Effects of dietary sheep, cow and goat milk solids on colitis in the interleukin-10 gene deficient mouse model of Inflammatory Bowel Disease.

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Abstract

Inflammatory Bowel Disease (IBD) is a group of chronic, immunologically-mediated gastrointestinal disorders resulting from interactions between environmental influences, host genetic susceptibility, and the intestinal microbiota. Dietary factors can ameliorate symptoms, providing a rationale for using targeted nutrition to alleviate symptoms. Food components, including milk-derived oligosaccharides and conjugated linoleic acid, have shown anti-inflammatory effects in IBD patients or animal models of IBD. Additionally, some ruminant milks are perceived by some IBD patients to have more beneficial effects on their symptoms (goat, sheep) than others (cow). Soy-based milk substitutes are perceived to be more beneficial than milk. No reports describe the effects of milk solids from different species on molecular pathways in the intestine that might explain differential effects in IBD. This thesis aimed to investigate the effects of dietary intervention with milk solids on the severity of colitis (histology) and molecular pathways (microarrays and qPCR) in the interleukin-10 gene deficient (*Il10*$^{-/-}$) mouse model of IBD.

First, laser microdissection (LMD) combined with microarrays was used to analyse colon epithelium gene expression in 6 and 12 week old *Il10*$^{-/-}$ mice fed a control diet. This indicated that intact colon was an appropriate tissue in which to study global changes in gene expression when colitis is established. It also showed that studying colon epithelium during the early stages of inflammation (6 weeks old) may identify molecular changes not seen in intact colon. Secondly, analysis of DNA methylation changes (both globally, and in specific inflammation-associated genes (*Ppara*, *Stat1* and *Tap2*)) in *Il10*$^{-/-}$ mouse colon showed that changes in total DNA methylation were correlated with changes in global gene expression, and changes in *Stat1* methylation during inflammation correlated with *Stat1* gene expression. However, these techniques had limitations for obtaining a global overview of molecular changes (DNA methylation) in response to dietary intervention in established inflammation (LMD) and therefore were not applied in the dietary intervention study. Finally, diets containing goat and cow whole milk solids (40% *w/w*) fed for 6 weeks had anti-inflammatory effects in the colon of 11-12 week old *Il10*$^{-/-}$ mice, shown by a reduction in colitis severity and immune-related gene expression. Further research is required to elucidate the physiological and molecular mechanisms of these anti-inflammatory effects.

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May it be when darkness falls your heart will be true.
You walk a lonely road. Oh, how far you are from home!
May it be the shadow's call will fly away.
May it be your journey on to light the day.
When the night is overcome, you may rise to find the sun.

Selected lyrics from the song "May it be" by Enya.
Featured in the film "The Fellowship of the Ring".

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ANOVA	analysis of variance
BCM7	β-casomorphin-7
C57BL/6J, C3H/HeJBir, BALBc, 129 SvEv, 129 Ola	various strains of inbred mice
CD	Crohn's disease
CIF	complex intestinal flora
CLA	conjugated linoleic acid
DSS	dextran sodium sulphate
EASE	expression analysis systematic explorer
EF	*E. faecalis/faecium* culture
EF.CIF	a 1:1 mixture of EF and CIF
FC	fold change
FDR	false discovery rate
GHS	general health score
GO	gene ontology
GSEA	gene set enrichment analysis
HIS	histological injury score
HPLC	high performance liquid chromatography
IBD	Inflammatory bowel disease
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IL	interleukin
IL10	interleukin-10
Il10$^{-/-}$	interleukin-10 gene deficient (mouse)
IPA	Ingenuity pathway analysis
Limma	linear models for microarray analysis
LMD	laser microdissection
LSD	least significant difference
MALDI-TOF	matrix-assisted laser desorption ionisation-time of flight (mass spectrometry)
MCT	medium chain triglycerides
MDR1	multi-drug-resistant gene/protein
Mdr1a$^{-/-}$	multi-drug-resistant gene deficient (mouse)
n-3/n-6	omega-3/6 (fatty acid)
NOD	nucleotide oligomerisation domain
PUFA	polyunsaturated fatty acids
qPCR	quantitative real-time polymerase chain reaction
REML	residual maximum likelihood
RIN	RNA integrity number
SPF	specific pathogen free
T_H	T-helper (cell)
TNBS	trinitrobenzenesulfonic acid
UC	ulcerative colitis

1 General introduction

Part of this chapter was published as a mini-review in Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis.

Russ, A., Barnett, M.P.G., McNabb, W.C., Anderson, R.C., Reynolds, G.W., & Roy, N.C. (2010). Post-weaning effects of milk and milk components on the intestinal mucosa in inflammation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *690*(1-2), 64-70.

1.1 Introduction

For most of human history, people have adapted their diets to available resources with one simple aim: survival. Over the last 10,000 years, advancements in agriculture have made it possible to produce a large quantity and variety of food [1]. People began to anticipate living longer than previous generations, making it important to consider long-term health when making dietary and other lifestyle choices. Many people now want to optimise their nutrition and long-term health, using modern knowledge of nutrition, physiology and genetics [2].

Traditional nutrition research has focussed on identifying and obtaining the dietary requirements that are necessary to prevent disease in the general population. A growing realisation that individual genetic differences could alter nutrient metabolism led to the development of nutrigenetic and nutrigenomic research. Nutrigenetics and nutrigenomics both involve studying the interaction between the diet and the genome and its effect on health: both areas of research are required to meet the long-term goal of improving health and prevention of disease, however, the approach and immediate goal of nutrigenetics and nutrigenomics research is different [3]. Nutrigenetics is the study of how an individual's specific genetic makeup affects their response to diet. It aims to identify and characterise gene variants associated with different responses to nutrients, and in many instances, relate this variation to disease states [3]. The field of nutrigenomics is an integrative science which seeks a genetic and molecular understanding of how nutrients affect the balance between health and disease by altering the expression of an individual's genetic makeup [2]. Compared to nutrigenetics, nutrigenomics is a broader study of how dietary components interact with the genome, transcriptome, proteome and metabolome, and how the resulting changes in physiological state relate to differences in the cellular responses of the biological system [3].

Personalised or targeted nutrition has the potential to prevent or mitigate chronic disease [2]. Chronic disease often involves inflammation. Cardiovascular disease, which is extensively studied in terms of nutrigenomic research [4], and inflammatory diseases of the intestine [5] all involve underlying inflammatory processes. These diseases are influenced and mediated by interactions between the environment (including diet) and variations in an individual's genetic sequence (Table 1.1).

The intestinal tract has a major role in health. It is where food and drink are digested and nutrients absorbed to support the body's growth, maintenance and repair mechanisms. The intestine also processes a large number of environmental micro-organisms, which are carried into the gastrointestinal tract on ingested substances, to prevent them causing intestinal or systemic infection. Any process, such as inflammation, that can impair the ability of the intestine to carry out these crucial activities can have a detrimental effect on overall health. These important activities (among others) are compromised in Inflammatory bowel disease (IBD).

The application of nutrigenomic science to IBD is a valuable and important step towards unravelling the environmental and genetic factors involved in IBD pathogenesis, and consequently enable application of personalised or targeted nutrition alongside other therapeutic approaches to maximise the health of people with IBD [6]. Discoveries made through the application of nutrigenomics to IBD research may also benefit people with other forms of intestinal dysfunction.

1.2 Inflammatory bowel disease

IBD is a general term for a group of diseases in humans that primarily affect the intestine and are chronic, relapsing, and immunologically-mediated with an as-yet unknown cause. The two main diseases of this type are Crohn's disease (CD) and ulcerative colitis (UC) [7]. IBD can have anything from a relatively minor to a severe impact on quality of life, with alternating periods of symptom existence (called the active phase) and absence (called the quiescent phase, or remission), and is increasing in prevalence worldwide [8]. Both CD and UC involve inflammation of the intestine, but they differ in the regions of intestine affected, the depth to which the inflammation extends across the intestinal wall, and the pattern of the lesions. In UC, inflammation occurs primarily in the colon, typically affects the more superficial layers (mucosa) only, and the lesions are continuous. In contrast, CD inflammation can occur throughout the intestine (including duodenum, jejunum, ileum, caecum and colon, but most commonly the terminal ileum), tends to be transmural (involving all layers of the intestinal wall) and is discontinuous [9].

Table 1.1 Diseases caused by interactions between genetics and diet and other environmental triggers. Some of the environmental and dietary factors thought to be involved in the pathogenesis of each disease are listed. + and – refer to positive or negative increase in risk associated with a particular factor.

Disease	Environmental factors	Effects of diet
Cardiovascular disease	Gestational environment [10]	Coffee intake (+/- depending on genetic makeup) [12]
	Psychosocial factors in the work environment [11]	
Inflammatory bowel disease	Cigarette smoking (+/- depending on whether Crohn's disease or ulcerative colitis) [13]	n-3 fatty acid intake (+) [15]
	Hygiene (high levels thought to increase risk) [14]	
Type II diabetes	Low level of physical activity (-) [16]	High fat intake (-) [17]
		Coffee intake (-) [18]
Bowel cancer	Low level of occupational physical activity (-) [19]	Low fibre intake (-) [19]
		High processed meat consumption and low fruit and vegetable intake (-) [20]

Many cases of IBD cannot be classified as either CD or UC due to the considerable overlap of both symptoms and pathology, in which case the diagnosis is "indeterminate IBD".

The aetiology of IBD is complex and not fully understood. It is thought to be caused by multiple environmental, genetic and microbial factors including: (1) the presence of bacterial antigens and adjuvants (substances that enhance immune responses to antigens without having antigenic effects themselves) within the intestine; (2) a host defect in immune regulation that allows immune responses against these antigens to occur where they should not; and (3) defective function of the mucosal barrier that allows (1) and (2) to interact [21].

1.2.1 Mucosal responses in IBD

The intestinal barrier, separating the luminal environment of the intestine from the rest of the body, is composed of four parts (Figure 1.1): a physical barrier, made up of the epithelial layer of cells; a chemical barrier of secreted mucus on the luminal surface of the epithelium; a microbial barrier, consisting of commensal bacteria, in the intestinal lumen and outer mucus layer; and the immunological barrier, consisting of immune cells present in the lamina propria. For a review, see [22].

1.2.1.1 Intestinal microbiota

A large number of micro-organisms, approximately 10 times as many cells as there are in the human body, are present in the intestine, particularly in the large intestine. While anatomically not part of the mucosa, at a functional level the intestinal microbiota plays an integral part in the mucosal response in IBD. Many bacterial species perform important roles in fermentation of dietary components that are unable to be digested and absorbed by the small intestine. These are in a symbiotic relationship with the human host, and are called the commensal microbiota. On the other hand, some species are detrimental to the host, such as pathogenic strains of *Escherischia coli* (which may be ingested in contaminated food and cause food poisoning). The mucosal immune system needs to be able to tolerate the commensal microbiota while defending against the pathogenic organisms.

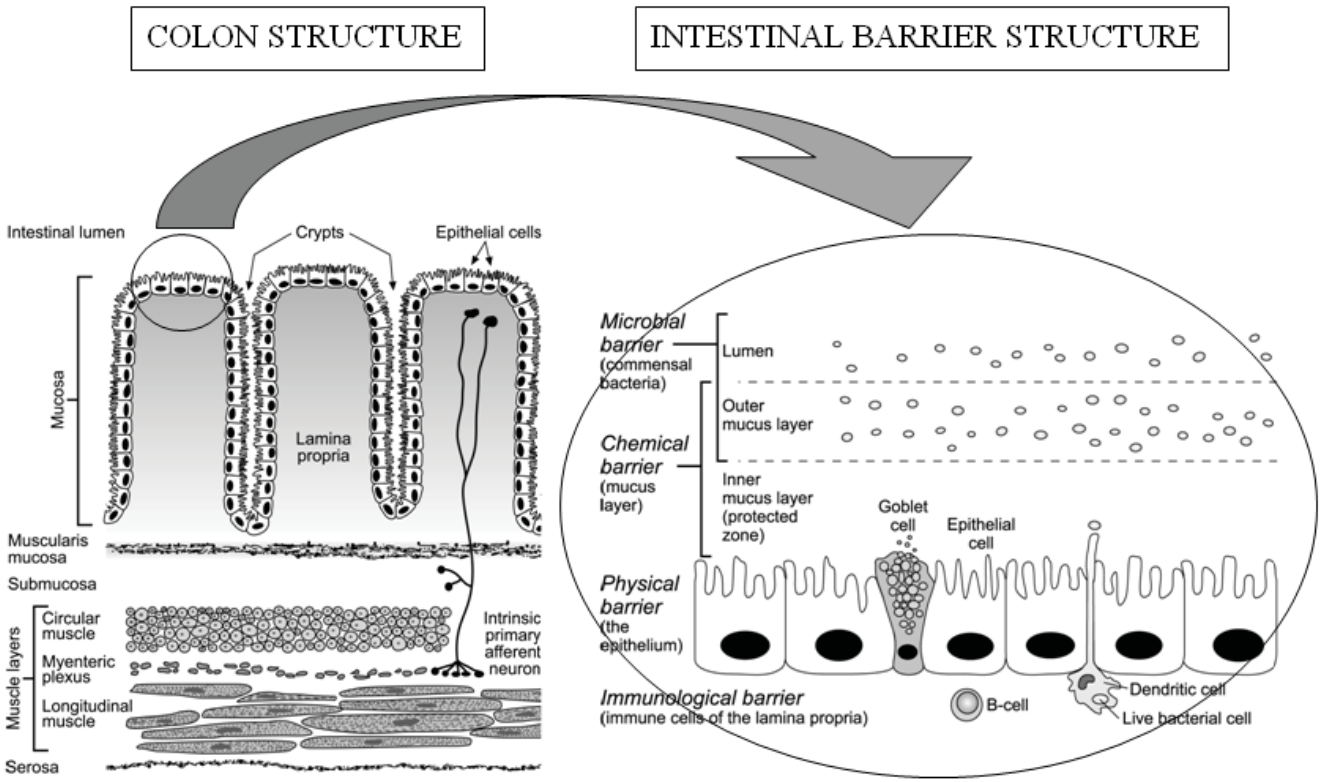


Figure 1.1 Structure of the colon and intestinal barrier. Figure adapted from Anderson et al. (2012) [22].

The intestinal microbiota has an important role in the pathogenesis of IBD, and may interact with dietary components to change the function of the epithelium and therefore affect the inflammatory process in the intestine. In IBD, there is an excessive inflammatory response to the intestinal microbiota [23, 24]. Samples from CD patients show an imbalance in ileal and colonic dendritic cell distribution and phenotype that may contribute to a lack of tolerance toward intestinal microbiota [25, 26]. There is some evidence that ileal dendritic cell imbalance and epithelial barrier dysfunction occur together in the early stages of ileitis in rats (a model of small intestinal inflammation) [26]. Bacteria known to be protective in IBD include strains of *Bifidobacteria*, *Lactobacillus*, *Bacteroides* and *Faecalibacterium* [27]. Bacteria known to have pro-inflammatory actions include some *Enterococcus* and *Helicobacter* strains [21].

An example of the interaction between the intestinal microbiota and the immunoregulatory role of intestinal epithelial cells [27] is the induction of regenerating islet-derived 3γ (REG3γ) gene and protein expression in Paneth cells by the Gram-negative commensal *Bacteroides thetaiotaomicron*, a bacterial species which is protective in IBD. REG3γ is an antimicrobial peptide produced by Paneth cells (a specialised type of intestinal epithelial cell) which is specific for certain Gram-positive bacteria. The REG3γ gene and protein often show increased expression levels in IBD. Many such interactions may occur in IBD.

1.2.1.2 Intestinal epithelium

The course of IBD includes active phases, where there are symptoms present, and quiescent phases where the patients are relatively free of symptoms. Active and quiescent phases also occur within the intestinal tissue, especially in CD, where some areas of tissue are affected while neighbouring areas are not (visibly) inflamed. Both transcellular (across) and paracellular (between) permeability are increased during active inflammation, while only paracellular permeability is enhanced during quiescent phases [28]. An increase in paracellular permeability in the intestinal epithelium can lead to chronic inflammation, whereas an increase in transcellular permeability coincides with bacterial translocation in active phases of intestinal inflammation [28].

The intestinal epithelium consists of a monolayer of columnar cells (epithelial cells) (Figure 1.2) held together by intercellular junctional complexes. Tight junctions are one of three types of junctional complexes that link one epithelial cell to another. They also regulate the passage of molecules between the cells via the paracellular pathway. The intestinal epithelium must provide an effective barrier to harmful macromolecules and microorganisms while allowing the mucosal immune system to sample luminal antigens [21].

Intestinal epithelial cells are the first line of defence against enteric antigens. Bacteria and lymphoepithelial interactions help to promote mucosal homeostasis [29]. Epithelial cells in the mammalian small intestine include: absorptive enterocytes (>80%), secretory goblet (16%), enteroendocrine (1%), Paneth (<3%) and stem cells at the crypt base [30], and immune cells which have migrated to the epithelium, such as intraepithelial lymphocytes. The colon differs from the small intestine (duodenum, jejunum and ileum) in that it has no Paneth cells and no villi [31].

Imbalances in mucosal immunity and perturbed epithelial barrier function seem to be critical for the manifestation of IBD. Continued and incorrect activation of the mucosal immune system occurs in IBD [32]. Factors which are responsible for this excessive activation are thought to include: altered tight junction structure and epithelial permeability, altered mucus composition [33] and pronounced infiltration of luminal microbiota resulting from loss of barrier integrity [32, 34].

An increase in intestinal permeability is a consistent finding in CD [35]. In CD patients, mechanisms for increased intestinal permeability include altered tight junction protein expression and increased epithelial apoptosis [21, 36]. Whether an early barrier defect in CD is exclusively determined by a primary alteration of the intestinal epithelium, or whether it occurs secondary to subclinical foci of inflammation that induce an increase in tight junction permeability via the secretion of mediators is unknown [37].

Epithelial cells play an important role in the initiation of inflammation and the tissue's response to bacteria, as well as controlling barrier permeability [38, 39]. Changes in the colonic epithelium, such as increased paracellular permeability and mucosal immune defence malfunction, are thought to be important in the initiation of inflammation [40, 41]. Changes in tissue pathology in the colon may be preceded by detectable changes in

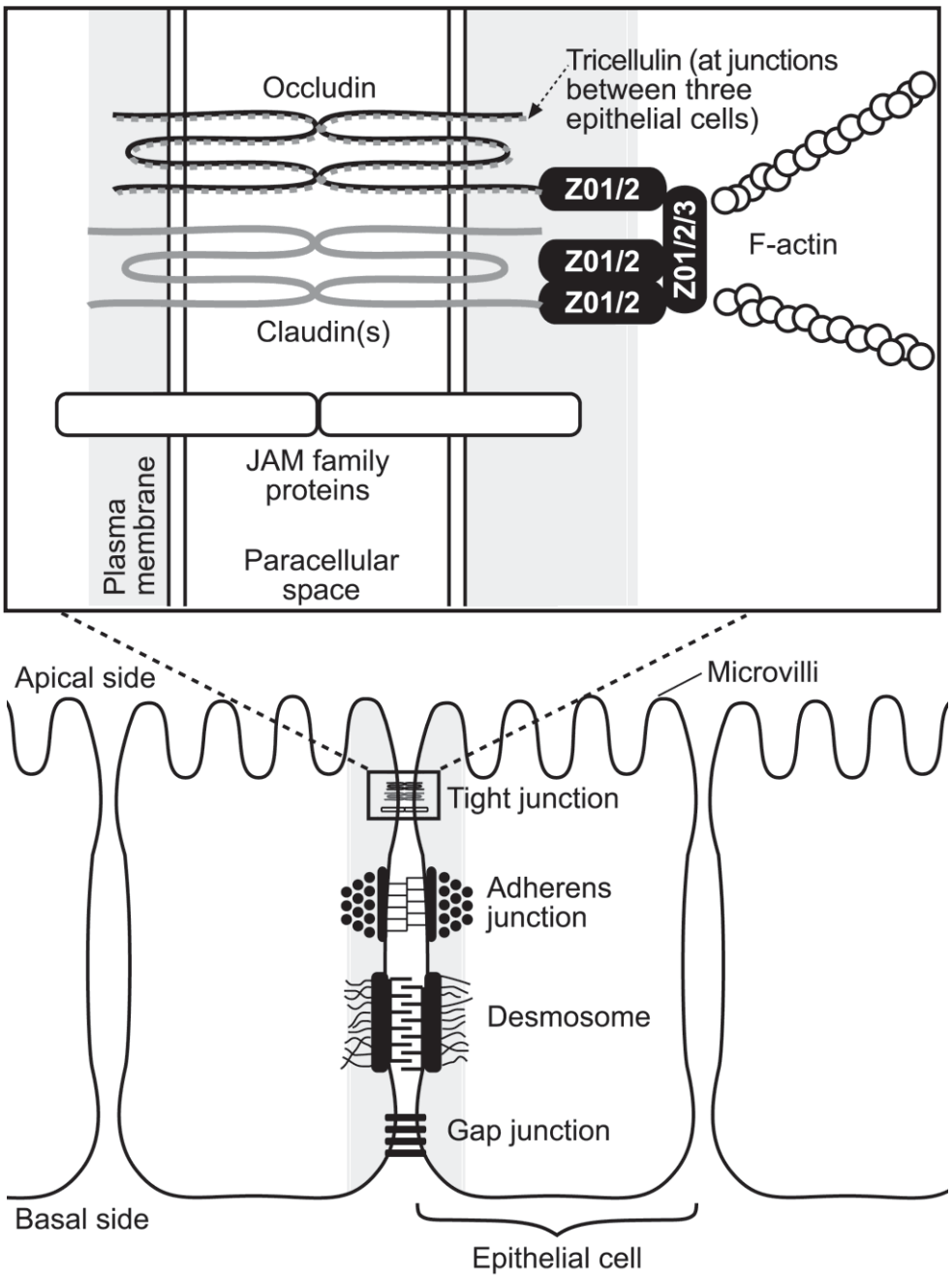


Figure 1.2 Intestinal intercellular junctions. Epithelial cells of the intestine are linked by intercellular junctions consisting of four components: tight junctions (that seal the paracellular space and control barrier function), adherens junctions (that are involved in cell-cell adhesion and cell signaling), desmosomes (that are involved in cell-cell-adhesion) and gap junctions (that connect the cytoplasm of two cells allowing for cellular communication). Tight junctions are formed from a branching network of strands, of which the major proteins are occludin and claudins [42]. Figure adapted from Anderson et al. (2012) [22].

gene expression of some immune-related signalling molecules [38, 39, 43, 44].

The intestinal epithelium is covered by a layer of mucus produced by the goblet cells located in the epithelial crypts in the colon and both villi and crypts in the small intestine (Figure 1.1). Goblet cell numbers increase distally along the intestine, with the highest numbers occurring in the colon and rectum [45]. Mucus is an important part of innate (non-antigen-specific) immune defence against pathogens and is also necessary for protection of the intestinal epithelium against physical damage [33]. Defects in the epithelial barrier, such as a lack of continuity in the epithelium or loss of mucus thickness, can result in an inflammatory response in the epithelium and underlying tissue, as foreign antigens can then enter the tissue from the lumen and provoke an immune response [33].

Disrupted barrier function is an important factor in the initiation of the inflammatory process. For example, mice with the nuclear factor-κB (NFκB) essential modulator gene (NEMO) knocked out in small intestinal and colonic epithelial cells ($NEMO^{IEC\text{-}KO}$) have a defect in NFκB signalling and subsequent impairment of the integrity and antimicrobial defence of the intestinal epithelium, which gives rise to chronic intestinal inflammation [40]. Disruption of epithelial integrity in colonic crypts occurs in these mice, followed by translocation of luminal bacteria into the lamina propria and activation of innate immune cells (e.g. dendritic cells and neutrophils) from 1-2 weeks after birth [40]. Inflammation progresses to the entire colon and involves T lymphocytes, and in the advanced stages, lymphoid follicles are present with surrounding dendritic cells. The lymphoid follicles and dendritic cells are co-localised with bacteria, suggesting that bacteria are still driving the immune response at this stage [40].

Increased interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα) production by T cells and macrophages occurs in IBD and these cytokines disrupt intestinal epithelial barrier integrity [46-48]. Increased levels of TNFα lead to increased production and activation of other pro-inflammatory cytokines and factors that promote intestinal inflammation [49]. Anti-TNFα antibody is a proven effective treatment for active CD [50-54].

Many pro-inflammatory actions of TNFα are mediated by the NFκB pathway [55]. In an *in vitro* human colon carcinoma cell line (Caco-2), a model of the small intestinal epithelium in terms of barrier properties and molecule uptake [56], TNFα induces an increase in paracellular permeability that is associated with NFκB-dependent down-regulation of zonula occludens (ZO)-1 protein expression and alterations in its junctional localisation [57]. Interleukin (IL)-1β induces an increase in tight junction permeability across Caco-2 cell monolayers, mediated by NFκB-dependent increases in myosin light chain kinase (MLCK) gene transcription and subsequent protein activity [55].

Decreased expression levels of enterocytic junctional proteins and their respective mRNAs is observed in actively inflamed tissue of IBD patients [36]. In tissues with inactive inflammation, only a few junctional molecules are affected (adherens junctional molecules E-cadherin and α-catenin), and expression of desmosomal and tight-junction associated proteins are relatively unchanged. In non-inflamed IBD tissues, junctional protein expression is similar to that seen in tissue from healthy controls [36]. Junctional molecule expression appears to be associated with the inflammatory process but it may not be a primary cause because tight junctions, not adherens junctions, control epithelial permeability. Expression of adherens junctions is more affected by active inflammation than expression of tight junctions and desmosomes [36].

Distribution of conductivity is consistent along colon biopsies from active CD tissue, indicating that focal epithelial lesions do not contribute to barrier dysfunction [58]. There are, however, reduced numbers of tight junction strands resulting in an increase in paracellular permeability [58]. The expression levels of occludin and the sealing tight junction proteins claudins 5 and 8 are decreased and redistributed away from the tight junction, while the abundance of the pore-forming tight junction protein claudin 2 is increased in IBD, leading to altered tight junction structure and barrier dysfunction [58]. Increased expression levels of claudin proteins 1 and 2 are positively correlated with inflammatory activity in IBD tissues [59]. TNFα released from lamina propria lymphocytes may contribute to the increased expression of claudin 2 protein in CD [58].

1.2.1.3 Intestinal immune system

Antigens are processed by various cell types in the intestinal epithelium [29]. Antigen enters through the M-cells (microfold or membranous cells) located in Peyer's Patches (found in the ileum) and is subsequently transferred to underlying dendritic cells, sampled by dendritic cells reaching into the lumen between enterocytes, or taken up by the enterocytes and processed further before being presented to the immune system (T and B cells and macrophages) [29]. Antigenic stimulation causes naive CD4+ T cells to differentiate into two subsets, T-helper-1 (T_H1) and T-helper-2 (T_H2) cells, which are characterised by different secreted cytokine profiles and different functions. A summary of the function of T cell subsets is presented in Table 1.2. T_H1 cells produce large quantities of IFNγ and mediate cellular immunity, whereas T_H2 cells are involved in humoral immunity (which involves the response of circulating antibody to antigen, as opposed to the response of cells such as macrophages and natural killer cells) [60].

Immune dysfunction in IBD can be broadly categorised into innate immune response, autophagy, and regulation of the interleukin-23 (IL23) pathway, as shown in Figure 1.3 [61]. In CD, there is increased production of T_H1-type cytokines in the colonic mucosa, which is related to increased activation of mucosal dendritic cells and macrophages. Membrane-associated Toll-like receptor (TLR) and intracellular (nucleotide oligomerisation domain; NOD) receptors are key mediators of innate host defence and have a pivotal role in the activation of antigen presenting cells such as macrophages and dendritic cells [24]. Autophagy is a process by which cells destroy their own components, for reasons including protection against infection through the destruction of intracellular bacteria [61].

IL23 [24, 62] appears to drive chronic inflammation by activating a subset of T cells characterised by the production of IL17, a pro-inflammatory cytokine that stimulates fibroblasts, endothelial cells, macrophages and epithelial cells to produce multiple inflammatory mediators including IL1, IL6, TNFα, nitric oxide synthase (NOS)-2, metalloproteases and chemokines [24, 60, 62]. IFNγ (produced by T_H1 cells) acts to suppress the differentiation of T_H17 cells, suggesting that T_H1 cells may play an immunoregulatory, rather than a pathogenic, role in the development of chronic inflammation [60].

Table 1.2 Functions of known T cell subsets. Types of T cell are grouped according to which part of the immune response they contribute. Adapted from: Dong, C., & Martinez, G. J. (2010). T cells: the usual subsets.

Type of T cell	Function
Innate immune response	
NKT cell	Both pro- and anti-inflammatory functions. Modulate immune responses in cancer, autoimmune disease, allergy, infection etc.
Cytotoxic T cell	Kill infected and transformed cells by secretion of perforin and granzymes which cause apoptosis. Generated in response to chronic antigen-mediated T cell receptor stimulation.
Innate and/or adaptive immune response	
γδ T cell	Have both pro- and anti-inflammatory functions and characteristics of both innate and adaptive immunity.
Inactive T cells	
Exhausted T cell	Express inhibitory receptors and lack effector cytokine production.
Anergic T cell	Functionally inactive – generation may be important for avoiding autoimmuine responses. Generated following T cell receptor activation in the absence of co-stimulatory signals.

Adaptive immune response: naive	
CD4+ αβ cell	Scan lymph nodes for the cognate antigen of MHC class II molecule complexes. Differentiate into effector or regulatory T cells when activated by APCs. Naïve T cells give rise to memory T cells when activated.
CD8+ αβ cell	Scan lymph nodes for the cognate antigen of MHC class I molecule complexes. Differentiate into CTLs and memory T cells when activated by APCs.
Adaptive immune response: regulatory	
T_R1 cell	Immunosuppression mediated by IL10 production. Generated from naïve T cells in the presence of TGFβ and IL27 or by some immune suppressive drugs.
Natural T_{Reg} cell	Mediate immunosuppression and tolerogenic responses. Generated in thymus.
Inducible T_{Reg} cell	Promote immune suppression and tolerance by both contact dependent and independent mechanisms. Generated by naïve T cells in periphery. In some cases, IL2 and TGFβ important for their differentiation.
Adaptive immune response: helper	
T_H1 cell	Promote protective immunity against intracellular pathogens by secreting IFNγ, which induces activation of macrophages and upregulation of iNOS. Development upregulated by IL12.
T_H2 cell	Promote humoral immune responses and host defence against extracellular pathogens. Can potentiate allergic disease. Development and maintenance regulated by IL4, IL25, and IL33

T_H9 cell	Involved in host defence against extracellular parasites, primarily nematodes: produce anti-inflammatory IL10 but also promote allergic inflammation. Recently characterised so role somewhat unclear.
T_H17 cell	Promote protective immunity against extracellular bacteria and fungi, mainly at mucosal surfaces, as well as autoimmune and inflammatory diseases. Generated in the presence of IL6 and TGFβ and/or IL21. Maintained by IL23 and IL1.
T_H22 cell	Involved in inflammatory skin diseases. Recently characterised so role somewhat unclear. Identity as independent T_H subset unconfirmed.
T_{FH} cell	Promote germinal centre responses and help with B cell class switching.
Adaptive immune response: memory	
Central memory T cell	Mount recall responses to antigen: lack immediate effector functions but rapidly proliferate and differentiate into effector T cell.
Effector memory T cell	Provide immediate protection on antigen challenge through rapid production of effector (inflammatory) cytokines (among other activities).
Intraepithelial lymphocytes	
CD8αα cell	Can have regulatory functions via their production of IL10 and TGFβ. Generated both in and outside of thymus. Express γδ or αα T cell receptors.

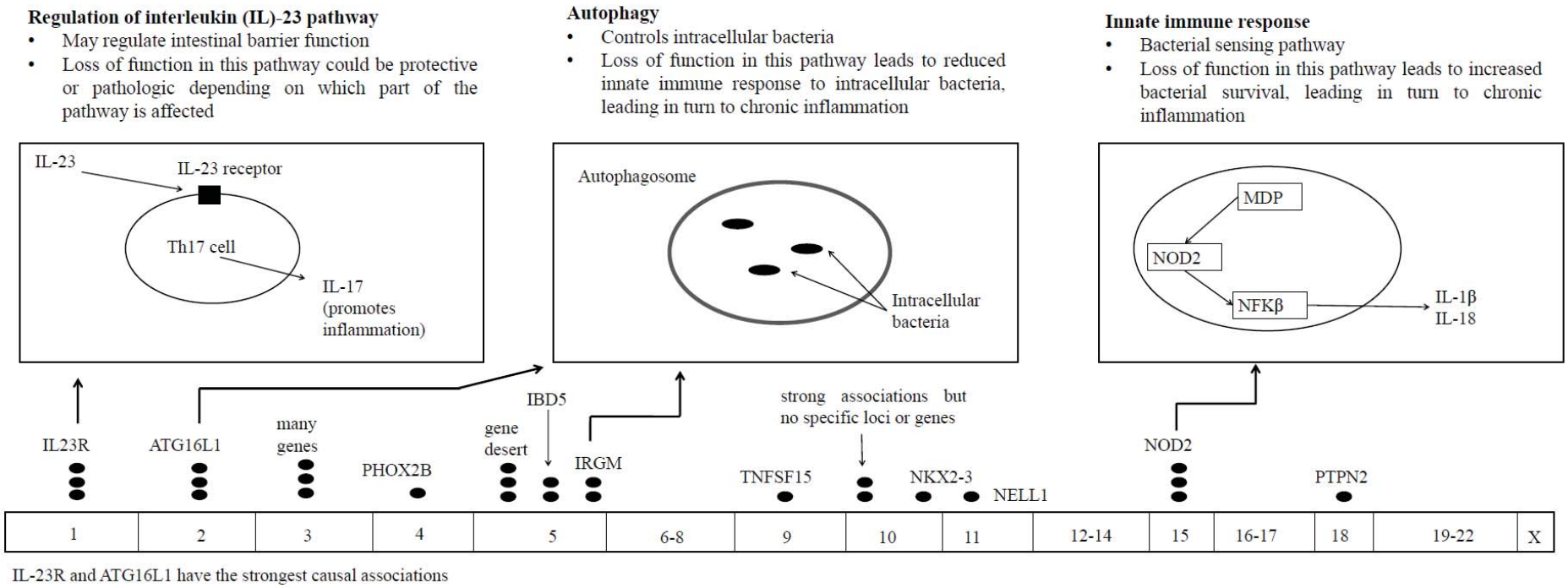


Figure 1.3 Genes associated with Crohn's disease. Chromosomal locations of the strongest and best replicated hits from the genome-wide association scans in CD are shown, including how these genes are thought to contribute to each of three major functional pathways linked to the pathogenesis of IBD. The line with segregations represents a chromosome, while the number of dots indicates the degree and breadth of support for a locus across studies, with three dots indicating very strong and independent confirmation. Adapted from Mathew (2008) [61].

Production of macrophage inflammatory protein 3α is dose-dependently increased by IL1β and TNFα in intestinal epithelial cell models (Caco-2 and HT-29) [63]. Levels of macrophage inflammatory protein 3α protein and corresponding mRNA were increased in tissues from CD patients compared to tissues from healthy controls or UC patients, with immunoreactivity in normal and IBD colon tissues principally associated with crypt and surface epithelial cells [63]. Macrophage inflammatory protein 3α production in enterocytes may play an important role in lymphocyte activation and recruitment to the colonic epithelium in IBD [63].

1.2.2 Animal models of IBD

Animal models of IBD enable the study of intestinal inflammation during early and intermediate as well as the later stages of disease development. Many of the findings about the pathogenesis of IBD have been obtained through the use of animal models, especially rodents.

1.2.2.1 Rodent models of IBD

The majority of animal models are rodents, which have the advantages of being relatively easily genetically manipulated, short-lived, quick to breed and easy to house in large numbers. Their environment (including factors such as diet) is also relatively easy to control and manipulate.

While findings from studies on intestinal inflammation using animal models must be cautiously extrapolated to human disease, many insights into the pathogenesis of IBD have been gained from rodent models [64]. These include: (1) the importance of antigenic, particularly microbial, stimulation [65]; (2) the importance of intestinal permeability defects in initiating and/or maintaining the mucosal inflammatory response [66]; (3) the role of genetic susceptibility in development of disease [67]; (4) the discovery that disease severity can be modulated by various dietary components (such as certain fatty acids) [68-70]; and (5) the complexity of the intestinal immune system and how imbalance between pro- and anti-inflammatory pathways can result in disease [23]. Because many aspects of the immune system appear to be involved, it is difficult to identify a definitive “IBD-causing” process [23].

Animal models of IBD fall into five categories (Table 1.3): (1) antigen- and microbiota-induced colitis [71]; (2) other inducible forms of colitis (chemical, immunological, and physical) [72, 73]; (3) genetic-based colitis models (transgenic and gene-deficient models) [74, 75]; (4) adoptive transfer models [76, 77]; and (5) spontaneous colitis models [78-80].

1.2.2.2 Interleukin-10 and the IL10 gene-deficient mouse

The intestinal immune system may be particularly vulnerable to changes in the regulatory mechanisms preventing immunopathologic reactions, due to it being continuously involved in immune responses against a variety of antigens [81]. *In vivo* and *in vitro* experiments suggest that IL10 plays a protective role in intestinal barrier function [43], and has an essential role in controlling intestinal immune responses directed against enteric antigens [81].

The IL10 gene-deficient (*Il10*$^{-/-}$) mouse is one example of a genetic-based colitis model of IBD. This type of model is useful for studying the role of genetic susceptibility to colitis and the interaction between genes and the environment in colitis development. The *Il10*$^{-/-}$ mouse has the IL10 gene inactivated by targeted mutation [81]. It is a commonly used, commercially available, rodent model of IBD. *Il10*$^{-/-}$ mice develop intestinal inflammation throughout any part of the small and large intestine (enterocolitis) with many of the same biochemical, histological and physiological characteristics seen in IBD, although the colon is most affected [65, 81, 82].

The mechanisms that underpin intestinal inflammation development in these mice have been reviewed [75], and inflammation in *Il10*$^{-/-}$ mice is characterised by a lack of regulatory T cell-derived IL10 suppression of T_H1 responses, resulting in dysregulated IL12 production and dominant T_H1 cytokine profiles with increased production of IFNγ and TNFα [75]. *Il10*$^{-/-}$ mice have been widely used to study the role of the microbiota, immune dysregulation and epithelial integrity in IBD pathogenesis.

A number of findings support the relevance of studying intestinal inflammation using the *Il10*$^{-/-}$ mouse model. In many genome-wide association studies in the human population, polymorphisms of the IL10 gene have not been strongly associated with development of CD [83], although a systematic genome-wide analysis of UC has

Table 1.3 Examples of animal models of IBD. The categories are: (1) antigen-induced colitis and colitis induced by the microbiota; (2) other inducible forms of colitis (chemical, immunological, and physical); (3) genetic-based colitis models (transgenic and gene-deficient models); and (4) adoptive transfer models; (5) spontaneous colitis models.

