overall dietary patterns remains unknown, and does show a need for future studies.

Table 1-1: Consumption of major dietary components and their susceptibility to the development of Ulcerative colitis and Crohn's disease

Food component	Ulcerative colitis			Crohn's disease		
	Decreased risk	Increased risk	Reference	Decreased risk	Increased risk	Reference
Total fat intake		+	Reif 1997 (110)		+ (sig)	Sakamoto 2005 (102)
		+	Geerling 2000 (107)		+	Amre 2007 (128)
		+ (sig)	Sakamoto 2005 (102)		+	Jantchou 2010 (117)
		+	Hart 2008 (105)			
		+	Jantchou 2010 (117)			
Saturated Fat		+	Reif 1997 (110)		+	Sakamoto 2005 (102)
		+	Geerling 2000 (107)		+	Amre 2007 (128)
		+	Sakamoto 2005 (102)			
MUFA		+ (sig)	Reif 1997 (110)		+ (sig)	Sakamoto 2005 (102)
		+ (sig)	Geerling 2000 (107)		+	Amre 2007 (128)
		+ (sig)	Sakamoto 2005 (102)			
		+	Hart 2008 (105)			
total PUFA		+	Reif 1997 (110)		+ (sig)	Sakamoto 2005 (102)
		+	Geerling 2000 (107)		+ (sig)	Amre 2007 (128)
		+	Sakamoto 2005 (102)			

Table 1-1: continued

		+	Hart 2008 (105)			
n-3 PUFA		+	Sakamoto 2005 (102)		+	Sakamoto 2005 (102)
			John 2010 (129)		+	Amre 2007 (128)
n-6 PUFA		+	Geerling 2000 (107)		+	Sakamoto 2005 (102)
		+	Sakamoto 2005 (102)		+	Amre 2007 (128)
Total carbohydrate		+ (sig)	Tragnone 1995 (111)		+ (sig)	Tragnone 1995 (111)
		+	Geerling 2000 (107)	+		Sakamoto 2005 (102)
	+		Sakamoto 2005 (102)	+		Amre 2007 (128)
		+	Hart 2008 (105)		+	Jantchou 2010 (117)
	+		Jantchou 2010 (117)		+ (sig)	Greeling (126)
Mono- and disaccharides		+ (sig)	Tragnone 1995 (111)		+ (sig)	Tragnone 1995 (111)
		+ (sig)	Reif 1997 (110)		+	Reif 1997 (110)
		+	Geerling 2000 (107)		+ (sig)	Mayberry (133)
	+		Hart 2008 (105)		+ (sig)	Silkoff (125)
		+ (sig)	Sakamoto 2005 (102)		+ (sig)	Lomer (131)
		+	Russel 1998 (132)		+ (sig)	Kasper (112)
					+	Persson (124)

Table 1-1: continued

Polysaccharides (starch)	+	+ (sig)	Tragnone 1995 (111) Hart 2008 (105)		+ (sig)	Tragnone 1995 (111)
Dietary fiber	+	+ +	Reif 1997 (110) Sakamoto 2005 (102) Hart 2008 (105)	+ + + (sig) +		Reif 1997 (110) Sakamoto 2005 (102) Amre 2007 (128) Persson 1992 (124)
Fruit	+ + +	+ +	Gilat 1987 (122) Higashi 1991 (123) Reif 1997 (110) Sakamoto 2005 (102) Halfvarson 2006 (127)	+ + +		Reif 1997 (110) Sakamoto 2005 (102) Amre 2007 (128)
Vegetables	+ +	+	Reif 1997 (110) Sakamoto 2005 (102) Halfvarson 2006 (127)	+	+ +	Reif 1997 (110) Sakamoto 2005 (102) Amre 2007 (128)
Total protein	+	+ + +	Reif 1997 (110) Geerling 2000 (107) Sakamoto 2005 (102) Hart 2008 (105)		+ + + (sig) +	Tragnone 1995 (111) Reif 1997 (110) Sakamoto 2005 (102) Amre 2007 (128)

Table 1-1 continued

Total protein cont.						
		+	Jantchou 2010 (117)		+	Jantchou 2010 (117)
		+	Joqwet (115)			
animal protein	+		Geerling 2000 (107)		+	Jantchou 2010 (117)
		+	Jantchou 2010 (117)		+	Shoda (101)
Total meat		+	Higashi 1991 (123)		+	Sakamoto 2005 (102)
		+	Sakamoto 2005 (102)			
		+	Jantchou 2010 (117)			
		+	Joqwet (115)			
Dairy		+ (sig)	Glasssman 1990 (130)	+		Sakamoto 2005 (102)
		+	Higashi 1991 (123)			
	+		Sakamoto 2005 (102)			
	+		Jantchou 2010 (117)			
Eggs		+	Higashi 1991 (123)		+	Reif 1997 (110)
		+	Reif 1997 (110)		+	Sakamoto 2005 (102)
		+	Sakamoto 2005 (102)	+		Halfarvson 2006 (127)
		+	Halfarvson 2006 (127)			
	+		Jantchou 2010 (117)			

This table has been adapted from Gentshew and Ferguson (2012) (gent) review paper. It provides a breakdown of the main dietary components and their association with the risk of developing Ulcerative colitis and Crohn's disease. Data is based on case control and prospective studies in various populations. Values bolded with (sig) were determined to be significant results as determined in each study.

1-5. Relevance and study aims

As mentioned above, there has been a rapid increase in the global incidence and severity of IBD over the past 50 years (12). This trend cannot be explained by genetics and immune dysregulation alone, therefore implicating the increasing role for environmental factors in the pathogenesis of this group of intestinal inflammatory disorders. Particulate matter exposure and diet (in particular, a western style diet high in fat and simple carbohydrates) are two potential environmental factors that have been suggested to be involved in the pathogenesis of IBD. Most evidence for their role is based on epidemiological studies and very little has been done to look at their direct role in intestinal inflammation and initiation of IBD.

Therefore the aims for this thesis include: **(1)** to determine the acute effects of orally administered PM on mucosal immune function; **(2)** examine chronic effects of orally administered PM on mucosal immune function, microflora composition, and IBD development in the IL10 deficient mouse model of colitis; **(3)** examine the role of a western style diet (i.e. high simple carbohydrates, high sugar) on intestinal immune function and microflora composition, and disease severity in the IL10 deficient mouse model of colitis;, and **(4)** to examine the combined effects of a western style diet in association

with PM exposure on mucosal immune function, microflora composition, and disease severity in the IL10 deficient mouse model of colitis.

1-6. Hypothesis

Based on previous epidemiological data, I hypothesize that oral exposure to PM and a western style diet will increase intestinal inflammation, thereby increasing the severity of disease in the mice.

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Chapter 2: Acute effects of particulate matter exposure on mucosal immune function and gut permeability

2-1. Introduction:

Particulate matter (PM) is a key pollutant in ambient air responsible for an assortment of negative health conditions associated with air pollution exposure in urban environments. It is generally referred to as PM_{10} (particles with an aerodynamic diameter less than 10μM), which is considered coarse particles, but also includes a fine ($PM_{2.5}$) particulate component (particles with an aerodynamic diameter less than 2.5μm) made up of combustion derived particles (1, 2). PM comes from sources such as vehicle exhaust, industrial emissions, road dust, forest fires, windblown soil etc. It is composed of a complex mixture of metals (e.g. aluminum, chromium, lead, vanadium, and zinc), ions, water and various polycyclic aromatic hydrocarbons (PAH); which are fuel burning by-products that have attracted a lot of attention because of their ability to generate reactive oxygen species (ROS) in target cells, and many are considered carcinogenic (3, 4) The exact health effect generated by PM will vary depending on the chemical composition, surface area and particle size.

Epidemiological studies have shown a strong association between ambient PM exposure and various adverse health effects, including an increased risk of stroke, myocardial infarction, arrhythmia, cardiac arrest, venous thrombosis, lung cancer and mortality. There is a direct effect on pulmonary

diseases such as pneumonia and asthma, and PM exposure leads to the

exacerbation of obstructive lung disease. The range of health effects is extensive

but believed to be predominantly confined to the respiratory and cardiovascular

systems. However emerging evidence now suggests PM exposure can also have

adverse consequences on other organ systems such as the gastrointestinal tract

(GI). There have been several reports linking exposure to air pollution with

inflammation in the gut (5) and epidemiological studies have shown an

association between PM exposure and the increased risk of appendicitis,

gastroenteritis in children, Crohn's Disease (CD) diagnosis in individuals younger

than 23 who were exposed to high concentrations of NO2 (a major component

of traffic related pollution associated with PM), increased hospitalizations in

Inflammatory Bowel Disease (IBD) patients, and higher risk of colon and liver

cancer (6-13). This suggests exposure to PM air pollution may be an important

environmental factor leading to exacerbations of inflammatory illness in the GI

tract.

Research on airborne pollutants has for the most part focused on the

respiratory effects after inhalation, as this is considered the primary route of PM

exposure, however the GI tract can also be exposed to high concentrations of

pollutants. The GI tract is exposed to a significant amount of the inhaled PM

when the particles are removed from the lungs via the mucocilliary clearance

system. The particles are transferred to the oral cavity, swallowed, travel

through the GI tract and are eliminated from the body in the feces (5). Intestinal

exposure also occurs through the ingestion of PM contaminated foods such as fruits and vegetables (14). One study estimated that in non-smokers, 12-18% of all PAH exposure occurred from the ingestion of vegetables contaminated with atmospheric particles (15). This suggests then that the intestinal tract has the ability to be exposed to PM at similar levels seen in the lungs, thus making the GI system an important route of PM exposure to consider.

The exact biological mechanisms underlying air pollution associated disease are poorly understood, but emerging data suggest the PAH and transition metal component of PM have the ability to induce reactive oxygen species and oxidative stress in bronchial and colonic epithelial cells (16-19). It is believed this process is what contributes to the mucosal inflammatory response observed in respiratory PM exposure studies. Little remains known as to how airborne PM affects gut immune function, therefore the aim of this study was to examine the effects of orally administered PM_{10} on intestinal mucosal and systemic immune function and on gut permeability. Based on results observed in respiratory studies, I hypothesize that oral exposure to PM will increase intestinal inflammation and alter gut permeability in WT mice.

2-2. Materials and Methods:

2-2-1. Particulate Matter (PM):

Ambient air particulate matter PM_{10} (EHC-93) was obtained from the videlon bag filters of the single pass air-purification system from the

Environment Health Center in Ottawa, Canada. Characteristics of the PM have been previously described (1,2) and are summarized in appendix 1: **table A-1**.

2-2-2. Animals and overview of PM treatment:

Adult Female 129 SvEv mice (~17-23g in weight) were each randomly assigned to one of 4 treatment groups (5-6 mice/group): PM (EHC-93) treatment for 7 or 14 days, or vehicle (ddH20) for 7 or 14 days. The mice were gavaged daily with 360ug of PM suspended in ddH20 or ddH20 only (control) depending on their particular treatment group. PM tends to aggregate and form clumps in aqueous solutions within a few minutes (20) therefore PM in ddH20 was sonicated and then vortexed immediately before ingestion. Permeability was measured by calculating lactulose and mannitol concentrations in the urine on the first (day 0), 7th, and 14th day of experimental treatment. Animals were sacrificed after day 7 or 14 and their spleen, colon, and small intestine were harvested. Portions of the colon and SI were cultured in RPMI 1640 media for 8hrs to examine cytokine secretion. Other sections of colon and SI were processed for gene expression profiles, while splenocytes were isolated for proliferation assays. The protocol for use of mice was approved by the Health Science Animal Care and Use Committee at the University of Alberta. Mice were kept in a temperature regulated day/night cycle with controlled humidity and temperature and had free access to food and water throughout the experiment.

2-2-3. Mucosal Cytokine secretion

Mucosal Immune function was examined by removing sections of the small and large intestine (approximately 5cm in length) from mice after 7 or 14 days of treatment. Sections were cultured in 1.5mL of RPMI 1640 media (10% heat inactivated fetal bovine serum, streptomycin (1g/ml), L-glutamine (2mM), 2 mercapto-ethanol (50uM), and non essential amino acids (10 mM) (Invitrogen) for 8hr at 35°C. Supernatant was collected and frozen at -80 and analyzed via a MesoScale Discovery kit to measure cytokine release. Specifically, IL1β, IL2, IL4 , IL5, CXCL1, IL10, IL12, IFNγ, and TNFα secretion were all measured using a mouse TH1/TH2 tissue culture MesoScale Discovery Kit (MesoScale Discovery) as per manufactures instructions. IL17 cytokine secretion was analyzed with an ELISA kit (R&D systems) as per manufacturer's protocol.

2-2-4. Gene Expression and pathway analysis

Total RNA was isolated from frozen small intestinal and colon samples using a modified TRIzol extraction procedure and purified using RNeasy columns (Qiagen). Briefly, frozen tissues were homogenized in 1mL of TRIzol (Invitrogen), then mixed with 200uL of chloroform and centrifuged at 1400rpm. The RNA containing aqueous layer was removed and purified by doubling the volume with 70% ethanol and passing it through the RNeasy spin column as per manufacturer instructions. RNA was quantified using a nanodrop 1000 spectrophotometer (Thermo Scientific). Single stranded cDNA was then synthesized from RNA using a High Capacity cDNA reverse transcription kit (Applied Biosystems) as per

manufactures instructions. Relative gene expression levels were measured using 96 plex mouse immune Taqman Low Density Arrays (TLDA) (Applied Biosystems) and DataAssist software (Applied Biosystems) was used to calculate the fold changed in gene expression. To determine biological relevance from gene expression data, Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com) (Ingenuity Systems) was used to identify specific gene network interactions. Only genes differentially expressed with a fold change of 1.5 within each treatment group compared to their control were selected for.

2-2-5. Cell isolation and Proliferation

Tritiated thymidine (^{3}H) was used to measure the proliferation of splenocytes isolated from female 129 SvEv mice after 7 and 14 days of treatment to examine the effects PM oral exposure has on systemic immune function.

Cell Isolation: Spleens were removed under sterile conditions and homogenized between two frosted glass slides. The tissue homogenate was then rinsed with 7mL of PBS and passed through a 70 μm nylon strainer (BD Bioscience) and centrifuged at 200 g for 5min. Cell pellets were resuspended in 1mL of PBS and red blood cells lysed. Splenocytes were centrifuged again at 200g for 5min, resuspended in 1mL of Imag™ buffer (PBS + BSA +0.09% Sodium Azide), and were then counted with a Beckman Coulter counter as per manufacturer instructions and diluted in RPMI media and plated at a concentration of 1x10^5 cells/well.

Cell plating/proliferation: The isolated splenocytes were incubated with RPMI 1640 media (10% heat inactivated fetal bovine serum, streptomycin (1g/ml), L-glutamine (2mM), 2 mercapto-ethanol (50uM), and non essential amino acids (10 mM) (Invitrogen)) alone as a negative control. A dose response with increasing concentrations of PM added to media (0.5mg/mL, 5mg/mL, or 50 mg/mL) was carried out. αCD3 was used as a positive control as it will activate T cells in the cell suspension resulting in optimal proliferation after 48hrs at 35°C. After 48hrs the cell cultures were centrifuged at 1000rpm resuspended in 100uL of warm media + ^{3}H thymidine (1/100 dilution) and incubated for another 24hr. Cells then harvested using an Inotech cell harvester and read on Wallac Microbeta Tilux Scintillation counter. Results are displayed as a ratio to αCD3 to normalize the data.

2-2-6. In vivo permeability

To examine if oral PM exposure had any effect on intestinal permeability, mice were placed in metabolic cages and urine was collected over a period of 22hrs and measured for lactulose/ mannitol excretion. Urine samples were collected in containers containing 100mL of 10% thymol solution (1g/10mL isopropanol) and 100mL of paraffin oil (to prevent the evaporation of the urine) 4 days prior to PM treatment (Day -4) (to provide a baseline excretion measurement), on the first day of experimental treatment (Day 0), and after 7 and 14 days of PM exposure (Day 7 and Day 14 respectively). Mice were fasted

(no food or water) for 4 hrs, then gavaged with 360ug of PM or vehicle (on days of PM treatment), and 0.2mL of a standard sugar probe (6g/ml lactulose, 4g/ml of mannitol). Mice were then housed individually in metabolic cages for a total of 22hrs to allow for urine collection. After 22 hrs, urine was collected and analyzed as per manufactures instructions by ion exchange high-performance liquid chromatography (HPLC) to calculate the lactulose/mannitol excretion ratio. HPLC chromatography was carried out by Chris Lukowski.

2-2-7. Statistical analysis

Data was analysed using a student T-test and ANOVA to compare the results from PM treated mice and their control treated counterparts. P-values <0.05 were considered significant.

2-3. Results:

2-3-1. PM exposure alters gene expression in both the small and large intestine

First we wanted to examine the effect PM would have on the mucosal immune response at the transcriptional level. Gene expression profiles were generated in small and large intestinal tissue using a TLDA panel specific for 96 genes related to common mouse immune pathways (specific gene functions summarised in appendix 1, section B). These genes encode for various molecules involved in intracellular signaling, apoptosis, antigen presentation, migration/adhesion, as well as cytokines and various transcription factors.

Within the small intestine, 11 genes showed a ≥ 1.5 fold change in expression after 7 days of PM treatment compared to control. Genes up-regulated included those involved in antigen presentation (B2m), neutrophil, monocyte and T cell migration (Sele, Cxcl10), and the messenger molecule Nos2 (Table 2-1). Genes down-regulated in PM treated mice include cytokines (IL13, IL12α, IL5, IFNγ), a chemokine involved in T cell trafficking to secondary lymph nodes (Ccl19), and a cell marker for B cells (CD19) (Table 2-1). After 14 days of treatment with PM we saw a ≥ 1.5 fold change in expression of 18 more genes. Up-regulated genes included cytokines (IL1β, IL4, IL12α, IL12β, TNF), chemokines and adhesion molecules for monocytes, basophils, T and B cells (Cxcl10, Cxcl19, Ccl2, Cxcl11), co-stimulatory receptor (CD40), B cell marker (CD19), and cell signalling and signal transduction molecules (Nos2, Socs1) (Table 2-1). Down-regulated genes included cytokines (IL17, IL5) and a neutrophil adhesion/migration molecule (Sele).

In PM treated mice, a total of 39 genes showed a ≥ 1.5 fold change in expression within the colon after 7 days of treatment compared to control mice. Genes up-regulated in these mice included pro-inflammatory cytokines (IL12α, IL12β, and IFNγ), monocyte and lymphocyte adhesion/migration molecules (Sele, Vcam1, Fn1, Ccl2, Ccl19, Ccr7), immune cell markers (CD4, CD19), and co-stimulatory receptors (CD80, CD86, CD40 and CD40lg) (Table2-2). Genes down regulated in response to PM at 7 days included various cytokines (IL4, IL6, IL10, IL17, TNF, Csf2), cell signalling and signal transduction molecules (Nos2, Socs1,

Smad7, CD38), transcription factors (NFκB, Tbx21, Stat1), and chemokines (Ccl 3, Ccl5, Cxcl11) (Table 2-2). We also saw a decrease in gene expression for the immune cell marker CD8a and the T cell receptor complex component CD3e. At 14 days, 12 genes showed a further change in expression within the colon compared to control mice. Within these mice, only the genes for the pro-inflammatory cytokines IL12, IL17, and IL2 were up-regulated (Table 2-2). Genes down- regulated in PM treated mice include cytokines (IL1α, IL1β, IL4, IL5), a transcription factor (Tbox 21), chemokines for monocytes and lymphocyte recirculation (Ccl5, Ccl19), and a component of MHC1 (B2m) which is needed for antigen presentation (Table 2-2). These data suggest an increase in CD4^{+} T helper cells, monocytes, and B cells within the colon after 7 days of PM exposure and a decrease in CD8^{+} effector cells with decreased NfκB pathway activity. At 14 days there appears to be some inflammation within the colon still but no longer monocyte migration and decreased antigen presentation.

The lists of differentially expressed genes were then analysed with Ingenuity Pathway Analysis (IPA) software to identify functional networks that represent biologically relevant cellular pathways. IPA revealed the genes differentially expressed in the SI in response to PM were initially associated with pathways involving immune cell development and function at day 7 (Figure 1a) which was followed by immune pathways associated with cell signalling, interaction, and movement by day 14 (Figure 2-1b). This suggests an initial influx of inflammatory cells within the SI in response to PM, followed by increased

antigen presentation and cell development and proliferation. The differentially

expressed genes in the colon of mice treated with PM identified three possible

pathways in the IPA database. The first pathway focused on cell signalling and

interaction, as well as cell mediated immune responses. This pathway

concentrates on NfκB down-regulation and the downstream effects of this

pathway. Here the down-regulation in NfκB is linked to decreased expression of

IL6, IL4, TNFα, Csf2 and Ccl5 (Figure 2-1c). IFNγ gene expression appears to be

up-regulated as a result of increased IL12, and decreased TBX21 and NFkB. The

second pathway focused on the co-stimulatory molecules CD80 and CD86 as well

as the co-receptors CD4 and CD8 for T cells (Figure 2-1d). This pathway is

associated with cell death, signalling, interaction and tissue development. STAT1

down-regulation is at the center of the third pathway (Figure 2-1e) and is

associated with the decrease in expression of SOCS1, Nos2, IL15, TBX21 and

increase of CCL19 and CD40/ CD40lg. This pathway centers around cell

movement, and immune cell trafficking. G-protein coupled receptor and RELE

also lay at the center of this network. Therefore genes differentially expressed at

day 7 in the colon are associated with cell signalling, interaction, movement and

death with further gene expression differences related to cell development, and

maintenance appearing by day 14 (Figure 2-1f).

Table 2-1: Gene expression levels in Small intestinal tissue after exposure to particulate matter for 7 and 14 days

Relative biological function	Symbol	Gene	7 Day exposure		14 Day exposure	
			Fold change	P value	Fold change	P value
Cytokine						
	Il1β	Interleukin 1B			**1.58***	**0.01**
	Il4	Interleukin 4			1.94	0.73
	Il5	Interleukin 5	-1.7	0.21	-1.64	0.66
	Il12α	Interleukin 12a	-2.11	0.52	4.66	0.33
	Il12β	Interleukin 12b			1.98	0.12
	Il13	Interleukin 13	-2.54	0.48		
	Il17	Interleukin 17			-1.94	0.67
	IFNγ	Interferon g	-1.58	0.74		
	TNF	Tumor necrosis factor			1.58	0.33
Cell Signalling	Nos2	Nitric Oxide synthase, inducible	1.6	0.51	1.58	0.67
	Agtr2	Angiotensin II receptor	-1.55	0.71	-1.55	0.69
Signal Transduction						
	Socs1	Suppressor of cytokine signalling 1			1.6	0.09
Cell Adhesion/Migration						
	Sele	Selectin E	7.8	0.09	-1.84	0.58
	Cxcl10	Chemokine (C-X-C motif) ligand 10	1.82	0.25	1.95	0.3
	Ccl19	Chemokine (C-C motif) ligand 19	-2.13	0.63	4.8	0.26
	Ccl2	Chemokine (C-C motif) ligand 2			2	0.11
	Cxcl11	Chemokine (C-X-C motif) ligand 11			1.77	0.22
	Ccr7	Chemokine (C-C motif) receptor 7			2.05	0.42

Table 2-1 cont. Gene expression levels in Small intestinal tissue after exposure to particulate matter for 7 and 14 days

Relative biological function	Symbol	Gene	7 Day exposure		14 Day exposure	
			Fold change	P value	Fold change	P value
Co-stimulatory molecule						
	Cd40	Cluster of Differentiation 40			2.1	0.14
Cell marker	Cd19	Cluster of Differentiation 19	-2.39	0.56	10.91	0.15
Antigen Presentation						
	B2m	Beta-2-Microglobulin	7.3	0.35		

Differential gene expression was measured in the small intestine of female 129 SvEv mice after 7 and 14 days of PM10 oral exposure using a TLDA mouse immune gene array. Genes were grouped according to relative effector functions as: cytokine, intracellular signalling and signal transduction, transcription factors cellular adhesion and migration, co-stimulation, cell surface markers, and antigen presentation. Values with * are statistically significant with a P<0.05.

Table 2-2: Gene expression levels in colon tissue after exposure to particulate matter for 7 and 14 days

Relative biological function	Symbol	Gene	7 Day exposure		14 Day exposure	
			Fold change	P value	Fold change	P value
Cytokine						
	Il1α	Interleukin 1a			-1.65	0.26
	Il1β	Interleukin 1B			**-1.75***	**0.05**
	Il2	Interleukin 2			1.59	0.66
	Il4	Interleukin 4	-2.96	0.53	-4.21	0.41
	Il5	Interleukin 5			-3.46	0.11
	Il6	Interleukin 6	-1.56	0.19		
	Il10	Interleukin 10	**-2.29***	**0.005**		
	Il12α	Interleukin 12α	2.28	0.61	6.44	0.15
	Il12β	Interleukin 12β	1.77	0.19		
	Il15	Interleukin 15	-1.54	0.18		
	Il17	Interleukin 17	-3.72	0.31	5.4	0.18
	IFNγ	Interferon γ	1.68	0.55		
	TNF	Tumor Necrosis Factor	**-1.73***	**0.04**		
	Csf2	Colony stimulating factor 2	**-3.68***	**0.03**		
Cell Signalling	Nos2	Nitric Oxide synthase, inducible	**-1.73***	**0.02**		
	Gzmb	Granzyme B	-3.34	0.28		
	Ece1	Endothelin converting enzyme 1	-1.86	0.09		
	Agtr2	Angiotensin II receptor	1.65	0.27	-1.89	0.14

Table 2-2 cont. Gene expression levels in colon tissue after exposure to particulate matter for 7 and 14 days

Relative biological function	Symbol	Gene	7 Day exposure		14 Day exposure	
			Fold change	P value	Fold change	P value
Signal Transduction						
	CD38	Cyclic ADP-ribose hydrolase	**-1.94***	**0.004**		
	Socs1	Suppressor of cytokine signalling 1	**-1.76***	**0.007**		
	Smad7		**-1.75***	**0.003**		
Transcription Factor						
	Tbx21	T-box21	-3.37	0.11	-1.85	0.3
	Nfkb1	Nuclear factor kappa-light chain enhancer of activated B cells 1	**-1.6***	**0.05**		
	Stat1	Signal transducer and activator of transcription	-1.54	0.1		
Cell adhesion/ migration						
	Fn1	Fibronectin 1	1.58	0.33		
	Ccl19	Chemokine (C-C motif) ligand 19	1.77	0.46		
	Ccl2	Chemokine (C-C motif) ligand 2	1.52	0.28		
	Ccr7	Chemokine (C-C motif) receptor 7	3.07	0.12		
	Ccl3	Chemokine (C-C motif) ligand 3	-1.59	0.38		
	Ccl5	Chemokine (C-C motif) ligand 5	-2.45	0.15	-1.66	0.14
	Cxcl11	Chemokine (C-X-C motif) ligand 11	-6.73	0.15		

Table 2-2 cont. Gene expression levels in colon tissue after exposure to particulate matter for 7 and 14 days

Relative biological function	Symbol	Gene	7 Day exposure		14 Day exposure	
			Fold change	P value	Fold change	P value
Cell adhesion/ migration	Ccl 13	Chemokine (C-C motif) ligand 13			-1.62	0.25
	Sele	Selectin E	2.04	0.54		
	Vcam1	Vascular cell adhesion molecule 1	**1.94***	**0.04**		
	Cxcr3	Chemokine (C-C motif) receptor 3	**-1.78***	**0.006**		
Co stimulatory receptor/ molecules	Icos	Inducible co-stimulator	1.6	0.07		
	Cd40	B cell surface antigen CD40	1.52	0.26		
	Cd40lg	CD40 ligand	1.88	0.35		
	Cd80	B7-1 antigen	**2.6***	**0.003**		
	Cd86	B7-2 antigen	**1.81***	**0.02**		
Cell marker	Cd4	T cell surface glycoprotein Th cells	**1.86***	**0.02**		
	Cd3e	T cell receptor complex, epsilon subunit	**-1.83***	**0.05**		
	Cd8a	T cell surface glycoprotein CD8 α	-1.72	0.24		
	Cd19	Differentiation antigen CD19	1.69	0.53		
Antigen Presentation	B2m	Beta-2-microglobulin			**-1.56***	**0.01**

Differential gene expression was measured in the colon of female 129 SvEv mice after 7 and 14 days of PM10 oral exposure using a TLDA mouse immune gene array. Genes were grouped according to relative effector functions as: cytokine, intracellular signalling and signal transduction, transcription factors cellular adhesion and migration, co-stimulation, cell surface markers, and antigen presentation. Values with * are statistically significant with a P<0.05

Fig 2-1a: IPA gene pathway: <u>small intestine-</u> day 7
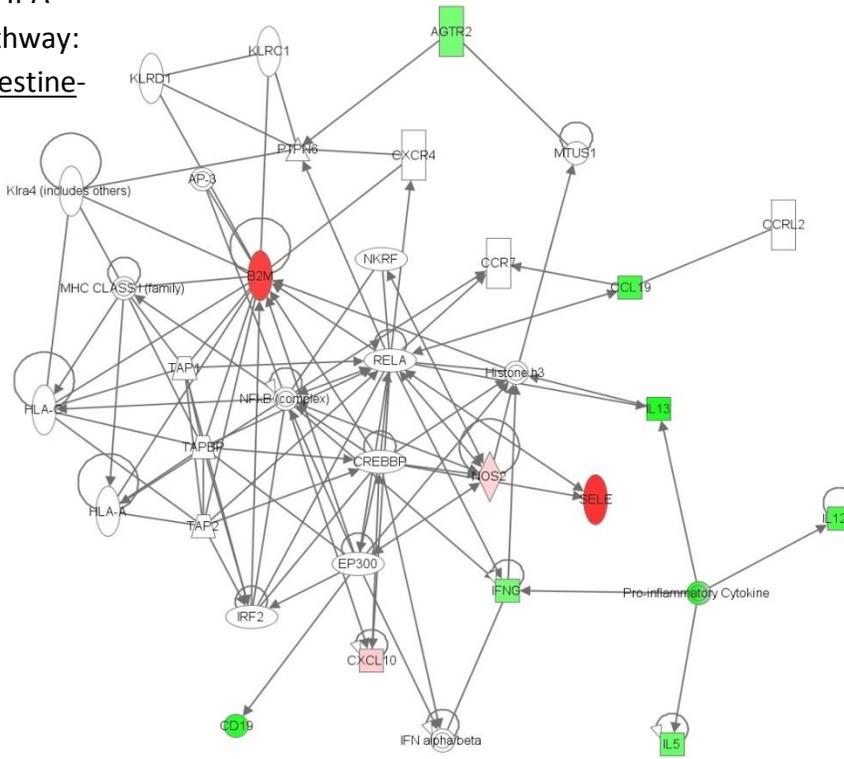

Fig 2-1b: IPA gene pathway-<u>small intestine</u> day 14

Fig 2-1c: IPA gene pathway 1- <u>colon</u> day 7
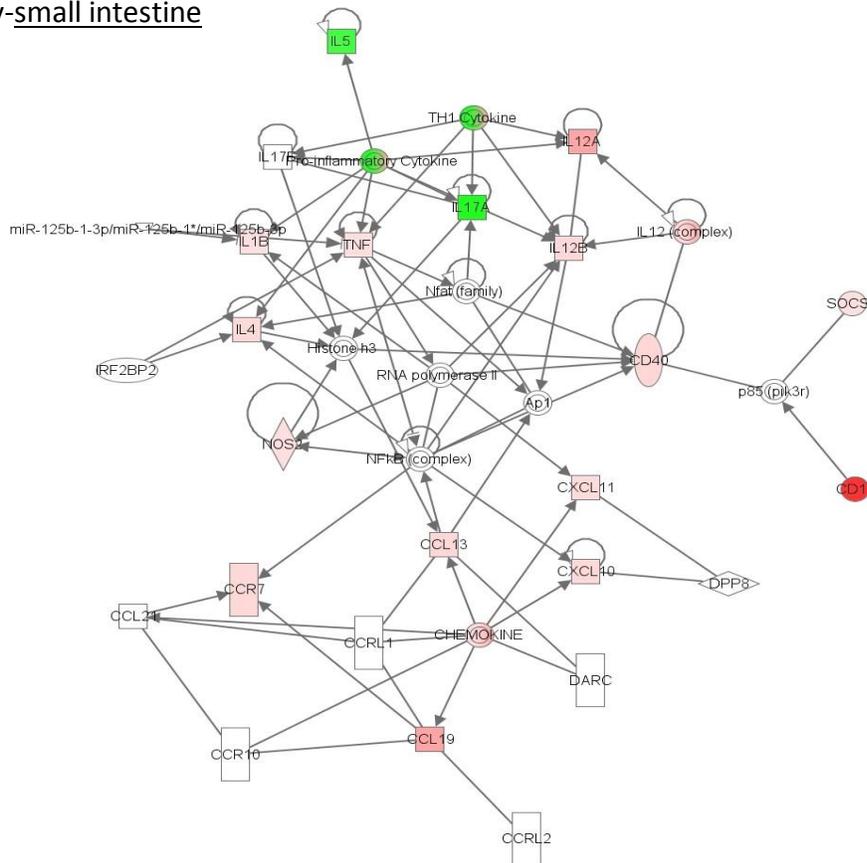
Fig 2-1d: IPA gene
pathway 2-

<u>colon</u> day 7
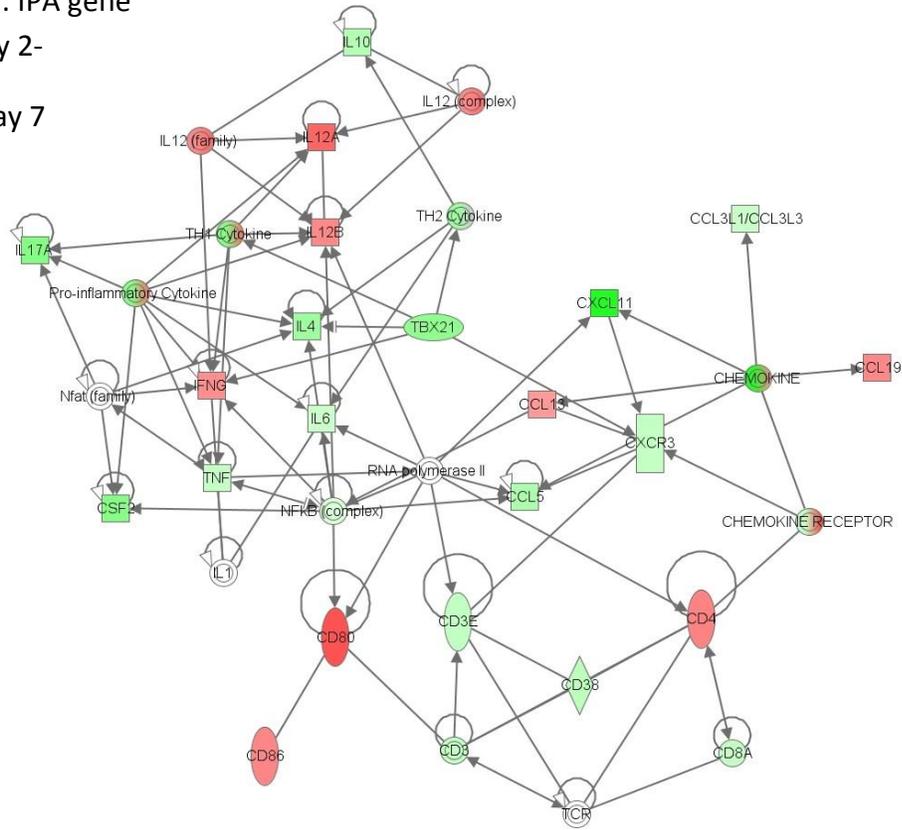
Fig 2-1e: IPA gene
pathway 3- <u>colon</u> day 7
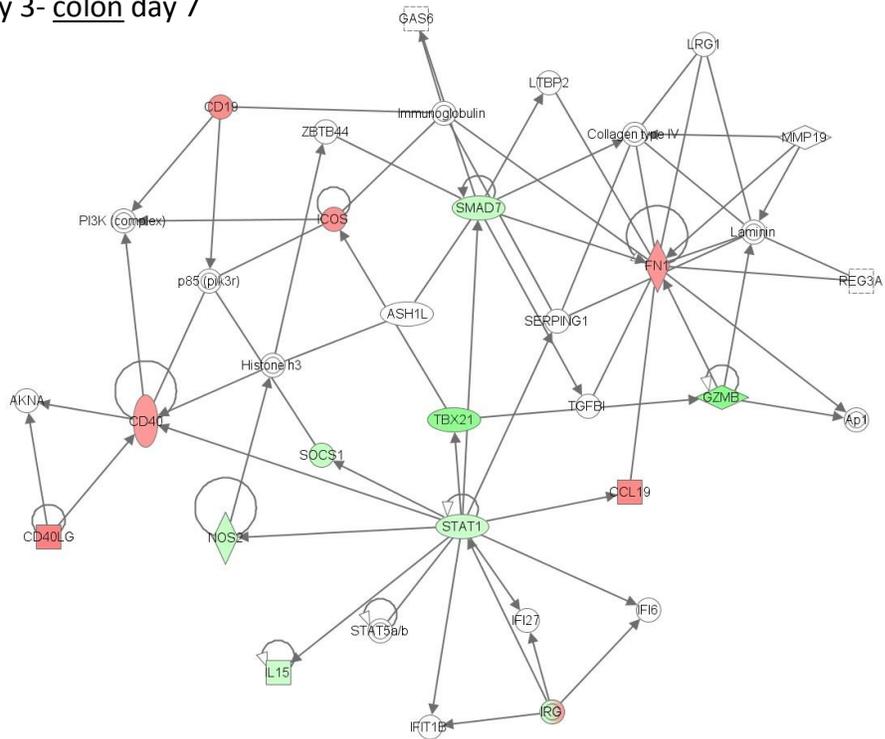
Fig 2-1f: IPA gene pathway- <u>colon</u> day 14

Legend: Up-regulated (green) / Down-regulated (red)

Cytokine/chemokin — Surface Receptor — Transcription Factor — Enzyme — G-protein coupled receptor — Other
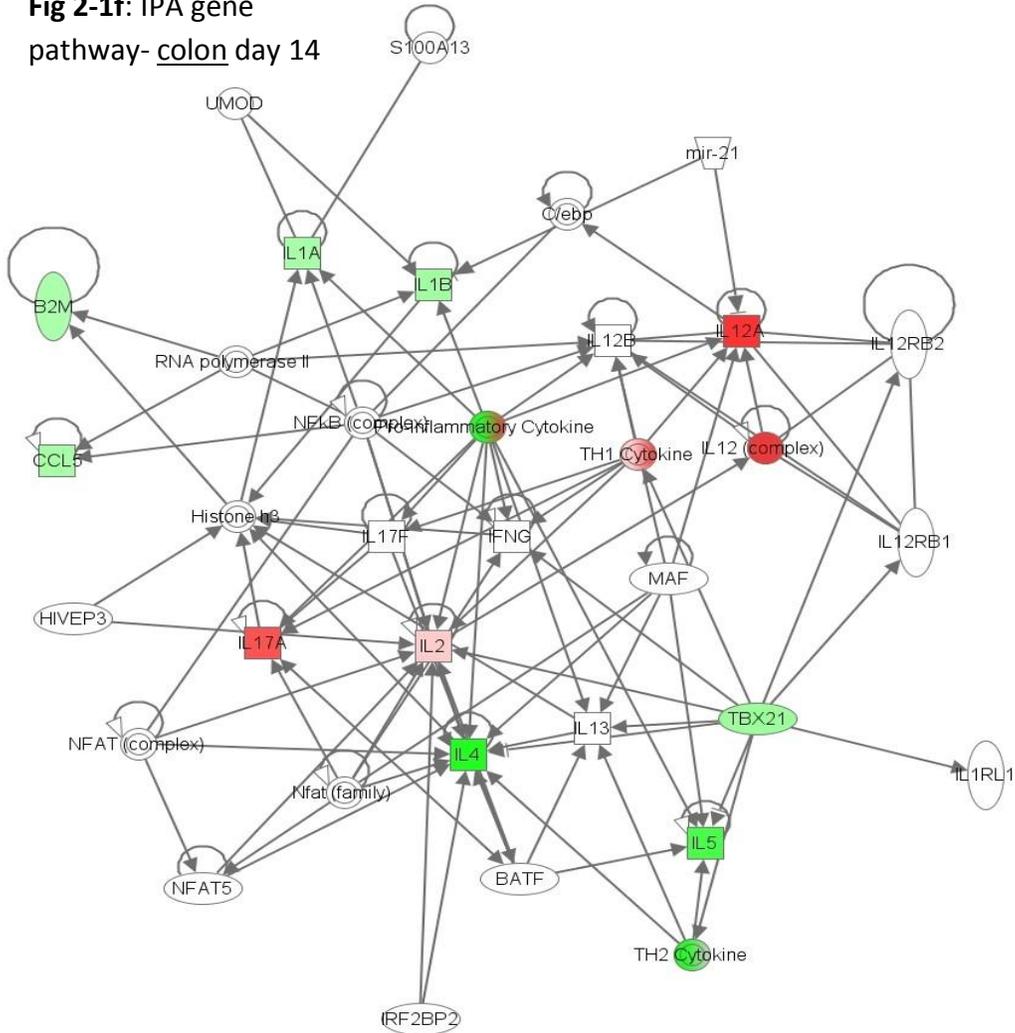
Figure2-1: IPA software analysis preformed on the up-regulated/down-regulated gene expression results in the small intestine and colon of mice treated with PM for 7 or 14 days compared to control. Arrows represent association between suggested genes in specific immune related pathways.

2-3-2. PM exposure elicits a transient increase in pro-cytokine secretion in the small intestine but not the colon

An increase in the secretion of pro-inflammatory cytokines (IL1b, IL2, IL12, IFNγ, and TNFα) was observed in the small intestines of mice exposed to the PM for 7 days compared to control mice (Figure 2-2). There was also an increase in CXCL1 secretion, which is a chemokine associated with intestinal inflammation, and an increase the anti-inflammatory cytokine IL5 in the small intestines of mice exposed to PM (Figure 2-2). After 14 days, there was no longer any difference in secretion of IL2, IL12, IL1β, IL10, TNFα and CXCL1 between mice exposed to PM and control. We did observe however, a significant decrease in IFNγ secretion in mice gavaged PM compared to their control after the 14 days (Figure2). We also saw no differences in IL17, and IL5 secretion within the first 14 days of PM exposure compared to controls in the small intestine. These data suggest that there is an acute TH1 inflammatory response within the small intestine in response to PM

The response within the colon to the PM appears to be quite different then that observed in the small intestine. There was an initial increase in IL5 secretion on day 7 and a significant decrease in IL2 secretion after 14 days in mice treated with PM (Figure 2-3). We observed no differences in secretion levels between mice exposed to PM and controls for IL1b, IL12 and CXCL1 (Figure 2-3). This could suggest that PM is not eliciting a TH1 inflammatory response

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within the colon as there was no increase in key inflammatory cytokines such as

IL12 which is important in driving the immune system towards a TH1 response.

We also saw no differences in IL17 within the first 14 days of PM exposure

compared to controls in the colon (Figure 2-3)

Figure2-2a

CXCL1 secretion

Control mice
PM mice

y-axis: **pg/mg of tissue** (0, 500, 1000, 1500, 2000, 2500)

x-axis: Day 7, Day 14

Day of treatment

Figure 2-2b

IL12 secretion

Control mice
PM mice

y-axis: **pg/mg of tissue** (0, 50, 100, 150, 200, 250)

x-axis: Day 7, Day 14

Day of treatment

Figure 2-2c

Il10 secretion

Figure 2-2d

IL1B secretion
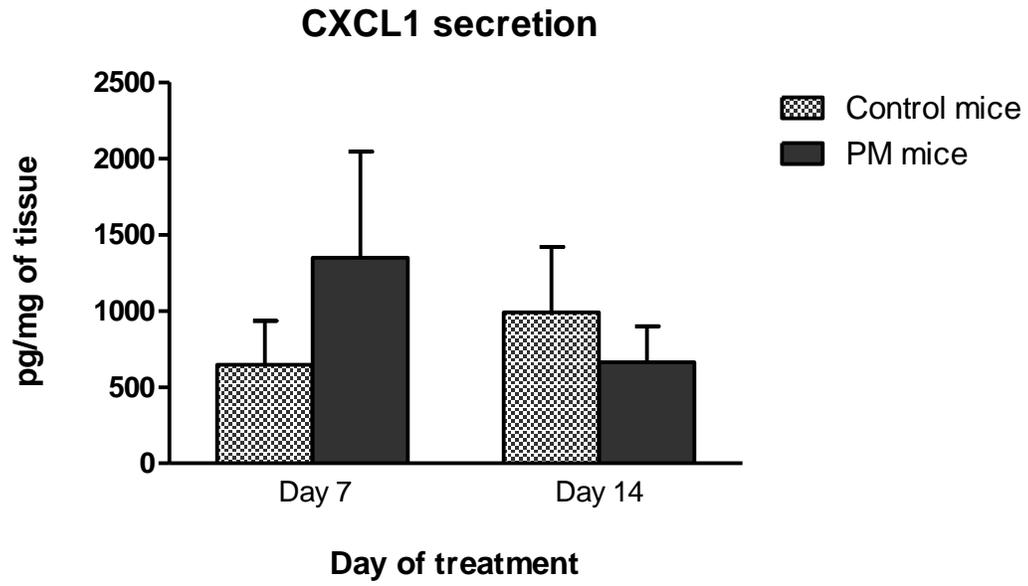
Figure 2-2e
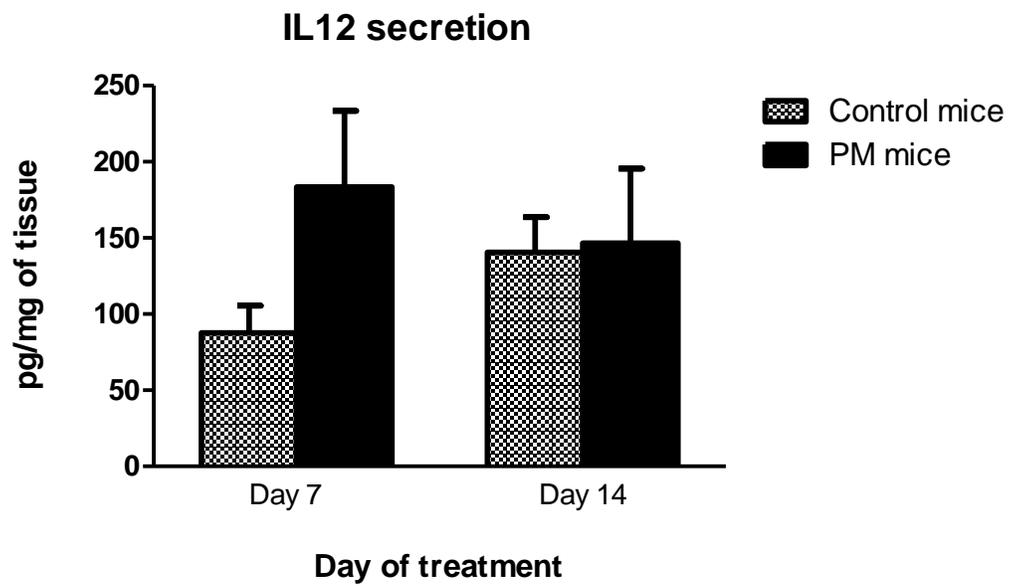
IL2 secretion
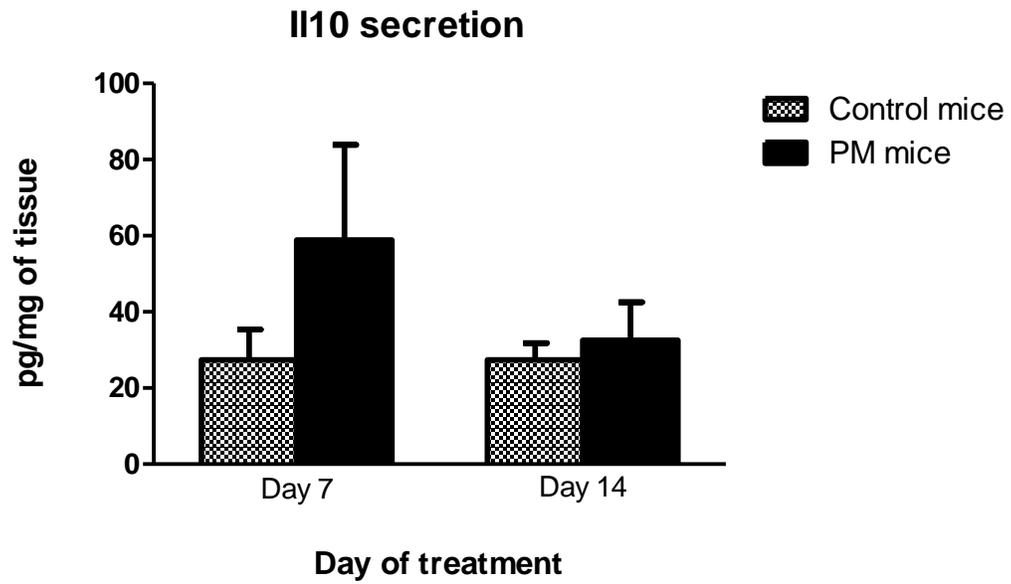
Figure 2-2f
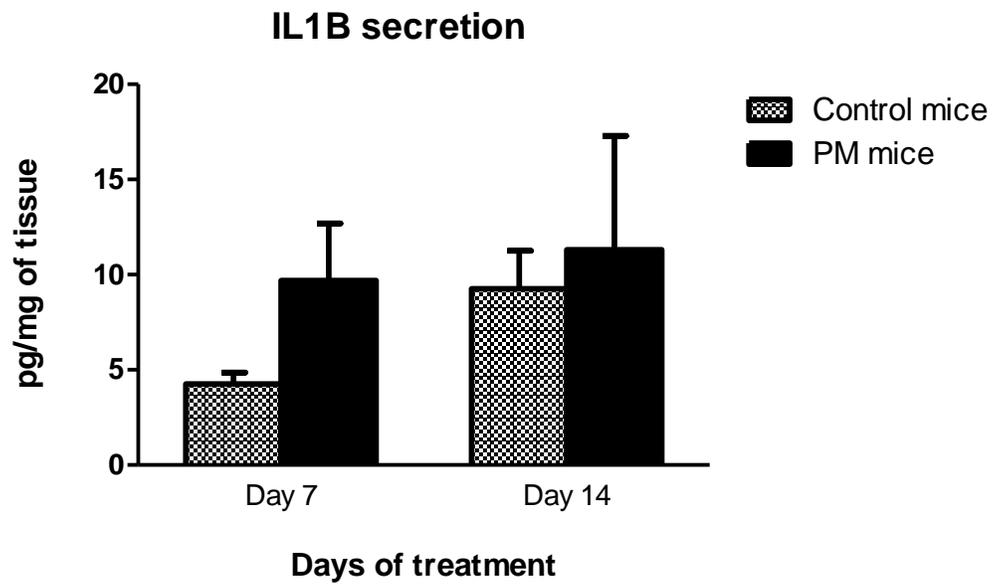
IFNg secretion
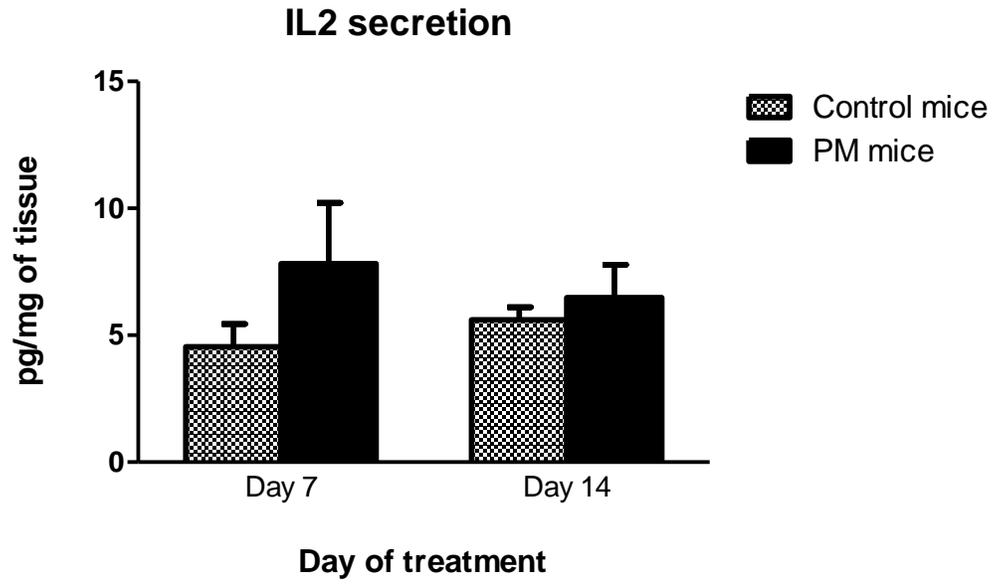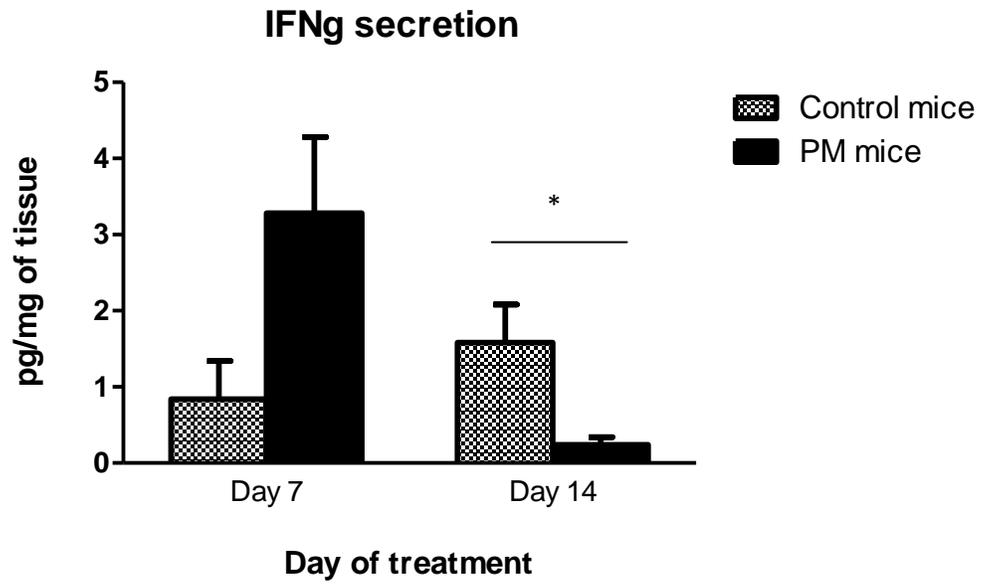
Figure 2-2g
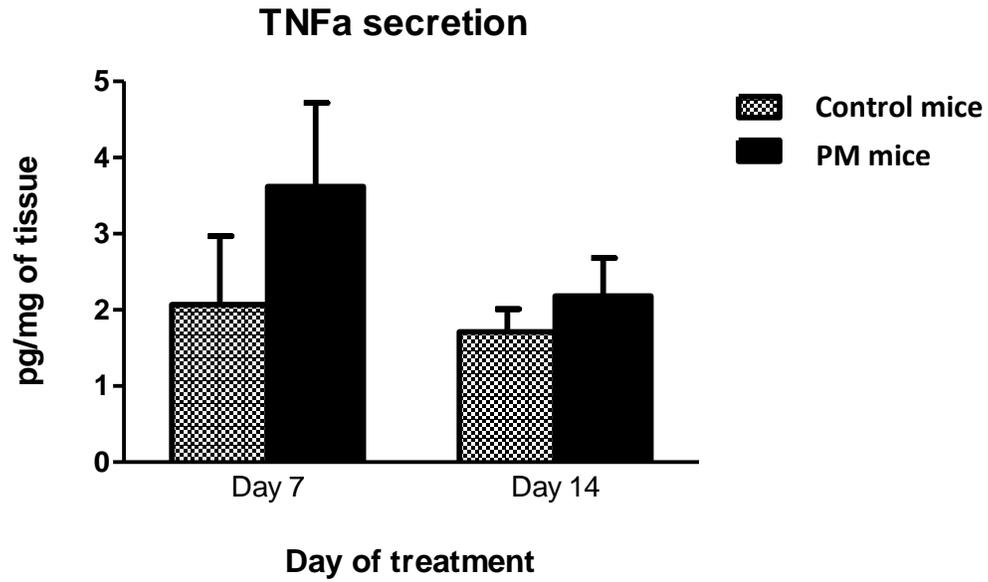
TNFa secretion

Figure 2-2: Small intestinal secretion levels for **a)** CXCL1, **b)** IL12, **c)** IL10, **d)** IL1β, **e)** IL2, **f)** IFNγ, and **g)** TNFα. Secretion was measured on day 7 and 14 of experiment, in both PM treated or control mice. Cytokine secretion levels are expressed in pg/mg of tissue to normalize the data,

Values displayed as mean ± SEM (n=5 in all groups)

 * Represents significance between PM treated mice and control at that time point (P<0.05)