Species	Mechanism of colitis induction	Category	Citation
Pig	dextran sodium sulphate (DSS)	2	[84]
Rat	dextran sodium sulphate (DSS)	2	[68]
Rat	trinitrobenzenesulfonic acid (TNBS)	2	[69, 85]
Mouse	*Il10*$^{-/-}$ gene deficient	1, 3 and 5	[75, 86]
Mouse	keratin 8 gene deficiency	1, 3 and 5	[87]
Mouse	dextran sodium sulphate (DSS)	2	[88] [89]
Mouse	deoxycholate	2	[90]
Mouse	genetic defects in both TGFβRII and IL-10R2 signaling	3 and 5	[91]
Mouse	multi-drug-resistant (MDR1) gene deficiency	3 and 5	[92]
Mouse	*CD45RB*^{Hi}T cell transfer into CB-17 SCID mice	4	[93]

reported an association between UC and a single nucleotide polymorphism (SNP) immediately flanking the IL10 gene [94], and *Il10* SNPs have been associated with paediatric onset CD [95]. In addition, a CD-associated NOD2 mutation was shown to suppress transcription of the *Il10* gene due to the inhibition of the activity of nuclear ribonucleoprotein hnRNP-A1 by the 3020insC Nod2 mutant protein [96]. An effective therapy for CD (anti-TNFα therapy) also ameliorates inflammation in *Il10*$^{-/-}$ mice [74]. Defective IL10 signalling has been found to define a subgroup of patients with IBD [97]. These findings indicate that *Il10*$^{-/-}$ mice are a relevant model in terms of both pathophysiology and genetic relevance to IBD.

The *Il10*$^{-/-}$ mice develop chronic enterocolitis that becomes apparent at 4-8 weeks of age when raised in conventional animal care conditions (i.e. conditions that are not germ-free, or specific-pathogen-free (SPF)). Intestinal permeability and antibody responses to systemic endotoxaemia increase during the development of colitis in *Il10*$^{-/-}$ mice (C57BL/6J background) [82]. *Il10*$^{-/-}$ mice also develop anaemia and growth retardation, probably explained by disturbance of nutrient absorption as a result of the alterations in microstructure and physiology of the small and large intestine (C57BL/6J background, with 129/Ola influence from stem cells used to generate the knockout) [81]. The anaemia seen in *Il10*$^{-/-}$ mice seems to be caused by iron deficiency, with other factors besides malabsorption probably contributing to the anaemia, such as overproduction of certain types of cytokines [81].

Exposure to micro-organisms has a major effect on disease progression in *Il10*$^{-/-}$ mice. *Il10*$^{-/-}$ mice housed in germ-free conditions do not develop any symptoms of intestinal inflammation, whereas those in conventional and specific pathogen free (SPF) conditions do (C57BL/6J x 129/Ola background) [65]. *Il10*$^{-/-}$ mice housed in SPF conditions have the same pathological changes in the proximal colon but different alterations in the duodenum and proximal jejunum compared to *Il10*$^{-/-}$ mice housed in conventional conditions, and the disease takes longer to develop (with weight loss evident from 7 weeks of age in SPF conditions versus 3-4 weeks of age for conventional conditions) and is less severe [65, 81].

The natural progression of enterocolitis in *Il10*$^{-/-}$ mice in SPF conditions has been characterised [98]. These mice have features reminiscent of the enterocolitis seen in human CD patients, but also exhibit some features atypical of CD (marked crypt

hyperplasia, rare occurrence of granulomas, fibrosis and lymphoid aggregates, and the absence of fissures, fistulae, and ileal inflammation). Enterocolitis in *Il10*$^{-/-}$ mice is associated with uncontrolled production of pro-inflammatory mediators by macrophages and T_H1-like cells including IL1α, TNFα, IL6, NO, and IFNγ [98].

Susceptibility to enterocolitis varies with background strain due to genetic differences. For example, C3H/HeJBir.*Il10*$^{-/-}$ mice are more susceptible than C57BL/6J.*Il10*$^{-/-}$ mice, and a first generation cross of these two strains produces mice that develop an intermediate degree of enterocolitis, confirming that susceptibility is heritable [67, 99]. C57BL/6J.*Il10*$^{-/-}$ mice are more prone to developing rectal prolapses than C3H/HeJBir.*Il10*$^{-/-}$ mice, and these can occur as early as 9 weeks of age, despite the occurrence of faecal bleeding and diarrhoea in C3H/HeJBir.*Il10*$^{-/-}$ mice but not C57BL/6J.*Il10*$^{-/-}$ mice [67]. *Il10*$^{-/-}$ mice on BALBc and 129 SvEv backgrounds develop more severe colitis than those on the C57BL/6 background [98].

1.2.2.3 The intestinal epithelium in *Il10*$^{-/-}$ mice

As in human IBD, increased small intestinal permeability appears to be an important aetiological factor in the development of colitis in *Il10*$^{-/-}$ mice, rather than merely coinciding with inflammation development. Treatment of *Il10*$^{-/-}$ mice (on 129 SvEv background) from 4 weeks of age with a zonulin receptor antagonist (AT-1001; a small peptide which reduces small intestinal permeability) results in reduced levels of histological injury in the colon as well as reduced colonic *ex-vivo* TNFα secretion at 17 weeks of age [43]. These findings support the idea that in IBD, as with other auto-immune diseases (type I diabetes, coeliac disease), dietary antigens can initiate disease through a mechanism involving increased intestinal permeability [43].

Colonic permeability to mannitol, a hydrophilic molecule that passively crosses the epithelium via the transcellular route, is increased *in vivo* in *Il10*$^{-/-}$ mice with normal enteric microbiota at 2, 4 and 10 weeks of age, both before and after inflammation development [66]. Increased permeability is also seen in the ileum at 2 weeks of age, but returns to normal by 6 weeks of age [66]. Levels of TNFα and IFNγ (cytokines that are known to disrupt epithelial barrier integrity) are increased in the ileal and colonic mucosa at 2 weeks of age. This may explain the increased intestinal permeability in these mice [66].

An apparent decrease in the number of goblet cells in the epithelium is a characteristic often reported in colitis. However, a study of goblet cell morphology in C57BL/6J.*Il10*$^{-/-}$ mice showed that the inflamed colon still contains high numbers of goblet cells with similar histochemical staining (by alcian blue) and mucin-2 (exclusively expressed by intestinal goblet cells with well-known function forming the extracellular mucus layer) and trefoil factor family peptide-3 (another goblet cell marker that is also important for epithelial restitution) expression patterns, although the cells are small and hypotrophic [100]. This finding suggests that goblet cells only appear to be depleted in number in colitis due to their altered morphology [100]. Goblet cell and enterocyte markers displayed region-specific changes in the large intestine of *Il10*$^{-/-}$ mice. For example, post-translational processing of mucin-2 was affected in the distal colon, reducing mucin-2 sulfation and perhaps reducing its protective properties [100].

Global gene expression analysis of the epithelium in *Il10*$^{-/-}$ mice provides further clues about the changes in function that occur during inflammation. Colon epithelial cells (cell type unspecified) were harvested by laser microdissection in *Il10*$^{-/-}$ mice at 11-13 weeks of age and analysed by microarray [101]. The expression levels of many chemokine genes were increased in mice with colitis relative to healthy controls. Expression of the LY6 [1] family of molecules was also increased and immunofluorescence confirmed that their expression was from epithelial cells and not from contamination by infiltrating immune cells. LY6 molecules may be useful targets by which to reduce the expression of chemokine genes in colitis [101, 102].

1.2.2.4 The intestinal microbiota in *Il10*$^{-/-}$ mice

Epithelial permeability of the intestinal tract is known to be influenced by pathogens and bacterial toxins through the modulation of tight junction proteins, and may also be influenced by the marked apoptosis that occurs during inflammation [34]. Increased small intestinal permeability has been observed before the development of colonic mucosal inflammation in the *Il10*$^{-/-}$ mouse model of IBD (129 SvEv background) [66].

[1] The LY6 molecules are a group of glycophosphatidyl inositol-anchored membrane proteins with ten conserved cysteines that are thought to be involved in cellular adhesion and signaling.

Disruption of ileal and colonic epithelial barrier integrity in *Il10*$^{-/-}$ mice can both initiate and perpetuate an inflammatory response following mono-association with otherwise non-disease-causing commensal bacteria [44]. Enteric bacteria are necessary for the development of colitis in *Il10*$^{-/-}$ mice, and rodent colitis models with different genetic abnormalities respond differently to the same defined bacterial stimuli [65].

The *Il10*$^{-/-}$ mice maintained in SPF conditions exhibit immune system activation and develop inflammation in the caecum and colon [65]. When maintained in germ-free conditions, *Il10*$^{-/-}$ mice do not develop colitis or immune system activation [65]. Intestinal permeability does not change in germ-free *Il10*$^{-/-}$ mice, as assessed by *in vivo* measurement of net water flux (as labelled polyethylene glycol) and mannitol clearance [66]. These findings are consistent with those of Kuhn et al. [81] and show that normal enteric bacteria are required for the development of spontaneous colitis in the *Il10*$^{-/-}$ mouse.

The age when a germ-free *Il10*$^{-/-}$ mouse is transferred into a non-sterile environment may affect the mucosal inflammatory response [65]. Whether germ-free mice move into an SPF environment at 3 weeks or 20-24 weeks of age affects the pattern and severity of inflammation, especially in the caecum, with the mice colonised at a younger age showing less severe inflammation [65]. In contrast, another study showed no difference in the degree of histological injury in adult versus neonatally colonised mice [103].

The role of bacteria in intestinal disease in *Il10*$^{-/-}$ mice has been studied [65] and reviewed [104]. Bacterial composition in the colon of *Il10*$^{-/-}$ mice changes as colitis progresses, demonstrated by raising mice in germ-free conditions then exposing them to the faecal microbiota of wild-type mice maintained in SPF conditions [105]. Generally, there appears to be a higher abundance of *Bacteroides* species, *Bifidobacterium animalis*, *Clostridium cocleatum*, and enterococci, and fewer *Eubacterium ventriosum* and members of the *Lactobacillus acidophilus* group, in colitic compared to non-colitic mice [105]. Changes in total number and composition of caecal bacteria are correlated with the severity of colon inflammation in *Il10*$^{-/-}$ mice [106, 107].

Enterococcus faecalis induces intestinal inflammation in germ-free *Il10*$^{-/-}$ mice on 129SEV background [108]. Inoculation of *Il10*$^{-/-}$ mice at 5 weeks of age (C57BL/6J background) with a mixture of bacterial cultures (including *E. faecalis* and

Enterococcus faecium strains) as well as microbes obtained from the intestinal tract of C57BL/BJ mice raised in conventional conditions, produces more consistent and reproducible mucosal inflammation in the colon by 12 weeks of age [109, 110].

Administration of probiotic strains of *Bifidobacterium infantis* and *Lactobacillus salivarius* reduced histological injury scores in *Il10*$^{-/-}$ mice with 129 Ola x C57BL/6J background [111], although another study found no prophylactic or therapeutic effect of *Lactobacillus salivarius* in either a DSS-treated or *Il10*$^{-/-}$ mouse model with the C57BL/6 background [112]. Treatment with the probiotic *Lactobacillus plantarum* decreased colonic paracellular permeability and reduced bacterial translocation and proinflammatory cytokine production in *Il10*$^{-/-}$ mice (129 Sv/Ev background) [113]. Treatment of *Il10*$^{-/-}$ mice (129 Sv/Ev background) with *Bifidobacterium infantis* conditioned medium for 30 days, beginning at 8 weeks of age, attenuated inflammation, normalised colonic permeability, and decreased colonic and splenic IFNγ secretion [114].

1.2.2.5 Histopathology of intestinal inflammation in *Il10*$^{-/-}$ mice

Profound histopathologic alterations are present in the intestinal tract and haemopoietic tissues of *Il10*$^{-/-}$ mice housed in conventional conditions [81]. The principal histopathologic finding is a chronic enterocolitis that involves the entire intestinal tract, with the caecum and proximal colon being particularly affected [67, 81, 115]. Mouse age and background strain, along with the type of microbiota the mouse is exposed to, affect the severity and location of the inflammation [65, 67, 98]. The intestinal pathology is characterised by a regionally variable pattern of mucosal inflammation associated with either hyper-regenerative or degenerative lesions of the intestinal epithelia [81]. The chronic inflammatory process causes excessive regenerative hyperplasia of the mucosa leading to a marked thickening of the intestinal wall, and the architecture of the mucosa is disturbed by the formation of abnormal crypt and villus structures, desquamation of apical epithelia, and multiple small superficial erosions, or ulcers, of the mucosa [81]. Goblet cells in the colonic crypts appear to be depleted, but in fact only appear to be depleted in number in colitis due to their altered morphology [100]. There is extensive lymphoplasmocytic and histiocytic infiltration of the lamina propria and submucosa with lymphocytes, plasma cells, macrophages, neutrophils,

occasional multinucleated giant cells and eosinophils, with diminished gut-associated lymphatic tissue [81].

Scoring of inflammatory lesions in mouse models of colitis typically involves examining lesion severity, degree of ulceration, hyperplasia and size of the area involved [99]. Mild hyperplasia consists of morphologically normal epithelium with increased crypt length, while moderate hyperplasia is characterised by increased crypt length (two or three times normal) with cell hyperchromasia, the appearance of decreased numbers of goblet cells, and branching in scattered individual crypts, and severe hyperplastic regions exhibit markedly thickened epithelium (four or more times normal), marked hyperchromasia of cells, few to no goblet cells, a high mitotic index of cells within the crypts, and numerous crypts with branching patterns [116]. An example of the histopathological changes that occur in colitis in *Il10*$^{-/-}$ mice is shown in Figure 1.4.

1.2.3 Gene expression changes in intestinal inflammation

1.2.3.1 Colon gene expression in whole tissue and selected mucosal cells

Changes in global gene expression in the inflamed intestine of IBD patients and mouse models of IBD have been useful to gain insight into the likely mechanisms underlying the initiation and maintenance of inflammation in the intestinal mucosa [76, 101, 117]. Many genes have increased expression levels in the colon of *Il10*$^{-/-}$ mice compared to control mice, and in older *Il10*$^{-/-}$ mice with colonic inflammation compared to younger mice with less severe inflammation [115]. The *Il10*$^{-/-}$ mice show a similar pattern of gene expression changes in inflammation compared to human IBD tissues, although changes in the mRNA abundance of genes known to be involved in IBD are also seen in other mouse models of colitis [76, 118]. Genes with increased expression levels in inflamed *Il10*$^{-/-}$ mice fall into some of the following categories: defence responses to biotic (for example, pathogenic) stimuli and stress, humoral and innate immune responses and antigen presentation [115].

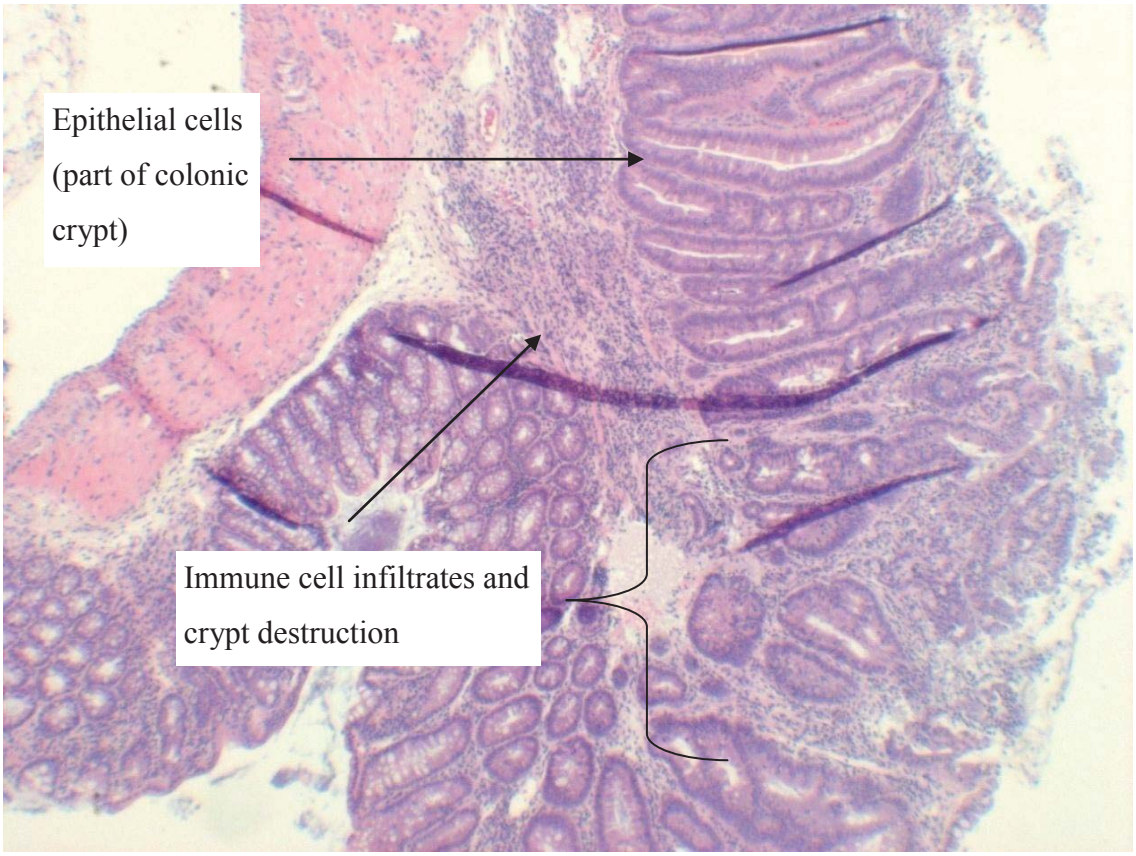


Figure 1.4 Histological section of the proximal colon from an *Il10*$^{-/-}$ mouse with colon inflammation. A large degree of infiltration of immune cells into the submucosa and mucosa can be seen, as well as crypt destruction. Stained with haematoxylin and eosin, viewed at 40x magnification. At higher magnification (100x), the appearance of goblet cell depletion can be seen in the crypts to the top right of the image (not shown).

However, in intact colon gene expression profiles, it is difficult to determine which regions of tissue are involved in the differentially expressed pathways. Studying gene expression changes in specific cell types or in regions of the colon may contribute to a better understanding of the role of particular cells in the complex processes involved in colon inflammation. Laser microdissection (LMD) is a technique that allows the removal of cell populations of interest from frozen tissue, enabling subsequent molecular profiling of these cells which is then reflective of their *in vivo* state [119-121]. Other methods of cell sorting must be performed immediately upon excision of the tissue, whereas LMD can be performed on stored frozen tissue.

Gene expression profiling combined with LMD with has not yet been widely applied to the study of cell function in the healthy and inflamed intestine. Various cell types in the colon may have as yet undefined actions in the development and maintenance of colon inflammation, such as that occurring in IBD. The use of LMD to study gene expression in these cells may lead to lines of investigation that could result in new hypotheses about the pathogenesis of IBD. Intestinal epithelial cells play an important role in the initiation of inflammation and the tissue's response to bacteria, as well as controlling barrier permeability [38, 39]. Changes in the intestinal epithelium, such as increased paracellular permeability and mucosal immune defence malfunction, are thought to be important in the initiation of inflammation [40, 41]. LMD could be used to further investigate the role of the epithelium in the development of inflammation by examining gene expression of signalling pathways in these cells in their *in vivo* state.

Differences between gene expression profiles in isolated epithelial cells compared to those in intact tissue would indicate the relative contribution of the epithelium (and that of recruited inflammatory cells) to the overall gene expression changes in a mouse colitis model. This concept has been used in the study of the development of *Helicobacter*-induced gastric lymphomas, using mRNA linear amplification and DNA microarrays to identify gene expression changes in mucosal cells or in immune cell infiltrates [122]. Molecular signatures were established that characterised the response of pure stomach-cell populations to *Helicobacter* infection *in vivo*. Gene expression results were obtained from whole stomach (at different stages of infection), and also lymphocytic and mucosal fractions. In the mucosal fraction, the expression of mucosal defence genes was increased, and in the lymphocytic fraction, the expression of many B

and some T cell genes was increased [122]. This study demonstrated that the likely cellular origin of particular transcripts could be identified by the analysis of gene expression in specific cell fractions.

Microdissection is also an important tool for studying the role of cell types that are difficult to study *in vitro*. There are no specific cell lines available for Paneth cells, so their function cannot be studied *in vitro.* LMD has allowed the study of relatively pure populations of Paneth cells and villus epithelial cells harvested from the CD-affected terminal ileum from patients undergoing surgical resection [123]. These authors show using *in situ* hybridisation and immunohistochemical localisation of NOD2 expression in Paneth cells that NOD2 mRNA was most abundant in the Paneth cell fraction compared to villus epithelial cells [123].

The majority of gene expression changes between healthy and inflamed colon may originate from infiltrating immune cells, potentially complicating the identification of important mucosal responses in inflammaton development. For example, neutrophils and recently recruited macrophages are responsible for production of interleukin-8 (IL8) in IBD, and IL8 mRNA and protein was shown by *in situ* hybridisation to occur in macrophages and neutrophils adjacent to ulceration in inflamed colon [124]. In endometriosis, separating out epithelial cells from leukocytes is important when interpreting gene expression profiles in inflamed tissue and their relevance to the pathology [125].

1.2.3.2 Epigenetic changes in intestinal inflammation

Gene expression is regulated at a number of levels, including the transcription level by proteins such as transcription factors, and also at the chromatin levels by reversible alterations in the availability of DNA for transcription, which are called epigenetic marks [126]. Epigenetic mechanisms mediate the interactions between the environment and an organism's genes, connecting environmental exposure with gene expression and function [127]. Epigenetic marks, unlike genetic variants such as mutations, are potentially reversible, despite being permanent enough to be "remembered" through multiple generations of cells [128]. The ability to modify epigenetic marks allows plasticity of phenotype in a fixed genotype, while the ability of epigenetic marks to

remain stable through generations of cells allows for programming of future generations in response to the environment of an ancestor [129].

Types of epigenetic marks include modifications of DNA, DNA-binding proteins, and histones that alter the structure of chromatin without altering the DNA nucleotide sequence (Figure 1.5) as well as small non-coding RNAs (microRNAs) [130]. Some of these epigenetic marks may be associated with heritable changes in gene function [131].

DNA methylation occurs at DNA sites where there is a cytosine nucleotide adjacent to a guanine nucleotide, called a CpG dinucleotide. Regions of DNA that contain a higher-than-average number of CpG sites are called CpG islands, which often occur at the start of a gene [132]. Methylation of CpG sites can lead to decreased gene transcription, especially methylation of CpG islands in the promoter region of a gene, while hypomethylation is associated with the over-expression of oncogenes in cancer cells [132].

Histones are the proteins around which DNA is packaged into chromatin, and their N-terminal tails can undergo a number of post-translational modifications such as methylation and acetylation which can alter the packaging of chromatin and increase or decrease the availability of a gene for transcription [133]. MicroRNAs are a class of small non-coding single-stranded RNA molecules about 21-22 nucleotides in length that regulate other genes by binding to mRNA and preventing translation [134]. MicroRNA genes are themselves regulated by chromatin modifications including DNA methylation, and microRNAs regulate the synthesis of chromatin modifiers such as histone methyltransferases and methyl CpG-binding proteins [133]. Epigenetic regulation of gene expression is therefore complex, and epigenetic modifications are interdependent.

Epigenetic mechanisms contribute to the translation of genetic and environmental factors into phenotype. Epigenetic patterns in various tissues, including the colon, are reorganised during gametogenesis, development [135] and aging [136]. Developmental changes in intestinal gene expression and epigenetic changes in carcinogenesis, as well as different incidence of gastrointestinal disease between monozygotic twins, points strongly towards a role for epigenetics in normal intestinal development and therefore also in intestinal pathology [135].

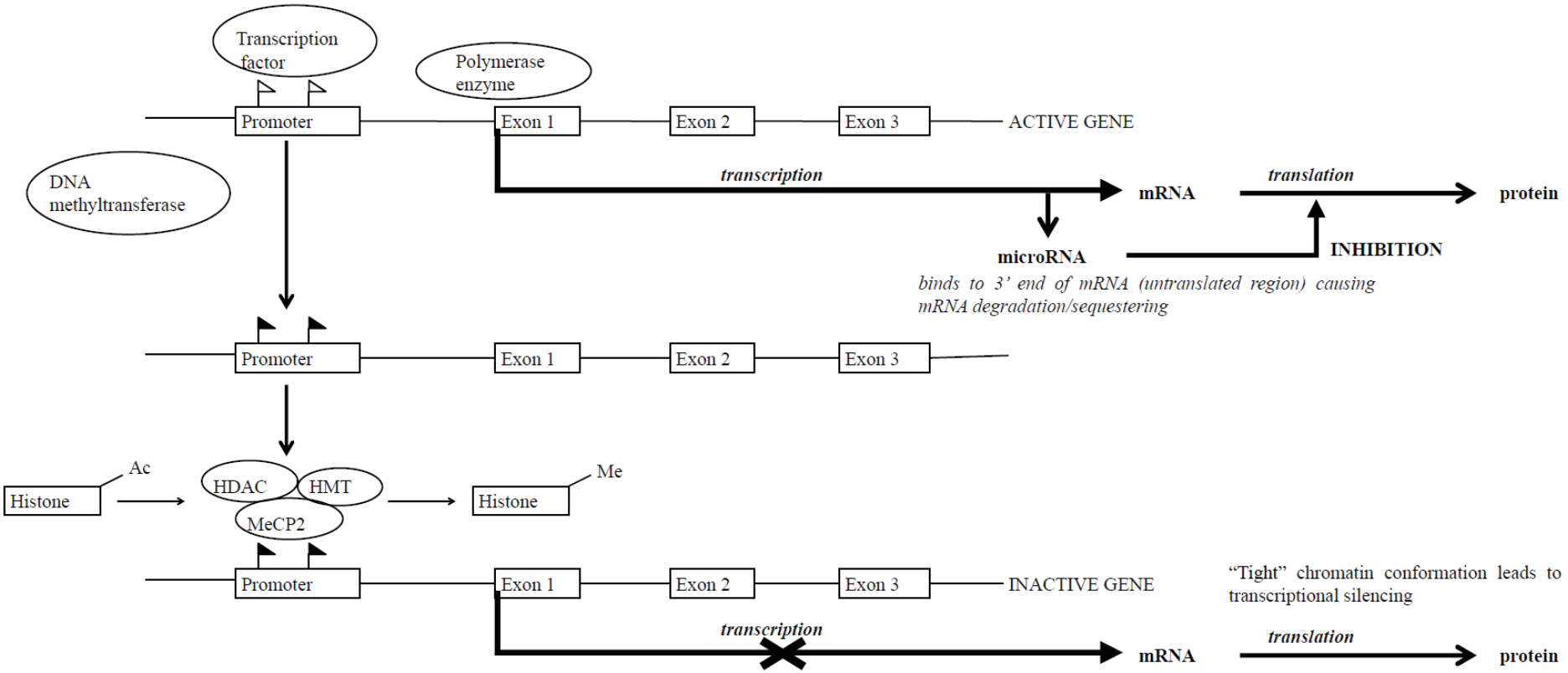


Figure 1.5 Epigenetic modulation of gene expression. Methylation of DNA at promoter regions generally represses gene transcription by recruiting enzymes that alter histone modifications leading to changes in chromatin structure. MicroRNAs are short RNA molecules that inhibit translation, thus affecting gene expression. HDAC = histone deacetylase; HMT = histone methyltransferase; MeCP2 = methyl-CpG binding protein 2. White triangles = nonmethylated CpG site; black triangles = methylated CpG site. Adapted from Gluckman et al., 2009 [130].

Inherited or acquired epimutations (changes in heritable chromatin marks) may be important in the aetiopathogenesis of IBD [137]. Differences in phenotype between monozygotic twins affected by IBD, and the results of genetic association studies, show that aberrant regulation of cytokine gene expression may have a role in IBD development [83, 137]. The proinflammatory cytokine TNFα (mRNA and protein expression of which is increased in IBD) is an example of how aberrant cytokine expression may relate to epigenetic mechanisms. Anti-TNFα antibodies ameliorate inflammation in IBD, and the expression of the TNFα gene is epigenetically regulated [138], and epigenetic regulation of TNFα may be important in IBD susceptibility [137]. Histone modifications have also been associated with other inflammatory diseases, such as lung inflammation [139, 140] and altered expression of various microRNAs is associated with IBD [141].

Alterations in DNA methylation have been detected in mucosal biopsies of IBD patients [142, 143]. Results from screening large numbers of methylated CpG islands suggested a causal relationship between age-related methylation and cancer progression in IBD patients [142]. These authors also studied methylation levels of specific genes in normal and dysplastic mucosa of UC patients, and their results suggest that methylation changes precede dysplasia in UC patients, but that methylation levels are not vastly altered in UC patients without evidence of dysplasia. These methylation changes may occur with cell aging in chronic intestinal inflammation and predispose individuals to the development of tumours, linking DNA methylation changes with chronic intestinal inflammation [142].

Epigenetic mechanisms are also implicated in immune function, which is another reason to consider that epigenetics may be important in IBD [144]. Epigenetic mechanisms are involved with T_H1/T_H2 differentiation, and one microRNA known to be involved in immune responses (miR-155) may have a role in up-regulating innate immunity [144]. DNA methylation can be affected during inflammation via DNA methyltransferase (DNMT)-1 being less able to recognise its methylcytosine substrate. Oxidative stress often occurs in persistent inflammation, and through a range of agents can cause the oxidation of 5-methylcytosine which can lead to loss of methylation. Reactive halogen compounds are a by-product of many chemical reactions produced by inflammatory processes and can react with cytosine to mimic methylcytosine [144]. Histone

deacetylase inhibitors have been shown in several studies to be effective for amelioration of chronic inflammatory disease [144].

Direct evidence for a role of epigenetics in IBD was shown in a mouse model of IBD predisposed to tumour development, mice that have glutathione peroxidase 1 and/or 2 knocked out [145]. It was found that inflammation creates a signature of aberrant DNA methylation, which is observed later in the malignant tissue [145]. Inflammation-mediated cytosine damage has been proposed as a mechanistic link between inflammation and the epigenetic alterations seen in cancer [146]. Changes in histone modifications were also observed in association with changes in methylation levels at CpG islands. The results indicated a role of histone H3 lysine-27 trimethylation and the Polycomb complexes in aberrant DNA methylation [145].

There is clearly a role for epigenetics in the carcinogenesis that is often a long-term consequence of chronic intestinal inflammation, such as the colorectal adenocarcinoma that occurs in *Il10*$^{-/-}$ mice as early as 3 months of age under SPF conditions [98]. However, epigenetic changes also occur in IBD in the absence of cancerous lesions. Alterations in the function of the *ABCB1* (also known as *MDR1*) gene are associated with UC and mice with the *Mdr1a* gene[2] knocked out are used as a model of intestinal inflammation [147, 148]. Promoter methylation of the *MDR1* gene is increased in inflamed rectal mucosa in UC, and is influenced by the *MDR1* C3435T polymorphism [149].

1.3 Dietary factors and intestinal inflammation

In addition to fuelling the body, nutrition impacts the functioning of the intestine in both health and disease. Nutrition is very important for maintaining optimal intestinal function, not just in the normal healthy intestine but also when disease of the gastrointestinal tract is present. IBD involves mild to severe changes in intestinal function, both in relatively inactive and active phases. Nutritional factors in infancy can alter the function of the intestine by changing microbiota composition [150] and

[2] In the mouse, two genes, *Abcb1a* and *Abcb1b*, encode the ABCB1 protein.

affecting the growth and development of the intestine [151]. Nutritional factors in adulthood can affect intestinal motility and permeability [152]. Dietary antigen may have a role in the development of IBD and dietary factors may also have a role in IBD development by modifying the enteric microbiota [153].

The importance of dietary factors in inflammation is demonstrated by the ability of enteral diets to induce remission in approximately 85% of CD patients after administration for 3-5 weeks [154]. Enteral diets are liquid and often administered via naso-gastric tube due to their poor palatability resulting in poor patient compliance if administered orally. These diets consist of protein, carbohydrate, fat and vitamins and minerals, and differ in terms of the intactness of the nutrients. Polymeric enteral diets include intact nutrients, such as proteins, while elemental enteral diets consist of hydrolysed nutrients, such as amino acids. Unfortunately, these diets cannot be used long-term due to compliance issues, making it important to investigate the role of palatable, commonly available foods in treating or preventing symptom relapse.

Nutritional factors which are known to beneficially affect the intestinal epithelial barrier include polyunsaturated fatty acids, zinc and polyphenols [155]. Some dietary components, such as curcumin [156-158] and n-3 polyunsaturated fatty acids [118, 159-161] have been demonstrated to have anti-inflammatory effects in mouse models of colitis, and in the case of n-3 fatty acids, in IBD patients [162]. A list of dietary components known to have beneficial effects on colitis in animal models, and their presumed mechanisms of action, is presented in Table 1.4.

Nutrition is an environmental factor that has been shown to modify epigenetic marks [128, 131, 163-165], and is also thought to influence the progression of IBD [23]. Nutritional factors can have profound effects on the expression of specific genes by epigenetic modification [166]. DNA methylation has been shown in a number of studies to be influenced by environmental and nutritional factors, including one study of methyl-donor supplementation in a mouse model of colitis [167]. Maternal methyl-donor supplementation in mice induces prolonged colitis susceptibility in the offspring [167]. This effect was seen in a chemical model of colitis, and was associated with epigenetic changes in the colon mucosa and microbiota [167]. This finding also supports the relevance of studying DNA methylation, rather than the other epigenetic modifications, for understanding the effect of diet on the inflammatory process.

Table 1.4 Anti-inflammatory foods. Dietary components with beneficial effects on intestinal inflammation in animal models of colitis and their presumed mechanisms of action.

Dietary component	Model	Effect	Mechanism of action or effects on gene expression if measured	Citation
Curcumin	*Mdr1a*$^{-/-}$ mice	Reduced colitis severity.	Possible antioxidant activity. Downregulation of genes involved with immune and inflammatory responses, altered expression of genes within xenobiotic and fibrosis development pathways.	[158]
n-3 polyunsaturated fatty acids	*Il10*$^{-/-}$ mice	Reduced colitis severity.	Activation of the *Ppara* gene that regulates the expression of pro-inflammatory and immunomodulatory genes and proteins. Altered expression of genes involved with xenobiotic metabolism, fatty acid metabolism, inflammatory and immune response and oxidative stress response pathways.	[118]
Conjugated linoleic acid	DSS-induced colitis in pigs	Delayed onset of colitis.	Increased abundance of colonic *Pparg* mRNA.	[84]

Medium-chain triglycerides (MCT)	*Il10*$^{-/-}$ mice	Reduced colitis incidence and colon damage. Reduced weight loss.	Decrease in colonic TLR-9 mRNA levels (sensor of commensal bacteria). Note that some of the effect may be due to reduction of n-6 PUFA in the diet, not merely addition of MCT.	[168]
Oligosaccharides	TNBS-induced colitis in rats	Reduction in colon weight and extent of necrotic lesions.	Increased colon mRNA levels of intestinal trefoil factor 3 (*Tff3*), a goblet cell specific bioactive peptide involved in maintenance and repair, particularly of epithelial cells.	[69]
Lactoferrin	DSS-induced colitis in mice	Reduced some histological signs of colitis.	Anti-inflammatory effect of the bactericidal region of the lactoferrin molecule.	[169]
Sheep milk solids	DSS-induced colitis in rats	Reduced colitis severity.	Altered caecal microbiota composition.	[170]
Antioxidants	DSS-induced colitis in mice	Improved diarrheoa and colon lesions, increased body weight. Normalisation of DSS-induced reduction of colon length.	Scavenging of reactive oxygen species, counteracting the reduction in antioxidant activity that occurs in colitis. The inhibition of NFκB may be involved.	[171]

1.4 Dietary milk and intestinal inflammation

1.4.1 Milk as an infant food

The unique feature of mammals is their ability to produce milk, on which their young rely for early postnatal nutrition and survival. Milk is a whole food secreted from modified sweat (mammary) glands by the females of all mammalian species specifically for the purpose of feeding their offspring. Mammals do not usually continue to consume milk into adulthood. However, over the last 10,000 years, humans have adopted ruminant milk as an adult food and some populations are able to tolerate and digest the lactose in milk through persistence of lactase enzyme activity into adulthood [172]. Archaeological evidence suggests that the earliest date for which there is direct evidence of ruminant milk consumption and processing by humans is about 9,000 years ago, in the Near East[3] and south-eastern Europe [173]. Processing of milk (such as fermenting it to form yoghurt) has allowed even people with lactose intolerance to gain nutritional benefits from ruminant milk.

The primary purpose of milk is to provide complete nutrition for the mammalian young, but it also plays an important role in development, particularly in that of the gastrointestinal tract [174-177]. It provides non-nutritional components that regulate both the intestinal microbiota and the infant's immune system [178]. These components (antimicrobial and immunoregulatory factors, antibodies, and immune cells) are part of the innate immune system and act to protect the infant from infection [179-182]. Well-described milk components may have additional roles or functions that have yet to be identified [183].

The importance of milk for infant growth and development is demonstrated by the documented advantages of breast-feeding for human babies, including short-term benefits during infancy and long-term benefits for adults [181]. Short-term benefits of

[3] According to the Merriam-Webster Dictionary and Thesaurus (http://www.merriam-webster.com/dictionary), accessed June 13, 2011, "Near East" refers to the countries of South West Asia & North East Africa and is sometimes used interchangeably with Middle East, which has become the more common term.

breast-feeding are seen in preterm babies, where the supply of anti-inflammatory factors in the mother's milk appears to be important for intestinal development [184]. Studies in human infants, newborn ruminants, and piglets show that enteral feeding, particularly of milk, induces a number of processes, including rapid maturation of the mucosa, secretion of some hydrolytic enzymes, nutrient absorption, intestinal motility and microbial colonisation of the gastrointestinal tract in preterm neonates [184].

During birth, the maternal microbiota colonises the infant's intestine, and the composition of the intestinal microbiota is further influenced by breast milk [185]. This has beneficial effects on the development of a healthy intestinal ecosystem [186]. Cross-fostering *Il10*$^{-/-}$ mice to a normal mother resulted in normal levels of colonic adherent bacteria and reduced colon inflammation. Conversely, cross-fostering of normal mice to an *Il10*$^{-/-}$ mother resulted in increased levels of colonic adherent bacteria and low levels of colon inflammation [150]. The timing of exposure of the intestinal immune system to specific microbes seems to be important for the development of tolerance to food antigens, suggesting that postnatal milk consumption may promote the development of the intestinal immune system [187].

1.4.2 Milk as an adult food

Intestinal inflammation can affect appetite, energy expenditure and enteric nutrient loss, resulting in malnutrition, therefore patients may need nutritional support [188], which milk and milk-based products may be able to provide. Milk and its components have evolved to have beneficial effects on gastrointestinal health in infants, with some of these also occurring in the adult. Milk may have some benefits beyond nutrition for people with intestinal inflammation.

Ruminant milks are often used to make infant formula as an alternative to human breast milk, and are also widely consumed by adults [189]. However, milk may not have the same benefits for adults as it does for infants. The gastrointestinal tract during neonatal growth and development differs from that of the adult [22], affecting the intestinal processing of milk components and their activity. For example, lactose intolerance can cause problems in adults [190] due to loss of the ability to synthesise the lactase enzyme in the small intestine. The development of allergies to other-species milk components (particularly proteins) can cause gastrointestinal problems in both infants and adults

[191, 192]. Ruminant milk is also commonly processed before consumption into many food formats, which could also affect the activity of its components.

Nutritional characteristics of milk have been extensively reviewed [189, 193-195]. Milk composition varies between species [195, 196] and breeds [197], is affected by nutrition and health status [198], and varies throughout lactation [199]. Feeding regimes and other farming practices may alter milk composition, nutritional value and health benefits [199, 200]. Compared with human milk, ruminant milk has less total carbohydrate and lactose, but more ash (a measure of mineral content), more protein, and varying levels of fat and water depending on species (see Table 1.5). Cow milk is the most commonly consumed milk in Western countries. Like human milk, cow milk contains a large variety of saturated and unsaturated fatty acids with differing potential health effects, protein of high nutritional value and a number of potentially beneficial bioactivities, as well as carbohydrate (including lactose and oligosaccharides), vitamins, minerals and antioxidants [189].

Goat and sheep milk are available for human consumption, but in New Zealand and other Western countries are generally produced in smaller quantities than cow milk. Goat milk has a similar composition to cow milk, but with some important differences, including differences in protein allergenicity, fat composition [193], and higher levels of active components such as oligosaccharides [201]. Goat milk provides better nutrient absorption and utilisation than cow milk [202-205] and has a lower pH and higher buffering capacity, which is beneficial for the treatment of ulcers [193, 194]. Sheep milk has higher total solids and energy content, and higher protein, fat, carbohydrate and ash than both goat and cow milk [206]. There appears to be little research into the health benefits of sheep milk in the human diet relative to goat and cow milk.

Goat milk is reported to be less allergenic than cow milk, and beneficial for some people with allergies to bovine milk proteins [191, 207]. The general classification of major goat milk proteins is similar to those of cow milk, but the goat milk proteins differ in genetic polymorphisms and their frequency in the population [193, 194, 208]. These polymorphisms are thought to be responsible for differences in digestibility, cheese-making properties and flavours of goat milk products [193]. They also affect calcium absorption [207], and may account for the discrepancy between studies on goat milk allergy [191, 207].

Table 1.5 Composition of whole milk from humans, cows, goats and sheep [189, 195, 201, 206, 209]. Average values are stated – note that data from other sources may be slightly different because composition varies with a number of factors including maternal nutrition, breed, and stage of lactation. Adapted from Russ et al., 2010.

Component weight (g per 100 g)		Goat	Cow	Sheep	Human
Energy (kJ)		288	256	451	291
Water		87.0	88.1	80.7	87.5
Protein	Total	3.6	3.2	6.0	1.0
	Casein	2.9	2.6	4.2	0.4
	Whey (by subtraction)	0.7	0.6	1.8	0.6
Fat	Total	4.1	3.3	7.0	4.4
	Saturated	2.7	1.9	4.6	2.0
	Monounsaturated	1.1	0.8	1.7	1.7
	Polyunsaturated	0.1	0.2	0.3	0.5
Carbohydrate	Total	4.5	4.5	5.4	6.9
	Oligosaccharides ($g.L^{-1}$)	0.25-0.30	0.03-0.06	0.02-0.04	5.00-8.00
Ash		0.8	0.7	1.0	0.2

Peptides formed when hydrolysing goat milk casein are less bitter than are those from cow milk casein. Goat milk casein micelles differ in sedimentation rate, solubilisation of β-casein, size, solvation, and heat stability. They also contain more calcium and phosphorus than those from cow milk. Goat milk also has higher levels of six essential amino acids (threonine, isoleucine, lysine, cystine, tyrosine, valine) [193]. The cysteine content has been suggested as one possible reason for improved intestinal absorption of copper (relative to cow milk) in studies of rats with malabsorption syndrome due to bowel resection [205].

Goat milk has a more beneficial fatty acid profile and distinct health benefits compared to cow milk. It is higher in short- and medium-chain fatty acids and mono- and poly-unsaturated fatty acids, and is lower in trans-fatty acids [193, 210]. Medium-chain triglycerides are saturated fatty acids with 6 to 12 carbons, with short-chain fatty acids having less than 6 carbons [211]. Higher levels of short- and medium-chain fatty acids in goat milk may be responsible for its beneficial effects on malabsorption syndrome and blood cholesterol [202, 205, 212, 213].

Relative to control and cow milk diets, goat milk diets have beneficial effects on the utilisation or bioavailability of copper and iron [205], calcium and phosphorus [212], zinc and selenium [203], and improve fat absorption in bowel-resected rats [202]. A number of explanations have been proposed for these effects, including the presence of higher concentrations of short and medium-chain fatty acids, cysteine, lysine, ascorbic acid, and greater protein solubility [202, 203, 205, 212, 213].

The medium-chain triglycerides in goat milk have been implicated in the improved effects of goat milk on fat metabolism and mineral utilisation compared to cow milk [202, 205, 213]. They do not require binding to proteins during digestion and transport, behave more like glucose than lipid in the liver, and the intestinal absorption of medium-chain triglycerides is more efficient than long-chain triglycerides. Medium-chain triglycerides are more readily used for energy, and stimulate bile and cholesterol secretion less than long-chain triglycerides. Increased medium-chain triglyceride intake contributes to reduced fat mass, improved insulin sensitivity, and may reduce intestinal injury (by lengthening intestinal epithelium and increasing number of goblet cells) and protect from hepatotoxicity [211]. Medium-chain triglycerides can have pro-

inflammatory effects, but overall they appear to be less inflammatory than long-chain triglycerides [211, 214].

Little published research exists on the nutritional value of sheep milk, although there is some information on composition and its products [195, 196, 206, 215]. Compared to goat and cow milk, sheep milk has more protein, fat, calcium, iron, magnesium, zinc, thiamin, riboflavin, vitamin B6, vitamin B12, medium chain fatty acids, monounsaturated fatty acids, and specific amino acids [206]. The high medium-chain triglyceride content of sheep milk may confer some of the same health benefits that have been reported for goat milk. Sheep milk also has smaller, more easily digestible fat globules than cow milk [215].

1.4.3 Milk and IBD patients

Ruminant milk may benefit patients with intestinal inflammation due to its high digestibility and ability to improve mineral utilisation, although factors such as milk protein allergenicity and lactose intolerance may mask or diminish these beneficial effects. Sheep and goat milk, with their high total solids, protein, essential amino acid, mineral and vitamin and medium-chain triglyceride content, may be useful for providing nutritional support in intestinal inflammation. The ingestion of dairy products (milk and yoghurt) is accompanied in healthy subjects by an overall decrease in the mRNA levels of inflammatory genes in white blood cells after 6 hours, suggesting that there is a net anti-inflammatory effect of dairy products including cow milk [216].

Milk may be beneficial for some IBD patients [217]. Generally, a greater proportion of IBD patients surveyed in New Zealand perceived goat and sheep milk to be beneficial for their wellbeing compared to cow milk, although overall, all of these milks were perceived to be more adverse than beneficial as shown in Figure 1.6 [217]. This could reflect differences in the response of each individual to the type of milk, which could be due to a number of factors including genotype, milk protein allergies, lactose intolerance [189], the form of IBD that the patient had (UC versus CD) [218, 219], or could have been due to psychological factors.

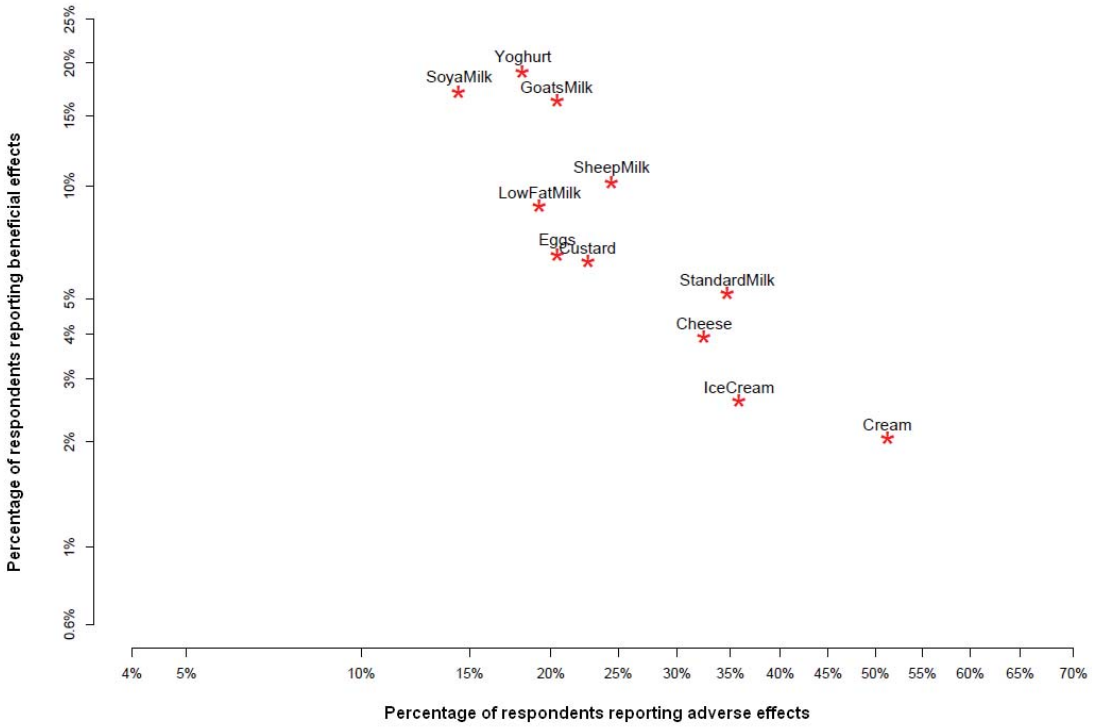


Figure 1.6 Dairy foods and IBD patients. Various dairy products ranked by IBD patients in terms of perceived adverse and beneficial effects on their intestinal function. Adapted from Triggs *et al.*, 2010 [217].

For example, IBD patients are often advised to remove milk from their diet due to a widely held belief that it is "bad" (exacerbates symptoms). However, it has been suggested that milk may be tolerated by many IBD patients, and that due to their nutritional value, dairy products may still be a useful part of their diet [190]. Goat milk has been promoted as having less allergenic effects than cow milk and containing larger amounts of some beneficial nutrients. This may have affected patient perception in the IBD patient survey [217]. Likewise, perception of soy-based milk substitutes, which were scored more highly beneficial than any of the ruminant milks [217], may have been affected by the marketing of soy as a healthy alternative to milk.

The questionnaire subjects' perception of cow milk differed between skimmed and whole milk, and in general, there was a trend for dairy products with higher fat content to score worse (decreased beneficial effects and/or increased adverse effects), which suggests that fat content of dairy foods may be important to the patient's wellbeing [217, 220]. Fat content of enteral diets for IBD patients has been proposed as an important factor in determining the effectiveness of enteral diet therapy [221]. Enteral diets with a low fat content (0.6-1.3% of total energy) were associated with a reduction of disease activity in a number of studies, while the use of diets with higher fat content (12-30% of total energy) tended to be associated with a poorer outcome [222]. Reduced availability of substrate for n-6 derived eicosanoid synthesis might be responsible for the beneficial effects of lower fat diets used to treat CD, as diets high in linoleic acid (a precursor of arachidonic acid, itself a precursor for pro-inflammatory eicosanoid synthesis) were associated with a less favourable outcome [221]. Lower amounts of long-chain triglycerides also appeared to be beneficial [223], however, the relationship between fat content and effectiveness of enteral diets in IBD is not consistent between studies [224]. It does seem likely that fat content and/or fat composition of diets has some impact on IBD, but it is unclear exactly how and to what extent [219, 222].

1.4.4 Soy "milk" and health

Soy "milk" is often substituted for ruminant milk when milk allergies or intolerance exist, for infants, children and adults. Soy foods have been claimed to have anti-inflammatory properties (among other health benefits): however, the evidence is inconclusive. Interest in soy in Western countries seems to have begun as part of attempts to create milk alternatives for infants and adults with intolerance to cow milk.

Neonatal piglets were used to investigate the possibility of allergy to soy proteins in infants who were allergic to cow milk proteins and the viability of soy products as an alternative. Neither intact nor hydrolysed soy protein elicit intestinal inflammation in neonatal piglets [225].

Concerns have been raised about the safety of soy diets, particularly with respect to its high isoflavone content which could in theory have oestrogen-like effects in the body. When fed to adult pigs as the sole source of dietary protein, soy was not associated with an increase in colonic cell proliferation [226] despite earlier reports of soy diets inducing colorectal carcinogenesis and increasing colonic cell proliferation [227, 228]. Clinical evidence suggests that soy-based infant formula adequately supports growth and development, and there is no conclusive evidence from animal, human infant or human adult studies to indicate that dietary isoflavones adversely affect human health or reproduction [229].

Soy has also been promoted as having immune-enhancing properties. High intakes of isoflavones (a phytoestrogenic substance) in middle-aged women appear to increase serum concentrations of IL6, an inflammatory mediator and generally pro-inflammatory cytokine [230], although soy is claimed to have anti-inflammatory properties. In end-stage renal disease patients on haemodialysis who have underlying systemic inflammation, 8 weeks of dietary intervention with soy resulted in reduced levels of C-reactive protein, IL6 and TNFα in blood (unstimulated by lipopolysaccharide), indicating a systemic anti-inflammatory response [231]. Other cytokines involved in the inflammatory response were not measured, so this study could not show whether the isoflavones in soy could selectively modulate other, potentially pro-inflammatory, cytokines. In contrast, another study found that 4 weeks of soy “milk” supplementation did not alter plasma markers of inflammation (IL6, IL1β, and TNFα) and oxidative stress in postmenopausal women compared to supplementation with reduced fat cow milk [232]. Overall, there is no compelling evidence that soy products reduce levels of IL6 or TNFα [232].

Soy-based diet had beneficial effects on inflammation-induced increases in pain sensitivity in rats relative to casein-based diet [233], suggesting soy has anti-inflammatory activity. The soy phytoestrogen genistein has been demonstrated to accelerate skin wound healing, associated with a dampened inflammatory response

[234]. Soy in combination with green tea may have anti-inflammatory effects in prostate cancer [235]. A soy protein-based diet had anti-inflammatory effects in the *Il10*$^{-/-}$ mouse model of colitis [236]. Soy and soy phytoestrogens appear to have some immunomodulatory properties, but there is no conclusive evidence yet to back claims of specific anti-inflammatory properties of soy.

1.4.5 Milk and intestinal function

Milk, whey and colostrum contain components which have been extracted from milk and used as food ingredients and found to enhance the immune system via both active and passive mechanisms. Active immune enhancement involves changing the cellular activities of the immune system, whereas passive enhancement involves molecules such as antibodies, oligosaccharides and glycoconjugates that can protect against pathogens. The role of dairy ingredients in enhancing immunity has been reviewed elsewhere [237].

A variety of non-specific secretory factors are present in milk: these are part of the innate immune defence system and include lipids, lactoferrin, lactoperoxidase, lysozyme and oligosaccharides [182, 185]. Human milk lipids inactivate viruses, protozoa and bacteria *in vitro*, although the mechanisms are unclear [238]. Antimicrobial activity depends on storage temperature (suggesting enzymatic activation is necessary) and requires the storage of lipid and aqueous fractions together [238]. Gastrointestinal digestive processes may play a role in activating milk lipids. Medium-chain and long-chain-unsaturated fatty acids are antiviral, but short-chain and other long-chain fatty acids are not [238]. While tri- and di-glycerides do not have antimicrobial activity, human milk (raw) contains lipases that can convert triglycerides to free fatty acids and monoglycerides, and these can have antimicrobial activity [238]. Whether these effects occur with ruminant milk that is fresh or processed for human consumption is unclear [238, 239].

A clinical study in healthy elderly people (60-83 years old) demonstrated that milk products have beneficial effects on innate immunity [240]. Subjects consumed processed, low fat, low lactose bovine milk twice daily for 6 weeks. In response to an increase in milk consumption, the subjects had a time-dependent increase in bactericidal activity of polymorphonuclear cells. The authors observed that this effect was not

unprecedented because milk contains a number of immunologically active proteins (such as α-lactalbumin, β-casein, caseinoglycopeptide and lactoferrin/lactoperoxidase) that are known to enhance phagocytic function, and that these results confirmed earlier unpublished studies by these authors where immune function was enhanced in mice fed milk-based diets.

Many reviews discuss the potential value of bovine colostrum for human health [241, 242]. Bovine colostrum modifies gastrointestinal tract growth, function and differentiation, and may help with growth and adaptation after bowel resection [243]. Studies have shown that colostrum: (1) can ameliorate the severity of non-steroidal anti-inflammatory-induced small intestinal injury in mice and humans [244, 245]; (2) may improve or prevent problems with intestinal permeability [246]; and (3) may reduce enteric bacterial overgrowth [244]. In some of these studies, bovine milk or whey protein was also used, but were less effective than colostrum [245, 246]. In rats, bovine colostrum and goat milk were equally effective at reducing hyperthermia-induced increases in gastrointestinal permeability, however, colostrum was effective at a lower dose than goat milk [247].

Oral administration of bovine colostrum to mice stimulates intestinal intraepithelial lymphocytes to polarise to T_H1-type [248]. Intestinal intraepithelial lymphocytes play an important role in mucosal immunity through surveillance of infected, premalignant and effete cells, maintenance of immune homeostasis (through regulation of inflammatory responses caused by infection and autoimmunity) and immunoregulation. Intestinal intraepithelial lymphocytes, which were isolated from Peyer's patches in colostrum-fed mice, showed T_H1-like responses characterised by intact IFNγ production but impaired IL4 production in response to triggering of T cell receptors. IL10 production was also impaired, while faecal immunoglobulin A (IgA) concentration and intestinal microbiota profile were unchanged. It was suggested that bovine colostrum may have directly stimulated intestinal intraepithelial lymphocytes to T_H1-biased responses and suppressed T_H2-biased responses. These changes could reduce allergies and help prevent infectious disease and malignancy in the intestine [248]. Exactly how, or whether, these effects may be useful in intestinal inflammation is unclear, although stimulation of T_H1 responses may be beneficial in UC-like colitis but not in CD-like

colitis, because UC appears to involve an excessive T_H2 response and CD an excessive T_H1 response [249].

A variant of bovine β-casein (termed A1), which is found more predominantly in certain breeds of cattle, releases a peptide opioid, β-casomorphin-7 (BCM7), on enzymatic digestion that binds to endogenous intestinal opioid receptors [250]. This peptide has been controversially proposed to have a role in the development of cardiovascular disease, diabetes, and autism [251-255]. The likelihood of release of BCM7 is lower in bovine A2 β-casein, and in sheep and goat β-casein (both of which are the A2 variant), due to the presence of a proline residue instead of a histidine at β-casein sequence position 67 [252]. BCM7 can reduce gastrointestinal motility [250] and can also stimulate mucin (mucus) production by intestinal goblet cells [256]. People with intestinal inflammation may be more susceptible to the systemic opioid effects of BCM7 due to the increased permeability of their intestinal epithelium. BCM7 can also have immunosuppressive effects, as it was able to inhibit human intestinal lymphocyte proliferation *in vitro* [257]. Some effects of BCM7, such as increased mucin secretion by goblet cells, could be beneficial in IBD, while others could be harmful.

Few studies have investigated the anti-inflammatory effects of whole or skimmed milk powder in animal models of IBD. Sheep milk has been shown to decrease histological injury when added at 11% (*w/w*) to the diet of rats with DSS-induced colitis [170]. As well as reduced histological injury, rats had increased caecal concentrations of butyric and propionic acids [170], which have anti-inflammatory activity in the colon [258]. At 56% (*w/w*) sheep milk solids in the diet, no beneficial effect was seen in the colon or in caecal short-chain fatty acid concentrations. Caecal digesta concentrations of beneficial microbial populations (lactobacilli, bifidobacteria and clostridia) were increased however, and the concentrations of potentially harmful bacteria (coliforms) were decreased, suggesting the benefits of sheep milk during DSS-induced colonic inflammation may be due to alterations in the composition of the caecal microbiota [170]. In contrast, *Il10*$^{-/-}$ mice fed skimmed cow and goat milk-based diets lost weight, developed diarrhoea, had no change in colitis severity as measured by histopathology, and had increased colonic mRNA levels of some inflammation-related genes compared to *Il10*$^{-/-}$ mice fed a soy protein-based diet [236].

1.4.6 Anti-inflammatory effects of specific milk components

1.4.6.1 Milk protein

Bovine milk contains a number of proteins with biological activities (including antimicrobial action and facilitation of nutrient absorption) as well as growth factors, hormones, enzymes, antibodies and immune stimulants. Bovine milk protein is approximately 80% casein and 20% whey. The bioactivities of whey and casein proteins and related peptides have been extensively investigated [259]. Casein binds calcium and phosphate, and clots in the stomach in order to facilitate digestion. Whey contains more water-soluble proteins, such as β-lactoglobulin, α-lactalbumin, bovine serum albumin and immunoglobulins. The value of bovine whey as a source of biologically active molecules for humans (when consumed as food ingredients) has been reviewed elsewhere [260]. Diets containing cheese powder and cheese whey protein alleviate DSS colitis in mice and rats [261, 262].

Some milk proteins are thought to be resistant to digestive enzymes present in the saliva, stomach, and pancreatic secretions, enabling the protein (or peptides derived from it) to retain function in the small intestine before being fully digested [189, 260]. Growth-promoting activity of the whey fraction of bovine milk has been observed in human foetal intestinal cells [263]. Changes in the concentrations of several growth factors were probably responsible for this effect because the changes in concentrations of TGF (transforming growth factor)-β, IGF (insulin-like growth factor)-I and IGF-binding proteins did not fully explain the results. Lactoferrin (a whey protein) has been shown to provide protection against T_H1 and T_H2-induced diseases via correction of T_H1/T_H2 imbalance [264], and specifically to mediate anti-inflammatory effects in DSS-induced colitis when given orally to mice [169]. Faecal lactoferrin is a sensitive and specific biomarker for the severity of intestinal inflammation, particularly in that of IBD, as faecal lactoferrin levels rise with increased neutrophil influx in the intestine [265, 266].

Transforming growth factor-β2 (TGF-β2) from bovine milk is thought to be responsible for the anti-inflammatory effects of Modulen IBD, an enteral polymeric diet developed by Nestlé (Vevey, Switzerland) to provide complete nutrition for IBD patients [267-272]. One study investigated whether Modulen could attenuate local and systemic

inflammation in a mouse model of IBD (*Il10*$^{-/-}$ mice on BALB/c background) compared to a standard enteral formula (with similar composition except it contained negligible amounts of TGF-β2) [272]. Body weight and haematocrit were higher in the Modulen-fed group at the end of the study. Colon histological injury scores, serum amyloid A and TNFα concentrations were lower, indicating that the Modulen diet had local and systemic anti-inflammatory effects [272]. Besides containing casein and whey proteins from bovine milk, Modulen is also higher in certain types of fat, such as medium-chain triglycerides, which are also at a higher concentration in goat and sheep milk than in cow milk, and are thought to improve digestibility and energy availability [193, 202, 205, 213].

Another milk protein having anti-inflammatory activity is milk fat globule epidermal growth factor-8 protein (MFG-E8) [273-275]. MFG-E8 plays an important role in the maintenance of intestinal epithelial homeostasis and the promotion of mucosal healing [276] and ameliorates TNBS and DSS-induced colitis in mice when administered intrarectally [273-275]. MFG-E8 also inhibits flagellin-mediated production of inflammatory cytokines in cultured intestinal epithelial cells by modulating NFκB activation, and modulates lipopolysaccharide-induced innate immune responses in macrophages [274]. These findings provide evidence of the role intestinal epithelial cells have in defence against bacterial pathogens during intestinal inflammation.

1.4.6.2 Milk fat

The health effects of milk fat have been reviewed previously [189]. Milk fat has a number of activities in the intestine and is also important for delivery of fat-soluble vitamins [239]. Reviews of the role of dairy product consumption in patients with cardiovascular disease suggest that there is no clear association between intake of dairy products and increased risk of cardiovascular disease, despite its high saturated fat content, perhaps due to the interaction of multiple factors in milk [277, 278]. Capric (also known as decanoic acid, 10:0) and lauric acid (dodecanoic acid, 12:0) are saturated fatty acids found in bovine milk. They are reported to inhibit cyclooxygenase enzymes I and II, which metabolise arachidonic acid (20:4, omega-6, written n-6) to produce pro-inflammatory eicosanoids [189]. Medium-chain triglycerides, which are present in higher concentrations in goat milk than cow milk, prevent TNBS-induced colitis in rats [279, 280] and also reduce the incidence of colitis in *Il10*$^{-/-}$ mice [168].

Unsaturated fatty acids in bovine milk include a number of polyunsaturated fatty acids (PUFA), the main ones being linoleic and α-linolenic acids [189]. Linoleic (18:2, omega-6, written n-6) and α-linolenic (18:3, n-3) acids can be converted into arachidonic acid and eicosapentaenoic acid (20:5, n-3) respectively, which can in turn be converted into metabolically active eicosanoids, compounds with a number of local functions including effects on platelet aggregation: prostaglandins, prostacyclins, thromboxanes and leukotrienes [189]. n-3 PUFA have anti-inflammatory properties: both n-6 and n-3 PUFA are required in the diet but the ratio n-6:n-3 should be 2:1 or lower, although the optimal ratio (from a therapeutic point of view) may vary with the disease [281]. For example, in one experiment using *Il10*$^{-/-}$ mice, dietary arachidonic acid (an n-6 fatty acid) was not pro-inflammatory and reduced endoplasmic reticulum stress, protecting colon enterocytes from oxidative stress [282]. Bovine milk has a low n-6:n-3 ratio compared to other non-marine foods [189, 283]. A decreased relapse rate for patients taking n-3 supplements has been shown in CD [284]. Eicosapentaenoic acid can block the conversion of n-6 PUFA into potentially harmful eicosanoids, and reduced the severity of colon inflammation compared to oleic acid (18:1, cis-9, n-9) when included in the diet of *Il10*$^{-/-}$ mice [118].

Bovine milk is an important dietary source of conjugated linoleic acid (CLA). CLA comprises about 0.7% of total fatty acids of whole bovine milk [285]. CLA is closely related to linoleic acid (which is 18:2, n-6, with double bonds at carbons 9 and 12) but has its between-carbon double bonds separated from each other by one single between-carbon bond. CLA has a number of isomers, not all of which have the same health effects. One of the most abundant isomers, with known anticancer properties, is the cis-9, trans-11 form. The conjugated nature of CLA confers chemical properties with different biological effects than linoleic acid (anti-inflammatory versus pro-inflammatory). CLA has immunomodulatory properties, confers protection against atherosclerosis and diabetes [286], has anticarcinogenic activity, and can beneficially modulate plasma cholesterol [189]. Milk also contains some vaccenic acid (18:1, trans-11, n-7), a trans-fatty acid that may have typical adverse trans-fatty-acid effects on blood lipids, but can also be converted into CLA [189]. Vaccenic acid comprises about 2-4% of the total fatty acids in milk from grass-fed cows [189]. CLA reduced colon inflammation and altered colonic peroxisome proliferator-activated receptor-responsive gene expression in a DSS pig model of IBD [159, 287, 288].

1.4.6.3 Milk carbohydrate

Milk carbohydrate consists of a mixture of small carbohydrate molecules (mono- and disaccharides, which are also known as sugars) and longer oligosaccharides. The predominant form of carbohydrate is the disaccharide lactose. All of these are simple sugars or chains (polymers) of simple sugars, and are used for energy by the newborn, but oligosaccharides in particular have other activities in the intestine.

Oligosaccharides probably exert indirect anti-infective effects via prebiotic activity (promoting the growth of beneficial bacteria in the large intestine) as well as competitively inhibiting the binding of pathogenic microbes to their target colon epithelial cells [289]. Human milk contains lactose-derived oligosaccharides, which are generally resistant to enzymatic hydrolysis and reach the colon intact, where they undergo bacterial fermentation. However, some intact oligosaccharides may be absorbed by the small intestine and subsequently inhibit leukocyte adhesion in a concentration-dependent manner [290]. Neutrally-charged human milk oligosaccharides are transported across the small intestinal epithelium by receptor-mediated transcytosis and paracellular pathways, whereas acidic human milk oligosaccharides cross via the paracellular route only [290]. These oligosaccharides may also be anti-inflammatory and contribute to a lower incidence of inflammatory disease in milk-fed infants [290].

The milk and/or colostrum of a number of domestic farm animals (including cows) also contains a number of acidic and neutral oligosaccharides, though the composition differs among species and stage of lactation. Goat milk has higher oligosaccharide content than bovine milk (Table 1.5). Oligosaccharides from goat milk reduced intestinal inflammation in DSS and TNBS-induced rat models of colitis [68, 69]. When some cellulose was substituted for oligosaccharides in the diet of adult male Sprague-Dawley rats with DSS-induced colitis (20 g per kg of diet), the expression levels of some genes including immune-related genes, growth factors, mucins and alkaline phosphatase (a marker of inflammation) were normalised. There were increased numbers of lactobacilli and bifidobacteria and decreased bacteroides, enterobacteria and coliforms in the faeces of oligosaccharide-supplemented rats [68].

In TNBS-induced colitis, oral dosing with goat milk oligosaccharides (500 mg per kg body weight per day, containing 5% lactose and virtually no salts) resulted in increased

weight gain and improved macroscopic appearance of the intestine in female Wistar rats approximately 8 weeks old (~200 g body weight), and a lower abundance of the pro-inflammatory inducible nitric oxide synthase and cyclo-oxygenase-2 proteins, and reduced expression level of the IL1β gene, demonstrating that the goat oligosaccharides had anti-inflammatory actions in the colon [69].

1.4.6.4 Milk antioxidants

Milk is a good source of antioxidants, with goat milk being particularly high in cysteine. Oxidant-mediated cellular injury appears to be important in the pathogenesis of IBD, as high levels of reactive oxygen species are implicated in tissue destruction, and oxidant exposure has been shown to damage enterocytes [171]. In IBD patients, regions of inflamed mucosal tissue contain many activated macrophages and neutrophils, which generate excessive amounts of reactive oxygen species. Milk diets inhibited oxidative and inflammatory stress in obese mice and humans [291].

Glutathione is required for normal intestinal function, as deficiency results in severe degeneration of the epithelial cells of the jejunum and colon [292]. In a chemically induced rat model of colitis, glutathione precursors attenuated colitis [292, 293]. In BALB/C male mice with DSS-induced colitis, three antioxidants (S-adenosylmethionine (a glutathione precursor), green tea polyphenols, and 2(R,S)-n-propylthiazolidine-4(R)-carboxylic acid (a cysteine prodrug involved in glutathione biosynthesis) reduced disease activity (each to a similar extent) with improvements in body weight, haematocrit, serum amyloid A, colon length, serum TNF-α, colonic and blood glutathione, and cytoskeletal architecture and lesions of the intestinal epithelium [171, 294]. These studies demonstrate the value of antioxidants for colitis, and while milk does not contain high concentrations of the particular antioxidants studied, it contains others that might have similar effects.

1.5 Conclusions and future perspectives

The pathogenesis of IBD is complex, and despite a large body of research, still poorly defined. It involves numerous factors, including diet, host genetic susceptibility, composition of the intestinal microbiota, function of the immune system in the intestinal mucosa, defects in the epithelial barrier, and epigenetic factors. Changes in gene

expression in affected tissue from mouse models of IBD can be used as an indicator of the net effect of these factors. There is much research into the role of the epithelium in intestinal inflammation, and on gene expression changes in inflamed IBD tissues, but as yet few studies report the changes in gene expression that occur in the intestinal epithelium in inflammation, which may further understanding of the epithelial signalling pathways involved in IBD pathogenesis.

Research into the role of epigenetic factors, which regulate gene expression, suggests that DNA methylation may be important in IBD. As epigenetic factors ultimately impact on gene expression, DNA methylation may be one mechanism by which the gene expression changes seen in inflamed IBD tissue occur. There is as yet little research into DNA methylation changes in mouse models of IBD and how they relate to the gene expression changes seen in intestinal inflammation. As DNA methylation is known to be affected by dietary factors, this is an important area to consider when elucidating the impact of dietary components such as milk on intestinal inflammation.

For IBD patients who can tolerate dairy products, milk may provide valuable nutritional support. Some components of milk may also have beneficial actions in the intestine, although these components may not be present in high enough concentrations in whole milk to provide substantial benefits. There is a large body of research into the general health benefits of milk and its components, but little research into potential health benefits for intestinal inflammation. The impact of whole milk on human health and the functionality of individual components needs to be investigated further to improve the benefits of milk components and reduce the potential adverse effects of these in intestinal inflammation.

1.6 Aims, approach and thesis structure

The first two main aims of this research are to investigate epigenetic and gene expression changes in the early and late stages of colon inflammation in the *Il10*$^{-/-}$ mouse model of IBD to:

1. Determine whether microdissection and subsequent microarray analysis of colon epithelial cells would enable the identification of pathways and processes related

to the inflammatory response that are not detected by microarray analysis of intact colon.

2. Investigate whether changes in global and gene-specific DNA methylation occur in a mouse model of IBD and could have a role in regulating the changes in colon gene expression profiles that occur in inflammation.

The third main aim of this research is to:

3. Investigate whether diets containing ruminant sheep and goat milk solids, or a milk substitute (soy "milk" solids), reduce inflammation and alter associated molecular pathways in the colon mucosa in a mouse model of IBD when compared to a diet containing cow milk solids, applying findings from Aims 1 and 2 where appropriate.

Prior to performing a dietary intervention experiment to assess the effects of milk and soy diets on colitis in a mouse model of IBD (the interleukin 10 gene-deficient or *Il10*$^{-/-}$ mouse), a mouse experiment was carried out to investigate the use of two types of analysis for gaining understanding of the molecular changes that occur in colitis. These methods could then be applied to future dietary intervention experiments in mouse models of IBD in order to further understand the molecular changes that occur in response to dietary components and how they may modify the pathophysiology of colitis.

First, to determine whether microdissection and subsequent microarray analysis of colon epithelial cells could be of value when studying the molecular pathways involved in colitis, a time-course experiment was performed in *Il10*$^{-/-}$ mice to describe gene expression changes before and after the development of intestinal inflammation in whole colon tissue and epithelial cell fractions (**Chapter 3**).

Secondly, to determine whether analysis of epigenetic mechanisms could provide insight into the mechanisms by which gene expression is altered in colitis, colon tissues from the time-course experiment were used to investigate the involvement of DNA methylation in the development of inflammation in *Il10*$^{-/-}$ mice (**Chapter 4**).

Thirdly, to further understand the effects of milk-based diets on colonic inflammation, a dietary intervention experiment was performed in *Il10*$^{-/-}$ mice. *Il10*$^{-/-}$ mice and C57BL/6 mice were fed a soy-based diet, or one of three ruminant milk-based diets and the severity of colitis and colon gene expression profiles assessed (**Chapter 5**). Techniques used in Chapters 3 and 4 were intended to be applied to tissues derived from the milk diet experiment where appropriate in order to further understand the mechanisms underpinning the effects of milk-based diets on colitis.

In **Chapter 6**, the main findings have been summarised and discussed. An outline of the thesis and experimental chapters is shown in Figure 1.7.

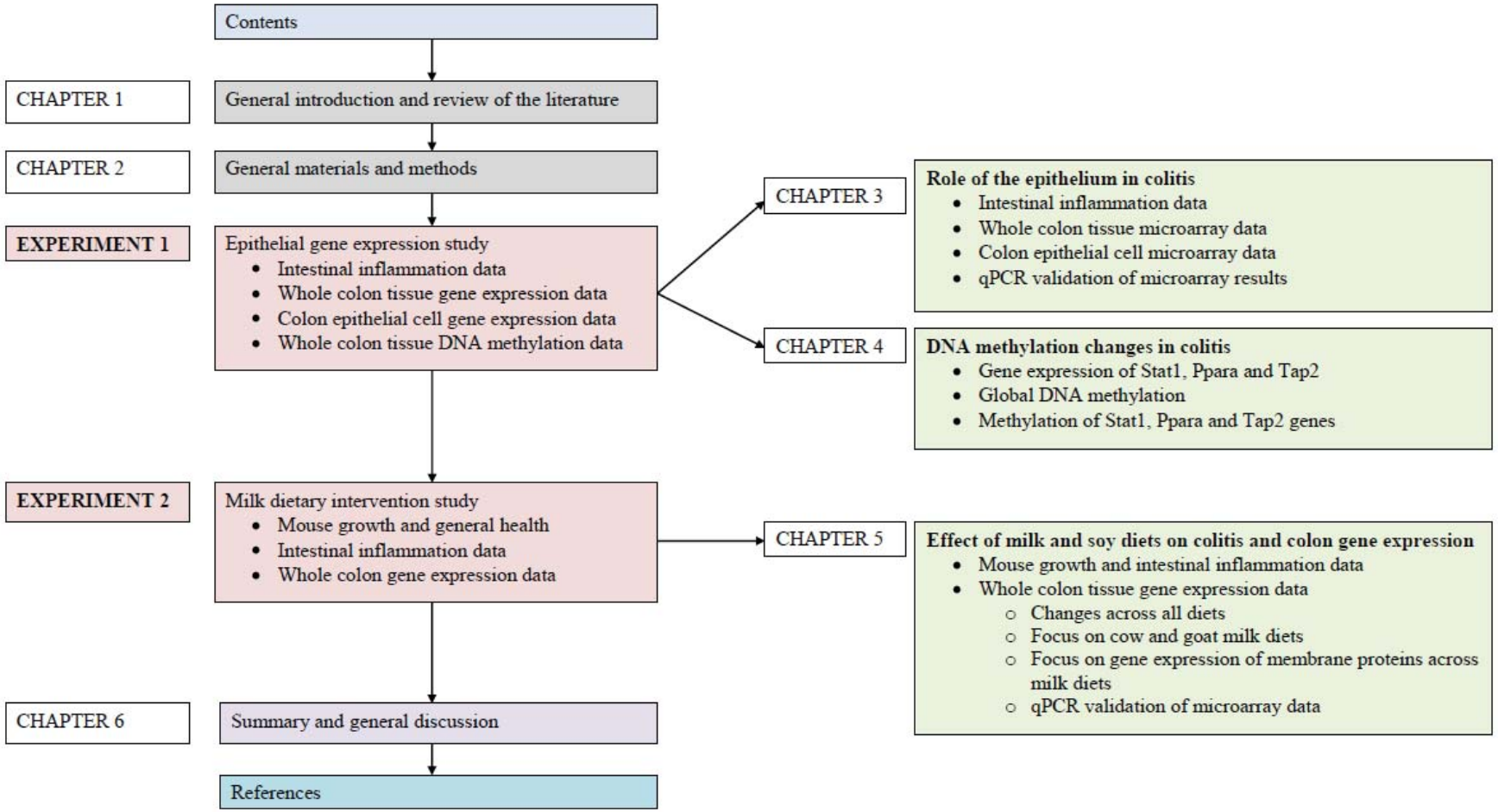


Figure 1.7 Thesis outline. Overall structure of the thesis and the experiments described within it.