Figure 2-3a

CXCL1 secretion

Figure 2-3b

IL12 secretion

Figure 2-3c

Il10 secretion

Figure 2-3d

IL1B secretion

Figure 2-3e

IL2 secretion

Figure 2-3f
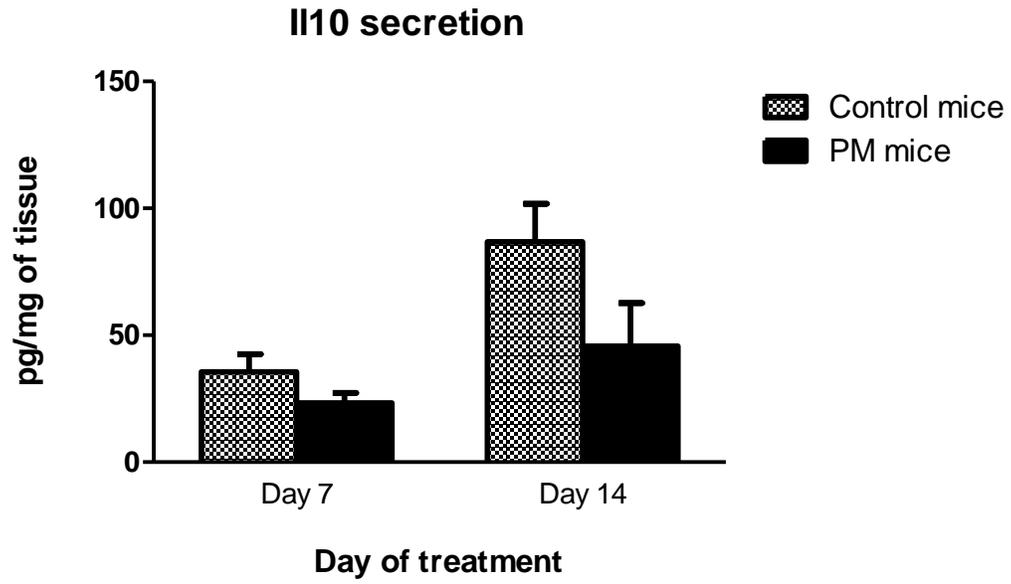
IL5 secretion
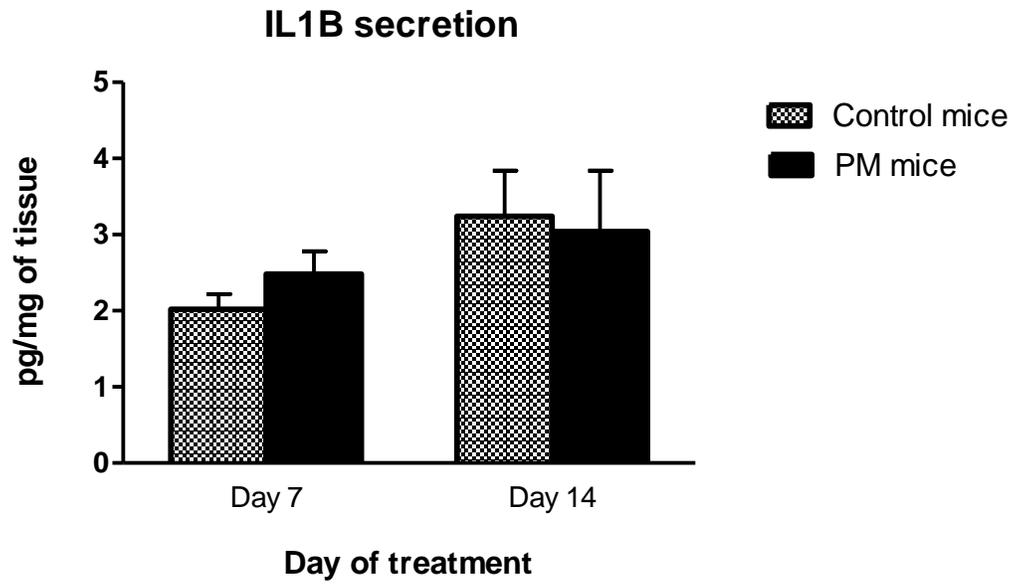
Figure 2-3g
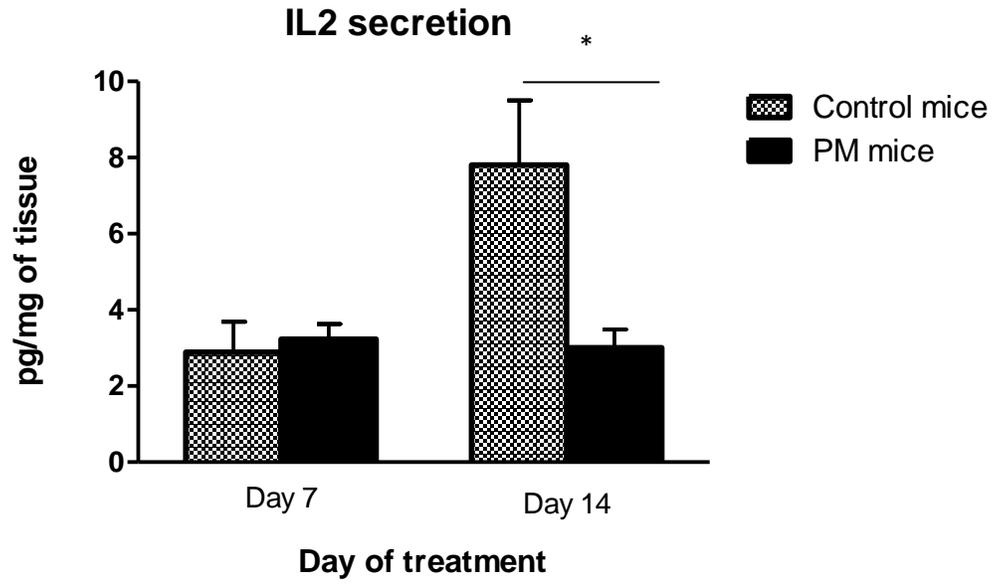
IFNg secretion
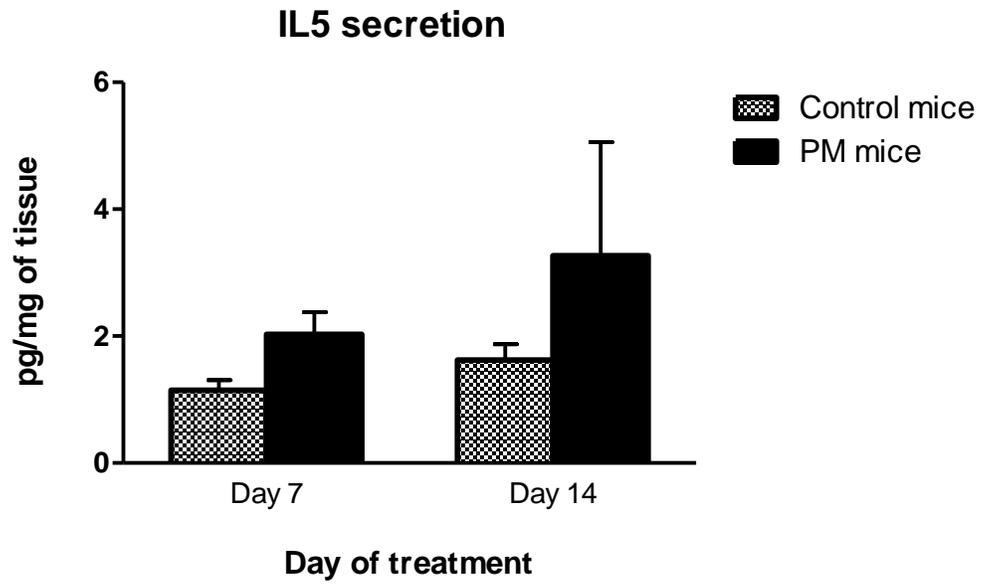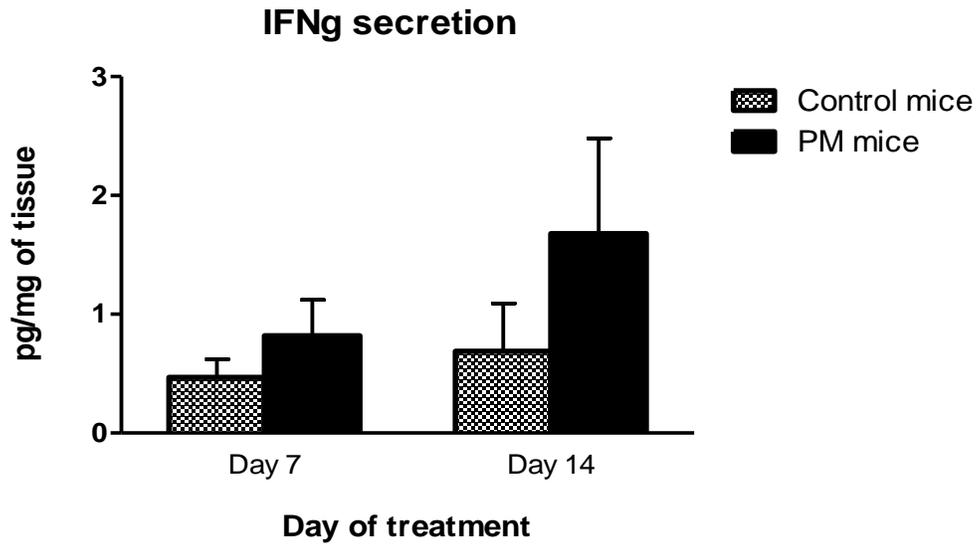
Figure 2-3h
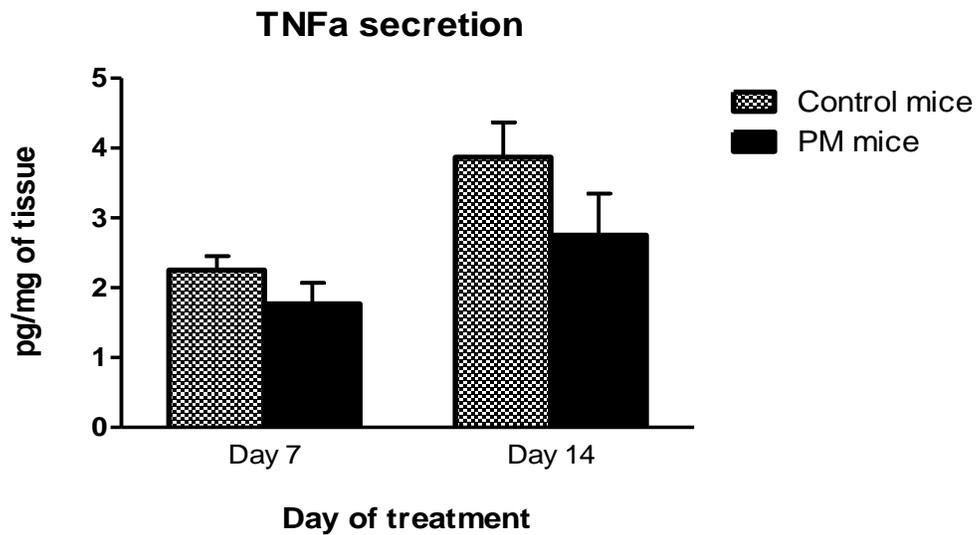
TNFa secretion

Figure 2-3: Colonic secretion levels for **a)** CXCL1, **b)** IL12, **c)** IL10, **d)** IL1β, **e)** IL2, **f)** IL5, **g)** IFNγ, and **h)**TNFα. Secretion was measured on day 7 and 14 of experiment, in both PM treated or control mice. Cytokine secretion levels are expressed in pg/mg of tissue to normalize the data. Values displayed as mean ± SEM (n=5 for all groups) * represents significance between PM treated mice and control at that time point (P<0.05)

2-3-3. PM exposure transiently alters intestinal permeability

PM has the ability to disrupt tight junction proteins and increase permeability in intestinal epithelial cell cultures (5). As well, we have shown that exposure to PM increased IFNγ and T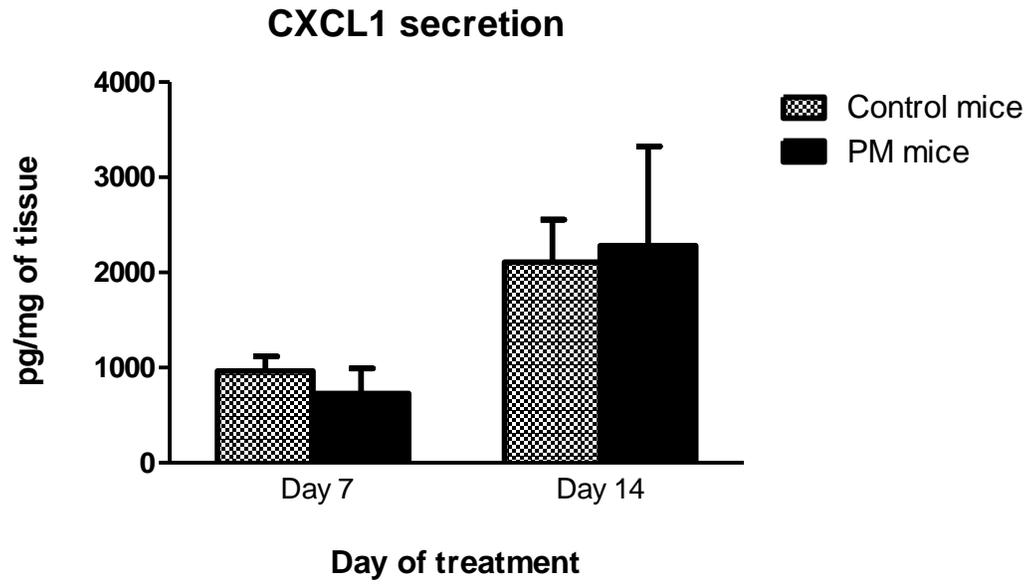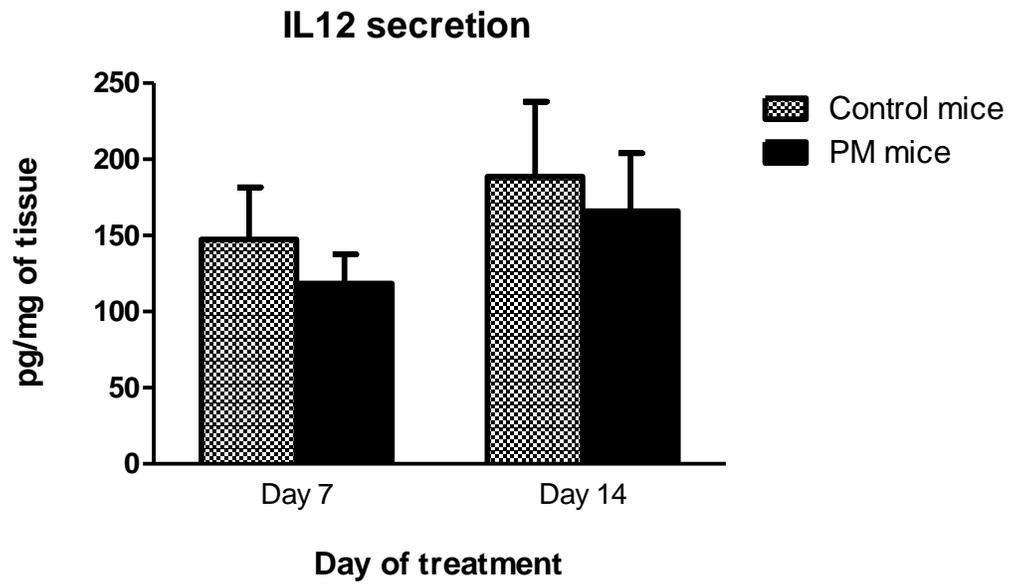NFα secretion in the small intestine of mice. These two cytokines also have the ability to disrupt intestinal barrier function and increase cell permeability within various cell lines (21-23). Therefore we wanted to see if intestinal permeability was altered in our mice in response to PM exposure. We measured permeability in the mice by examining the lactulose/mannitol (Lac/Man) excretion levels in the urine on days 7 and 14 of treatment. The higher the ratio, the more sugar excreted in the urine, the more permeable the intestine. There was a trending increase (although statistically insignificant) in the Lac/Man ratio at 7 days in mice gavaged PM compared to control mice (Figure 2-4). The concentration of sugar in the urine of PM treated mice at 14 days is similar to that seen in the control mice. This suggests a possible transient increase in intestinal permeability in response to the PM.

Figure 2-4
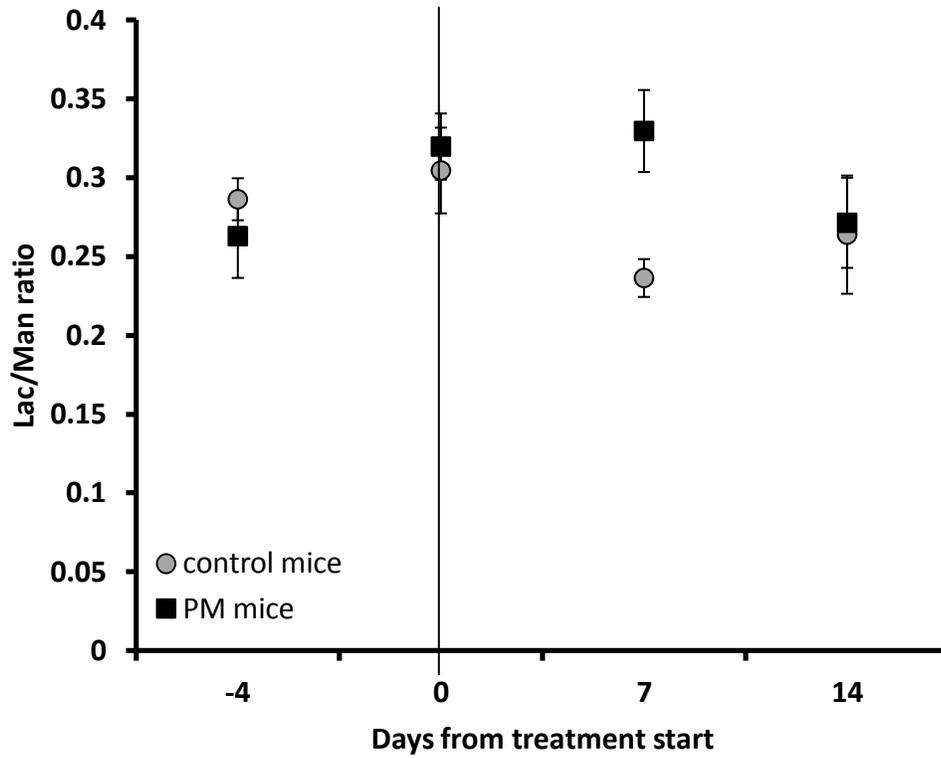
Figure 2-4: Intestinal permeability (Lac/Man ratio) of WT mice gavaged with vehicle (control) or 360ug of PM for 7 or 14 days. Samples were taken prior to experimental treatment (day -4) as a measure of baseline sugar excretion, start of experimental treatment (day 0), and on day 7 and 14 of treatment.

Values displayed as mean ± SEM (n=5 for all groups)

2-3-4. PM exposure alters systemic immune function

Our previous results indicate oral exposure to PM can initiate an inflammatory reaction within the intestinal mucosa. This lead us to ask the question whether or not ingested PM could also produce a systemic immune response in the mice. To measure this, splenocytes were isolated from vehicle or PM treated mice on day 7 and 14. The splenocytes ability to proliferate in response to PM particles was then measured *in vitro* and displayed in Figure 2-5 as a ratio of anti-CD3 activation to normalize the data. There was a significant increase in proliferation of splenocytes from 7 day vehicle treated mice towards 0.5ug/mL of PM, while a similar, increase in splenocytes proliferation in response occurred to 5ug/mL of PM (Figure 2-5). This suggests the particles are able to elicit a systemic immune response. Splenocytes isolated from PM treated mice also proliferated in response to 0.5 and 5 μg/mL of PM (although this was not statistically significant, p value of 0.07 and 0.2 respectively) (Figure 2-5). Interestingly, splenocytes from the PM treated mice proliferated less to the 0.5 and 5ug/mL concentration of PM particles then splenocytes from vehicle treated mice (Figure 5). This could suggest possible immunosuppression is occurring in response to the PM in mice that have been continuously exposed to the particles. Similar results were observed in splenocytes isolated at day 14 (Figure 2-5).

Figure 2-5a

Day 7 proliferation

Figure 2-5b

Day 14 proliferation
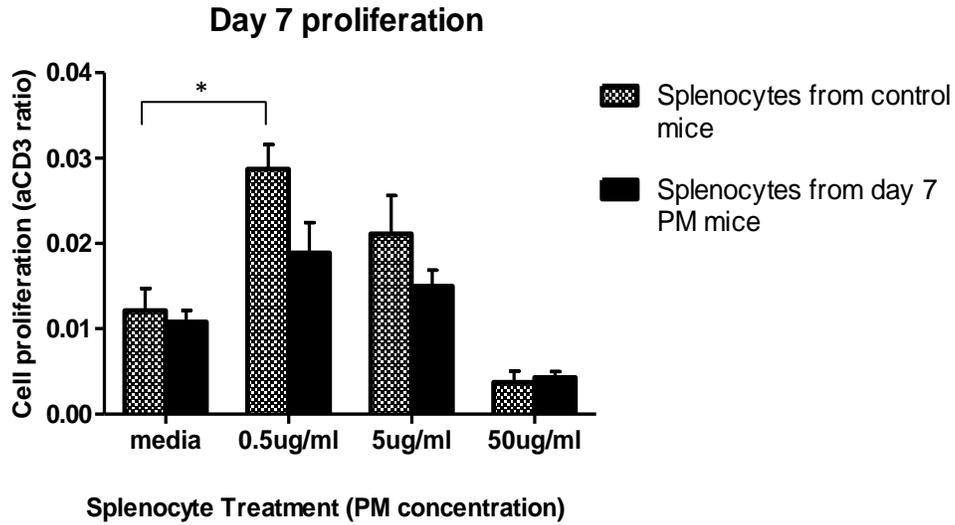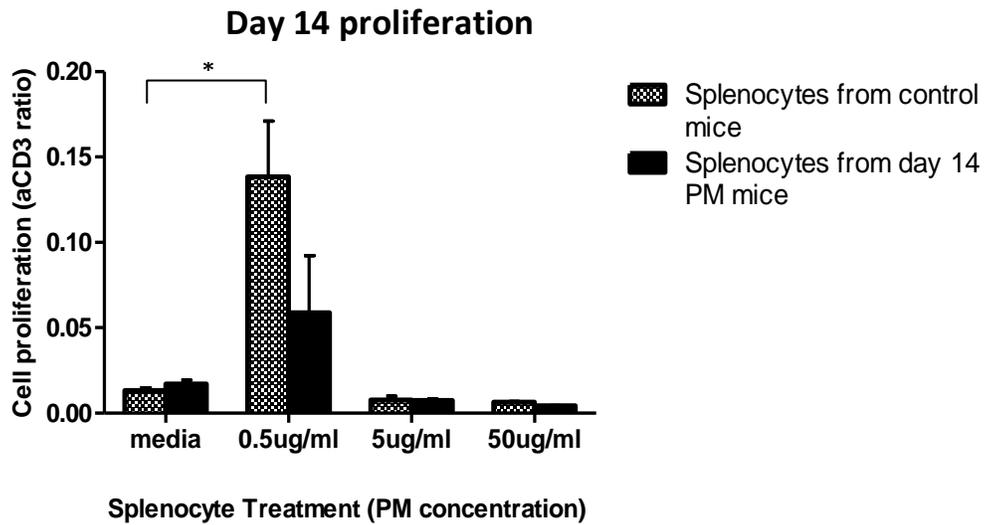
Figure 2-5: Splenocytes were isolated from PM treated mice or control mice on **a)** day 7 and **b)** day 14 or experimental protocol. Splenocytes were then incubated with media alone, aCD3, and increasing concentrations of PM solution for 24 hrs and measured for cellular proliferation. Data is expressed as a ratio of aCD3 proliferation for normalization. Values displayed as mean ± SEM (n=5)

*represents significance between splenocytes incubated with PM solution and splenocytes incubated in media alone. P<0.05

2-4. Discussion:

Until recently, most of the adverse health effects associated with airborne pollutants were believed to be confined to the respiratory and cardiovascular systems alone, but several reports have now linked air pollution particles with negative health effects in the GI tract (5-13). These studies however, have focused mainly on the ability of the particles to cause DNA damage and oxidative stress in colon epithelial cells and less on the intestinal immune response that can follow; therefore we wanted to examine the acute effects of oral exposure to particulate matter (PM_{10}) on mucosal and systemic immune function and intestinal permeability.

We demonstrate at both the level of transcription and protein translation the ability for PM_{10} to initiate an acute intestinal inflammatory response in WT 129SvEv mice. Similar to previous studies involving the bronchial epithelial response to PM (24-26), we saw an increase in tissue secretion for various Th1 and Th2 cytokines, up-regulation of adhesion molecules along with their complementary ligands on inflammatory cells, and the recruitment and activation of various leukocytes including neutrophils, macrophages, and lymphocytes within the small and large intestines. PM_{10} also transiently increased gut permeability and altered systemic immune function.

Cytokine secretion and relative gene expression in the small and large intestine of mice exposed to PM was measured to determine if PM could elicit an

intestinal immune response in the mucosa. Any response to a foreign antigen (Ag) will always involve the release of signalling molecules such as cytokines and chemokines as well as the activation of various immune cells in order to protect the tissue from invading organisms. In a normal intestinal immune response, antigen presenting cells (APC) will recognize this process and present a foreign Ag to nearby T cells in order to initiate a localized inflammatory response to the stimuli. This process is regulated by the cytokines present in the surrounding environment, as they provide the signals needed to initiate intracellular signalling pathways resulting in changes in gene expression and differentiation of these T cells into different lineages to create a customized, efficient immune response with Ag specific effector functions (27, 28). After 7 days of treatment, mice exposed to PM had a high amount of IL12 secreted from their small intestinal tissue. IL12 is predominantly produced by macrophages and dendritic cells (DC) in response to antigenic stimulation and is the key cytokine driving a T helper 1 (Th1) cell mediated effector response in naive T cells (29, 30). The Th1 type inflammatory response is characterised by increased secretion of IFNγ, TNFα and other pro-inflammatory cytokines such as IL1β, IL2, IL12, and IL18 from Th1 cells, natural killer cells (NK) and APC (27). At 7 days we also observed an increase in the pro-inflammatory cytokines IL1B, IL2, IFNγ, and TNFα in small intestinal tissue of mice treated with PM_{10}. This suggests an initial recognition and Th1 type inflammatory response to the PM by the mucosal immune system within the small intestine.

A cellular immune response within the tissue is also characterized by an increased activation of macrophages, enhanced T cell proliferation, and leukocyte infiltration (28). The increased secretion of IL12, NOS2 gene expression, and IL1B secretion (which is a pro-inflammatory cytokine, released mainly form activated macrophages and stimulates T cell proliferation by inducing IL2) (31), all imply an increase in macrophage activation in the small intestine of mice treated with PM for 7 days. IL2 is produced mainly by activated CD4 T cells in response to Ag stimulation (27). It is a key growth factor for T cells, thus suggests T cell proliferation in small intestinal tissue at day 7 of PM exposure. CXCL1 is a chemokine that attracts neutrophils and is considered a classic marker of intestinal inflammation. A high amount of CXCL1 was secreted from the small intestine of PM treated mice, and gene expression analysis revealed a 7 fold increase in Sele mRNA, a gene that is up-regulated in endothelial cells and is responsible for the accumulation of neutrophils to the site of inflammation. We also saw an increase in CXCL10 transcription, which is a chemokine that attracts monocytes, NK cells and T cells. This suggests there is an infiltration of various innate effector cells such as neutrophils, macrophages, NK cells as well as influx of T cells to the inflamed tissue in response to the PM at 7 days of treatment, indicating an acute Th1 mediated immune response within the small intestine.

The cytokine secretion and gene expression results within the small intestine are consistent with previous studies examining the effect of PM and

diesel exhaust on airway inflammation (32-35). Combustion derived PM has been reported to induce acute airway inflammation characterized by increased cytokine and chemokine secretion from bronchial epithelium and macrophages, up-regulation of endothelial and leukocyte adhesion molecules, and the migration of neutrophils and other inflammatory cells into the airways (24-26,39-41).

After 14 days of PM exposure however, I no longer observed the markers of the acute Th1 driven inflammation in the small intestinal tissue as secretion of IL1β, IL2, IL12, TNFα, IL10, and CXCL1 all returned to basal levels and a significant decrease in IFNγ secretion was observed. One possible explanation for the decrease in pro-inflammatory cytokine secretion could be due to increased production of the anti-inflammatory cytokine IL10 at day 7 in mice treated with PM. Anti-inflammatory molecules are normally released during an immune response to prevent unnecessary bystander damage to surrounding tissue (36) because prolonged inflammation can cause widespread cell death and lead to chronic inflammation. These molecules favour a Th2 type response and act by limiting the expansion of lymphocytes and return activated macrophages to a normal resting state thereby attenuating mucosal inflammation (36). IL10 in particular is a Th2 cytokine that has the ability to inhibit Ag presentation and the effector functions of activated macrophages as well as down regulate IFNγ and IL2 production by Th1 lymphocytes (37). Increased secretion of IL10 could then

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be responsible for the significant decrease in IFNγ and decrease in IL2 production measured at day 14 of PM treatment.

The cytokine secretion and gene expression profile observed at day 7 is indicative of an acute inflammatory response driven by innate inflammatory mediators. After 14 days however, there appears to be a shift towards an adaptive type response characterized by an increase in lymphocyte mediated effector functions. This can occur when the innate response is unable to clear a foreign Ag from the inflamed tissue or there is continuous stimulation by an Ag (as is the case in our study). The acute inflammatory response can't be resolved and prolonged inflammation occurs, causing a progressive shift in the effector cells present in the tissue towards macrophages and lymphocytes with Ag specific mediated functions (27). Gene expression analysis of small intestinal tissue from PM treated mice could indicate a shift in the type of immune cells present from the innate effector cells such as neutrophils and monocytes to adaptive immune cell types (i.e. lymphocytes). The decreased level of Sele mRNA and secretion of CXCL1 indicate neutrophils are no longer being attracted to the small intestine. We also observed an increase in Ccl2, CxcL10, and Cxcl11 gene expression at day 14, which have chemotactic activity for monocytes and activated T cells. There was also an increase in gene expression for the chemokine receptor Ccr7, which is expressed on activated T and B lymphocytes and is involved in their migration to and from inflamed tissue. This could then

suggest a decrease in the innate inflammatory cell response and an increase in adaptive immune response to PM after 14 days of continuous exposure.

I demonstrated that there was no increase in secretion for the Th2 mediated cytokines IL4 and IL5 in the small intestine at 7 days. IL4 is the driving signal needed to differentiate naive T cells towards the Th2 immune pathways, and IL5 is involved in B cell differentiation, and therefore these cytokines are needed promote a humoral type immune response which is effective against extracellular pathogens (27). At 7 days there was a decrease in the gene expression of the B cell receptor CD19, and a decrease in IL13 and IL5 mRNA which are involved in B cell maturation, differentiation and growth respectively; therefore, this suggests there is no Th2 response occurring within the small intestine in response to PM after 7 days of exposure. It's important to note that the effect a particular cytokine has on directing the immune response is dependent on the timing of its release, its abundance, the abundance of the cytokines receptor present on surrounding cells, and the local environment in which the cytokine is present (38). Therefore even with the presence of IL10 cytokine in the small intestine there can still be a Th1 mediated cell response occurring at day 7 of PM exposure. Gene expression data suggest a possible shift towards the Th2 humoral immune response after 14 days of PM treatment however. There was a 10 fold increase in CD19 mRNA in treated mice after 14 days of PM exposure. CD19 is a receptor on B cells that lowers the threshold needed for activation; therefore its expression is indicative of B cell presence.

There was also an increase in CD40 expression, which is the receptor involved in

T cell dependent immunoglobulin (Ig) class switching, memory B cell and

germinal center formation (28). Increased expression of these genes could

suggest initiation at the transcriptional level of B cell activity in the small

intestine after PM exposure. This is consistent with several studies examining the

effect of particulates on the respiratory system. Increased B cell recruitment

along with increase in Ig class switching have all been previously demonstrated in

bronchial epithelial cells (30,42), and some have reported the down regulation

of Th1 cytokines like IFNγ, and an increase in Th2 related cytokines and

chemokines in the bronchial mucosal of healthy volunteer exposed to diesel

exhaust (43-45)

The colon appeared to respond differently to the particles than the small

intestine. For instance, the acute inflammation observed at day 7 in the small

intestine which was characterized by pro-inflammatory cytokine secretion and

innate effector cell migration was not observed in the colon at the same time

point. There was no increase in the secretion of the pro-inflammatory cytokines

IL2, IL12, and TNFα as well as the chemokine CXCL1, which is a marker of

neutrophil infiltration and therefore intestinal inflammation. PM exposure also

resulted in significant decreases in Csf2 (which is a growth factor needed for the

production, differentiation, and function of granulocytes and macrophages) (28)

and NOS2 gene expression which along with the lack of IL12 (which is produced

by macrophages and DC in response to antigenic stimulation) cytokine secretion

could suggest a decrease in macrophage production and effector function in colonic tissue. There was also a decrease in Ccl5 expression, which is involved in migration of monocytes and eosinophils from the blood, and ligation with its receptor also activates eosinophils. Neutrophil, macrophage and granulocyte infiltration and effector function are the key cellular mediator in acute inflammation, they are required for initial recognition of a foreign Ag and release the inflammatory mediators needed to activate the mucosal immune response. Without their presence an acute inflammatory response is likely not occurring in the colonic tissue at day 7 of PM exposure

Gene expression analysis in the colon indicates the major cellular components present in the tissue are lymphocytes. There was an increase in transcription of the B cell markers CD19 and CD40, as well as CD40lg on activated T cells on day 7. Engagement of CD40 with CD40lg results in immunoglobulin class switching, memory B cell development and B cell germinal center formation (46). This indicates increased B cell activation and proliferation in the colon in response to PM exposure. There was also a significant increase in CD80 (B7.1) and CD86 (B7.2) expression and increased CD4 transcription after 7 days of PM exposure in the colon. CD80 and CD86 are co-stimulatory molecules present on APC and are essential for T cell activation and proliferation. They interact with either CD28 or CTLA4 on T cells to regulate the immune response. Interaction with CTLA4 transmits inhibitory signals to the T cells while CD28 engagement is crucial for complete and sustained activation of CD4+ T cells (28). The increased

expression of CD4 could then suggest interaction with CD28 on T cells to increase

CD4+ T cell proliferation. As well the increased gene expressions of Icos (which

enhances T cell response to foreign Ag and is essential for efficient interaction

between B and T cells) and IL12 (growth factor for activated T cells) could

indicate increased CD4+ T cell activation, proliferation and effector function.

After 7 days of PM exposure, an increase in gene expression was seen for

molecules involved in cell adhesion and migration of macrophages and activated

lymphocytes. Specifically there was an increase in lymphocyte expression of Ccr7

and colonic expression of its ligand Ccl19. Ccl19 is responsible for attracting Ag

engaged B cells and CCr7+ T memory cells towards the colon (46). This could

suggest migration of Ag specific lymphocyte towards the colon in mice exposed

to PM.

Gene array analysis also suggests a decrease in a Th1 effector response in

the colon. There was a significant decrease in Cxcr3 mRNA, which is expressed on

activated Th1 cells and its ligand CxcL11, suggesting a decrease in the migration

of Th1 cells in the colon at day 7. There was a decrease in CD8a expression, the

co-receptor present on cytotoxic T cells, which are the main effector cell in an Ag

specific Th1 mediated immune responses (28). A significant decrease in B2M

gene expression was also observed in colonic tissue after 14 days of PM

exposure. B2M is a component of MHC class 1, which is involved in Ag

presentation/interaction with CD8 + T cells. This suggests a decreased ability to

present Ag to cytotoxic T cells within the colon of PM. Overall this indicates a

decrease in Th1 cell migration and CD8 effector cell function in the colon after 7 days of PM exposure.

Interestingly I observed a significant decrease in expression of the nuclear transcription factor, NFκB in the colon of mice exposed to PM for 7 days. NFκB is present in almost all cells and an important transcription factor involved in the regulation of inflammation, apoptosis, and proliferation (47). In T cells, activation of the NFκB pathway requires ligation of the T cell receptor by a particular stimulus which initiates intracellular signalling pathways allowing NFκB to enter the nucleus and activate genes involved in T cell development, maturation and proliferation (47). However there was a significant decrease in the expression of CD3e, which is the component of the T cell receptor (TCR) complex involved in signal transduction. Decreased CD3e expression could then lead to decreased NFκB activation in T cells and therefore suggests a decreased ability for resident naive T cells within the colonic mucosa to undergo differentiation and proliferation.

The decreased expression of NFκB is inconsistent with other studies, which found the particles had the ability to generate free radicals and reactive oxygen species (ROS) which in turn activated NFκB in the bronchial epithelium and colonic epithelial cell lines (48,5). This difference can be due to the fact that NFκB expression was measured at the level of transcription in this study, while most studies measure NFκB activity at the protein level. Another reason for this

inconsistency could be due to the type of PM and exposure methods used in other studies. For instance, most *in vivo* studies examined the effect of $PM_{2.5}$ or diesel exhaust particulate (DEP) specifically on bronchial epithelial cells whereas this study looks at PM_{10} (49, 30). The amount of polycyclic aromatic hydrocarbons (PAH) present in $PM_{2.5}$ and diesel exhaust (almost entirely PAH) is a lot higher than the percentage in PM_{10} (20% PAH component) (32). The response of bronchial epithelial cells to air pollutants has been shown to vary depending on the principle components of the particles (50). This means the exact composition of particulates present in the PM will have different effects on the type of cellular response it can initiate. As well the NFκB activity was usually examined between 1hr and 24 hrs after a single PM or diesel exhaust exposure (51, 49). The mice in this study were exposed to PM on a daily basis and tissue was only analysed after day 7 and day 14 of treatment. If the activation of NFκB occurs quickly after the first exposure to PM, the window of increased NFκB transcription could easily be missed, as it is known the level of NFκB activity fluctuates throughout the immune response (52).

A significant decrease in IL2 cytokine secretion occurred in the colon of mice exposed to PM for 14 days. As previously mentioned, IL2 is produced mainly by activated CD4 T cells in response to Ag stimulation and is a key growth factor needed for T cell proliferation. This decrease suggests a lack of proliferation in naive T cells in the colonic tissue, which is consistent with the gene expression data at day 7. The significant decrease in CD3 and NFκB

observed at day 7 would prevent activation of the signalling pathways in naive T cells responsible for IL2 release, thereby blocking T cell development and proliferation. Interestingly there was also a significant decrease in IL1B gene expression at day 14 in the colon. This cytokine is important in stimulating T cell proliferation by inducing IL2 release. This suggests the presence of Ag specific lymphocytes present in the colon were probably activated and underwent Ag specific proliferated in the small intestine, then migrated to the colon, and not the result of differentiation and proliferation of resident naive T cells already present in the colon.

Various human exposure studies have demonstrated the ability for combustion-derived pollutants to initiate an acute inflammatory response in the airways, characterized by neutrophil, lymphocyte, and granulocyte influx and pro-inflammatory cytokine and chemokines secretion (50,24-26). Unfortunately, without flow cytometry or immunohistochemistry analysis, it cannot be confirmed by these studies whether there was an increase in these inflammatory cells within the small and large intestine; however an increase in gene expression of various endothelial and leukocyte adhesion molecules and cell surface molecules in response to PM exposure was demonstrated. This suggests an increase in acute cellular mediated effector cells in the small intestine and lymphocyte migration into the colon of mice treated with PM. These studies also reported up-regulation of similar endothelial adhesion molecules such as Vcam1 and cytokines in the bronchial epithelial cells as was observed in the intestinal

mucosa (35, 53-55). Some studies have also demonstrated in decrease in Th1 cytokine expression such as IFNγ and a shift towards a Th2 immune response with increased B cell infiltration and production of Th2 cells (56,57) which is similar to what was observed in intestinal mucosa in response to PM exposure.

The pro-inflammatory response associated with PM exposure is dependent on the transition metals and PAH component of air pollution. Presence of transition metals and PAH in PM_{10} is believed to generate reactive oxygen species (ROS) and oxidative stress in bronchial and colonic epithelial cells, thereby stimulating intracellular signalling pathways involved in inflammation (16-19,32-34). PAH have been directly associated with increased migration of various effector cells such as on lymphocytes, mononuclear cells, neutrophils, and eosinophils, and triggering the release of pro-inflammatory cytokines and chemokines (44). It has also been associated with B cell recruitment and IgE production (42). EHC-93 consists of about 20% PAH, and is made up of various transition metals such as chromium, lead, vanadium, and zinc (Appendix 1: **Table A**). This suggests the PM used in these studies should have similar effects in the induction of oxidative stress and DNA damage within the epithelium, thus initiating a mucosal immune response.

The acute inflammatory response initiated by PM in the small intestine was also accompanied by a transient increase in intestinal permeability. This increase in permeability might be a direct result of the PM itself or a secondary effect

caused by the activation of various inflammatory mediators by the particulate. PM has been shown to generate oxygen free radicals which induce oxidative stress in the epithelia resulting in increased permeability (48,58). PM has also been shown to disrupt tight junction structure in the Caco-2 intestinal epithelial cell line and decrease transcription of the tight junction proteins ZO-1 and occludin in the small intestine of mice exposed to PM (5). This implies that PM has the direct ability to increase intestinal permeability.

An acute inflammatory response in the intestine also has the ability to temporarily disrupt the epithelial barrier, specifically with increased secretion of IFNγ and TNFα. These two cytokines have been shown on numerous occasions to disrupt barrier function therefore increasing permeability in epithelial cell lines (21-23). Incubation of these cytokines with intestinal epithelial monolayers also results in the reorganization of many tight junction proteins including ZO-1, occludin, claudin-1, and claudin-4 (59). IFNγ has been shown to increase mucosal permeability by damaging the tight junctions between intestinal epithelial cells and can induce expression of various adhesion molecules which increase the migration of inflammatory cells (60). TNFα can also indirectly increase permeability by inducing the production of various proteases in intestinal stromal cells, which degrade the lamina propria extracellular matrix (61). The increased secretion of IFNγ in both the small and large intestine and the increased TNFα in the small intestine suggest the acute inflammatory response initiated in response to PM exposure along with the direct effect of the particles

themselves on intestinal epithelium may have contributed to the transient increase in intestinal permeability observed at day 7.

Disruptions in intestinal barrier can result in increased translocation of various luminal Ag including bacteria and bacterial components, food and other luminal products into the intestinal mucosa. The increased permeability observed at day 7 then could imply an increased transfer of luminal Ag into the interstium occurred, exposing the mucosal immune system to an influx of bacterial Ag and possibly PM particles as well. An increase in bacterial Ag presentation to mucosal immune cells could provide another explanation for the shift towards an adaptive immune response observed at day 14 in the small intestine.

An inflammatory driven alteration of barrier function in the epithelium might also increase the systemic translocation of the particles. Increased mucosal exposure could then increase systemic tolerance towards the particles. This could be one explanation for the decreased proliferation of splenocytes from mice treated with PM for 7 and 14 days to the PM particles in vitro.

Results from this study are consistent with other studies demonstrating the ability of particulate matter exposure to cause inflammation in the gut (5). Here I demonstrated PM_{10} had the ability to initiate an acute inflammatory response in the small intestine, which was characterized by an increase in tissue secretion for various Th1 and Th2 cytokines, up-regulation of adhesion molecules

along with their complementary ligand on inflammatory cells, and the recruitment and activation of various leukocytes including neutrophils, macrophages, and lymphocytes within the small and large intestines. PM_{10} also transiently increased gut permeability and altered systemic immune.

This has several implications regarding gastrointestinal disease. For instance, mice exposed to PM had a significant decrease in IL10 gene expression at day 14 and decreased secretion within the colon at days7 and 14. This anti-inflammatory cytokine is important in attenuating mucosal inflammation (28), and mice that lack IL10 develop colitis (62). At day 7 of PM exposure there was an increase in acute inflammation and intestinal permeability which could allow an influx of luminal Ag including bacteria into the mucosa. In genetically predisposed individuals, this influx of bacterial Ag along with a decreased ability to attenuate an inflammatory response could act as an environmental trigger and cause an exacerbation of gut inflammation.

Interestingly, diesel exhaust has been shown to increase susceptibility to bacterial pulmonary infections through the impairment of microbial defence mechanisms (63). Data from these studies suggest a similar effect occurs in the colon in response to PM exposure. At day 14 there was decreased expression of Th1 and $CD8^+$ effector T cells in the colon. Triggering the CD8 T cell pathway of the Th1 response produces a significant increase in pro-inflammatory cytokines and proliferation of Ag specific T cell clones allowing for an effective cell

mediated effector response against intracellular bacteria (28). There was also a significant decrease in B2M gene expression, which is a component of MHCI, which is involved in Ag presentation to CD8$^+$ T cells. Without an effective CD8$^+$ cytotoxic T cell response, the colon may not be able to mount an effective immune response against invading bacteria. This means pollutants my predispose individuals to intestinal inflammatory conditions such as IBD by altered immune function and its interaction with intestinal bacteria. However, histopathology and immunohistochemistry will need to be done in future studies to confirm these results.

In conclusion, I have demonstrated the ability for orally ingested airborne pollutants to elicit and acute inflammatory response in the small intestine which is accompanied by an increase in intestinal permeability, and which is followed by subsequent alterations in large intestinal immune function. These results therefore confirm my overall hypothesis which stated that oral exposure to PM would increase intestinal inflammation and alter permeability, and thus provide a possible mechanism whereby airborne particulate matter may play a role in triggering or exacerbating gastrointestinal disease.

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Chapter 3: Long term effects of particulate matter exposure on mucosal immunity and intestinal microflora in the mouse model of colitis.

3-1. Introduction:

Having shown an effect of short-term PM treatment on gut permeability and immune function, this next series of experiments was aimed at determining the long-term effects of PM treatment in both a wild-type mouse and in a mouse model of colitis and to determine if PM treatment would alter the gut microbiome. I hypothesized that because PM treatment altered permeability, treatment of the IL-10 deficient mouse would result in a more severe colitis and that this would be associated with changes in the gut microbiota.

3-2. Materials and Methods:

3-2-1. Particulate Matter (PM):

Ambient air particulate matter (EHC-93) was obtained from the videlon bag filters of the single pass air-purification system from the Environment Health Center in Ottawa, Canada. The characteristics of the PM have been previously described (1,2), and are presented in appendix 1 -**Table A-1**.

3-2-2. Animals and long term PM exposure experimental setup:

Adult Female 129 SvEv WT and 129 SvEv IL10 deficient (IL10$^{-/-}$) mice were each randomly assigned to the control or PM exposure treatment groups. Control WT and IL10$^{-/-}$ mice were placed on a standard mouse chow (Laboratory Rodent diet 5001, Lab Diet). PM treated WT and IL10$^{-/-}$ mice were placed on the same standard mouse chow treated with PM$_{10}$ (EHC-93) such that each mouse received a daily dose of 360ug of PM. The PM chow was made weekly by adding 0.09g of EHC-93 to 1kg of 5001 Lab Diet chow. Mice were placed on the two diets for a total of 35days with free access to the food and water. At sacrifice, portions of the colon and small intestine were homogenized to examine tissue expression of various cytokines. Cecal contents were also collected at the end of the experiment to study short chain fatty acid concentration, and stool samples were collected from all mice prior to (day 0) and following (day 35) treatment and analyzed for microbial composition. The protocol for use of mice was approved by the Health Science Animal Care and Use Committee at the University of Alberta, and animals were kept in a regulated day/night cycle with controlled humidity and temperature and had free access to food and water throughout the experiment.

3-2-3. Mucosal Cytokine expression:

Mucosal Immune function was examined by removing sections of the small and large intestine from both WT and IL10 deficient mice after 35 days of

treatment and snap freezing samples at -80 until analysis. Tissue samples were homogenized and examined for tissue expression as follows: briefly, frozen tissue samples were thawed in RIPA buffer with protease inhibitor (20ug/mL Phenylmethylsulfonyl fluoride, 15ug/mL Aprotinin, 2ug/mL Pepstatin A, 2 ug/mL Leupeptin) on ice. Samples were then topped up to 500uL with the RIPA buffer and sonicated for 15 sec and placed on ice to homogenize the tissue. The samples were then centrifuged at 10000rpm for 10min and supernatant was collected and snap frozen for later cytokine analysis. IL1Bβ IL2, IL4, IL5, CXCL1, IL10, IL12, IFNγ, and TNFα tissue expression was analyzed using a Th1/Th2 tissue culture MesoScale Discovery Kit (MesoScale Discovery) as per manufactures instructions. IL13 and IL17 cytokine expression was examined using standard ELISA kits (R& D systems) as per manufacturer's protocol.

3-2-4. Microbial analysis:

Changes in microbial composition were determined through the use of Terminal Restriction Fragment Length Polymorphism (T-RFLP). This was preformed from fecal samples collected on day 0 (prior to PM treatment) and day 35 (following treatment) of WT and IL10-/- mice on the chow alone or chow + PM. T-RFLP analysis was performed by Matt Emberg.

Briefly, total DNA was extracted from the fecal samples using a FastDNA Spin Kit for FECES (MP Bio) as per manufacturer's instructions. The broad range forward primer 6-FAM-8F (Applied Biosystems) (5'-AGAGTTTGATCCTGGCTCAG-

3') and broad range reverse primer 926R (Applied Biosystems) (5'-

AGAAAGGAGGTGATCCAGCC-3') were used to amplify 16S rRNA by PCR. The PCR

was performed using 50ng DNA, cycling conditions were: 94°C for 2 min followed

by 35 cycles of 94°C 1 min, 56°C 1 min, 72°C 1 min, and a final 10 min extension

at 72 °C. Every PCR run included a DNA-free template control, and amplification

was confirmed with visualization of a single 920kb PCR product on 1% agarose

gel. A Qiagen MinElute PCR purification kit (Qiagen) was used to purify the

amplicons, as per manufacturer's instructions. The Hpa II restriction enzyme

(Promega) was used to digest amplicon DNA (200-300ng, determined with a

Nanodrop spectrophotometer) (Thermo Scientific), the digestion occurred for

16hr at 37°C. With each sample, 100ng of HPA II digested fragments were

resolved in duplicate using a 3130XL Genetic Analyzer (Applied Biosystems).

Fragment length normalization was achieved by running an internal

ROX1000DBA marker to separate each sample. Bionumerics 6.0 software

(Applied Maths) was used to normalize the fluorescently labeled terminal

fragment lengths and select peaks of interest, which are associated, in silico,

with fragment lengths of known bacteria using Microbial Community Analysis 3

(MiCA; Shyu,2007) and Ribosomal Database Project v.9 (RDP; Cole, 2009). MiCA

takes the fragment lengths and primer data and associates the likely bacterial

sequence with each band in the trace. RDP uses the list generated from MICA to

cluster the sequences and establish likely bacteria and abundances. Fragments

with peaks between 25 and 650 base pairs in length are used in the community

composition and cluster analysis. Principle component/clustering analysis was then preformed on each sample and based on the band patterns alone from each trace to identify specific clusters.

3-2-5. Short chain fatty acid analysis:

Cecal contents were collect at sacrifice (day 35) from WT and IL10-/- mice on the control chow and chow with PM and flash frozen and stored at -80 °C for short chain fatty acid concentration analysis.

To begin, volatile fatty acids needed to be extracted from the cecal contents. Briefly, 0.1N HCl is added to the cecal material and allowed to shake (180x g) overnight at 25°C. The sample is then vortexed and 5mL of diluents collected and added to 1mL of meta-phosphoric acid (HPO_3, 25% w/v in distilled H_2O). The samples are mixed and frozen overnight, then thawed, vortexed, and centrifuged at 3000x g for 20min. Up to 2 mL of supernatant is then transferred into a GC vial (PerkinElmer) and sealed to be analyzed by gas chromatography by Dr. M.G. Ganzle (University of Alberta). Concentration of acetate, propionate, butyrate, isobutyrate, isovalerate, valerate and caproate was determined using external standards (sigma), and isocaproate was used as internal standard.

3-2-6. Statistical analysis

Data was analysed using a student T-test and ANOVA to compare the results from PM treated mice and their control treated counterparts. P-values <0.05 were considered significant.

3-3. Results:

3-3-1. Long term PM exposure elicits an increase in pro-inflammatory cytokine expression in the colon of WT mice

Following 35 days of treatment there was a significant increase in the expression of T helper 1 (Th1) related cytokine IL12 in WT mice exposed to PM compared to controls in the small intestine (Figure 3-1). This would suggest a Th1 cell mediated response towards the particles after long term exposure; however no increase was observed in the expression of the pro-inflammatory cytokines IFNγ, TNFα, and IL1β, which are all characteristic of an acute Th1 mediated inflammatory response (Figure 3-1). There was also a trending decrease in CXCL1 expression compared to control chow mice, which is a chemokine associated with intestinal inflammation caused by neutrophil influx into the tissue. Long term PM exposure had no effect on the expression of the pro-inflammatory cytokine IL17 (Figure 3-2). IL2, IL4 and IL5 were not detected in either control or PM treated mice, suggesting a possible lack of Th2 cell immune response within the small intestine of WT mice.

In the colon, there was a significant increase in the pro-inflammatory cytokines TNFα, IFNγ, and IL12 as well as a trending increase in IL-1β (Figure 3-1) and IL17 (Figure 3-2) expression in WT mice exposed to PM for 35 days compared to control. This suggests chronic Th1 related inflammation in the colon and possibly the involvement of Th17 cells long term PM treatment. There was no change in expression for the chemokine CXCL1 and Th2 mediated cytokines IL5 (Figure 3-1) and IL13 (Figure 3-2) in response to long term PM exposure, and IL2 and IL4 were not detected in either control or PM treated mice suggesting a lack of a Th2 mediated immune response within the colon.

Figure 3-1a CXCL1
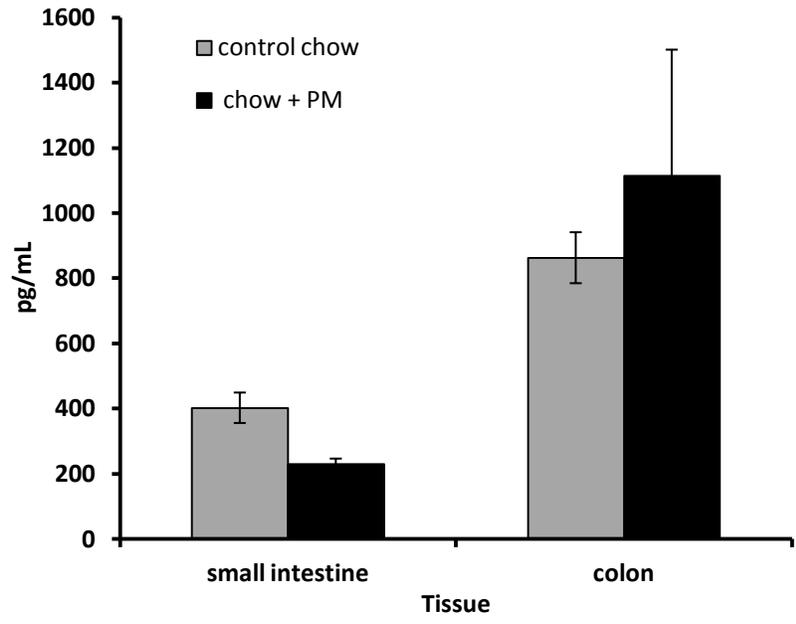
Figure 3-1b IL12
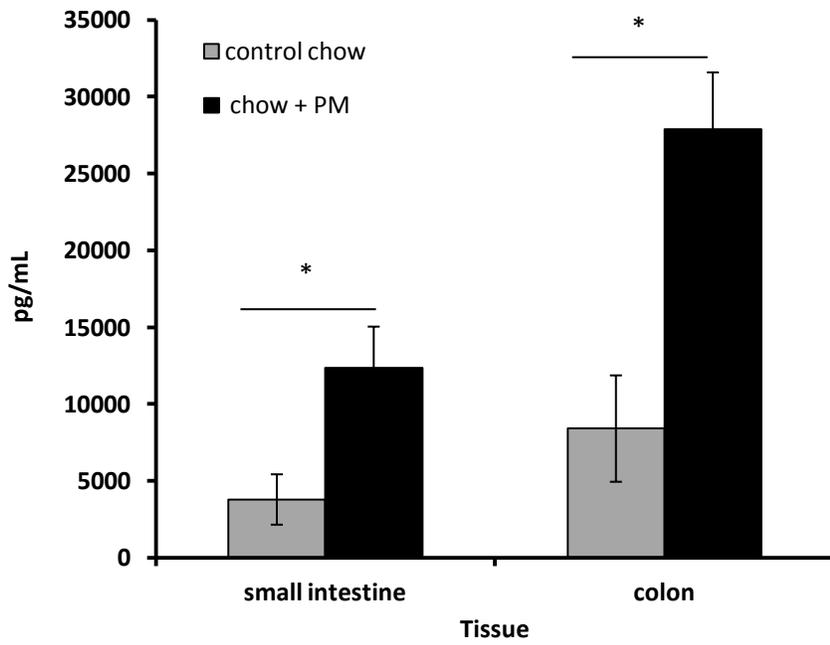
Figure 3-1c TNFα
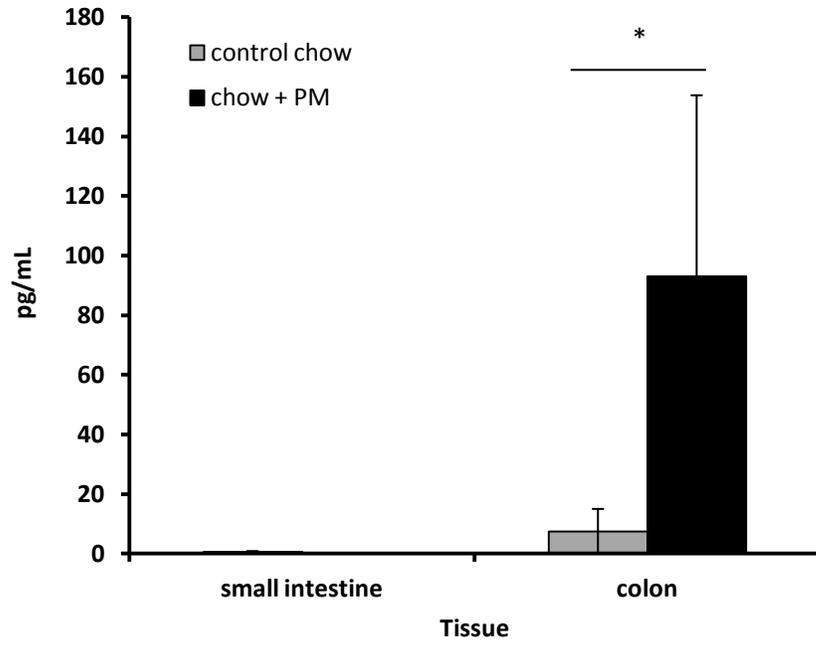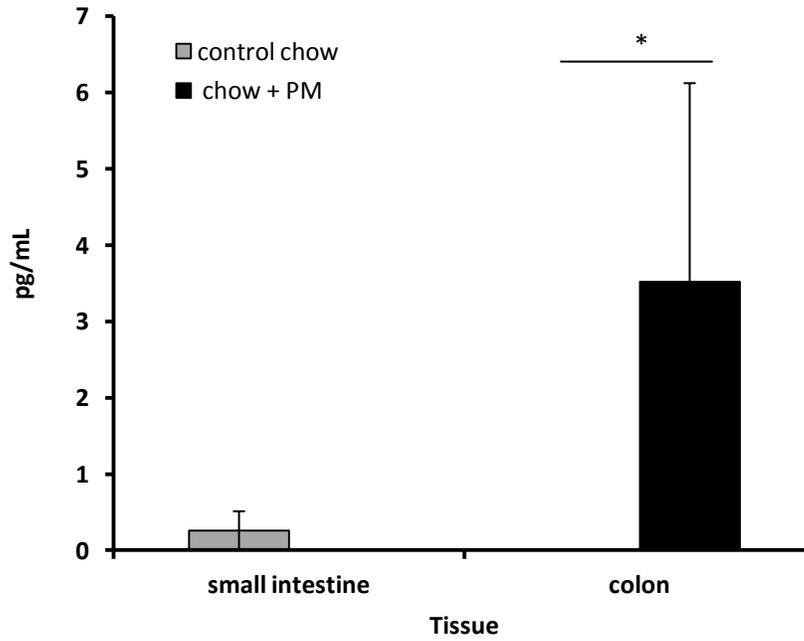
Figure 3-1d IFNγ
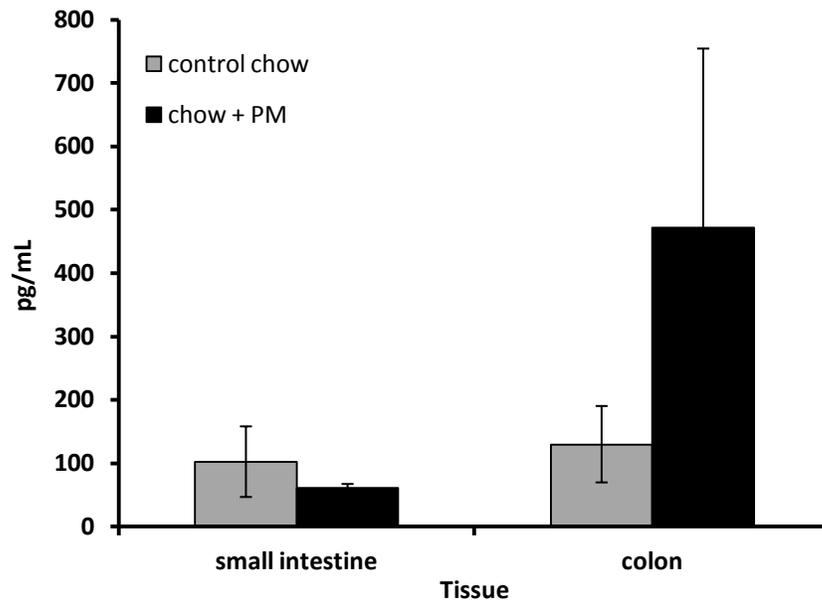
Figure 3-1e IL1B

Figure 3-1f IL5
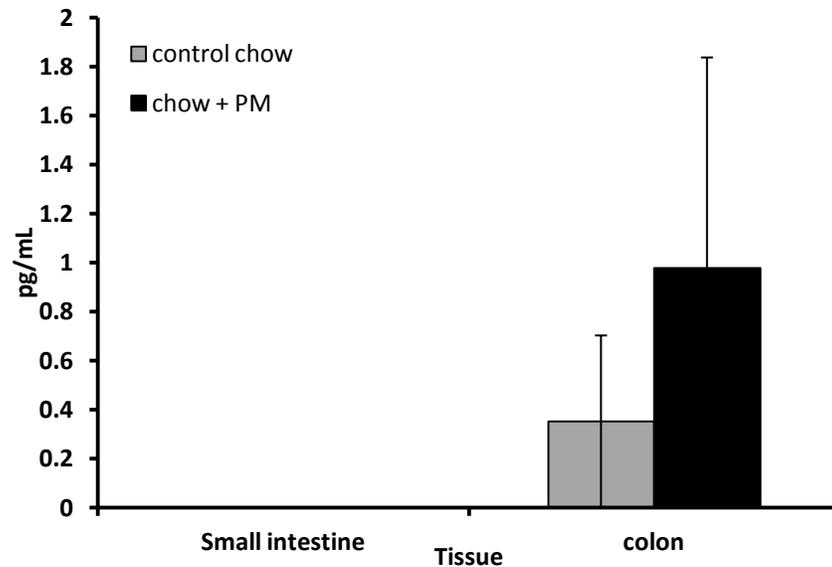
Figure 3-1: a) CXCL1 **b)** IL12, **c)** TNFα, **d)** IFNγ **e)** IL1β and **f)** IL5 expression from small and large intestine tissue homogenate of WT mice after 35 days on standard mouse chow (control) or mouse chow with PM. Samples were measured with a Th1/Th2 MesoScale discovery kit. No expression IL2 and IL4 was measured in control of PM treated mice (data not shown) * represents significance with P ≤0.05. Values are means ± SEM. (n=3-7 for all groups)
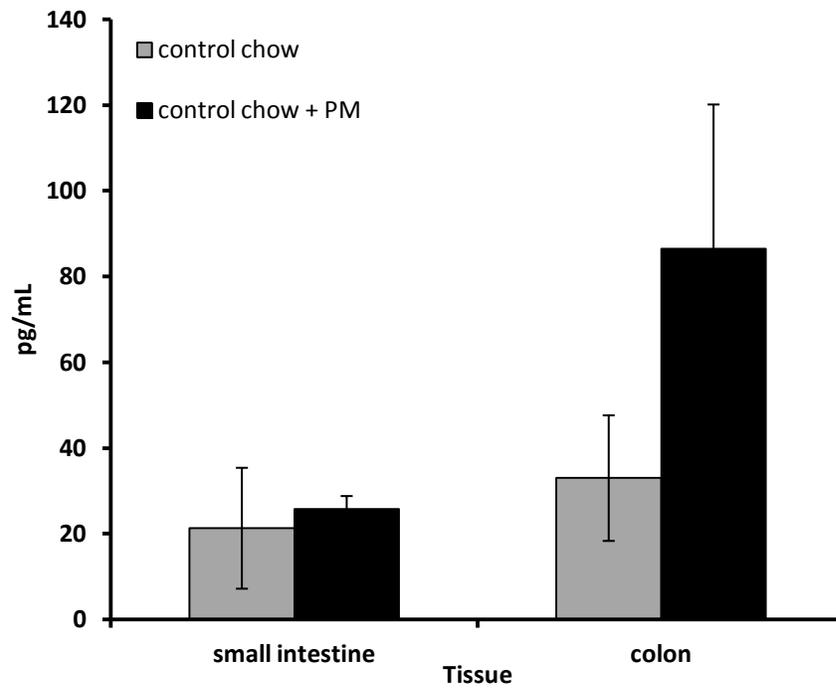
Figure 3-2: IL17 expression from small intestinal and colonic tissue homogenate in WT mice placed on chow alone or chow with PM for 35 days. Cytokine expression was measured with an IL17 ELISA kit. Values are means ± SEM

n=3-7 for all groups

3-3-2. Long term PM exposure alters microbial composition in WT mice

Microbial analysis was performed on the fecal samples taken on day 0 (prior to treatment) and on day 35 (following treatment) using the non-culture method of terminal restriction fragment length polymorphism (T-RFLP). Principle component analysis (PCA) was performed and demonstrated shifts in the bacterial population over the 35 days in PM treated mice that were different then the changes that occurred in the control mice over the 35 days (Figure 3-3). This suggests that the dietary exposure to PM has the ability to alter microbial composition. The Shannon-Wiener index was used to determine if PM exposure altered bacterial diversity in WT mice. There appears to be a trending increase in the index over the 35 days for WT mice placed on the PM chow (Figure 3-4). This could indicate an increase in microbial diversity in response to PM.

Differences were observed in many phyla both in WT mice on control chow and in mice on PM treatment. However, due to the large variability between individual mice, most of these differences did not reach statistical significance. There was a trending increase in the bacterial phylum *Firmicutes* at day 35 compared to day 0 in control chow WT mice (Figure 3-5); however in mice fed the chow with PM there was a decrease in *Firmicutes* at day 35. The phylum *Bacteroidetes* was decreased in the control mice only fed chow at day 35 compared to day 0 yet there was an increase in these bacteria at day 35 compared to day 0 in PM treated mice. There was an increase in *Actinobacteria*

and *Verrucomicrobia* at day 35 compared to day zero in both the control mice

and PM treated mice (Figure 3-3). *Tenericutes and Protobacteria* were increased

in mice fed chow only at day 35 compared to day 0, while there was a trending

decrease observed in these bacteria at day 35 in mice fed the chow with PM

(Figure 3-3). Finally, there were significant decreases at day 35 in unclassifiable

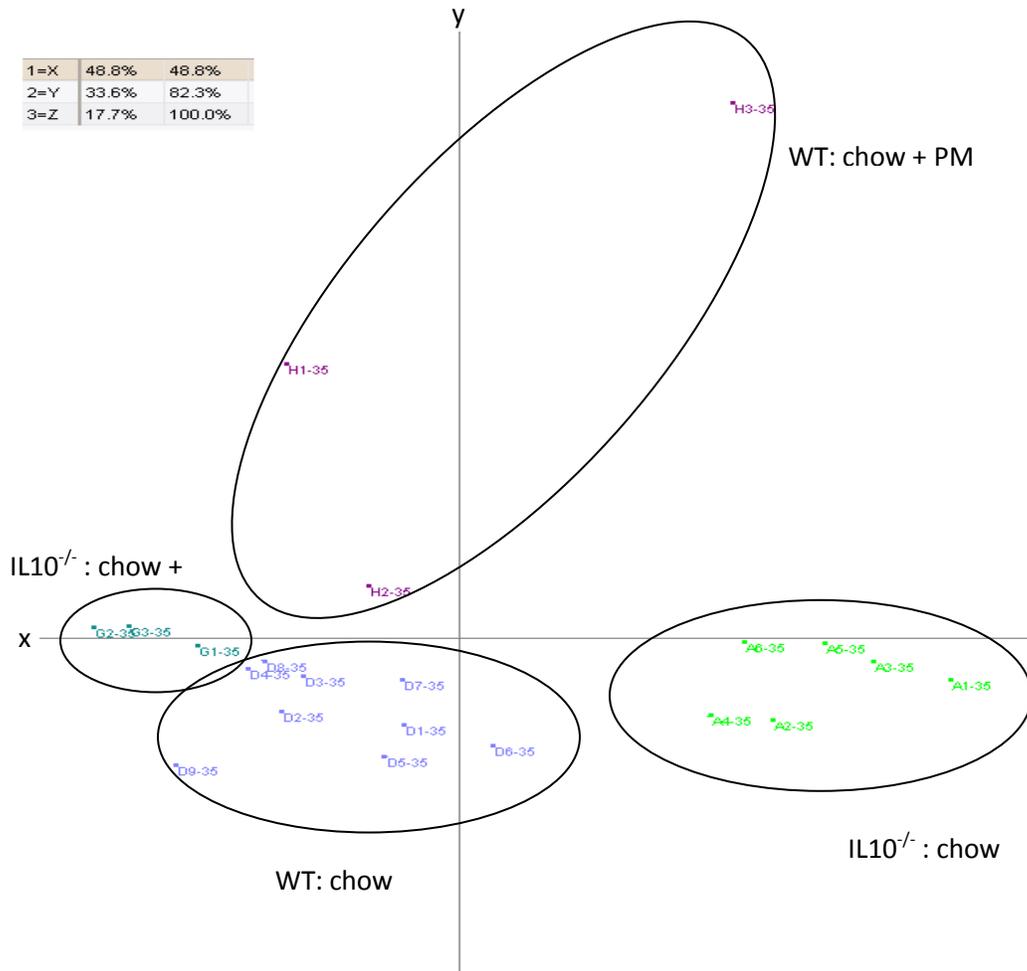bacteria compared to day zero in both mice fed chow only and in mice fed chow

with PM.

1=X	48.8%	48.8%
2=Y	33.6%	82.3%
3=Z	17.7%	100.0%

Figure 3-3: PCA plot examining changes in the microbial composition in IL10$^{-/-}$ and WT mice after 35 days on control chow or chow + PM. Results are based on the band patterns obtained during the TRFLP analysis of stool samples. Each dot represents one mouse. **A**- IL10$^{-/-}$ control, **D**- WT control, **G**- IL10$^{-/-}$ on chow + PM, **H**- WT mice on chow + PM.
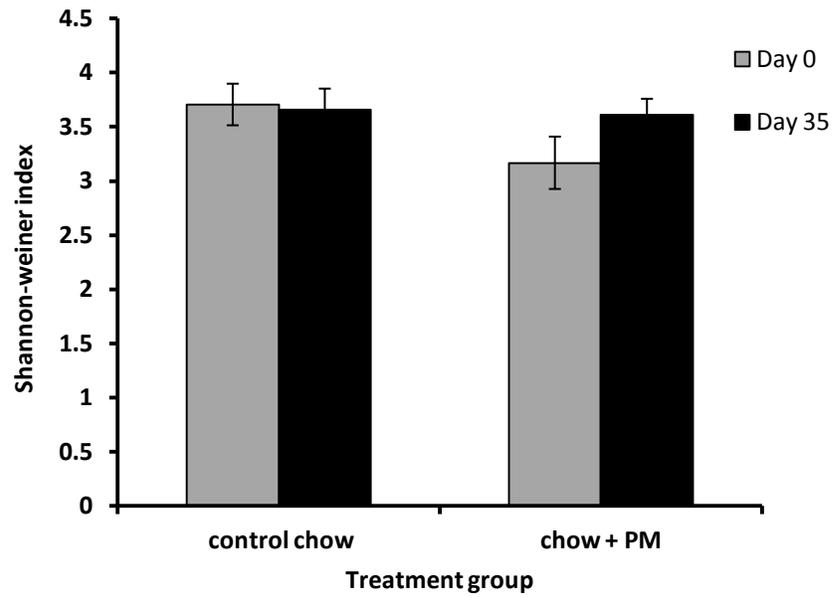
106

Figure 3-4: Shannon-Weiner index of microbial diversity: microbial diversity was determined from fecal samples taken prior to (Day 0) and following (day 35) treatment in WT mice fed control chow or control chow + PM.

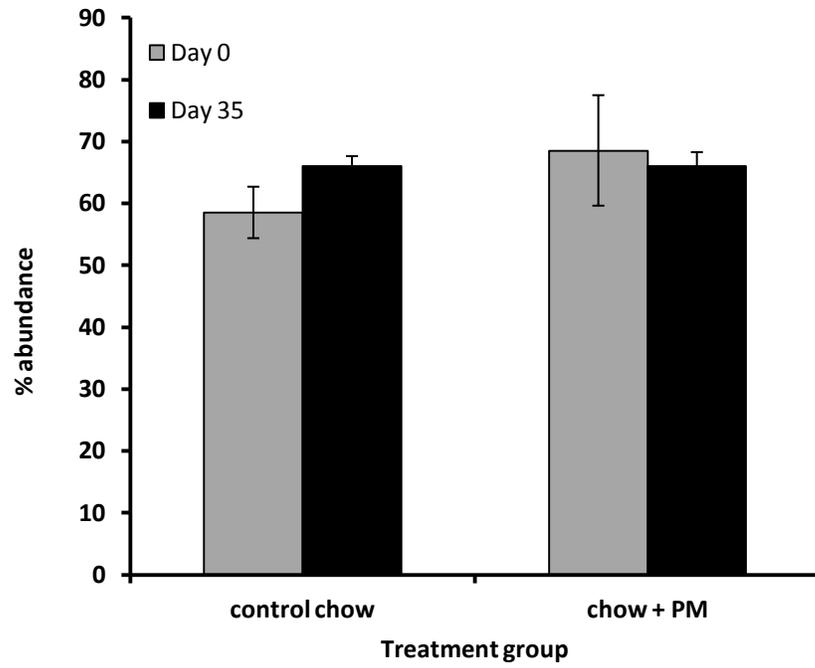Samples are means ± SEM (n=3-7)

Figure 3-5a *Firmicutes*
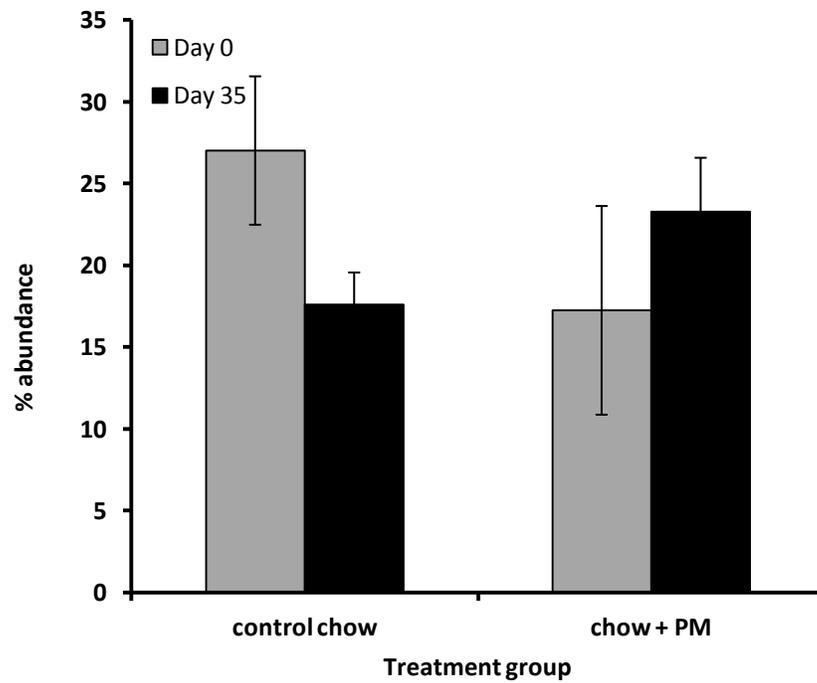

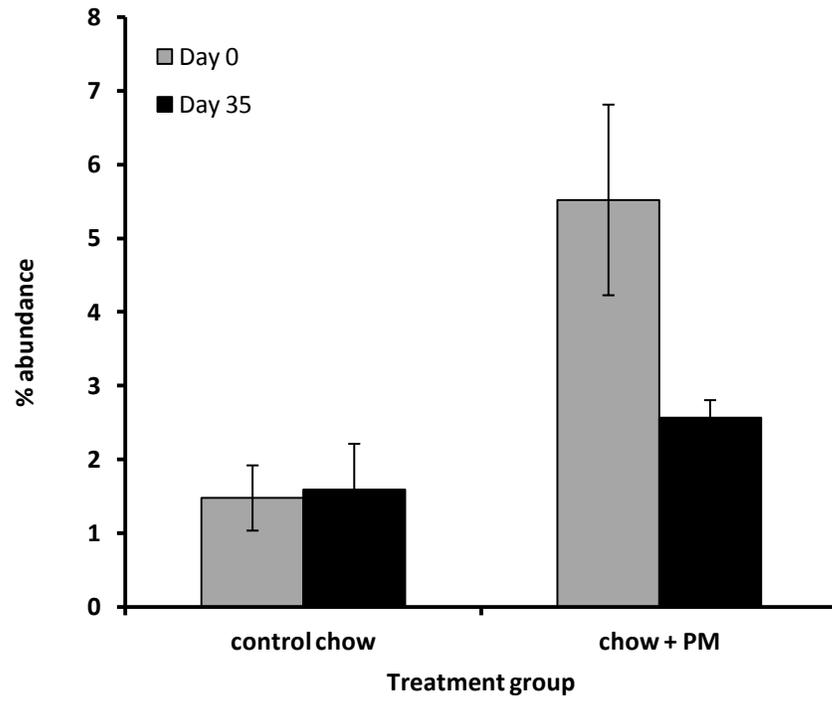**Figure 3-5b** *Bacteroidetes*

Figure 3-5c *Tenericutes*
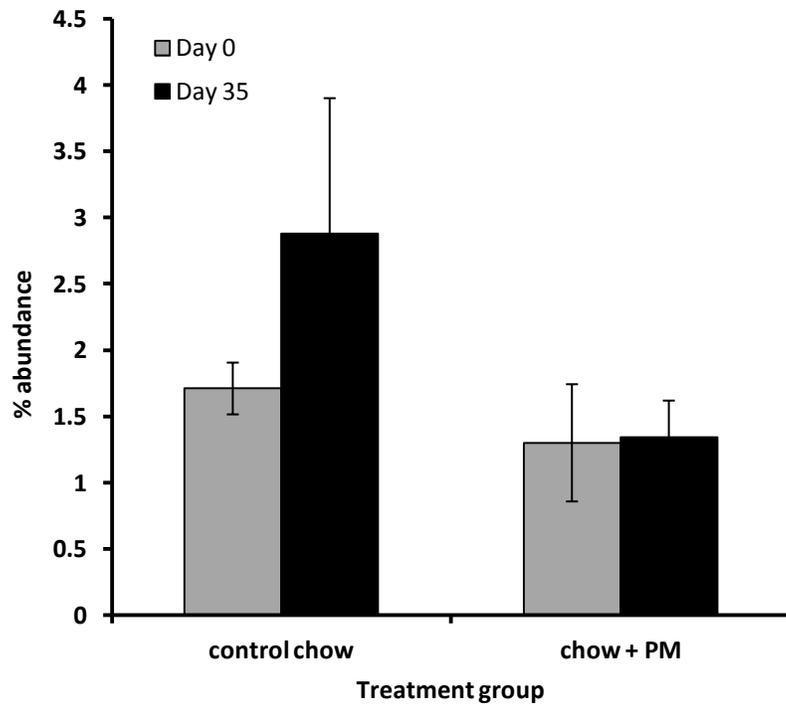

Figure 3-5d *Actinobacteria*
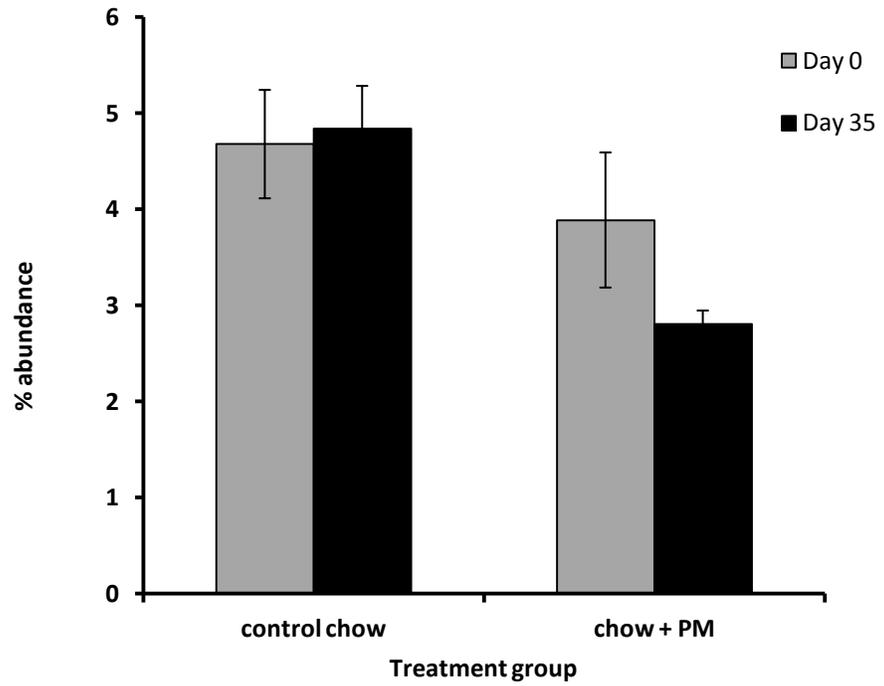
Figure 3-5e *Protobacteria*
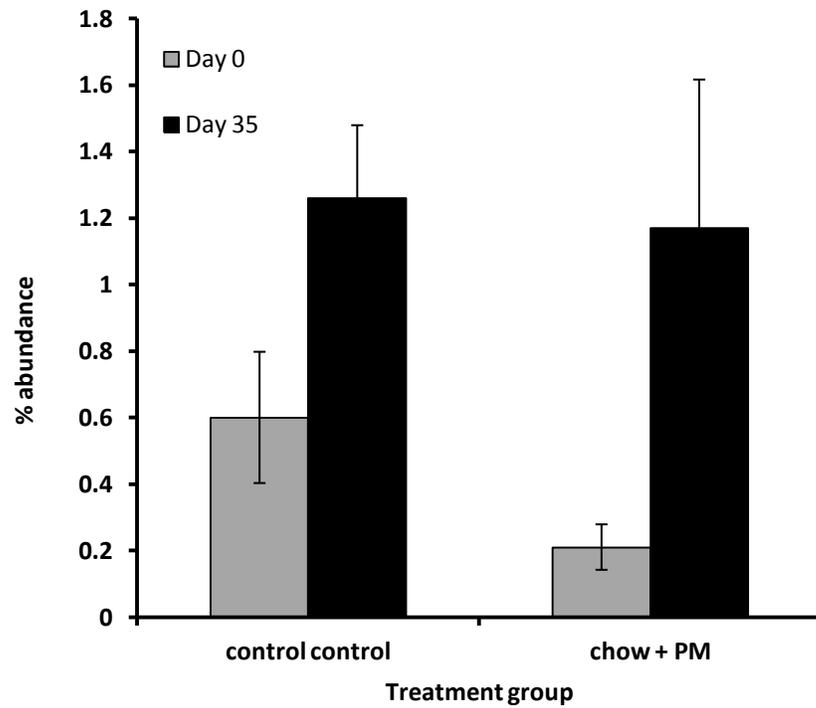

Figure 3-5f *Verrucomicrobia*
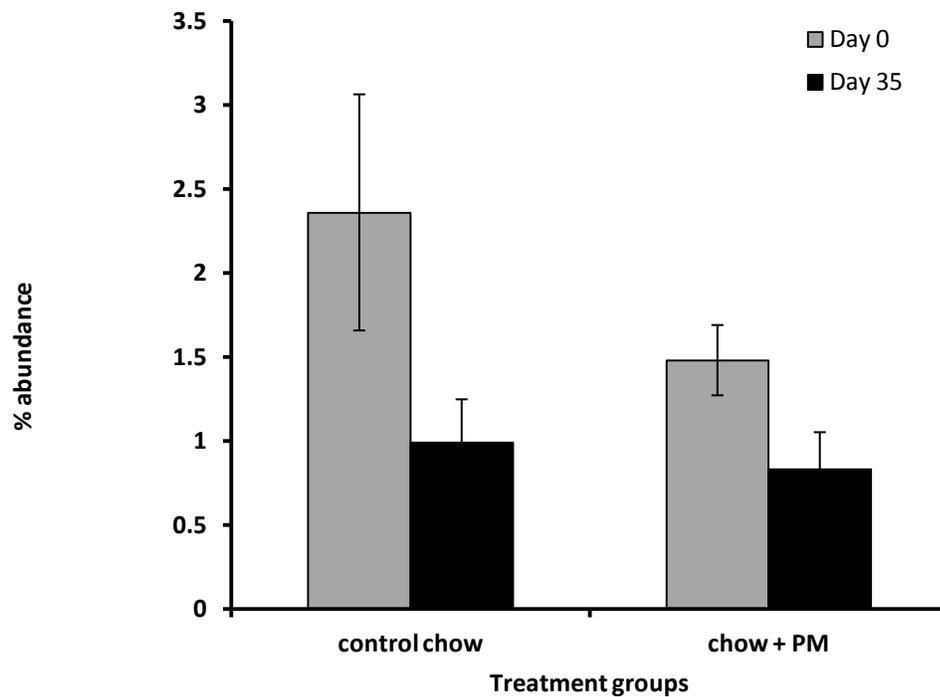
Figure 3-5g Unclassified bacteria

Figure 3-5: T-RFLP analysis preformed on stool samples taken prior to (Day 0) and following (day 35) treatment in WT mice placed on a standard chow with or without PM. Data is presented as the bacteria phylum: **a)** *Firmicutes,* **b)** *Bacteroidetes,* **c)** *Tenericutes,* **d)** *Actinobacteria,* **e)** *Proteobacteria,* **f)** *Verrucomicrobia,* **g)** unclassified bacteria.

Values are means ± SEM (n=3-7 for all groups)

3-3-3. Short chain Fatty Acid composition altered in response to long term PM exposure

Short chain fatty acid (SCFA) analysis was performed on cecal contents from WT mice after 35 days on the control chow or PM chow treatment. There was an observable increase in the concentration of isobutyric in the cecal contents of WT mice exposed to PM for 35 days compared to control. There was also a trending decrease in butyric and valeric acid concentration after 35 days in PM treated WT mice (Figure 3-6). No changes in concentrations were observed for acetate, propionic, caproic, and isovaleric acid in response to PM exposure (Figure 3-6).
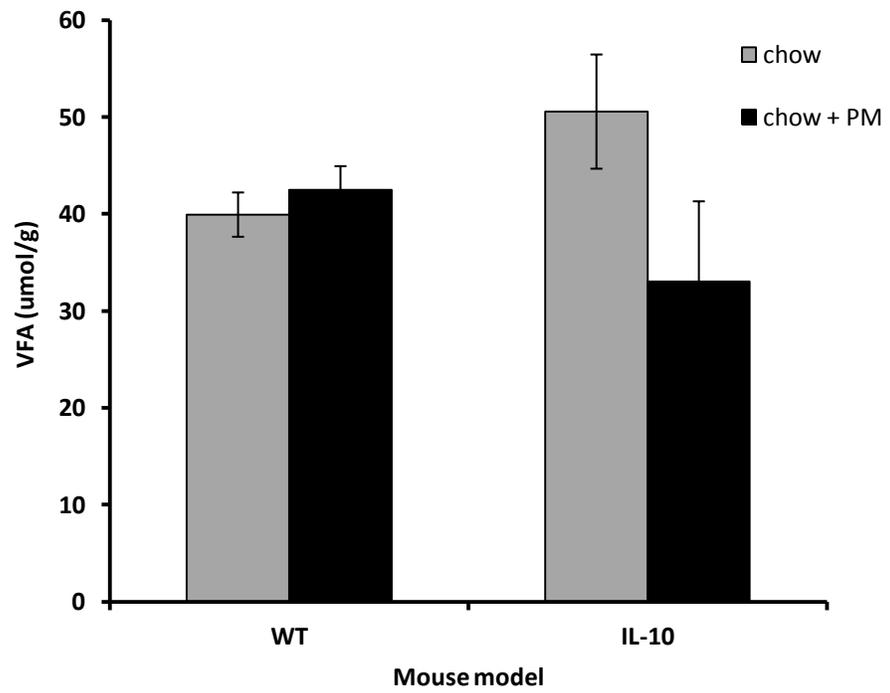
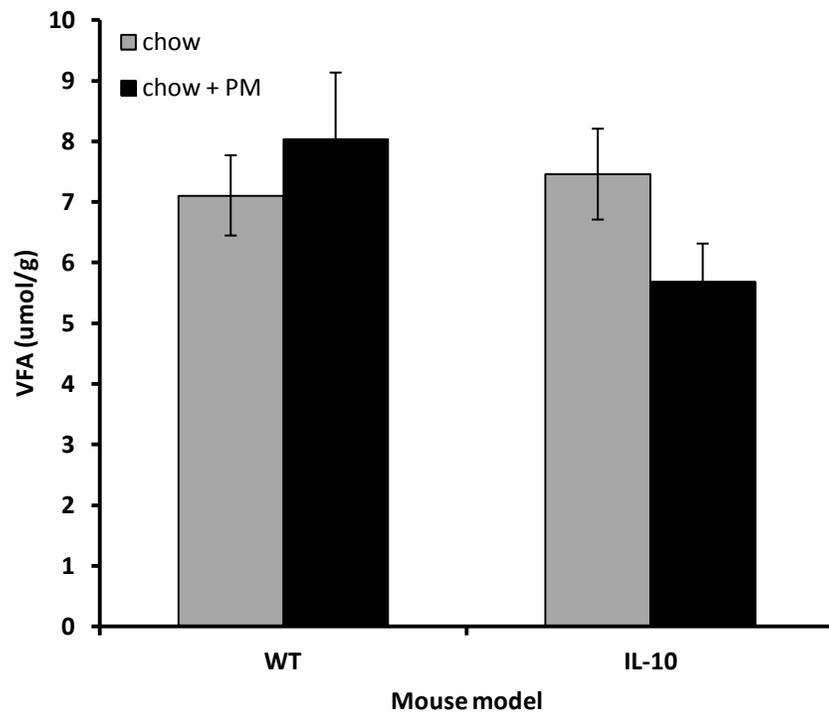**Figure 3-6a** Acetate

Figure 3-6b Propanoic acid

Figure 3-6c Isobutyric acid
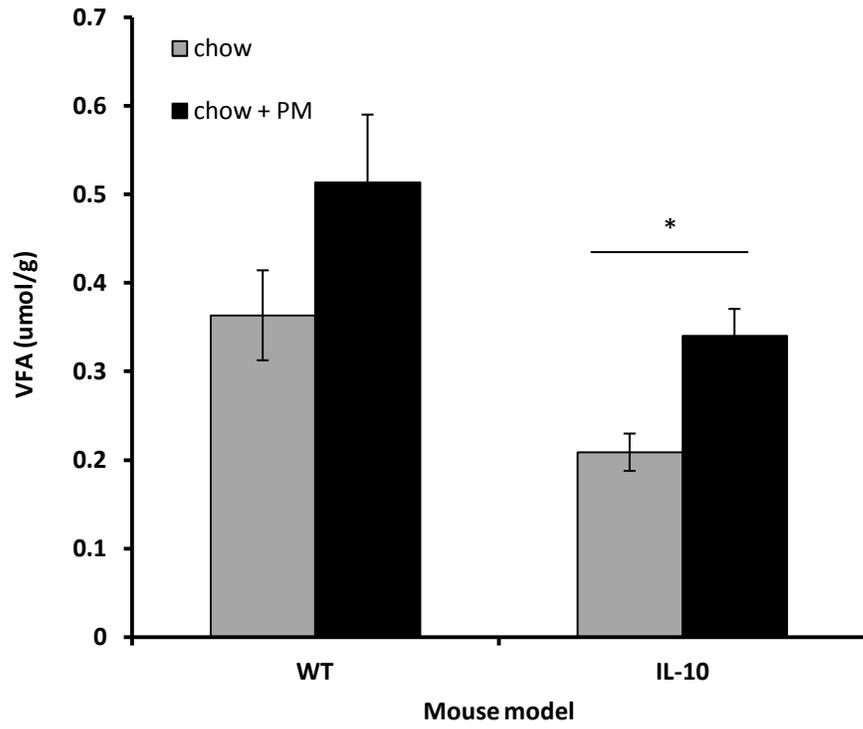
Figure 3-6d Butyric acid
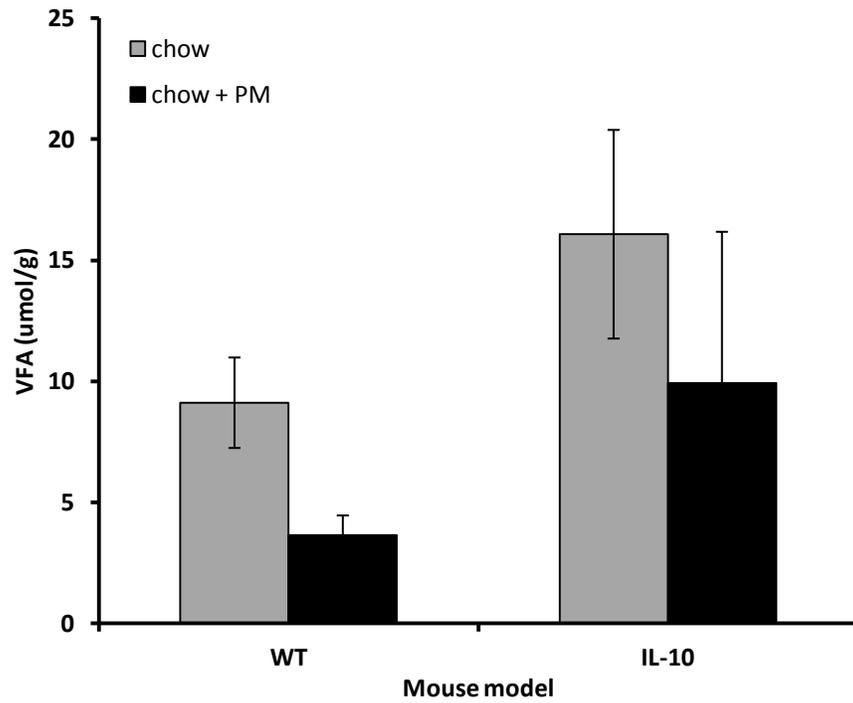
Figure 3-6e Isovaleric acid

Figure 3-6f Valeric acid

Figure 3-6g Caproic acid

Figure 3-6: Short chain fatty acid analysis: **a)** Acetate **b)** Propionic **c)** Isobutyric **d)** Butyric **e)** Isovaleric **f)** Valeric and **g)** Caproic acid analysis from cecal contents of WT and IL10 deficient mice after 35 days on standard mouse chow or chow with PM

Values are means ± SEM (n=3-7 for all groups)

3-3-4. Long term PM exposure in IL10 deficient mice significantly increases IL17 expression in the colon

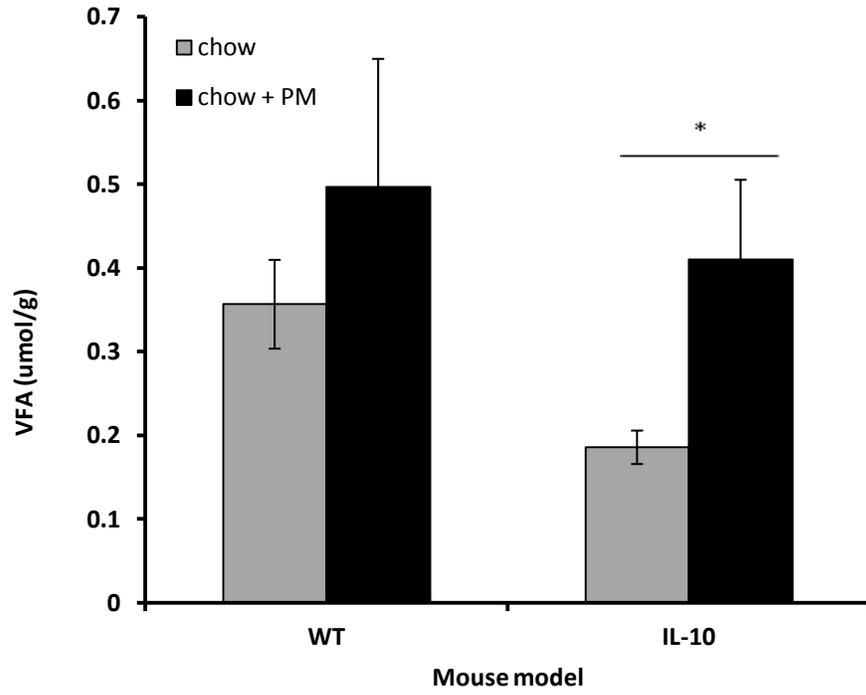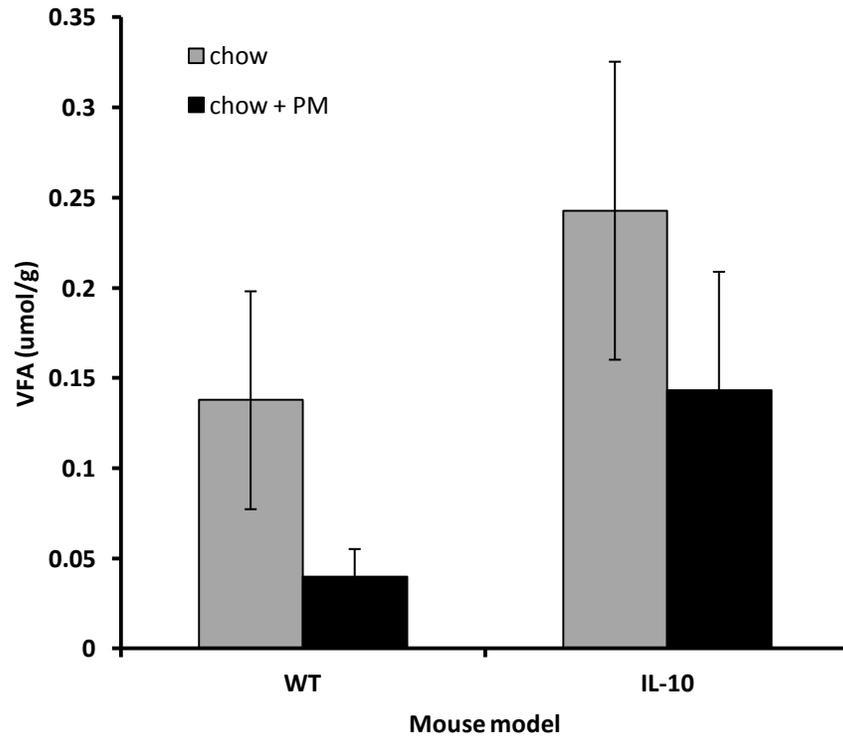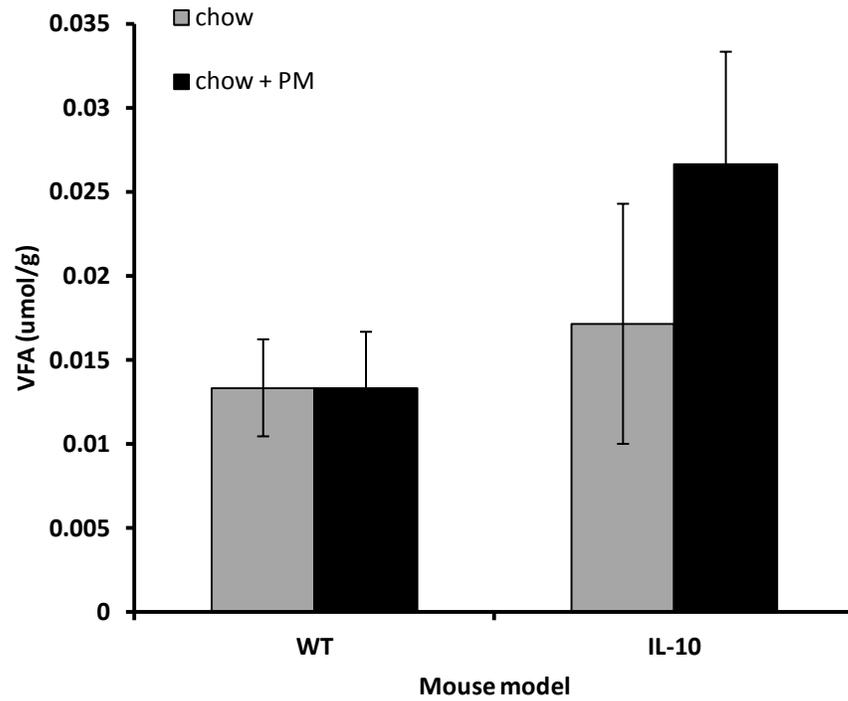Next I wanted to examine the effects of PM exposure on the IL10$^{-/-}$ mouse to compare the immune pathogenesis of particulates in an established model of colitis compared to WT mice. IL10$^{-/-}$ mice were given the same standard mouse chow with or without PM that was given to WT mice for a total of 35 days, and tissue samples were taken from the small and large intestine to examine cytokine expression.

There was an observable decrease in the Th2 cell mediated cytokine IL4 and in the inflammatory chemokine CXCL1 in the small intestines of IL10$^{-/-}$ mice fed the PM chow compared to control chow mice (Figure 3-7). No change in IFNγ, IL1β, IL5, IL12, TNFα (Figure 3-7), IL13 or IL17 (Figure 3-8) expression was detected in IL10$^{-/-}$ mice exposed to PM. This could suggest a lack of inflammatory response towards the particles within the small intestine in the IL10$^{-/-}$ mouse model of colitis.

In the colon of IL10$^{-/-}$ mice exposed to PM, there was a significant increase in expression of the pro-inflammatory cytokine IL17 (Figure 3-8), suggesting the possibility of a Th1 or Th17 T cell mediated immune response towards the particles within the colon. However, since there was an observable decrease in the expression of IFNγ, IL1β and IL2, the latter possibility is more likely. In addition there was an increasing expression for the Th2 cell mediated

cytokine IL13 (Figure 3-8), which suggests the possibility of B cell activation

within the colon in response to PM. No change in CXCL1, IL12, and TNFα (Figure

3-7) further imply a lack of Th1 mediated immune response, as well as neutrophil

infiltration or eosinophil proliferation within the colon of IL10$^{-/-}$ mice exposed to

PM.

Figure 3-7a CXCL1
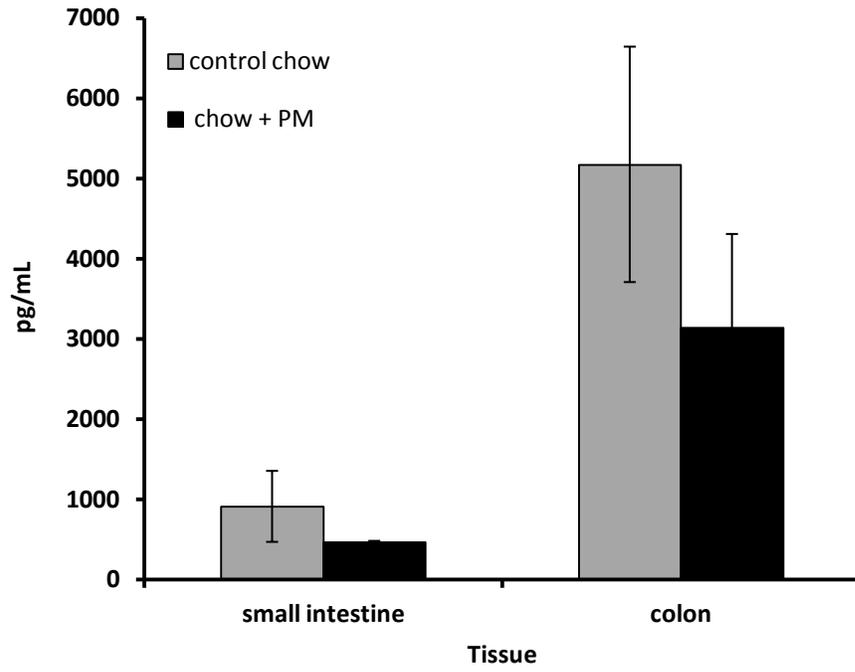
Figure 3-7b IL12
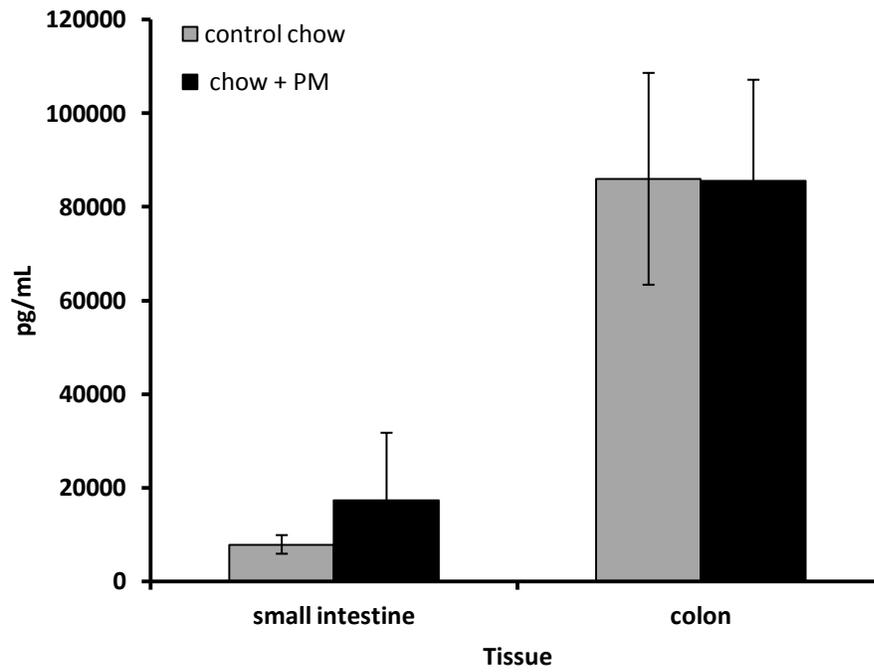
Figure 3-7c TNFα
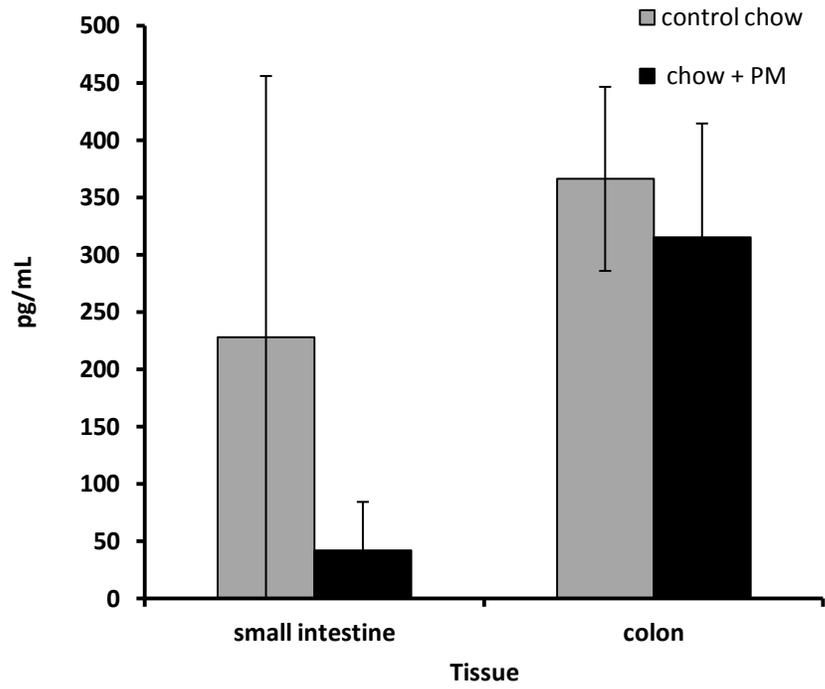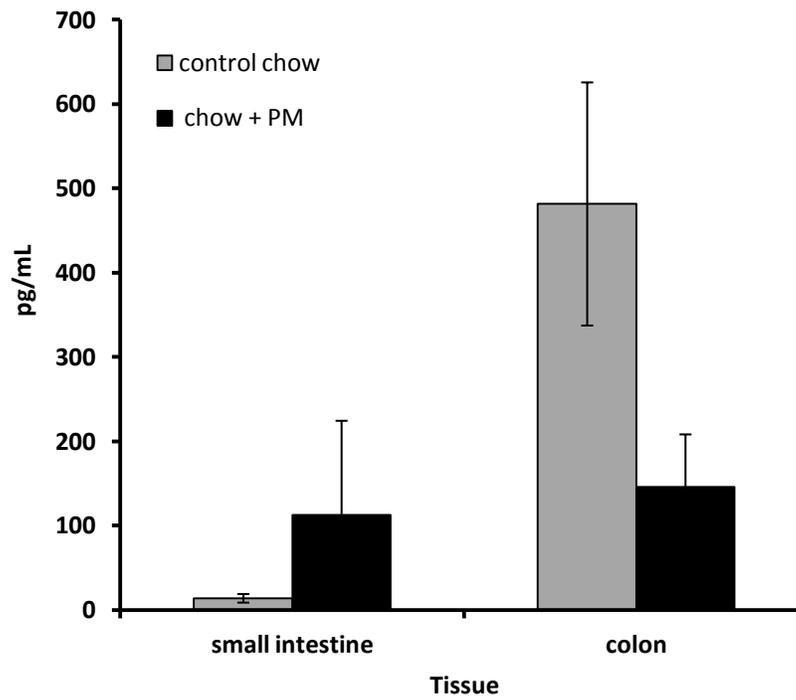
Figure 3-7d IFNγ
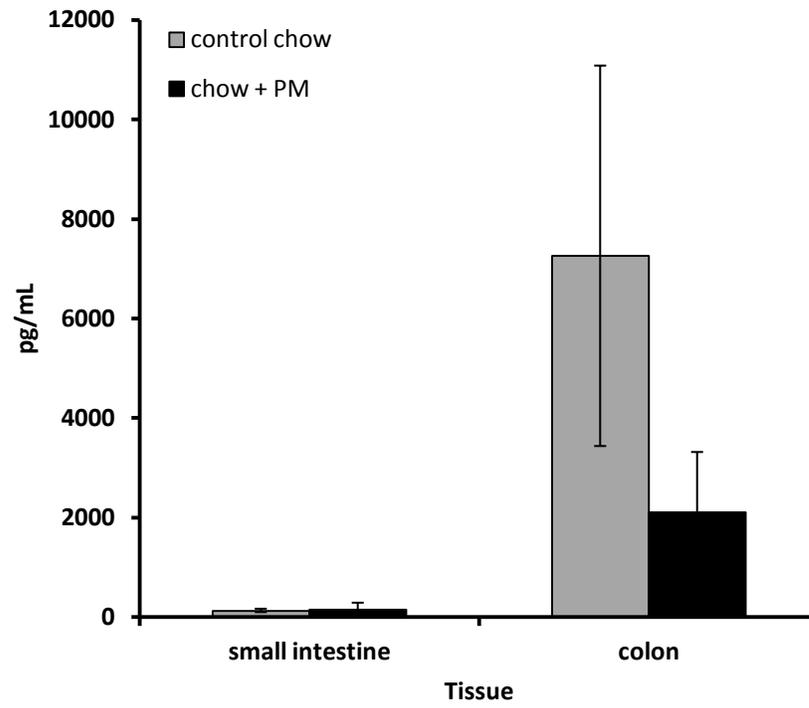
Figure 3-7e IL1β
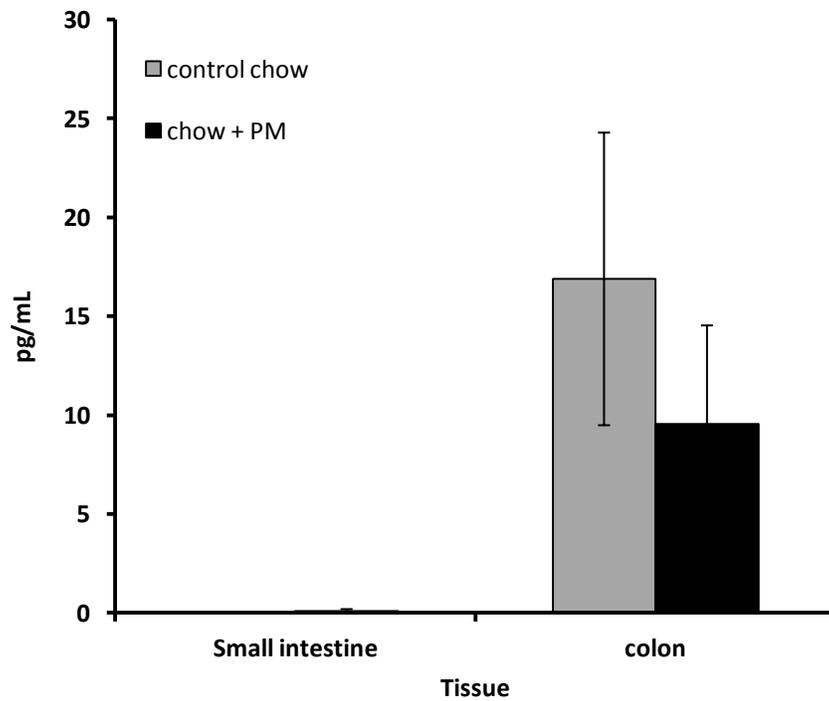
Figure 3-7f IL5

Figure 3-7g IL4
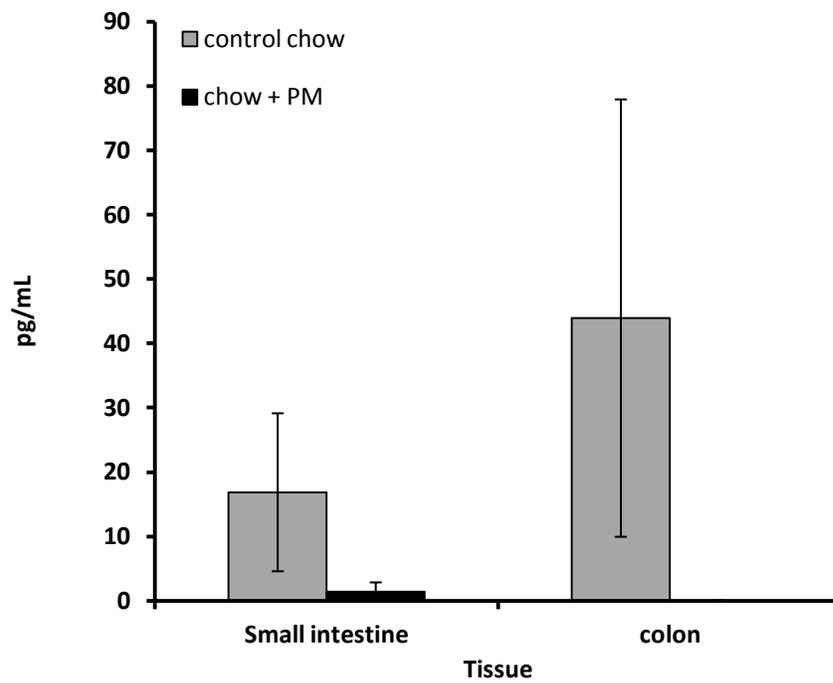
Figure 3-7: a) CXCL1 **b)** IL12, **c)** TNFα, **d)** IFNγ **e)** IL1β **f)** IL5 and **g)** IL4 expression from small and large intestinal tissue homogenate of IL10 deficient mice after 35 days on standard mouse chow (control) or mouse chow with PM. Samples were measured with a Th1/Th2 MesoScale discovery kit.

Values are means ± SEM (n=3-7 for all groups)

Figure 3-8a IL13
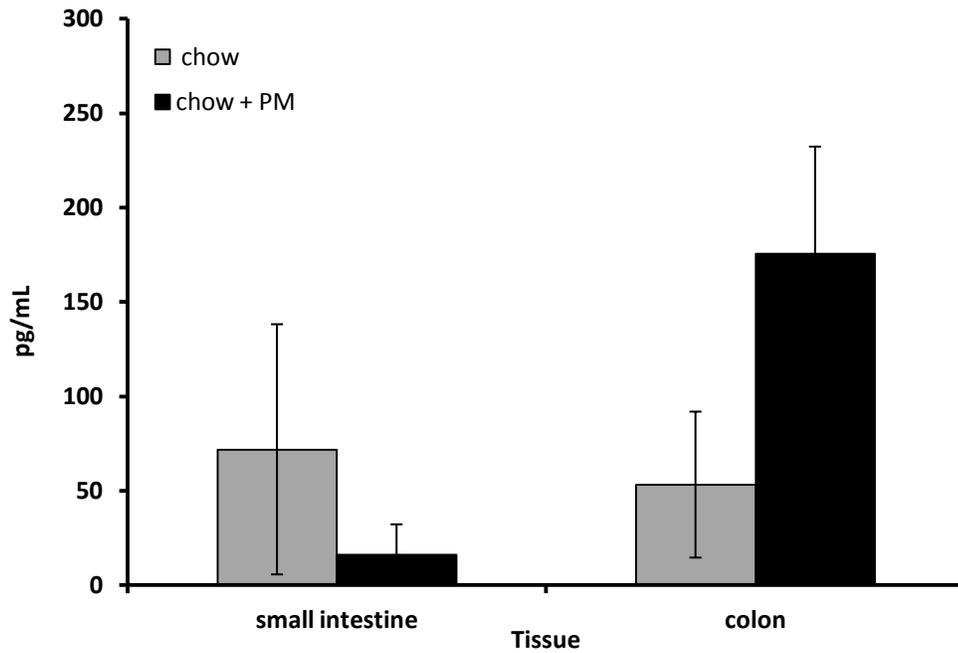
Figure 3-8b IL17
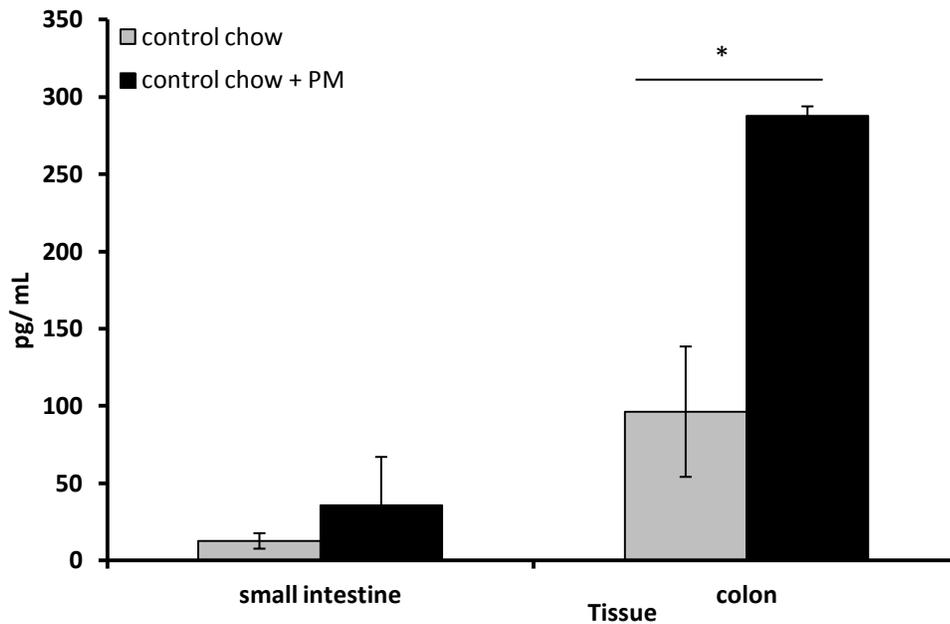
Figure 3-8: **a)** IL13 and **b)** IL17 ELISA expression from small intestinal and colon tissue homogenate of IL10 deficient mice after 35 days of treatment with control chow or chow + PM. Cytokines were examined with standard ELISA kits.

* represents significance with P ≤0.05. Values are means ± SEM (n=3-7 for all groups)

3-3-5. Long term PM exposure alters microbial composition in IL10$^{-/-}$ mice

Microbial analysis was performed on fecal samples from day 0 and day 35 control and PM treated IL10$^{-/-}$ mice using T-RFLP. Principle component analysis (PCA) was performed and confirmed a shift within the bacterial composition in response to the PM that was different to the change in bacteria occurring overtime within the IL10$^{-/-}$ mice in general (Figure 3-5).