2 Methods

2.1 Introduction

Two mouse experiments were conducted to fulfil the aims and objectives outlined in the introductory chapter. Colon tissues from these mice were then used for histology analysis, gene expression analysis, laser microdissection and DNA methylation analysis. Tissues were also collected for protein expression analysis and protein extracted, however the protein data is not presented in this thesis. All methods for the mouse experiments and subsequent molecular analyses are described in this chapter.

2.2 Mouse experiments

Two mouse experiments were carried out as part of this research. One experiment, the "time-course experiment" (Chapters 3 and 4) was carried out to investigate colon gene expression and DNA methylation in *Il10*$^{-/-}$ mice pre- and post-inflammation. The other experiment, the "milk diet experiment" (Chapter 5) was carried out to determine whether ruminant milks could modify the development of colitis in *Il10*$^{-/-}$ mice, and if so, to investigate colon gene expression changes to gain insight into the mechanisms by which the diets impacted on molecular pathways and signalling in the colon.

2.2.1 Experimental design

Both experiments were conducted according to a factorial design (Figure 2.1), with two strains of mice (*Il10*$^{-/-}$ mice, the IBD model, and C57BL/6J mice, the control strain) and either two time points (pre- and post-inflammation) or four milk-based and two control diets. Experiments were approved by the Ruakura (time-course experiment; application 11343) or Grasslands (milk diet experiment; application 11562) Animal Ethics Committees, and carried out in accordance with the New Zealand Animal Welfare Act 1999.

2.2.2 Mice

The *Il10*$^{-/-}$ mice used are designated B6.129P2-*Il10*^{tm1Cgn}/J. These mice and the background strain, C57BL/6J, were sourced from the Jackson Laboratory, Maine, USA [81], imported under MAF Import Permits (time-course experiment permit number 2007032320; milk diet experiment permit numbers 200903-6837, 7029, and 7151) and ERMA approval GMC001197.

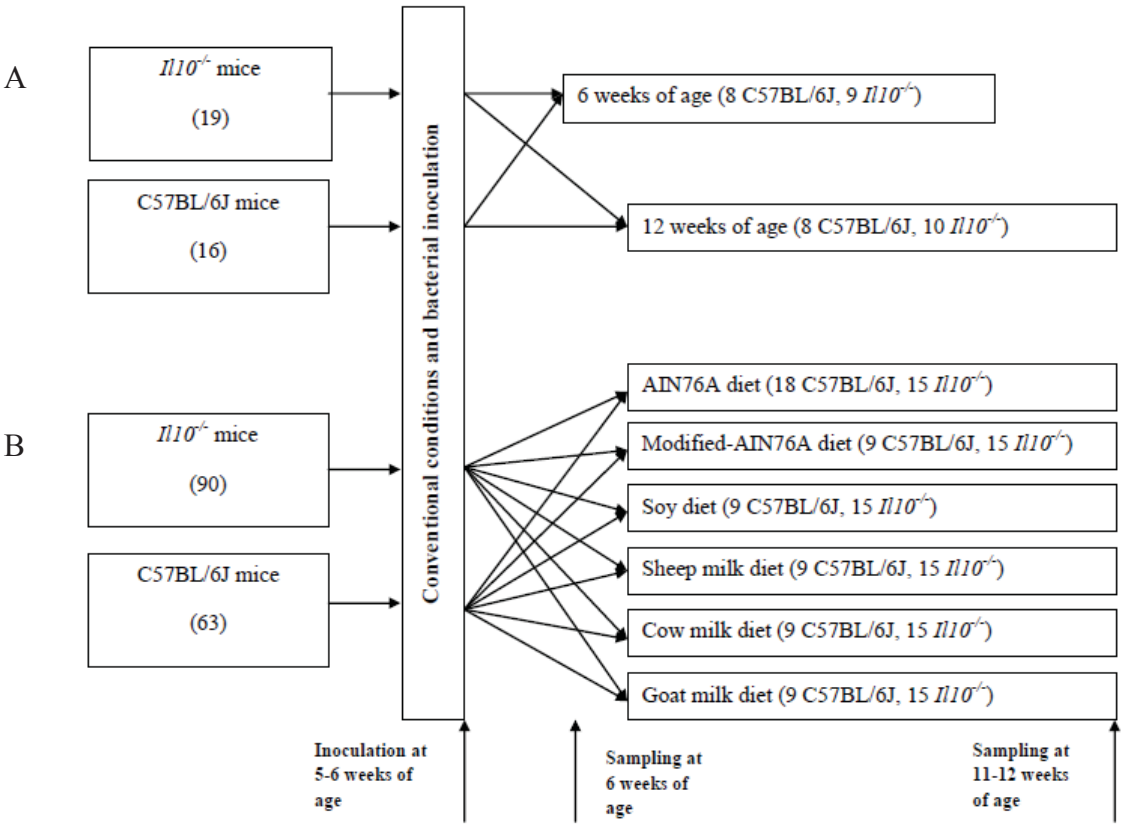


Figure 2.1 Experimental designs for mouse experiments. Designs of the (A) time-course experiment and (B) milk diet experiment are shown. Mice were housed in conventional conditions upon arrival into the facility, and bacterial inoculation occurred within the following week.

The mice were kept in SPF conditions at the Jackson Laboratory and then transferred into conventional conditions upon arrival at the Small Animal Colony, AgResearch Ruakura (time-course experiment) or the Ulyatt-Reid Small Animal Facility, AgResearch Grasslands (milk diet experiment) at approximately 4-5 weeks of age.

2.2.2.1 Time-course experiment

Nineteen *Il10*$^{-/-}$ mice (C57BL/6J background) were purchased from the Jackson Laboratory (Maine, USA) and 16 control C57BL/6JArc mice were obtained from the Small Animal Colony (AgResearch Ruakura, Hamilton, New Zealand). The C57BL/6J mice for this experiment were originally to be obtained from the Jackson Laboratory, but due to difficulties with shipping, replacements had to be obtained in New Zealand at the beginning of the experiment. The data for age and number of animals per treatment are shown in Table 2.1.

2.2.2.2 Milk diet experiment

All mice were sourced from the Jackson Laboratory (Maine, USA) at 4 weeks of age. This experiment was carried out in three identical blocks due to limitations of mouse availability from the supplier. Each diet group (per block) consisted of *Il10*$^{-/-}$ (n = 5) and C57BL/6J mice (n = 3), giving a total of n = 15 *Il10*$^{-/-}$ mice and n = 9 C57BL/6J mice per treatment. More C57BL/6J mice (n = 6) were included in the AIN-76A diet group (giving a total of 18) in order to enable between-batch comparisons using the C57BL/6J mice and diet. For blocks 1 and 2, all mice were 29 days ± 3 days of age on arrival, while the mice in block 3 were 30 days ± 3 days of age on arrival because they arrived one day later due to shipping issues. All mice were 11 weeks old at sampling (+ 0-2 days due to the three-day sampling period) except for three mice sampled early for welfare reasons.

2.2.3 Diet

Fresh pre-weighed food was provided and refusals measured 3-4 times weekly. Five grams of food per day was provided throughout the experiments, which typically meant 10 g was provided every two days. The mice always ate less than 5 grams per day, therefore this was sufficient to provide *ad libitum* intake throughout the study.

Table 2.1 Mouse data at the beginning of the time-course experiment.

Strain	Age at sampling	Number	Date of Birth	Experiment start day (1st weight measurement)	Age at beginning of experiment	Age at sampling
Pre-inflammation						
Il10$^{-/-}$	6 weeks	9	25/09/2007 ± 3 days	29/10/2007 (= Day 1)	32 ± 5 days	35 ± 5 days (3 days after inoculation)
C57BL/6J	6 weeks	8	3 x 27/09/2007 5 x 10-24/09/2007	30/10/2007 (= Day 2)	39 ± 7 days	42 ± 7 days
Post-inflammation						
Il10$^{-/-}$	12 weeks	10	25/09/2007 ± 3 days	29/10/2007	32 ± 5 days	70 ±3 days (38 days after inoculation)
C57BL/6J	12 weeks	8	1 x 27/09/2007 7 x 10-24/09/2007	30/10/2007	39 ± 7 days	77 ± 7 days
Mice used for harvesting of conventional intestinal flora for inoculation on 30/10/2007						
C57BL/6J	6 weeks	3	10-24/09/2007	-	-	42 ± 7 days

2.2.3.1 Time-course experiment

All mice were fed non-sterile powdered AIN-76A diet which was made in-house at Crop & Food Research, Palmerston North, New Zealand (NZ) [295-297]. This diet was used for two reasons: (1) to ensure that development of inflammation followed the expected course [115]; and (2) to enable comparison between both experiments. AIN-76A composition is shown in Table 2.2.

2.2.3.2 Milk diet experiment

Sheep, goat, cow and soy solids were incorporated into modified AIN-76A diets, at a concentration of 40% milk solids by weight. A milk and soy-free semi-purified rodent diet (also based on AIN-76A) was used as a control. Diets were formulated by Research Diets, Inc. (New Jersey, USA) to ensure accurate formulation and to allow preparation of the diets in pellet form. The macronutrient composition of the milk solids was analysed prior to diet formulation, to determine total fat, protein, and carbohydrate, caloric value, moisture, ash, and total and available lysine.

An AIN-76A diet was included for comparison with other in-house experiments using this mouse model, and confirmed that the *Il10*$^{-/-}$ mouse model worked as expected, and a modified-AIN-76A diet was included as a composition-control for the milk and soy diets. The milk, soy and modified-AIN-76A diets had the same amounts of fibre, vitamins and minerals incorporated. Total protein, fat and carbohydrate levels were kept as consistent as possible across all diets by adjusting the basal AIN-76A components as necessary. Energy per gram of diet was between 18.1 and 19.1 kJ for the milk and modified-AIN-76A diets. The soy diet was lower in energy (16.6 kJ, similar to the 16.3 kJ of the AIN-76A diet), due in part to the fibre content of the soy powder.

2.2.3.2.1 Diet formulation considerations

Diets enriched with milk components have been fed to both rats and mice in published studies [68, 84, 171, 272, 279]. However, there is limited data on the effects of feeding whole milk in rodent models of chronic inflammation [170, 236]. This made it difficult to determine how the diets were to be formulated. Whole milk solids were incorporated into the base AIN-76A diet at a level that provided up to 20% (*w/w*) protein (required

Table 2.2 Composition of experimental diets.

	AIN-76A		**Modified-AIN-76A**		**Soy diet**		**Sheep milk diet**		**Cow milk diet**		**Goat milk diet**	
	g	kcal	g	kcal	g	kcal	g	kcal	g	kcal	g	kcal
Component (%)												
Protein	20	21	22	21	21	20	22	21	22	21	21	21
Carbohydrate	66	68	54	50	50	50	50	46	51	48	53	52
Fat	5	12	14	30	13	30	16	33	15	31	12	27
Total		100		100		100		100		100		100
Calculated energy (kJ/g)	16.3		18.1		16.6		19.1		18.5		18.0	
Test component												
Soy powder	0	0	0	0	400	1680	0	0	0	0	0	0
Goat milk powder	0	0	0	0	0	0	0	0	0	0	400	2236
Cow (A1) milk powder	0	0	0	0	0	0	0	0	400	2350	0	0
Sheep milk powder	0	0	0	0	0	0	400	2476	0	0	0	0
Protein												
Casein[1]	200	800	0	0	0	0	0	0	0	0	0	0
DL-Methionine[2]	3	12	0	0	0	0	0	0	0	0	0	0
Egg whites (dried)	0	0	200	800	58.9	235.6	72.6	290.4	101	404	93	372
Protein (from milk)	0	0	0	0	0	0	127.4	509.6	99	396	108.8	435.2
Protein (from Soy, NOW)	0	0	0	0	141	564	0	0	0	0	0	0
Carbohydrate												
Cornstarch[3]	150	600	248	992	137.3	549.2	117.2	468.8	103	412	139	556

Maltodextrin 10	0	0	75	300	75	300	75	300	75	300	75	300
Sucrose[4]	500	2000	150	600	150	600	150	600	150	600	150	600
Carbohydrate (from milk)	0	0	0	0	0	0	99.8	399.2	131.6	526.4	131.4	525.6
Carbohydrate (from Soy, NOW)	0	0	0	0	114.5	458	0	0	0	0	0	0
Fat												
Corn Oil[5]	50	450	130	1170	49	441	0	0	0	0	0	0
Fat (from milk)	0	0	0	0	0	0	143.8	1294	136	1224	119.2	1073
Fat (from Soy, NOW)	0	0	0	0	79.3	713.7	0	0	0	0	0	0
Other												
Mineral Mix[6]	35	0	35	0	35	0	35	0	35	0	35	0
Vitamin Mix[6]	10	40	10	40	10	40	10	40	10	40	10	40
Choline Bitartrate[2]	2	0	2	0	2	0	2	0	2	0	2	0
Biotin, 1%	0	0	0.4	0	0.4	0	0.4	0	0.4	0	0.4	0
Cellulose[7]	50	0	50	0	50	0	50	0	50	0	50	0
Ash weight	0	0	0	0	0	0	19.6	0	21.4	0	28	0
Moisture weight	0	0	0	0	0	0	9.4	0	12	0	12.6	0
Fibre (in 400 g soy)	0	0	0	0	44	0	0	0	0	0	0	0
Sodium (in 400 g soy)	0	0	0	0	7.9	0	0	0	0	0	0	0
Soy weight unaccounted for	0	0	0	0	13.3	0	0	0	0	0	0	0
Total	1000	3902	900.45	3902	967.7	3846	912.25	4175	926.45	4106	954.45	4104

All components except the soy and milk powders were sourced from Research Diets, Inc. (New Jersey, USA) for the milk diet experiment. Milk and soy products were sourced from: Blue River Dairy Products Ltd., Invercargill, NZ; Dairy Goat Co-operative (NZ) Ltd., Hamilton, NZ; Ruakura Research Centre, AgResearch Ltd., Hamilton, NZ; NOW Foods, Bloomingdale, Illinois, USA. Supplier information is provided for the time-course experiment according to the superscripts given in the table: [1]Alacid, lactic casein 30 mesh, NZMP Ltd., Wellington, NZ; [2] Sigma-Aldrich Inc., St. Louis, MO, USA; [3] Golden Harvest, Primary Foods Ltd., Auckland, NZ; [4] Chelsea, New Zealand Sugar Company Ltd., Auckland, NZ; [5] Tradewinds, Davis Trading, Palmerston North, NZ; [6] Bio-Serv, Frenchtown, New Jersey, USA; [7] Arbocell B600, J. Rettenmeier & Sohne Gmbh, Rosenberg, Germany. Weights do not add to 1000 g because the diet composition was calculated in terms of energy (as close as possible to 3902 kJ).

for mouse growth) and kept the fat content less than or equal to 20% (*w/w*) to avoid possible negative effects of high fat diets on colitis. The aim was to use the highest possible dose as it was unknown at what level of incorporation milk solids would exert their effects, if any. The modified-AIN-76A diet was used to control for altered composition of the milk diets, while the AIN-76A diet was used as an in-house control for inflammation severity between experiments using this mouse model.

The milks each had different macronutrient compositions (i.e. different proportions of solids, protein, fat, carbohydrate), therefore balancing the diets required careful consideration about what features the diets were to be balanced for in order to test the differences in overall composition on development of colitis. It was decided to balance total protein, fat and carbohydrate, using a percentage of milk solids that provided the maximum desired amount of fat. The remaining protein and carbohydrate that was required to balance the diets was supplemented from the same source that was used in the modified-AIN-76A control diet. This ensured that the composition or profile of each macronutrient from milk/soy was tested rather than the proportions of each macronutrient, which would be better tested using purified diets. The same amount of the standard AIN-76A vitamin and mineral mix was added to each diet, allowing the differences in vitamin and mineral content in the milk solids to remain in the diets.

The high fat content of whole milk solids posed a problem for diet formulation. The fat content of AIN-76A is 5% (*w/w*), and in order to keep fat at this level, diets could contain only about 15% milk solids (assuming a typical milk solids fat content of 30%). Diets higher than 20% fat may exacerbate colitis [170]. The target fat content of the diets in this experiment was therefore 20% or less. The type of fat used in the diets could affect the course of inflammation, so it was decided to use corn oil in the modified-AIN-76A diet to be consistent with the AIN-76A diet, and add a percentage of solids to the milk/soy diets that kept the final fat content similar without making any corrections to the fat content by adding an extra fat source.

A protein source that did not contain milk or soy components was required for the modified-AIN-76A diet, and overall, egg white appeared to be the best choice. Like milk solids, dried whole egg and powdered beef consist of less than 50% protein and significant amounts of fat, making them a poor choice for a protein source in a semi-purified diet. Dried egg white, however, consists of over 80% protein and negligible fat,

so is much closer to the composition of casein. Beef, egg and soy products also have potential biological or nutritional effects that may not be well-defined. For example, beef promotes intestinal non-haeme iron absorption, egg white has been shown to promote non-haeme iron absorption at an intermediate level while soy protein inhibits absorption [298]. Soy protein was not a suitable alternative protein source for the control diet as it may have anti-inflammatory effects [231]. Amino acid mixtures of similar composition to the bovine casein can be substituted in the AIN-76A diet; however, this would not be appropriate as a control for a whole protein source because feeding free amino acids does not support whole-body protein homeostasis as well as the corresponding casein diet, which could affect intestinal function and gene expression [299]. Final diet composition is shown in Table 2.2.

2.2.3.2.2 Final diet details

Soy milk powder was sourced from NOW® Foods (Bloomingdale, Illinois, USA). This soy milk powder was made from whole organic soybeans. The sheep and goat whole milk powder was supplied by Blue River Dairy Products Limited (Invercargill, NZ) and the Dairy Goat Co-operative (NZ) Ltd, respectively. Bovine milk was collected as close to peak lactation as was possible (November-December 2008) from dairy cows at the Ruakura Research Centre (AgResearch Ltd, Hamilton, NZ) and dried in one block sufficient for possible follow-up experiments (resulting in approximately 8-12 kg of dried milk powder). These precautions ensured that variation in milk composition according to breed, species [300], stage of lactation, nutrition and health status [199] was minimised.

Cow milk with pure β-casein was used to remove β-casein variability. Using A1 cow milk meant that the milk composition was defined and replicable with respect to β-casein variants. The likelihood of release of the heptapeptide β-casomorphin-7 (BCM7) is lower in cow A2, sheep and goat milk due to an amino acid substitution at β-casein sequence position 67 [252].

All milk solids were prepared by pasteurisation, homogenisation and spray-drying, which is the usual method for preparing milk powder for human consumption [301] and shipped from New Zealand to Research Diets, Inc. (New Jersey, USA). All diets were formulated by Research Diets Inc., colour-coded then pelleted using a cold extrusion

process (in order to avoid heat-related changes in diet constituents). Internal temperatures may reach 18-32ºC for a few seconds during this process. Pellets were then dried at 18ºC in the dark for two days. Upon arrival, diets were stored (air-tight) at room temperature in a cool dry vacuum until fed (in order to maintain the dryness of the milk powder component).

Milk composition was tested by the Nutrition Laboratory at Massey University (Palmerston North, NZ) for protein, fat, energy and carbohydrate content prior to diet formulation, and similarly, the composition of each diet, including lactose content, was tested at the end of the experiment by AsureQuality (Auckland, NZ) to check the formulation accuracy.

2.2.4 Housing

Mice were housed in conventional conditions, in a room with 12 hour light/dark cycle, and controlled temperature (22 ± 1 °C) and humidity (approximately 50%). Mice were contained in shoebox-style polycarbonate cages with wire lids, lined with recycled paper litter with tissue for nest building and plastic tube or hut for environmental enrichment. Water was provided *ad libitum* (refreshed weekly) and cages were cleaned and autoclaved weekly. Mice were checked every day for general appearance and behaviour, and disease symptoms (such as weight loss, diarrhoea, and inactivity) using the General Health Score (GHS) [302], and weighed 3-4 times weekly. Mice which reached a minimum welfare cut-off score assessed by observations of appearance and behaviour (GHS of 3 or higher out of 5; a score of 1 was considered healthy) were promptly euthanased.

2.2.5 Bacterial inoculation

Note: Gavage was performed by Ric Broadhurst (AgResearch Ruakura, Hamilton, NZ) for the time-course experiment, and by Leigh Ryan (AgResearch Grasslands, Palmerston North, NZ) for the milk diet experiment.

Mice were inoculated with a mixture of *Enterococcus* species [108] and conventional intestinal microbiota [75] at approximately 5 weeks of age in order to produce reliable and consistent levels of colon inflammation by 12 weeks of age [110]. Mice were inoculated via oral gavage using a 1 mL syringe with 22 gauge, 25 mm long gavage

needle with a small bend in the end to prevent the needle entering the trachea. Each mouse was given 200 μL of a 1:1 (*v/v*) mixture of each of an *E. faecalis/faecium* culture (EF) and complex intestinal flora (CIF) solution (which in the 1:1 combination are abbreviated to EF.CIF solution).

Enterococcus strains were subcultured onto fresh Slanetz and Bartley medium (Oxoid Ltd., Hampshire, UK), incubated at 42°C for 48 hours, transferred to Todd-Hewitt Broth (Oxoid Ltd., Hampshire, UK) and incubated at 37°C for 24 hours, then collected by centrifugation (3000 x g at 4°C for 10 minutes), resuspended in sterile 1 x phosphate buffered saline (PBS; pH = 7.30 ± 1.5) and pooled. The *Enteroccocus* solution contained (on average) approximately 8.5 x 10^{10} colony forming units per 100 μL, and consisted of 12 *E. faecalis* and *E. faecium* strains isolated from calves and chickens (AgResearch, Palmerston North, NZ).

The CIF solution was prepared from the digesta of 3 age-matched control mice (C57BL/6J) that were raised under conventional conditions in the Ruakura Small Animal Colony (AgResearch, Hamilton, NZ). Mice from this facility were used for each experiment to ensure consistency of the intestinal microbiota used for the inoculation. The intestinal tract (pyloric sphincter to caecum) was removed and digesta collected and suspended in sterile 1x PBS. This solution was immediately mixed in a 50:50 *v/v* combination with the *Enterococcus* culture solution and administered to the mice within 60 minutes. Mice were carefully monitored after the procedure for any signs of aspiration of gavage solution into the lungs or physical damage from the gavage procedure. No mice suffered any adverse effects.

2.2.6 Sampling

Note: Euthanasia and cardiac puncture was performed by Ric Broadhurst (AgResearch Ruakura, Hamilton, NZ) for the time-course experiment, and by Leigh Ryan (AgResearch Grasslands, Palmerston North, NZ) for the milk diet experiment.

The time-course of inflammation in *Il10*$^{-/-}$ mice (inoculated as described above) was previously investigated and it was demonstrated that colon inflammation increases from about 6 weeks of age and plateaus at 11 weeks of age [115]. Mice were euthanased at around 5-6 weeks for the pre-inflammation time-point (post-inoculation, no inflammation detected by histopathology) or 11-12 weeks for the maximal-

inflammation time-point. For the milk diet experiment, only the latter sampling time was used, and the mice were sampled approximately 6 weeks after inoculation, i.e. 11-12 weeks of age.

Immediately prior to sampling, mice were fasted for 14 hours overnight, re-fed for 2 hours and fasted again for 2 hours in order to minimise variation in food intake and gene expression due to differences in intake [303]. Each mouse was sampled over approximately 15 minutes so the fasting and re-feeding was staggered by one hour in groups of 4-5 mice.

Mice were weighed just prior to euthanasia via CO_2 asphyxiation and cervical dislocation. Blood was immediately collected by cardiac puncture (0.5 - 1 mL), using a 21G ½ inch (0.5 x 16mm) needle and 3mL syringe containing approximately 100µL of a 0.5 mole/L solution of ethylenediaminetetraacetic acid (EDTA), pH 8.0 (GIBCO 15575-038). The blood was transferred to a 2 mL centrifuge tube and centrifuged at 2,000 g for 3 minutes (at room temperature). Blood plasma (the supernatant) was transferred into separated tubes and snap-frozen in liquid nitrogen and stored at -80°C. Spleen, liver, thymus, kidneys, and skeletal muscle were snap-frozen in liquid nitrogen and stored at -80°C, however these samples (and plasma) were not used in the research presented here. The intestine was carefully removed and opened lengthwise while being rolled around a plastic 10 mL pipette, then flushed with cold 0.9% NaCl to remove digesta, laid out on ice cold stainless steel tray and sectioned.

The intestine of each mouse was sectioned as shown in Figure 2.2. Sections of small intestine (duodenum, jejunum, ileum) and colon were fixed in 10% phosphate buffered formalin for histology and stored at room temperature. One set of each tissue was taken for gene expression analysis and placed in 1 mL of RNAlater (Ambion Inc., Austin, Texas, USA) to improve preservation of RNA integrity, stored at 4°C overnight, and snap-frozen the next morning after removal of excess liquid for long-term storage at -80°C. Another set for each tissue type was snap-frozen in liquid nitrogen, then stored at -80°C, for analysis of DNA methylation. An intact section of each intestinal tissue was kept from the proximal end of each section for embedding in Optimal Cutting Temperature medium (Tissue-Tek OCT, Sakura Finetek U.S.A., Inc., Torrance, California, USA) and stored at -80°C for gene expression analysis of laser microdissected cells.

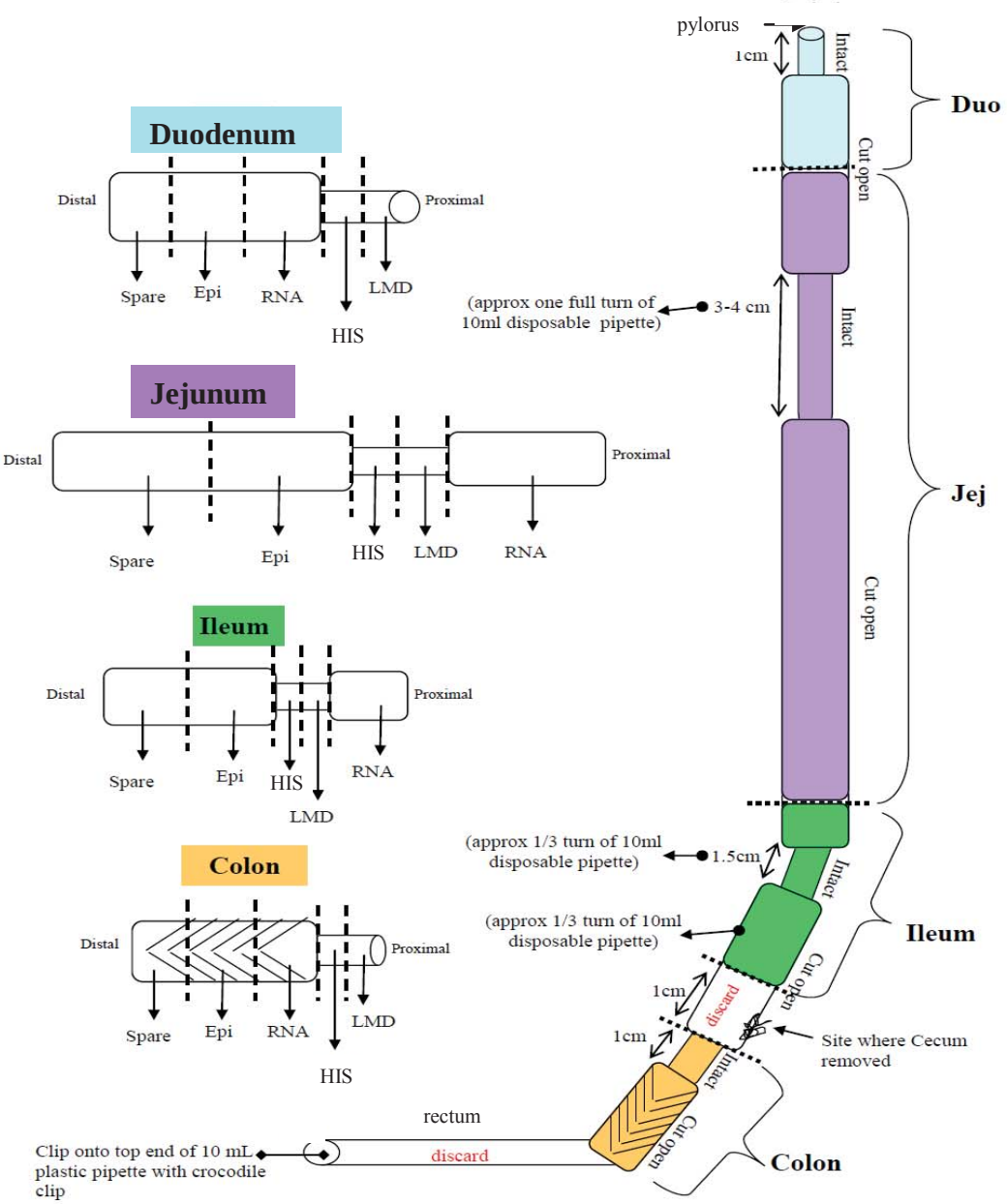


Figure 2.2 Intestinal sectioning. Diagram of mouse intestine (duodenum, jejunum, ileum and colon) showing how each one was sectioned into the samples taken for analysis of gene expression (RNA), DNA methylation (Epi), gene expression analysis of microdissected cells (LMD) and histological injury (HIS).

2.2.7 Evaluation of intestinal inflammation

2.2.7.1 Measurement of intestinal area

For the time-course experiment, the intestine of each mouse was photographed before sectioning as a secondary measure of intestinal inflammation. The gastrointestinal tract (GIT) was unrolled from the pipette onto an ice-cold stainless steel tray with attached ruler, to enable subsequent estimation of intestinal area using imaging software. Corel Paint Shop Pro Photo XI was used to prepare the images, and the images were analysed using ImageJ software [304]. Due to sampling time pressure, data was obtained for most mice at the 12-week sampling time, but only three for each strain at the 6-week sampling time. Observations of the appearance of the intestine were also recorded at sampling for both the time-course experiment and the dietary intervention experiment, as another indicator of the degree and location of intestinal inflammation in each mouse.

2.2.7.2 Histological injury score

Note: Scoring of sections was performed by William Zhu (University of Auckland, Auckland, NZ). For the milk diet experiment, Kelly Armstrong (AgResearch Grasslands, Palmerston North, NZ) also scored the sections.

Inflammation in all intestinal regions (with the exception of the caecum in the time-course experiment) was determined by histopathology. For each mouse, one (time-course experiment) or three (milk diet experiment) serial sections were scored for each tissue: duodenum, jejunum, ileum, caecum, and colon. Sectioning and staining to visualise cellular architecture was performed by histology laboratories at either the University of Auckland (time-course experiment) or Massey University (milk diet experiment). Tissues were fixed in 10% phosphate-buffered formaldehyde, dehydrated using a series of ethanol concentrations for 1 hour each (70%, 80%, 95%, 100%), incubated for 1 hour in xylene, embedded in paraffin, sectioned to 5 μm thickness, and placed on a glass slide. Sections were then deparaffinised with xylene, rehydrated with 100% and 95% ethanol, stained with haematoxylin for 3-5 minutes, rinsed, placed into 1% HCl in 70% ethanol, rinsed, placed into 95% ethanol, and stained with eosin for 1-4 minutes. Finally, they were dehydrated twice in 95% and 100% ethanol and xylene and enclosed in mounting medium (Permount) and placed on slides. The slides were examined by light microscopy.

For the time-course experiment, scores from 0-3 were assigned for each aspect of inflammation (0 = no change from normal tissue, 3 = lesions involve most areas and all layers including mucosa, muscle and omental fat). The characteristics scored were inflammatory cell infiltration (monocytes, neutrophils, fibrin exudation, lymphangiecstasis), tissue destruction (enterocyte loss, ballooning, oedema, mucosal atrophy), and tissue repair (hyperplasia, angiogenesis, granulomas). Total histological injury score (HIS) for each section = Σ ([inflammatory cell infiltration score] x 2) + [tissue destruction] + [tissue repair].

Scoring for the milk diet experiment was performed with adjustments made to the original method in an attempt to provide a more sensitive and informative scoring system adapted for research purposes (as opposed to a more clinical focus). Due to the low HIS scores in some inflamed *Il10*$^{-/-}$ mice in the time-course experiment, three, not one, sections were stained per intestinal region, and scoring represented an average or overall picture of the three sections. This was intended to reduce the risk of missing inflamed areas due to the patchy nature of the colitis. Scores from 0-10 were assigned for each aspect of inflammation (with increased number up to 10 reflecting increased deviation from normal, and 0 reflecting no deviation). The characteristics scored were crypt hyperplasia, aberrant crypts, crypt injury, crypt loss, goblet cell loss, crypt abscess, lymphoid aggregates, sub-mucosal thickening, hyperchromasia (an indicator of carcinogenesis), surface loss, monocytes and macrophages, neutrophils, plasma cells and lymphocytes. Total histological injury score (HIS) for each section = Σ [½ x (10 crypt and mucosal features) + ½ x ((monocytes & macrophages x 4) + (neutrophils x 2) + (plasma cells & lymphocytes x 4))]. Scoring for the milk diet experiment was performed independently by two researchers blinded to the treatments. Because these scores were consistent, only those from the researcher who scored the time-course experiment were used in order to be consistent between both experiments.

2.2.8 Statistical analysis of weight, intake and histology scores

Note: For the time-course experiment, ANOVA for colon HIS was performed by Zaneta Park (AgResearch Grasslands). For the milk diet experiment, REML analysis for weight and intake data was performed by Dr John Koolaard (AgResearch Grasslands).

For the time-course experiment, initial and final weight and intake data, and intestinal area, were analysed using the general analysis of variance (ANOVA) function in GenStat edition 11 (VSN International Ltd, Hemel Hempstead, Hertfordshire, UK). Values were excluded for a particular mouse on a particular day if transcription errors were detected. Initial and final weights were analysed on days 1-2 and 39 respectively, and initial and final intakes were analysed on days 4 and 39 respectively. Differences between means were considered significant when probability was less than 0.05 ($P < 0.05$).

For the milk diet experiment, statistical analysis of weight and intake data was done using the Residual Maximum Likelihood (REML) function in GenStat edition 11 (VSN International Ltd, Hemel Hempstead, Hertfordshire, UK) because the data did not meet statistical requirements for ANOVA (i.e. a balanced design with equal variances). This experiment was run in 3 blocks or batches, equal in treatment distribution and mouse number but overlapping in time by 2 weeks, so "block" was included in the model for each analysis. Graphs were made using SigmaPlot v10.0 (Systat Software, Inc., San Jose, California, USA). Maximum 5% least significant difference (LSD) was used to determine whether differences between groups were significant ($P < 0.05$). Block 3 mouse data was adjusted by one day to allow straightforward graphical representation of all blocks together, because these mice arrived one day late. All mice were individually weighed, caged and fed the treatment diets for the first time on the day after arrival, designated day 1.

Statistical analysis of histology scores was performed using GenStat edition 11. For the time-course experiment, colon histological injury scores were (log + 0.05)-transformed (due to the high number of zero values in the data) prior to running unbalanced ANOVA analysis. For the milk diet experiment, duodenum and colon scores were square-root transformed prior to REML analysis, while caecum, jejunum and ileum scores did not require transformation (according to an examination of the residual plots). A REML analysis was appropriate for the milk diet experiment because the data did not meet statistical requirements for ANOVA. Differences between means were considered significant when $P < 0.05$. HIS were graphed using SigmaPlot v10.0.

2.3 Analyses of gene expression in the whole colon

Note: Microarray experimental design structure was generated by Zaneta Park (AgResearch Grasslands).

Microarrays are a tool for monitoring the expression of a large number of genes in parallel, typically the entire known genome of an organism [305]. DNA microarrays consist of a predefined arrangement of a large number of probe sequences that serve as hybridisation templates for RNA or DNA fragments generated from a tissue sample. This method allows for monitoring of simultaneous gene expression levels across the entire genome in a particular tissue, an essential tool for studying the effects of dietary or other interventions in disease at the tissue or cell level [306]. The workflow for analysis of colon gene expression is shown in Figure 2.3.

2.3.1 Microarray experimental design

A reference design was used for each microarray experiment, which involved hybridising an experimental sample labelled with Cy3 dye with a reference RNA sample labelled with Cy5 dye to each microarray. The design was generated using CycDesigN 4.0 (CycSoftware Ltd, Ranfurly, NZ). Colon HIS was used to select 6 mice per treatment such that the full range of HIS in the experiment were represented. When mice had similar scores, representative animals were randomly selected for microarray. Treatments were randomised and evenly spread across microarray slides so that array or slide effects could be accounted for in the analysis.

2.3.1.1 Time-course experiment

The microarray experimental design for the intact colon microarray analysis is shown in Table 2.3. For the colon epithelial cell microarrays, a number of substitutions were required due to samples having unacceptably low RNA integrity. All substitutions were between mice with the same or very similar HIS (between HIS of 0 and 1). The final microarray design with mice used for each microarray experiment is shown in Table 2.4.