The Shannon-Wiener index was again performed to determine if PM exposure altered bacterial diversity. There was no difference observed in the Shannon-Wiener index for both IL10$^{-/-}$ mice on the control diet or treated with PM after 35 days of treatment (Figure 3-10). This suggests no difference in microbial diversity in response to long term PM exposure.

Differences were observed in many phyla both in IL-10$^{-/-}$ mice on control chow and in mice on PM treatment. However, again due to the large variability between individual mice, most of these differences did not reach statistical significance. In both IL10$^{-/-}$ mice fed the control chow or chow with PM, there was an increase in *Firmicutes* and *Spirocheates* and an observable decrease in *Bacteroidetes* and *Proteobacteria* at day 35 compared to day 0 (Figure 3-9). *Actinobacteria, Ternicutes* and unclassified bacteria were all increased in IL10$^{-/-}$ mice placed on control chow, while being decreased in mice fed chow with PM. There was a decrease in *Verrucomicrobia* in control IL10$^{-/-}$ mice, but an increase in mice that were fed chow with PM (Figure 3-9).

Figure 3-9a *Firmicutes*
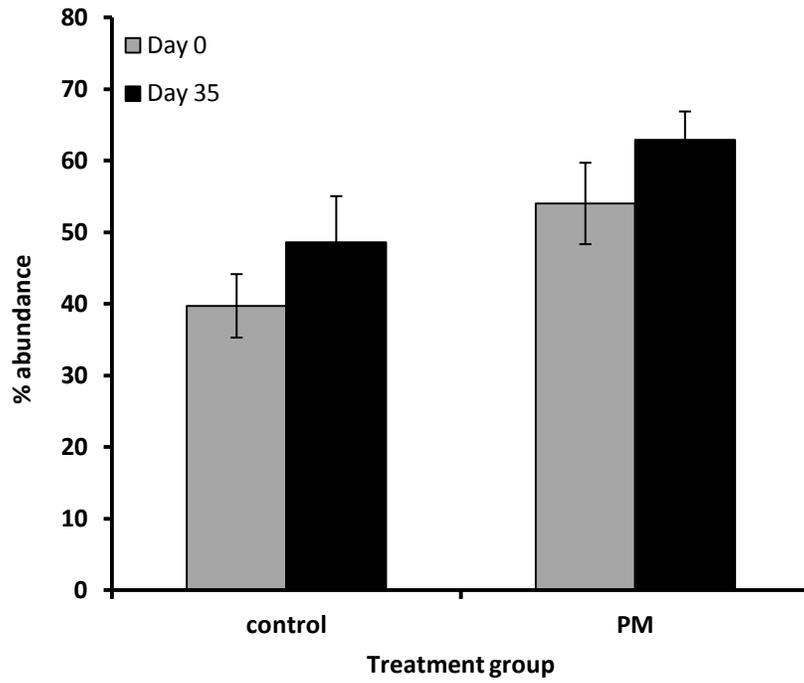
Figure 3-9b *Bacteroidetes*
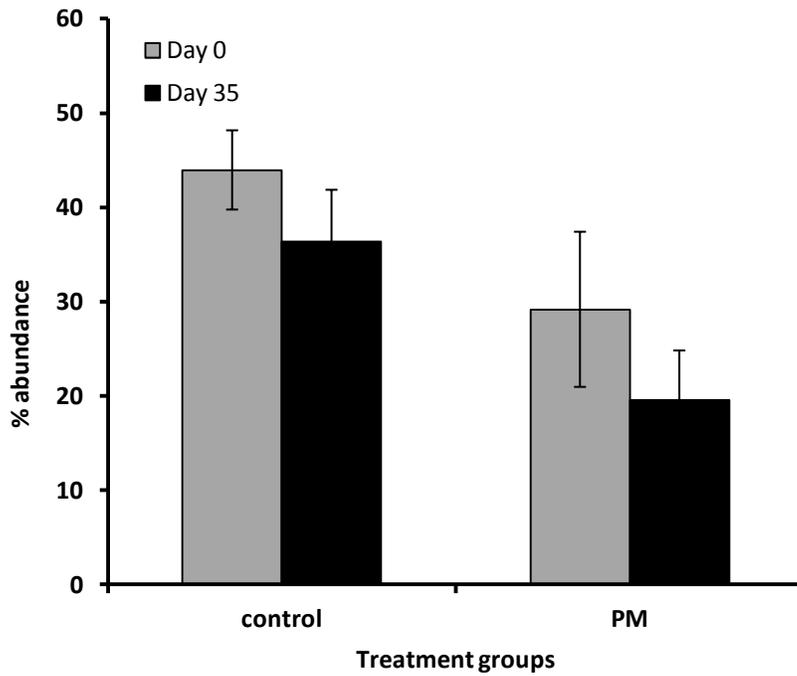
Figure 3-9c *Actinobacteria*
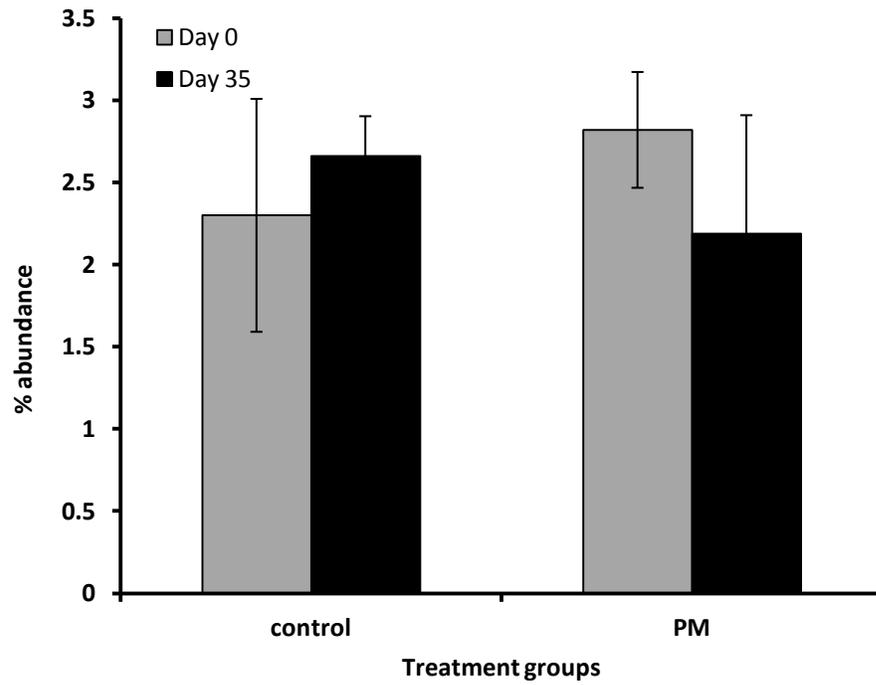
Figure 3-9d *Verrucomicrobia*
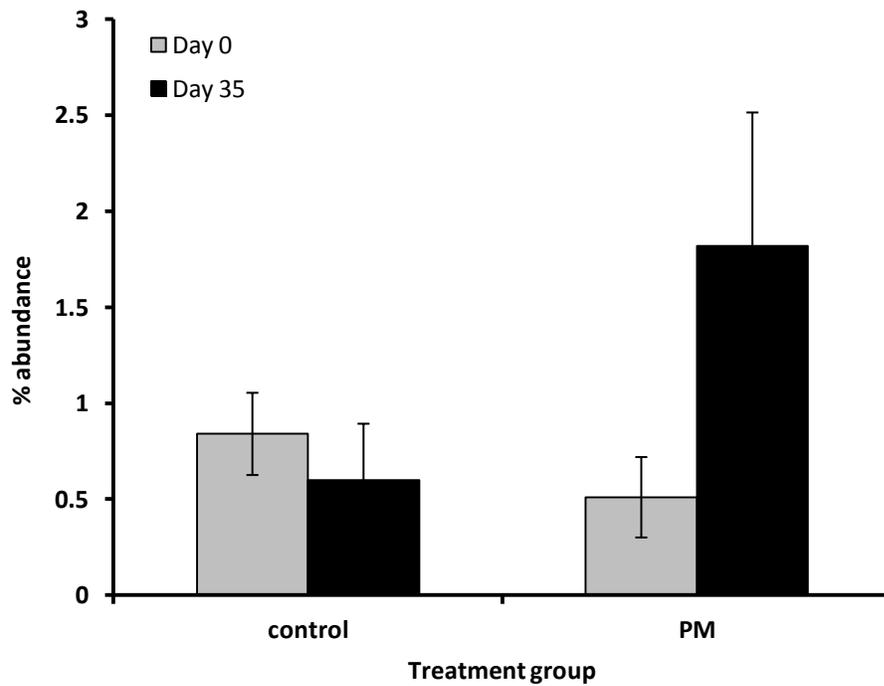
Figure 3-9e *Tenericutes*
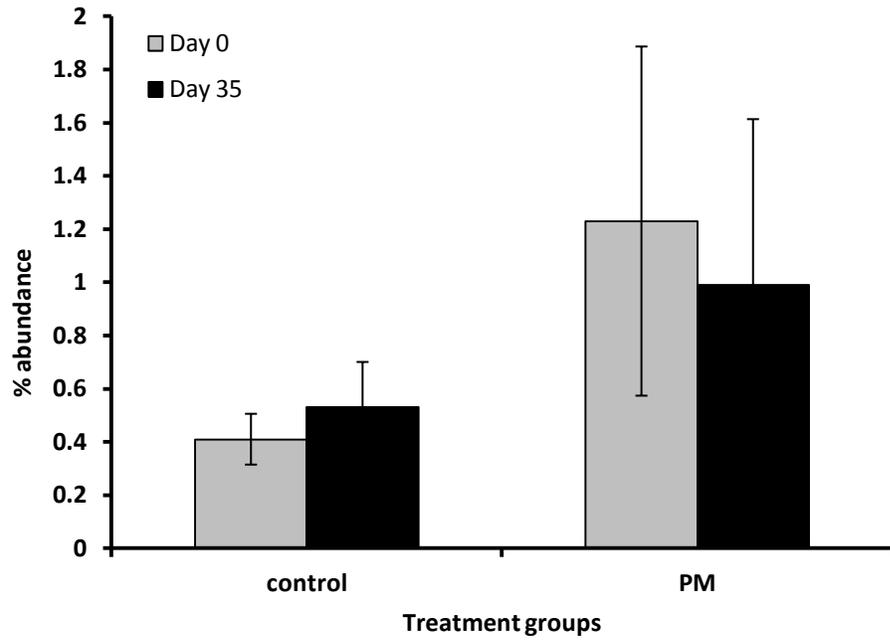
Figure 3-9f *Spirocheates*
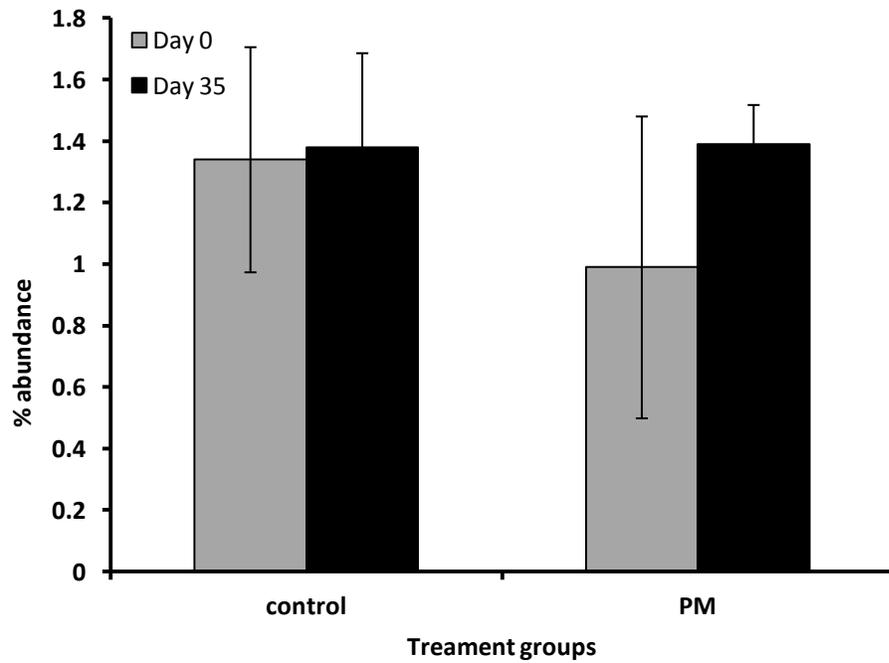
Figure 3-9g *Proteobacteria*
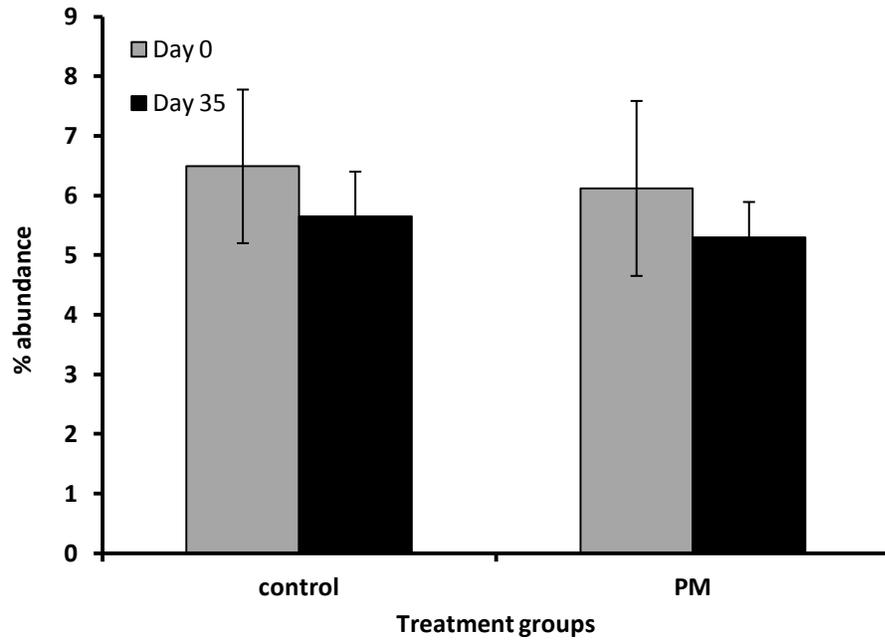
Figure 3-9h Unclassified bacteria
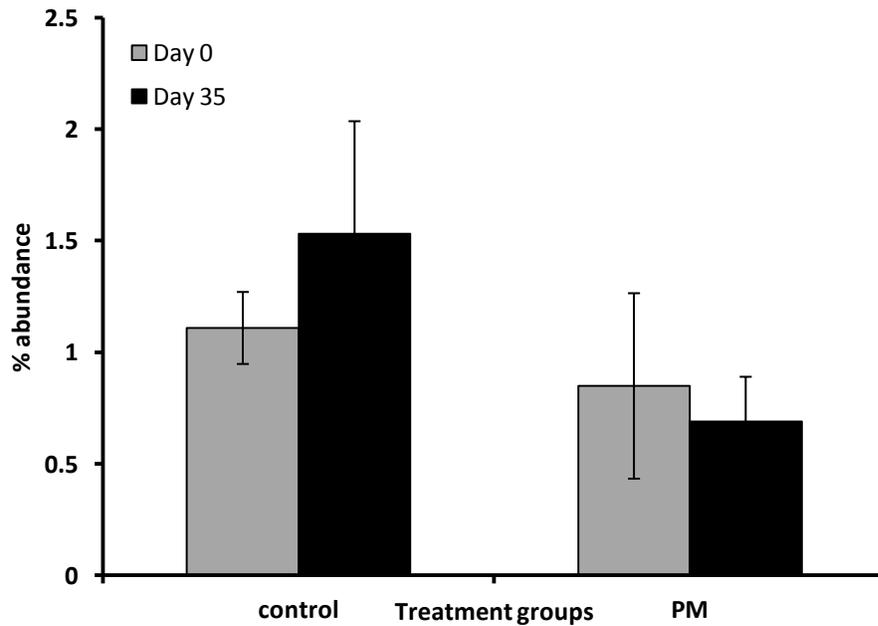
Figure 3-9: T-RFLP analysis preformed on stool samples taken prior to (Day 0) and following (day 35) treatment in IL10$^{-/-}$ mice placed on a standard chow with or without PM. Data is presented as the bacteria phylum: **a)** *Firmicutes,* **b)** *Bacteroidetes,* **c)** *Actinobacteria,* **d)** *Verrucomicrobia,* **e)** *Tenericutes,* **f)** *Spirocheates,* **g)** *Proteobacteria,* and **h)** unclassified bacteria. Values are means ± SEM (n=3-7 for all groups)
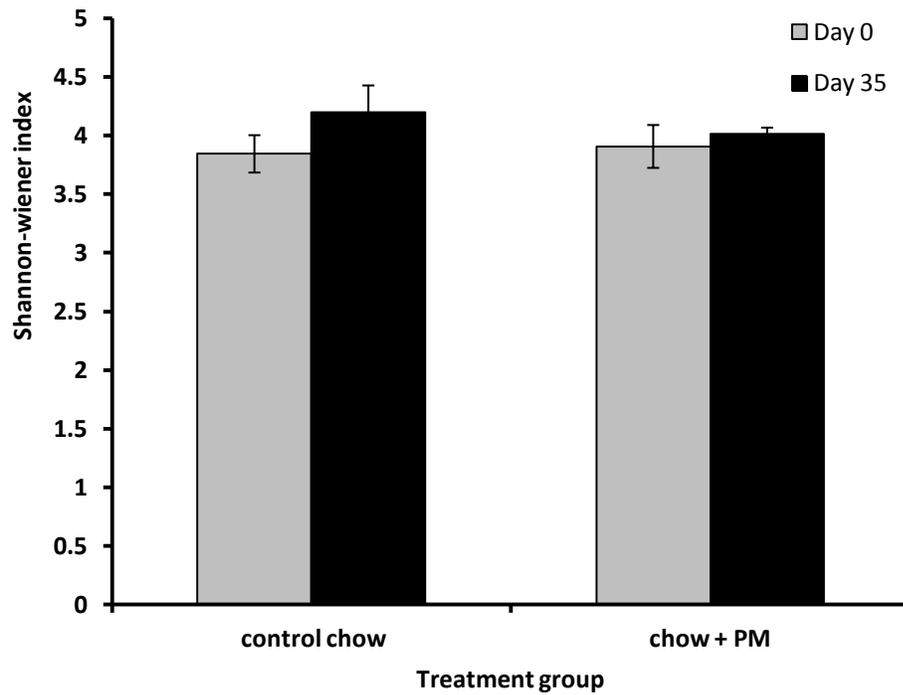
Figure 3-10: Shannon-Weiner index of microbial diversity: microbial diversity was determined from fecal samples taken prior to (Day 0) and following (day 35) treatment in IL10$^{-/-}$ mice fed control chow or control chow + PM.

Values are means ± SEM (n=3-7 for all groups)

3-3-6. Short chain Fatty Acid composition is significantly altered in IL10$^{-/-}$ mice in response to long term PM exposure

Short chain fatty acid (SCFA) analysis was performed on the cecal contents of IL10$^{-/-}$ mice on control chow or chow with PM for 35. There was a significant increase in concentration for the branched fatty acids isobutyric and isovaleric in IL10$^{-/-}$ mice exposed to PM compared to control (Figure 3-6). There was also an observable yet insignificant decrease in acetate and propionic acid concentration in mice fed PM chow compared to control mice. No changes were observed for butyric, caproic, and valeric acid in response to long term PM exposure.

3-4. Discussion:

Recent epidemiological studies have shown strong associations between air pollution particles and various inflammation related diseases of the gastrointestinal tract, including a possible role in the development of inflammatory bowel disease (IBD) (3-10). Research on particulate matter (PM) and diesel exhaust have shown the particles have the ability to cause DNA damage and oxidative stress and increased permeability in colonic epithelial cells (11-15). As well, short term oral exposure to PM resulted in acute inflammation within the small intestine and increased intestinal permeability *in vivo* [Chapter2]. This suggests exposure to PM in air pollution may be an important environmental factor leading to the exacerbation of inflammatory illnesses in the

gut. The long term effects of particulate exposure however, are not well understood, especially in relation to intestinal health. Therefore the aim of this study was to examine the effect of long term PM exposure on the mucosal immune response and microbial composition in both WT and the IL10$^{-/-}$ mouse model of colitis. In the present study I demonstrate that long term exposure to PM$_{10}$ has the ability to alter the expression of various T-helper (Th1) and T-helper (Th2) associated cytokines and modulate microbial composition in WT and IL10$^{-/-}$ mice.

In WT mice exposed to PM for a total of 35 days, there was an increase in colonic tissue expression of the pro-inflammatory cytokines IFNγ, TNFα, interleukin 12 (IL12), and IL17. This suggests a chronic Th1 or a Th17 cell mediated immune response within the colon of WT mice after long term exposure to PM. Chronic inflammation occurs when the immune response is unable to clear a foreign Ag from the inflamed tissue, or it is under continuous stimulation from an Ag, and results in unresolved and prolonged inflammation within the tissue (16). Chronic inflammation is mediated by mononuclear cells such as monocytes/macrophages and lymphocytes, and requires the production of IFNγ for the maintenance of tissue inflammation (16). The apparent adaptive immune response triggered within the colon of WT mice in response to acute PM exposure (Chapter 2), followed by the significant increase in INFγ, TNFα, and IL12 colonic expression after long term exposure, and strongly suggests long term oral

exposure to PM has the ability to trigger low grade chronic inflammation within the colonic tissue.

Short chain fatty acid (SCFA) analysis revealed alterations in the concentrations of isobutyric, butyric and valeric acid in WT mice after 35 days of PM exposure. Of particular note was the decrease in butyrate concentration, which is important in colonic epithelial cell metabolism (17). Butyrate is also important in colonic mucosal health; specifically it is involved in induction of host defense peptides, intestinal motility, and involved in cell proliferation and differentiation within the basal crypts in the colon (18). It also demonstrates anti-inflammatory properties (17,19,20) and has been tested as a treatment in intestinal inflammatory diseases such as IBD (19,20). There is also recent evidence suggesting a role for butyrate in the enhancement of barrier function by stimulating mucin production in colonic epithelial cell lines (21,22). Depletion of butyrate has been shown to result in decreased barrier function and increased susceptibility to inflammation in the colon (17,19). As well it has been suggested Ulcerative Colitis (UC) may be associated with mucosal defects in the metabolism of butyrate (23). The decrease in butyrate production, although not quite statistically significant, could therefore make the mice more susceptible to bacterial infections and colonic inflammation by decreasing colonic epithelial cell health.

SCFA's such as butyrate are the end products of bacterial fermentation of the undigested carbohydrates in the intestine (17, 19). The amount of SCFA production is dependent on type of substrate, transit time through the intestine, and the amount of and type of bacteria present in the colon (17, 19). The alterations in SCFA production in response to PM exposure lead us to examine the effects of the particles on gut microflora composition. I was able to demonstrate, through terminal restriction fragment length polymorphism (T-RFLP) and principle component analysis (PCA) of fecal samples taken prior to and following PM treatment, changes in the microbial population within the WT mice. I found the alteration in microflora composition to differ from the changes occurring over the 35 days in control WT mice. Unfortunately due to the limitations of this study, I am unsure if the change in intestinal microflora was caused by the PM directly, or indirectly due to altered mucosal immune response in PM treated mice, or both. Interestingly, I found no decrease in the phylum *Firmicutes*, which contain the main species of bacteria responsible for butyrate production (2). Therefore altered bacterial population alone can't be responsible for the effect on SCFA production. Recent reports indicate that butyrate deficiency can occur as a result of decreased butyrate uptake in inflamed mucosa (25). It is then possible that the chronic inflammation observed in the colon of WT mice after long term PM exposure could be reducing the ability of colonocytes to take up the butyrate which leads to decreased production.

These have strong implications regarding gastrointestinal inflammatory disorders, including IBD. In IBD the Th1/Th2 balance becomes disturbed, for instance CD is considered a Th1 and Th17 cell mediated immune response characterized by chronic inflammation, granuloma formation and increased production of various pro-inflammatory cytokines including IL12, IFNγ and TNFα (26,27). Our data has demonstrated the ability for oral PM exposure to induce Th1 inflammation initially in the small intestine [refer chapter 2] and Th1/Th17 chronic inflammation in the colon after continuous long term exposure. . Although the methods of PM administration differed between acute and long term exposure studies, both still demonstrate the ability for the airborne particulate's ability to alter mucosal inflammation in WT mice. This could suggest that in individuals with the genetic predisposition, PM exposure could initiate mucosal inflammation and act as a trigger in IBD pathogenesis.

Therefore, I also wanted to examine the effect of PM exposure on the IL10$^{-/-}$ model of chronic gut inflammation. In this model, disease progression depends upon the presence of intestinal bacteria; if the mice are housed in germ free environments, they will not develop disease. A large amount of data also implicates a role for gut microflora in the pathogenesis of IBD in humans (28), and because analysis in WT mice demonstrated a shift in microbial population and possible increase in diversity in response to long term PM exposure, I wanted to examine the effect of PM on the intestinal microflora in IL10$^{-/-}$ mice after long term exposure. Although no change in microbial diversity was

observed, PCA discriminant analysis and T-RFLP revealed a shift in the microbial

composition in response to PM exposure. *Verrucomicrobia* is a novel phylogenic

group of bacteria that have been recently identified, and although relatively less

abundant are still known to play important roles in human health (29). This

suggests PM could be having either a direct or secondary effect on microbial

composition. It has been previously shown that exposure to combustion derived

particles has the ability to increase susceptibility to bacterial and viral pulmonary

infections (30,31); therefore, along with the ability to alter microbial

composition, this may suggest PM exposure can subject individuals to increased

risk of bacterial infection, along with altered immune function to act as a trigger

for intestinal inflammatory diseases.

After 35 days of PM exposure there was a significant increase in IL17

expression in colon of IL10$^{-/-}$ mice. This is an important result because IL17 is a

pro-inflammatory cytokine known to be involved in several chronic inflammatory

diseases, including IBD (32-35). The increased expression of IL17 in the tissue

suggests an increase in Th17 cells in the colon in response to PM exposure, which

could imply the ability to exacerbate gut inflammation and worsen disease

severity in genetically predisposed individuals. There was also an increase in IL13

expression in the colonic tissue after long term exposure to PM in the IL10

deficient mice. This has important implications in UC patients which is associated

with the Th2 mediated response, and in particular increased IL13 production

(36). Previous studies have demonstrated the ability for combustion derived

particles to enhance Th2 cytokine production such as IL13 in the bronchial epithelium (37). Interestingly I did not observe an increase in IL12, TNFα and IFNγ expression in the IL10$^{-/-}$ mice in response to long term PM exposure which was observed in the WT mice. This could be because the IL10$^{-/-}$ mouse already has increased expression of these cytokines (38,39).

SCFA analysis demonstrated a significant increase in the branched chain fatty acids isobutyric and isovaleric acid concentrations in IL10$^{-/-}$ mice exposed to PM for 35 days compared to control. Interestingly, branched chain fatty acids (BCFA) are thought to be toxic for colonic epithelial cells (40). Individuals with IBD already have severe colonic damage caused by the chronic inflammation and granuloma formation, therefore increased production of BCFA might intensify the inflammation within the colon and worsen disease progression.

It is believed that IBD development occurs as the result of environmental, genetic, and immunological factors. Specifically, immune deregulation in genetically predisposed individuals might result in loss of tolerance towards resident microflora causing uncontrolled inflammation. In general, I was able to demonstrate the ability for orally ingested PM to elicit inflammation within the mucosa, and alter microbial composition within the gastrointestinal tract of WT mice and exacerbate gut inflammation in the IL10$^{-/-}$ mouse. This provides some evidence to support my hypothesis that oral PM exposure can increase inflammation within genetically susceptible individuals. This suggestes,

therefore, that environmental factors such as airborne pollutants might have the

ability to act as a trigger, initiating inflammation and exacerbating

gastrointestinal disease. These findings provide a mechanism whereby airborne

particulate matter may play a role in gastrointestinal disease.

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Chapter 4: Effects of a western style diet on mucosal immune function and intestinal microflora

4-1. Introduction:

The role of diet has also long been associated with IBD etiology, due to the location of the inflammation, the fact that the intestinal tract is in constant exposure to the dietary antigens, and the effect of diet on microbial composition.

Several dietary factors have been implicated as causative agents in disease pathogenesis, including diets high in simple sugars or starches, and increased consumption of total fats and protein (1-9). This type of diet is classically considered a western style diet (i.e. high in refined sugars and fat, and low in fruits, vegetables, and fibre), and is becoming more prevalent in countries such as Japan and China. It is believed such a diet could affect intestinal inflammation via a variety of mechanisms, including the direct effect of dietary antigens on the intestinal mucosa and indirect effects through the alteration of enteric microflora (10,11).

The aim of this study was to directly examine the effect of a western style diet (i.e. high in sugar and fat, and low in fibre) on mucosal immune responses and microflora composition in the IL10 gene deficient mouse model of colitis. I

hypothesize that a western diet will increase the severity of disease in the IL-10 knockout mouse.

4-2. Methods:

4-2-1. Animals and dietary treatment experimental setup:

Following weaning, female wild type (WT) and IL10 gene deficient (IL10$^{-/-}$) mice on the 129SvEv background, were randomly divided into two dietary treatment groups: a western style diet (33% fat and 24% simple sugars) and a standard mouse chow (n=7-9 mice in each group) for 35 days. The western diet was composed of 850g/Kg of TD. 06206 basal mix (Tekland diet) and supplemented with 150g/Kg of fat from sunflower oil (2% total fat) (Safeway brand), flax oil (2% of total fat) (Gold Top organic brand) and lard (96% of total fat) (Tenderflake) standard mouse chow (Laboratory Rodent Diet 5001, Lab Diet). Both the western diet and standard mouse chow (Laboratory Rodent Diet 5001, Lab Diet) were prepared fresh every 2 weeks and stored at -4 °C until use. Nutritional composition is displayed in Tables 4-1 and 4-2. Mice had free access to food and water throughout the experiment; amount of food eaten was determined by weighing of food weekly. Mice were weighed once a week and fecal samples collected and snap-frozen for microbial analysis. At the end of the 35 days, mice were sacrificed and the weight of the cecum along with the weight and length of the small and large intestine measured. Sections of the small and large intestine were collected for mucosal cytokine expression and histology.

Cecal contents were also collected to examine short chain fatty acid concentration.

The protocol for use of mice was approved by the Health Science Animal Care and Use Committee at the University of Alberta, and animals were kept in a regulated day/night cycle with controlled humidity and temperature.

4-2-2. Histological injury score

On day 35, IL10$^{-/-}$ and WT mice on chow and western diet, were sacrificed and sections of the large intestine were harvested, flushed with cold PBS, cut longitudinally and fixed in 10% buffered formalin. These samples were then embedded in paraffin wax, sectioned at 4um and stained with haematoxylin and eosin (H&E) for examination. Slides were assessed by a pathologist in a blinded fashion and assigned a histological score for intestinal inflammation. This score was based on the sum of 4 scoring criteria: mucosal ulceration (enterocyte injury) (score of 0-3), epithelial hyperplasia (score of 0-3), and lymphocyte and neutrophil lamina propria infiltration (score of 0-2 each).

4-2-3. Mucosal cytokine expression

To assess mucosal immune function, sections of the small and large intestine were removed from WT and IL10$^{-/-}$ mice, flushed with cold PBS and snap frozen at -80°C for analysis. Tissue samples were then homogenized to determine cytokine expression levels within the tissue. Briefly, samples were

thawed on ice in 500uL of RIPA buffer with protease inhibitor (20ug/mL

Phenylmethylsulfonyl fluoride, 15ug/mL Aprotinin, 2ug/mL Pepstatin A, 2 ug/mL

Leupeptin), and sonicated for 15sec and placed on ice. The homogenized tissues

were then centrifuged at 10000rpm for 10min and supernatants collected for

cytokine analysis. IL1β, IL2, IL4, IL5, CXCL1, IL12, IFNγ, and TNFα expression were

measured using a Th1/Th2 tissue culture MesoScale Discovery Kit (MesoScale

Discovery) as per manufacturer's instructions. IL13 and IL17 cytokine expression

was examined using enzyme-linked immunosorbent assay (ELISA) (R& D systems)

as per manufacturer's protocol.

4-2-4. Microbial analysis

Changes in microbial composition were determined in fecal samples

collected on days 0 and 35 through the use of Terminal Restriction Fragment

Length Polymorphism (T-RFLP) which was performed by Matt Emberg. Total

DNA was extracted from the fecal samples using a FastDNA Spin Kit for FECES

(MP Bio) as per manufacturer's instructions. The broad range forward primer 6-

FAM-8F (Applied Biosystems) (5'-AGAGTTTGATCCTGGCTCAG-3') and broad range

reverse primer 926R (Applied Biosystems) (5'-AGAAAGGAGGTGATCCAGCC-3')

were used to amplify 16S rRNA by PCR. The PCR was performed using 50ng DNA,

cycling conditions were: 94°C for 2 min followed by 35 cycles of 94°C 1 min, 56°C

1 min, 72°C 1 min, and a final 10 min extension at 72 °C. Every PCR run included a

DNA-free template control, and amplification was confirmed with visualization of

a single 920kb PCR product on 1% agarose gel. A Qiagen MinElute PCR

purification kit (Qiagen) was used to purify the amplicons, as per manufacturer's

instructions. The Hpa II restriction enzyme (Promega) was used to digest

amplicon DNA (200-300ng, determined with a Nanodrop spectrophotometer)

(Thermo Scientific), the digestion occurred for 16hr at 37°C. With each sample,

100ng of HPA II digested fragments were resolved in duplicate using a 3130XL

Genetic Analyzer (Applied Biosystems). Fragment length normalization was

achieved by running an internal ROX1000DBA marker to separate each sample.

Bionumerics 6.0 software (Applied Maths) was used to normalize the

fluorescently labeled terminal fragment lengths and select peaks of interest,

which are associated, *in silico,* with fragment lengths of known bacteria using

Microbial Community Analysis 3 (MiCA; Shyu,2007) and Ribosomal Database

Project v.9 (RDP; Cole, 2009). MiCA takes the fragment lengths and primer data

and associates the likely bacterial sequence with each band in the trace. RDP

uses the list generated from MICA to cluster the sequences and establish likely

bacteria and abundances. Fragments with peaks between 25 and 650 base pairs

in length are used in the community composition and cluster analysis. Principle

component/clustering analysis was then preformed on each sample and based

on the band patterns alone from each trace to identify specific clusters.

Bionumerics 6.0 software was also used to calculate the Shannon-weiner index

based on abundance data, to determine microbial diversity.

4-2-5. Short chain fatty acid analysis

Cecal contents were collect at sacrifice on day 35 from WT and IL10-/-mice and snap frozen and stored at -80 °C for short chain fatty acid concentration analysis. Briefly, 0.1N HCl was added to the cecal material and allowed to shake (180x g) overnight at 25°C. The sample was then vortexed and 5mL of diluents collected and added to 1mL of meta-phosphoric acid (HPO_3, 25% w/v in distilled H_2O). The samples were mixed and frozen overnight, and then thawed, vortexed, and centrifuged at 3000x g for 20min. Supernatant was then transferred into a GC vial (PerkinElmer) and sealed for analysis by gas chromatography by Dr. M.G. Ganzle (University of Alberta). Concentration of acetate, propionate, butyrate, isobutyrate, isovalerate, valerate and caproate was determined using external standards (sigma), and isocaproate was used as internal standard.

4-2-6. Statistical analysis

Data was analysed using a student T-test and ANOVA to compare the results from mice placed on a western diet to their control treated counterparts. Calculated P-values <0.05 were considered significant.

4-3. Results:

4-3-1. Morphological and histological analysis

IL10 $^{-/-}$ mice placed on the control chow demonstrated a decrease in their overall body weight over the 35 days compared to WT mice (Figure 4-1), and although not quite significant, there is an observable increase in their colon weight/length ratio which is associated with a higher colonic weight compared to WT mice on the control chow (Table 4- 3). These mice also had a higher overall histopathology score which is characterized by a trending increase in enterocyte injury and lymphocyte and neutrophil infiltrate within the colon compared to WT mice (Table 4-4). While these results were not significant it is important to note that 86% of IL10$^{-/-}$ mice demonstrated some degree of enterocyte injury or had immune cell infiltrate compared to WT mice which had no epithelial cell damage or neutrophil infiltration within the colonic tissue. These results suggest the IL10$^{-/-}$ mice are beginning to show signs of disease. This is consistent with previous studies which have demonstrated a decrease in body weight accompanied by an increase in histological injury and an increase in colon weight/length ratio which is associated mainly with an increased colon weight. This is suggestive of chronic inflammation within the tissue and indicative of disease in the mice (12-14). No change in the small intestine weight or length was observed for WT and IL10$^{-/-}$ mice (Table 4-3). IL10$^{-/-}$ mice placed on the western style diet gained significantly more weight over the 35 days then their control chow fed counterparts (Figure 4-1). This weight gain was similar to the weight gain associated with WT mice placed on the western diet (Figure 4-1). The increased weight gain occurred even though these mice consumed less food each week and had a decrease in the

intake of kcal/g of food eaten compared to their control chow counterparts (Figure 4-1). These mice also displayed a significant decrease in their colon weight/length ratio compared to chow fed IL10$^{-/-}$ mice which was the result of a significant decrease in colonic weight (Table 4-3). Interestingly, we observed a trending decrease in the histopathological score for IL10$^{-/-}$ mice on the western diet compared to IL10$^{-/-}$ mice on the chow diet (Table 4-4). In these mice, there was a lack of enterocyte injury (table 4-4) and all but one mouse had no neutrophil infiltration in the colon. These results suggest a decrease in disease progression or severity in IL10$^{-/-}$ mice exposed to the high fat, high carbohydrate western diet.

WT mice placed on the western diet also demonstrated an increase in weight compared to chow fed mice. There was also a significant decrease in their colon weight/length ratio (Table 4-3); however this was due to significant decreases in the measurements for both colonic weight and length (Table 4-3). No change in weight or length was observed in the small intestine of WT and IL10$^{-/-}$ mice (Table 4-3).

I also observed a significant decrease in cecum weight in WT mice and a trending, not quite significant decrease in IL10$^{-/-}$ mice placed on the western diet compared to their control chow counterparts (Table 4-3). This could suggest a reduction in fermentation within the colon in WT and IL10$^{-/-}$ mice on the western diet.

Table 4-1. Nutrition breakdown of control chow and western diet

Diet	Protein (%)	Carbohydrate (%)					Fat (%)	Kcal/g
		Sugar*		Fiber		Starch		
Control chow	29	monosaccharide	0.52	NDF#	15.1	31.9	13	4.1
		disaccharide	5.71					
Western diet	26	monosaccharide	23.9	NDF#	5	20.3	33	4
		disaccharide	0.029					

*sugar composition within: **control chow**: monosaccharide (glucose 0.22%, fructose 0.3%), disaccharide (sucrose 3.7%, lactose 2.01%).**western diet**: monosaccharide (glucose 23.9%), disaccharide (sucrose 0.03%)

NDF composition differs between control chow and western diet. **Control chow**: cellulose, hemi-cellulose, and lignin. **Western diet**: cellulose

Protein, carbohydrate, and fat values displayed as % of total diet

Table 4-2: Fatty acid composition of control chow and western diet (measured by Miriam Jacome)

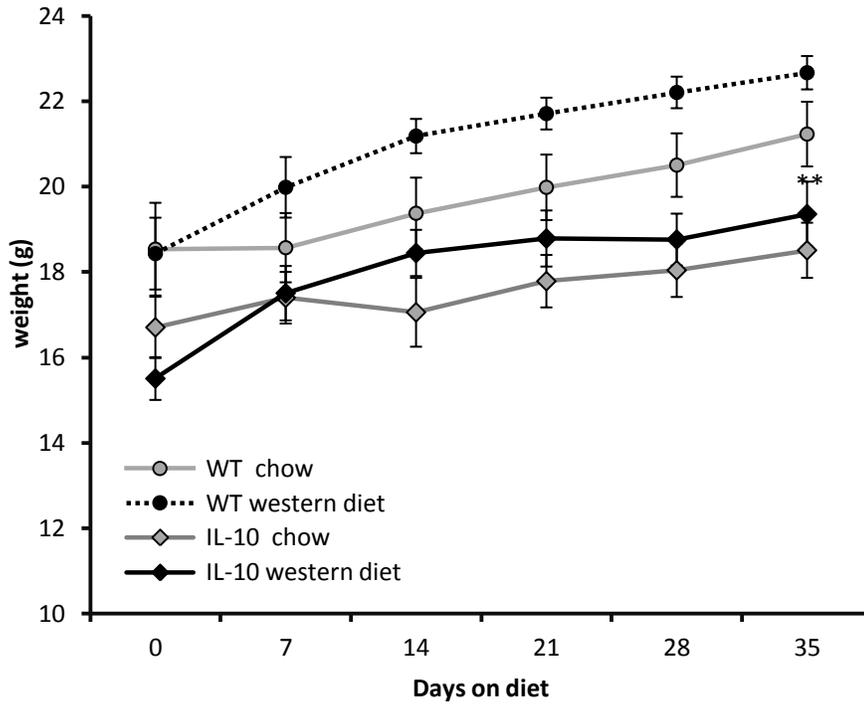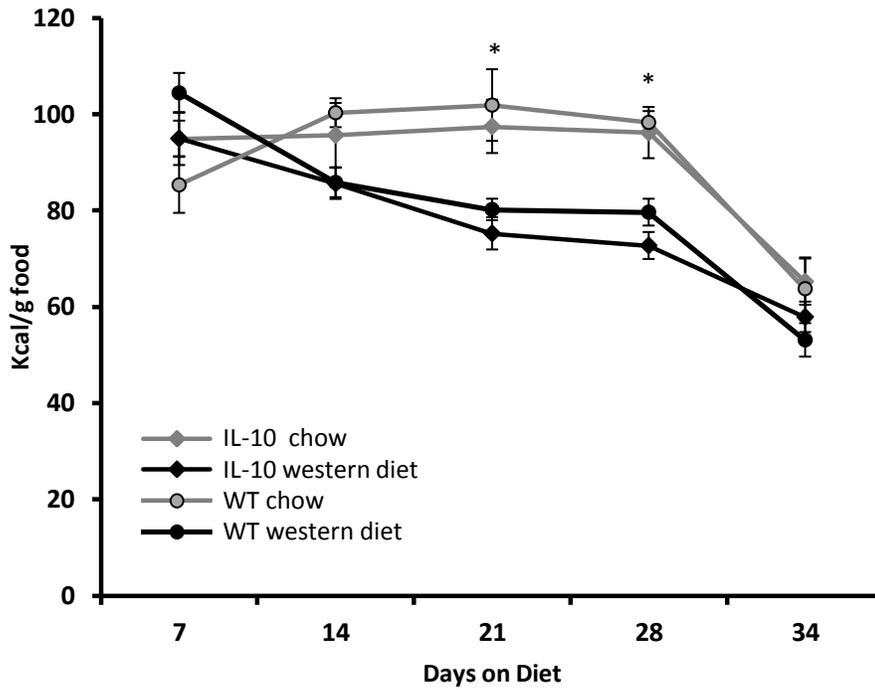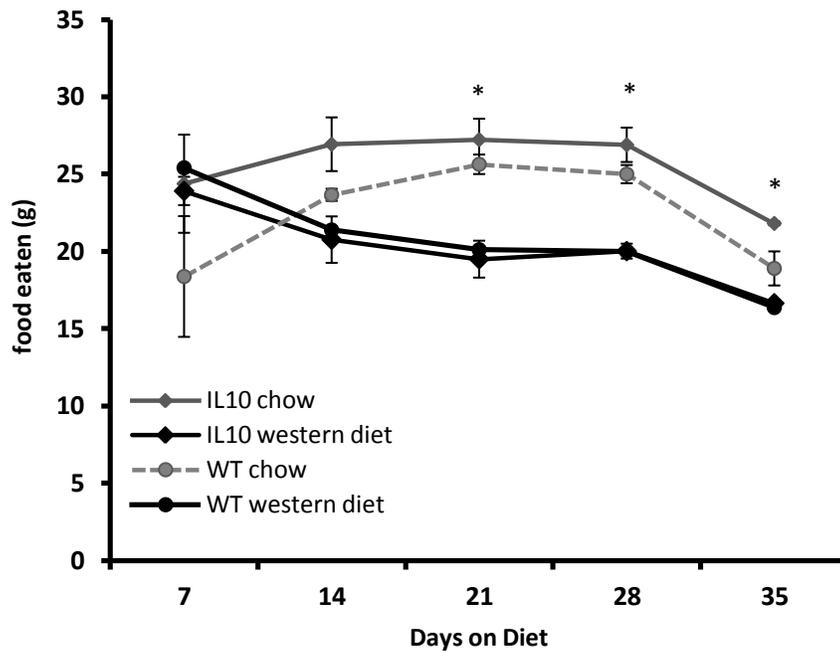|	Chow diet	Western diet
C14:0 (Miristic acid)	0.84	1.03
C16:0 (Palmitic acid)	26.16	26.34
C16:1	1.42	1.80
C18:0 (Estearic acid)	13.37	16.88
C18:1 (Oleic acid)	27.20	34.85
C18:2 n6 (Linoleic acid)	23.93	15.33
C18:3 n3 (Linolenic acid)	2.85	1.59
C20:0	0.46	0.27
C20:1	0.79	0.79
C20:2 n6	0.38	0.59
C20:4 n6 (Araquidonic acid)	0.36	0.08
C20:5 n3 (EPA)	1.42	0.16
C22:6 n3 (DHA)	0.81	No Detectable
Summaries		
Saturated fatty acids (SFA)	40.83	44.80
MUFA	29.41	37.44
PUFA	29.76	17.76
SFA/PUFA	0.73	0.40
n6	24.29	15.41
n3	5.08	1.76
n6/n3	4.78	8.76

Figure 4-1a

Figure 4-1b

Figure 4-1c

Figure 4-1: Measurements relating to weight and food consumption in WT and IL10$^{-/-}$ on the standard mouse chow or western diet. Values displayed as mean ± SEM **a)** Overall weight gain (g) over 35 days for mice placed on the western diet and control chow. ****** represents a significant overall increase in weight over the 35 days (P<0.05) **b)** Amount of kcal consumed per g of food eaten each week ***** represents significant decrease in kcal per g of food consumption on western diet in WT and IL10$^{-/-}$ mice (P<0.05)   **c)** Measurement of food eaten each week over the 5 weeks. *****represents significant decrease in food consumption that week for both WT and IL10$^{-/-}$ mice on western diet (P<0.05)

Table 4-3: Small and large intestine and cecum measurements for IL10$^{-/-}$ and WT mice on a standard mouse chow or western diet for 35 days

Group	Small intestine			Colon			Cecum
	weight (g)	length (cm)	Weight/length (%)	weight (g)	length (cm)	Weight/length (%)	Weight (g)
WT + chow (n=3)	0.719 ± 0.03	30.1 ± 0.9	2.18 ± 0.08	0.221 ± 0.005	8.5 ± 0.12	2.5 ± 0.09	1.25 ± 0.036
WT + western (n= 3)	0.665 ± 0.04	34.2 ± 1.7	2.14 ± 0.13	0.116 ± 0.004**	7.0 ± 0.31**	1.81 ± 0.09**	1.10±0.0047*
IL10$^{-/-}$ + chow (n= 6)	0.879 ± 0.04	36.8 ± 3.1	2.47 ± 0.09	0.353 ± 0.07	7.93 ± 0.3	3.57 ± 0.06	1.17 ± 0.018
IL10$^{-/-}$ + western (n=6)	0.767 ± 0.07	36.5 ± 1.6	2.25 ± 0.01	0.136 ± 0.02#	7.37 ± 0.52	2.19 ± 0.02#	1.13 ± 0.020

*represents significance for cecum weight between WT mice on western diet and control chow (P<0.05) **represents significance for colon measurements between WT mice on western diet and control chow (P<0.05)

#represents significance for colon measurements between IL10-/- mice on western diet and control chow (P<0.05)

Values are presented as mean ± SEM

Table 4- 4: Histological score for IL10$^{-/-}$ and WT mice on the standard mouse chow or western diet for 35 days

Group	Histology					
	# of affected mice	Enterocyte injury (0-3 units)	Epithelia hyperplasia (0-3 units)	Lamina Propria Mononuclear Infiltrate (0-2 units)	Lamina Propria neutrophil Infiltrate (0-2 units)	Total histological injury (0-10 units)
WT + chow (n=3)	1	0	0	0.33 ± 0.19	0	0.33 ± 0.19
WT +western (n= 3)	2	0	0	0.67± 0.38	0	0.67 ± 0.38
IL10$^{-/-}$ + chow (n= 6)	6	0.71 ± 0.27	0	1.14 ± 0.43	0.43 ± 0.16	2.29 ± 0.86
IL10$^{-/-}$+ western (n=6)	6	0.38 ± 0.13	0.14 ± 0.14	0.71 ± 0.29	0.14 ± 0.14	1 ± 0.38

Values displayed as mean ± SEM

4-3-2. Western style diet alters immune function in IL10 deficient mice

Small and large intestinal tissue homogenate was analyzed for expression of various T-helper (Th) 1 and Th2 related cytokines in order to determine if exposure to the western style diet alters mucosal immune function. Compared to WT mice, IL10$^{-/-}$ mice expressed significantly higher levels of the pro-inflammatory cytokines interleukin (IL) 12, TNFα, and IFNγ, and a trending increase in IL17 and IL1β, within the colon (Figure 4-2). There was also a significant increase in the chemokine CXCL1, which is a key marker of intestinal inflammation and associated with neutrophil infiltration into the tissue (Figure 4-2). These increases are indicative of inflammatory cell presence and active inflammation involving the Th1 and Th17 mediated immune pathways within the colon of IL10$^{-/-}$ mice at day 35.

Interestingly, when IL10$^{-/-}$ mice were placed on the western diet, there was a significant decrease in the expression of IL12, TNFα, IFNγ, IL2 and CXCL1 (Figure 4-2). This suggests a decreased inflammation in the colons of IL10$^{-/-}$ mice on a western diet. I also observed a trending decrease in the Th2 cell mediated cytokines IL4, IL5, and IL13. This could suggest a decrease in the overall mucosal immune response within the colon of IL10$^{-/-}$ mice placed on a western style diet.

Within the small intestine of control IL10$^{-/-}$ mice, IL-1β expression was significantly increased compared with WT mice (Figure 4-3). IL-10$^{-/-}$ mice on the western diet had increased expression levels for the Th1 mediated cytokines IL12

and IFNγ, (Figure 4-3). There was no effect of the western diet on small

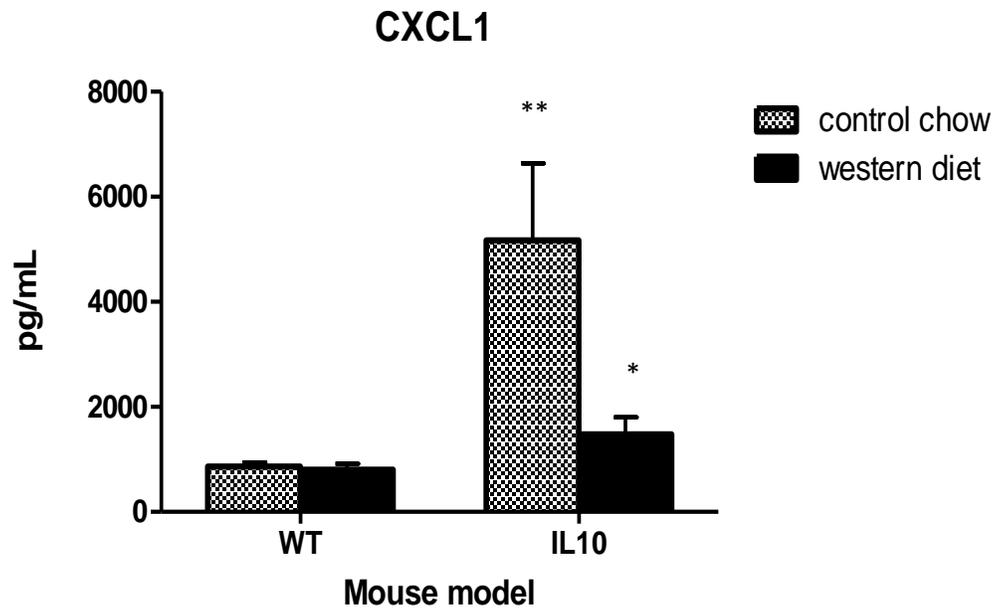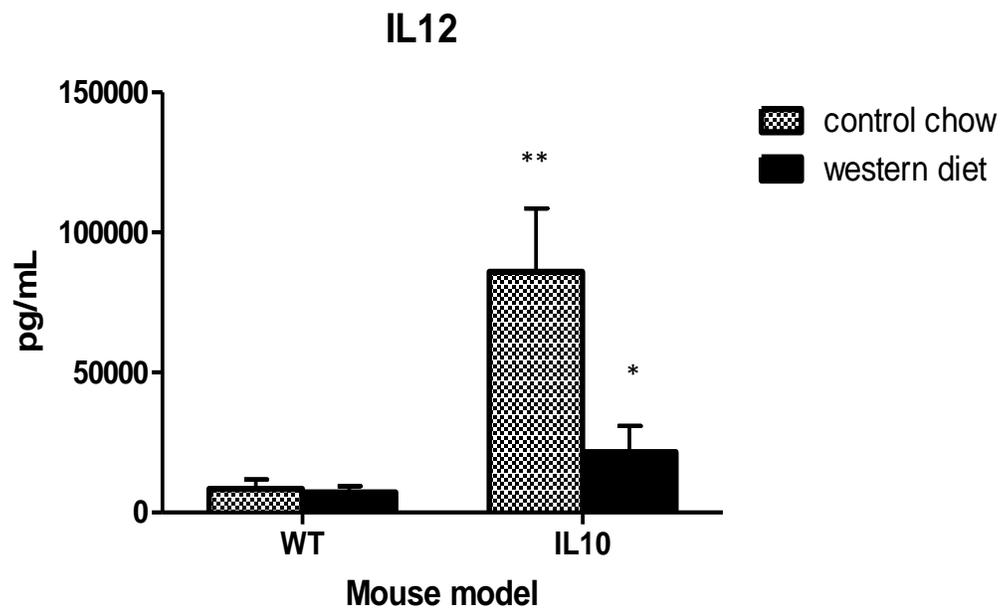intestinal immune function in WT mice.
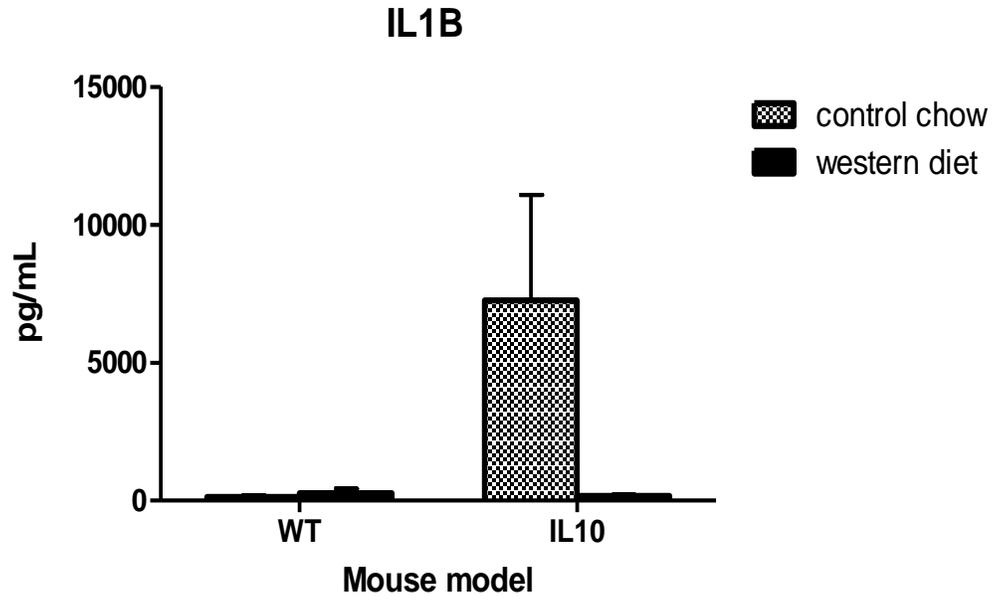
Figure 4-2a

CXCL1

Figure 4-2b

IL12

Figure 4-2c

IL1B

Figure 4-2d

TNFa
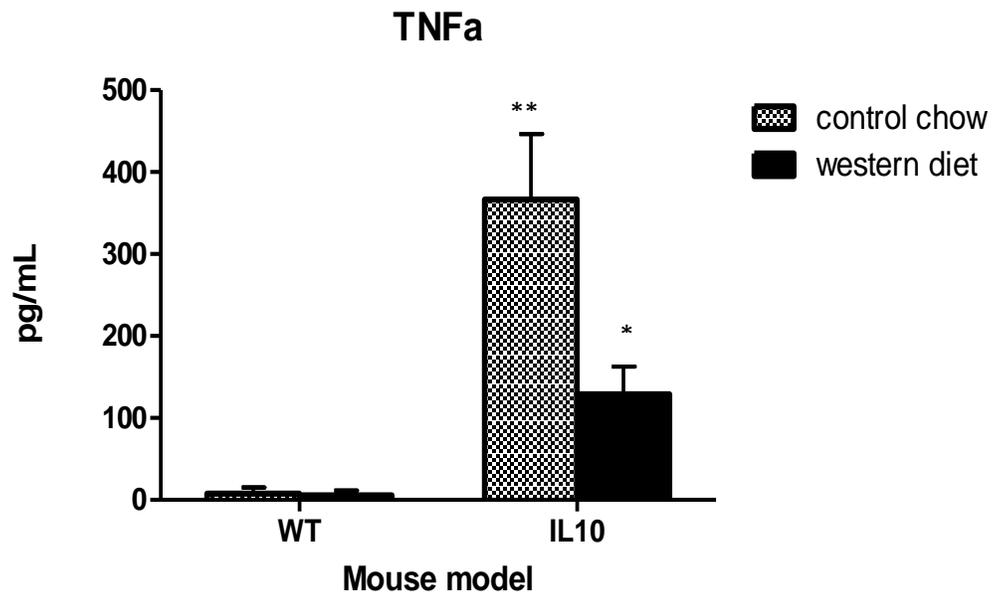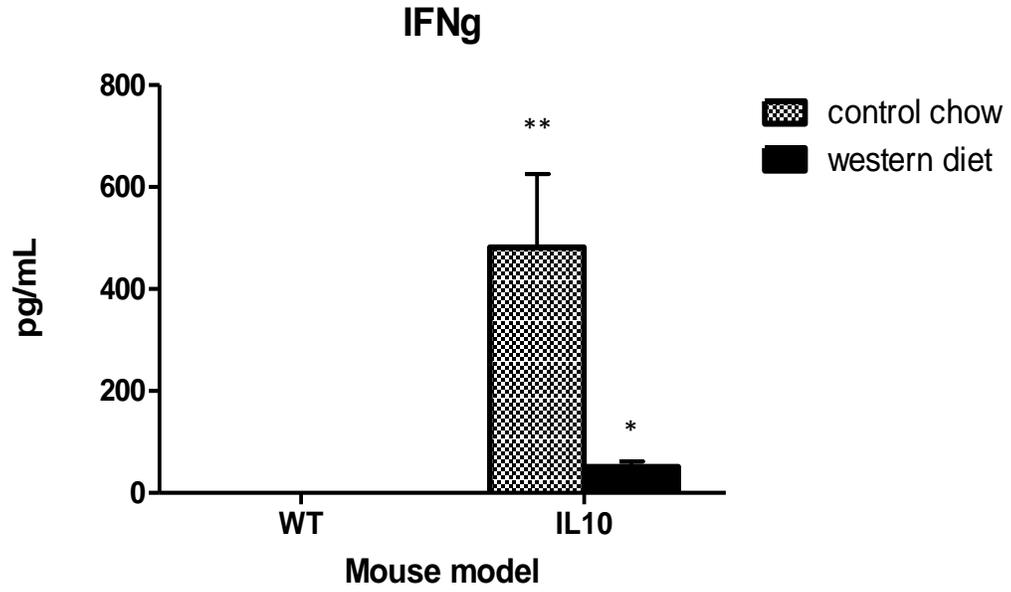
Figure 4-2e

Figure 4-2f

Figure 4-2g

IL4

Figure 4-2h

IL5

Figure 4-2i

IL13

Figure 4-2j

IL17
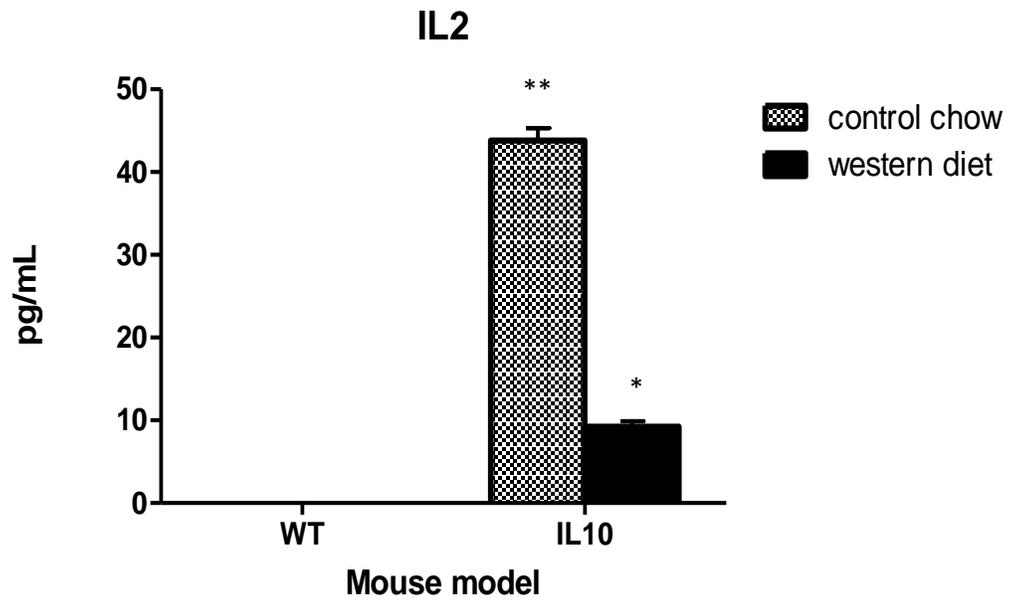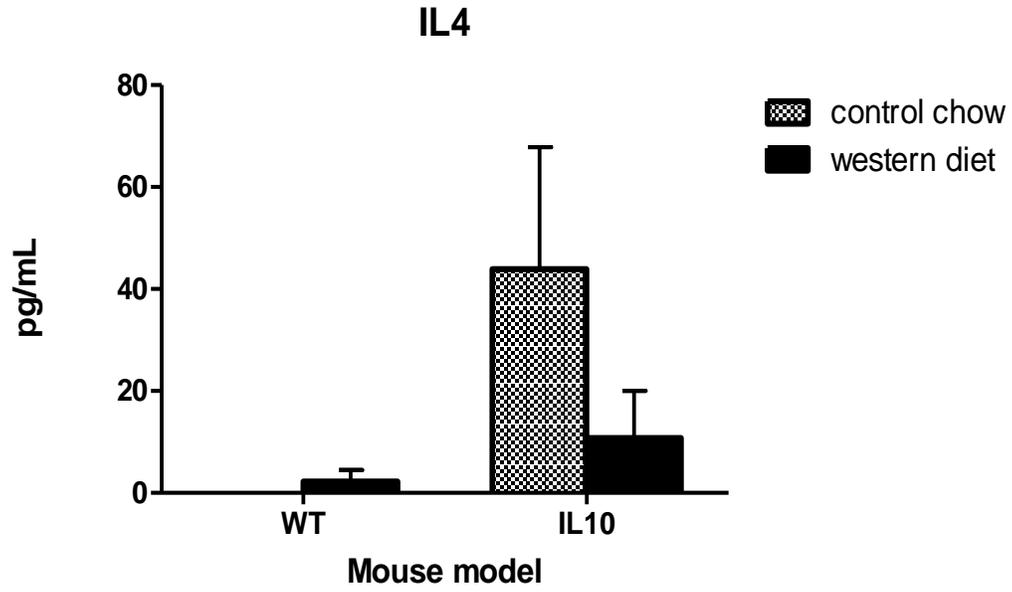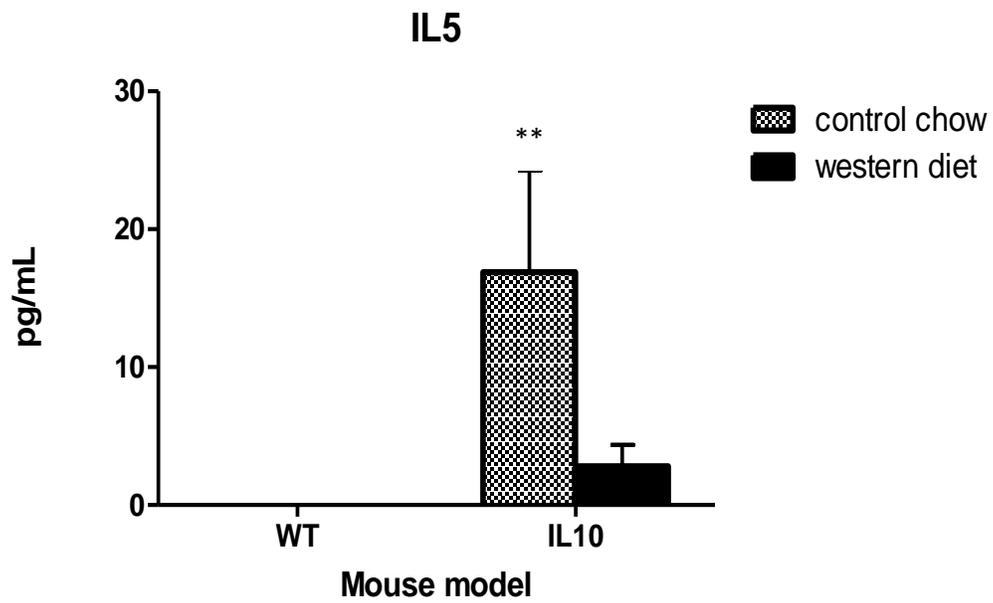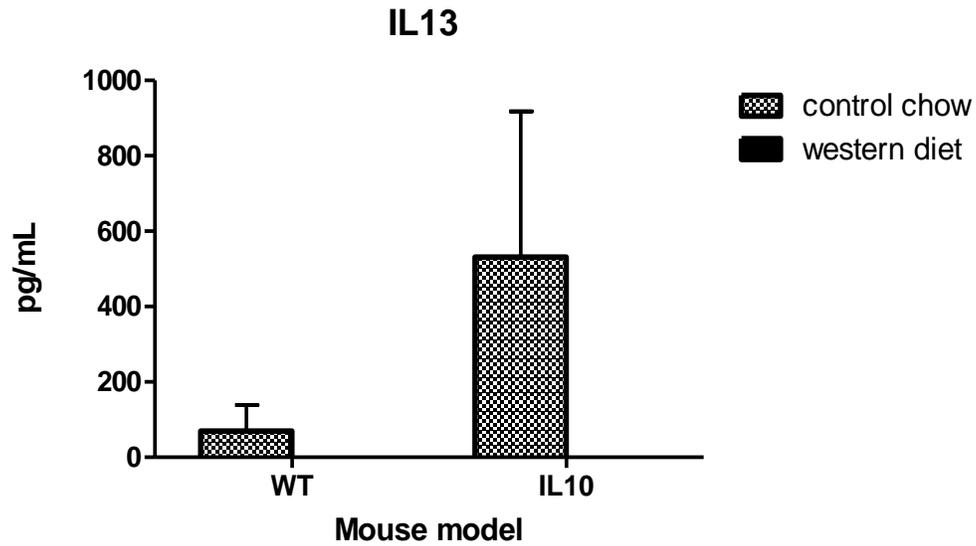
Figure 4-2: Cytokine and chemokine expression from colonic homogenate of WT and IL10$^{-/-}$ mice after 35 days on the western diet or control chow. **a)** CXCL1 chemokine expression, **b)** Interleukin (IL) 12, **c)** IL1b, **d)** TNFα, **e)** IFNγ, **f)** IL2, **g)** IL4, **h)** IL5, **i)** IL13, and **j)** IL17. Values displayed as mean ± SEM (n= 7-8 for all groups).   * Significance between western diet treated mice and control chow (P<0.05)

** Significant difference between WT and IL10$^{-/-}$ mice (P<0.05)

Figure 4-3a
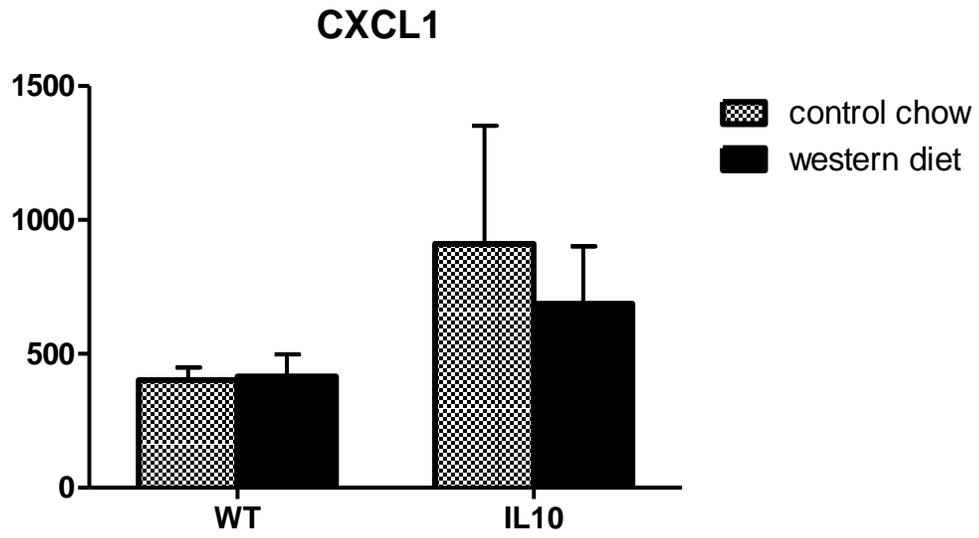
Figure 4-3b
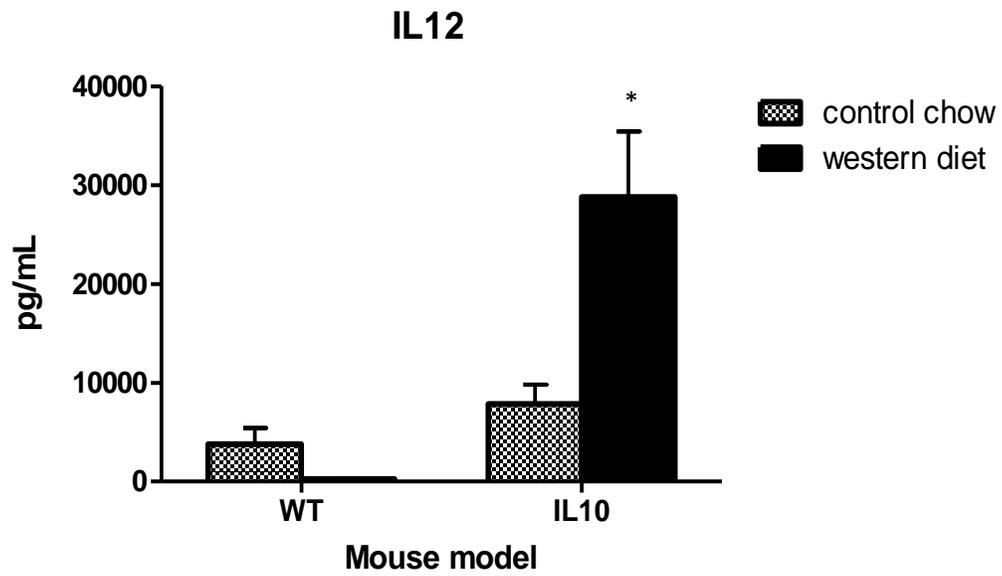
Figure 4-3c
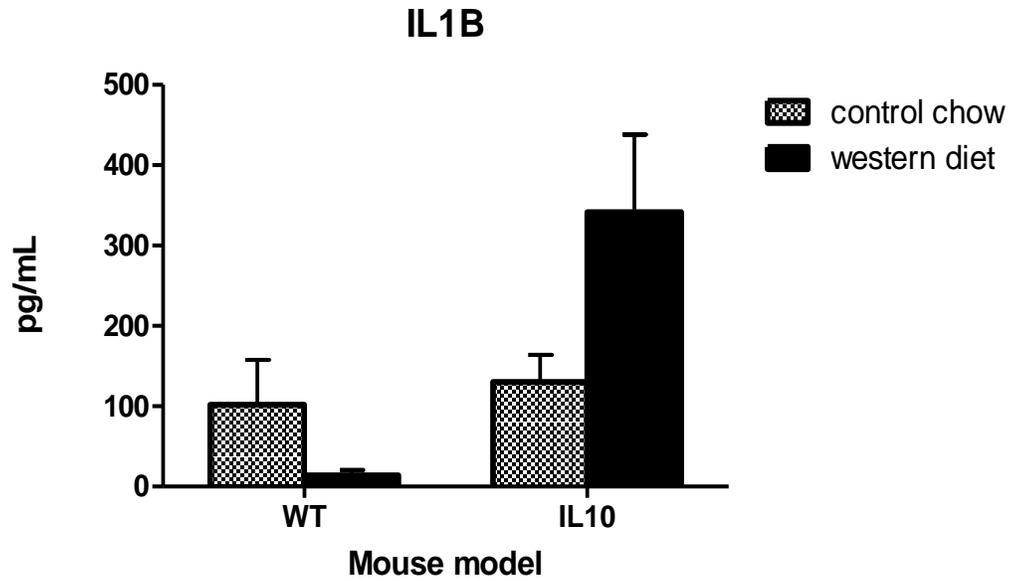
Figure 4-3d
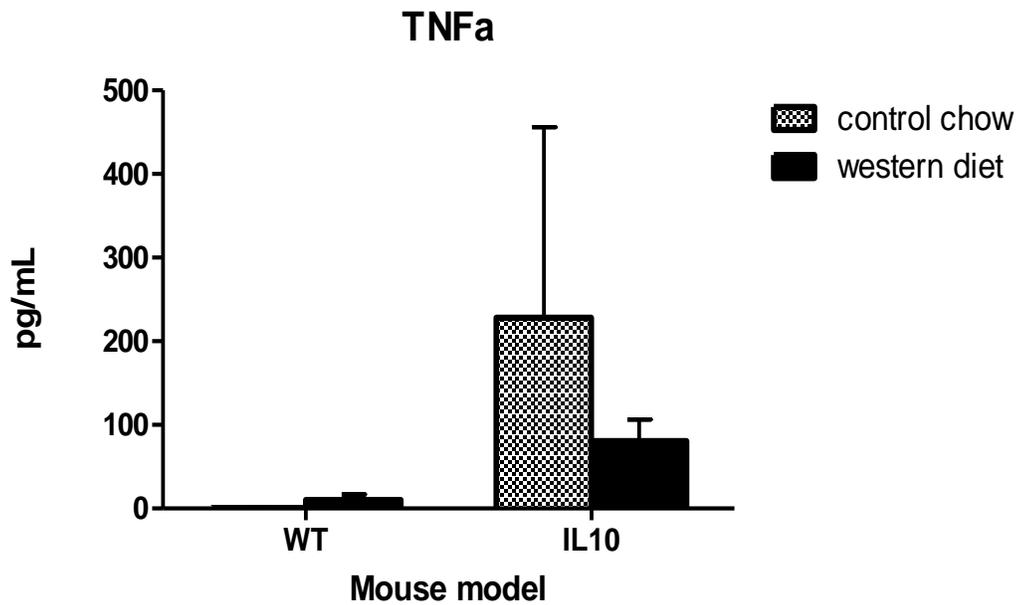
Figure 4-3e
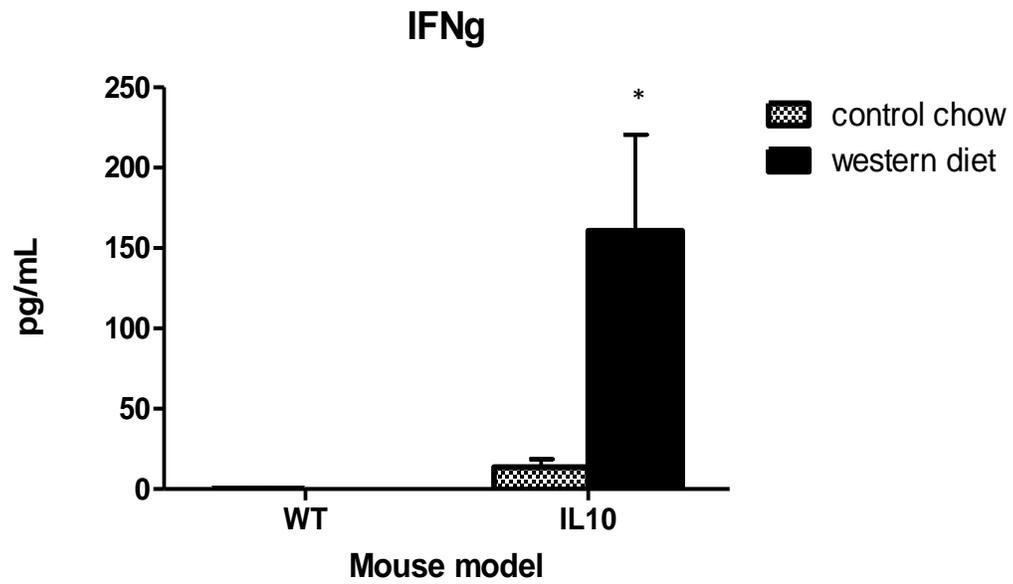
Figure 4-3f

Figure 4-3g

IL5

Figure 4-3h

IL13
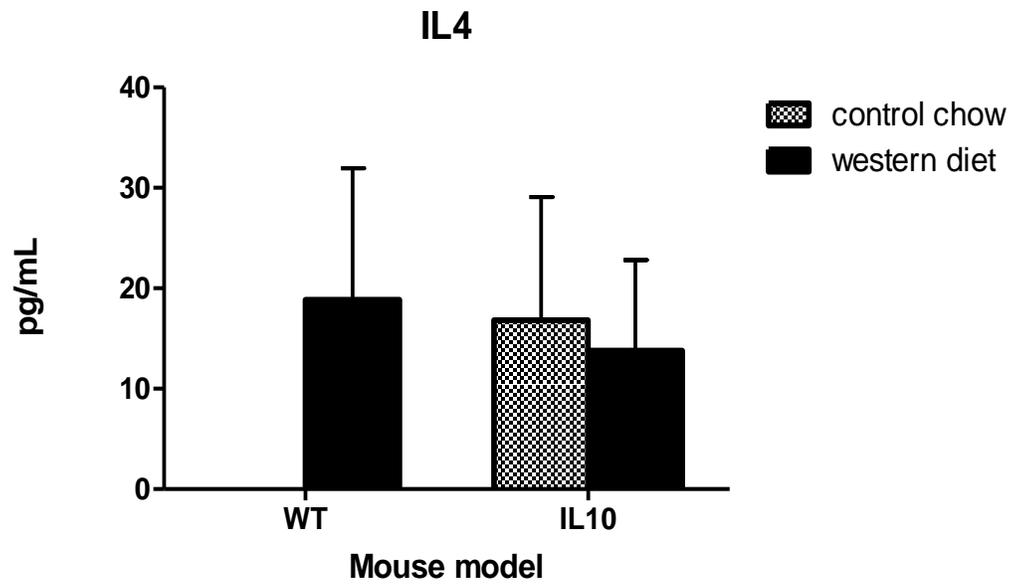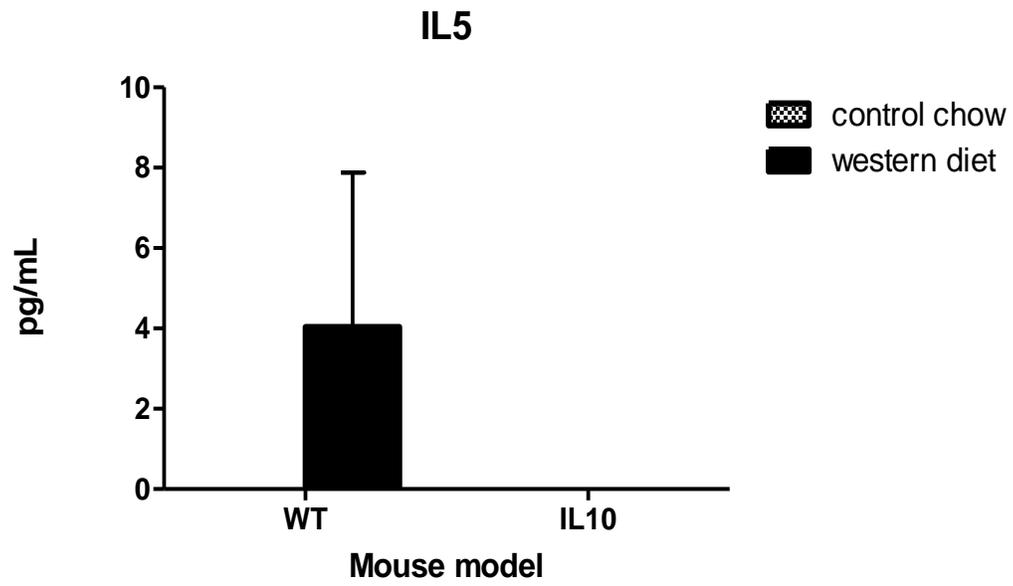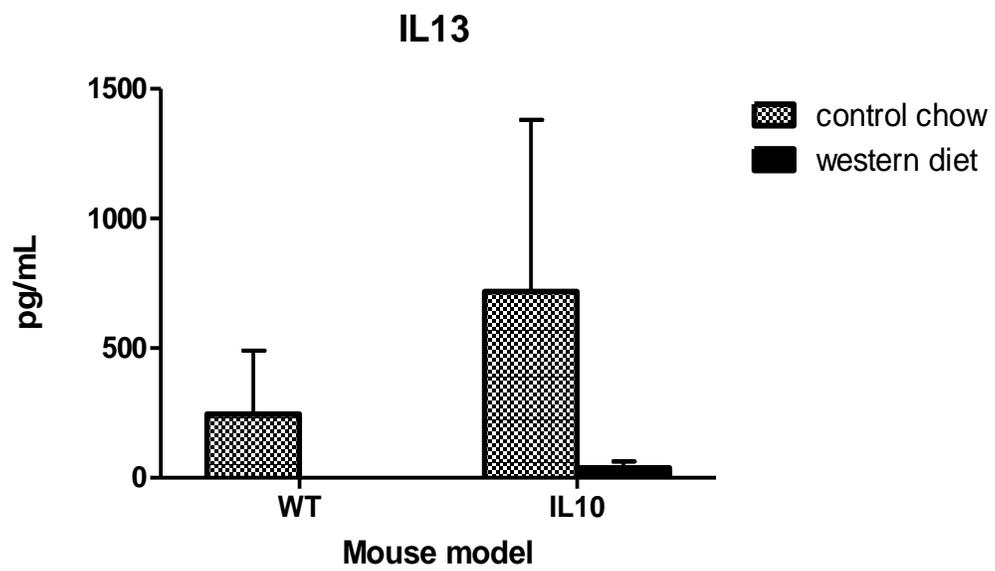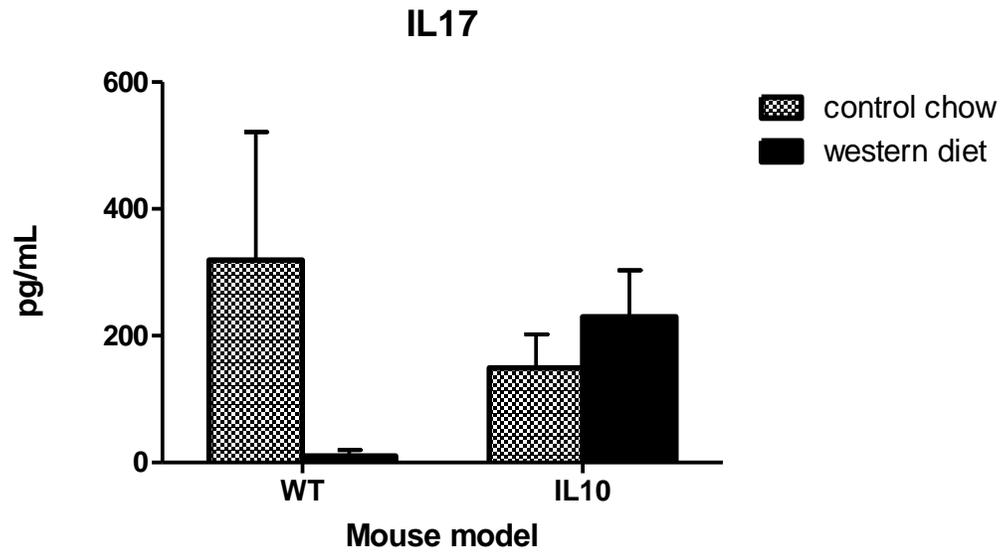
Figure 4-3i

Figure 4-3: Cytokine and chemokine expression from small intestinal tissue homogenate of WT and IL10$^{-/-}$ mice after 35 days on the western diet or control chow. **a)** CXCL1 chemokine expression, **b)** Interleukin (IL) 12, **c)** IL1b, **d)** TNFα, **e)** IFNγ, **f)** IL4, **g)** IL5, **h)** IL12, and **i)** IL17. There was no IL2 detected in the small intestine. Values displayed as mean ± SEM (n= 8)

* Significance between western diet treated mice and control chow P<0.05

4-3-3. Western style diet alters microbial composition in IL10$^{-/-}$ mice

Having shown that IL-10$^{-/-}$ mice on the western diet had attenuated disease and cytokine secretion in the colon, I then sought to determine if microbial composition of the colon was also altered by the western diet. Microbial composition of fecal pellets was measured in samples taken prior to day 0 and following treatments at day 35 using the non-culture method of Terminal restriction fragment length polymorphism (T-RFLP).

Western diet consumption resulted in significant alterations within the microbial community of IL10$^{-/-}$ mice. Principle component analysis (PCA) confirmed that there were significant alterations in the microbiome of IL10$^{-/-}$ mice on the western diet compared to control IL10-/- mice on the chow (Figure 4-6). To determine if microbial diversity was also altered by the western diet, the Shannon-Weiner index was determined. There was a significant decrease in the index in IL10$^{-/-}$ mice placed on the western diet compared to their control chow counterparts (Figure 4-7). This suggests a significant decrease in overall microbial diversity in response to the western diet. Specifically, there was a significant decrease in the abundance of the bacterial phyla *Bacteroidetes* and *Spirocheates*, and a significant increase in *Ternicutes* and unclassifiable bacteria in IL10$^{-/-}$ after 35 days on the western diet (Figure 4-4). There was also a trending increase in the *Firmicutes* phyla, although this was not quite statistically significant (Figure 4-4). Further T-RFLP analysis suggests the significant decrease

in *Bacteroidetes* may have been due to the reduced abundance of *Bacteroidia* in particular and the increase in *Tenericutes* the result of increased *Mollicute* abundance in the IL10-/- mice on the western diet (Table 4-5). Within the *Firmicutes*, there was an increased abundance of *Erysipelotrichi* and *Bacili*; however this was also accompanied by decreased *Clostriadia* abundance, which may explain why the overall abundance of *Firmicutes* was not significantly increased in IL10-/- mice fed the western diet (Table 4-5).

Western diet exposure also altered microbiota within WT mice, although these changes were different than those observed in the IL10$^{-/-}$ mice. In WT mice, there was a significant increase in the overall abundance of *Verrucomicrobia* phylum after 35 days on the western diet compared to control chow (Figure 4-5). Within the phylum *Firmicutes*, there was increased *Clostridia* abundance in response to western diet exposure; there were also increases in *Betaproteobacteria* and *Deltaproteobacteria* within the *Proteobacteria* phylum in WT mice placed on the western diet (Table 4-6). PCA analysis again confirms alterations within the bacterial population for WT mice placed on the western diet compared to their control chow counterparts (Figure 4-6). There was also a trending, yet insignificant decrease in the Shannon-Weiner index, which could suggest decreased microbial diversity in response to the western diet (Figure 4-7). This suggests a western style diet can alter the microbial community in both IL10$^{-/-}$ and to a lesser extent, WT mice.

Figure 4-4a
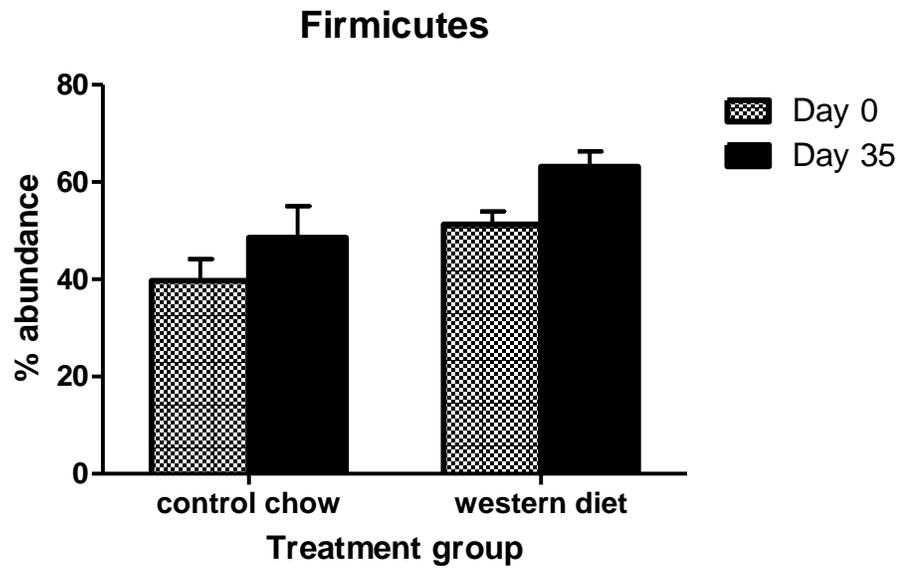
Firmicutes
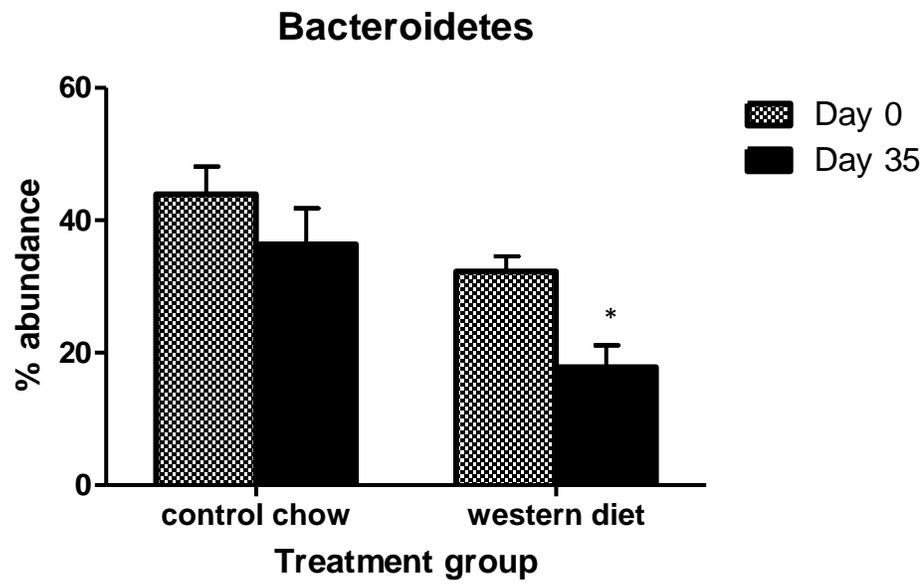
Figure 4-4b

Bacteroidetes

Figure 4-4c

Tenericutes

Figure 4-4d

Actinobacteria

Figure 4-4e

Proteobacteria

Figure 4-4f

Verrucomicrobia

Figure 4-4g

Spirocheates

Figure 4-4h

Fusobacteria

Figure 4-4i

Unclassified bacteria
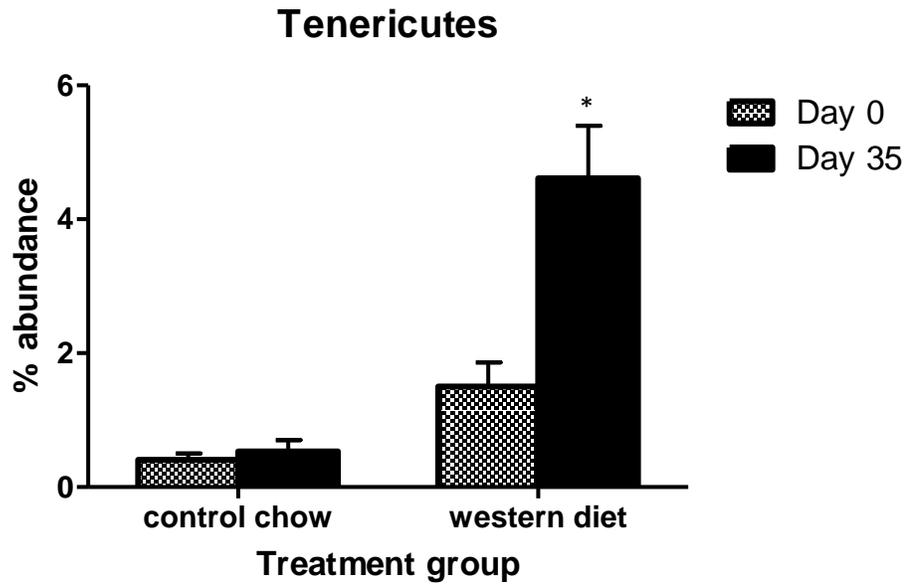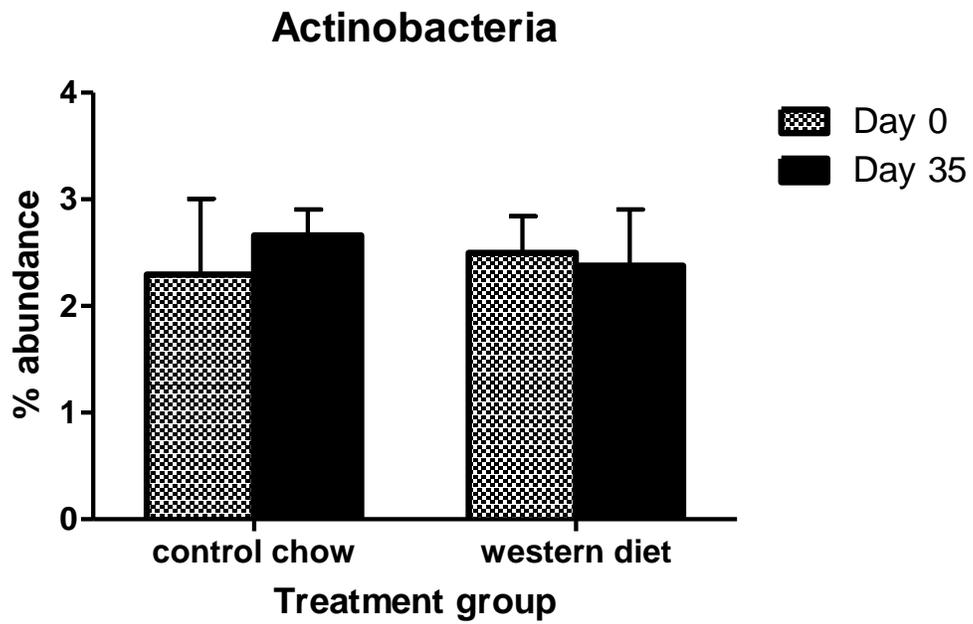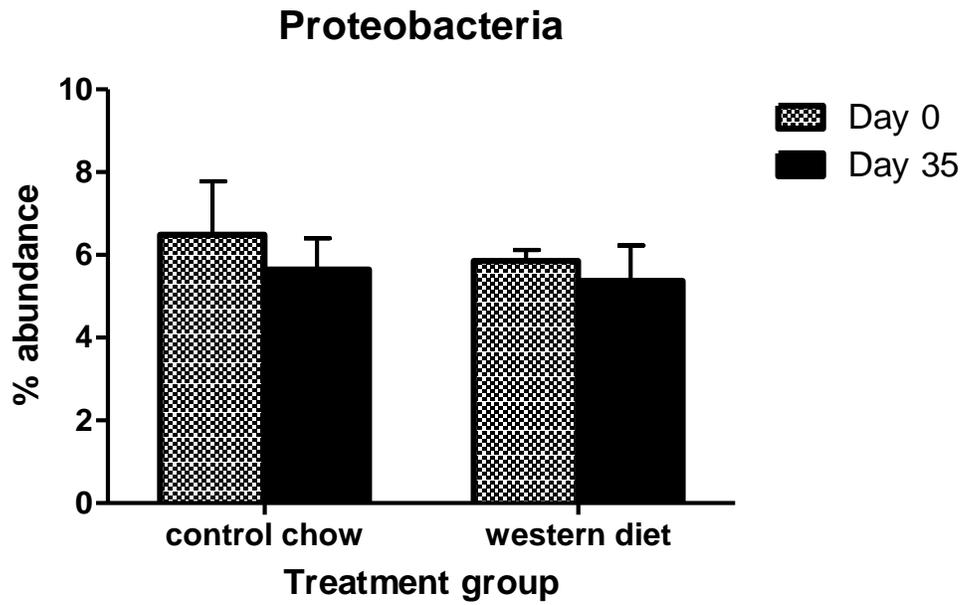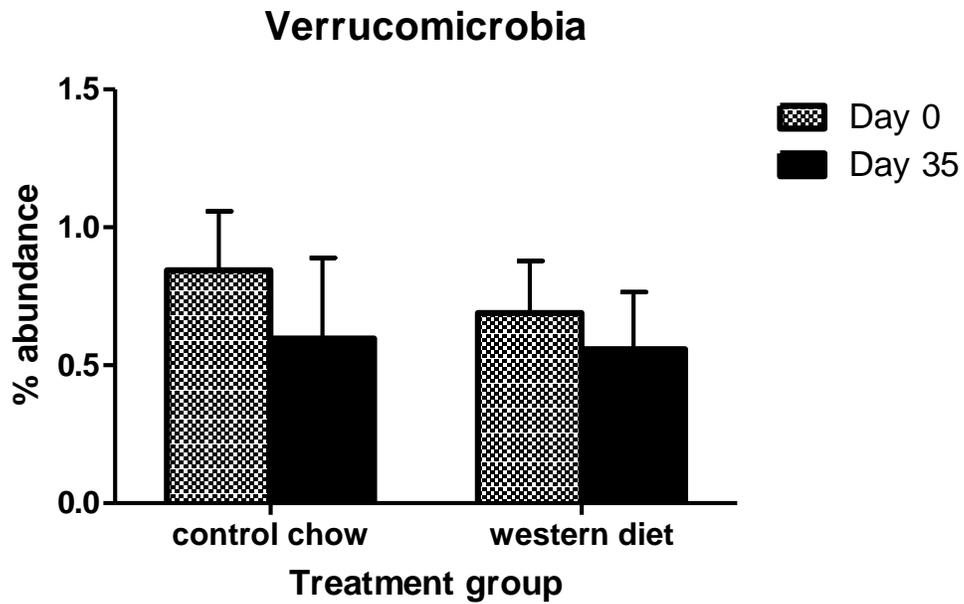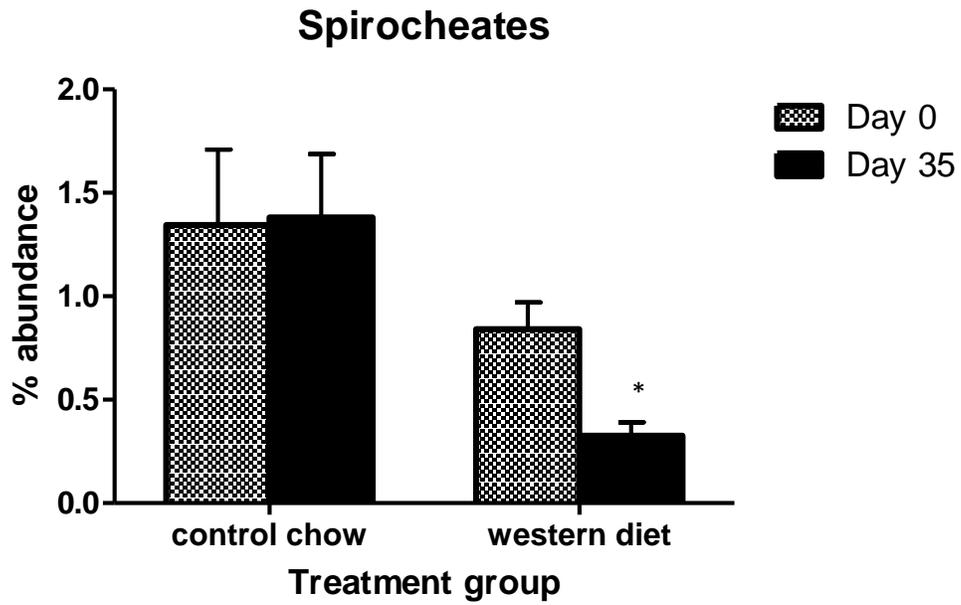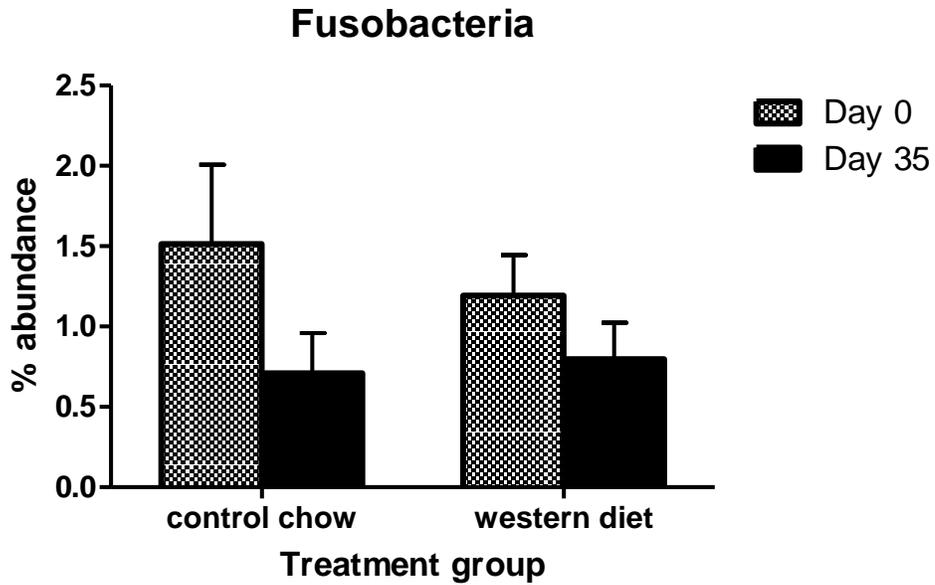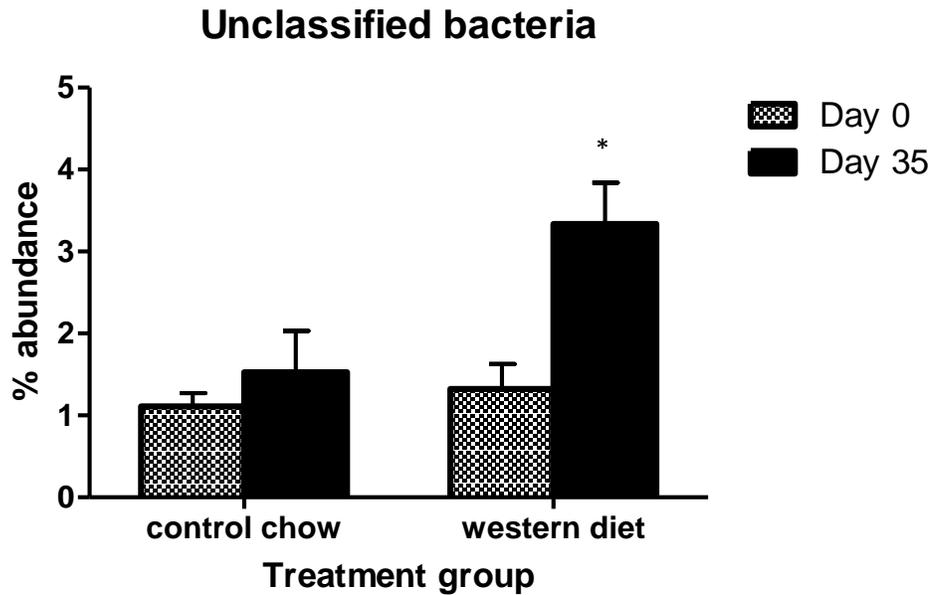
Figure 4-4. Microbial analysis performed on fecal samples prior to treatments (day 0) and following treatments (day 35) for IL10$^{-/-}$ mice on the control chow and western diet. Analysis was performed using T-RFLP, and bacteria phyla are displayed as % abundance. Bacteria phyla: **a)** *Firmicutes*, **b)** *Bacteroidetes,* **c)** *Tenericutes*, **d)** *Actinobacteria*, **e)** *Proteobacteria*, **f)** *Verrucomicrobia*, **g)** *Spirocheates*, **h)** *Fusobacteria*, **g)** unclassified bacteria.

* Significance between day 0 and day 35 samples p<0.05

Values displayed as mean ± SEM (n= 7-8)

Figure 4-5a
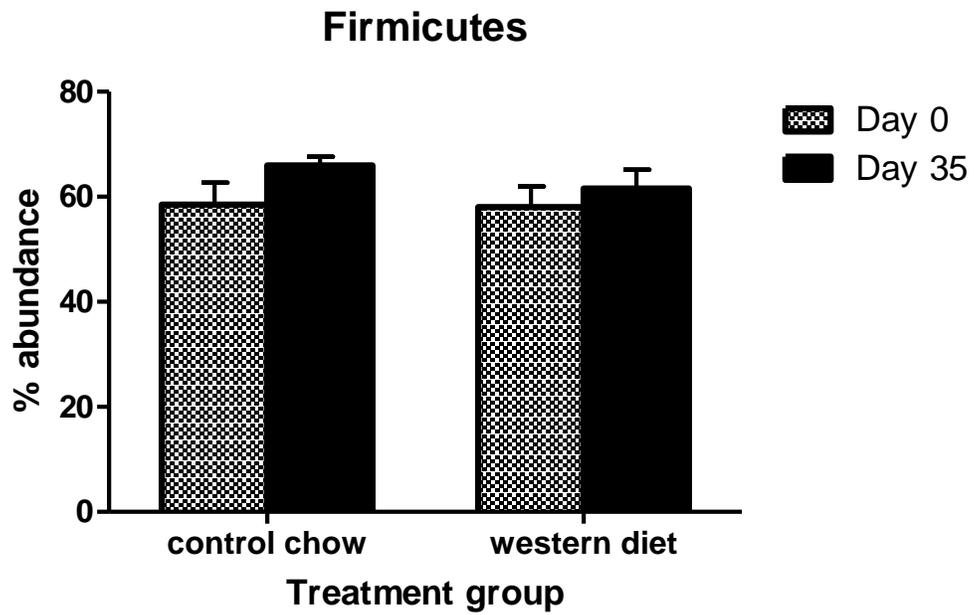
Firmicutes
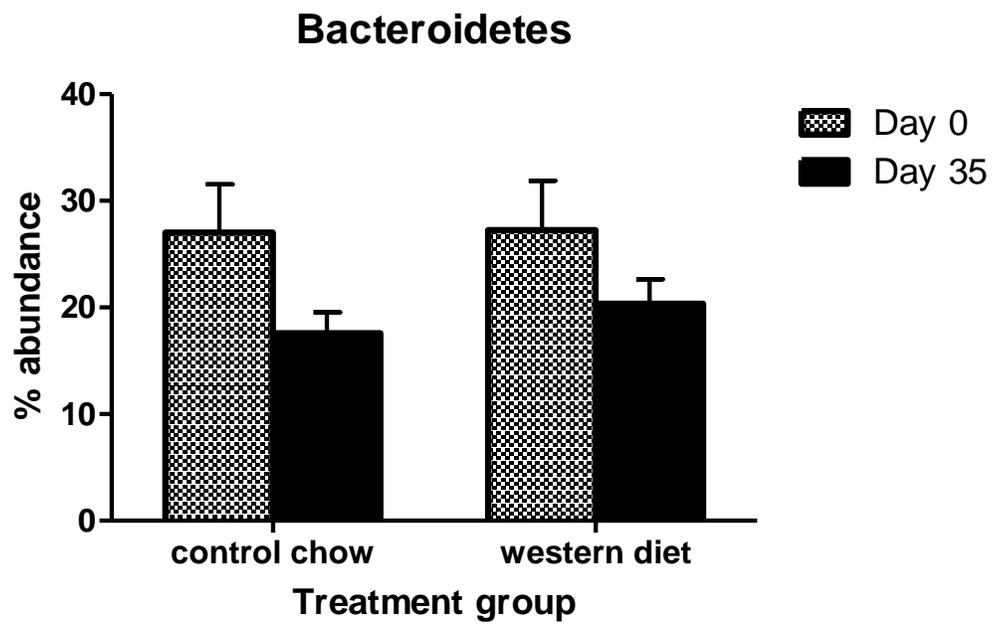
Figure 4-5b

Bacteroidetes

Figure 4-5c

Tenericutes

Figure 4-5d

Actinobacteria

Figure 4-5e

Proteobacteria

Figure 4-5f

Verrucomicrobia

Figure 4-5g

Spirocheates

Figure 4-5h

Fusobacteria

Figure 4-5i

Unclassified bacteria
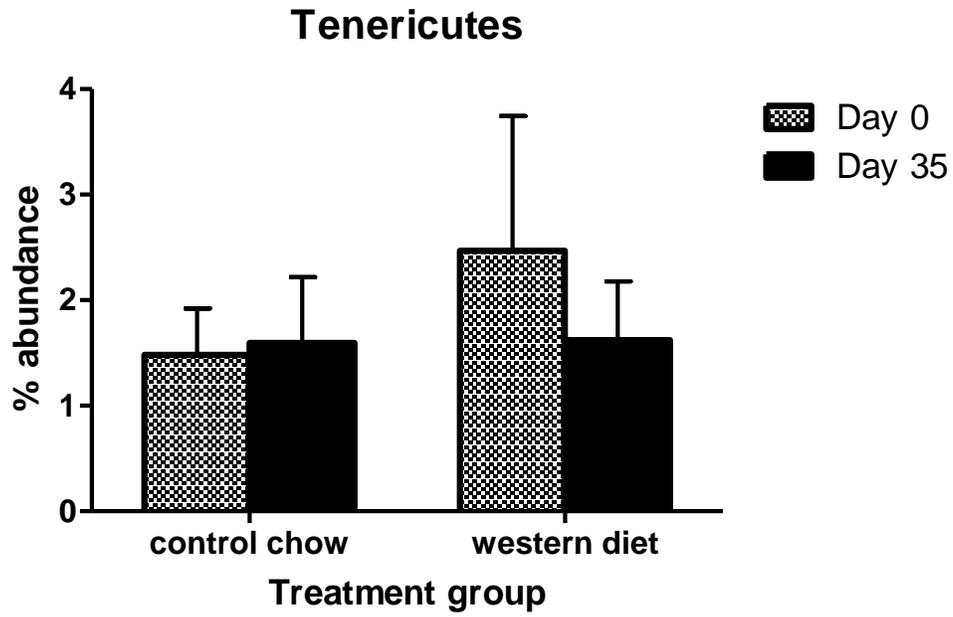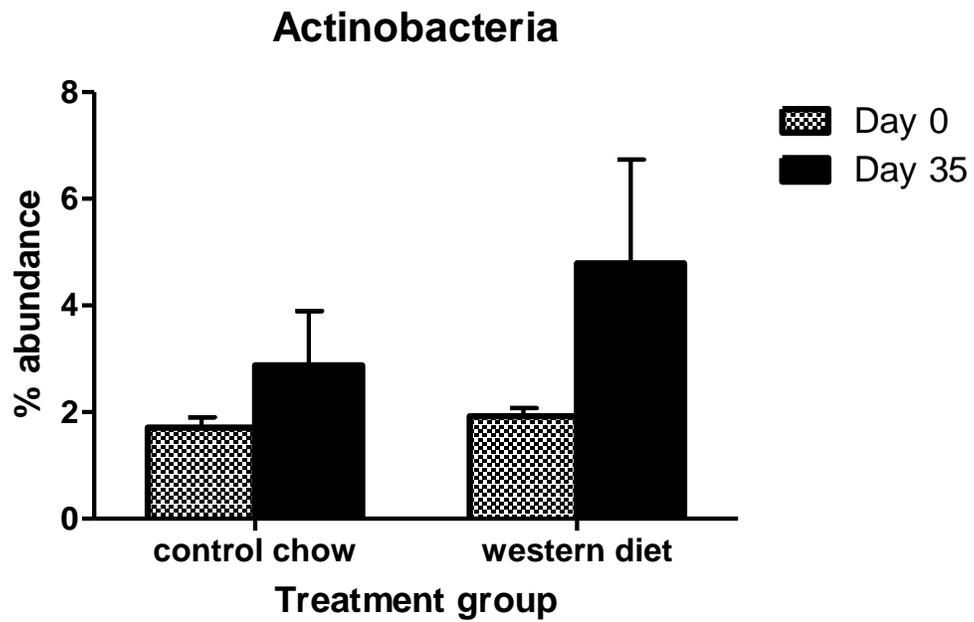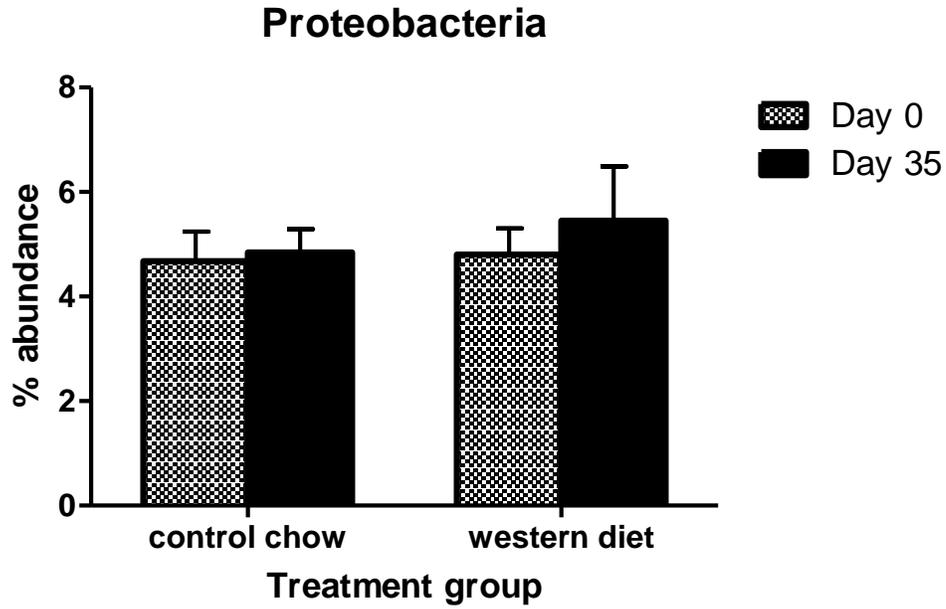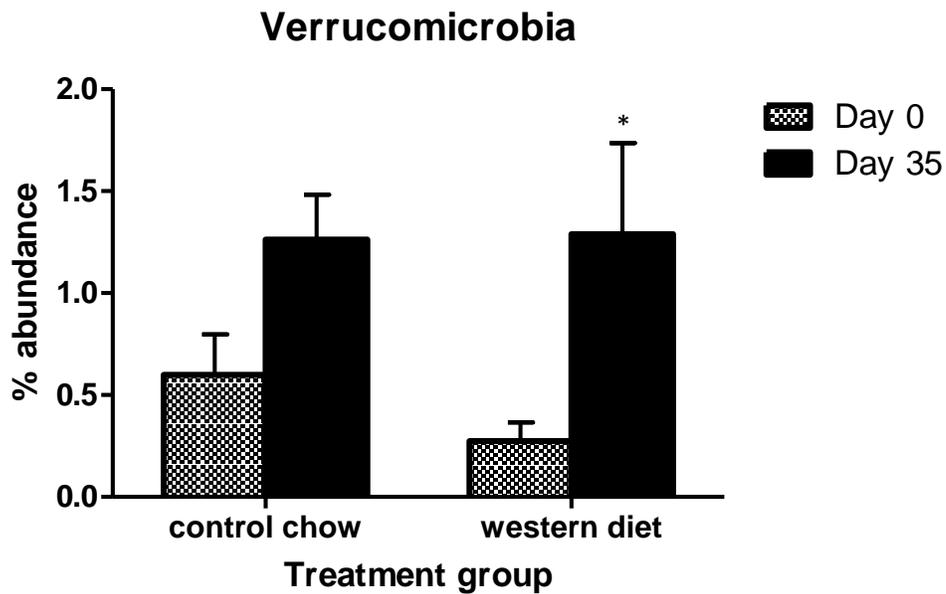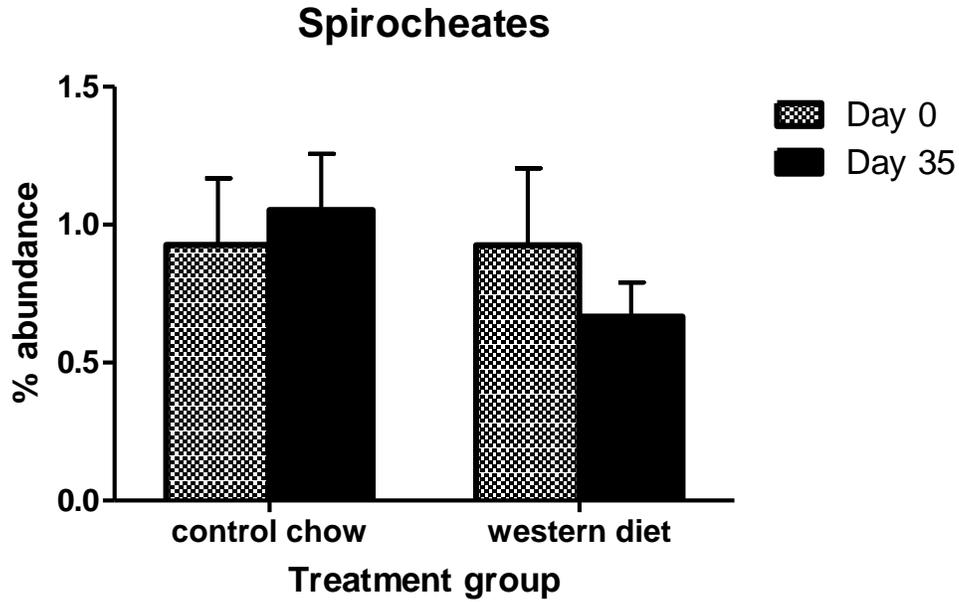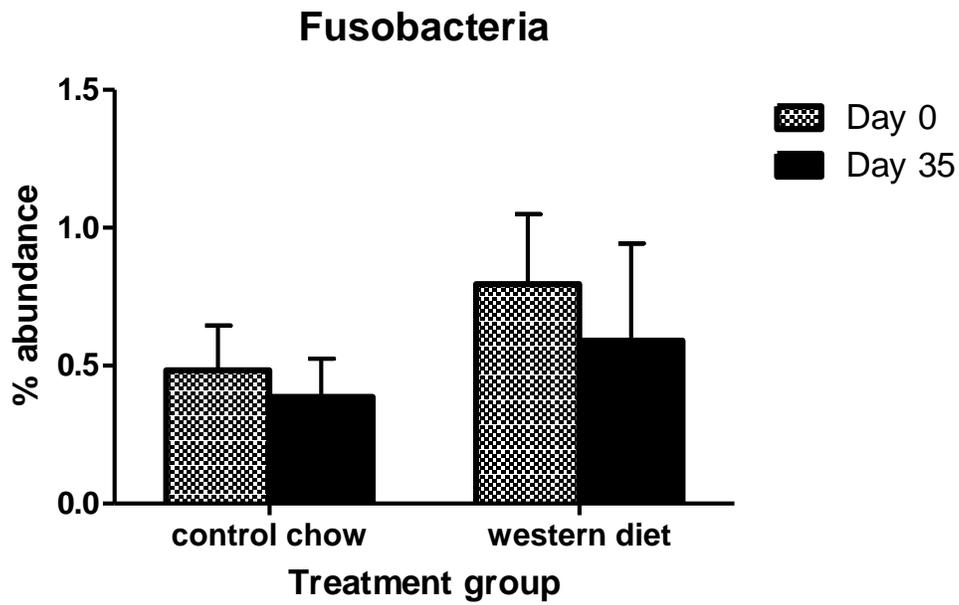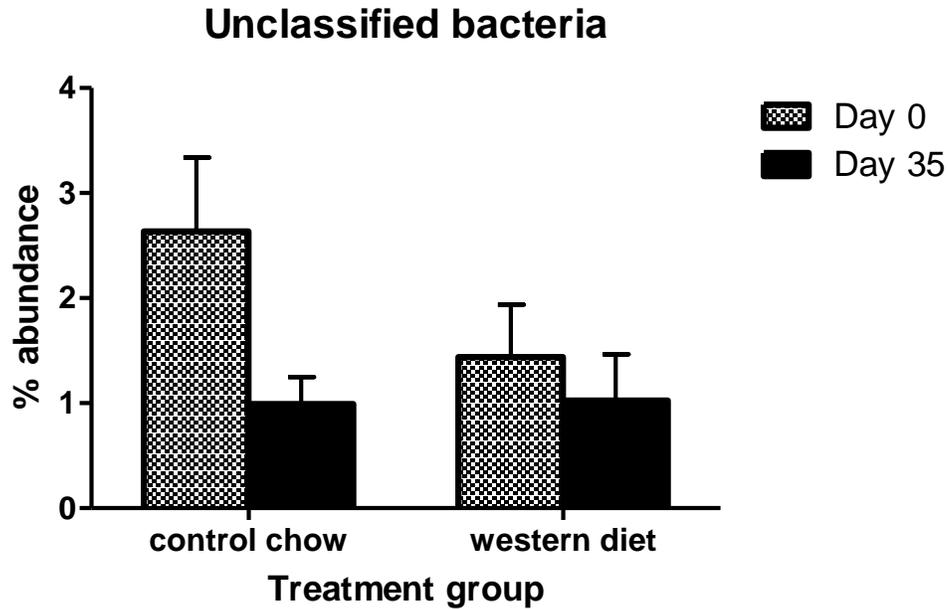
Figure 4-5. Microbial analysis performed on fecal samples prior to treatments (day 0) and following treatments (day 35) for WT mice on the control chow and western diet. Analysis was performed using T-RFLP, and bacteria phyla are displayed as % abundance. Bacteria phyla: **a)** *Firmicutes*, **b)** *Bacteroidetes,* **c)** *Tenericutes*, **d)** *Actinobacteria*, **e)** *Proteobacteria*, **f)** *Verrucomicrobia*, **g)** *Spirocheates*, **h)** *Fusobacteria*, **g)** unclassified bacteria.