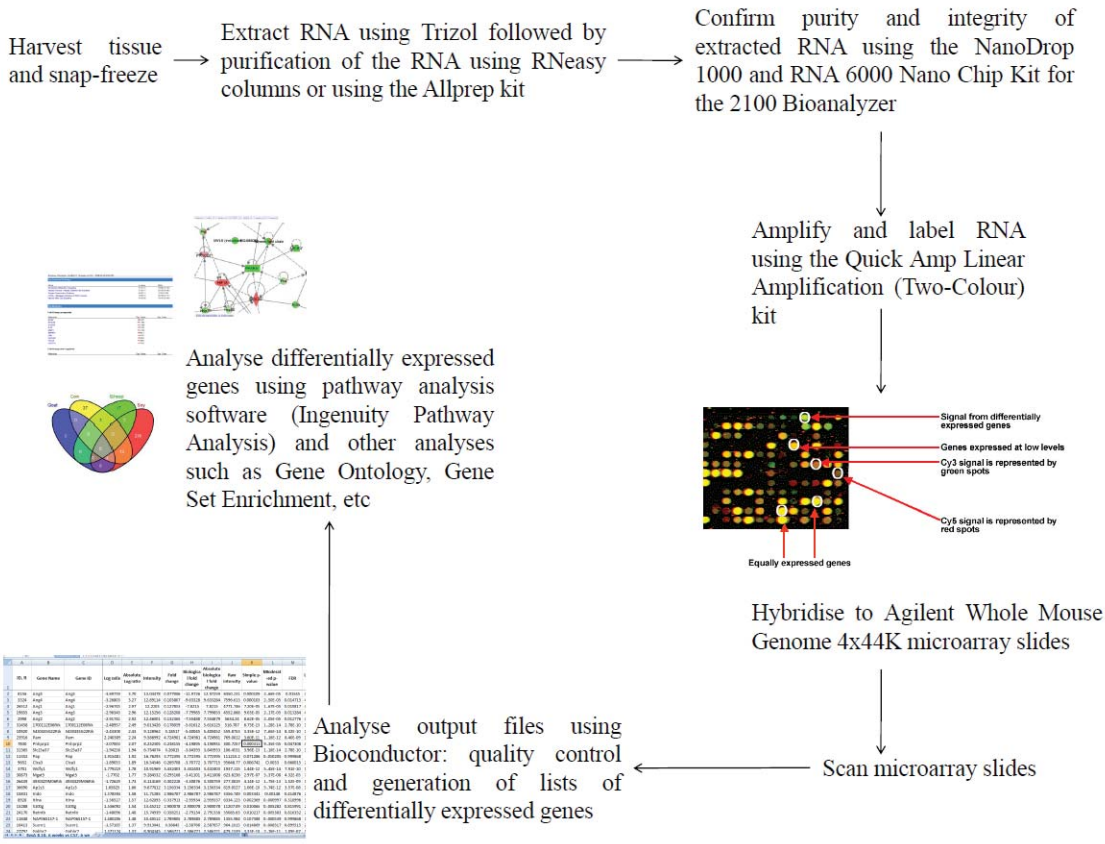


Figure 2.3 Gene expression analysis workflow. Main steps involved in performing gene expression analysis on colon tissue.

Table 2.3 Intact-colon microarray design for the time-course experiment. Mouse number and corresponding total histology scores of each mouse are shown for the intact colon analysis. IL10 is used as an abbreviation for an *Il10*$^{-/-}$ mouse.

Slide	Array 1			Array 2			Array 3			Array 4		
	Treatment	**HIS**	**Mouse**	**Treatment**	**HIS**	**Mouse**	**Treatment**	**HIS**	**Mouse**	**Treatment**	**HIS**	**Mouse**
1	C57BL/6J 12 weeks	0.5	25	IL10 6 weeks	0.5	16	IL10 12 weeks	4	12	C57BL/6J 6 weeks	0.5	20
2	IL10 6 weeks	0	2	C57BL/6J 12 weeks	1	21	C57BL/6J 6 weeks	0.5	30	IL10 12 weeks	6	17
3	IL10 12 weeks	0.5	10	C57BL/6J 6 weeks	0.5	35	C57BL/6J 12 weeks	0.5	29	IL10 6 weeks	0	7
4	IL10 12 weeks	2	13	IL10 6 weeks	0.5	18	C57BL/6J 6 weeks	1	28	C57BL/6J 12 weeks	0.5	31
5	C57BL/6J 6 weeks	0	22	C57BL/6J 12 weeks	0.5	24	IL10 12 weeks	4.5	1	IL10 6 weeks	1	9
6	C57BL/6J 6 weeks	0	26	IL10 12 weeks	9.5	15	IL10 6 weeks	0.5	14	C57BL/6J 12 weeks	0	32

Table 2.4 Colon epithelial cell microarray design for the time-course experiment. IL10 is used as an abbreviation for an *Il10*$^{-/-}$ mouse, and C57 for a C57BL/6J mouse.

Slide	Array 1	Array 2	Array 3	Array 4
Microarray design showing treatments				
1	C57, 12 weeks	IL10, 6 weeks	IL10, 12 weeks	C57, 6 weeks
2	IL10, 6 weeks	C57, 12 weeks	C57, 6 weeks	IL10, 12 weeks
3	IL10, 12 weeks	C57, 6 weeks	C57, 12 weeks	IL10, 6 weeks
4	IL10, 12 weeks	IL10, 6 weeks	C57, 6 weeks	C57, 12 weeks
5	C57, 6 weeks	C57, 12 weeks	IL10, 12 weeks	IL10, 6 weeks
6	C57, 6 weeks	IL10, 12 weeks	IL10, 6 weeks	C57, 12 weeks
Microarray design showing mice used: intact colon				
1	25	16	12	20
2	2	21	30	17
3	10	35	29	7
4	13	18	28	31
5	22	24	1	9
6	26	15	14	32
Microarray design showing mice used: colon epithelial cells				
1	25	16	12	20
2	2	33	30	17
3	8	23	29	5
4	13	18	28	31
5	22	25	4	3
6	27	15	14	34

2.3.1.2 Milk diet experiment

Treatments were randomly assigned ensuring that all treatments were evenly represented across the slides and the four arrays within each slide, as shown in Table 2.5. Colon histology score was also taken into account, with the scores stratified into the top half ("high") and bottom half ("low") of the range within a treatment. Three animals were randomly selected from each stratum for each treatment. This ensured that the full range of histology scores was represented in each treatment, preventing potential bias in the gene expression results, as well as enabling the comparison of gene expression data for high and low-scoring histology scores.

2.3.2 Colon tissue extraction

For the time-course experiment, total RNA and protein was extracted from up to 20 mg sections of whole proximal colon using Trizol (Invitrogen, Carlsbad, California, USA), followed by purification of the RNA using RNeasy columns (Qiagen Inc., Valencia, California, USA) and precipitation and cleanup of the protein according to the Trizol manufacturer's protocol. For the milk diet experiment, total RNA, DNA, and protein were extracted using the AllPrep kit (Qiagen Inc., Valencia, California, USA), in order to obtain DNA from the same sample as RNA to allow for subsequent analysis of DNA methylation. Protein pellets were stored at -20°C for later use for proteomic analysis. Use of this kit yielded superior RNA samples, in terms of purity and quantity, compared to the Trizol method used in the first experiment.

The purity and yield of RNA was assessed using the NanoDrop 1000 (Thermo Scientific, Wilmington, Delaware, USA). RNA Integrity Number (RIN) was measured, and concentration estimated, for each sample using the RNA 6000 Nano Chip Kit for the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Samples were only used in subsequent analyses if they were of high purity (absorbance ratios A260:A280 greater than 1.8 and A260:A230 greater than 1.6) and integrity (RIN greater than 8.0). Where there were impurities present, indicated by a low A260:A230, the samples were further purified using ammonium acetate precipitation using glycogen (Ambion Inc., Austin, Texas, USA) as a carrier to increase yield of precipitated RNA. Concentrations of RNA used in downstream analysis were from those obtained using

Table 2.5 Microarray design for the milk diet experiment. IL10 is used as an abbreviation for an *Il10*$^{-/-}$ mouse, and C57 for a C57BL/6J mouse. Low and high describe the category of colon histology score.

Slide	Array 1	Array 2	Array 3	Array 4
1	C57 Goat High	IL10 Soy High	IL10 Modified-AIN-76A Low	C57 Cow Low
2	IL10 Sheep Low	IL10 Cow High	C57 AIN-76A Low	C57 Soy High
3	C57 Modified-AIN-76A High	C57 Sheep Low	IL10 Goat High	IL10 AIN-76A Low
4	IL10 AIN-76A High	C57 Goat Low	C57 Soy Low	IL10 Modified-AIN-76A High
5	IL10 Soy Low	C57 Cow High	C57 Modified-AIN-76A Low	IL10 Sheep High
6	C57 AIN-76A High	IL10 Goat Low	IL10 Cow Low	C57 Sheep High
7	C57 Sheep High	IL10 AIN-76A Low	C57 Cow High	IL10 Cow Low
8	C57 Soy Low	IL10 Modified-AIN-76A High	IL10 Soy Low	C57 Goat High
9	IL10 Goat Low	C57 Modified-AIN-76A Low	IL10 Sheep High	C57 AIN-76A High
10	C57 Cow Low	C57 Soy High	IL10 AIN-76A High	IL10 Goat High
11	IL10 Modified-AIN-76A Low	C57 AIN-76A Low	C57 Sheep Low	IL10 Soy High
12	IL10 Cow High	IL10 Sheep Low	C57 Goat Low	C57 Modified-AIN-76A High
13	C57 Cow Low	IL10 Soy Low	IL10 AIN-76A High	C57 Soy High
14	IL10 Cow High	C57 Modified-AIN-76A Low	C57 Goat High	C57 Sheep Low
15	C57 AIN-76A High	IL10 Goat High	IL10 Sheep Low	IL10 Modified-AIN-76A Low
16	IL10 Soy High	C57 AIN-76A Low	IL10 Goat Low	C57 Modified-AIN-76A High
17	C57 Goat Low	IL10 Sheep High	C57 Soy Low	IL10 Cow Low
18	IL10 Modified-AIN-76A High	C57 Sheep High	C57 Cow High	IL10 AIN-76A Low

the NanoDrop because this method is considered more accurate than the estimate provided obtained by the Bioanalyzer.

2.3.3 Microarray hybridisation and scanning

Extracted colon RNA was amplified using the Low Input Linear Amplification (Two-Colour) kit for the time-course experiment, and the updated version of the same kit, the Quick Amp Linear Amplification (Two-Colour) kit (Agilent Technologies, Santa Clara, California, USA), for the milk diet experiment. The resulting cRNA was hybridised to mouse 44K oligonucleotide two-colour arrays (Whole Mouse Genome Oligo Microarray Kit, 4 x 44K; version 1) (Agilent Technologies, Santa Clara, California, USA) according to the manufacturer's instructions. Each slide contained four identical arrays, each containing probes for over 41,000 well-characterised mouse genes and transcripts from public databases, including RefSeq, RIKEN, NIA, Ensembl, UCSC Goldenpath and Unigene, designed to represent transcripts for all known genes in the mouse genome.

The purity and fluorescent intensity of the amplified and labelled cRNA was assessed using the NanoDrop 1000 (Thermo Scientific, Wilmington, Delaware, USA). The amplification and labelling procedure was repeated if a sample did not meet the requirements of specific activity > 8.0 pmol Cy3 or C5 per μg of cRNA and yield > 825 ng. Samples were also run on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) using the RNA 6000 Nano Chip Kit to confirm that a range of fragment lengths were present.

Amplified cDNA from each mouse was labelled with Cy3 dye and hybridised to one array, along with Cy5-labelled reference cDNA that was prepared in one large pool from total RNA extracted from normal healthy Swiss mouse tissue (including small intestine, colon, kidney, and liver of adult mice, plus foetus tissue). Hybridisation was performed using the Gene Expression Hybridization kit (Agilent Technologies) according to the manufacturer instructions. Ten mM cyanine (Cy) 3-CTP and Cy 5-CTP dye was obtained from Perkin-Elmer (Boston, MA, USA) for the intact colon microarrays in the time-course experiment whereas the Cy dye was included in the Agilent amplification kits for the subsequent microarray analyses. Slides were scanned using the DNA microarray scanner G2565CA (Agilent Technologies, Santa Clara,

California, USA) according to the manufacturer's instructions. Array images (TIFF files) were uploaded into Agilent Feature Extraction Software (version 8.5.1.1 (intact colon arrays) or 10.10.1.1 (epithelial and milk diet arrays)), which generated numerical intensity data for each probe, as well as a quality control report for each array. The data files generated by the feature extraction software were used for further analysis of gene expression data.

2.3.4 Microarray data quality control

Note: Microarray quality control analysis and generation of differentially expressed gene lists was performed by Zaneta Park (AgResearch Grasslands), except for the epithelial arrays which were analysed by Paul Maclean (AgResearch Ruakura).

Two forms of quality control were applied to the microarray data. Quality control reports were produced automatically by the Agilent Feature Extraction software. These were a good pre-analysis indicator of the technical quality of each array. Further extensive quality control and generation of gene lists from feature extraction data was performed using the Bioconductor R package (R 2.12.1; R Foundation for Statistical Computing, Vienna, Austria).

Background correction was not necessary for any of the microarray experiments due to homogeneous hybridisation [307]. No background correction is the best option to use if the spatial patterns observed for the background intensities are not the same as those observed for the foreground intensities, which was the case in all experiments. Examination of intensity boxplots, spatial images, density plots, and MA plots showed that common background correction methods were less appropriate than no background correction. Comparison of pathways using Ingenuity Pathway Analysis (IPA) between the AIN-76A *Il10*$^{-/-}$ versus C57BL/6J comparisons for each experiment, as well as between the same comparison in the milk diet experiment for background correction and no background correction, also showed that the results were robust between experiments and background correction methods. The use of no background correction for each intact-colon microarray experiment was therefore deemed to be appropriate.

Analysis of the raw microarray data was performed using the Linear models for microarray analysis (Limma) package for Bioconductor [308, 309] due to its greater flexibility compared with the Agilent analysis software. The quality of the data was

assessed for each individual array and each slide using diagnostic box plots, density plots, and spatial images generated from the raw data. All arrays and slides that met quality control criteria were included in the downstream analysis. Flagged spots were removed from the analysis. Spots were excluded when they failed any of these criteria in the foreground: poor quality green spots; poor quality red spots; multiple copies of the gene tiled and this one differs (green); and multiple copies of the gene tiled and this one differs (red).

Intensity ratio values for all spots were normalised using a global loess smoothing procedure to remove the effect of systematic variation on the microarrays [309, 310]. Dark corner, E1A, EQC, bright corner and negative control spots were removed prior to normalisation.

2.3.5 Generation of lists of differentially expressed genes

The normalised data from the arrays of each treatment group (diet or age, and mouse strain) were averaged. For each treatment comparison, a list of differentially expressed probe sets was generated by calculating a moderated t-statistic and false discovery rate (FDR) for each probe set using the Limma package. The Limma library implements an empirical Bayes approach to assign differential gene expression. Expression changes were considered significant when biological fold change (FC) was greater than 1.5 and FDR was less than 0.05. Biological fold change is a recalculation of fold change (where negative expression is scaled from 0 to 1, and positive expression is scaled from 1 up) to present both positive and negative fold changes from 1 in each direction to make interpretation more intuitive. The Venny software was used to generate Venn diagrams showing the number of genes each treatment comparison has in common (http://bioinfogp.cnb.csic.es/tools/venny/index.html) [311].

For the intact colon and colon epithelial cell microarrays, four gene lists of differential expression were generated, and for the milk diet experiment, 10 gene lists of differential expression were generated, comparing each diet to the modified-AIN-76A diet for each strain of mice (Table 2.6). In addition, the comparison between *Il10*$^{-/-}$ and C57BL/6J mice on AIN-76A diet was also analysed to compare results against those of previous experiments in this mouse model.

Table 2.6 Differentially expressed gene lists generated for each microarray analysis.

Time-course experiment

		C57BL/6J	***Il10*$^{-/-}$**
Strain	**Age**	**12 weeks**	**12 weeks**
C57BL/6J	6 weeks	■	
	12 weeks	■	■
Il10$^{-/-}$	6 weeks		■
	12 weeks	■	■

Milk diet experiment

		C57BL/6J	***Il10*$^{-/-}$**
Strain	**Diet**	**Modified-AIN-76A**	**Modified-AIN-76A**
C57BL/6J	AIN76A	■	
	Modified-AIN76A	■	
	Soy	■	
	Sheep	■	
	Cow	■	
	Goat	■	
Il10$^{-/-}$	AIN76A		■
	Modified-AIN76A	■	■
	Soy		■
	Sheep		■
	Cow		■
	Goat		■

Black fill represents a comparison for which a gene list was generated.
Grey fill represents an invalid comparison between the same treatment.
No fill represents a comparison that is not presented in this thesis.

2.3.6 Quality control analysis for array and hybridisation day effects

Graphing the gene expression data using principle components analysis (not shown) highlighted a "hybridisation day" effect in the microarray data from the time-course experiment. The bulk of the comparisons were done between-slide, and the treatments were evenly spread across the slides, so these effects, if present, were anticipated to have a negligible effect on the fold changes and probabilities. However, subsequent to this discovery, all microarray experiments were checked for slide, array, and day effects. The data was modelled using a linear mixed effects model [312] using the lme4 package [313]. The model was fitted using a REML algorithm to minimise the effects of day and slide. The model was: log2 intensity for gene = (Strain x Inflammation x Diet) + random (Day/Slide). The fixed effects for the model were: Strain x Inflammation x Diet. The random effects for the model were: Day/Slide. In the model equation, "x" denotes interaction, "+" summation and "/" nesting.

2.3.7 Pathway and gene set analysis

2.3.7.1 Pathway analysis

Ingenuity Pathway Analysis, IPA (IPA Version 9.0, Ingenuity Systems, Inc., Redwood City, California, USA; www.ingenuity.com), was used to perform network and pathway analyses. The full Limma-generated gene lists for each treatment comparison were uploaded into IPA.

Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the dataset. Molecules from the dataset that met the absolute fold change cutoff of 1.5 and were associated with a canonical pathway in Ingenuity's Knowledge Base were considered for the analysis. The significance of the association between the dataset and the canonical pathway was measured in two ways: 1) a ratio of the number of molecules from the dataset that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. Pathways were considered to be significant when at least 10% of the genes from a particular pathway were differentially expressed in the microarray dataset; and 2) Fisher's exact test was used to calculate the probability that the association between the

genes in the dataset and the canonical pathway is explained by chance alone. A probability less than 0.05 was considered significant.

Upload settings for datasets were “flexible format”, “all identifiers”, FC and FDR as “observation” and Agilent probe name as “identifier”, and array platform was set as “whole mouse genome 4 x 44K”. Core analysis settings were: direct and indirect relationships, endogenous chemicals included, 140 molecules per network, 25 networks per analysis, data sources, species and tissues and cell lines were each set to “all”, and confidence was set as “experimentally observed”. Duplicates were resolved using median fold change, and molecules coloured using FC. The number of network eligible molecules was calculated using the following cutoffs: absolute FC greater than 1.5 and FDR less than 0.05.

Datasets were examined for top functions and disease processes, as well as top canonical pathways. Pathways of interest were overlaid with expression values from each dataset for visual comparison. It was noted during analysis that when comparing changes between two datasets, the intensity of expression shown in diagrams of canonical pathways in IPA are relative to the set of expression values in that comparison. Hence, while the colour intensity of each set of pathways are relevant to that one dataset, they may not be directly comparable between datasets. Figure 2.4 provides an example of the type diagram that can be produced using IPA to examine gene networks.

2.3.7.2 Over-representation analysis

The Expression Analysis Systematic Explorer (EASE) version 2.0 tool was developed to automate the process of biological theme determination for lists of genes [314]. Lists of significantly differentially expressed genes (FDR < 0.05, absolute biological FC > 1.5) for each treatment comparison were uploaded into EASE. The three basic functions of EASE are: theme discover; customisable linking to online tools; and the creation of descriptive annotation tables. EASE software uses the gene ontologies or the gene associations provided by Gene Ontology (GO) Consortium members to perform a hypergeometric test on a list of genes to see whether the list of genes is significantly over-represented among GO terms compared to all genes [314].

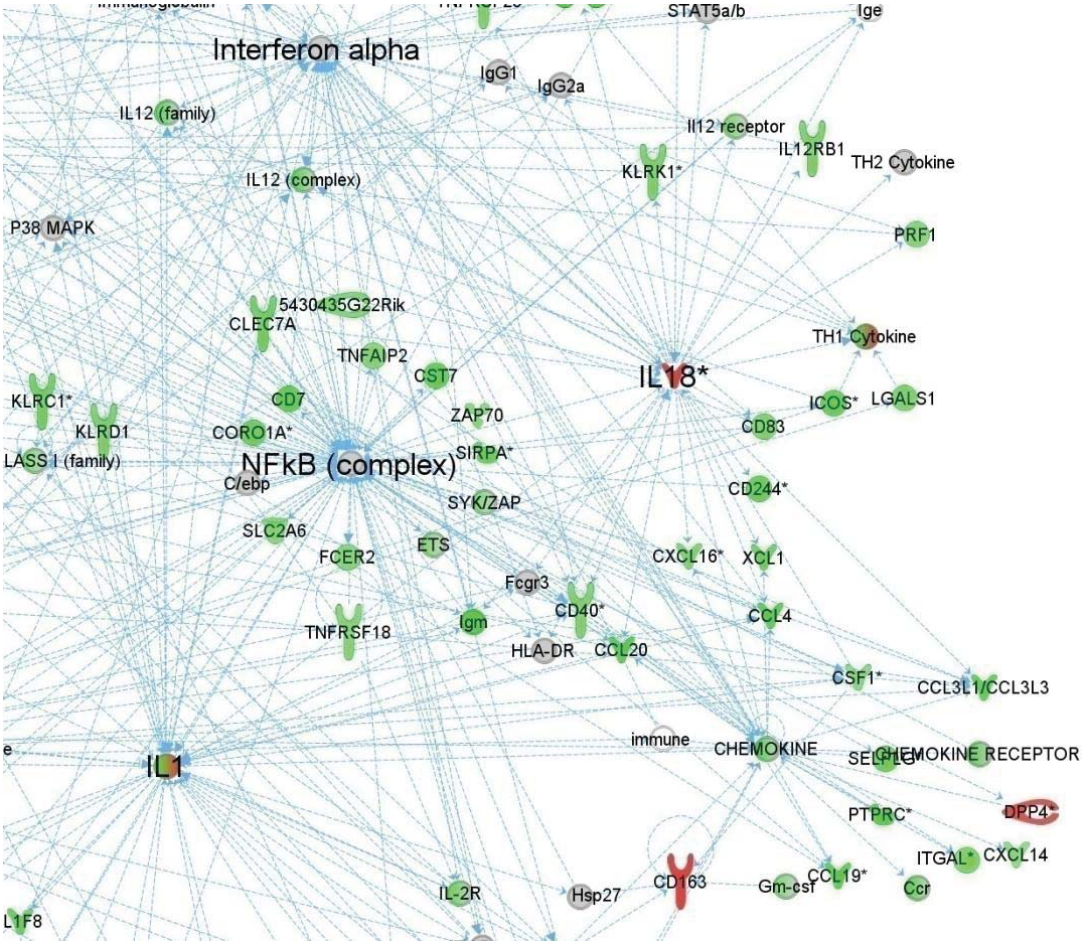


Figure 2.4 Example of a network diagram generated in IPA. *denotes genes that are detected two or more times on an array. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as a line. All relationships are supported by at least one reference from literature. Red and green coloured nodes indicate degree of fold change, with red indicating an increase in expression and green indicating a decrease in expression. Colour intensity is correlated with the degree of change in expression with greater intensity representing a higher expression level. Nodes are displayed with various shapes that represent the functional class of genes.

EASE calculates over-representation with respect to the total number of genes assayed, and gene identifiers are converted such that a single gene represented by more than one identifier receives only one "vote" for each of the categories it belongs to. Two statistical measures may be used: the one-tailed Fisher exact probability or a variant of this called the EASE score. The EASE score is recommended because it is a conservative adjustment to the Fisher exact probability and therefore favours more robust categories by penalising the significance of categories supported by fewer genes. EASE calculates a wide variety of probability corrections, including Bonferroni-type methods, FDR and bootstrap methods [314]. EASE scores less than 0.05 were considered significant.

2.3.7.3 Gene set enrichment analysis

Note: GSEA was performed by Dr Wayne Young (AgResearch Grasslands).

Gene set enrichment analysis (GSEA), unlike the analysis methods described previously, analyses entire gene sets, rather than genes which have been determined already to be differentially expressed. GSEA examines the probability value ranking of all genes in a dataset (the entire genome in microarray experiments) to determine whether they belong to a biologically meaningful set such as a KEGG pathway, i.e. they have probability (P value) ranks that are not randomly distributed. GSEA moves away from a gene-centric view, where genes are considered significantly differentially expressed (or not) based on probability, to a method where genes are still ranked according to probability, but what is important is how a set of genes within a biologically meaningful pathway behave [315]. GSEA was performed using the Bioconductor R package (R 2.12.1; R Foundation for Statistical Computing, Vienna, Austria) on the normalised data. Only pathways with probability less than 0.05 were considered significant.

2.3.8 Generation of heatmaps and measures of similarity between treatments

Note: Heatmaps for the time-course experiment were generated by Paul Maclean (AgResearch Ruakura) and for the milk diet experiment by Dr Wayne Young (AgResearch Grasslands).

Hierarchical clustering was used to generate heatmaps. Heatmaps were created using the gplots library and heatmap.2 command in R. To create the heatmap of immune-related genes in intact colon and colon epithelium for the time-course experiment, presented in Chapter 3 (Figure 3.4), a list of immune-related genes was generated. In IPA, all genes present in immune-related networks for the comparison *Il10*$^{-/-}$ mice at 12 weeks of age versus *Il10*$^{-/-}$ mice at 6 weeks of age, in both intact colon and colon epithelium, were combined into a list. This list was further limited to only those genes that were differentially expressed by at least 2-fold in the comparisons *Il10*$^{-/-}$ mice at 12 weeks of age versus *Il10*$^{-/-}$ mice at 6 weeks of age and *Il10*$^{-/-}$ mice at 12 weeks of age versus C57BL/6J mice at 12 weeks of age in intact colon or epithelium. A fold change of 2 was chosen to limit the number of genes used. Data was restricted to the genes with the highest range of expression values across the treatments of interest in order to restrict the number of rows on the heatmap. Heatmaps were generated that included the four treatments in the time-course experiment and both intact colon and epithelium gene expression data. A measure of the similarity between treatments, the Pearson correlation coefficient, was calculated using a correlation analysis in R.

The set of genes annotated to the GO term GO:0031224 “Intrinsic to membrane” was downloaded from the AMIGO website to create the heatmap of membrane proteins for the milk diet experiment, presented in Section 5.5.6.4.3. Heatmaps were generated from the downloaded gene set for the comparisons of interest. Expression of this gene set in the comparison of interest was analysed using a Rotation Gene Set Test (ROAST). ROAST is part of the limma package in R, and tests the significance of the entire set of genes by testing the hypotheses of whether the expression of all the genes tends to be increased, decreased or a mixture of both [316].

2.4 Analysis of gene expression in colon epithelium

2.4.1 Introduction

Examining cells dissected from frozen tissue preserves the influence of surrounding cells and the luminal environment on gene expression [31]. LMD is a technique that allows specific regions of tissues and/or phenotypically similar populations of cells to be removed from frozen tissue, enabling subsequent molecular profiling of these cells which is reflective of their *in vivo* state [119, 120, 317].

There are two types of laser microdissection systems. Laser capture microdissection (LCM) involves using a laser to melt a thermolabile polymer onto the target cells so they separate from the tissue when the cap of the sample tube is removed [119]. Laser (or laser-assisted) microdissection (LMD) also involves the use of a laser to separate target cells from the surrounding tissue, but they are captured by lowering the tube lid down to the target cells which are picked up by static electricity on the cap [318]. The research presented in this thesis was done using LMD rather than LCM. An overview of the workflow is shown in Figure 2.5.

Cells obtained by LMD can, depending on the quantity of cells and the number of target molecules, be used for analysis of protein expression and DNA methylation. Gene expression and DNA sequence analysis can be performed on a very small number of cells because DNA and RNA can be copied using polymerase chain reaction (PCR) techniques to produce much larger quantities of the target material.

2.4.2 RNA preparation from colon epithelial cells

The section embedding, cutting, staining and microdissection steps required trouble-shooting in order to produce RNA of sufficient quantity and quality for gene expression analysis. Also, appropriate protocols needed to be selected for extraction, amplification, quantification and qualitative assessment of RNA integrity for the yields obtained of colon epithelial cells, which were much lower that the yields obtained from intact colon (by approximately a factor of 1000).

2.4.2.1 Tissue embedding

Embedding of samples needed to be rapid to retain RNA integrity and needed to preserve tissue morphology to enable cell identification. Lengths of intestine were placed on stainless steel blocks on dry ice and embedding medium (Tissue-Tek OCT, Sakura Finetek U.S.A., Inc., Torrance, California, USA) placed at the base. Tissue was frozen from the base up and further medium was added until the tissue was encased. This maintained tissue shape as well as rapidly freezing the tissue. RNA was extracted from fresh cut sections to determine whether RNA integrity was maintained during the embedding procedure.

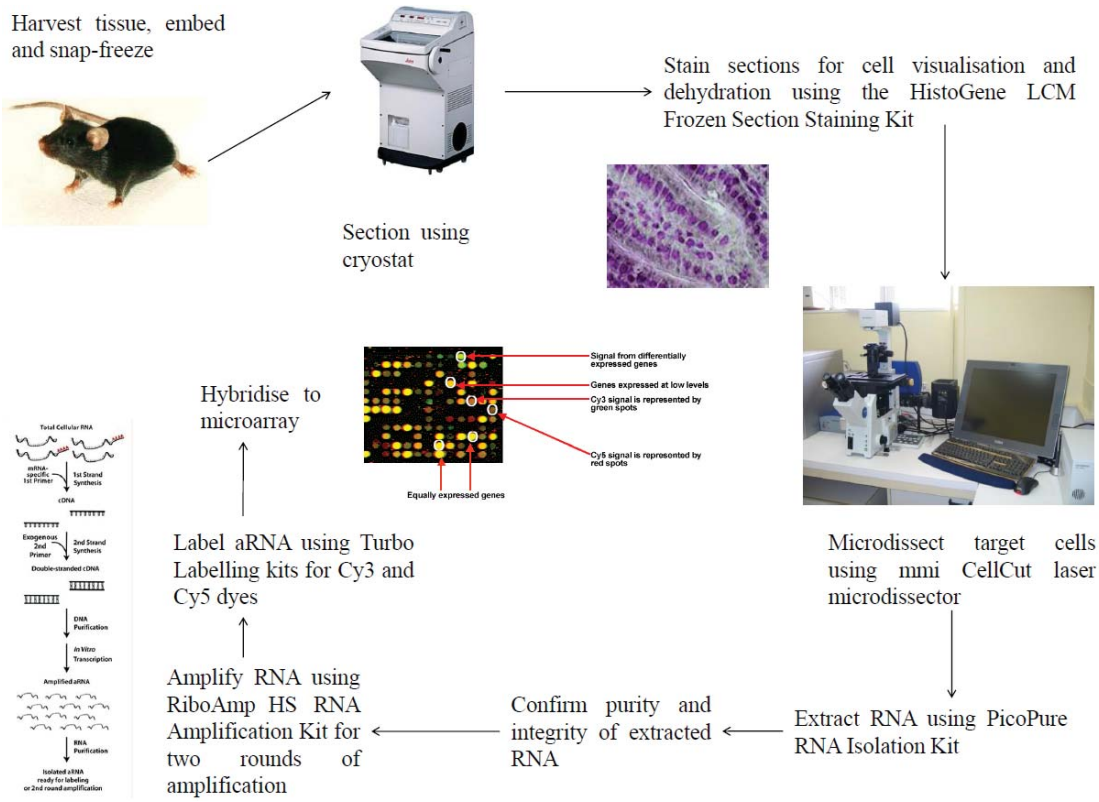


Figure 2.5 Laser microdissection workflow. Main steps involved in obtaining RNA from target cell types (colon epithelial cells) using laser microdissection and performing gene expression analysis.

2.4.2.2 Staining and dehydration of sections

The staining procedure needed to fix the tissue to the slide and allow proper incorporation of stain into the cells. The stain needed to produce good visualisation of tissue architecture to enable detection and cutting of the epithelial cells while maintaining intact RNA. The staining process also needed to be fast and dehydrate the cells, with microdissection typically done within 30 minutes in order to minimise rehydration until the sample was placed in lysis buffer for RNA extraction.

Initially, a standard histological stain, haematoxylin (10% *w/v*) and eosin Y (0.5% *w/v*), was trialled (ProSciTech, Queensland, Australia). From fresh frozen tissue, the RINs were superior to 8.0 without stain, and between 7.0-7.6 with stain. However, microdissected cells yielded poor quality RNA, so the HistoGene LCM Frozen Section Staining Kit (Arcturus Bioscience Inc., Mountain View, California, USA) was trialled, which involves a rapid, single-step staining procedure, with subsequent improvements in RNA quality.

To improve the degree of cell dehydration during the staining procedure the eosin Y/cresyl violet staining procedure was used, with the stains made up with ethanol (4% cresyl violet (*w/v*) in 75% ethanol (70% *v/v*) and 0.5% eosin Y (*w/v*) in 95% ethanol (30% *v/v*)) and aqueous solutions removed from the staining protocol as much as possible [319]. Removal of water steps as recommended by Clement-Ziza *et al.* [319] from the staining protocol yielded RNA with acceptable electropherogram ribosomal mRNA peaks, and therefore of sufficient integrity for microarray analysis. The Histogene one-step haematoxylin-based stain (without water steps) yielded slightly lower RIN than the eosin Y/cresyl violet stain used by Clement-Ziza *et al.*, but better visualisation.

The Histogene stain was chosen due to its improved visualisation of the cells of interest, but was used without the post-stain water wash step from the manufacturer's protocol. The final xylene wash was also excluded due to it causing the slide membrane to become brittle and negatively affecting sample cutting and pickup by the cap. The two 100% ethanol steps in the Histogene protocol preceding the final xylene wash appeared to dehydrate the sections adequately as acceptable RINs were obtained from these sections.

2.4.2.3 Sample preparation and microdissection

Note: Sample embedding, sectioning, staining and microdissection was performed by Jason Peters (AgResearch Grasslands).

Samples were embedded using a methodology that ensured that the colon was maintained in its natural shape in cross-section resulting in excellent visualisation on the laser microdissector. Immediately on removing the intestinal tissue from the animal, the tissue was rinsed with saline, trimmed and embedded on a stainless steel block placed on dry ice (3-4 colon lengths per block), in a 'face down' orientation so that it was supported in an optimal position for cryosectioning. The embedding material was poured over and allowed to slowly freeze. Samples were stored at -85°C prior to cryosectioning, which was undertaken using a Leica CM1950 cryostat (Leica Microsystems Gmbh, Wetzler, Germany), and sections were then placed on an RNase-free metal-framed membrane slide (Molecular Machines and Industries AG, Zurich, Switzerland).

Staining was carried out using the HistoGene LCM Frozen Section Staining Kit (Arcturus Bioscience Inc., Mountain View, California, USA) according to the manufacturer's instructions with a minor modification, the removal of water steps, and with all equipment RNaseZap (Ambion Inc., Austin, Texas, USA) and UV-treated to destroy any DNA, RNA or protein (such as RNase), prior to use. All stain solutions were kept in a cold block to minimise activity of any contaminating RNases. All staining equipment was treated with UV light prior to use to destroy any contaminating nucleic acids or proteins. Slides were stored in a dessicator until microdissection was performed to prevent rehydration of the cells and reactivation of endogenous RNases that could compromise sample RNA integrity. Microdissection was performed using the mmi CellCut laser microdissector (Molecular Machines and Industries AG, Zurich, Switzerland) within 45 minutes of the end of the staining procedure.

2.4.2.4 RNA extraction of microdissected cells

RNA was extracted from the colon sections using the PicoPure RNA Isolation Kit (Arcturus Bioscience Inc., Mountain View, California, USA) following the manufacturer's instructions (User Guide Version D) and including the recommended DNase treatment using the RNase-free DNase Set (Qiagen Inc., Valencia, California,

USA). This extraction kit is validated for small cell samples, such as those obtained by LMD, allowed for elution in a small volume (11 – 30 µL) which was important due to the small sample size obtained from mouse colon epithelium. Other column-based RNA extraction kits, such as the Qiagen AllPrep or RNeasy kits are designed for elution volumes of 30-50 µL. The Arcturus RNA extraction and amplification kits have been used for extraction and amplification of RNA from microdissected cells by other researchers with good results [31, 122].

The integrity of the extracted total RNA was verified using the RNA 6000 Pico Chip Kit for the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Many researchers report the Bioanalyzer electropherograms or gel-like images in their publications but do not report a RIN for each sample. Pico chip RINs are not as well validated by Agilent as Nano chip RINs. This made it difficult to determine how appropriate the RNA quality was for downstream work, but as a general rule, the aim was to see two strong ribosomal RNA peaks, or at least a RIN of close to 6.0.

Yields from microdissected cells are often too low to use spectrophotometric methods, specifically, the NanoDrop 1000 (Thermo Scientific, Wilmington, Delaware, USA), to measure total RNA concentration and purity via absorbance at 230, 260 and 280 nm. However, although RNA purity could not be measured in these small samples, the Picopure extraction kit is designed to yield high purity RNA that is ready for amplification, and the use of the DNase and subsequent wash steps ensured the RNA was free from contaminants, such as genomic DNA.

The Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, California, USA) was used in addition to the Bioanalyzer concentration estimate to quantify samples. The Qubit RNA Assay Kit is able to measure smaller amounts of RNA than the spectrophotometric NanoDrop method (being accurate for initial sample concentrations from 250 pg/µL to 100 ng/µL). It is also more accurate than the spectrophotometric and Bioanalyzer methods because it directly measures RNA even in the presence of other molecules (DNA, salts, solvents, proteins, and free nucleotides) that would confound spectrophotometric methods. However, RNA quantity in some samples was still too low to enable accurate measurement with the Qubit. The Qubit result was used to calculate RNA input for the amplification – where the sample was too low in concentration to be measured (original sample < 4 ng/µL), the maximum volume (10 µL) of sample was used.

2.4.3 RNA amplification and labelling

Extracted total RNA (30 ng in a maximum volume of 10 μL) was linearly amplified using the RiboAmp HS RNA Amplification Kit for two rounds of amplification (Arcturus Bioscience Inc., Mountain View, California, USA). Amplified cRNA (10 μg in a maximum volume of 40 μL) was labelled for microarray hybridisation using the Turbo Labelling kits for Cy3 and Cy5 dyes (Arcturus Bioscience Inc., Mountain View, California, USA). These kits have been used previously with good results [320, 321].

2.4.4 Microarray hybridisation

Hybridisation of the samples to the microarrays and quality control analysis of the data was performed according to the methods outlined in Sections 2.3.3 and 2.3.4. The REML analysis was not required for the epithelial cell data because the arrays were more homogenous than the other microarray experiments.

2.4.5 Microarray data analysis

Analysis of microarray data including quality control analysis, generation of lists of differentially expressed genes, and pathway analyses were performed as described in Sections 2.3.5-2.3.7.

2.5 qPCR validation of microarray results

The expression of 6 genes was quantified using real-time quantification polymerase chain reaction analysis (qPCR) for each microarray experiment. For the intact colon microarrays and the epithelial cell microarrays in the time-course experiment (Chapter 3), the genes selected for analysis were: Peroxisome proliferator-activated receptor α (*Ppara*; NM_011144; PrimeTime assay ID Mm.PT.51.7020742), Signal transducer and activator of transcription 1 (*Stat1*; NM_009283; Mm.PT.51.19067632), Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP) (*Tap2*; NM_011530; Mm.PT.51.9501990.gs), Interleukin 18 (*Il18*; NM_008360; Mm.PT.51.11096643), Matrix metalloproteinase 3 (*Mmp3*; NM_010809; Mm.PT.51.16575457.gs), and S100 calcium binding protein G (*S100g*; NM_009789; Mm.PT.51.7385865). For the milk diet experiment, the genes selected for analysis were: *Stat1*, *Il18*, *Mmp3*, *S100g*, Glucagon

(*Gcg*; NM_008100; Mm.PT.51.17070389), and Chemokine (C-C motif) ligand 19 (*Ccl19*; NM_011888; Mm.PT.51.8417829.g).

For qPCR analysis, 500 ng of total RNA was reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems by Life Technologies, Carlsbad, California, USA) according to manufacturers' instructions. For the epithelial cell samples, 500 ng of amplified RNA resulting from two rounds of amplification of the total extracted RNA was reverse transcribed. The cDNA was stored at -20°C prior to the determination of the expression levels of each gene. Expression levels of each gene were determined using manufacturer-optimised primer/probe mixes, PrimeTime qPCR Assays (20x; Integrated DNA Technologies (IDT), Coralville, Iowa, USA) and TaqMan Fast Advanced Master Mix (2x; Applied Biosystems) on the RotorGene 6000 qPCR instrument (Qiagen, distributed by Bio-Strategy Ltd, Auckland, NZ). Expression levels of the target genes were assessed relative to the Calnexin (*Canx*; NM_007597) reference gene, which was not differentially expressed between treatments according to microarray analysis and has been used for microarray validation by qPCR in other mouse models of colitis [118, 148, 322].

All qPCR analyses (no-template controls and samples) were prepared as duplicate 10 µl reactions comprising a 9.0 µl aliquot of master mix (5.0 µl of TaqMan Fast Advanced Master Mix, 0.5 µl of PrimeTime qPCR assay for the gene of interest, 3.5 µl of nuclease-free water), and 1 µl of cDNA (at a 1 in 10 dilution in nuclease-free water). The thermal profile used was 50°C for 2 minutes, 95°C for 20 seconds and 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Each run was performed twice. The data were normalised to the reference gene and analysed for expression level changes using the Relative Expression Software Tool (REST) software version 2.0.13 [323]. Data are presented as relative fold changes (equivalent to the FC values presented for the microarray results), 95% confidence interval and probability (P) values for each gene and treatment comparison. Data were considered significant when absolute FC was greater than 1.5 and probability was less than 0.05.

2.6 Analysis of DNA methylation in the colon

The workflow for the analysis of both global and specific gene methylation is shown in Figure 2.6.

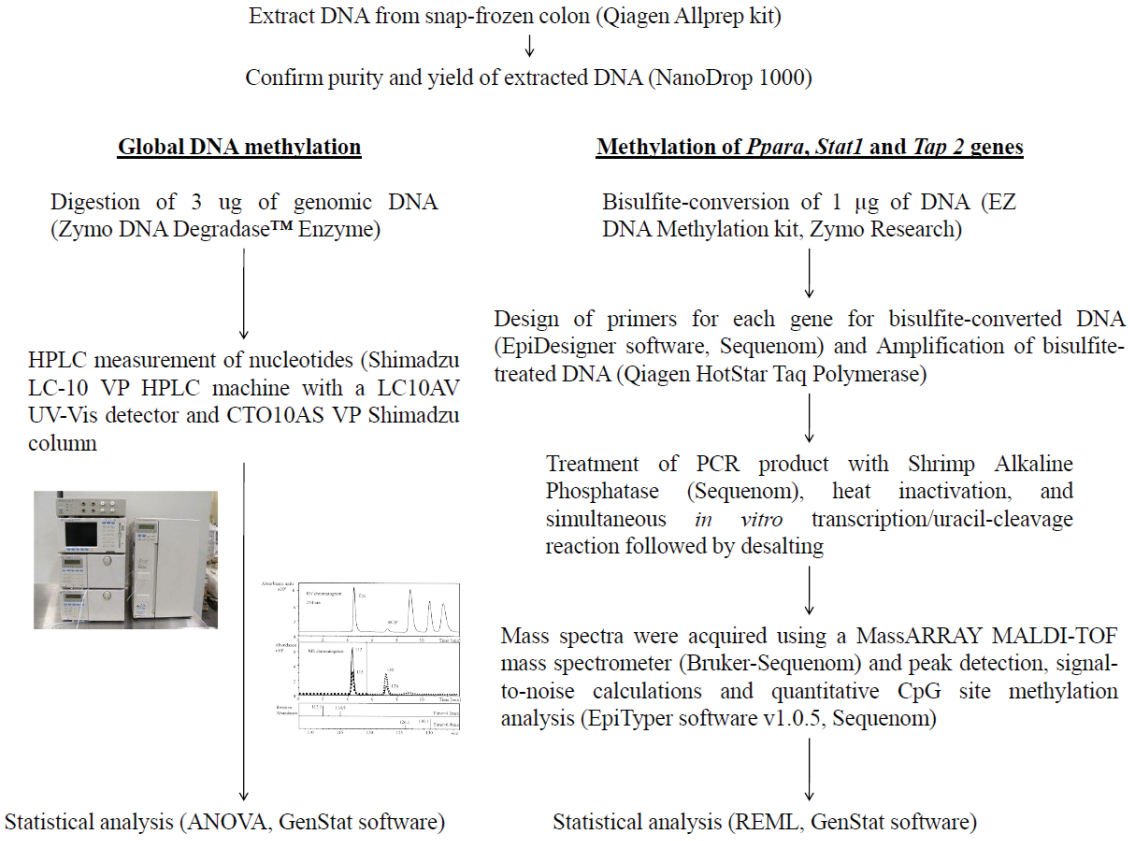


Figure 2.6 DNA methylation analysis workflow. Main steps involved in performing DNA methylation analysis on colon tissue.

2.6.1 Colon tissue DNA extraction

DNA from up to 20 mg colon tissue samples was extracted using the Qiagen Allprep kit (Qiagen, Hilden, Germany) as described previously (section 2.3.2). The purity of the extracted DNA was assessed using the NanoDrop 1000 (Thermo Scientific, Wilmington, Delaware, USA). Samples were only used in subsequent analyses if they were of high purity (A260:A280 > 1.8; A260:A230 > 1.6). Where the purity was too low, the samples were further purified using ammonium acetate precipitation with the use of glycogen (Ambion, Inc., Applied Biosystems, Foster City, California) as a carrier to increase yield of precipitated RNA.

2.6.2 Global DNA methylation

Analysis of global DNA methylation was performed on mouse colon DNA using enzymatic digestion followed by measurement of nucleotides via High Performance Liquid Chromatography (HPLC) [324-327] using a method adapted from that of Ramsahoye (2002) specifically to be suitable for low DNA sample input [328].

2.6.2.1 Genomic DNA digestion

Note: DNA digestion was performed by Kelly Armstrong (AgResearch Grasslands) with assistance from the candidate.

Three µg of each sample was digested in triplicate using the Zymo DNA Degradase Enzyme, with an activity of 10 units/µL (Zymo Research, Irvine, California, USA), using 7.5 µL buffer plus 3.0 µL enzyme plus water to make the final reaction volume to 75 µL. The manufacturer instructions were modified from the recommendation of using 1µL of enzyme for digestion of up to 5 µg genomic DNA to 3µL of enzyme for digestion of 3 µg genomic DNA [328]. Samples were incubated for 3 hours at 37^{o}C and loaded onto an Ultrafree-MC spin column with 0.45 µm pore size (Merck Millipore International, Merck KGaA, Darmstadt, Germany) and centrifuged at 100 x g for 5 minutes to remove cellular debris. Sample volume was then adjusted to 75 µL to accommodate losses from the columns and NanoDrop measurement.

2.6.2.2 HPLC measurement of nucleotides

Note: HPLC analysis was performed by Bryan Treloar (AgResearch Grasslands).

Samples were pipetted into 250 µL glass-bottomed vials, which were put into 8 mm amber vials and placed into the sample loader of a Shimadzu LC-10 VP HPLC machine with a LC10AV UV-Vis detector and CTO10AS VP Shimadzu column oven (Shimadzu Corporation, Kyoto, Japan). Sample detection was at 280 nm (UV), column temperature was 6^{o}C, and flow rate was 1 mL/minute, with injection volume of 50 µL and run time of 21 minutes. Mobile phase was 50 mM diammonium hydrogen orthophosphate, pH 4.1 (97%), and 100% acetonitrile (3%), using isocratic reverse-phase HPLC. The column used was of the brand Luna, particle size 5µm, phase C8, pore size 100Å, length 150 mm, internal diameter 4.6 mm (part number OOF-4040-EO; Phenomenex, Inc., Torrance, California, USA).

Nucleotide standards for deoxy-thymidine monophosphate (dTMP), deoxy-cytosine monophosphate (dCMP), deoxy-adenosine monophosphate (dAMP), deoxy-guanosine monophosphate (dGMP), and methylated deoxy-cytosine monophosphate (5-mdCMP) (Millenium Science Pty Ltd., Surrey Hills, Melbourne, Australia) were run through the HPLC at varying concentrations to create standard curves relating the area under the curve to sample concentration. Concentrations of each nucleotide in the test samples were calculated from the curves using linear regression, then the percentage of methylation calculated as: % 5-mdCMP = [5-mdCMP/(dCMP + 5-mdCMP)] x 100.

2.6.2.3 Statistical analysis

Total methylation levels were analysed using two-way ANOVA in Genstat v11. Triplicate measurements were converted to means prior to ANOVA as exploratory analysis revealed the absence of a block effect of the triplicate measurements. A probability value of less than 0.05 was considered significant, and a value of less than 0.10 was considered a trend.

2.6.3 DNA methylation of specific genes

2.6.3.1 Gene selection

A list of genes that were differentially expressed in IBD and mouse models was compiled from literature and in-house experiments using mouse models of IBD. MethPrimer software (http://www.urogene.org/methprimer/) was used to scan these genes for CpG islands and suitable primer sets for bisulfite-modified DNA undergoing

PCR [329]. The list was narrowed down to three genes which had 5 or more CpG islands and suitable primers: *Stat1*, *Tap2*, and *Ppara*. Each of these genes was differentially expressed in *Il10*$^{-/-}$ mice in the time-course experiment described in Chapter 3.

2.6.3.2 Methylation analysis of Ppara, Stat1, and Tap2 genes

Note: Sample preparation and spectrometric analysis were performed by Leticia Castro (The University of Auckland). Design of Sequenom primers was performed by Leticia Castro and Cameron Maclean (AgResearch).

DNA was extracted as described for the global DNA methylation analysis (section 2.6.1). Only samples that provided enough DNA in the primary elution for both global DNA methylation and specific gene methylation were used for global methylation analysis. DNA methylation of the key genes in colon tissue was assessed using the Sequenom MassARRAY Compact System (www.sequenom.com). This system is an efficient high-throughput method for determining quantitative DNA methylation analysis from various tissue types using base-specific cleavage of DNA and matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry [330]. Both hypo- and hyper-methylation are detected with high precision and reproducibility [330, 331].

One μg of DNA was bisulfite-converted using an EZ DNA Methylation kit (Zymo Research). Sequenom EpiDesigner software was used to design PCR primers specific for bisulfite-converted DNA. Each reverse primer contained a T7-promoter tag for *in vitro* transcription (5`-cagtaatacgactcactatagggagaaggct-3`), and the forward primer was tagged with a 10mer to balance Tm (5`-aggaagagag-3`). Bisulfite-treated DNA was PCR amplified (Qiagen HotStarTaq Polymerase) and treated with Shrimp Alkaline Phosphatase (Sequenom), heat inactivated, and a simultaneous *in vitro* transcription/uracil-cleavage reaction was performed. Transcription cleavage products were desalted and spotted on a 384-pad SpectroCHIP (Sequenom) using a MassARRAY nanodispenser (Samsung). Mass spectra were acquired using a MassARRAY MALDI-TOF MS (Bruker-Sequenom) and peak detection, signal-to-noise calculations and quantitative CpG site methylation analysis were performed using proprietary EpiTyper software v1.0.5 (Sequenom).

2.6.3.3 Statistical analysis

Note: Statistical analysis was performed by Dr Harold Henderson (AgResearch Ruakura).

For fragments containing a single CpG site, DNA methylation state was calculated by the ratio of methylated to un-methylated fragments. For cleavage products containing multiple CpG sites the average methylation status of the fragment was reported. Differences in methylation levels at individual sites (or cleavage products containing multiple CpG sites) were analysed using residual maximum likelihood (REML; GenStat Release 14.1). A probability value of less than 0.05 was considered significant.

3 Gene expression changes in the microdissected colon epithelium are similar to those of intact colon in the *Il10*$^{-/-}$ mouse

Part of the introductory material presented in this chapter, along with a brief description of the LMD method development for gene expression profiling as described in Chapter 2, has been accepted as a book chapter:

Russ, A.E., Peters J.S., McNabb, W.C. and Roy, N.C. (2012). *The importance of cell-specific gene expression patterns for understanding nutrient and gene interactions in Inflammatory Bowel Diseases.* In: Nutrigenetics and nutrigenomics in functional foods and personalised nutrition, Lynnette R Ferguson (Ed.), CRC Press, ISBN: 978-143-987-680-0 (accepted).

The results presented in this chapter have been submitted to *PLOS ONE*:

Russ, A.E., Peters J.S., McNabb, W.C., Barnett, M.P.G., Anderson, R.C., Park, Z., Zhu, S., Maclean, P., Young, W., Reynolds, G.W., and Roy, N.C. (submitted). Gene expression changes in the colon epithelium are similar to those of intact colon during late inflammation in interleukin-10 gene deficient mice.

3.1 Abstract

Epithelial cells play an important role in the protection of the colon mucosa from the resident microbiota and are involved in the initiation and maintenance of intestinal inflammation in addition to their role in nutrient and water absorption. LMD is a technique that allows the extraction of specific cell types, such as colonic epithelial cells, to analyse gene expression. LMD of colon epithelial cells followed by microarray analysis could be of more value than microarray analysis of intact colon for determining which pathways are active in the colon mucosa in the early and late stages of inflammation due to increased sensitivity to changes in specific cell populations. An experiment was performed using microarray analysis of intact colon samples and microdissected colon epithelial cell samples from *Il10*$^{-/-}$ and C57BL/6J mice at 6 and 12 weeks of age to study the molecular changes that occur in early and late inflammation stages in colon epithelium of a mouse model of colitis. Results showed that intact colon and colon epithelial cell gene expression profiles were similar in terms of pathways between *Il10*$^{-/-}$ and C57BL/6J mice at 12 weeks of age and between *Il10*$^{-/-}$ mice at 12 and 6 weeks of age. More immune-related pathways were identified at 6 weeks of age in epithelial cells than intact colon. This suggests that LMD and targeting of specific cell types may be of particular use when studying the early stages of inflammation before the intestinal morphology is detectably altered, by increasing analysis sensitivity to mucosal gene expression changes.

3.2 Introduction

Laser microdissection is a technique that allows pure populations of cells to be removed from frozen tissue, enabling subsequent molecular profiling of these cells which is then reflective of their *in vivo* state [119-121]. An example of how LMD can contribute to understanding the development of a disease is a study of the development of *Helicobacter*-induced gastric lymphomas [122]. The authors demonstrated that gene expression patterns in lymphocytic and mucosal fractions differed from each other, and from expression patterns in whole stomach. These findings confirm that the likely cellular origin of particular transcripts could be identified by analysis of gene expression in specific cell fractions, thus providing more information about the relationship between a particular pathology and associated gene expression patterns.

Gene expression analysis in the large intestine has been performed in a number of studies using mouse models of IBD and biopsy tissues of IBD patients [76, 332, 333]. Such analyses have mostly been performed on intact colon tissue and provide useful clues as to the molecular pathways involved in the initiation and maintenance of inflammation. However, the colon is a complex tissue with distinct layers (mucosa, submucosa, muscular layers and serosa) and numerous cell types in each layer. The epithelial layer of the colon mucosa contains enterocytes, goblet cells, stem cells and intraepithelial lymphocytes. The cellular location of gene expression signals cannot be determined in intact colon sections and expression signals from a small population of cells may be missed entirely, even if they are an important signal within that population, due to noise from changes in expression from more abundant cell types. The ability to analyse gene expression profiles in specific cell types or tissue regions using LMD may provide a much more detailed understanding of the function of the various cells and types of tissue within the colon in inflammation. As yet, there are no reports of global gene expression profiles in colon epithelium before and after the development of colitis.

Gene expression analysis of intact colon includes a large number of infiltrating immune cells, which may be associated with tissue damage and the gross changes in intestinal morphology that occur in inflammation, but may be less involved with the initiation and maintenance of the pro-inflammatory signals that underly inflammation. Epithelial cells play an important role in the initiation of inflammation and the intestine's response to bacteria [38, 39]. Changes in the colonic epithelium, such as increased paracellular permeability and mucosal immune defence malfunction, are thought to be important in the initiation of inflammation [40, 41]. Disruption of the epithelial barrier allows entry of bacterial antigen into the mucosa, and when the mucosal immune response is unable to limit the infection, an uncontrolled immune response to the lumenal microbiota, leading to an inappropriate inflammatory response, may result. The central role of the epithelium in inflammation development makes an understanding of the cellular changes occurring in the epithelial layer in colitis a required foundation for studying the effects of food components on the inflammatory process in IBD.

3.3 Aims and hypothesis

The hypothesis of this study was that microdissection and subsequent microarray analysis of colon epithelial cells would enable the identification of pathways and processes related to the inflammatory response that are not detected by microarray analysis of intact colon.

The first aim of this study was to characterise global gene expression changes in intact colon tissue before and after inflammation developed in *Il10*$^{-/-}$ mice (at 6 and 12 weeks of age, respectively). This was intended to better understand the molecular changes occurring in the colon in the early stage of inflammation, before any measurable histological changes in inflammatory phenotype occur. Whole colon profiles were assessed at 12 weeks to confirm that the gene expression changes occurring in this model are similar to other gene expression profiles in colitis and previous experiments using this model in our laboratory.

The second aim of this study was to examine whether the global gene expression profile of colon epithelial cells is similar to that of whole colon tissue, and if not, describe the differences between the profiles with the aim of determining whether intact colon provides a good approximation of the changes in the epithelium in inflammation, or whether inflammation-related changes may not be detected in intact colon. If many inflammation-related changes are detected in the colon epithelium that are undetectable in intact colon, this would suggest that future studies of the pathogenesis of colitis and/or the impact of dietary components or other treatments on the disease process should include analysis of specific regions of colon tissue or specific cell types in addition to, or instead of, intact tissue.

3.4 Methods

The mouse experiment was carried out according to Section 2.2., the microarray analysis was performed according to Section 2.3, the LMD work was performed according to Sections 2.4, and the qPCR validation was performed according to Section 2.5.

3.5 Results

3.5.1 Bodyweight, feed intake, and body condition of mice

All mice sampled at 6 weeks of age had a GHS of 1 (healthy) at the beginning of the experiment, which did not change for the duration of the experiment. At the beginning of the experiment, the body weight of the C57BL/6J mice was lower than that of the *Il10*$^{-/-}$ mice (means of 16.5 *vs.* 18.6 g respectively, P = 0.03; Table 3.1). At 6 weeks of age, the mean weight of the C57BL/6J mice was still lower than that of the *Il10*$^{-/-}$ mice (means of 17.7 *vs.* 19.6 g respectively, P = 0.02; Table 3.1). Only one intake measurement was made for this group of mice.

The mice sampled at 12 weeks also had a GHS of 1 (healthy) at the beginning and end of the experiment. The *Il10*$^{-/-}$ mice developed minor changes in the fur condition on the back of their neck, with it becoming thinner and duller towards the end of the experiment. However, no other external changes or differences between strains were observed. The body weight of the *Il10*$^{-/-}$ mice sampled at 12 weeks of age was not different to that of the C57BL/6J mice at the beginning (P = 0.08) and end (P = 0.50) of the experiment (Table 3.1). Mean food intake was slightly higher for the C57BL/6J mice at the beginning of the experiment (P = 0.04), but similar for both strains at the end of the experiment (P = 0.40).

3.5.2 Histological injury score

Duodenum histology scores were low and showed no correlation between treatments, as expected, while jejunum and ileum scores were zero for all mice. Colon histology results for each sampling time and mouse strain are shown in Table 3.2 while the intestinal area and colon HIS data for each mouse are shown in Table 3.3. Colon HIS were not different between *Il10*$^{-/-}$ and control mice at 6 weeks of age (0.17 *vs.* 0.28; P > 0.05), but were significantly higher for *Il10*$^{-/-}$ mice compared to C57BL/6J mice (3.00 *vs.* 0.28, P < 0.05) at 12 weeks.

No mice of either strain at 6 weeks of age and/or any C57BL/6J mice at 12 weeks of age had any visible signs of inflammation throughout the length of their intestinal tract. All

Table 3.1 Mean body weight and food intake data for all mice at the beginning and end of the experiment.

Parameter	Mean initial	SD	P values for between-strain differences	Mean final	SD	P values for between-strain differences
Body weight (g)						
6 week old C57BL/6J mice	16.5	2.0	0.03	17.7	1.7	0.02
6 week old *Il10*$^{-/-}$ mice	18.6	1.6		19.6	1.4	
12 week old C57BL/6J mice	17.0	1.6	0.08	23.7	1.9	0.50
12 week old *Il10*$^{-/-}$ mice	18.8	1.8		24.2	1.3	
Food intake (g/day)						
6 week old C57BL/6J mice	3.4	0.6	0.27	ND	ND	ND
6 week old *Il10*$^{-/-}$ mice	3.1	0.4		ND	ND	ND
12 week old C57BL/6J mice	3.7	0.7	0.04	3.0	0.2	0.40
12 week old *Il10*$^{-/-}$ mice	3.0	0.6		3.1	0.3	

SD: standard deviation

ND: no data

Table 3.2 Mean colon histological injury score and mean intestinal area for each strain at each time point.

Strain	Age (weeks)	Number of mice	Mean colon histological injury score	Number of samples available for intestinal area	Mean intestinal area (mm^2)	Mean intestinal area per gram body weight (mm^2/g)
Il10$^{-/-}$	6	9	0.17	3	1534	83.5
	12	10	3.00 *	9	1923 *	86.1 *
C57BL/6J	6	8	0.28	3	1502	95.7
	12	8	0.28	8	1399	61.9

* denotes a mean that is significantly ($P < 0.05$) different for *Il10*$^{-/-}$ mice compared to C57BL/6J mice at 12 weeks of age. Predicted means for colon histological injury score were obtained from unbalanced ANOVA analysis performed on (log+0.05)-transformed data in GenStat v11, then predicted means were back-transformed to give the data presented here.

Table 3.3 Intestinal inflammation data for individual mice. Data for intestinal area and colon histological injury score for each mouse, obtained for most mice at the 12-week sampling time, but for only three of each strain at the 6-week sampling time.

Mouse	Age (weeks)	Strain	Final bodyweight from day of sampling (g)	Intestine (mm^2)	Intestinal area per gram body weight (mm^2/g)	Colon histological injury score
5	6	*Il10*$^{-/-}$	20.5	1752.5	85.5	0
14	6	*Il10*$^{-/-}$	17.6	1325.9	75.3	0.5
19	6	*Il10*$^{-/-}$	17.0	1522.9	89.6	0
20	6	C57BL/6J	16.7	1758.2	105.3	0.5
26	6	C57BL/6J	14.5	1286.9	88.8	0
28	6	C57BL/6J	15.7	1461.1	93.1	1
1	12	*Il10*$^{-/-}$	23.8	1902.6	79.9	4.5
4	12	*Il10*$^{-/-}$	21.1	2187.5	103.7	4.5
6	12	*Il10*$^{-/-}$	23.2	1885.3	81.3	3
8	12	*Il10*$^{-/-}$	23.7	1941.5	81.9	0.5
10	12	*Il10*$^{-/-}$	21.5	1585.4	73.7	0.5
11	12	*Il10*$^{-/-}$	24.2	1960.1	81.0	7.5
12	12	*Il10*$^{-/-}$	19.6	2028.1	103.5	4
15	12	*Il10*$^{-/-}$	22.1	1757.7	79.5	9.5
17	12	*Il10*$^{-/-}$	22.8	2061.5	90.4	6
21	12	C57BL/6J	21.6	1163.5	53.9	1
24	12	C57BL/6J	21.4	1394.8	65.2	0.5
25	12	C57BL/6J	21.5	1196.1	55.6	0.5
29	12	C57BL/6J	20.4	1283.7	62.9	0.5
31	12	C57BL/6J	25.7	1365.3	53.1	0.5
32	12	C57BL/6J	24.0	1496.8	62.4	0
33	12	C57BL/6J	25.0	1583.4	63.3	0.5
34	12	C57BL/6J	21.7	1704.8	78.6	0

Il10$^{-/-}$ mice at 12 weeks had some intestinal thickening, indicating the presence of inflammation throughout the intestine. Intestinal area, with and without taking into account mouse weight at sampling, was significantly larger for *Il10*$^{-/-}$ mice than C57BL/6J mice at 12 weeks of age ($P < 0.05$) (Table 3.2).

3.5.3 Colon gene expression by microarray analysis

The data discussed in this chapter have been deposited in NCBI's Gene Expression Omnibus [334] and are accessible through GEO Series accession number GSE39859 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39859).

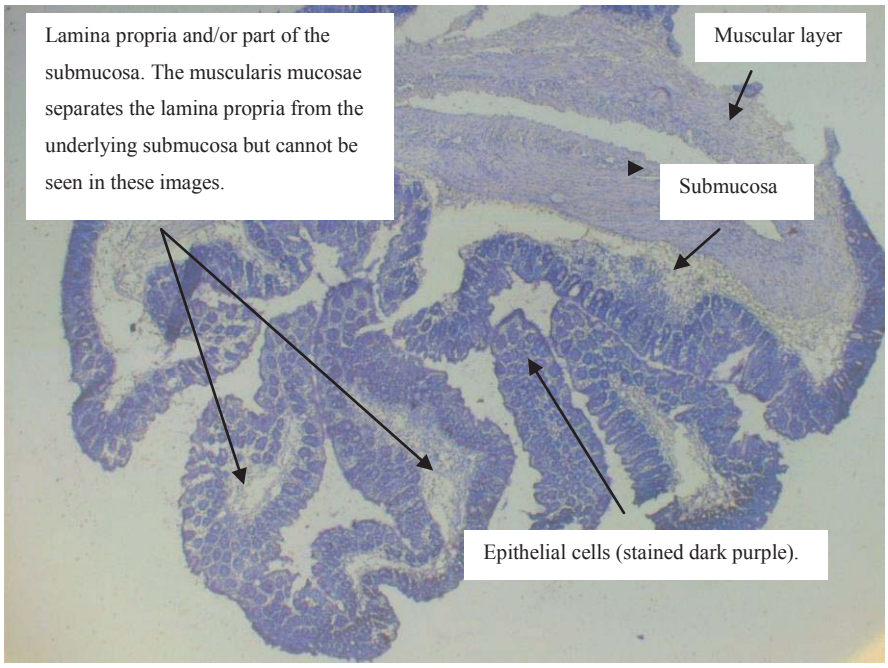
3.5.4 Cell harvesting for colon gene expression analysis

For the intact colon microarrays, RNA was extracted from a short segment (<20 μg) of proximal colon. For the epithelial cell microarrays, RNA was extracted from epithelial cells that were microdissected from sections of proximal colon. Intact colon and epithelial cell RNA were therefore taken from the same region of the colon, the proximal end, and where possible, from the same mice. An example of which cells were dissected out for the epithelial cell microarray analysis from a non-inflamed mouse is shown in Figure 3.1, and an example of cells dissected from an inflamed mouse is shown in Figure 3.2.

3.5.4.1 Quality control analysis of between-slide effects

For the intact colon microarrays, REML analysis combined with IPA clustering analysis was run as a quality control check to determine whether the results were significantly different depending on the day the slides were hybridised. Graphs drawn from principle components analysis showed a significant difference between the three slides hybridised on the first day, and the three slides hybridised on the second day. The resulting corrected gene list for the comparison "*Il10*$^{-/-}$ *vs.* C57BL/6J mice at 12 weeks of age" for intact microarray was similar to the uncorrected gene list in terms of top genes, degree of change in expression of these top genes (Table 3.4), and pathways. The uncorrected data was thus used for further analysis. Data from the epithelial arrays was consistent between hybridisation batches so no correction was required.

A



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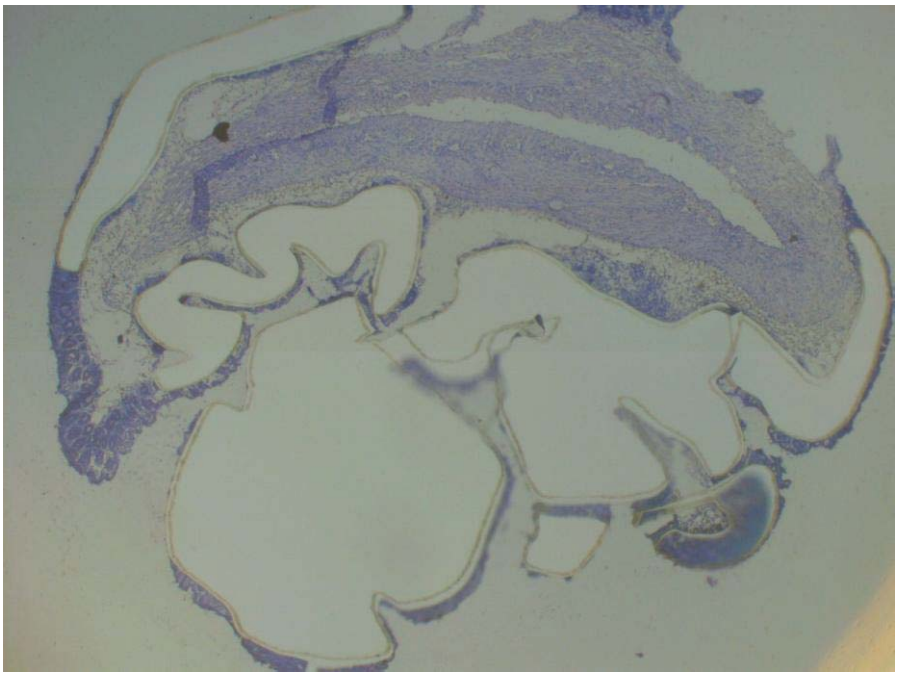


Figure 3.1 Non-inflamed mouse colon section. Colon section for mouse 18, an *Il10*$^{-/-}$ mouse sampled at 6 weeks of age, before microdissection of epithelial cells (A) and after microdissection of epithelial cells (B). The lamina propria was taken from this section along with the epithelial cells but is mostly connective tissue in the non-inflamed mice. Stained with Arcturus Histogene stain, viewed using 4x objective.

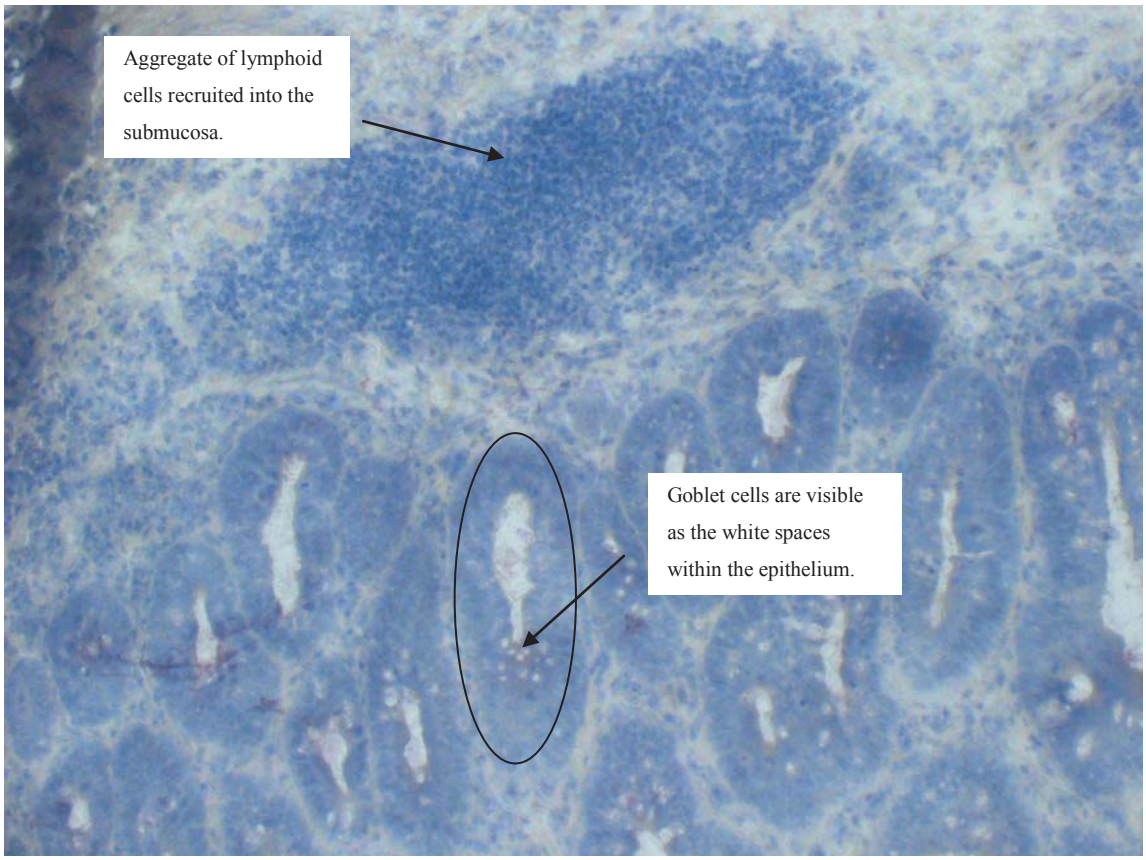


Figure 3.2 Inflamed mouse colon section. Colon section from mouse 17, an *Il10*$^{-/-}$ mouse sampled at 12 weeks of age, before microdissection of epithelial cells. The aggregate of immune cells is visible in the mucosa, which is typical for CD-like colitis, such as that seen in this mouse model. The circle outlines epithelial cells in a colonic crypt, which were cut out for analysis of gene expression. Stained with Arcturus Histogene stain, viewed using 20x objective.

Table 3.4 Corrected and non-corrected gene expression data. Top 20 differentially expressed genes (ordered by degree of fold change) for the comparison "*Il10*$^{-/-}$ mice at 12 weeks of age vs. C57BL/6J mice at 12 weeks of age" in intact colon before and after correction for microarray slide effects. "FC" is used as an abbreviation for degree of fold change; "FDR" is used as an abbreviation for false discovery rate.

		Non-corrected data		**Corrected data**	
Gene ID	**Gene name**	**FC**	**FDR**	**FC**	**FDR**
S100a9	S-100 calcium binding protein	35.94	<0.001	35.63	<0.001
Pap	Pancreatitis-associated protein	31.36	<0.001	30.75	<0.001
Reg3g	Regenerating islet-derived 3γ	30.18	<0.001	29.85	<0.001
S100g	S-100 calcium binding protein	-20.95	<0.001	-21.16	<0.001
S100a8	S-100 calcium binding protein	18.82	<0.001	18.79	<0.001
NAP065157	unknown	16.74	<0.001	16.85	<0.001
Cxcl5	Chemokine (CXC motif)	15.95	<0.001	15.88	<0.001
Igh-1a	Immunoglobulin heavy chain	15.17	<0.001	15.20	<0.001
Iigp1	Interferon inducible GTPase 1	14.99	<0.001	15.03	<0.001
Irg1	Immunoresponsive gene 1	14.56	<0.001	14.67	<0.001
Ighg	Immunoglobulin heavy chain (γ polypeptide)	14.47	<0.001	14.48	<0.001
Il1b	Interleukin 1β	14.28	<0.001	Not in top 20	
Cxcl9	Chemokine (CXC motif)	13.94	<0.001	13.75	<0.001
Irg1	Immunoresponsive gene 1	13.64	<0.001	13.68	<0.001
Mmp3	Matrix metalloprotease 3	13.64	<0.001	13.61	<0.001
Slc28a3	Solute carrier family 28 (sodium-coupled nucleoside transporter), member 3	12.87	<0.001	12.83	<0.001
Chi3l3	Chitinase 3-like 3	12.75	<0.001	12.84	0.003
Cxcl10	Chemokine (CXC motif)	11.87	<0.001	11.74	0.003
Indo	Indoleamine 2,3-dioxygenase 1	11.26	<0.001	11.33	<0.001
Ubd	Ubiquitin D	10.95	<0.001	10.99	<0.001
Tgtp	T-cell specific GTPase	Not in top 20		10.51	<0.001

3.5.4.2 Differentially expressed genes

Within this thesis, a differentially expressed gene is considered to be one which has a higher or lower level of expression (i.e. higher or lower level of transcribed mRNA) in one treatment relative to another, above 1.5 times and with a FDR of less than 0.05. Most gene expression analyses, such as those performed by IPA and EASE software, focus on only those genes which are considered differentially expressed between treatments, as these are typically considered to be of most biological interest. However, non-differentially expressed genes may be important also, particularly when they are part of a set of genes that is more highly expressed overall in one treatment than another than would be expected by chance. For this reason, GSEA was also performed on this data to identify sets of genes that may be highly or lowly expressed in one treatment regardless of whether specific genes in the set were considered to be differentially expressed. Due to the large size of many of the data sets, for the IPA data, the top 5 significant pathways or networks are presented, and for other data such as EASE or GSEA, the top 20 pathways or gene sets are presented (in order of ascending probability value, unless otherwise stated).

In intact colon, the highest number of differentially expressed genes (FC > 1.5; FDR < 0.05), 3493, as well as the highest fold changes, occurred in 12 week old *Il10*$^{-/-}$ mice compared to 12 week old C57BL/6J mice (Table 3.5). A similar number of genes, 2309, were differentially expressed in 12 week old *Il10*$^{-/-}$ mice compared to 6 week old *Il10*$^{-/-}$ mice. In contrast, a lower number of genes were differentially expressed in C57BL/6J mice at 12 weeks compared to those at 6 weeks (251), and in 6 week old *Il10*$^{-/-}$ mice compared to 6 week old C57BL/6J mice (65). Fewer genes were differentially expressed for each comparison in the epithelial cell microarrays than the intact colon microarrays. The highest numbers of differentially expressed genes in epithelial cells also occurred in the 12 week old *Il10*$^{-/-}$ mice compared to 12 week old C57BL/6J mice (888) and 12 week old *Il10*$^{-/-}$ mice compared to 6 week old *Il10*$^{-/-}$ mice (385).