* Significance between day 0 and day 35 samples $p<0.05$

Values displayed as mean ± SEM (n=8)

Table 4-5: Microbial composition from fecal samples taken prior to (day 0) and following (day 35) dietary treatment (control chow or western diet) for IL10$^{-/-}$ mice

Phyla	Class	Control chow		Western Diet	
		Day 0	Day 35	Day 0	Day 35
Firmicutes	% of Total	39.72	48.58	51.32	63.2
	Clostridia*	88.98	90.57	88.29	80.29
	Erysipelotrichi*	2.8	2.88	3.09	8.24
	Bacilli*	2.6	1.66	2.37	3.5
Bacteroidetes	% of Total	43.94	36.35	32.25	17.84
	Bacteroidia*	61.07	60.67	55.57	43.48
	Sphingobacteria*	0.85	0.8	0.74	2.35
	Flavobacteria*	2.83	2.85	5.45	8.43
Proteobacteria	% of Total	6.49	5.65	5.85	5.37
	Alphaproteobacteria*	13.02	16.54	21.01	10.04
	Betaproteobacteria*	13.93	16.55	22.98	39.9
	Deltaproteobacteria*	24.33	15.94	8.9	4.49
	Gammaproteobacteria*	48.29	49.85	41.11	43.31
Actinobacteria	*Actinobacteria*	2.296	2.66	2.5	2.38
Fusobacteria	*Fusobacteria*	1.51	0.71	1.19	0.8
Spirochates	*Spirochates*	1.34	1.38	0.84	0.33
Tenericutes	*Mollicutes*	0.41	0.53	1.5	4.61
Verrucomicrobia	*Verrucomicrobiad*	0.83	0.6	0.68	0.56
Unclassified bacteria	Unclassified bacteria	1.11	1.53	1.32	3.33

*represents % of phyla.

Table 4-6: Microbial composition from fecal samples taken prior to (day 0) and following (day 35) dietary treatment (control chow or western diet) for WT mice

Phyla	Class	Control chow		Western Diet	
		Day 0	Day 35	Day 0	Day 35
Firmicutes	% of Total	58.51	65.99	58.02	61.56
	Clostridia*	90.14	90.42	85.84	88.05
	Erysipelotrichi*	1.73	2.36	4.59	1.91
	Bacilli*	1.6	2.23	3.91	5.03
Bacteroidetes	% of Total	27.02	17.59	27.24	20.36
	Bacteroidia*	63.4	72.3	57.72	62.1
	Sphingobacteria*	0.36	0.45	2.02	0.93
	Flavobacteria*	5.25	2.71	4.51	3.26
Proteobacteria	% of Total	4.67	4.84	4.8	5.45
	Alphaproteobacteria*	24.71	7.72	21.14	5.45
	Betaproteobacteria*	16.7	14.66	15.16	16.74
	Deltaproteobacteria*	6.705	5.71	13.92	17.04
	Gammaproteobacteria*	51.34	71.06	48.55	58.31
Actinobacteria	Actinobacteria	1.71	2.88	1.93	4.79
Fusobacteria	Fusobacteria	0.48	0.39	0.39	0.59
Spirochates	Spirochates	0.93	1.05	0.93	0.67
Tenericutes	Mollicutes	1.8	1.72	1.11	2.71
Verrucomicrobia	Verrucomicrobiad	0.5	1.26	0.54	1.29
Unclassified bacteria	Unclassified bacteria	2.63	0.99	1.44	1.03

*represents % of phyla

1=X	54.2%	54.2%
2=Y	27.8%	82.1%
3=Z	17.9%	100.0%
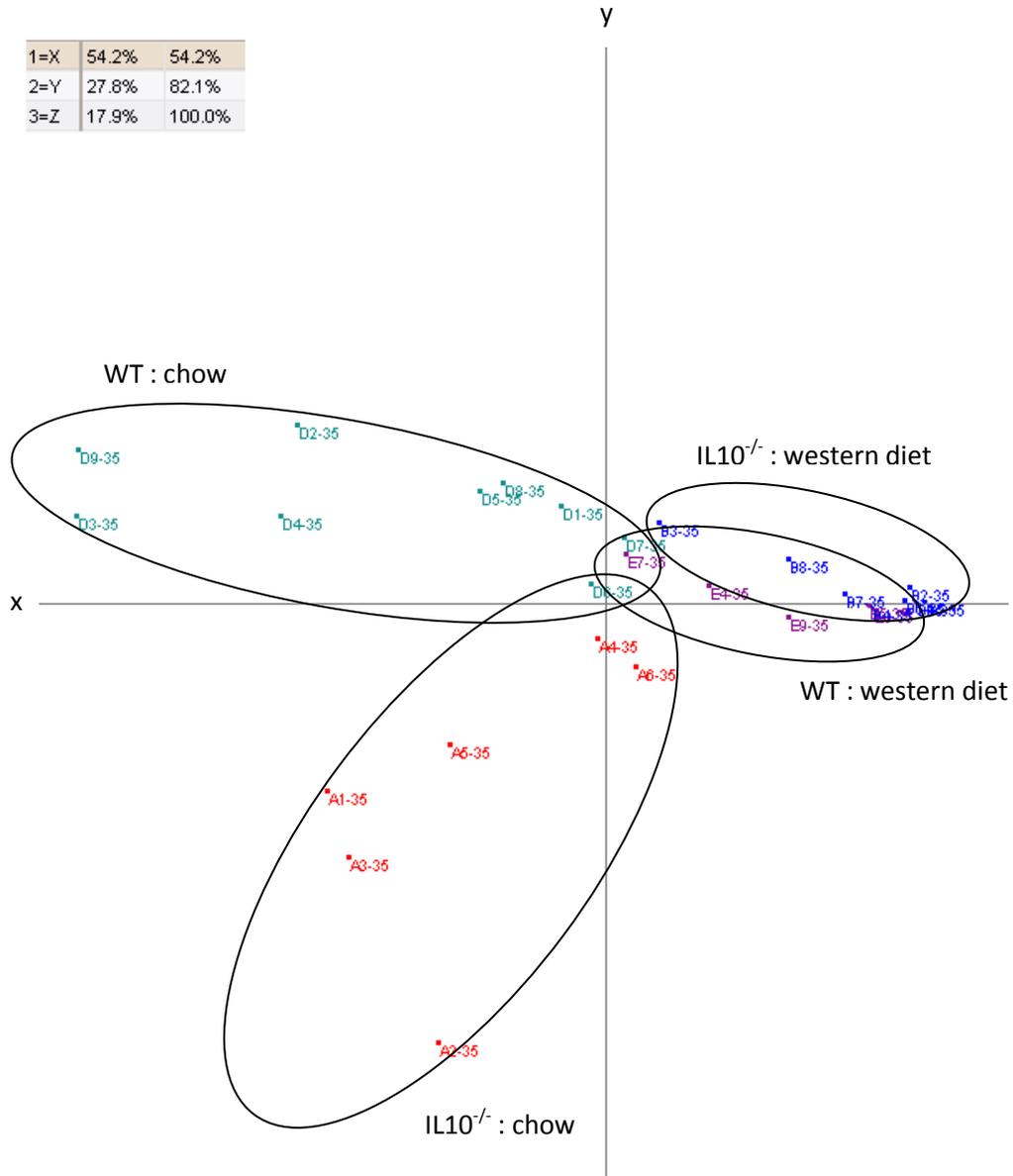
Figure 4-6: PCA plot examining changes in the microbial composition in IL10$^{-/-}$ and WT mice after 35 days on control chow or chow + PM. Results are based on the band patterns obtained during the TRFLP analysis of stool samples. Each dot represents one mouse. **A-** IL10$^{-/-}$ control, **B-** IL10$^{-/-}$ western diet, **D-** WT control, **H-** WT western diet

Figure 4-7a

Microbial diversity in IL10$^{-/-}$ mice

Figure 4-7b

microbial diversity in WT mice
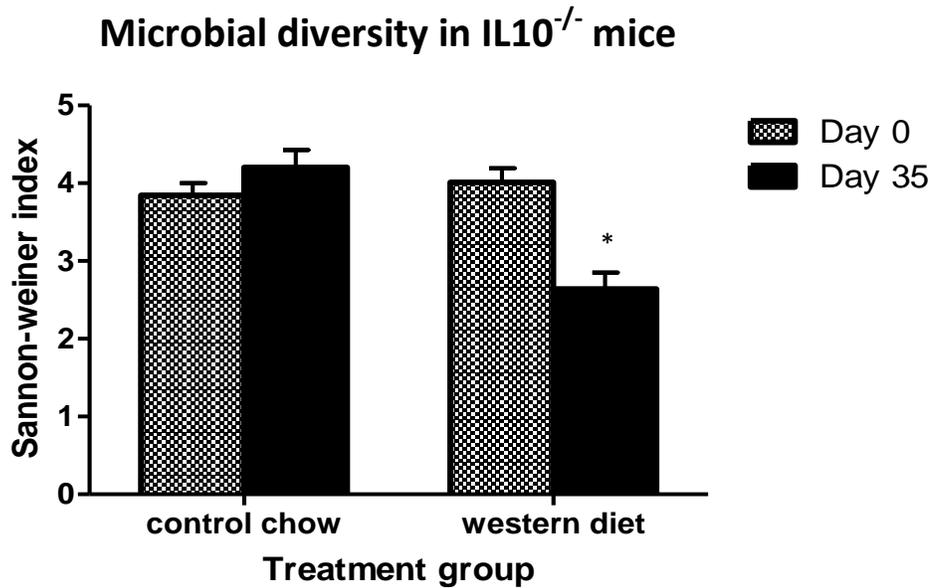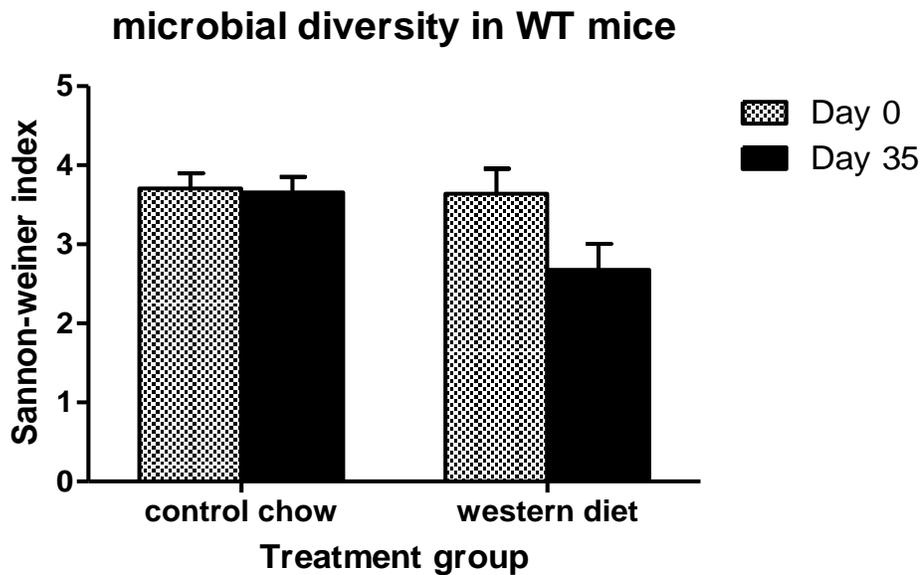
Figure 4-7: Shannon-Weiner index of microbial diversity, calculated from fecal samples taken prior to (day 0) and following dietary treatment with standard control chow or western diet in **a)** IL10$^{-/-}$ mice, **b)** WT mice. Values displayed as mean ± SEM (n= 7-8)

 * Significance between day 0 and day 35 samples $p<0.05$

4-3-4. Western diet alters short chain fatty acid production in IL10$^{-/-}$ and WT mice

Measurements of short chain fatty acids (SCFA) was done on cecal contents from both IL10$^{-/-}$ and WT mice after 35 days on the control chow or western diet. In IL10$^{-/-}$ mice placed on the western diet, there was a significant decrease in acetate, propanoic, butyric, and caproic acid production compared to their control chow counterparts (fig 4-8). There was also a trending decrease in valeric acid in mice placed on the western diet for 35 days, (Figure 4-8). There was an observable, increase in the branched chain fatty acids isobutyric and isovaleric acid in IL10$^{-/-}$ placed on the western diet compared to those placed on chow (Figure 4-8). This would indicate significant alterations in fermentation within IL10$^{-/-}$ mice placed on a western style diet.

Similar results were also observed in the WT mice. I demonstrated there was a significant decrease in acetate, propanoic, butyric, and caproic acid, accompanied by a trending increase in isovaleric acid production in WT mice placed on the western diet compared to their control chow counterparts (Figure 4-8). This suggests significant alterations in fermentation within WT mice on a western diet as was seen for the IL-10$^{-/-}$.

Figure 4-8a

Acetate

Figure 4-8b

Propanoic acid

Figure 4-8c

Butyric acid

Figure 4-8d

Valeric acid

Figure 4-8e

caproic acid
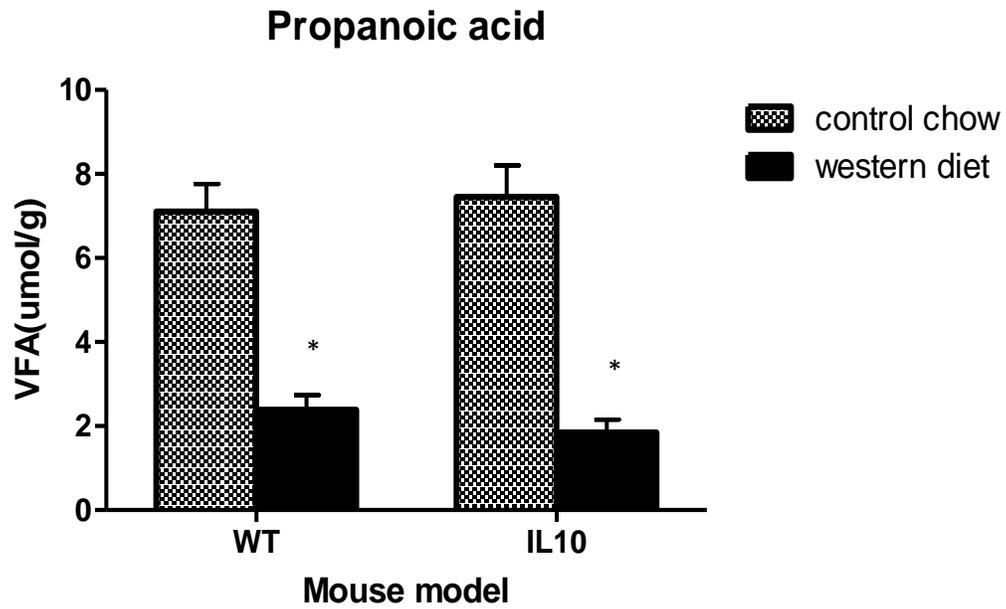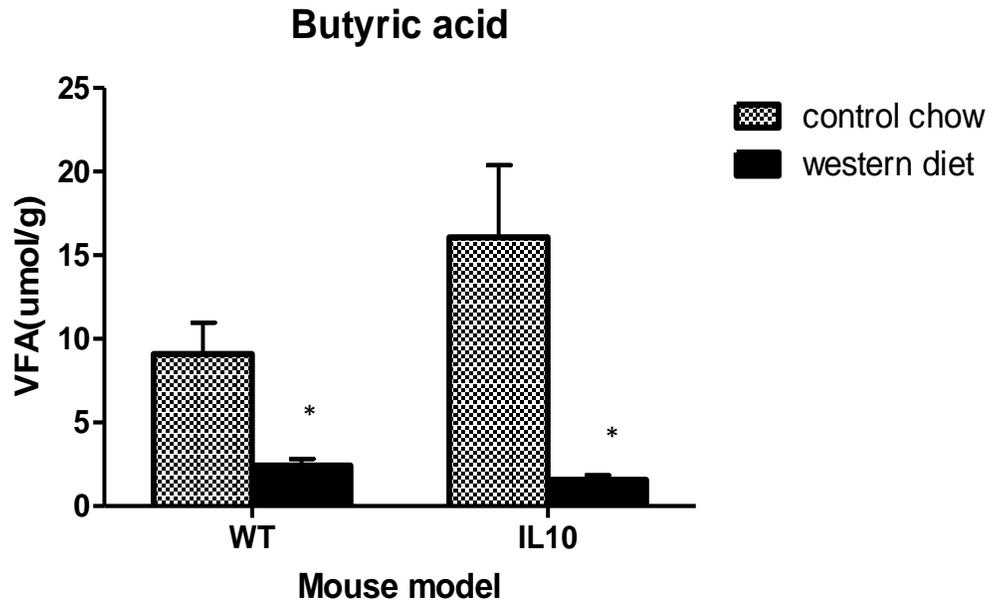
Figure 4-8f
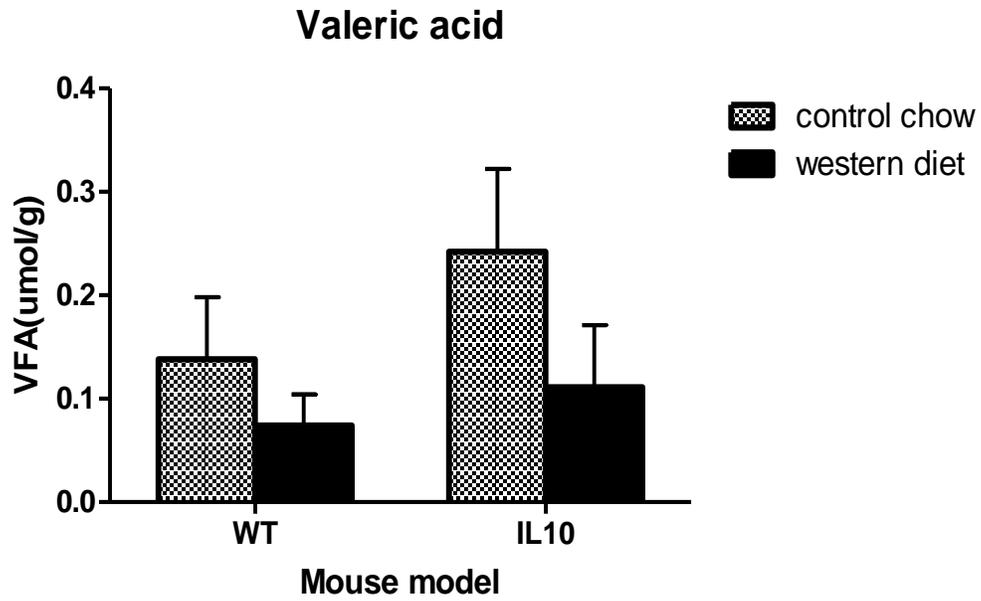
Isobutyric acid
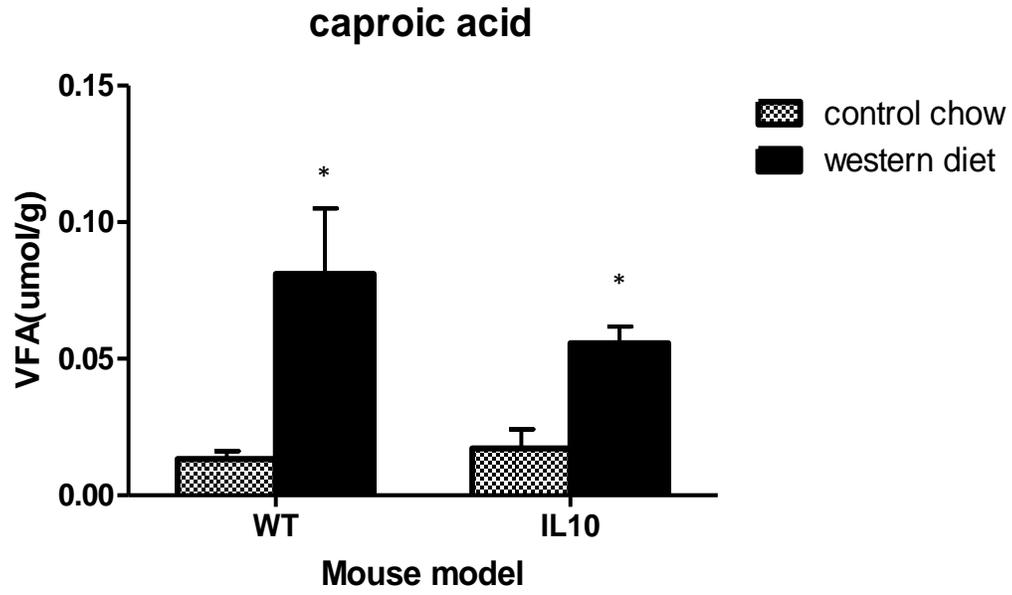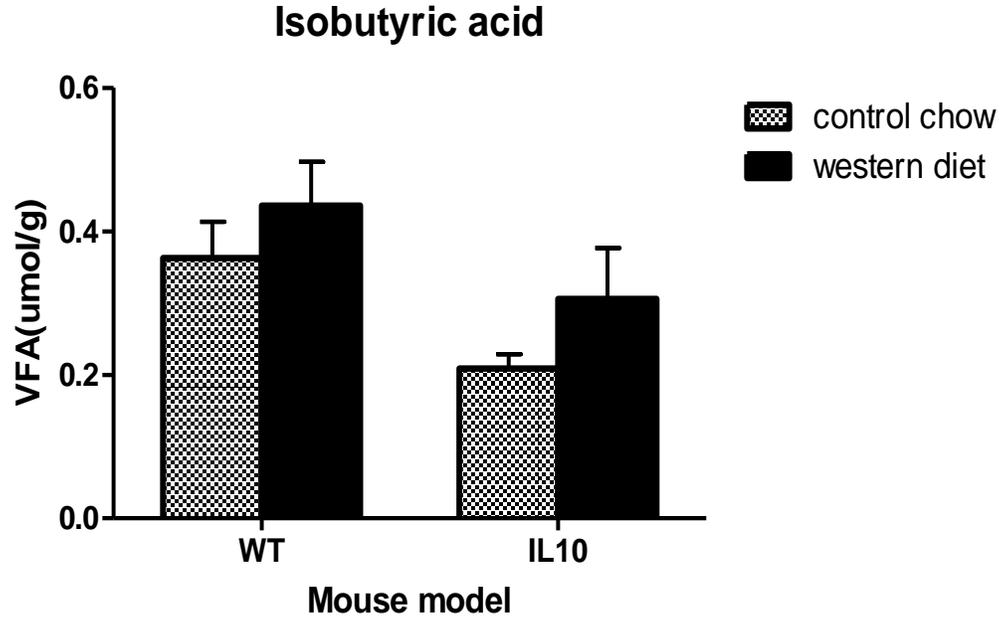
Figure 4-8g
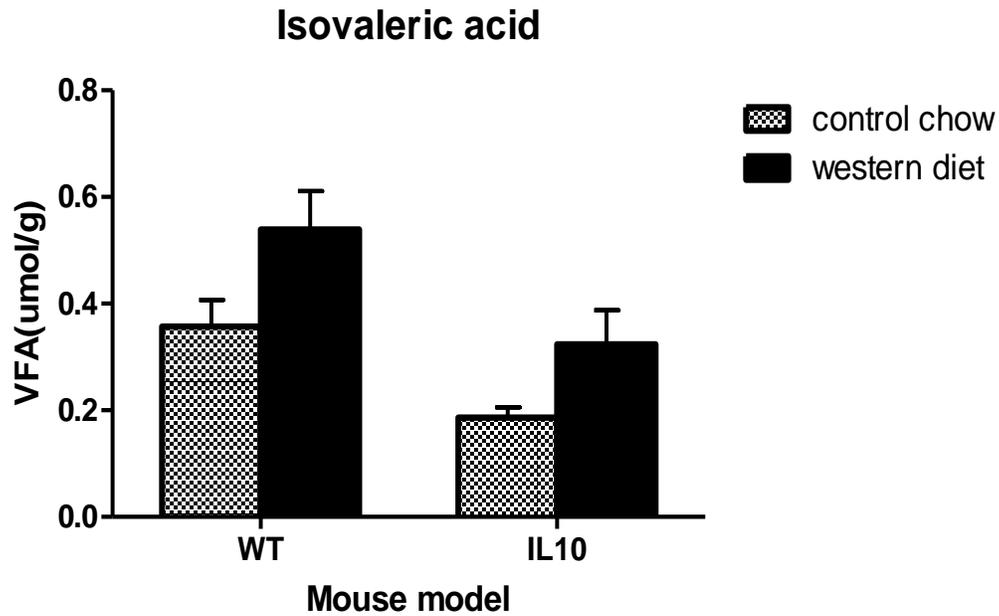
Isovaleric acid

Figure 4-8. Short chain fatty acid concentration from cecal contents of IL10-/- and WT mice on day 35 of control chow or western diet. **a)** acetic acid, **b)** propanoic acid, **c)** butyric acid, **d)** valeric acid, **e)** caproic acid, **f)** isobutyric acid, **g)** isovaleric acid

* Significance between control chow and western diet $p<0.05$

Values displayed as mean ± SEM (n= 7-8)

4-4. Discussion:

Environmental risk factors are an essential component in the pathogenesis of inflammatory bowel disease (IBD) and are believed to be largely responsible for the rapid increase in incidence observed around the world. Various epidemiological studies have suggested dietary factors in particular are important environmental factors associated with increased risk of disease (1,10). Of particular interest is the "western style" diet, which is characterized as having high fat and carbohydrate content (1). This type of diet has recently been associated with increased risk of ulcerative colitis (UC) (8,15), and is believed to contribute to the high incidence of IBD in western industrialized nations (16,17); however the exact mechanism as to how this type of diet contributes to disease pathogenesis remains relatively unknown. Therefore, I wanted to examine the effect of a diet rich in fat and simple sugars (western diet), on the mucosal immune system and intestinal microbiota of the IL10 gene deficient (IL10$^{-/-}$) mouse model of colitis.

I was able to demonstrate that treatment of IL10$^{-/-}$ mice with a western style diet, rich in fat and simple carbohydrates (CHO), resulted in attenuated intestinal inflammation as characterized by the significant reduction in pro-inflammatory cytokine expression in the large intestine and decreased histolopathological scores. This suggests an overall reduction in disease severity in response to the western diet in the IL10$^{-/-}$ mouse model of colitis. The western

diet also significantly altered microflora composition and diversity, and

significantly decreased short chain fatty acid (SCFA) production in the cecum.

The IL10^{-/-} mouse is a common model used to study IBD because these

mice develop enterocolitis with similar characteristics to human CD when

housed in conventional conditions [18,]. This is also a proven model to examine

the interaction between immune, environmental and genetic factors in the

development of colitis (19). To demonstrate that our mice did in fact develop

disease, WT and IL10^{-/-} mice on the standard mouse chow were examined for

overall body weight, colonic weight, cytokine expression levels, and

histopathology. When IL10^{-/-} mice develop colitis they tend to have decreased

body weight and increased colon weight compared to WT mice, anemia,

increased mucosal expression of various pro-inflammatory cytokines such as IL1,

TNFα, IL6 and IFNγ in their colon, and increased histopathology associated with

epithelial hyperplasia and transmural leukocyte infiltration (19,20). I found that

by the end of experimental treatment (day 35), the IL10^{-/-} mice on the standard

mouse chow were beginning to develop colitis, which was demonstrated by

reduced overall body weight and accompanying increase in the weight/length

ratio of the colon compared to WT mice. The increase in the colon weight/length

ratio was due to a significant increase in the overall weight of the tissue which

could suggest edema in the colon (14). There was also a significant increase in

colonic expression of the pro-inflammatory cytokines IFNγ, TNFα, and IL12, and

in the chemokine CXCL1 in IL10^{-/-} mice compared to WT mice, which suggest T

helper (Th) 1 mediated inflammation within the colon of control IL10$^{-/-}$ mice.

There was an overall increase in the histopathology score for the colon of IL10$^{-/-}$

mice compared to WT, which was mostly the result of increased enterocyte

injury and leukocyte and neutrophil tissue infiltration; even though these results

were not significant it should be noted that all but one (83%) IL10$^{-/-}$ mouse

demonstrated some level of enterocyte injury compared to none in the WT mice.

It is important to note that in our colony, 129SvEv IL10$^{-/-}$ mice only begin to

develop colitis around 12 weeks of age; the mice used in this experiment were

between the ages of 9 and 12 wks by the end of treatment with the majority

being 10 wks old. This means the majority of our mice were only at the beginning

stages of developing colitis, which can help to explain why I did not see

significantly higher histopathological scores in the IL10$^{-/-}$ mice compared to WT

mice, and why none of the mice demonstrated any epithelial hyperplasia.

Younger mice begin developing colitis with only small areas of inflammation in

the cecum, ascending and transverse colon and are characterized with small

increases in mononuclear and neutrophil infiltration; however as the mice age,

the disease becomes more severe with increased colonic inflammation and

increased transmural leukocyte infiltrate and epithelial hyperplasia (19).

Therefore the trending increase in histological damage accompanied by the

significant decrease in body weight and significant increase in colonic weight and

pro-inflammatory cytokine expression within the colon of IL10$^{-/-}$ mice suggest the

development of inflammation and disease within these mice, and perhaps if the

mice were examined at a slightly older age, there would be significant increases in histopathology also observed.

Interestingly, when the IL10$^{-/-}$ mice were placed on the western diet for 35 days, there was an overall decrease in colonic inflammation and disease severity compared to control IL10$^{-/-}$ mice placed on the standard mouse chow. I demonstrated that at the end of the 35 day treatment period, IL10$^{-/-}$ mice on the standard chow were in varying stages of developing colitis (depending on their age), however IL10$^{-/-}$ mice placed on the western diet didn't appear to show any signs of disease in the colon. These mice gained significantly more weight over the 35 days, even though they consumed significantly less food and less kcal/g of food each week compared to their control chow counterparts. They also displayed a significant reduction in their colon weight, which could suggest decreased immune cell and fluid infiltrate within the colonic tissue. As mentioned above, decreased overall body weight and increased colonic weight are key characteristics associated with disease in the IL10$^{-/-}$ mouse model of colitis; therefore the weight gain and reduced colonic weight could indicate decreased disease severity in response to the western diet. We also observed a trending decrease in the overall histology score in the colon of IL10$^{-/-}$ mice on the western diet compared to their control chow counterparts. This was mainly the result of a decreased enterocyte injury score, in which none of the mice on the western diet displayed any epithelial damage, and a decrease in neutrophil infiltration. Although these results were not significant due to the varying level of

disease severity within control IL10$^{-/-}$ mice, it does appear that there is less

innate inflammatory cell infiltrate and less epithelial damage in the colons of

mice in response to the western diet which is the opposite of what I

hypothesized. It remains to be shown what would happen if these mice were

placed on this diet for a longer period of time.

IL10$^{-/-}$ mice placed on the western diet demonstrated a significant

decrease in colonic expression of the pro-inflammatory cytokines IL12, TNFα,

and IFNγ, and trending decreases in IL1β and IL17 compared to control IL10$^{-/-}$

mice. The significant decrease in expression of these cytokines, which in many

cases returned to similar levels of that observed in WT mice, suggests decreased

inflammation in the colonic mucosa in response to a western diet. This can have

important implications for CD and UC patients, which similarly to IL10$^{-/-}$ mice,

express high levels of TNFα, IFNγ, and IL17 amongst other pro-inflammatory

cytokines in their intestinal tissue which results in ulcerations and lesions within

the tissue (21). If the western style diet was able to similarly reduce colonic

expression of these cytokines, then these patients would have decreased

intestinal inflammation and possibly decreased intestinal injury as well. A

significant decrease in CXCL1 chemokine expression was observed, which is a

chemo-attractant for neutrophils. This would suggest an overall reduction in

neutrophil colonic infiltration, which is consistent with the histological data. A

trending decrease was also observed for the Th2 mediated cytokines IL4, IL5, and

IL13, which are required for stimulation, proliferation, and antibody class

switching of B cells, thus are important for humoral type immune responses. This would indicate that the western diet appeared to have an overall immunosuppressive effect on the colonic mucosal immune system. This could have serious consequences on the mice`s ability to fight intracellular and extracellular pathogens within the colon, thus making them more susceptible to infection.

There was a significant increase in IL12 and IFNγ expression, and although not quite significant, an increase in IL1β expression as well in the small intestine of IL10$^{-/-}$ mice on the western diet compared to their control chow counterparts. These are all pro-inflammatory cytokines involved in a Th1 cell mediated immune response. IL12 is a key cytokine involved in the differentiation of naive T cells into Th1 type immune cells, while IFNγ is important in the maintenance of chronic inflammation (22); therefore it appears as if there was chronic Th1 cell mediated inflammation within the small intestinal tissue of IL10$^{-/-}$ mice in response to the western diet. Interestingly, this cytokine profile was not observed in the WT mice on the western diet, which could suggest that the change in diet resulted in chronic small intestinal inflammation only in genetically predisposed individuals. These data could also suggest the intestinal inflammation normally observed in IL10$^{-/-}$ mice shifts from the colon to the small intestine when these mice are placed on a western diet.

The lack of inflammation and overall attenuation of disease severity in the colon of IL10$^{-/-}$ mice on the western diet was a surprising and unexpected result, and the opposite of what I hypotheszied, since increased consumption of fats and sugars have previously been associated with increased risk of IBD development in humans and animal models. For example, several studies have found that high sucrose consumption is associated with increased risk of IBD development (8,23-25), while others have suggested increased overall fat intake, monounsaturated fatty acid (MUFA), omega (n)-6 polyunsaturated fatty acid (PUFA), and decreased n-3 PUFA are associated with increased incidence of CD an UC (15,23,26). However the limitation with many case control studies like these is that they rely heavily on patient recall of the pre-diseased diet which can sometimes allow for biased answers and in many cases result in confounding results, thus making most of the epidemiological data inconclusive (10, 27). The discrepancy between our results and previous studies can also be due to the diet that I used. The test diet is meant to represent a typical diet consumed in western societies, characterized by having a high percentage of fat and sugar, and decreased complex carbohydrates and fiber. However, most studies only look at the effect of one nutritional component within a western style diet at a time. These studies therefore cannot take into account the possible effect of dietary interactions on the host physiology. Because I was studying such a complex diet, it is possible the various components within the diet are

interacting to create a different response than what would be observed with each component alone.

Although most of the studies would suggest components of our diet would result in enhanced intestinal inflammation and therefore increased disease severity, a few scientific and epidemiological papers have suggested certain elements of our western diet can actually decrease inflammation and have been linked with protective roles for IBD. For instance, oleic acid, which is present in a higher percentage in the western diet used in these studies compared to the control chow, has previously demonstrated protective properties in the DSS induced model of colitis by decreasing the overall disease activity score, when present in higher concentrations (28, 29). It is possible the increased oleic acid acted as a ligand for the nuclear receptor peroxisome proliferator- activated receptor (PPAR) γ which can then inhibit NFκB associated inflammation (30). This could then be one reason I observed attenuated colonic injury and a significant reduction in the pro-inflammatory cytokine expression in the colon. Epidemiological studies have also associated Mediterranean style diets which are high in MUFA, with decreased risk of CD development (1), in a similar fashion, our western diet also has a higher percentage of MUFA compared the control chow, which could suggest the MUFA component is playing a role in the reduced disease severity in the IL10$^{-/-}$ mice. Several studies have suggested increased consumption of linoleic acid is associated with increased risk of developing IBD (9, 26, 31) and others have reported higher fibre

consumption in CD patients (10). The western diet used in these studies is lower in both linoleic acid and fibre compared to control chow which could suggest the lack of these dietary components are playing a role in the disease reduction in the IL10$^{-/-}$ mice.

It is possible the decreased colonic mucosal immune response and diminished disease severity in the IL10$^{-/-}$ mice on the western diet was the result of secondary effects caused by significant changes in microbial composition in these mice. There is strong evidence to support the hypothesis that intestinal microflora are involved in the pathogenesis of IBD (32-37). For instance, intestinal inflammation fails to develop in IL10$^{-/-}$ mice when they are kept in germ free conditions (38-40), and microbial profiles in IBD patients are significantly different from those of healthy subjects (1, 41-43). It is believed that the changes in microbial composition, which may favour more aggressive bacteria over protective species, may result in onset of intestinal inflammation in genetically predisposed individuals (44,45), thus suggesting any alteration in bacteria composition can have varying effects on the initiation and overall maintenance of colitis.

I observed significant changes in the diversity and composition of the intestinal microflora of IL10$^{-/-}$ mice on the western diet, which is not surprising considering intestinal bacteria rely on dietary and host derived nutrients for survival. Previous studies have shown that alterations in amount and type of fat

and CHO within a diet can result in compositional changes in the intestinal microflora (46-48). It is possible therefore that the changes in microbial composition caused by the western diet favoured the survival of protective bacteria over aggressive species. For example, bacteria in the phylum *Bacteroidetes* are believed to be involved in intestinal inflammation (54,55) and are associated with human CD (49-51). We observed a significant decrease in the abundance of this phylum in IL10$^{-/-}$ mice after 35 days on the western diet. This could suggest the decreased inflammation in the colon of IL10$^{-/-}$ mice on the western diet could be the result of decreased abundance of *Bacteroidetes*. As well increased abundance of *Actinobacteria*, and *Proteobacteria* accompanied by decreased *Firmicutes* are also associated with IBD patients (42,52,53). There was no increase in abundance of *Actinobateria* and *Proteobacteria* in the IL10$^{-/-}$ mice on the western diet. I also observed a trending increase in the phylum *Firmicutes* and in particular *Bacilli* and *Clostridium* which contain bacteria that have shown protective effects towards IBD (54,55). In particular *Faecalibacterium prausnitzii* which is in the class *Clostridia*, has been shown to decrease colitis in mice and has demonstrated anti-inflammatory effects in CD patients (56). Due to the limitations of our bacterial analysis I cannot confirm an increase in *F. Prausnitzii* in particular, but an overall increase in the class *Clostridia* was observed. This could suggest that an overall protective change in the microbial composition within IL10$^{-/-}$ mice on the western diet occurred which could be responsible for the decreased inflammation within the colon.

Lastly, it's important to mention the results from the SCFA analysis, which

demonstrated that there was a significant reduction in acetate, butyrate,

propanoate, and caproic acid concentration in WT and IL10$^{-/-}$ mice on the

western diet. This is not that surprising since SCFA are for the most part

produced from fermentation of undigested CHO, particularly resistant starches

and dietary fibre (57), and the western diet contains very little fiber and reduced

amount of complex carbohydrates compared to the standard mouse chow. The

amount of SCFA production depends on the type and amount of bacteria in the

colon, the composition of the diet, and the time it takes to pass through the

gastrointestinal tract (57). The alterations observed in the intestinal microflora

in response to the western diet accompanied by the changes in the dietary

components themselves could result in reduced bacterial fermentation, resulting

in decreased SCFA production. There was a decrease in cecum size in mice on the

western diet, which could confirm a decrease in bacteria fermentation in these

mice, which results in the reduced SCFA production. SCFA are important for

colonic health, butyrate in particular, which is the major energy source for

colonic epithelial cells ((57). Depletion of butyrate results in decreased barrier

function and increased susceptibility of colonocytes to inflammation (58,59).

There also appears to be decreased levels of butyrate and acetate in IBD patients

compared to healthy subjects (60). This could suggest that although there is

decreased disease activity and inflammation within the colon of IL10$^{-/-}$ mice on

the western diet, the colonic epithelial cells themselves might still be in an unhealthy state.

In these studies, I have demonstrated in the IL10$^{-/-}$ mouse model of colitis, that a western style diet, high in fat and simple CHO, attenuated mucosal inflammation in the colon. However, surprisingly, increased inflammation in the small intestine, suggesting that the type of diet consumed might alter the phenotype of disease in IBD patients. The immunosuppressive effects observed in the colon of IL10$^{-/-}$ mice on the diet included a reduction in both the Th1 and Th2 arms of the immune system, which could then make these mice more susceptible to infection because they may no longer be able to mount an effective immune response against invading pathogens. Therefore future studies will need to be done to examine the long term effects of such a diet on the health of these mice.

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Chapter 5: Effects of western diet in combination with dietary exposure to particulate matter on mucosal immune function and intestinal microflora in a mouse model of colitis

5-1. Introduction:

As mentioned in previous chapters, environmental risk factors are essential components in the pathogenesis of inflammatory bowel disease (IBD), and are believed to be the primary cause of the rapid increase in worldwide incidence (1,2). Of particular interest, is the role of airborne particulate matter (PM), and the high fat, high sugar western style diet, which have both been hypothesized in the pathogenesis of IBD.

I previously demonstrated in earlier chapters that dietary exposure to PM_{10} and a western style diet, each on their own, in different ways, have the ability to alter the expression of various T-helper (Th) 1 and Th2 mediated cytokines, and intestinal microbial composition in both WT mice and in the $IL10^{-/-}$ mouse model of colitis. Since individuals in western nations are normally exposed simultaneously to the western style diet and PM_{10}, I wanted to examine the role of these two factors in combination on mucosal immune function and microbial composition in WT and $IL10^{-/-}$ mouse model of colitis. Because of the results observed in previous chapters, I hypothesized that exposure to PM and a

western style diet would increase intestinal inflammation and alter microbial composition in IL10$^{-/-}$ mice.

5-2. Methods:

5-2-1. Particulate Matter (PM):

Ambient air particulate matter (EHC-93) was obtained from the videlon bag filters of the single pass air-purification system from the Environment Health Center in Ottawa, Canada. The characteristics of the PM have been previously described (3,4), and are presented in Appendix 1- **Table A-1**. EHC-93 is classified as PM$_{10}$, therefore is made up of both coarse and fine particulate components.

5-2-2. Animals and experimental setup:

Following weaning, female wild type (WT) and IL10$^{-/-}$ mice were randomly divided into four treatment groups: a western style diet (33% fat and 24% simple sugars) with or without PM$_{10}$, or a standard mouse chow with or without PM$_{10}$ (n=7-9 mice in each group) for 35 days. The western diet was composed of 850g/Kg of TD. 06206 basal mix (Tekland diet) and supplemented with 150g/Kg of fat from sunflower oil (2% total fat) (Safeway brand), flax oil (2% of total fat) (Gold Top organic brand) and lard (96% of total fat) (Tenderflake) standard mouse chow (Laboratory Rodent Diet 5001, Lab Diet). Both the western diet and standard mouse chow (Laboratory Rodent Diet 5001, Lab Diet) were prepared fresh every 2 weeks and stored at -4 °C until use. Nutritional composition is

summarized in Tables 4-1 and 4-2. Mice on the PM diet received a daily dose of

~360ug of PM. Mice had free access to food and water throughout the

experiment; amount of food eaten was determined by weighing of food weekly.

Mice were weighed once a week and fecal samples collected and snap-frozen for

microbial analysis. At the end of the 35 days, mice were sacrificed and the weight

of the cecum along with the weight and length of the small and large intestine

measured. Sections of the small and large intestine were collected for mucosal

cytokine expression and histology. Cecal contents were also collected to examine

short chain fatty acid concentration.

The protocol for use of mice was approved by the Health Science Animal

Care and Use Committee at the University of Alberta, and animals were kept in a

regulated day/night cycle with controlled humidity and temperature.

5-2-3. Histological injury score

On day 35, IL10$^{-/-}$ and WT mice on chow and western diet +/- PM, were

sacrificed and sections of the large intestine were harvested, flushed with cold

PBS, cut longitudinally and fixed in 10% buffered formalin. These samples were

then embedded in paraffin wax, sectioned at 4um and stained with haematoxylin

and eosin (H&E) for examination. Slides were assessed by a pathologist (Dr.

Aducio Thiesen, Dept of Pathology) in a blinded fashion and assigned a

histological score for intestinal inflammation. This score was based on the sum of

4 scoring criteria: mucosal ulceration (enterocyte injury) (score of 0-3), epithelial

hyperplasia (score of 0-3), and lymphocyte and neutrophil lamina propria infiltration (score of 0-2 each).

5-2-4. Mucosal cytokine expression

To assess mucosal immune function, sections of the small and large intestine were removed from WT and IL10$^{-/-}$ mice, flushed with cold PBS and snap frozen at -80°C for analysis. Tissue samples were then homogenized to determine cytokine expression levels within the tissue. Briefly, samples were thawed on ice in 500uL of RIPA buffer with protease inhibitor (20ug/mL Phenylmethylsulfonyl fluoride, 15ug/mL Aprotinin, 2ug/mL Pepstatin A, 2 ug/mL Leupeptin), and sonicated for 15sec and placed on ice. The homogenized tissues were then centrifuged at 10000rpm for 10min and supernatants collected for cytokine analysis. IL1β, IL2, IL4, IL5, CXCL1, IL12, IFNγ, and TNFα expression were measured using a Th1/Th2 tissue culture MesoScale Discovery Kit (MesoScale Discovery) as per manufacturer's instructions. IL13 and IL17 cytokine expression was examined using enzyme-linked immunosorbent assay (ELISA) (R& D systems) as per manufacturer's protocol.

5-2-5. Microbial analysis

Changes in microbial composition were determined in fecal samples collected on days 0 and 35 through the use of terminal restriction fragment length polymorphism (T-RFLP). T-RFLP was done by Matt Emberg. Total DNA was extracted from the fecal samples using a FastDNA Spin Kit for FECES (MP Bio) as

per manufacturer's instructions. The broad range forward primer 6-FAM-8F

(Applied Biosystems) (5'-AGAGTTTGATCCTGGCTCAG-3') and broad range reverse

primer 926R (Applied Biosystems) (5'-AGAAAGGAGGTGATCCAGCC-3') were used

to amplify 16S rRNA by PCR. The PCR was performed using 50ng DNA, cycling

conditions were: 94°C for 2 min followed by 35 cycles of 94°C 1 min, 56°C 1 min,

72°C 1 min, and a final 10 min extension at 72 °C. Every PCR run included a DNA-

free template control, and amplification was confirmed with visualization of a

single 920kb PCR product on 1% agarose gel. A Qiagen MinElute PCR purification

kit (Qiagen) was used to purify the amplicons, as per manufacturer's instructions.

The Hpa II restriction enzyme (Promega) was used to digest amplicon DNA (200-

300ng, determined with a Nanodrop spectrophotometer) (Thermo Scientific),

the digestion occurred for 16hr at 37°C. With each sample, 100ng of HPA II

digested fragments were resolved in duplicate using a 3130XL Genetic Analyzer

(Applied Biosystems). Fragment length normalization was achieved by running an

internal ROX1000DBA marker to separate each sample. Bionumerics 6.0 software

(Applied Maths) was used to normalize the fluorescently labeled terminal

fragment lengths and select peaks of interest, which are associated, *in silico,* with

fragment lengths of known bacteria using Microbial Community Analysis 3

(MiCA; Shyu,2007) and Ribosomal Database Project v.9 (RDP; Cole, 2009). MiCA

takes the fragment lengths and primer data and associates the likely bacterial

sequence with each band in the trace. RDP uses the list generated from MICA to

cluster the sequences and establish likely bacteria and abundances. Fragments

with peaks between 25 and 650 base pairs in length are used in the community composition and cluster analysis. Principle component/clustering analysis was then preformed on each sample and based on the band patterns alone from each trace to identify specific clusters. Bionumerics 6.0 software was also used to calculate the Shannon-Weiner index based on abundance data, to determine microbial diversity.

5-2-6. Short chain fatty acid analysis

Cecal contents were collect at sacrifice on day 35 from WT and IL10$^{-/-}$ mice and snap frozen and stored at -80 °C for short chain fatty acid concentration analysis. Briefly, 0.1N HCl was added to the cecal material and allowed to shake (180x g) overnight at 25°C. The sample was then vortexed and 5mL of diluents collected and added to 1mL of meta-phosphoric acid (HPO$_3$, 25% w/v in distilled H$_2$O). The samples were mixed and frozen overnight, then thawed, vortexed, and centrifuged at 3000x g for 20min. Supernatant was then transferred into a GC vial (PerkinElmer) and sealed for analysis by gas chromatography by Dr. M.G. Ganzle (University of Alberta). Concentration of acetate, propionate, butyrate, isobutyrate, isovalerate, valerate and caproate was determined using external standards (sigma), and isocaproate was used as internal standard.

5-2-7. Statistical analysis

Statistical analysis was done using a student T-test or ANOVA to compare the results from mice on the western diet with PM to control mice on chow or mice on western diet alone. Calculated P-values <0.05 were considered significant.

5-3. Results:

5-3-1. Morphological and histological analyses reveal that IL10$^{-/-}$ mice on the western + PM diet have attenuated disease by day 35

There was a significant decrease in the colonic weight and length of IL10$^{-/-}$ mice on the western + PM diet compared to control mice which was accompanied by an overall reduction in the weight of the small intestine (Table 5-1). These mice also had a lower overall colonic histopathology score, which was characterized by reduced enterocyte injury, and lymphocyte and neutrophil infiltration within the colon compared to control mice (Table 5-2). These results suggest there is decreased colonic disease severity in IL10$^{-/-}$ mice in response to a western + PM diet.

WT mice placed on the western diet also demonstrated a significant decrease in colon weight and length in response to the western + PM diet compared to the control fed mice (Table 5-1). There was a significant decrease in small intestinal weight, however, this was accompanied by significant increases in length which left no overall change in the weigh/length ratio in response to the western + PM diet compared to their control mice on the chow (Table 5-1).

There was also no difference in colonic histology between control mice and western + PM treated mice (Table 5-2). I also observed a significant decrease in cecum weight in WT placed on the western +PM diet compared to their control chow counterparts (Table 5-1). This could suggest a reduction in fermentation within the colon in mice exposed to both a high fat, high sugar diet, and PM.

Table 5-1: Small intestine, colon, and cecum measurements for IL10$^{-/-}$ and WT mice on a standard mouse chow, PM chow, western diet, or western + PM diet for 35 days

mouse strain	Group	Small intestine			Colon			Cecum
		weight (g)	length (cm)	Weight/length (%)	weight (g)	length (cm)	weight/length (%)	weight (g)
WT mice	Control chow (n=5)	0.719 ± 0.03	30.1 ± 0.9	2.18 ± 0.08	0.221 ± 0.005	8.5 ± 0.12	2.5 ± 0.09	1.25 ± 0.036
	chow + PM (n=3)	0.799 ± 0.03	37.3 ± 1.3	2.14 ± 0.02	0.251 ± 0.012	8.27 ± 0.75	3.12 ± 0.48	1.18 ± 0.023
	western (n= 6)	0.665 ± 0.04	34.2 ± 1.7	2.14 ± 0.13	0.116 ± 0.004[a]	7 ± 0.31[a]	1.81 ± 0.09[a]	1.10 ± 0.0047[a]
	western + PM (n=6)	0.645 ± 0.01[a,c]	35.1 ± 1.4[a]	2.19 ± 0.16	0.118 ± 0.009[a,b]	6.6 ± 0.32[a,b]	1.71 ± 0.04[a,b]	1.10 ± 0.0043[a,b]
IL10 mice	control chow (n= 6)	0.879 ± 0.04	36.8 ± 3.1	2.47 ± 0.09	0.353 ± 0.07	7.93 ± 0.3	3.57 ± 0.62	1.17 ± 0.018
	chow + PM (n= 3)	0.824 ± 0.08	37.6 ± 0.8	2.19 ± 0.19	0.345 ± 0.11	8.3 ± 0.42	4.41 ± 1.17	1.18 ± 0.013
	western (n=6)	0.767 ± 0.07	36.5 ± 1.6	2.25 ± 0.01	0.136 ± 0.02[a]	7.37 ± 0.52	2.19 ± 0.02[a]	1.13 ± 0.020
	western + PM (n=6)	0.557 ± 0.15[a]	35.7 ± 0.9	1.73 ± 0.38	0.118 ± 0.01[a,b,c]	7.1 ± 0.15[a,b]	1.59 ± 0.08[a,b]	1.15 ± 0.026

Values are displayed as mean ± SEM. [a] significance between control chow mice and respective treatment group of mice (p<0.05). [b] significance between western + PM diet mice and PM chow mice (p<0.05). [c] significance between western + PM diet mice and western diet mice (P<0.05)

Table 5-2: Colonic histology scores for IL10$^{-/-}$ and WT mice on the Control mouse chow, Chow + PM, Western diet, or Western +PM diet for 35 days

mouse strain	Group	Histology					
		# of affected mice	Enterocyte injury (0-3 units)	Epithelia hyperplasia (0-3 units)	Lamina Propria Mononuclear Infiltrate (0-2 units)	Lamina- Propria neutrophil Infiltrate (0-2 units)	Histological score (0-10 units)
WT mice	control chow (n=3)	1	0	0	0.33 ± 0.19	0	0.33 ± 0.19
	chow + PM (n=3)	0	0	0	0	0	0
	western (n= 3)	2	0	0	0.67 ± 0.38	0	0.67 ± 0.38
	western + PM (n=3)	1	0	0	0.33 ± 0.19	0	0.33 ± 0.19
IL10 mice	Control chow (n= 7)	6	0.71 ± 0.27	0	1.14 ± 0.43	0.43 ± 0.16	2.29 ± 0.86
	chow + PM (n=3)	2	1.00 ± 0.58	0.33 ± 0.19	1 ± 0.58	0.67 ± 0.38	3 ± 1.73
	western (n=6)	5	0	0.14 ± 0.14	0.71 ± 0.29	0.14 ± 0.14	1 ± 0.38
	western + PM (n=8)	6	0.25 ± 0.09	0.13 ± 0.004	1 ± 0.35	0.13 ± 0.04	1.50 ± 0.53

Values are displayed as mean ± SEM

5-3-2. A western diet in combination with PM exposure results in an overall reduction in mucosal immune function

Small intestinal and colonic tissue were homogenized and analyzed for expression of various T-helper (Th) 1 and Th2 related cytokines to determine the effect a western diet in combination with PM would have on mucosal immune function.

Compared to WT mice, untreated IL10$^{-/-}$ mice expressed significantly higher levels of the pro-inflammatory cytokines IL12, TNFα, and IFNγ, and a trending increase in IL17 and IL1β, within the colon (Figure 5-1). There was also a significant increase in the chemokine CXCL1, a key marker of intestinal inflammation and associated with neutrophil infiltration into the tissue (Figure 5-1). These increases suggest the presence of inflammatory cell infiltrates and active Th1 and Th17 mediated inflammation within the colons of IL10$^{-/-}$ mice at day 35.

When IL10$^{-/-}$ mice were placed on the western + PM diet, there was a significant decrease in colonic expression of IL12, TNFα, IFNγ, and CXCL1, as well as a trending decrease in IL17, IL1β compared to control mice (P values : 0.23 and 0.06 respectively) (Figure 5-1). This suggests decreased inflammation in the colons of IL10$^{-/-}$ mice when placed on a western diet with PM. There was also no expression of the Th2 mediated cytokines IL4 and IL5 and an insignificant decrease in IL13 (Figure 5-1). This then suggests a decrease in overall mucosal

immune response within the colon of IL10$^{-/-}$ mice exposed to PM while on a western diet.

These results were similar to that observed with the western diet alone. In these mice, there was a significant decrease in the colonic expression of IL12, TNFα, IFNγ, IL2, and CXCL1, as well as a trending decrease in IL4, IL5, and IL13 compared to controls (Figure 5-1). On the other hand, a significant increase in IL17 expression, with no effect on CXCL1, IL12, and TNFα was observed in PM treated mice compared to control (Figure 5-1). This would suggest that the reduced overall mucosal immune function observed in the colon of IL10$^{-/-}$ mice in the PM + western diet treatment group is the result of the western diet alone, however, these mice also demonstrated a significant decrease in expression of IL1β and IFNγ compared to the western diet group. This implies that a western diet in combination with PM exposure results in an overall decrease in colonic immune function.