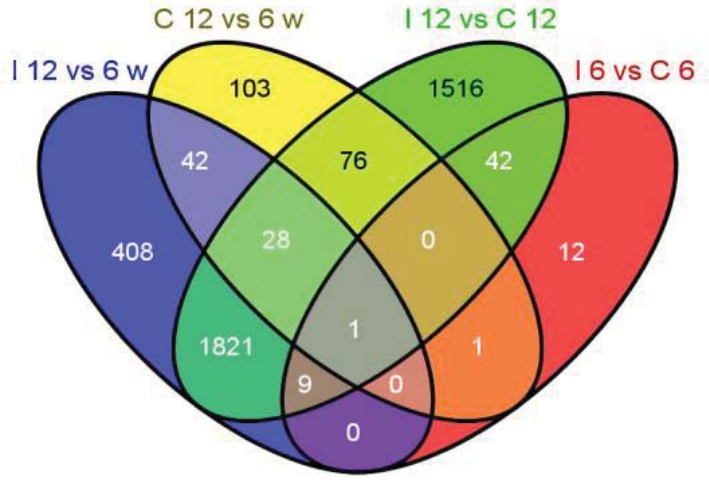
The distribution of differentially expressed genes between groups for both intact colon and colon epithelium are shown in the Venn diagram in Figure 3.3. In the intact colon, the vast majority of differentially expressed genes occurred in the comparison 12 week old *Il10*$^{-/-}$ mice *vs.* 12 week old C57BL/6J mice and approximately half of these genes were shared with the comparison 12 week *vs.* 6 week old *Il10*$^{-/-}$ mice.

Table 3.5 Numbers of differentially expressed genes for each treatment comparison for both intact colon and colon epithelial cell microarray experiments.

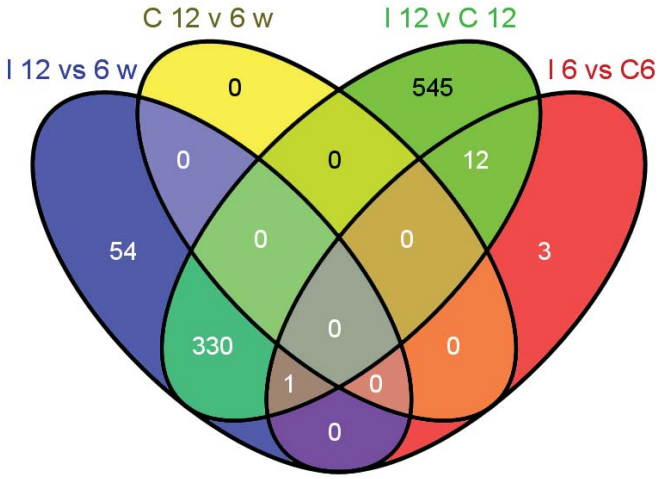
Treatment comparison	**Number of genes differentially expressed in intact colon**	**Number of genes differentially expressed in colon epithelial cells**
Il10$^{-/-}$ mice at 12 weeks old *vs.* C57BL/6J mice at 12 weeks old	3493	888
Il10$^{-/-}$ mice at 12 weeks old *vs.* *Il10*$^{-/-}$ mice at 6 weeks old	2309	385
C57BL/6J mice at 12 weeks old *vs.* C57BL/6J mice at 6 weeks old	251	0
Il10$^{-/-}$ mice at 6 weeks old *vs*. C57BL/6J mice at 6 weeks old	65	16

Figure 3.3 Overlap of differentially expressed genes between treatments. Venn diagrams show the numbers of differentially expressed genes in each comparison and how many are shared between each comparison, for intact colon (A) and colon epithelium (B). Treatments are identified in the diagram as: *Il10*$^{-/-}$ mice = I; C57BL/6J mice = C; weeks of age = w.

A



B



Note: Each colour arbitrarily represents a different treatment. Numbers are of differentially expressed genes within each treatment comparison and each microarray experiment (intact colon and epithelial cells). Numbers in (B) are not subsets of the equivalent treatment comparison in (A), although there is some overlap in the gene lists for each comparison between intact colon and colon epithelium.

Approximately 400 and 100 genes were unique to the 12 *vs.* 6 week comparisons for *Il10*$^{-/-}$ mice and C57BL/6J mice, respectively. In the colon epithelium, over half of the genes differentially expressed in the 12 week old *Il10*$^{-/-}$ mice *vs.*12 week old C57BL/6J mice were shared with the comparison 12 week *vs.* 6 week old *Il10*$^{-/-}$ mice.

A list of immune-related differentially expressed genes was plotted in a heatmap to visually show the clustering of treatments by similarity in gene expression profile (Figure 3.4). Immune-related genes accounted for many of the differentially expressed genes for the *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* C57BL/6J mice at 12 weeks of age and the *Il10*$^{-/-}$ mice at 12 weeks of age *vs. Il10*$^{-/-}$ mice at 6 weeks of age comparisons in both intact colon and colon epithelium. The gene expression profiles of *Il10*$^{-/-}$ mice at 12 weeks of age for intact colon and colon epithelium were very similar to each other (Pearson correlation coefficient = 0.89), while the remaining profiles were clustered together by tissue type. In the other mice, tissue type (intact colon versus colon epithelium) contributed to the differentiation between expression profiles to a greater extent. All C57BL/6J mice and 6-week-old *Il10*$^{-/-}$ mice had similar profiles within intact colon and colon epithelium.

In the intact colon gene expression profiles, the 6-week-old C57BL/6J and *Il10*$^{-/-}$ mice were more similar to each other (Pearson correlation coefficient = 0.97) than to 12-week-old C57BL/6J mice (Pearson correlation coefficients = 0.96 and 0.91), whereas in the epithelium, 6-week-old C57BL/6J mice were more similar to 12-week-old C57BL/6J mice (Pearson correlation coefficient = 0.97) than the 6-week-old *Il10*$^{-/-}$ mice (Pearson correlation coefficient = 0.94). This shows that in the epithelium, there is a greater difference evident in gene expression profiles in this subset of immune-related genes between *Il10*$^{-/-}$ mice and C57BL/6J mice at 6 weeks of age.

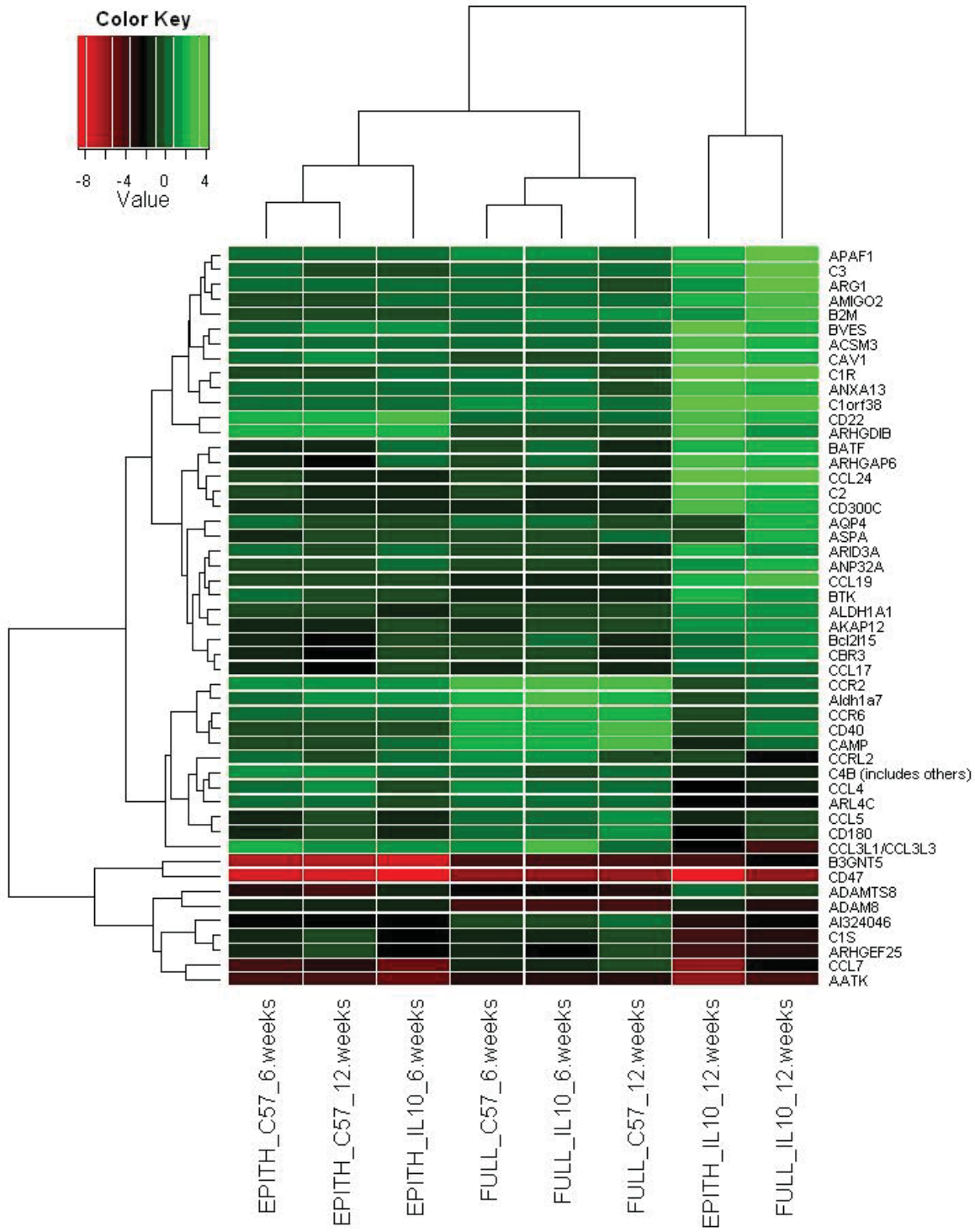


Figure 3.4 Immune-related gene expression profiles in intact colon and epithelium. Heatmap of a subset of differentially expressed immune related genes from the *Il10*$^{-/-}$ mice at 12 weeks vs. C57BL/6J mice at 12 weeks comparison, showing the gene expression profile of each treatment comparison in both intact colon and colon epithelium. Expression values for each gene are averages from 6 arrays per treatment.

3.5.5 Pathway analysis

The top five canonical pathways are presented in Table 3.6 for each comparison for intact colon and colon epithelium. Pathways are only presented if they reached the criteria for significance that are described in Chapter 2. The only comparisons for which significant pathways were identified for intact colon or colon epithelium were *Il1*$^{-/-}$ mice at 12 weeks of age versus C57BL/6J mice at 12 weeks of age and *Il10*$^{-/-}$ mice at 12 weeks of age versus C57BL/6J mice at 6 weeks of age. The percentages of genes in a pathway that were differentially expressed in any treatment comparison were smaller for the colon epithelium data (10-22%) than the intact colon (25-52%).

There was overlap within the top 5 pathways in each treatment comparison for intact colon and colon epithelium. In the epithelium, apart from one pathway, fatty acid metabolism, the top 5 pathways were all related to immune functioning for each treatment comparison in the epithelium. In intact colon and epithelium, the top 5 pathways were related to immune function: communication between innate and adaptive immune cells; altered T and B cell signalling in rheumatoid arthritis; graft-versus-host disease signalling; T helper cell differentiation; dendritic cell maturation; allograft rejection signalling; and LPS/IL-1 mediated inhibition of RXR function.

A network of the molecules from the top 5 differentially expressed canonical pathways in the colon epithelium for *Il10*$^{-/-}$ mice at 12 weeks of age compared to 6 weeks of age is shown in Figure 3.5. The same network overlaid with expression values for the same comparison in intact colon is shown in Figure 3.6. As the network diagrams show, these genes had similar patterns of expression in both intact colon and colon epithelium. One major difference was that the abundance of IL18 mRNA was reduced in the colon epithelium of *Il10*$^{-/-}$ mice at 12 weeks of age compared to 6 weeks of age but was not differentially expressed for the intact colon. These findings suggest that the gene expression profiles in *Il10*$^{-/-}$ mice at 12 weeks of age were similar between intact colon and epithelium alone, but there were some differences.

3.5.6 Gene set enrichment analysis

Differentially expressed genes clustered into few gene sets in *Il10*$^{-/-}$ *vs.* C57BL/6J mice at 6 weeks of age, 3 in intact colon and 6 in colon epithelium (Table 3.7). This was not

Table 3.6 Top five canonical pathways for intact colon and colon epithelium. Canonical pathways were generated by IPA from the gene lists for each treatment comparison. The comparisons were: *Il10*$^{-/-}$ mice at 12 weeks of age vs. C57BL/6J mice at 12 weeks of age; *Il10*$^{-/-}$ mice at 12 weeks of age vs. *Il10*$^{-/-}$ mice at 6 weeks of age; C57BL/6J mice at 12 weeks of age vs. C57BL/6J mice at 6 weeks of age; and *Il10*$^{-/-}$ mice at 6 weeks of age vs. C57BL/6J mice at 6 weeks of age.

Part of colon	Canonical pathway	Percentage of genes in pathway that were observed in this gene list	P value
Il10$^{-/-}$ mice, 12 vs. 6 weeks			
Intact colon	Communication between innate and adaptive immune cells	36	9.54×10^{-24}
	Altered T and B cell signalling in rheumatoid arthritis	42	1.40×10^{-20}
	Graft-versus-host disease signalling	50	2.73×10^{-17}
	T helper cell differentiation	44	1.62×10^{-16}
	Dendritic cell maturation	25	3.68×10^{-15}
Colon epithelium	Dendritic cell maturation	10	1.95×10^{-11}
	T helper cell differentiation	18	1.06×10^{-10}
	Altered T and B cell signalling in rheumatoid arthritis	15	1.28×10^{-10}
	Graft-vs-host disease signalling	22	2.11×10^{-10}
	Type I diabetes mellitus signalling	12	3.64×10^{-09}
Il10$^{-/-}$ mice vs. C57BL/6J mice, 12 weeks			
Intact colon	Communication between innate and adaptive immune cells	38	7.42×10^{-19}
	Dendritic cell maturation	31	4.49×10^{-16}
	Altered T and B cell signalling in rheumatoid arthritis	45	1.19×10^{-15}
	Allograft rejection signalling	29	5.82×10^{-14}
	Graft-vs-host disease signalling	52	6.70×10^{-14}
Colon epithelium	LPS/IL-1 mediated inhibition of RXR function	14	2.92×10^{-12}
	Fatty acid metabolism	13	5.78×10^{-12}
	Allograft rejection signalling	16	4.12×10^{-11}
	Communication between innate and adaptive immune cells	16	4.23×10^{-10}
	Dendritic cell maturation	13	6.00×10^{-10}

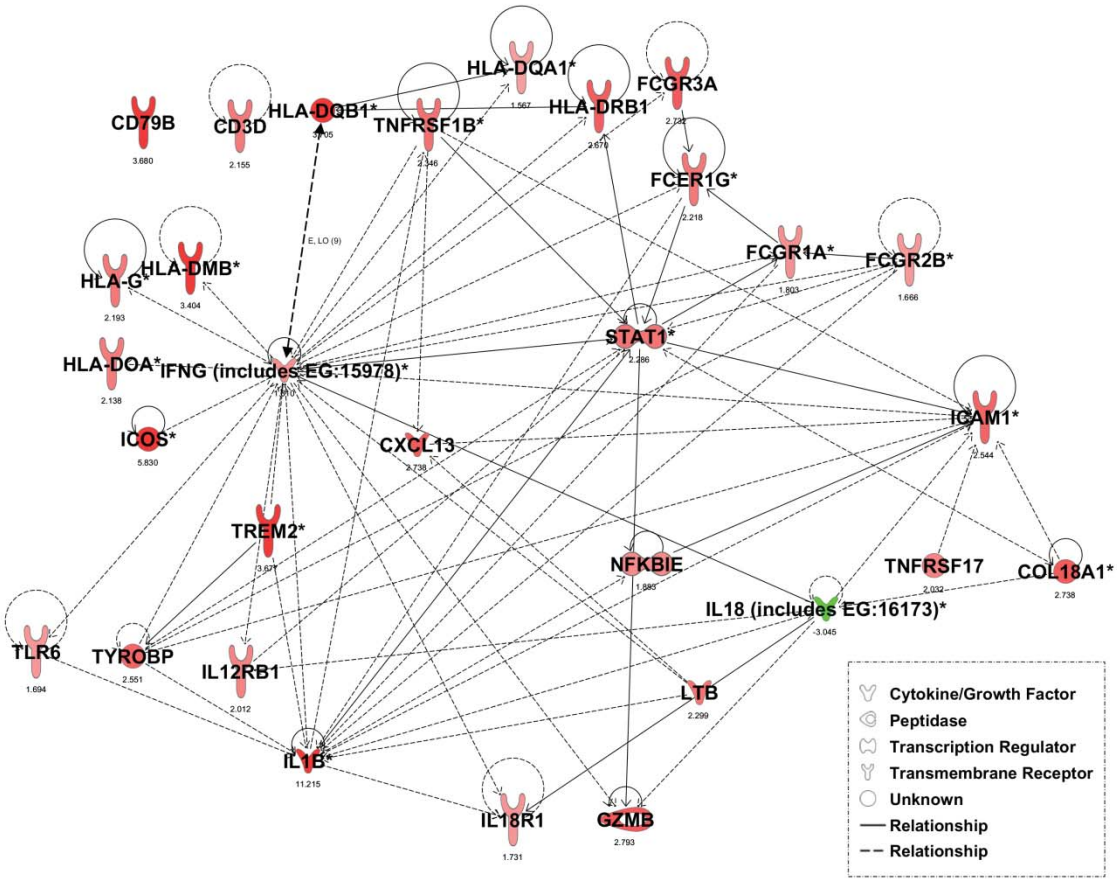


Figure 3.5 Gene expression in the colon epithelium. Network diagram of genes from the top five differentially expressed canonical pathways in *Il10*$^{-/-}$ mice at 12 weeks of age vs. 6 weeks of age in the colon epithelium. Molecules coloured in green represent a gene with decreased expression in *Il10*$^{-/-}$ mice at 12 weeks of age relative to in *Il10*$^{-/-}$ mice at 6 weeks of age, while red indicates an increase in expression (see Section 2.3.7.1 for more detail about interpretation of network diagrams). Legend indicates interpretation of molecule shape and line type; numbers beneath molecules indicate fold change values.

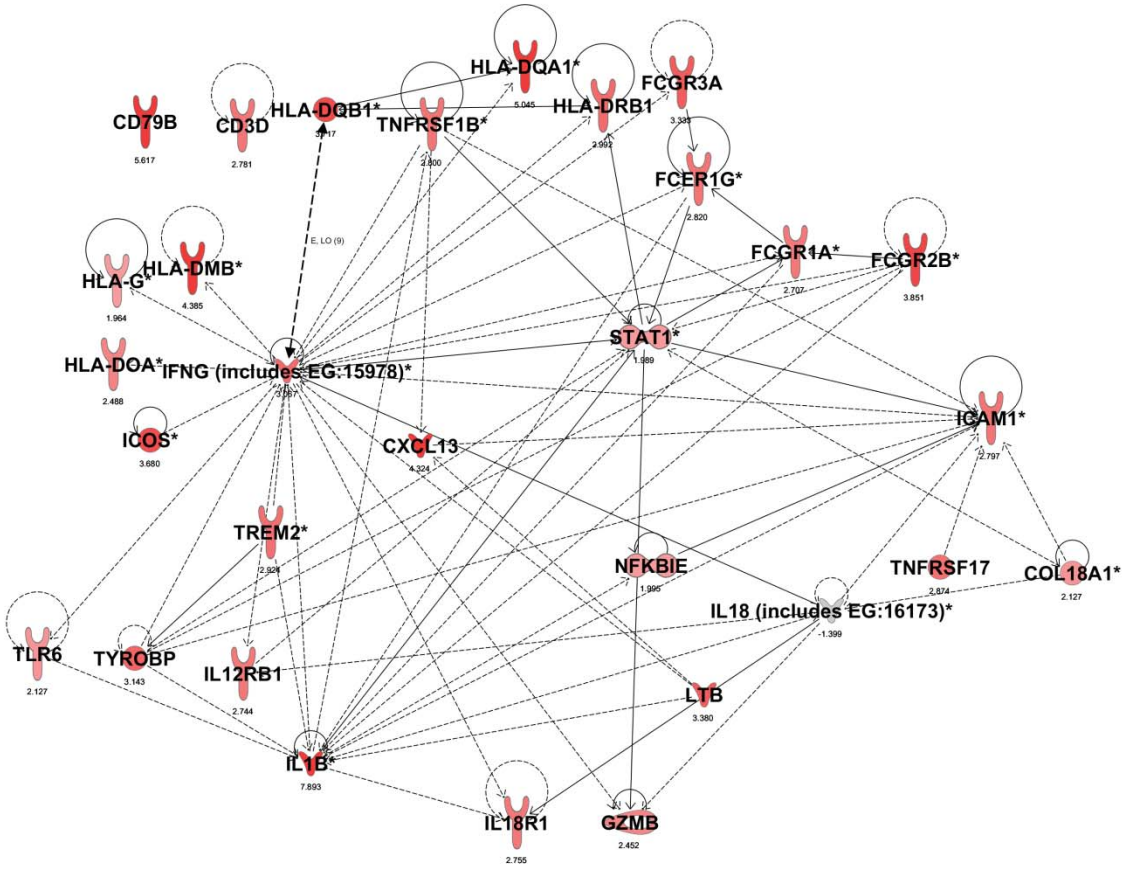


Figure 3.6 Gene expression in the intact colon. Network diagram shown in Figure 3.5 overlaid with expression values for the same treatment comparison in intact colon.

Table 3.7 Gene sets from enrichment analysis for *Il10*$^{-/-}$ mice at 6 weeks of age *vs.*C57BL/6J mice at 6 weeks of age for intact colon and colon epithelium.

Pathway ID	Pathway name	P value
Intact colon		
mmu00520	Amino sugar and nucleotide sugar metabolism	0.016
mmu04141	Protein processing in endoplasmic reticulum	0.020
mmu04914	Progesterone-mediated oocyte maturation	0.031
Colon epithelium		
mmu00565	Ether lipid metabolism	<0.001
mmu04062	Chemokine signalling pathway	0.004
mmu04914	Progesterone-mediated oocyte maturation	0.020
mmu05144	Malaria	0.033
mmu04110	Cell cycle	0.035
mmu04060	Cytokine-cytokine receptor interaction	0.048

surprising because there was no difference in colon HIS between these groups. In the colon epithelium, 2 of the 6 gene sets were immune-related (chemokine signalling pathway, and cytokine-cytokine receptor interaction) while in the intact colon, no immune-related gene sets were identified. This may indicate that targeting epithelial cells can increase sensitivity for detecting immune changes that occur early in the inflammatory process.

Differentially expressed genes clustered into few gene sets in C57BL/6J mice at 12 weeks of age *vs.* C57BL/6J mice at 6 weeks of age: 3 sets in intact colon and 4 sets in colon epithelium (Table 3.8). These gene sets were related to cell membrane function and cell signalling and likely represented age and growth-related changes in the colon of the C57BL/6J mice.

Differentially expressed genes clustered into a large number of gene sets in *Il10*$^{-/-}$ *vs.* C57BL/6J mice at 12 weeks of age, and in the *Il10*$^{-/-}$ mice at 12 weeks *vs.* 6 weeks, which was expected because there was a change in colon HIS. A number of these pathways were related to immunity and cancer. There were fewer metabolism-related gene sets in *Il10*$^{-/-}$ mice at 12 weeks *vs.* 6 weeks than *Il10*$^{-/-}$ *vs.* C57BL/6J mice at 12 weeks of age. Overall, differentially expressed genes clustered into similar gene sets in the colon epithelium and intact colon for both comparisons of *Il10*$^{-/-}$ mice at 12 weeks *vs.* C57BL/6J mice at 12 weeks (Table 3.9) and *Il10*$^{-/-}$ mice at 6 weeks (Table 3.10).

3.5.7 Over-representation analysis

In *Il10*$^{-/-}$ mice *vs.*C57BL/6J mice at 6 weeks of age, there were no over-represented gene categories generated in the colon epithelium, and only 3 in the intact colon: these were cell differentiation, cytokine metabolism and cytokine biosynthesis. In C57BL/6J mice at 12 weeks *vs.* 6 weeks of age, there were no over-represented gene categories generated in the colon epithelium, although there were 24 in the intact colon. None of these gene categories were immune-related: instead, they were related to normal cell function and included processes related to cell functions such as enzyme and transporter activity, and cellular components, such as cytoskeleton and cell membrane structure. The colon grows and develops with age in C57BL/6J mice (as with *Il10*$^{-/-}$ mice), which may explain why there were over-represented processes in intact colon in C57BL/6J

Table 3.8 Gene sets from enrichment analysis for C57BL/6J mice at 12 weeks of age *vs*. C57BL/6J mice at 6 weeks of age for intact colon and colon epithelium.

Pathway ID	Pathway name	P value
Intact colon		
mmu04540	Gap junction	0.002
mmu04080	Neuroactive ligand-receptor interaction	0.005
mmu04670	Leukocyte transendothelial migration	0.020
Colon epithelium		
mmu00512	O-Glycan biosynthesis	0.012
mmu04070	Phosphatidylinositol signaling system	0.020
mmu00562	Inositol phosphate metabolism	0.039
mmu03010	Ribosome	0.040

Table 3.9 Top 20 gene sets from enrichment analysis for *Il10*$^{-/-}$ mice at 12 weeks of age *vs*. C57BL/6J mice at 12 weeks of age for intact and colon epithelium.

Pathway ID	Pathway name	P value
Intact colon		
mmu00051	Fructose and mannose metabolism	<0.001
mmu00520	Amino sugar and nucleotide sugar metabolism	<0.001
mmu00562	Inositol phosphate metabolism	<0.001
mmu00565	Ether lipid metabolism	<0.001
mmu00601	Glycosphingolipid biosynthesis - lacto and neolacto series	<0.001
mmu04010	MAPK signaling pathway	<0.001
mmu04012	ErbBsignaling pathway	<0.001
mmu04060	Cytokine-cytokine receptor interaction	<0.001
mmu04062	Chemokine signaling pathway	<0.001
mmu04115	p53 signaling pathway	<0.001
mmu04120	Ubiquitin mediated proteolysis	<0.001
mmu04142	Lysosome	<0.001
mmu04145	Phagosome	<0.001
mmu04150	mTORsignaling pathway	<0.001
mmu04210	Apoptosis	<0.001
mmu04370	VEGF signaling pathway	<0.001
mmu04512	ECM-receptor interaction	<0.001
mmu04514	Cell adhesion molecules (CAMs)	<0.001
mmu04612	Antigen processing and presentation	<0.001
mmu04620	Toll-like receptor signaling pathway	<0.001
Colon epithelium		
mmu00565	Ether lipid metabolism	<0.001
mmu00601	Glycosphingolipid biosynthesis - lacto and neolacto series	<0.001
mmu04010	MAPK signaling pathway	<0.001
mmu04060	Cytokine-cytokine receptor interaction	<0.001
mmu04062	Chemokine signaling pathway	<0.001
mmu04145	Phagosome	<0.001
mmu04210	Apoptosis	<0.001
mmu04270	Vascular smooth muscle contraction	<0.001
mmu04370	VEGF signaling pathway	<0.001
mmu04514	Cell adhesion molecules (CAMs)	<0.001
mmu04612	Antigen processing and presentation	<0.001
mmu04620	Toll-like receptor signaling pathway	<0.001
mmu04621	NOD-like receptor signaling pathway	<0.001
mmu04623	Cytosolic DNA-sensing pathway	<0.001
mmu04630	Jak-STAT signaling pathway	<0.001
mmu04640	Hematopoietic cell lineage	<0.001
mmu04650	Natural killer cell mediated cytotoxicity	<0.001
mmu04660	T cell receptor signaling pathway	<0.001
mmu04662	B cell receptor signaling pathway	<0.001
mmu04664	Fc epsilon RI signaling pathway	<0.001

Table 3.10 Top 20 gene sets from enrichment analysis for *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* *Il10*$^{-/-}$ mice at 6 weeks of age for intact and colon epithelium.

Pathway ID	Pathway name	P value
Intact colon		
mmu00601	Glycosphingolipid biosynthesis - lacto and neolacto series	<0.001
mmu04012	ErbBsignaling pathway	<0.001
mmu04060	Cytokine-cytokine receptor interaction	<0.001
mmu04062	Chemokine signaling pathway	<0.001
mmu04142	Lysosome	<0.001
mmu04144	Endocytosis	<0.001
mmu04145	Phagosome	<0.001
mmu04210	Apoptosis	<0.001
mmu04370	VEGF signaling pathway	<0.001
mmu04514	Cell adhesion molecules (CAMs)	<0.001
mmu04612	Antigen processing and presentation	<0.001
mmu04620	Toll-like receptor signaling pathway	<0.001
mmu04621	NOD-like receptor signaling pathway	<0.001
mmu04630	Jak-STAT signaling pathway	<0.001
mmu04640	Hematopoietic cell lineage	<0.001
mmu04650	Natural killer cell mediated cytotoxicity	<0.001
mmu04660	T cell receptor signaling pathway	<0.001
mmu04662	B cell receptor signaling pathway	<0.001
mmu04664	Fc epsilon RI signaling pathway	<0.001
mmu04666	Fc gamma R-mediated phagocytosis	<0.001
Colon epithelium		
mmu00562	Inositol phosphate metabolism	<0.001
mmu04060	Cytokine-cytokine receptor interaction	<0.001
mmu04062	Chemokine signaling pathway	<0.001
mmu04145	Phagosome	<0.001
mmu04210	Apoptosis	<0.001
mmu04514	Cell adhesion molecules (CAMs)	<0.001
mmu04612	Antigen processing and presentation	<0.001
mmu04620	Toll-like receptor signaling pathway	<0.001
mmu04621	NOD-like receptor signaling pathway	<0.001
mmu04640	Hematopoietic cell lineage	<0.001
mmu04650	Natural killer cell mediated cytotoxicity	<0.001
mmu04660	T cell receptor signaling pathway	<0.001
mmu04662	B cell receptor signaling pathway	<0.001
mmu04664	Fc epsilon RI signaling pathway	<0.001
mmu04666	Fc gamma R-mediated phagocytosis	<0.001
mmu04672	Intestinal immune network for IgA production	<0.001
mmu04730	Long-term depression	<0.001

mmu04940	Type I diabetes mellitus	<0.001
mmu05140	Leishmaniasis	<0.001
mmu05142	Chagas disease	<0.001

mice. However, the epithelium in C57BL/6J mice may function similarly at 6 weeks and 12 weeks of age, explaining why no processes were over-represented in colon epithelium. Many immune gene categories were over-represented in both intact colon and colon epithelium for the other two comparisons, *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* C57BL/6J mice at 12 weeks of age (Table 3.11) and *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* 6 week old *Il10*$^{-/-}$ mice (Table 3.12). The top 20 pathways for these comparisons were similar between intact colon and colon epithelium, containing many immune-related pathways and defence against external stimuli or stress response pathways.

Approximately 45% of the differentially expressed genes in the colon epithelium were also differentially expressed in intact colon for the comparisons *Il10*$^{-/-}$ mice at 12 weeks *vs. Il10*$^{-/-}$ mice at 6 weeks, and *Il10*$^{-/-}$ mice at 12 weeks *vs.* C57BL/6J mice at 12 weeks. Therefore, the similarity in pathways, processes and gene sets between intact colon and colon epithelium in these comparisons must be due to fewer than 50% of the differentially expressed genes in the colon epithelium, or many of the differentially expressed genes in the epithelium belong to similar immune-related pathways. In the *Il10*$^{-/-}$ mice at 6 weeks *vs.* C57BL/6J mice at 6 weeks, 27% of differentially expressed genes in the colon epithelium were also differentially expressed in intact colon.

3.5.8 qPCR validation of microarray results

The qPCR results confirmed the microarray FC and FDR/P values for the six genes measured (Table 3.13). Three genes were differentially expressed in the qPCR results but not in the microarray results (*Il18*, intact colon, *Stat1* and *S100g* in colon epithelium). This was expected to occur for some genes due to the increased sensitivity of the qPCR method of measuring gene expression, and the direction of fold change was consistent between both methods.

Two genes, *Ppara* and *Mmp3* did not amplify in the colon epithelium samples, despite amplifying well in the intact colon samples. The calibrator used in each run and the success of these genes in the intact colon as well as the success of the other four genes in the colon epithelium samples confirmed that there were no technical issues with the primer/probe mix, the qPCR enzymes, or the cDNA synthesis. The lack of amplification

Table 3.11 Top 20 over-represented gene categories for the comparison *Il10*$^{-/-}$ mice versus C57BL/6J mice at 12 weeks for intact colon and colon epithelium.

System	Gene Category	EASE
Intact colon		
GO Biological Process	response to biotic stimulus	<0.001
GO Biological Process	defense response	<0.001
GO Biological Process	immune response	<0.001
GO Biological Process	response to external stimulus	<0.001
GO Biological Process	response to pest/pathogen/parasite	<0.001
GO Biological Process	response to wounding	<0.001
GO Biological Process	humoral immune response	<0.001
GO Molecular Function	signal transducer activity	<0.001
GO Biological Process	response to stress	<0.001
GO Cellular Component	extracellular	<0.001
GO Biological Process	inflammatory response	<0.001
GO Biological Process	cell communication	<0.001
GO Biological Process	innate immune response	<0.001
GO Molecular Function	receptor binding	<0.001
GO Biological Process	humoraldefense mechanism	<0.001
GO Biological Process	cellular defense response	<0.001
GO Cellular Component	extracellular space	<0.001
GO Biological Process	signal transduction	<0.001
GO Biological Process	chemotaxis	<0.001
GO Biological Process	taxis	<0.001
Colon epithelium		
GO Biological Process	response to biotic stimulus	<0.001
GO Biological Process	defense response	<0.001
GO Biological Process	immune response	<0.001
GO Biological Process	response to external stimulus	<0.001
GO Biological Process	response to pest/pathogen/parasite	<0.001
GO Biological Process	inflammatory response	<0.001
GO Biological Process	innate immune response	<0.001
GO Biological Process	response to wounding	<0.001
GO Molecular Function	catalytic activity	<0.001
GO Biological Process	response to stress	<0.001
GO Molecular Function	chemokine receptor binding	<0.001
GO Molecular Function	chemokine activity	<0.001
GO Molecular Function	chemoattractant activity	<0.001
GO Molecular Function	G-protein-coupled receptor binding	<0.001
GO Molecular Function	oxidoreductase activity	<0.001
GO Biological Process	humoral immune response	<0.001
GO Biological Process	lipid metabolism	<0.001
GO Biological Process	response to chemical substance	<0.001
GO Molecular Function	oxidoreductase activity, acting on CH-OH	<0.001
GO Molecular Function	cytokine activity	<0.001

Table 3.12 Top 20 over-represented gene categories for the comparison *Il10*$^{-/-}$ mice, 12 versus 6 weeks, for intact colon and colon epithelium.

System	Gene Category	EASE score
Intact colon		
GO Biological Process	defense response	<0.001
GO Biological Process	response to biotic stimulus	<0.001
GO Biological Process	immune response	<0.001
GO Biological Process	response to external stimulus	<0.001
GO Biological Process	response to pest/pathogen/parasite	<0.001
GO Biological Process	response to wounding	<0.001
GO Molecular Function	signal transducer activity	<0.001
GO Biological Process	humoral immune response	<0.001
GO Biological Process	response to stress	<0.001
GO Biological Process	cell communication	<0.001
GO Biological Process	inflammatory response	<0.001
GO Biological Process	innate immune response	<0.001
GO Cellular Component	extracellular	<0.001
GO Biological Process	humoraldefense mechanism	<0.001
GO Biological Process	cellular defense response	<0.001
GO Cellular Component	plasma membrane	<0.001
GO Biological Process	taxis	<0.001
GO Biological Process	chemotaxis	<0.001
GO Molecular Function	receptor activity	<0.001
GO Biological Process	response to chemical substance	<0.001
Colon epithelium		
GO Biological Process	response to biotic stimulus	<0.001
GO Biological Process	defense response	<0.001
GO Biological Process	immune response	<0.001
GO Biological Process	response to external stimulus	<0.001
GO Biological Process	response to pest/pathogen/parasite	<0.001
GO Biological Process	humoral immune response	<0.001
GO Biological Process	inflammatory response	<0.001
GO Biological Process	response to stress	<0.001
GO Biological Process	innate immune response	<0.001
GO Biological Process	response to wounding	<0.001
GO Molecular Function	signal transducer activity	<0.001
GO Biological Process	humoraldefense mechanism	<0.001
GO Biological Process	antimicrobial humoral response	<0.001
GO Cellular Component	extracellular	<0.001
GO Molecular Function	receptor activity	<0.001
GO Biological Process	antimicrobial humoral response	<0.001
GO Molecular Function	cytokine activity	<0.001
GO Biological Process	signal transduction	<0.001
GO Molecular Function	defense/immunity protein activity	<0.001
GO Biological Process	taxis	<0.001

Table 3.13 qPCR validation of microarray results. Table shows fold changes (FC) and P values (for qPCR data) or false discovery rates (FDR; for microarray data) for the six genes validated using qPCR for both intact colon and colon epithelium. All genes that were differentially expressed in the microarrays were differentially expressed according to qPCR and all fold changes were in the same direction in both qPCR and microarray. * denotes genes that were significantly differentially expressed according to qPCR but not microarray.