In WT mice, there was no change in colonic cytokine expression in mice on the western diet + PM compared to control, which is similar to the results observed in the western diet alone mice (Figure 5-1). However, there was a reduced expression of IL1β, TNFα, IL17, CXCL-1, and IL12 compared to PM treated mice (Figure 5-1). This suggests that in the WT mice, the effect of the diet on mucosal immunity is stronger than PM exposure.

Within the small intestines of IL10$^{-/-}$ mice, there was a significant increase

in IFNγ expression and a trending increase in IL17 (P value 0.16) in mice on the

western + PM diet compared to control mice (Figure 5-2). There was no change

in cytokine expression levels within the small intestine of WT mice on the

western + PM diet compared to control (Figure 5-2). This could suggest low

grade chronic inflammation within the small intestine of IL10$^{-/-}$ mice in response

to a western diet with concurrent exposure to PM.
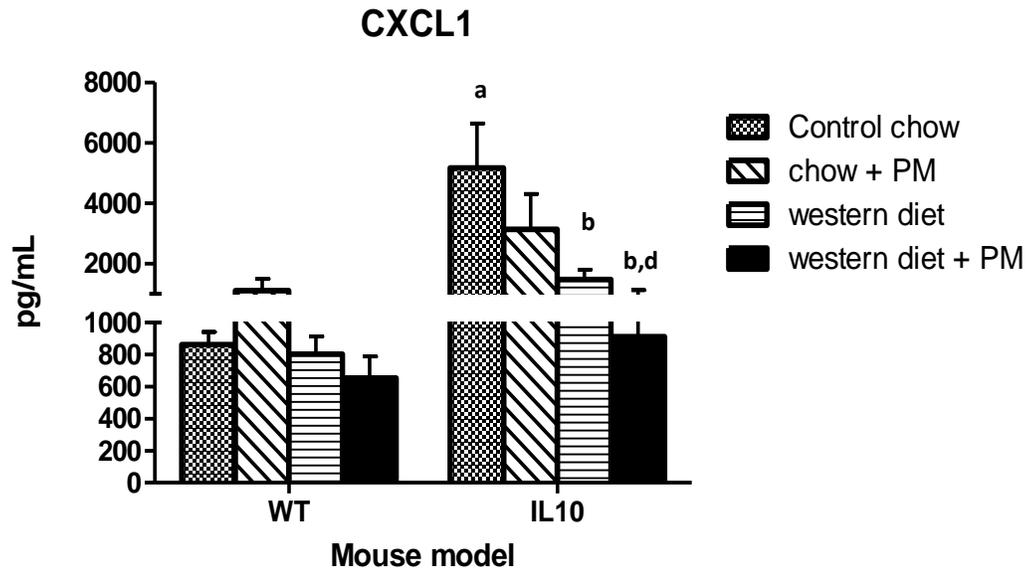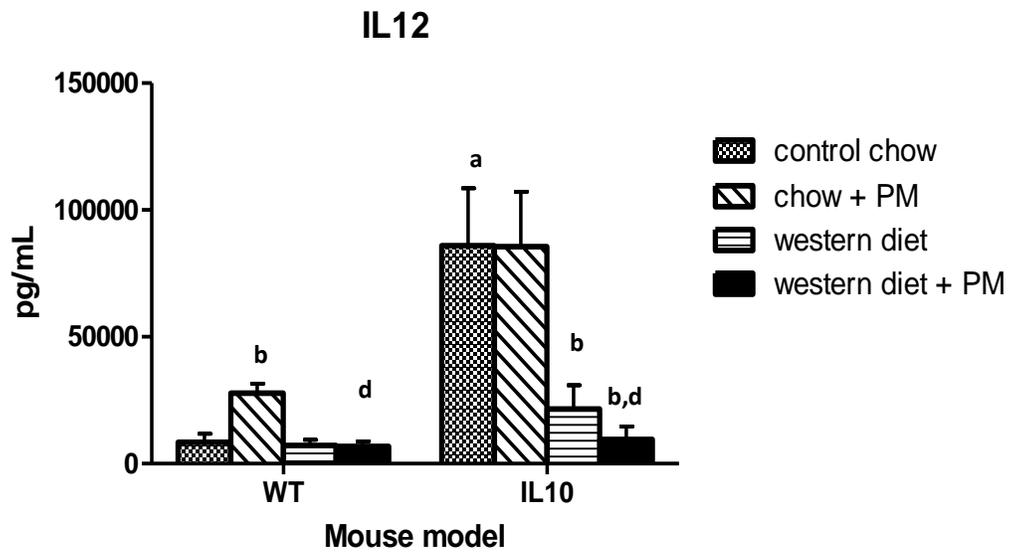
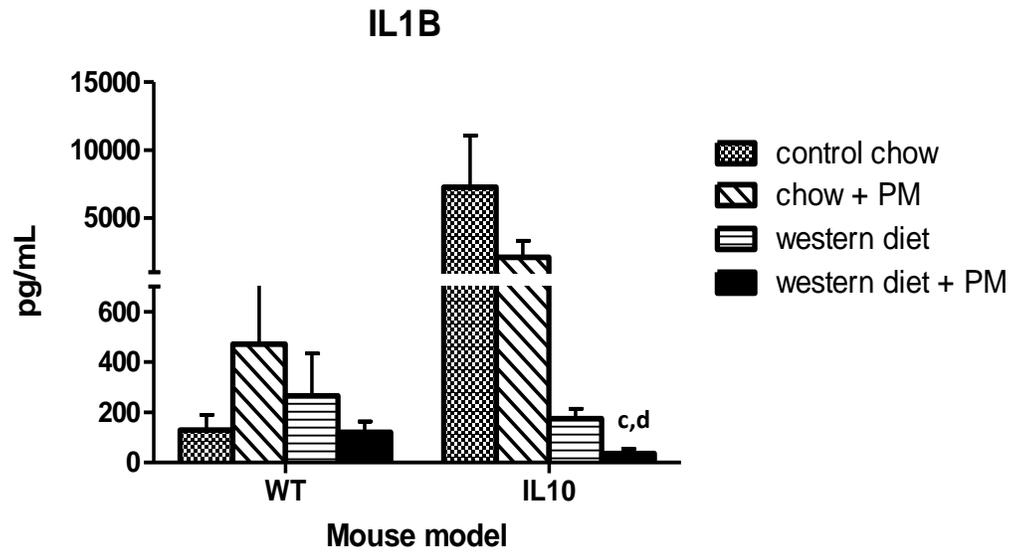**Figure 5-1a**

CXCL1

Figure 5-1b

IL12

Figure 5-1c

IL1B

Figure 5-1d

TNFa
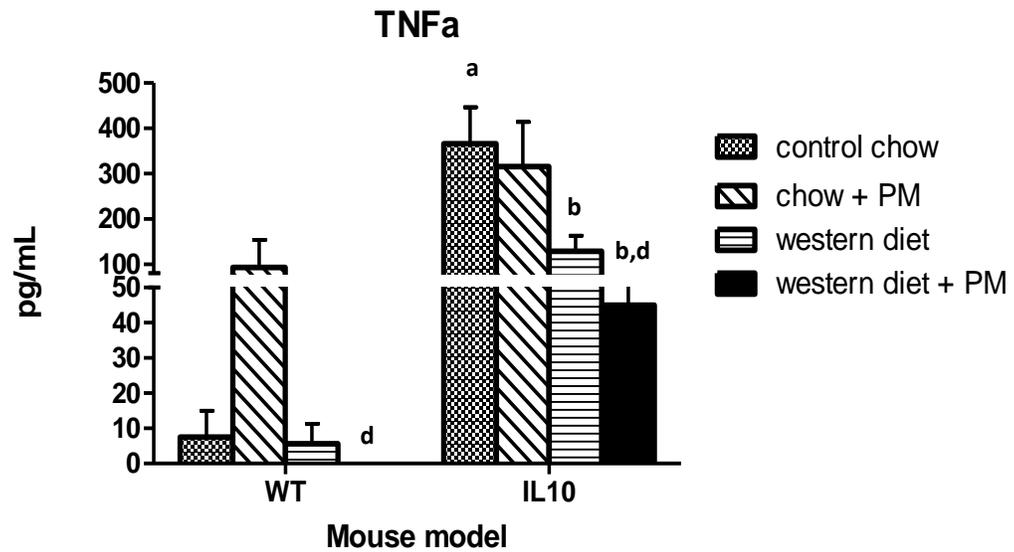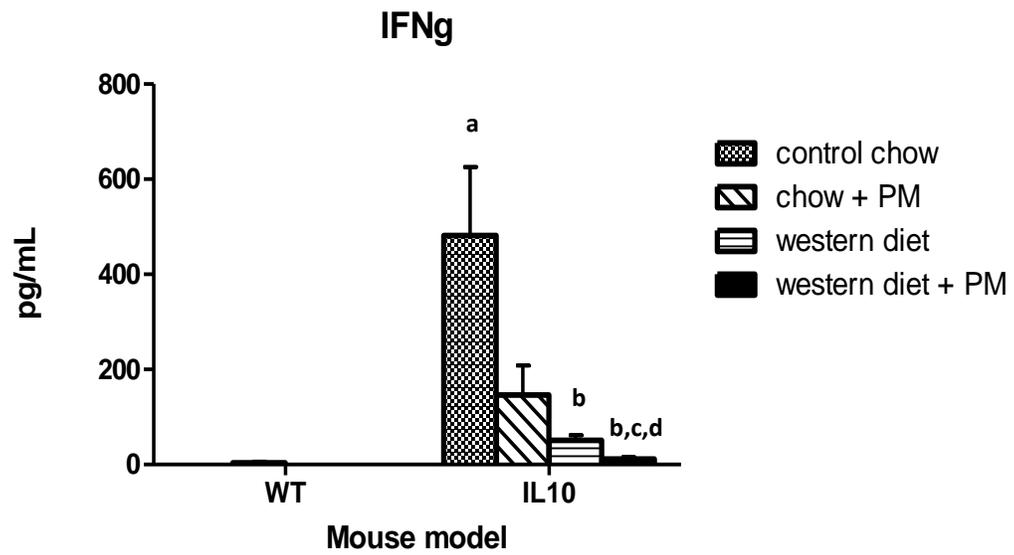
Figure 5-1e

Figure 5-1f

Figure 5-1g

IL2

Figure 5-1h

IL4

Figure 5-1i

IL5

Figure 5-1j

IL13
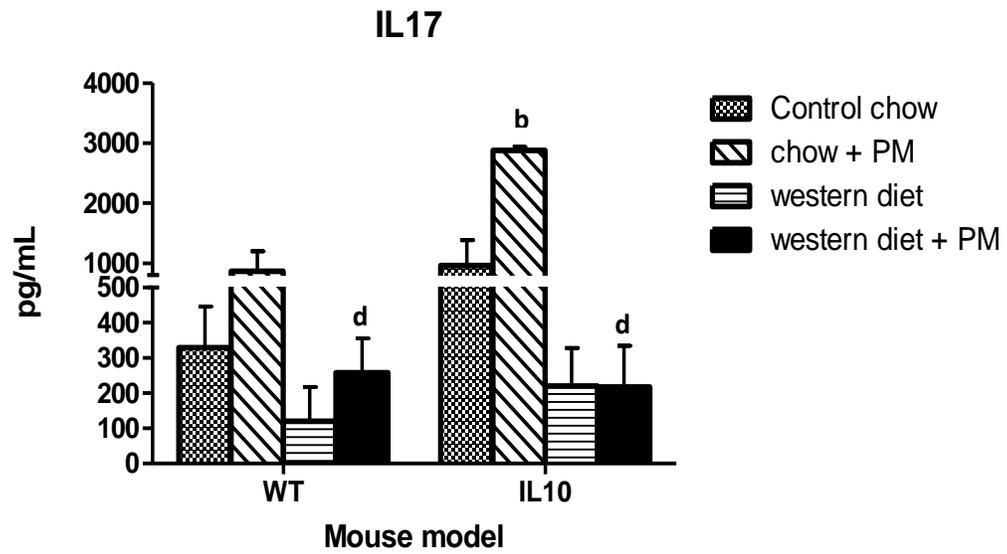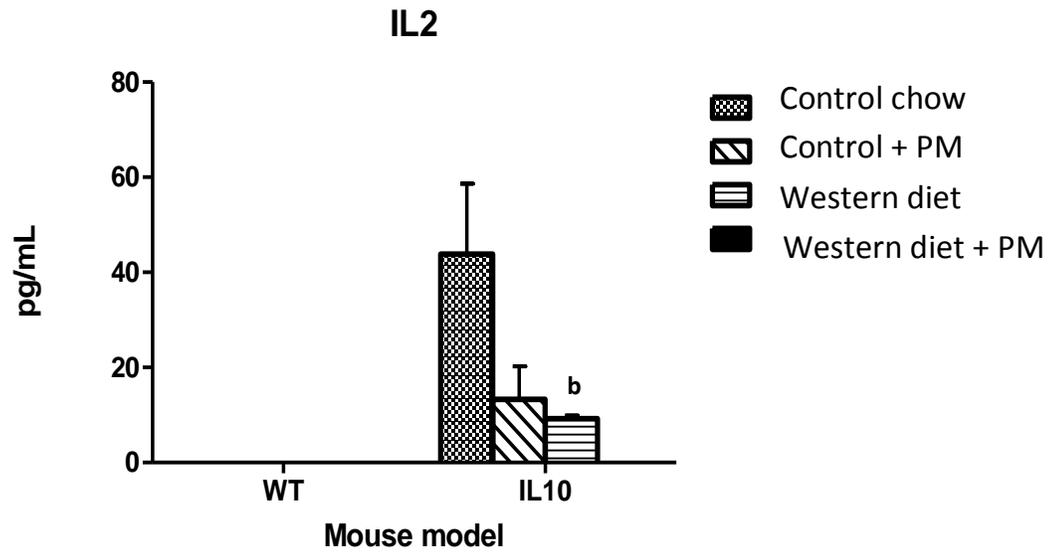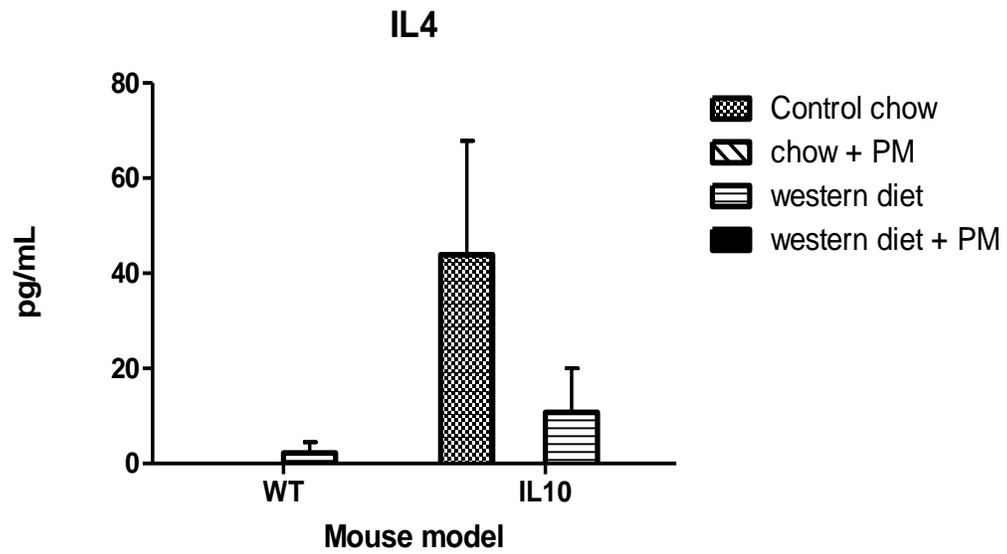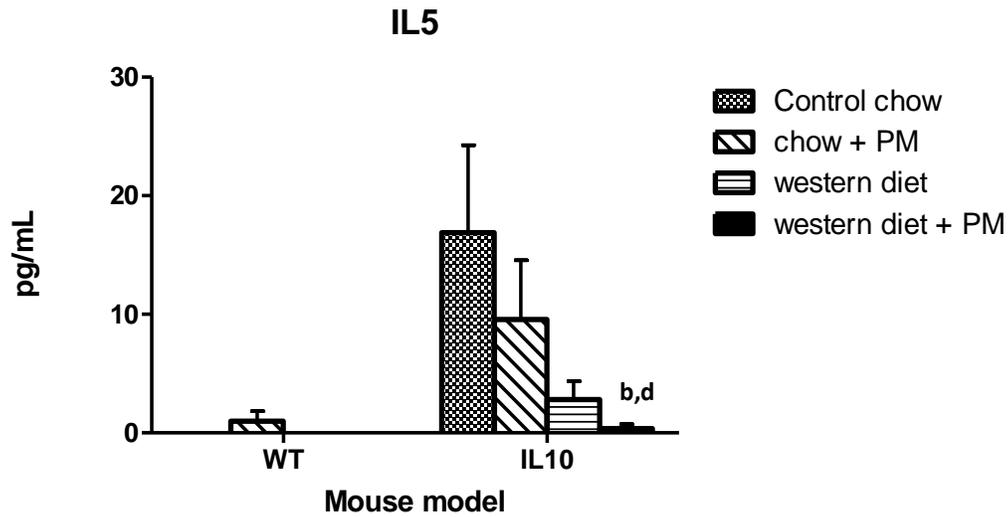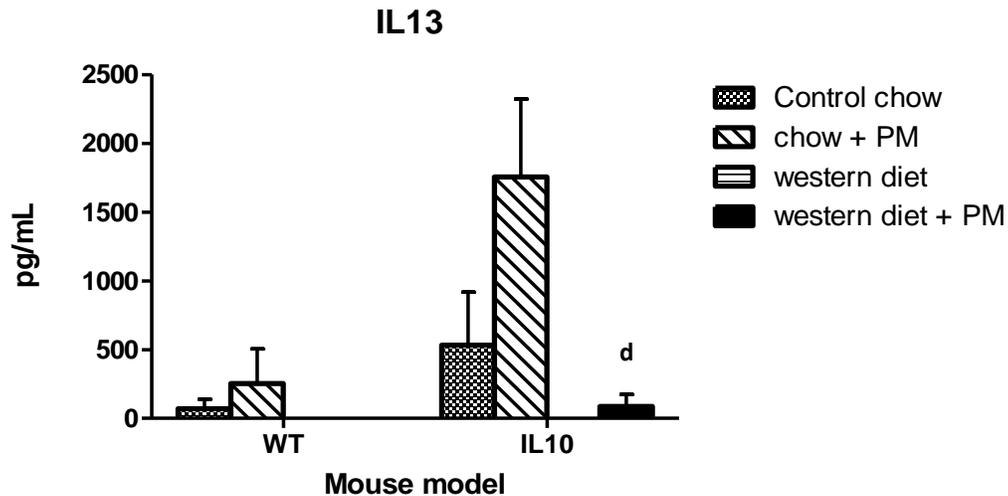
Figure 5-1: Cytokine and chemokine expression from colonic homogenate of WT and IL10$^{-/-}$ mice after 35 days on the PM chow, western diet, western + PM, or control chow. **a)** CXCL1 chemokine expression, **b)** Interleukin (IL) 12, **c)** IL1β, **d)** TNFα, **e)** IFNγ, **f)** IL17, **g)** IL2, **h)** IL4, **i)** IL5, and **j)** IL13. Values displayed as mean ± SEM (n=3-9).

statistical significance (P<0.05) between: [a] IL10$^{-/-}$ mice and WT mice on the control chow, [b] control chow mice and respective treatment group, [c] western + PM diet mice and western diet mice, [d] western + PM diet mice and PM chow mice

Figure 5-2a

CXCL1

Figure 5-2b

IL12

Figure 5-2c

IL1B

Figure 5-2d

TNFa

Figure 5-2e

IFNg

Figure 5-2f

IL17

Figure 5-2g

IL13
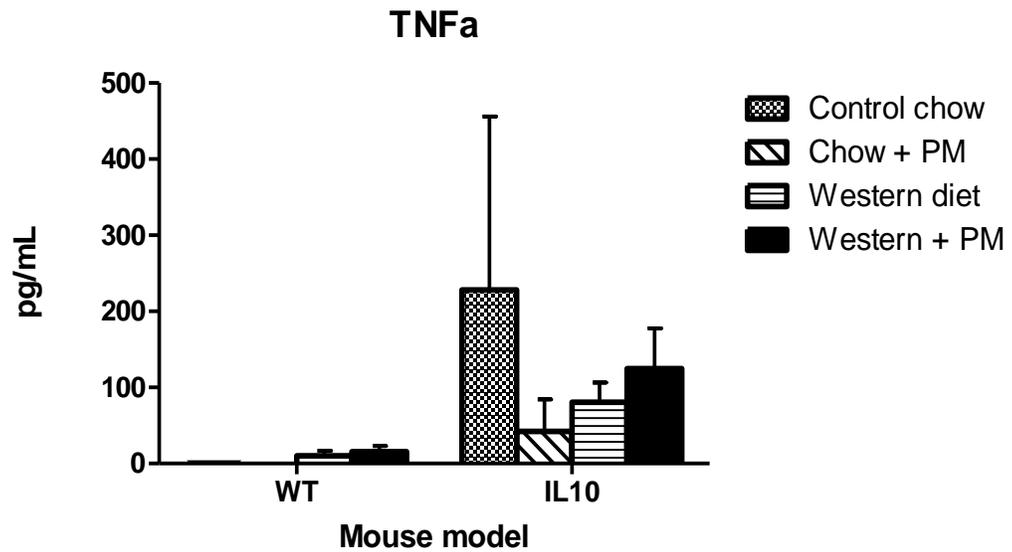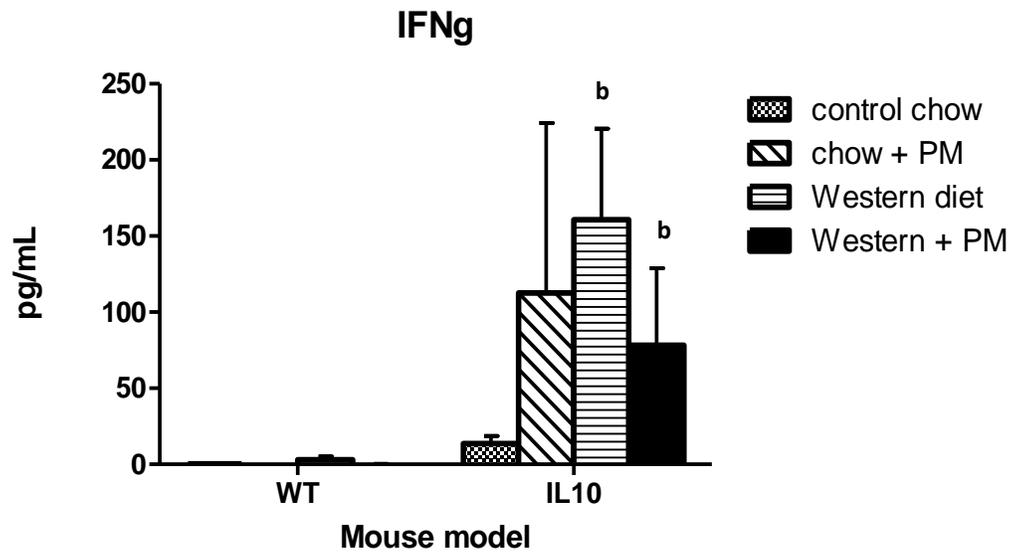
Figure 5-2: Cytokine and chemokine expression from small intestinal tissue homogenate of WT and IL10$^{-/-}$ mice after 35 days on the PM chow, western diet, western + PM diet, or control chow. **a)** CXCL1, **b)** IL 12, **c)** IL1β, **d)** TNFα, **e)** IFNγ, **f)** IL17, and **g)** IL13.

Values displayed as mean ± SEM (n=3-9)

Statistical significance (P<0.05) between: [a] IL10$^{-/-}$ mice and WT mice on the control chow, [b] control chow mice and respective treatment group, [c] western + PM diet mice and western diet mice, [d] western + PM diet mice and PM chow mice

5-3-3. Western style diet in combination with oral PM exposure uniquely alters microbial composition in IL10$^{-/-}$ mice

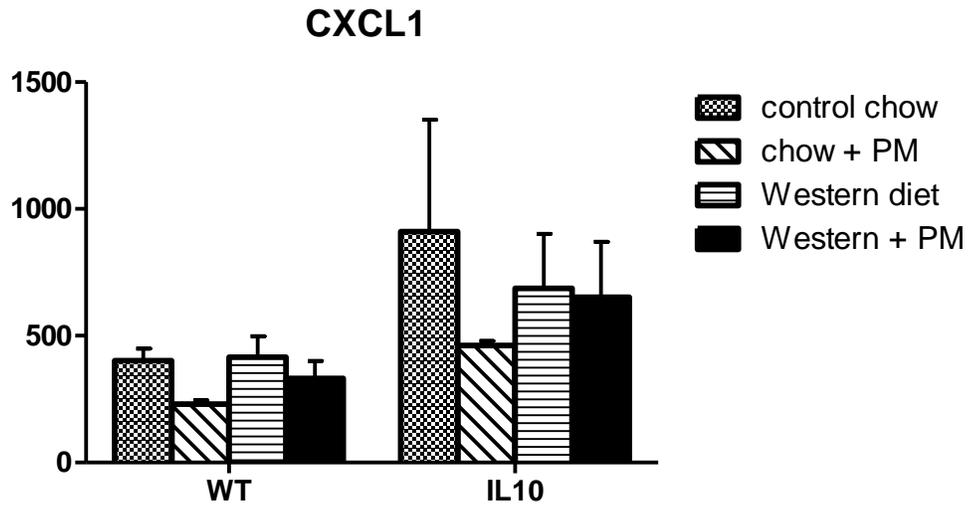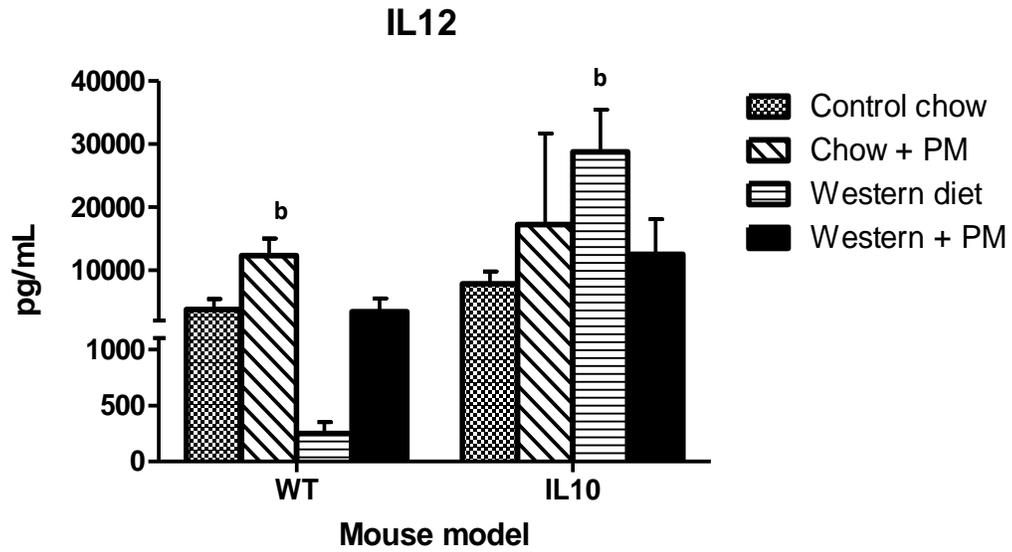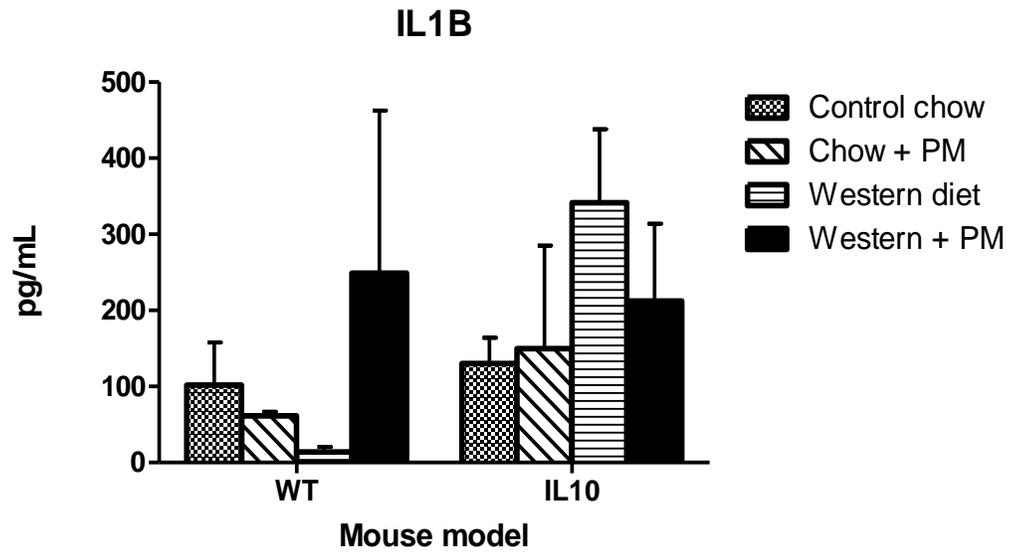I demonstrated that IL-10$^{-/-}$ mice on the western + PM diet had attenuated disease and cytokine secretion, therefore I then wanted to determine if the microbial composition within the colon could also be altered by these environmental factors. Microbial composition was determined from fecal pellets taken prior to (day 0) and following treatments (day 35) and analysed using the non-culture method of Terminal restriction fragment length polymorphism (T-RFLP).

Consumption of the western + PM diet resulted in significant alterations within the microbial community of IL10$^{-/-}$ mice. Specifically, I observed a significant increase in abundance of *Tenericutes* and unclassifiable bacteria, and a significant decrease in *Bacteroidetes* after 35 days of treatment (Figure 5-3). Although no change in abundance was observed with *Proteobacteria* overall, I did observe an increase in the class of *Alphaproteobacteria* in response to the western + PM diet, which didn't occur with western diet or PM treated mice respectively (Table 5-3). Principle component analysis (PCA) confirms that there were alterations in the bacterial population in IL10$^{-/-}$ mice on the western +PM diet compared to control, western, and PM treated mice respectively (Figure 5-6).

To determine if microbial diversity was also altered in these mice, the Shannon-Weiner index was determined. There was a significant decrease in the index in IL10^{-/-} mice on the western + PM diet after 35 days compared to control mice (Figure 5-5).

Western + PM diet also altered microbiota composition in WT mice. There was a significant increase in the abundance of *Verrucomicrobia*, with a trending increase in *Tenericutes* and *Actinobacteria*, as well as reduced *Proteiobacteria* after 35 days of treatment compared to control mice (Figure 5-4). Interestingly, there was a decrease in *Sphirocheate* and unclassifiable bacterial abundance in the control mice over the 35 days, which was not observed in the mice on the western + PM diet (Figure 5-4). Within the phylum *Firmicutes*, there was increased *Erysipelotrichi* abundance after 35 days on the western + PM diet, which was not observed in the western diet mice, or PM treated mice alone (Table 5-4). Similar to what was observed in the IL10^{-/-} mice, there was again an increase in *Alphaproteobacteria* in western + PM diet mice (Table 5-4). PCA analysis confirmed that there were alterations in the bacterial population of WT mice on the western + PM diet compared to the control chow, and PM counterparts, however there was substantial overlap with the western diet only mice (Figure 5-7). Again, there was a trending decrease in overall microbial diversity in the WT mice on the western + PM diet (Figure 5-5). This suggests a combination of a western diet with PM exposure can significantly

alter microbial composition in WT and IL10$^{-/-}$ mice differently than each of these

two factors can on their own.

Figure 5-3a
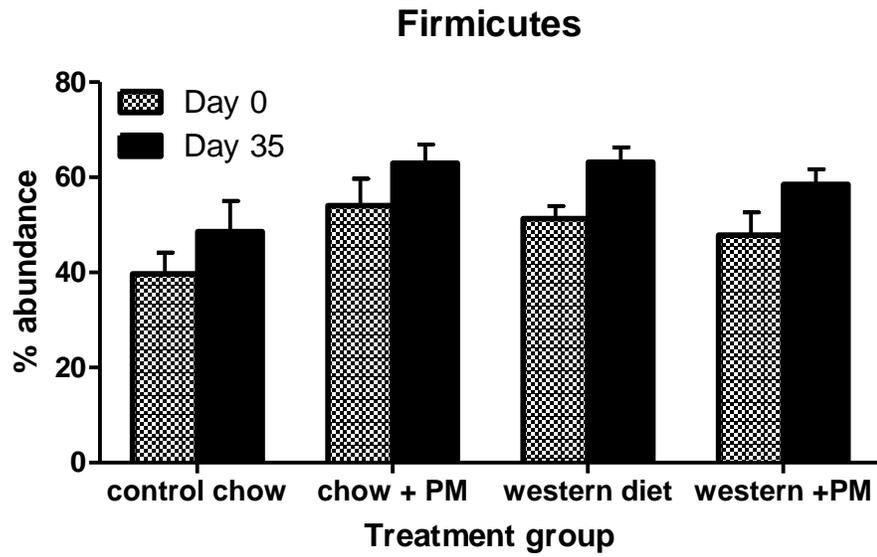
Firmicutes

Figure 5-3b

Bacteroidetes

Figure 5-3c

Tenericutes

Figure 5-3d

Actinobacteria

Figure 5-3e

Proteobacteria

Figure 5-3f

Verrucomicrobia

Figure 5-3g

Unclassified bacteria

Figure 5-3h

Spirocheates
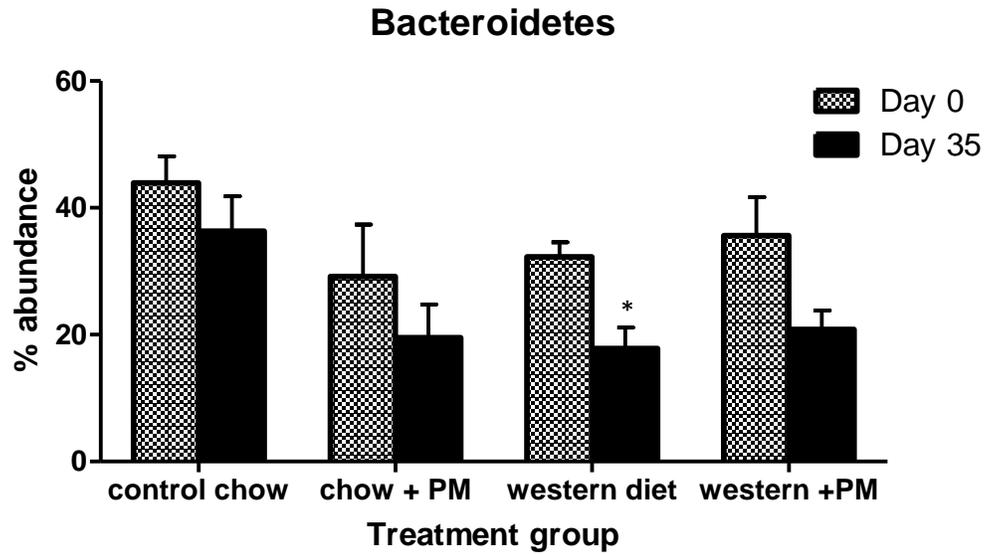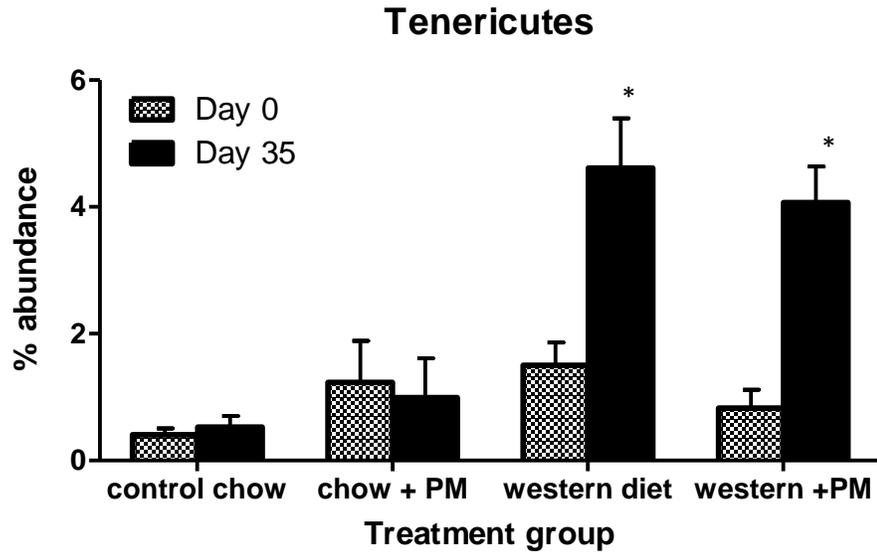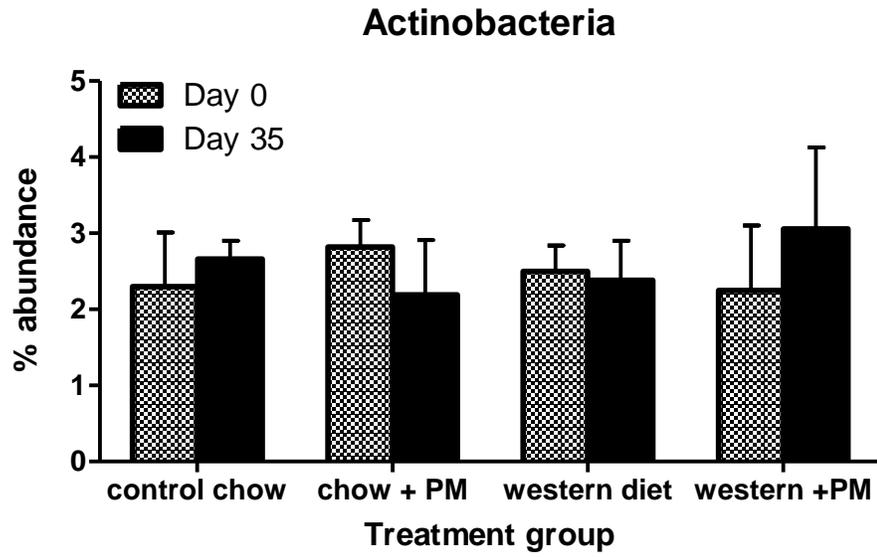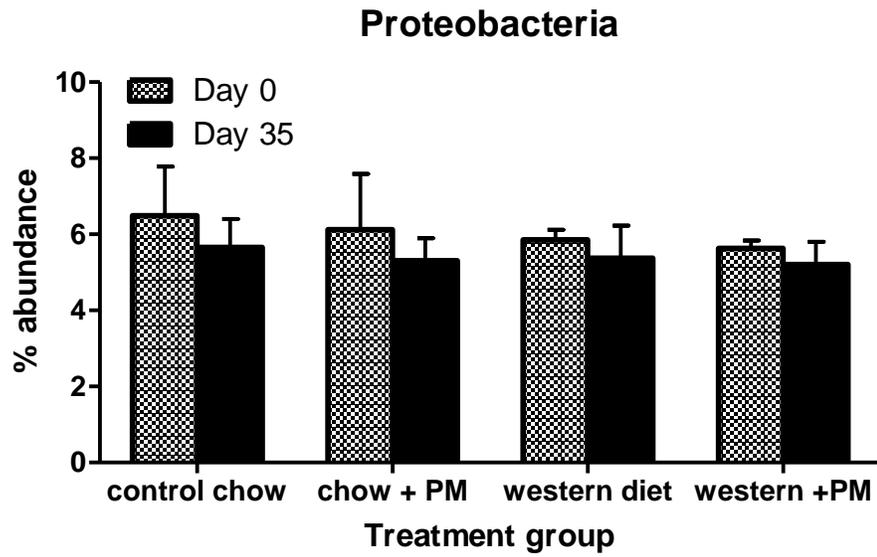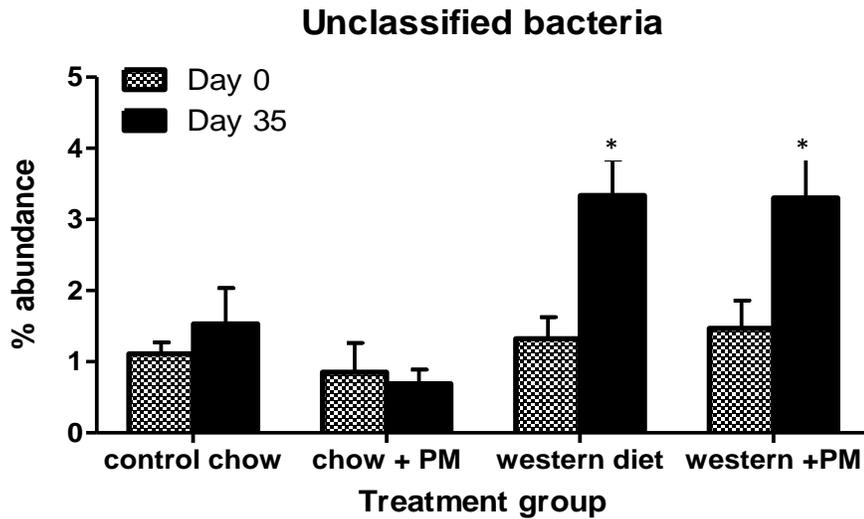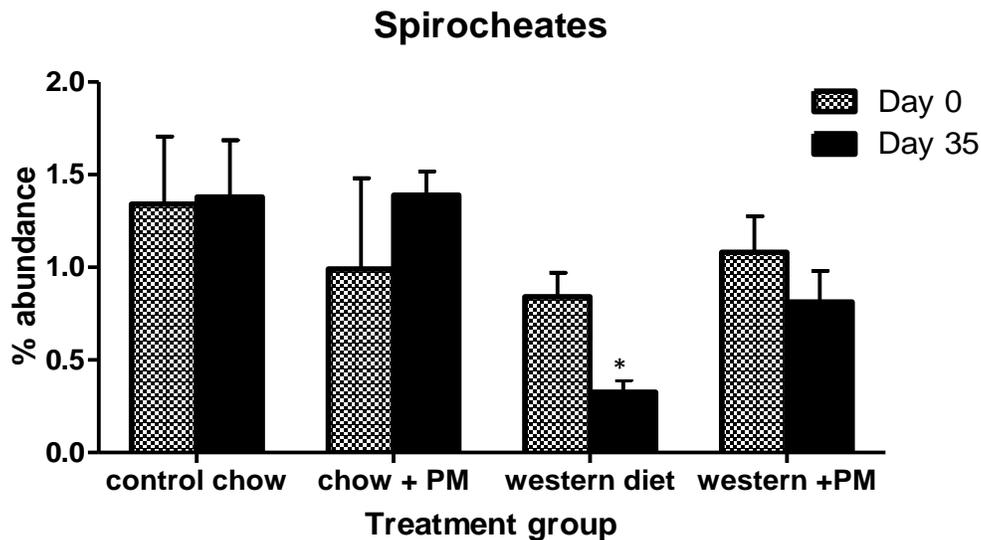
Figure 5-3: Microbial analysis performed on fecal samples taken prior to treatments (day 0) and following treatments (day 35) for IL10$^{-/-}$ mice on the control chow, PM chow, western diet, and western + PM diet. Analysis was performed using T-RFLP, and bacteria phyla are displayed as % abundance. Bacteria phyla: **a)** *Firmicutes*, **b)** *Bacteroidetes,* **c)** *Tenericutes*, **d)** *Actinobacteria*, **e)** *Proteobacteria*, **f)** *Verrucomicrobia*, **g)** unclassified bacteria, and **h)** *Spirocheates.* Values displayed as mean ± SEM (n= 7-8). ***** Significance between the day 0 and day 35 samples (p<0.05)

Table 5-3: Changes in microbial composition from fecal samples after 35 days on control chow, PM chow, western diet, or western + PM diet as analyzed with TRFLP in IL10$^{-/-}$ mice

Phyla	Class	Treatment group			
		Chow	Chow + PM	Western diet	Western + PM
Firmicutes	% of Total	48.58	62.96	63.2	58.49
	Clostridia*	90.57	90.42	80.29	78.44
	Erysipelotrichi*	2.88	2.2	8.24	10.94
	Bacilli*	1.66	1.71	3.5	2.55
Bacteroidetes	% of Total	36.35	19.54	17.84	20.83
	Bacteroidia*	60.67	57.38	43.48	55.4
	Sphingobacteria*	0.8	1.53	2.35	1.05
	Flavobacteria*	2.85	2.63	8.43	3.56
Proteobacteria	% of Total	5.65	5.3	5.37	5.2
	Alphaproteobacteria*	16.54	8.8	10.04	21.42
	Betaproteobacteria*	16.55	9.28	39.9	25.35
	Deltaproteobacteria*	15.94	7.83	4.49	8.45
	Gammaproteobacteria*	48.85	73.55	43.31	41.85
Actinobacteria	Actinobacteria	2.66	2.19	2.38	3.06
Spirochates	Spirochates	1.38	1.39	0.33	0.813
Tenericutes	Mollicutes	0.53	0.99	4.61	4.07
Verrucomicrobia	Verrucomicrobiad	0.6	1.82	0.56	1.06
Unclassified Bacteria	Unclassified bacteria	1.53	0.69	3.33	3.3

*represents % of phyla

Figure 5-4a
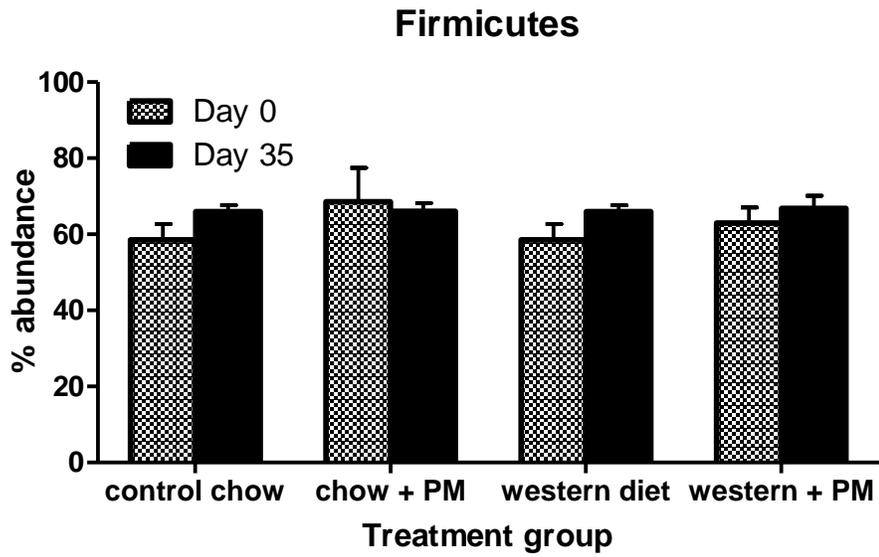
Firmicutes

Figure 5-4b

Bacteroidetes

Figure 5-4c

Tenericutes

Figure 5-4d

Actinobacteria

Figure 5-4e

Proteobacteria

Figure 5-4f

Verrucomicrobia

Figure 5-4g

Unclassified bacteria

Figure 5-4h

Spirocheates
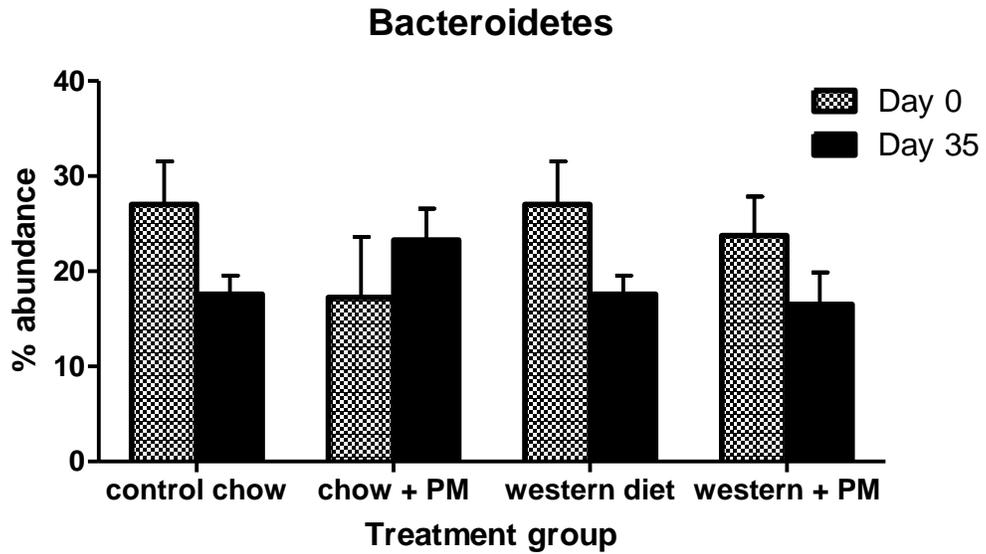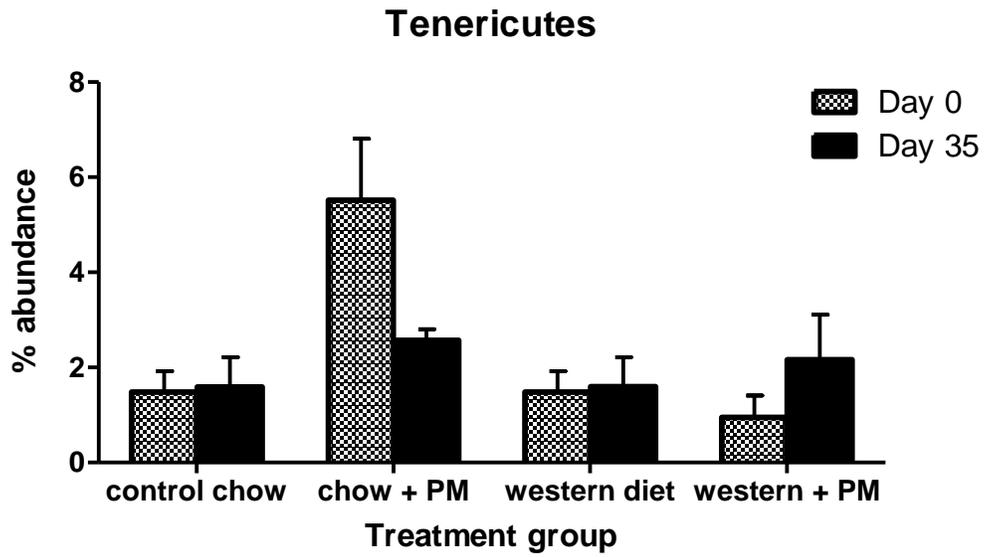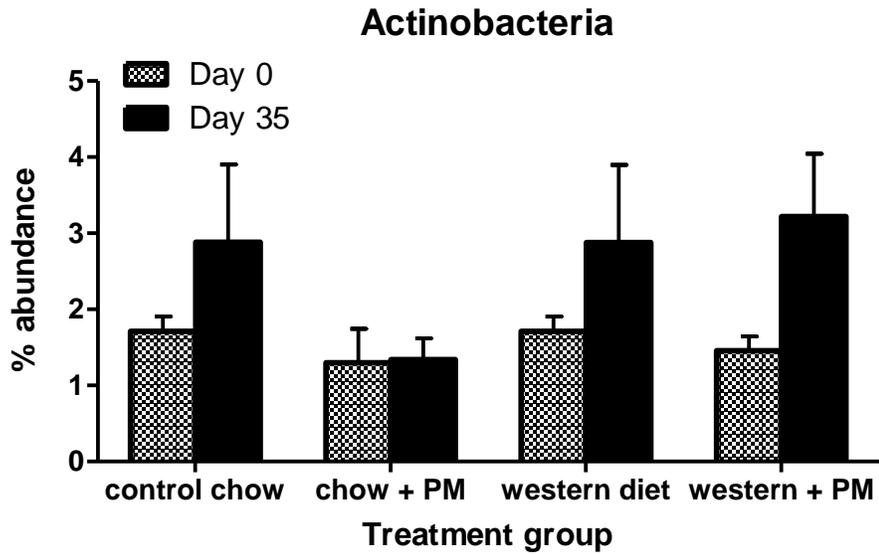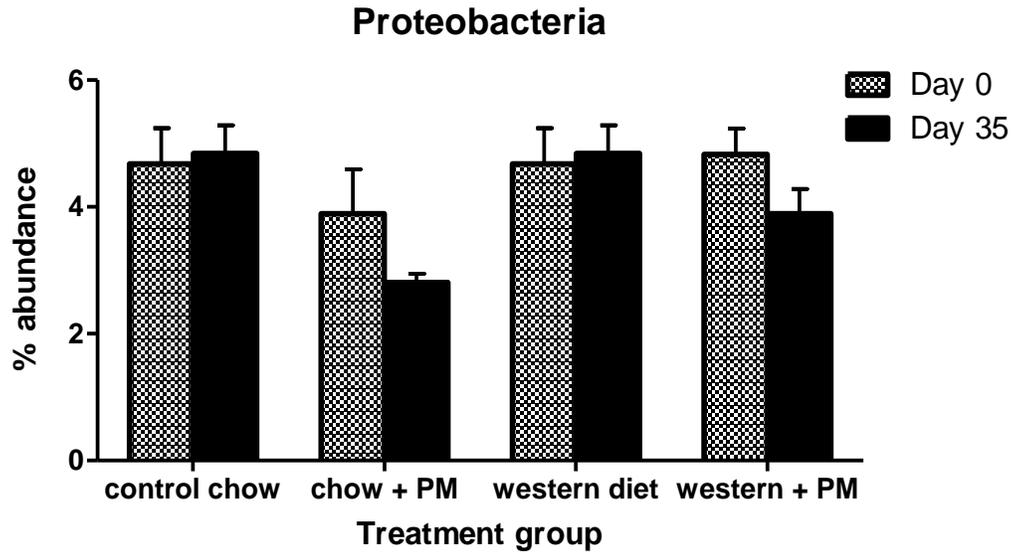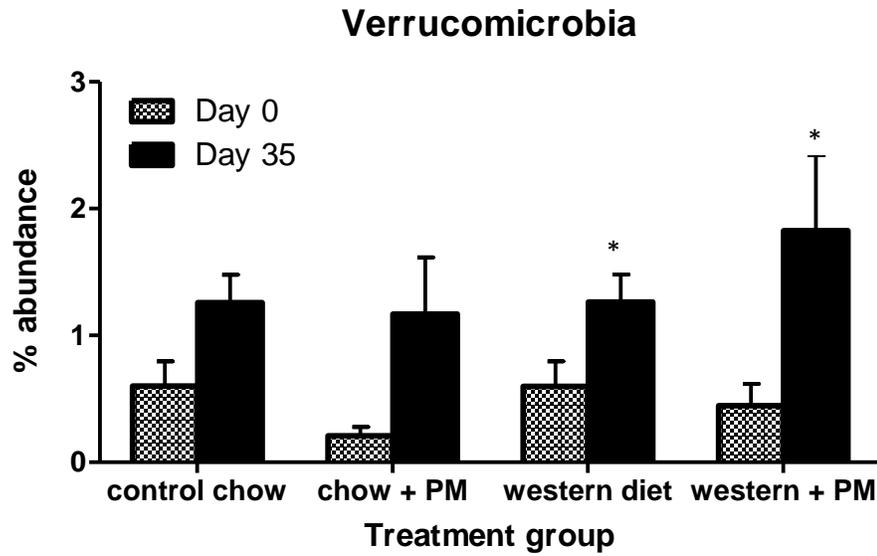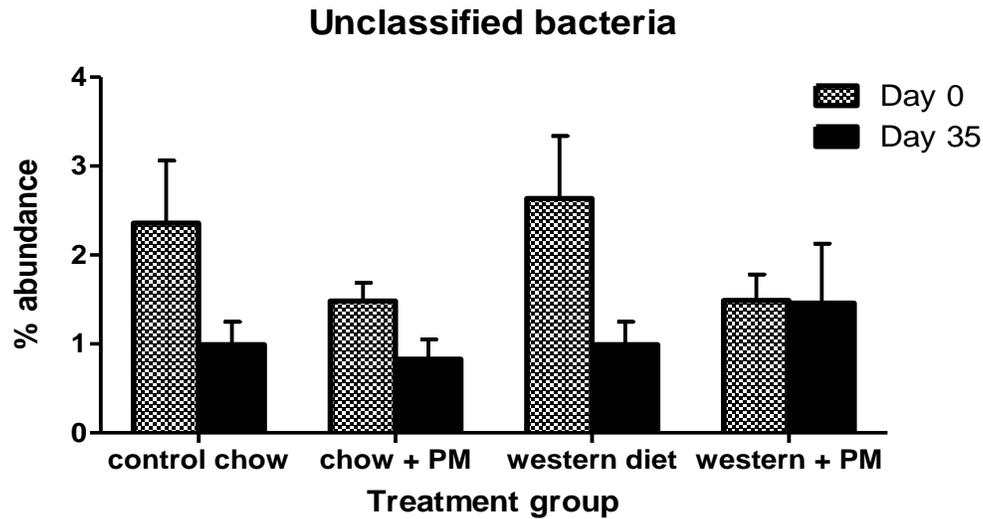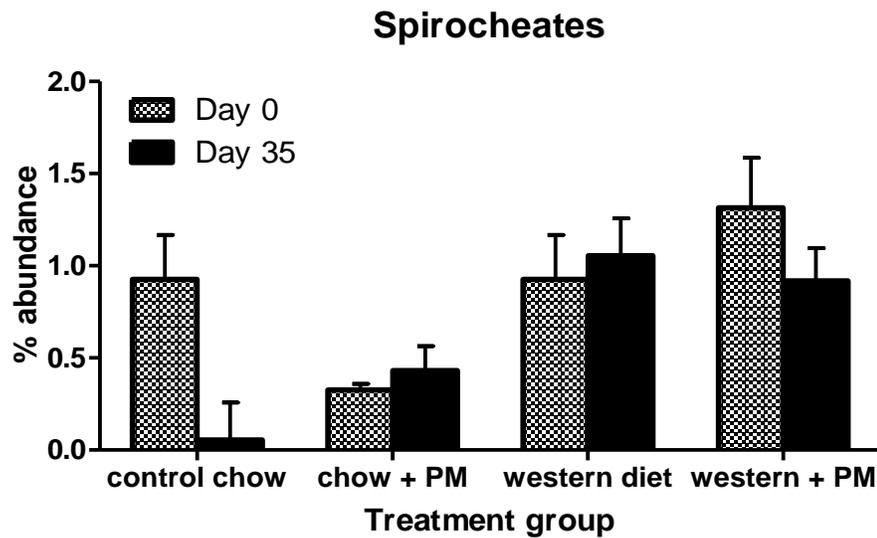
Figure 5-4: Microbial analysis performed on fecal samples taken prior to treatments (day 0) and following treatments (day 35) for WT mice on the control chow, PM chow,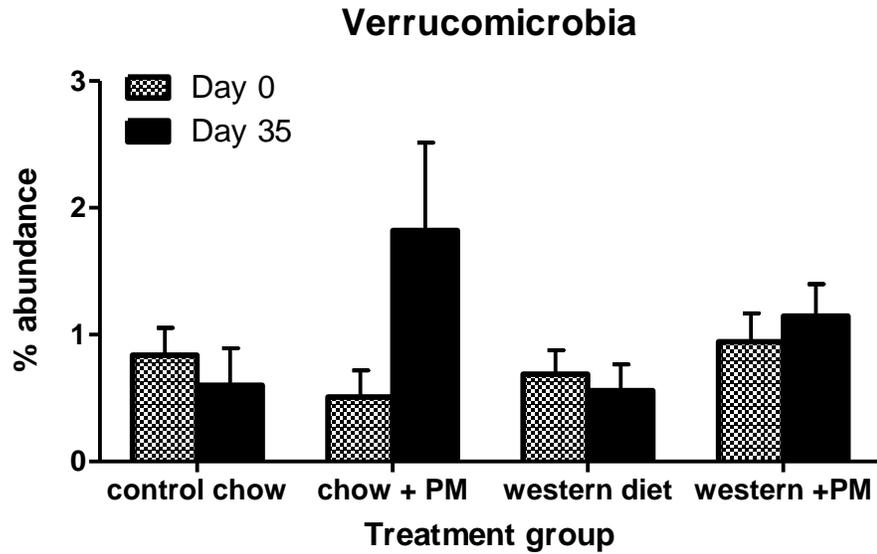 western diet, and western + PM diet. Analysis was performed using T-RFLP, and bacteria phyla are displayed as % abundance. Bacteria phyla: **a)** *Firmicutes*, **b)** *Bacteroidetes,* **c)** *Tenericutes*, **d)** *Actinobacteria*, **e)** *Proteobacteria*, **f)** *Verrucomicrobia*, **g)** unclassified bacteria, and **h)** *Spirocheates.* Values displayed as mean ± SEM (n= 7-8) ***** represents significance between the day 0 and day 35 samples P<0.05

Table 5-4: Changes in microbial composition from fecal samples after 35 days on control chow, PM chow, western diet, or western + PM diet as analyzed with TRFLP in WT mice

Phyla	Class	Treatment group			
		Chow	Chow + PM	Western diet	Western + PM
Firmicutes	% of Total	65.92	66.02	61.56	66.81
	Clostridia*	90.42	88.51	88.05	85.38
	Erysipelotrichi*	2.36	2.61	1.91	5.32
	Bacilli*	2.23	2.25	5.03	1.77
Bacteroidetes	% of Total	17.59	23.3	20.36	16.52
	Bacteroidia*	72.3	51.5	62.1	62.68
	Sphingobacteria*	0.45	0.95	0.93	1.16
	Flavobacteria*	2.71	7.19	3.26	2.61
Proteobacteria	% of Total	4.84	2.81	5.45	3.89
	Alphaproteobacteria*	7.72	8.22	5.45	17.73
	Betaproteobacteria*	14.66	15.73	16.74	17.34
	Deltaproteobacteria*	5.71	31.31	17.04	9.67
	Gammaproteobacteria*	71.06	44.53	58.31	54.4
Actinobacteria	Actinobacteria	2.88	1.34	4.79	2.6
Spirochates	Spirochates	1.05	1.39	0.67	0.84
Tenericutes	Mollicutes	1.59	2.57	1.62	2.38
Verrucomicrobia	Verrucomicrobiad	1.26	1.17	1.62	1.83
Unclassified bacteria	Unclassified bacteria	0.99	0.83	1.03	1.31

*represents % of phyla

Figure 5-5a

microbial diversity in IL10 mice

Figure 5-5b

microbial diversity in WT mice
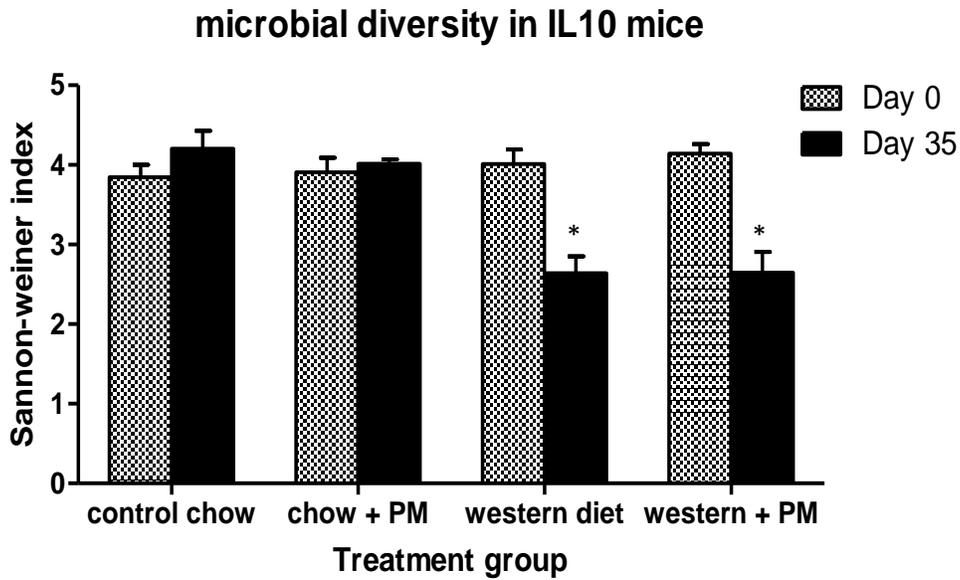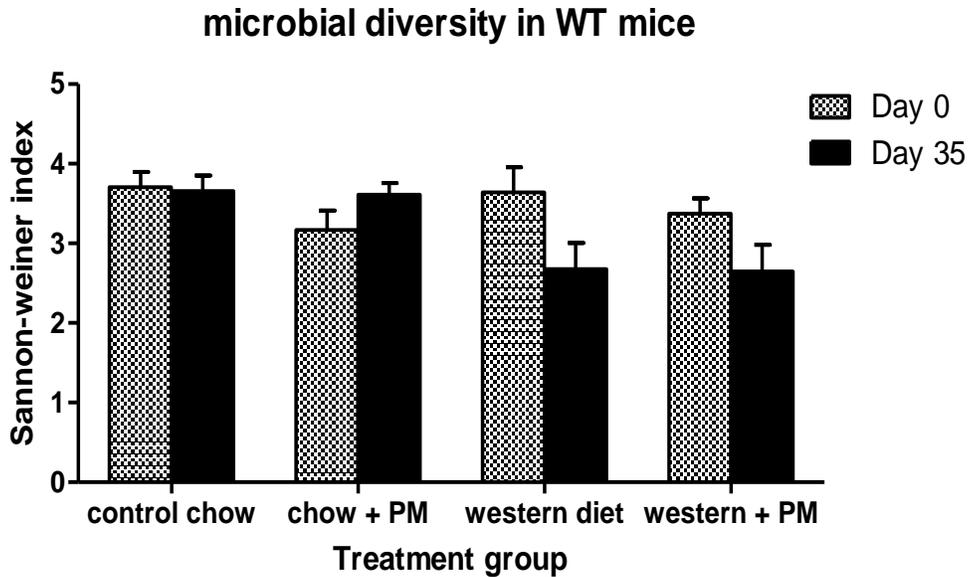
Figure 5-5: Shannon-Weiner index of microbial diversity, calculated from fecal samples taken prior to (day 0) and following dietary treatment (day 35) with control chow, PM chow, western diet, or western + PM diet in **a)** IL10$^{-/-}$ mice, **b)** WT mice. Values displayed as mean ± SEM (n= 7-8) * represents significance between day 0 and day 35 samples p<0.05

Figure 5-6
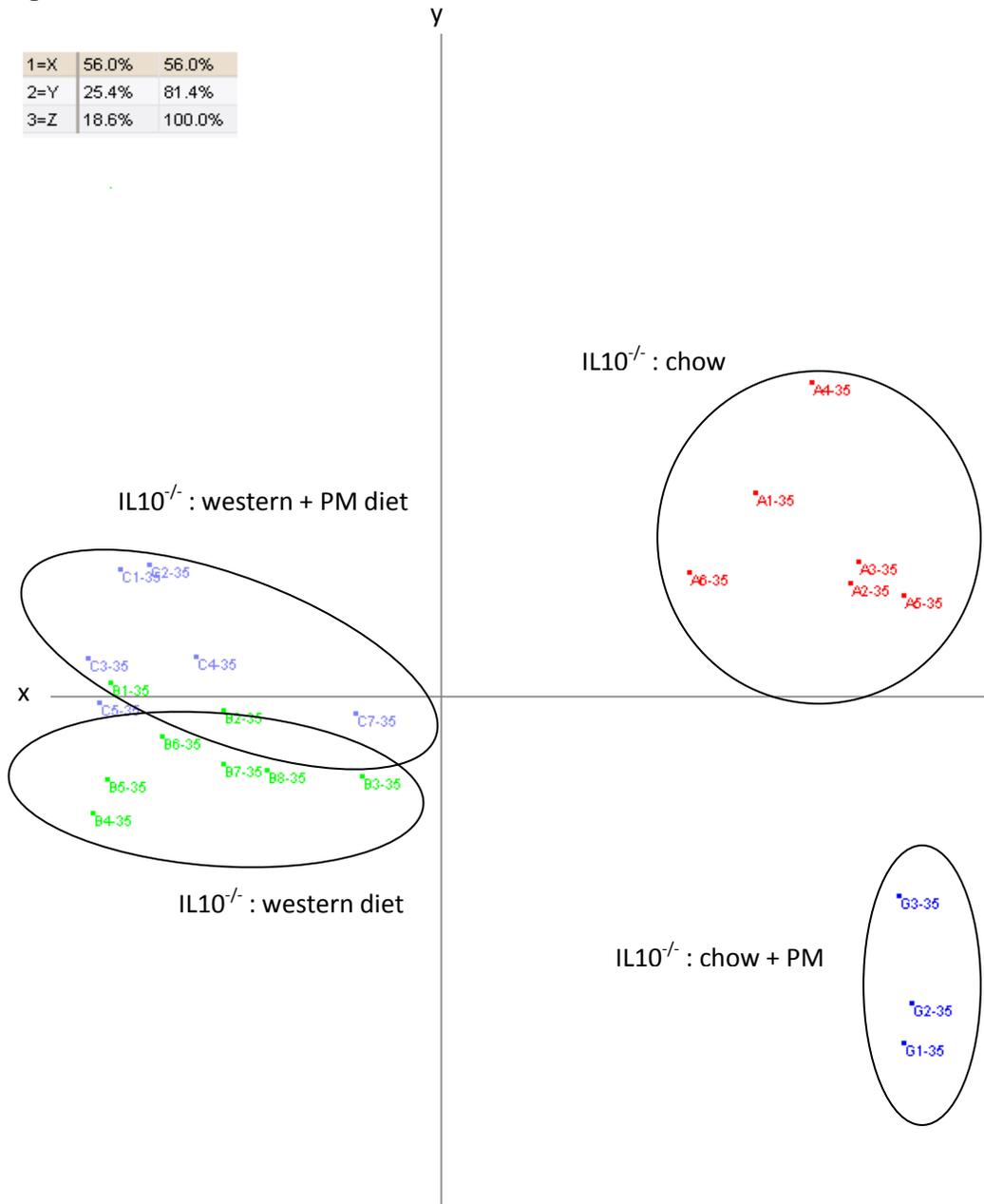
Figure 5-6: PCA plot examining changes in the microbial composition in IL10$^{-/-}$ mice after 35 days on control chow, PM +chow, western diet, or western + PM diet. Results are based on the band patterns obtained during the TRFLP analysis of stool samples. Each dot represents one mouse. **A**- Control chow, **B**- western diet **G**- IL10$^{-/-}$ on chow + PM, **C**- western + PM chow.

Figure 5-7
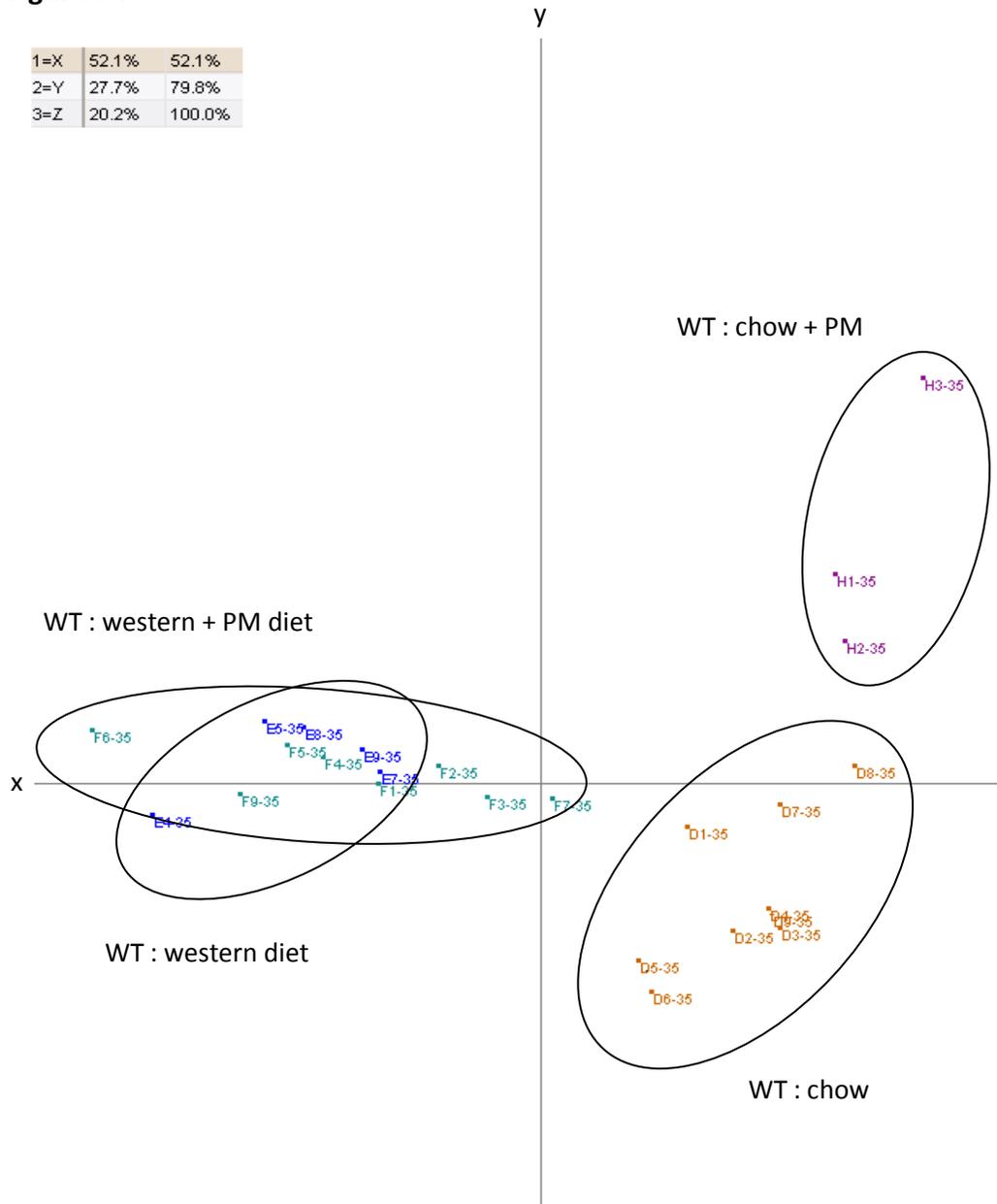
Figure 5-7: PCA plot examining changes in the microbial composition in WT mice after 35 days on control chow, PM +chow, western diet, or western + PM diet. Results are based on the band patterns obtained during the TRFLP analysis of stool samples. Each dot represents one mouse. **D**- Control chow, **E**- western diet **H**- IL10$^{-/-}$ on chow + PM, **F**- western + PM chow.

5-3-4. Western + PM diet alters short chain fatty acid production in IL10$^{-/-}$ and WT mice

Short chain fatty acid (SCFA) analysis was performed on cecal contents from both IL10$^{-/-}$ and WT mice after 35 days of treatment. In IL10$^{-/-}$ mice placed on the western + PM diet, there was a significant decrease in butyrate and valerate concentration compared to control mice (Figure 5-8). This was different than what was observed in mice placed only on the western diet or PM chow respectively. Mice on the western diet demonstrated a significant reduction in acetate, propanoate, butyrate and caproic acid, and PM treated mice had significant increase in the branched chain fatty acids (BCFA) isobutyrate and isovalerate, compared to control (Figure 5-8). Production of acetate, isobutyrate, and isovalerate were not altered in mice on the western + PM diet, which suggests that the combination of the two factors results in a completely different response from the bacteria, resulting in altered fermentation within the IL10$^{-/-}$ mice.

In WT mice, there was a significant decrease in acetate, propanoate, and butyrate, along with a significant increase in isovaleric and caproic acid, and trending increase in isobutyrate concentration in mice placed on the western +PM diet (Figure 5-8). This was different then the response observed in the IL10$^{-/-}$ mice, which suggests there is different interactions with the western + PM diet between healthy control WT mice, and colitis prone IL10$^{-/-}$ mice.

Figure 5-8a

Acetate

Figure 5-8b

Propanoic acid

Figure 5-8c

Isobutyric acid

Figure 5-8d

Butyric acid

Figure 5-8e

Isovaleric acid

Figure 5-8f

Valeric acid

Figure 5-8g

caproic acid
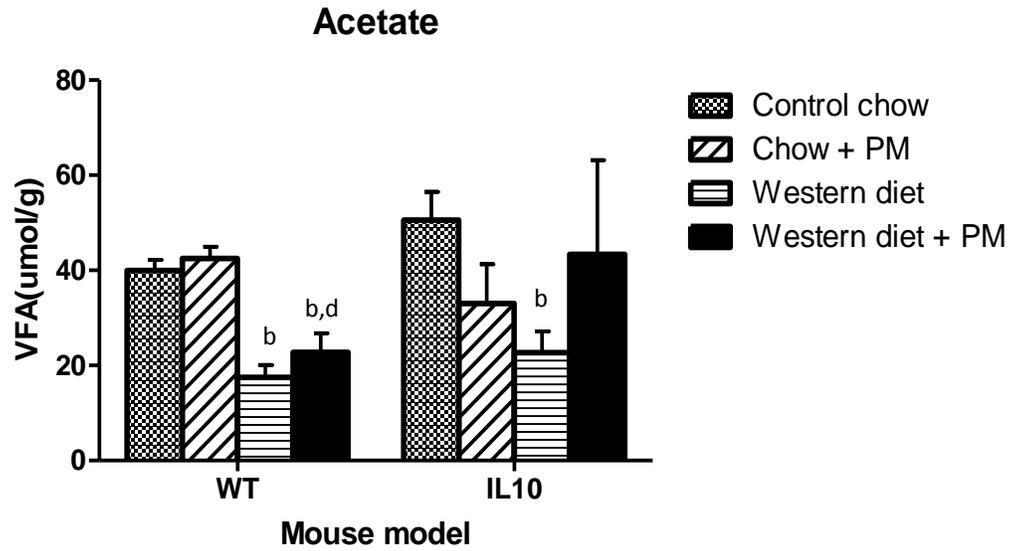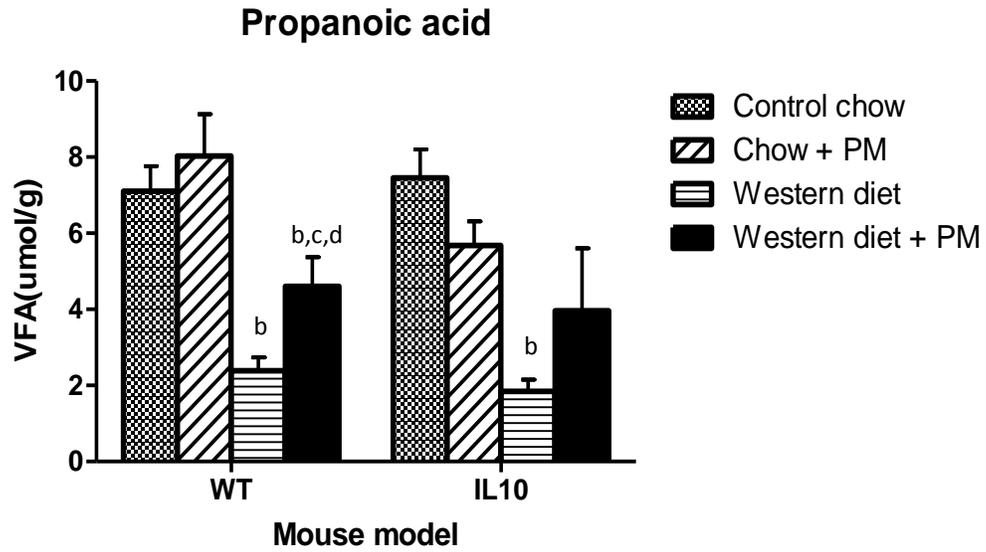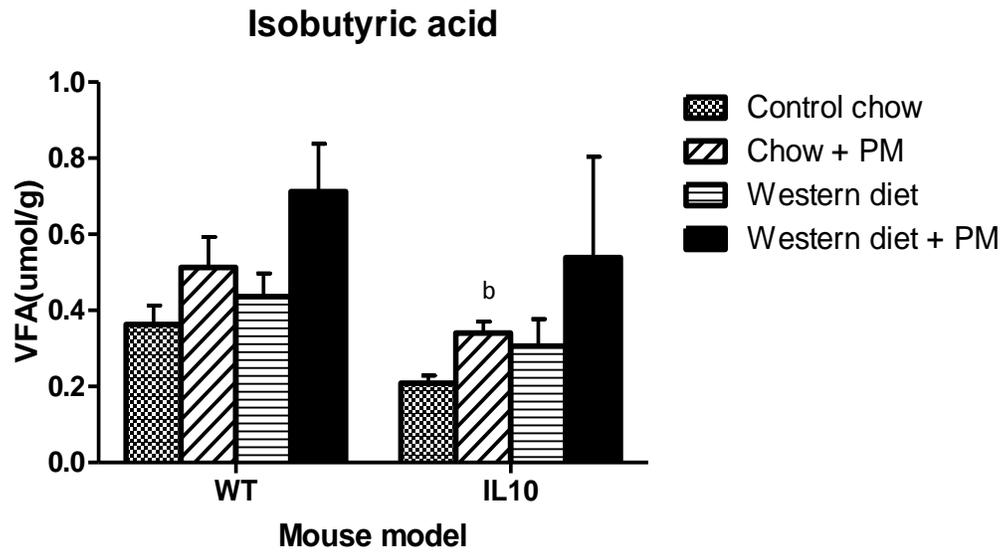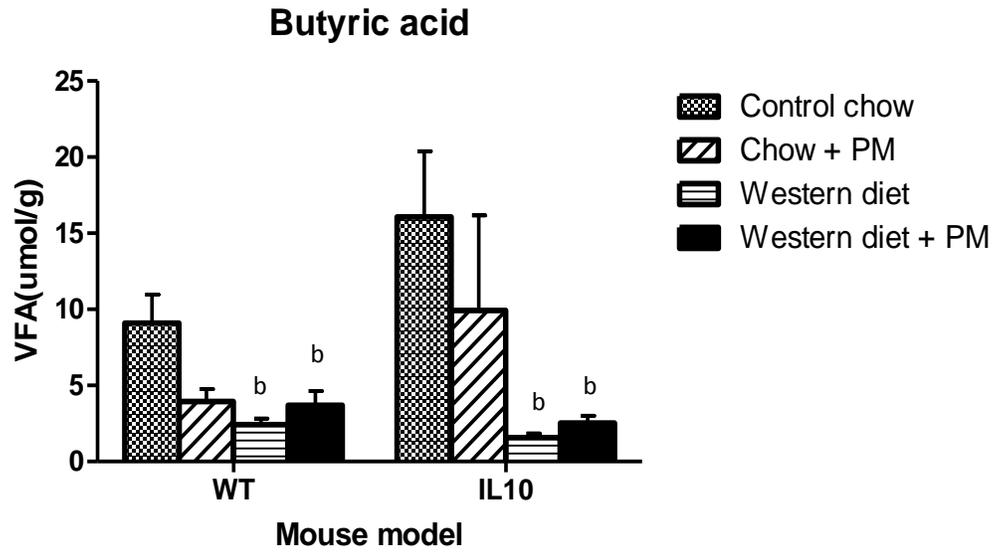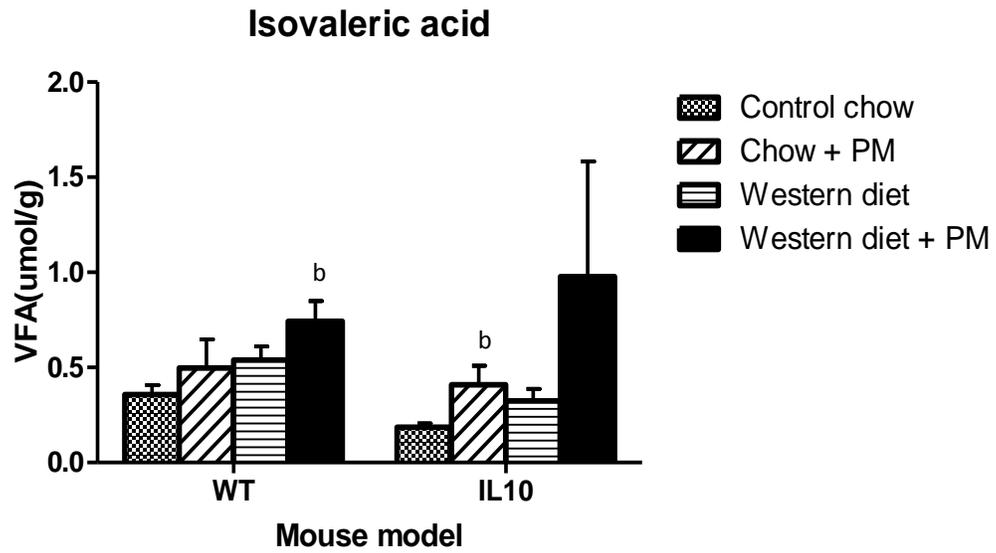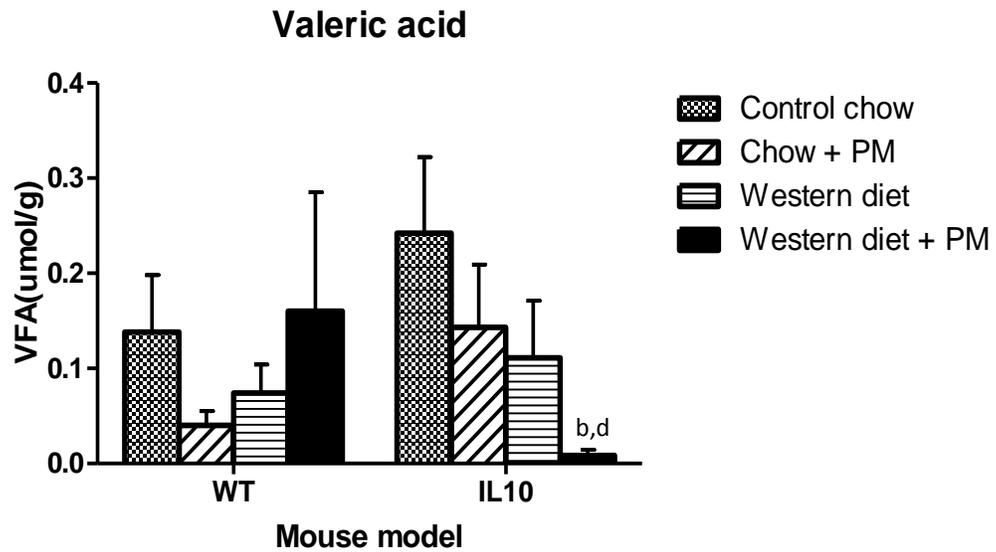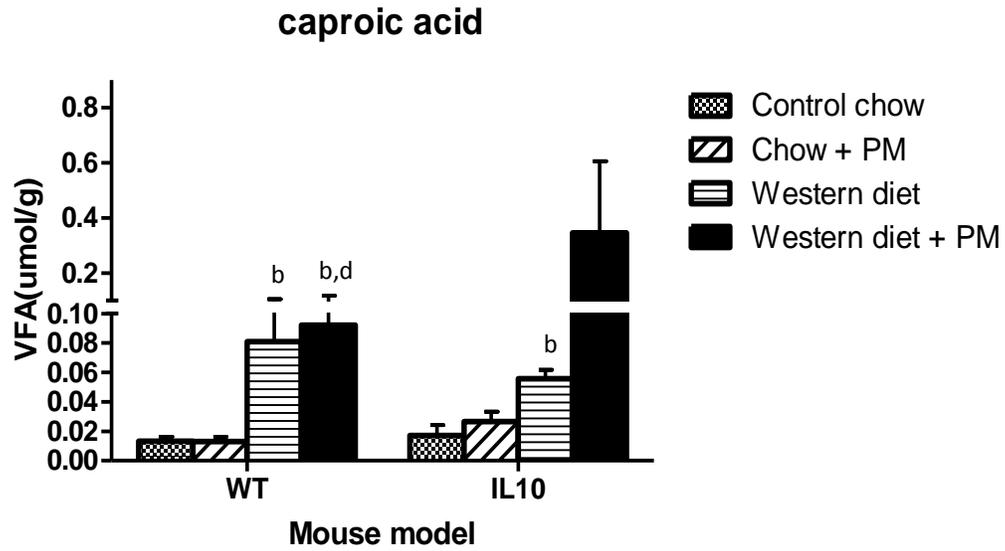
Figure 5-8: Short chain fatty acid concentration (SCFA) from cecal contents of IL10$^{-/-}$ and WT mice after 35 days on control chow, PM chow, western diet, or western + PM diet. SCFA: **a)** acetic acid, **b)** propanoic acid, **c)** isobutyric acid, **d)** butyric acid, **e)** isovaleric acid, **f)** valeric acid, **g)** caproic acid. Values displayed as mean ± SEM (n= 7-8)

Statistical significance (P<0.05) between: [a] IL10$^{-/-}$ mice and WT mice on the control chow, [b] control chow mice and respective treatment group, [c] western + PM diet mice and western diet mice, [d] western + PM diet mice and PM chow mice

5-4. Discussion:

As mentioned in previous chapters, environmental risk factors are essential components in the pathogenesis of inflammatory bowel disease (IBD), and are believed to be the primary cause of increased incidence around the world. High fat, high sugar (western diet) diets and airborne particulate matter (PM) are two such factors that are associated with increased risk of disease. I have been able to demonstrate in previous chapters that dietary exposure to PM_{10} and a western style diet, each on their own, have the ability to alter in different ways, cytokine expression of various T-helper (Th) 1 and Th2 related cytokines, and microflora composition in the $IL10^{-/-}$ mouse. Since individuals in western nations are typically exposed to both a western style diet and PM at the same time, I wanted to examine the role of these two factors in combination on the mucosal immune system and intestinal microflora composition in the $IL10^{-/-}$ mouse model of colitis.

In this chapter, I demonstrate that treatment of $IL10^{-/-}$ mice with a western style diet (rich in fat and simple carbohydrates (CHO)) in combination with airborne PM, resulted in attenuated colonic inflammation, characterized by a significant reduction of pro-inflammatory cytokine expression in the large intestine and decreased histopathological scores. This suggests there is an overall reduction in colonic disease severity in the $IL10^{-/-}$ mice in response to the western + PM diet. I was also able to demonstrate a significant alteration in microfloral composition and diversity, and short chain fatty acid (SCFA)

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production in the cecum in response to the western + PM diet. Interestingly, these results differed in WT mice, and between mice treated with the western diet or PM alone.

As I have demonstrated in the previous chapter, the untreated IL10$^{-/-}$ mice have begun to develop colitis by day 35 of the experiment, as characterized by the increasing histopathological scores, increased colonic weight, and increased expression of various pro-inflammatory cytokines and chemokines compared to control WT mice. Interestingly, when IL10$^{-/-}$ mice were placed on the combined western + PM diet, there was a significant decrease in colonic weight and a trending decrease in the overall histology score. Specifically I observed a decreasing trend in enterocyte injury and neutrophil infiltration, and although this was not significant, it should be noted only 17% of the mice had some sort of injury. These results were not significant because control IL10$^{-/-}$ mice were in varying stages of disease. This does suggest, however, that there is less inflammatory cell infiltrate and damage within the colons of mice on a western + PM diet. In future studies, it would be of interest to run the experiments longer so that all the IL10$^{-/-}$ mice can develop disease, and perhaps these results would become more dramatic.

These results were very similar to what was observed in IL10$^{-/-}$ mice on the western diet alone, in which there was also a significant reduction in colonic weight with reduced epithelial damage and neutrophil infiltrate. In PM treated

mice on the other hand, there was a trending increase in the colonic histological

score which was the opposite of what was observed in the western + PM treated

mice. Therefore it could be that the results I observed with the western + PM

diet are simply the result of the western diet alone, which could be having a

stronger effect on the physiology of the mouse intestinal tract. Interestingly

though, there was a significant decrease in the colonic weight of the western +

PM mice compared to the western alone mice, which could suggest PM is still

having some sort of effect on the health of the colon.

I was able to demonstrate that treatment of IL10$^{-/-}$ mice with western +

PM diet resulted in a significant reduction expression for the pro-inflammatory

cytokines IL12, TNFα, and IFNγ, along with a trending decrease in IL1β and IL17

compared to control IL10$^{-/-}$ mice. These are Th1 effector cell mediated cytokines

that are expressed in high levels in the intestinal tissue of CD and UC patients,

and typically result in ulcerations and lesions (5). The decreased expression of

these cytokines in the colonic tissue can have important implications for patients

suffering from CD, such that if this western diet, in combination with PM was

able to similarly reduce colonic inflammation, then these patients could have

decreased intestinal injury and perhaps disease severity. There was also a

significant decrease in CXCL1 chemokine expression in the colon of IL10$^{-/-}$ mice on

the western + PM diet compared to control mice. CXCL1 is a chemoattractant for

neutrophils, therefore reduced expression suggests less neutrophils are being

recruited to the colonic tissue, which is consistent with the histological data.

There was also a lack of IL2, which is a Th1 mediated cytokine needed for T cell

activation and proliferation, being expressed in the colon of these mice.

Therefore this suggests there is an overall lack of inflammation within the colon

of IL10$^{-/-}$ mice on the western + PM diet.

In IL10$^{-/-}$ mice placed on the western + PM diet, there was a decrease in

the expression the Th2 cell mediated cytokines IL4, IL5, and IL13, which are

needed a humoral type immune response. This then suggests that the western +

PM diet not only reduced the pro-inflammatory response, but instead had

overall immunosuppressive effects within the colon.

Although there appears to be immune suppression in the colon of IL10$^{-/-}$

mice on the western + PM diet, in the small intestine, the significant increase in

IFNγ expression and trending increase in IL17, suggests low grade chronic

inflammation within this tissue. These are both pro-inflammatory cytokines that

have been known to be involved in several chronic inflammatory diseases,

including IBD (6-9). Increased expression of IL17 suggests an increase in Th17

cells in the tissue, and IFNγ, which is involved in the maintenance of

inflammation (10,11), suggests there is chronic Th1 inflammation in the small

intestine in response to the western + PM diet in IL10$^{-/-}$ mice. These results were

not observed in the small intestine of WT mice on the western + PM diet, which

suggests, these two factors in combination with each other only have the ability

to exacerbate small intestinal inflammation and possibly worsen disease severity in genetically predisposed individuals.

The cytokine results are similar to what was observed in the IL10$^{-/-}$ mice on the western diet alone. Specifically there was significant colonic reduction in CXCL1, IL12, TNFα, IFNγ, and IL2 expression, with trending decreases in IL1β, IL17, IL4, IL5, and IL13 compared to control mice. This suggests, similar with the western + PM diet mice, an overall reduction in mucosal immune function in the colon of IL10$^{-/-}$ mice on a western diet. The colonic cytokine profile in IL10$^{-/-}$ mice exposed to PM however, differed, in these mice there was no significant decrease in the above cytokines and instead expressed significantly higher IL17 in the colon compared to control mice.   There was also a significant decrease in the expression of IL1β, TNFα, IFNγ, and IL17 in IL10$^{-/-}$ mice on the western + PM diet compared to those on PM alone. This suggests the effects on mucosal immune function appear to be the more of a result of interaction of the western diet with the host then the host with PM.

I observed significant changes in the microbial diversity and composition in IL10$^{-/-}$ mice on the western + PM diet compared to control mice. This is not surprising since intestinal bacteria rely heavily on dietary nutrients for their survival, and the diet between the control mice and the western + PM mice was altered dramatically, thus changing they type of nutrients the bacteria were exposed to. In particular, altering the amount of fat and CHO within a diet will

result in compositional changes in intestinal bacteria (12,13). It is possible, that by increasing the amount of fat and digestible CHO in the diet, this has decreased the amount of food available for the bacteria, which could have resulted in decreased overall bacterial abundance within the colon. Although I did not measure for total bacteria, I did observe a decrease in diversity of bacteria, which could be indicative of decreased abundance as well. Decreased bacteria in the colon could in part be responsible for the lack of inflammation in this tissue within the IL10$^{-/-}$ mice, since it has been shown that presence of bacteria are important for initiating inflammation in this mouse model of colitis (14). In future studies it would be of interest to examine total bacterial numbers and see if they were affected by this change in diet.

Interestingly, there was also a difference in the bacterial composition of western + PM diet mice compared to western diet or PM mice alone in the IL10$^{-/-}$ mice. Any changes in abundance observed in this group of mice, were very dissimilar to changes observed in PM mice at the end of the 35 days. There was a closer resemblance to the bacterial profile observed in the western diet alone mice, however, principle component analysis (PCA) performed on the samples demonstrated that there was changes in the bacterial composition of western + PM mice that differed from the changes observed in control, PM, or western diet mice respectively, as these bacteria easily separated out from the other groups. TRFLP also demonstrated this difference, for example there was an increase in the abundance of *Alphoproteobacteria* in the western + PM mice compared to

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control mice. While in PM and western diet alone mice each demonstrated a decrease in abundance of this class of bacteria compared to control. Therefore it appears as though presence of both environmental factors in combination has a completely different effect on the enteric microflora then they do alone.

Lastly, it's important to mention that WT and IL10$^{-/-}$ mice on the western + PM diet had a significant reduction in butyrate production compared to their respective controls. This is not that surprising, since short chain fatty acid (SCFA) are the fermentation products of undigested CHO, particularly resistant starches and dietary fibre (15), and the western + PM diet has very little fibre and complex CHO compared to the standard mouse chow. SCFA production is dependent on the type and amount of bacteria in the colon, the composition of the diet, and transit time through the GI tract (15). This could then suggest that the decreased production of butyrate and other SCFA's could be the result of the altered microbial composition, deceased bacterial numbers in the cecum and colon, or caused by a lack of fermentable nutrients in the diet. I hypothesize, however, that it is a combination of all of these factors that results in the reduced SCFA production. Interestingly there was a decrease in cecum size observed in the mice on the western + PM diet, which could confirm a decrease in bacteria fermentation in these mice. Because of the various health benefits associated with butyrate production (16,17), the decrease in butyrate concentration in mice on the western + PM diet could suggest, that although

there is no inflammation within the colonic tissue in these mice, the colonic

epithelial cells themselves might be in an unhealthy state.

Again, differences were observed in the SCFA profile between mice on

the western + PM diet and those on western diet or PM alone. This is mostly

likely due to the differences in the bacterial composition associated with each

treatment group, which suggests that the combination of these two

environmental factors has the ability to induce a completely new response in the

mice.

Environmental factors such as diet and airborne pollutants have recently

been suggested to play a role in the development of IBD, however exactly how

they are involved in disease development has yet to be determined. I have

previously been able to demonstrate that PM and western diet, each, on their

own, have the ability to differentially modulate intestinal immune function and

microbial composition. It was believed that the two factors in combination with

each other would result in a combined effect and increased overall intestinal

inflammation. However, when mice were placed on the western + PM diet,

similar as to what was observed with the western diet alone mice, there was

colonic immunosuppression and increased small intestinal inflammation. This

suggests the presence of the western diet has a stronger effect on mucosal

immune function than the particulates. Interestingly, microbial analysis revealed

completely different changes in bacterial composition in mice exposed to the

two factors in combination. This suggests that the interaction between the particles and the high fat diet alters the way the bacteria would respond to each of these factors alone. Future studies will therefore need to be done to examine how this interaction is altering microbial function. These results demonstrate that these two environmental factors have a part in altering intestinal immune function and microbial composition, however what role those changes play in the pathogenesis remains unknown.

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Chapter 6. Conclusions and Discussion

6-1. Overview:

Inflammatory bowel disease (IBD) is a group of chronic gastrointestinal (GI) disorders with increasing incidence worldwide (1-3). Although the exact pathogenesis of IBD remains unknown, there is evidence to suggest that it is the result of a combination of an inappropriate immune response to enteric flora, genetic predisposition and environmental factors (4-7).

Diet is an environmental factor that has been suggested to play a role in IBD pathogenesis. Dietary antigens directly interact with intestinal epithelium and mucosal immune system, alter microbial composition, lead to insulin resistance and impair barrier function (8-11). Therefore, in a genetically predisposed individual, certain diets may act to initiate or exacerbate intestinal inflammation leading to the development of IBD. Increased fat intake and specific fatty acids may contribute to IBD through a direct effect of fatty acids on the intestinal inflammatory response (2). Previous studies have shown an association between high sugar and starch consumption with the development of insulin resistance and chronic inflammation (12,13). This suggests that there is specific dietary factors have the ability to alter immune function which could increase susceptibility to IBD.

Microbes are required for the development, health and function of the gastrointestinal tract including the mucosal immune system. There is significant

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epidemiological and experimental evidence to suggest that because of this close association with the mucosal immune system, enteric bacteria and their products are key environmental factors in intestinal dysregulation and initiation of GI inflammation (14-17). Although the exact role bacteria play in the pathogenesis of IBD is unknown, it is hypothesized that IBD is due to a persistent intestinal infection with an unidentified organism, an alteration in bacterial composition, a defective mucosal barrier and/or a dysfunctional host immune response to bacteria.

Particulate matter (PM) is a component of air pollution that has been implicated in an increasing number of health conditions and, like enteric flora, may be involved in the pathogenesis of IBD. Sources of PM include vehicle exhaust, road dust, forest fires, and industrial emissions (18) and it is composed of a mixture of metals and polycyclic aromatic hydrocarbons. PM has been shown to activate inflammation associated transcription factors NFκB and AP-1 (19,20) and has been associated with the development of gastroenteritis in children, appendicitis, colorectal cancer and Crohn's disease in individuals that are exposed to traffic related pollutants (21-28). However, the exact biological mechanism by which PM mediates intestinal disease is still poorly understood. It is thought that PM may act in a similar way as in the respiratory tract by causing oxidative stress and alter mucosal barrier function thus allowing for the translocation of particles and enteric bacteria from the lumen triggering a cycle of inflammation in a genetically susceptible individual (29).

Based on these principles there are four main goals that comprise this thesis: first, to examine the acute effects of oral exposure to particulate matter (PM10) on mucosal and systemic immune function and intestinal permeability in WT mice, second, to examine the effect of long term exposure of PM on the mucosal immune response and microbial composition in both WT and IL10 deficient mice, third, to examine the effect of a diet rich in fat and refined sugars (western diet) on the mucosal immune system and intestinal microbiota of the IL10 deficient mouse and, finally, to determine the effect of a combination of western diet and PM on the mucosal immune system and intestinal microbial composition in the guts of WT and IL10 deficient mice. I hypothesized that oral exposure to PM and a western style diet will increase intestinal inflammation and severity of disease in WT and IL10 knockout mice.

6-2. Conclusions:

In these studies I was able to demonstrate, at the level of transcription and protein translation, the ability of orally ingested PM to initiate an acute intestinal inflammatory response in WT 129SvEv mice. There was an increase in tissue secretion for various Th1 and Th2 cytokines, up-regulation of adhesion molecules—along with their complementary ligands—on inflammatory cells and recruitment of various leukocytes in the small and large intestines. In addition to this, PM increased gut permeability and altered systemic immune function. These findings provide a mechanism whereby airborne particulate matter may

play a role in triggering or exacerbating gastrointestinal inflammatory disease.

Because the long term effects of PM exposure are not well understood, I next examined the effect of long term (35 day) PM exposure on the mucosal immune response and microbial composition in both WT and the IL10 knockout (IL10$^{-/-}$) mouse model of colitis. I demonstrated that long term PM has the ability to alter the expression of various cytokines associated with Th1 and Th2 helper cells and can modulate the microbial composition in both WT and IL10 deficient mice. Although such modulation was not entirely consistent with the short term exposure of PM on WT mice, I still demonstrate a clear ability of PM to elicit/exacerbate inflammation in the mucosa of WT and genetically predisposed (IL10$^{-/-}$) mice as well as alter the microbial composition in both.

Since the pathogenesis of IBD is strongly associated with diet, I next examined the effect of a western diet on the mucosal immune system and enteric flora in the IL10$^{-/-}$ mouse model of colitis. I found that the western diet significantly altered the intestinal microflora composition and diversity as well as decreased short chain fatty acid production in the cecum. However, contrary to what I hypothesized, this type of diet fed to IL10$^{-/-}$ mice, resulted in an attenuation of colonic inflammation and reduction of pro-inflammatory cytokine expression. However, another unexpected finding was the observation that IL10$^{-/-}$ mice on the western diet appeared to develop inflammation in the upper small intestine. These findings may be the result of studying the combined effect of

multiple macronutrient variables at a single time therefore future studies would need to be done to address the response to changes in individual nutrient components alone. Furthermore, it may be possible that certain elements of this western diet, (such as oleic acid or decreased linoleic acid and fibre) may be able to attenuate inflammation, or the changes in enteric flora in response to such a diet may have been protective effect in the colon, while shifting the disease into the small intestine. Another possibility is that the western diet altered bile salt composition, which together with changes in microflora caused inflammatory responses in the upper small intestine where bile salt concentrations are the highest.

Lastly, I examined the role of the western diet in combination with PM exposure, since both factors are typically associated with westernized nations. Similar to results from the western diet study, IL10$^{-/-}$ mice given a western + PM diet demonstrated an overall reduction in colonic inflammation and disease severity, as determined by the decreased expression of pro-inflammatory cells and histopathological score in the large intestine. These mice also demonstrated significant alterations in microbial composition and function while on the western + PM diet. Interestingly, these were separate changes than those observed in mice on the western diet or PM chow alone, suggesting the combination of these two factors together, alters their characteristics in some way, that they can differentially affect enteric microbes.

Overall I have been able to demonstrate that PM and a western style diet have the ability to alter intestinal immune function and microbial composition, which if occurred in a genetically predisposed individual, could exacerbate underlying conditions, resulting in uncontrolled intestinal inflammation and possibly act as a trigger in IBD.

6-3. Limitations:

6-3-1. IL10$^{-/-}$ mice

Animal models are essential tools used to study the pathogenesis of various human diseases, especially when human testing is limited or believed to be unethical. In this study I used the IL10 gene deficient (IL10$^{-/-}$) mouse as a surrogate for human IBD, specifically for Crohn's disease (CD) as this is an animal model that closely mimics human disease. IL10 is a regulatory cytokine that characteristically downregulates Th1 cytokines, MHC class II molecules, NFκB expression and other cell surface antigens involved in adaptive immunity. Because of this, IL10$^{-/-}$ mice are unable to regulate immune responses, leading to a tendency toward excessive inflammation that is characteristic of CD. Despite being an excellent model, there are limitations to its use as a substitute for human disease. First, the gut microbes that inhabit mice are different from those found in humans. Thus, the changes that I saw in gut microbes in mice may not occur in humans. There are also some discrepancies between the adaptive immune system of mice and humans, and while the lack of IL 10 in mice yields a

mouse form of CD, the pathogenesis of human CD is much more complex and

likely involves the dysregulation of many different factors. Similarly, IL10$^{-/-}$ mice

do not show the exact same symptomatology as CD patients. Therefore the

results obtained in these chapters might not necessarily be transferable CD in

humans.

6-3-2. Particulate matter

Particulate matter is the component of air pollution that has been

implicated in the development of mucosal inflammation in both respiratory and

gastrointestinal mucosa. Despite this implication, it is unclear what the optimal

concentration for PM is in *in-vivo* studies (30). In this study, the mice received

approximately 360ug of PM/day (through consumption in food or direct oral

gavage). It has been reported that ambient PM concentrations within large

American cities averages around 80ug/m^3 (31), however concentrations

continuously peak well above 1000ug/m^3 (33). In addition, particle composition

is highly variable and can change according to climate conditions and may even

vary within the same day (31). With such a wide range in composition, exposure

concentrations, and the fact that it is still unknown as to how much of the

inhaled particulates localize to the GI tract, makes it hard to determine an

optimal concentration for exposure for the mice. However, given that similar

studies have used around 200ug of PM/day, it is possible the concentration used

in this study is an overestimation of the concentrations individuals are exposed

to on a daily basis. This concentration was chosen, however, to make sure the entire GI tract would be exposed to the particle and to determine if that exposure could initiate an intestinal response. Further studies will need to be done to determine if PM induces similar effects on GI function and microbial composition at lower exposure levels or by intermittent exposure.

6-4. Future directions

There are many questions that remain unresolved regarding the pathogenesis of IBD. The protective anti-inflammatory effect of a western diet observed in the colons of $IL10^{-/-}$ mice was an unexpected result. This could possibly be due to the fact that in our western diet there were anti-inflammatory elements, such as linoleic acid, that could have contributed to such a result. Future studies should address the ability of individual macromolecules (protein, fat and sugar) and other western diet components in causing an anti- or pro-inflammatory response in $IL10^{-/-}$ mice. Furthermore, the combination of western diet with particulate matter still resulted in a decrease in inflammation in $IL10^{-/-}$ mice—another unexpected result. It may be that the anti-inflammatory components of the diet "overpower" the pro-inflammatory effects of PM. Because of these unexpected results, it will be interesting to see how changes in individual dietary components will affect the intestinal immune function. Future studies will also need to be done to examine the long term effects of the western

diet to determine if long term exposure really does prevent the onset of disease

in the IL10$^{-/-}$ mice.

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Appendix 1

Section 1-A: PM$_{10}$ (EHC-93) composition

Table A-1: PAH, Ion, and Metal composition of EHC-93

PAH composition	µg of PAH/g particle
Acenaphtene	0.2
Anthracene	0.54
Benzo[a]anthracene	1.1
Benzo[b]fluoranthene	2.78
Benzo[ghi]perylene	1.52
Benzo[a]pyrene	0.95
Benzo[e]pyrene	1.09
Chrysene	1.66
Indeno[1,2,3cd)pyrene	1.19
Fluoranthere	2.47
Phenanthrene	1.83
Perylene	0.28
Pyrene	2.11

Ion composition	µg of ions/g particle
Sulfate ion	45 x 103

Metal composition	µg of metals/g particle (% solubility in water)
Aluminum	10 x 103 (2%)
Chromium	42 (3%)
Copper	845 (17%)
Iron	15 x 103 (1%)
Lead	7 x 103 (4%)
Magnesium	7 x 103 (14%)
Nickel	67 (7%)
Vanadium	90 (0%)
Zinc	10 x 103 (46%)

Table adapted from Vincent (1) and Vincent (2).Data is expressed in ug/g of particulate material.

Section 1-B: Mouse Th1/Th2 immune Panel- Taqman Low density Array

Information from Gene Cards - http://www-bimas.cit.nih.gov/cgi-bin/cards/

Cytokines

IL1α – Cytokine. Member of the interleukin 1 cytokine family. Involved in various immune responses, inflammatory processes and hematopoiesis. Produced by monocytes and macrophages. Stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity. Involved in the inflammatory response and is an endogenous pyrogen. Can stimulate the release of prostaglandin and collagenase.

IL1β - Cytokine. Produced by activated macrophages. Stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity. Involved in the inflammatory response and is an endogenous pyrogen. Can stimulate the release of prostaglandin and collagenase.

IL2 – Cytokine. Important for the proliferation of T and B lymphocytes. Produced by T-cells in response to antigenic or mitogenic stimulation. Required for T-cell proliferation and other activities crucial to regulation of the immune response. Stimulates B-cells, monocytes, lymphokine-activated killer cells, natural killer cells, and glioma cells.

IL4 – Cytokine. Pleiotropic cytokine produced by activated T cells. B-cell and activator (as well as other cell types. Co-stimulator of DNA-synthesis and induces the expression of class II MHC molecules on resting B-cells. Enhances both secretion and cell surface expression of IgE and IgG1. Regulates the expression of the low affinity Fc receptor for IgE (CD23) on both lymphocytes and monocytes.

IL5 – Cytokine. Acts as a growth and differentiation factor for both B cells and eosinophils. Main regulator of eosinopoiesis, eosinophil maturation and activation. Elevated production of this cytokine is reported to be related to asthma or hypereosinophilic syndromes. Induces terminal differentiation of late-developing B-cells to immunoglobulin secreting cells.

IL6 – Cytokine. Involved in the final differentiation of B-cells into Ig-secreting cells. Induces myeloma and plasmacytoma growth and nerve cells differentiation. Is an acute phase reactant and induces other acute phase reactants.

IL10 – Cytokine. Produced primarily by monocytes and by lymphocytes. Down-regulates the expression of Th1 cytokines, MHC class II Ags, and costimulatory molecules on macrophages. Enhances B cell survival, proliferation, and antibody production. Can block NF-kappa B activity. Regulates JAK-STAT signaling pathway.

IL12α – Cytokine. Acts as a growth factor for activated T and NK cells. enhance the lytic activity of NK/lymphokine-activated Killer cells, and stimulate the production of IFN-gamma by resting PBMC.

IL12β – Cytokine. Associates with IL23A to form IL-23. Functions in innate and adaptive immunity. IL-23 may constitute with IL-17 an acute response to infection in peripheral tissues. Activates the Jak-Stat signalling cascade. Stimulates memory rather than naïve T-cells and promotes production of proinflammatory cytokines. Induces autoimmune inflammation.

IL13 – Cytokine. Produced primarily by activated Th2 cells. Involved in several stages of B-cell maturation and differentiation. Up-regulates CD23 and MHC class II expression, and promotes IgE isotype switching of B cells. Down-regulates macrophage activity, thereby inhibits the production of pro-inflammatory cytokines and chemokines.

IL15 – Cytokine. Regulates T and natural killer cell activation and proliferation. This cytokine and interleukin 2 share many biological activities. The number of CD8+ memory cells is shown to be controlled by a balance between this cytokine and IL2. Induces the activation of JAK kinases, as well as the phosphorylation and activation of transcription activators STAT3, STAT5, and STAT6.

IL17 – Cytokine. Pro-inflammatory cytokine produced by activated T cells. Regulates the activities of NF-kappaB and mitogen-activated protein kinases. Stimulates the expression of IL6 and cyclooxygenase-2 (PTGS2/COX-2). Enhances the production of nitric oxide (NO).

IFNγ – Cytokine. Member of the type II interferon family. Soluble cytokine with antiviral immunoregulatory and anti-tumor properties. Potent activator of macrophages.

TNFα – Cytokine. Secreted by macrophages and can induce cell death of certain tumor cell lines. Potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion. Implicated in induction of cachexia, under certain conditions it can stimulate cell proliferation and induce cell differentiation.

Chemokine

CXCL10 – Chemokine. Stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression. Chemotactic for monocytes and T-lymphocytes.

CXCL11 – Chemokine. Chemotactic for interleukin-activated T-cells but not unstimulated T-cells, neutrophils or monocytes. Induces calcium release in activated T-cells. May play an important role in CNS diseases which involve T-cell recruitment. May play a role in skin immune responses.

CCL2 – Chemokine. Chemotactic factor that attracts monocytes and basophils but not neutrophils or eosinophils. Augments monocyte anti-tumor activity. Implicated in the pathogenesis of diseases characterized by monocytic infiltrates, like psoriasis, rheumatoid arthritis or atherosclerosis.

CCL3 – Chemokine. Involved in the acute inflammatory state in the recruitment and activation of polymorphonuclear leukocytes. Inflammatory and chemokinetic properties.

CCL5 – Chemokine. Secreted proteins involved in immunoregulatory and inflammatory processes. Chemoattractant for blood monocytes, memory T-helper cells and eosinophils. Causes the release of histamine from basophils and activates eosinophils.

CCL19 – Chemokine. May play a role in inflammatory and immunological responses and normal lymphocyte recirculation and homing. May be important in trafficking of T-cells in thymus, and T-cell and B-cell migration to secondary lymphoid organs.

Chemokine receptor

CCR7 – Chemokine receptor. Receptor for the MIP-3-beta chemokine. Member of the G protein-coupled receptor family. Expressed in various lymphoid tissues and activates B and T lymphocytes. Controls the migration of memory T cells to inflamed tissues, as well as stimulates dendritic cell maturation.

CXCR3 – Chemokine receptor.   Receptor for CXCL9, CXCL10 and CXCL11 and mediates the proliferation of human mesangial cells (HMC). Isoform 2 is a receptor for CXCL4 and also mediates the inhibitory activities of CXCL9, CXCL10 and CXCL11 on the growth of human microvascular endothelial cells (HMVEC). Isoform 2 may play a role in angiogenesis. Isoform 3 mediates activity of CXCL11.

Cell adhesion molecules

Sele – Adhesion molecule. Cell-surface glycoprotein having a role in immunoadhesion. Mediates in the adhesion of blood neutrophils in cytokine-activated endothelium through interaction with PSGL1/SELPLG. May have a role in capillary morphogenesis. Is thought to be responsible for the accumulation of blood leukocytes at sites of inflammation by mediating the adhesion of cells to the vascular lining.

Fn1 – Adhesion molecule. Fibronectins bind cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin. Fibronectins are involved in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape. Interaction with TNR mediates inhibition of cell adhesion and neurite outgrowth

Vcam1 – Adhesion molecule.   Important in cell-cell recognition. Appears to function in leukocyte-endothelial cell adhesion. Interacts with the beta-1 integrin VLA4 on leukocytes, and mediates both adhesion and signal transduction. The VCAM1/VLA4 interaction may play a pathophysiologic role both in immune responses and in leukocyte emigration to sites of inflammation.

Co-stimulatory molecules

CD40 - Essential in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation.

CD40lg - Expressed on the surface of T cells. It regulates B cell function by engaging CD40 on the B cell surface. A defect in this gene results in an inability to undergo immunoglobulin class switch and is associated with hyper-IgM syndrome.

CD80 - B-lymphocyte activation antigen B7-1 (formerly referred to as B7) provides regulatory signals for T lymphocytes as a consequence of binding to the CD28.

CD86 - Receptor involved in the costimulatory signal essential for T-lymphocyte proliferation and interleukin-2 production, by binding CD28 or CTLA-4. May play a critical role in the early events of T-cell activation and costimulation of naive T-cells, such as deciding between immunity and anergy that is made by T-cells within 24 hours after activation.

Icos - Enhances all basic T-cell responses to a foreign antigen, namely proliferation, secretion of lymphokines, up-regulation of molecules that mediate cell-cell interaction, and effective help for antibody secretion by B-cells. Essential both for efficient interaction between T and B-cells and for normal antibody responses to T-cell dependent antigens.

Cell markers

CD3 - The protein encoded by this gene is part of the T-cell receptor/CD3 complex (TCR/CD3 complex) and is involved in T-cell development and signal transduction.

CD4 - Accessory protein for MHC class-II antigen/T-cell receptor interaction. May regulate T-cell activation. Induces the aggregation of lipid rafts.

CD8 - Found on most cytotoxic T lymphocytes that mediates efficient cell-cell interactions within the immune system. The CD8 antigen acts as a co-repressor with the T-cell receptor on the T lymphocyte to recognize antigens displayed by an antigen presenting cell (APC) in the context of class I MHC molecules.

CD19 - Assembles with the antigen receptor of B lymphocytes in order to decrease the threshold for antigen receptor-dependent stimulation

Antigen presentation

B2M - Beta-2-microglobulin is a serum protein found in association with the major histocompatibility complex (MHC) class I heavy chain on the surface of nearly all nucleated cells.

References

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