	***Il10*$^{-/-}$ mice, 12 weeks *vs.* *Il10*$^{-/-}$ mice, 6 weeks**					***Il10*$^{-/-}$ mice, 12 weeks *vs.* C57BL/6J mice, 12 weeks**			
	Gene	**qPCR FC**	**qPCR P value**	**Microarray FC**	**Microarray FDR**	**Gene**	**qPCR FC**	**qPCR P value**	**Microarray FC**	**Microarray FDR**
Intact colon	Ppara	-2.6	<0.001	-3.0	<0.001	Ppara	-2.6	<0.001	-2.4	0.002
	Stat1	2.0	<0.001	2.1	0.033	Stat1	3.1	<0.001	3.6	<0.001
	S100g	-62.5	<0.001	-99.2	<0.001	S100g	-15.6	<0.001	-21.0	<0.001
	Il18	-1.3	0.009*	-1.4	0.361	Il18	1.1	0.113	1.1	0.764
	Mmp3	6.6	<0.001	10.7	<0.001	Mmp3	5.3	<0.001	13.6	<0.001
	Tap2	1.9	<0.001	1.6	0.029	Tap2	2.2	<0.001	2.4	<0.001
Colon epithelium	Stat1	2.9	<0.001*	1.6	0.785	Stat1	6.3	<0.001	4.2	<0.001
	S100g	-6.5	<0.001*	-1.2	0.997	S100g	-4.3	0.032	-10.9	0.001
	Il18	-2.0	0.107	2.0	0.456	Il18	1.1	0.982	-1.4	0.768
	Tap2	1.6	0.488	-1.2	0.997	Tap2	-1.1	0.953	1.2	0.988

was consistent across epithelial samples for both genes, although three samples for *Mmp3* amplified well in duplicate across both runs and one sample for *Ppara* amplified well for one run. These were different samples but all from the *Il10*$^{-/-}$ mice at 12 weeks of age, the group for which both genes were expected to be more highly expressed. In addition, for the *Tap2* gene, one sample did not amplify in either run and three other samples did not amplify consistently across duplicates/runs, suggesting the cause of these failed amplifications may be sample-related. Due to the very low cDNA abundance of these genes in these samples, the analysis was not repeated using undiluted cDNA as it appeared unlikely to improve the result. Average intensity values for these genes on the epithelial microarrays were similar to those of the other genes and intact colon microarrays, indicating that low abundance in the original RNA sample is unlikely to explain the amplification failure. Repeating the cDNA synthesis with a larger RNA input might improve the result by increasing abundance, although the consistently low abundance across samples suggests this may be unlikely. Repeating the amplification of the original extracted RNA might improve abundance, but this was not performed due to the limited quantity of remaining starting material.

3.6 Discussion

Colon histology results confirmed that the mice used in this experiment developed the desired phenotype of colon inflammation, as expected in this mouse model [110, 115, 322]. The gene expression patterns in 12 week old *Il10*$^{-/-}$ mice compared to age-matched C57BL/6J mice, or 6 week old *Il10*$^{-/-}$ mice, support the findings of other studies of colon gene expression in the *Il10*$^{-/-}$ model [110, 118, 335] and in other mouse models of intestinal inflammation [76]. The present results confirm that these mice are a relevant model in which to study the gene expression profile of the colon epithelium during colitis.

From a list of 32 genes whose expression is known to be altered in both the inflamed intestinal mucosa in IBD patients [336-339] and mouse models [76], the majority were also differentially expressed in this experiment. These genes included many interleukins, chemokine receptor and ligands, metalloproteases, and commonly recognised genes in studies of IBD and other inflammatory diseases, such as lymphotoxin-β, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, and some of the S100 calcium binding proteins (S100a8 and 9). These genes, and whether or not they

were differentially expressed in intact colon or colon epithelium, in *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* *Il10*$^{-/-}$ mice at 6 weeks of age, or *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* C57BL/6J mice at 12 weeks of age, are shown in Table 3.14.

The majority of these genes were differentially expressed in the intact colon (91% for the comparison *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* C57BL/6J mice at 12 weeks of age and 84% for the comparison *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* *Il10*$^{-/-}$ mice at 6 weeks of age), confirming that the pathology of the mouse model used here bears many similarities (in terms of colon gene expression) to IBD and other commonly used mouse models of IBD [76, 115]. In the colon epithelium, 69% of these genes were differentially expressed for *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* C57BL/6J mice at 12 weeks of age, compared to 91% in intact colon, suggesting that the gene expression profiles were similar, but not identical, in colon epithelium compared to intact colon. However, in *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* *Il10*$^{-/-}$ mice at 6 weeks of age, only 34% of these genes were differentially expressed in the colon epithelium compared to 84% in intact colon, indicating that there were differences in the gene expression profile between epithelium and intact colon in *Il10*$^{-/-}$ mice, from the early stage of colitis to when colitis was fully developed.

Genes whose expression level is known to be increased in IBD were increased in the colon epithelium of *Il10*$^{-/-}$ mice at 12 weeks of age, including: cytokine and cytokine receptor genes (*IFN-γ*, *Ltβ, IL-6, IL-16, IL-18R1*), and chemokine and chemokine receptor genes (*CCR2, CCL2, 3, 4, 5, 17, CXCR3, CXCL1, 5, 10)*. Chemokines control leukocyte trafficking and the migration of leukocytes into sites of inflammation is crucial for the pathogenesis of experimental colitis. Increased gene and protein expression of chemokines occurs in IBD [340, 341]. The results from this experiment reinforce findings that the colon epithelium produces chemokine signals to induce an influx of leukocytes in acute mucosal inflammation [342].

Genes involved in tissue remodelling (*Mmp3, 7, 9, 14, Timp1*) were also expressed in the epithelium in inflamed *Il10*$^{-/-}$ mice at 12 weeks of age. MMPs are involved with tissue remodelling, angiogenesis, and promotion of leukocyte extravasation and their expression is increased in IBD [343, 344]. The activity of MMP1, 3 and 9 is controlled

Table 3.14 Genes highly expressed in IBD patients and their expression in intact colon or epithelium. Genes identified as having increased expression levels in colon tissue in CD and/or UC patients with indication of whether they were also differentially expressed in the *Il10*$^{-/-}$ mice at 12 week of age *vs.* C57BL/6J mice at 12 weeks or *vs. Il10*$^{-/-}$ mice at 6 weeks of age. A shaded square indicates that the gene was differentially expressed in a particular gene list (black = gene is differentially expressed in intact colon; grey = is differentially expressed in colon epithelium) while a blank space indicates that the gene was not significantly differentially expressed in the dataset.

	***Il10*$^{-/-}$ vs.C57BL/6J mice, 12 weeks**		***Il10*$^{-/-}$ mice, 12 vs.6 weeks**	
IBD-related genes	**Intact colon**	**Colon epithelium**	**Intact colon**	**Colon epithelium**
Tumour necrosis factor; *TNF*				
Interferon-γ; *IFNγ*				
Lymphotoxin β; *Ltb*				
Interleukin-6; *IL-6*				
Interleukin-16; *IL-16*				
Interleukin-18 receptor 1; *IL-18R1*				
Interleukin-22; *IL-22*				
Chemokine receptor 2; *CCR2*				
Chemokine receptor 7; *CCR7*				
Chemokine (C-C motif) ligand 2; *CCL2*				
Chemokine (C-C motif) ligand 3; *CCL3*				
Chemokine (C-C motif) ligand 4; *CCL4*				
Chemokine (C-C motif) ligand 5; *CCL5*				
Chemokine (C-C motif) ligand 7; *CCL7*				
Chemokine (C-C motif) ligand 11; *CCL11*				
Chemokine (C-C motif) ligand 17; *CCL17*				
Chemokine (C-C motif) ligand 20; *CCL20*				
Chemokine (C-X-C motif) receptor 3; *CXCR3*				

Chemokine (C-X-C motif) ligand 1; *CXCL1*				
Chemokine (C-X-C motif) ligand 5; *CXCL5*				
Chemokine (C-X-C motif) ligand 10; *CXCL10*				
Matrix metalloprotease 3; *Mmp3*				
Matrix metalloprotease 7; *Mmp7*				
Matrix metalloprotease 9; *Mmp9*				
Matrix metalloprotease 14; *Mmp14*				
Tissue inhibitor of metalloproteinase 1; *Timp1*				
Regenerating islet-derived 3γ; *Reg3γ*				
Pancreatitis-associated protein; *Pap*				
S-100 calcium binding protein A8: *S-100a8*				
S-100 calcium binding protein A9: *S-100a9*				
ATP-binding cassette, subfamily B, 1a; *Abcb1a*				
Prostaglandin-endoperoxide synthase 2; *Ptgs2*				
TOTAL DIFFERENTIALLY EXPRESSED GENES (OUT OF 32)	29	22	27	11

by tissue inhibitor of metalloproteinase (TIMP-1), the expression of which is also increased in colitis [344]. Increased production of MMPs has a role in tissue damage in IBD and some studies show that genetic variation in MMP genes may play a role in interindividual differences in UC susceptibility and clinical outcome [345]. The results of this experiment suggest that increased expression levels of gene transcripts for MMP and TIMP1 proteins in IBD occurs in the epithelium.

Other genes whose expression levels are known to be increased in IBD and that were increased in the *Il10*$^{-/-}$ mice at 12 weeks of age in colon epithelium were the S-100 calcium binding proteins A8 and A9 (calgranulin A and B) (*S100a8, 9).* These proteins, S100A8 and S100A9, form a heterodimer called calprotectin, a pro-inflammatory mediator involved in acute and chronic inflammation [346, 347]. Increased expression levels of these genes in colitis may reflect neutrophil infiltration [76]. However, the expression of these genes in the colon epithelium in this experiment suggests colon epithelial cells (enterocytes, goblet cells, enteroendocrine, and stem cells, and/or intraepithelial lymphocytes) must be a source of *S100a8* and a*9* transcripts unless the dissected epithelial cell samples were contaminated with substantial numbers of neutrophils from the lamina propria, however, every effort was made to exclude cells of the lamina propria during microdissection. This finding is in agreement with studies that showed that *S100a8* and *S100a9* are expressed in mucosal epithelium under inflammatory conditions and in inflammation-associated cancer [346, 347].

As shown in the qPCR results (Table 3.13) and network diagrams (Figures 3.5 and 3.6), the *Stat1* gene had higher mRNA levels in inflamed *Il10*$^{-/-}$ mice in both intact colon and colon epithelium. *Stat1* gene expression levels are increased in the colon in IBD, leading to increased abundance and activity of the STAT1 protein [348]. The inhibition of STAT1 may be an important part of the anti-inflammatory effect of glucocorticoids [348]. In intact colon, the *Ppara* gene had lower mRNA abundance in inflamed *Il10*$^{-/-}$ mice, in agreement with previous studies [115], while the *Tap2* gene had higher mRNA abundance in inflamed *Il10*$^{-/-}$ mice. DNA methylation levels of these three genes may be a mechanism by which their expression is modulated in inflammation, and will be described in Chapter 4.

From the list of IBD-related genes, those that were not differentially expressed in the colon epithelium were: Tumour necrosis factor (TNF), IL6, interleukin-22, Chemokine (C-C motif) ligand (CCL)-4, 7, 11, 17, and 20, Chemokine (C-X-C motif) receptor 3 (CXCR3) Chemokine (C-X-C motif) ligand (CXCL)-1, 5 and 10; Matrix metalloprotease (*Mmp*)-7, 9, and 14, Tissue inhibitor of metalloproteinase-1 (*Timp1*), Regenerating islet-derived 3γ (*Reg3γ*), Pancreatitis-associated protein (*Pap*), ATP-binding cassette, subfamily B (MDR/TAP), 1a (*Abcb1a*), and Prostaglandin-endoperoxide synthase 2 (*Ptgs2*). The lack of differential expression of *Abcb1a* in the epithelium is surprising, considering that it codes for a membrane-associated transporter protein whose expression is altered in UC [349] and mouse models of colitis [76], it was differentially expressed in intact colon, and would be expected to be found in the epithelium.

Overall, genes that have been identified in other studies as being differentially expressed in colitis were well-represented in these microarray experiments for both intact colon and colon epithelium. When genes were not differentially expressed in a particular treatment comparison, closely related genes were often differentially expressed. For example, in colon epithelium for *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* C57BL/6J mice at 12 weeks of age, *Mmps 7, 9* or *14* were not differentially expressed but *Mmp10* was. Also, while *TNF* itself was not differentially expressed, TNF receptors were. This indicates that intact colon samples provide a good indication of changes in gene expression profile that are occurring in the mucosa, in terms of gene sets and pathways.

The epithelial cell data was more sensitive to immune changes at 6 weeks of age between strains. When designing an experiment to analyse gene expression changes in *Il10*$^{-/-}$ mice in the early stages of colitis, it may be worth considering analysing the colon epithelium instead of intact colon. However, the colon epithelium had fewer differentially expressed genes from the group of 32 known IBD-related genes than did intact colon, particularly in the treatment comparison *Il10*$^{-/-}$ mice at 12 weeks of age *vs.* *Il10*$^{-/-}$ mice at 6 weeks of age. This may indicate that some of these IBD-associated genes are more strongly expressed in non-epithelial cell types in colitis.

The mRNA transcripts from the immune genes that were differentially expressed in the colon epithelium between inflamed *Il10*$^{-/-}$ mice and the other non-inflamed mice were likely to come from the enterocytes which form the majority of the cell set collected, or from intraepithelial lymphocytes (IEL), although some contamination by immune cells in the lamina propria was possible. Future gene expression profiling of the colon epithelium should include a comparison of microdissected lamina propria gene expression profiles for genes known to be predominantly expressed in each compartment as a plausibility check for the specificity of the cell fractions although this would not rule out some degree of cross-contamination [117]. It may be impossible to avoid harvesting infiltrating immune cells alongside the colon epithelium in inflammation due to the epithelial layer damage and disruption that occurs [81].

Relative to other types of T cell, little is known about the phenotypes and functions of the many types of IEL. IELs are more abundant in the large intestine [350] and release cytokines upon activation that contribute to the activation and the recruitment of innate immune cells [351, 352]. IELs produce cytokines, chemokines and growth factors, and are important in maintaining the epithelial barrier [351]. In humans, the colon IEL population consists predominantly of $\gamma\delta^+$ $CD4^-$ $CD8^+$ T cells, although some $CD4^+$ or double positive T cells are also present [352]. In mice, intestinal IELs consist of T cells bearing αβ T cell antigen receptors (40-70% of the IEL population) and those bearing γδ T cell antigen receptors (30-60% of the IEL population) [353]. γδ IEL biological functions include promotion of wound repair, immunoregulation and interaction with the other cell types of the epithelial layer, and maintenance of homeostasis with the intestinal microbiota including release antimicrobial peptides upon epithelial injury [354]. IELs have profound cytotoxic activity against intestinal epithelial cells in inflammatory conditions [352], which may be associated with the intestinal mucosal injury that occurs in IBD.

The relative contribution of the IELs to the gene expression profile of the colon epithelium in *Il10*$^{-/-}$ mice is unknown. Whether the numbers of IELs were higher in epithelial tissue of inflamed colon of *Il10*$^{-/-}$ mice compared to non-inflamed mice is also unknown. It is likely, based on the higher percentage of $IL23R^+$ $CD8^+$ IEL observed in CD and UC patients compared to healthy controls [352], that some types of IEL are present in increased numbers in the inflamed colon, if not the total number of all types.

Increased pro-inflammatory cytokine secretory activity of IELs during colitis could explain why so many immune genes have increased expression levels in epithelia from inflamed mouse colon.

3.7 Conclusions

Results from microarray analysis of intact colon in this experiment are consistent with other data in this and similar models indicating the suitability of the samples collected in this experiment for studying epithelial cell function in colitis. The findings from this study indicate that when analysed at the pathway level, intact colon is an appropriate tissue in which to examine gene expression changes in the mucosa in fully-developed colitis, as it produces similar gene expression profiles to the epithelium alone. They do indicate, however, that studying epithelial cell function, as opposed to intact colon function, may be more relevant when studying the earlier stages of inflammation because isolation of specific cells may improve sensitivity or the ability to detect early changes in cell function that may lead to the development of inflammation.

The results presented here indicate that the study of gene expression profiles in specific cell types could be of particular benefit in the early stages of colon inflammation to help elucidate the early changes in mucosal function, but perhaps less useful in fully developed inflammation. The expression of genes can be controlled by epigenetic mechanisms, and epigenetic mechanisms such as DNA methylation have been implicated in the development of IBD. To further investigate mucosal changes in the early stages of inflammation, in particular those that could influence gene expression, intact colon samples from the time-course experiment were used to measure DNA methylation in the early and late stages of inflammation development (Chapter 4).

4 Global and gene-specific DNA methylation is altered in the *Il10*$^{-/-}$ mouse colon in early and late inflammation

4.1 Abstract

The involvement of epigenetic mechanisms in the pathogenesis of IBD is ill-defined, but may be an important factor in understanding the pathogenesis of colitis. The first objective of this study was to measure global DNA methylation using HPLC to test the hypothesis that global methylation levels would be decreased in *Il10*$^{-/-}$ mice at 12 weeks of age compared to C57BL/6J mice. The second objective was to measure the methylation levels of selected regions of the *Tap2*, *Stat1* and *Ppara* genes using MALDI-TOF mass spectrometry to test the hypothesis that changes observed in *Stat1*, *Tap2* and *Ppara* gene expression in the *Il10*$^{-/-}$ mouse model are associated with differential methylation of CpG sites within key regulatory regions of these genes. Global methylation was increased in *Il10*$^{-/-}$ mice at 6 weeks of age compared to *Il10*$^{-/-}$ mice at 12 weeks of age and C57BL/6J mice, and methylation levels of specific CpG sites within an intronic region, but not the proximal promoter region, of the *Stat1* gene were reduced in *Il10*$^{-/-}$ mice at 12 weeks of age compared with C57BL/6J mice at 12 weeks of age. This study showed that DNA methylation levels were altered in the *Il10*$^{-/-}$ mouse model of IBD and these alterations correlated with changes in the expression of genes that occur in inflammation.

4.2 Introduction

Epigenetic mechanisms are implicated in inflammatory conditions such as IBD. Genetic variation alone accounts for only approximately 25% of IBD heritability [355], indicating that epigenetic mechanisms might contribute to heritability to a large extent. The different incidence of IBD in monozygotic twins is supporting evidence for the role of epigenetic mechanisms in the development of colitis, as are the alterations in global and gene-specific DNA methylation that have been detected in mucosal biopsies of IBD patients [142, 143] and in a mouse model of colitis [145].

Global hypomethylation accompanied by gene-specific hypermethylation is known to occur in both cancer and aging [356]. In a DSS model of colitis, three CpG islands are involved in induction of aberrant DNA methylation in colon epithelial cells, and their methylation levels were increased from 8 weeks after DSS treatment until the development of colon cancers 7 weeks later [357]. Inflammation triggered by the DSS treatment appeared to be responsible for induction of methylation [357], suggesting that

inflammation-associated changes in DNA methylation are also likely to occur in other models of colitis.

Peroxisome proliferator-activated receptor alpha (*Ppara*), signal transducer and activator of transcription 1 (*Stat1*) and the transporter 2, ATP-binding cassette, sub-family B (*Tap2*) genes were shown to have altered expression levels during inflammation in the *Il10*$^{-/-}$ mouse model of IBD [110, 118] (Chapter 3) and contain CpG islands, suggesting their potential for epigenetic regulation by DNA methylation. The *Ppara* gene has been identified as a central (hub) gene in the inoculated *Il10*$^{-/-}$ mouse model [118]. *Ppara* expression is known to be modified by DNA methylation. Methylation at an intergenic CpG island 50 kb upstream of *Ppara*, a region considered likely to be an enhancer for Ppara (chr15: 85,514,715-85,514,920), is associated with decreased *Ppara* gene expression [358]. Increased *Ppara* mRNA expression is associated with decreased CpG methylation in the *Ppara* promoter in the liver [359]. Alterations in methylation state may thus influence the mRNA levels of *Ppara*, and these methylation changes may be modulated by colitis.

The *Stat1* gene encodes a protein from the STAT family [360]. STAT proteins are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. STAT1 can be activated by various ligands including the inflammation-associated cytokines IFNγ and IL6 and mediates the expression of a variety of genes important for cell viability in response to different cell stimuli and pathogens. STAT1 protein activation and expression is increased in the intestinal mucosa in IBD, although more so in CD than UC [348]. Glucocorticoids, a common therapy during active phases of IBD, inhibit phosphorylation and activation of STAT1, suggesting that inhibition of STAT1 may be an important part of the anti-inflammatory effect of glucocorticoids [348]. Changes in methylation patterns of the *Stat1* gene associated with altered STAT1 protein expression have been observed in a cancer study [360].

The *Tap2* gene encodes a membrane-associated protein member of the MDR/TAP subfamily, part of the superfamily of ATP-binding cassette (ABC) transporters. The Tap2 protein is involved in antigen presentation, forming a heterodimer with Tap1 in order to transport peptides from the cytoplasm to the endoplasmic reticulum. The *Tap2* gene may be involved with the genetic heterogeneity of IBD, with some indication that

the response of IBD patients to steroid therapy varies with some *Tap2* polymorphisms [361]. Transcription of the *Tap2* gene can be increased by demethylation of the promoter region induced by treatment with 5-azacytidine, an inhibitor of DNA methyltransferase [362].

4.3 Aims and hypotheses

The first objective of this study was to test the hypothesis that global methylation levels are decreased in *Il10*$^{-/-}$ mice at 12 weeks of age relative to C57BL/6J mice at 12 weeks and *Il10*$^{-/-}$ mice at 6 weeks of age. This was investigated by measuring global DNA methylation using high performance liquid chromatography (HPLC) to determine whether an increase or decrease in global DNA methylation is observed between control C57BL/6J and *Il10*$^{-/-}$ mice, and whether methylation levels vary in early and later stages of inflammation.

The putative anti-inflammatory role of the *Ppara, Stat1*, and *Tap2* genes, and the potential for their expression to be mediated by methylation, suggested the hypothesis that changes observed in *Stat1, Tap2* and *Ppara* gene expression in the *Il10*$^{-/-}$ mouse model are associated with differential methylation of CpG sites within key regulatory regions of these genes. The second objective of this study was to measure the methylation levels of selected regions of the *Tap2*, *Stat1* and *Ppara* genes using bisulfite treatment of DNA followed by MALDI-TOF mass spectrometry to determine whether there is an increase or decrease in the methylation levels of these genes between control C57BL/6J and *Il10*$^{-/-}$ mice, and whether methylation levels vary in early and later stages of inflammation.

4.4 Methods

The mouse experiment was carried out as described in Sections 2.2-2.5. The DNA extraction was carried out as described in Section 2.6.1, the global methylation analysis by HPLC as described in Section 2.6.2, and the specific gene methylation analysis by MALDI-TOF mass spectrometry as described in Section 2.6.3.

4.5 Results

4.5.1 Global DNA methylation

There was an age-related change in global DNA methylation levels in the *Il10*$^{-/-}$ mice. Global methylation levels were not different between *Il10*$^{-/-}$ (4.78 %) and C57BL/6J (4.67 %) mice (5% LSD = 0.17 %, P = 0.17). However, global methylation levels were different between the two sampling ages, 6 (4.82 %) and 12 (4.64 %) weeks (5% LSD = 0.17 %, P = 0.04). There was a trend for the *Il10*$^{-/-}$ mice to have higher levels of global methylation at 6 weeks of age (4.95 %) compared to *Il10*$^{-/-}$ mice at 12 weeks (4.63 %) and C57BL/6J mice at 6 weeks (4.67 %) and 12 weeks (4.66 %) (P = 0.07 for the interaction between mouse strain and sampling age). Differences between the *Il10*$^{-/-}$ mice at 6 weeks and the other mice exceeded the maximum 5% LSD of 0.24.

4.5.2 Methylation of sites within genes of interest

Two regions of the *Ppara* gene were assessed for methylation at CpG sites within a CpG island; Ppara_pp12, at genomic co-ordinates chr15:85564996-85565378, and Ppara_pp37, at chr15:85565771-85566091. Two regions of the *Stat1* gene were assessed for methylation levels at CpG sites within a CpG island; Stat1_pp3, at genomic co-ordinates chr1:52175244-52175553, and Stat1_int1_2, at chr1:52177118-52177592. Two regions of the *Tap2* gene were assessed for methylation at CpG sites; Tap2_5p_15, at genomic co-ordinates chr17:34340976-34341368, and Tap2_int2_16, at chr17:34342763-34343150.

Of the 6 gene regions analysed, only 3 yielded usable data. Table 4.1 summarises the quality of data obtained by Sequenom analysis. This shows that EpiDesigner primers targeting *Tap2*, and those targeting the pp_12 region of the *Ppara* gene, failed to generate reliable PCR products following bisulfite conversion. For each of these primer sets less than 50% of the CpG analyses gave usable data. Due to cost and material constraints, further methylation analysis of these genes was not performed. Table 4.2 shows details of the three sites analysed successfully.

4.5.2.1 Methylation of *Ppara*

Only the Ppara_pp37 region yielded quality data. As Figure 4.1 shows, no significant differences were observed (either with respect to time or genotype) in the methylation of CpG sites within the analysed regions of *Ppara*.

4.5.2.2 Methylation of *Stat1*

Both regions analysed yielded quality data. As Figure 4.2 shows, no significant differences were observed (either with respect to time or genotype) in the methylation levels of CpG sites within the proximal promoter region of *Stat1*. However, within the analysed intron there was an overall pattern of reduced methylation in *Il10*$^{-/-}$ mice at 12 weeks of age compared with C57BL/6J mice at 12 weeks of age ($P < 0.001$). Specifically, CpGs 1, 4-5 and 12 showed a significant reduction in methylation levels. The apparent reduction in methylation levels when comparing 12 week old *Il10*$^{-/-}$ mice with 6 week old mice of the same genotype was not significant ($P = 0.21$).

4.5.2.3 Methylation of *Tap2*

Neither of the two regions assessed yielded quality methylation data, as shown by Table 4.1.

4.6 Discussion

This study showed that global methylation levels in colon tissues of *Il10*$^{-/-}$ mice were higher at an early stage of inflammation (6 weeks of age) compared to when inflammation was established (12 weeks of age). The reduction in global methylation levels as the inflammation progressed was linked to changes in the mRNA levels of many genes in inflammatory pathways, including *Ppara*, *Stat1* and *Tap2*. These genes belong to pathways responsible for glucocorticoid receptor signalling, antigen presentation, and production of nitric oxide and reactive oxygen species in macrophages. Many genes in inflammatory pathways have increased levels of transcription in inflammation, although some like *Ppara* have reduced transcription (Figure 4.3). Activation of the transcription factor STAT1 enhances inflammatory gene transcription, while activation of PPARα can negatively regulate the induction of inflammatory responses via interaction with transcription factors [363].

Table 4.1 Summary of data available following Sequenom analysis of amplicons within the *Stat1*, *Ppara* and *Tap2* genes.

Gene	***Stat1***		***Ppara***		***Tap2***	
Amplicon	**int1_2**	**pp_3**	**pp_12**	**pp_37**	**5p_15**	**int2_16**
No. of samples	28	28	28	28	28	28
No. samples with usable spectra	26	28	14	28	16	18
Usable spectra (%)	92.9	100	50	100	57.1	64.3
CpGs within amplicon	4	5	8	13	5	5
Total CpGs analysed	112	140	224	364	140	140
CpGs with data	91	128	98	364	58	46
Usable data (%)	81.3	91.4	43.8	100	41.4	32.9

"No. of samples" is the total number of individual mice from which DNA was available for Sequenom EpiTyper analysis.

"No. samples with usable spectra" refers to those samples for which at least one CpG site within the amplicon in question returned valid data.

"Usable spectra (%)" is calculated by the number of samples with usable spectra divided by the total number of samples (n = 28) multiplied by 100.

"CpGs within amplicon" refers to those CpG sites for which any data were generated.

"Total CpGs analysed" is the number of CpGs within the amplicon multiplied by the total number of samples analysed.

"CpGs with data" is the total number of CpGs that returned a valid reading within that amplicon across all samples analyses.

"Usable data (%)" shows the valid data as a percentage of the total CpGs analysed, again within the amplicon in question across all samples analysed.

Table 4.2 CpG sites analysed for regions of the *Stat1* and *Ppara* gene that yielded good quality methylation data. Methylation sites which could not be distinguished from each other due to the location of a cleavage site are noted in the "Comment" column.

Gene	Chromosome	Sequenom Amplicon	CpG site	Location on chromosome	Location relative to transcription start site	Comment
Stat1	1	pp3	1	52,175,291	-991	
Stat1	1	pp3	2	52,175,330	-952	ND
Stat1	1	pp3	3	52,175,337	-945	
Stat1	1	pp3	4	52,175,352	-930	
Stat1	1	pp3	5	52,175,368	-914	
Stat1	1	pp3	6	52,175,391	-891	
Stat1	1	pp3	7	52,175,406	-876	ND
Stat1	1	int1_2	1	52,177,152	870	
Stat1	1	int1_2	2	52,177,180	898	ND
Stat1	1	int1_2	3	52,177,189	907	
Stat1	1	int1_2	4	52,177,224	942	CpGs 4 and 5 reported as
Stat1	1	int1_2	5	52,177,227	945	single methylation event
Stat1	1	int1_2	6	52,177,246	964	ND
Stat1	1	int1_2	7	52,177,284	1,002	ND
Stat1	1	int1_2	8	52,177,318	1,036	ND
Stat1	1	int1_2	9	52,177,328	1,046	ND
Stat1	1	int1_2	10	52,177,411	1,129	ND
Stat1	1	int1_2	11	52,177,455	1,173	ND
Stat1	1	int1_2	12	52,177,464	1,182	
Ppara	15	pp_37	1	85,565,792	-202	CpGs 1, 2 and 3 reported
Ppara	15	pp_37	2	85,565,810	-184	as single methylation
Ppara	15	pp_37	3	85,565,820	-174	event
Ppara	15	pp_37	4	85,565,827	-167	CpGs 4 and 5 reported as
Ppara	15	pp_37	5	85,565,840	-154	single methylation event
Ppara	15	pp_37	6	85,565,849	-145	ND
Ppara	15	pp_37	7	85,565,854	-140	
Ppara	15	pp_37	8	85,565,863	-131	CpGs 8, 9 and 10
Ppara	15	pp_37	9	85,565,866	-128	reported as single
Ppara	15	pp_37	10	85,565,868	-126	methylation event
Ppara	15	pp_37	11	85,565,876	-118	
Ppara	15	pp_37	12	85,565,885	-109	ND
Ppara	15	pp_37	13	85,565,889	-105	ND
Ppara	15	pp_37	14	85,565,896	-98	ND
Ppara	15	pp_37	15	85,565,899	-95	ND
Ppara	15	pp_37	16	85,565,901	-93	ND

Ppara	15	pp_37	17	85,565,906	-88	
Ppara	15	pp_37	18	85,565,914	-80	CpGs 18, 19, 20 and 21
Ppara	15	pp_37	19	85,565,916	-78	reported as single
Ppara	15	pp_37	20	85,565,925	-69	methylation event
Ppara	15	pp_37	21	85,565,929	-65	
Ppara	15	pp_37	22	85,565,931	-63	CpGs 22 and 23 reported
Ppara	15	pp_37	23	85,565,933	-61	as single methylation event
Ppara	15	pp_37	24	85,565,939	-55	ND
Ppara	15	pp_37	25	85,565,945	-49	CpGs 25, 26, 27 and 28
Ppara	15	pp_37	26	85,565,958	-36	reported as single
Ppara	15	pp_37	27	85,565,962	-32	methylation event
Ppara	15	pp_37	28	85,565,967	-27	
Ppara	15	pp_37	29	85,565,975	-19	ND
Ppara	15	pp_37	30	85,565,987	-7	ND
Ppara	15	pp_37	31	85,565,993	-1	ND
Ppara	15	pp_37	32	85,566,003	9	ND
Ppara	15	pp_37	33	85,566,009	15	ND
Ppara	15	pp_37	34	85,566,021	27	CpGs 34 and 35 reported
Ppara	15	pp_37	35	85,566,035	41	as single methylation event
Ppara	15	pp_37	36	85,566,037	43	
Ppara	15	pp_37	37	85,566,049	55	CpGs 37 and 38 reported
Ppara	15	pp_37	38	85,566,055	61	as single methylation event
Ppara	15	pp_37	39	85,566,061	67	

ND = no data

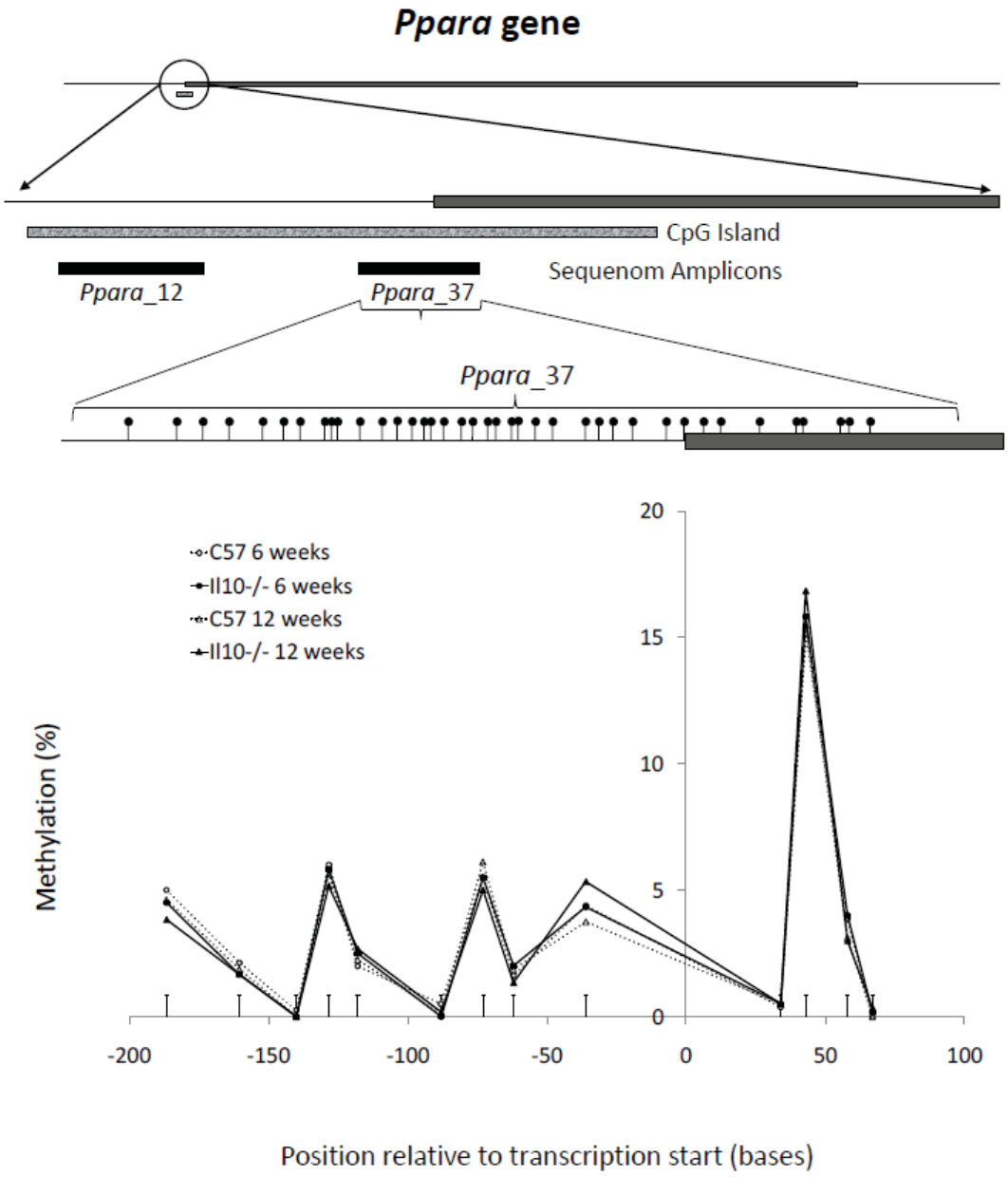


Figure 4.1 Methylation levels of CpG sites associated with the *Ppara* gene. Percentage of methylation is shown for each group at each CpG site within the *Ppara* amplicon that yielded quality methylation data. Error bars along the horizontal axis represent standard error of difference (SED). CpG sites are shown as black circles above vertical lines along the Ppara_37 region of the gene. Where CpG sites could not be distinguished and were reported as a single event, the mean distance from the transcription start site was used to plot the methylation percentage. C57 = C57BL/6J mice.

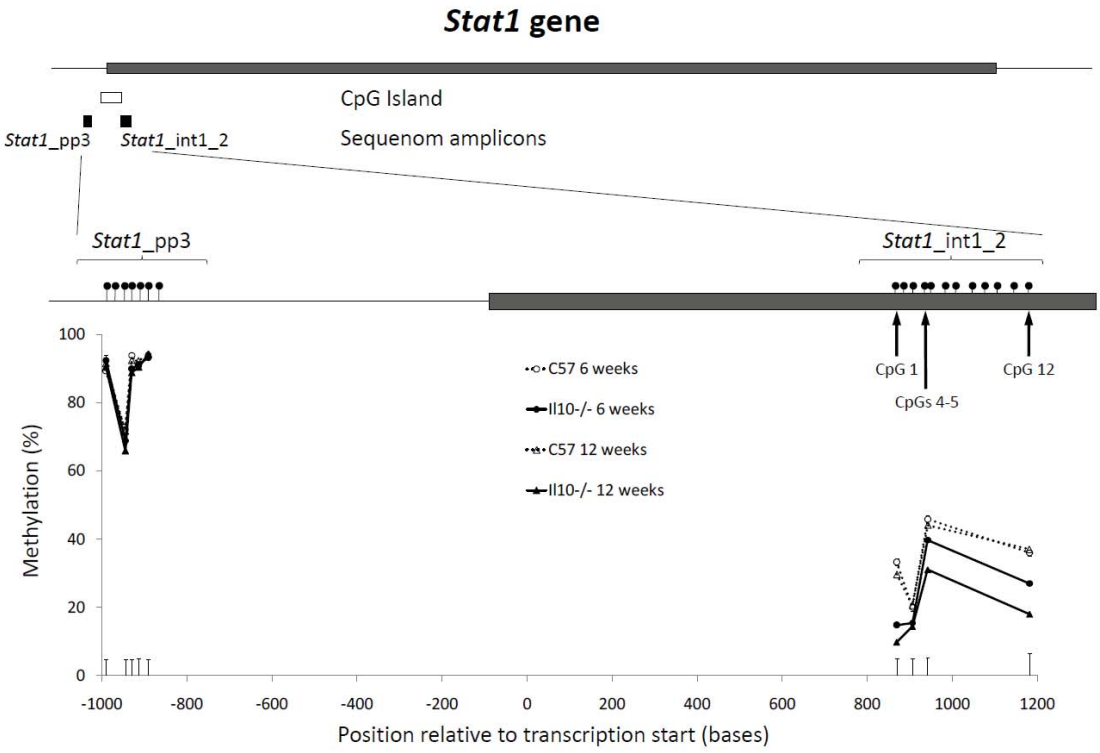


Figure 4.2 Methylation levels of CpG sites associated with the *Stat1* gene. Percentage of methylation is shown for each group at each CpG site within the promoter and intron amplicons of the *Stat1* gene. Error bars along the horizontal axis represent standard error of difference. CpG sites are shown as black circles above vertical lines along the Stat1_pp3 and Stat1_int1_2 regions of the gene. Where CpG sites could not be distinguished and were reported as a single event, the mean distance from the transcription start site was used to plot the methylation percentage. C57 = C57BL/6J mice.

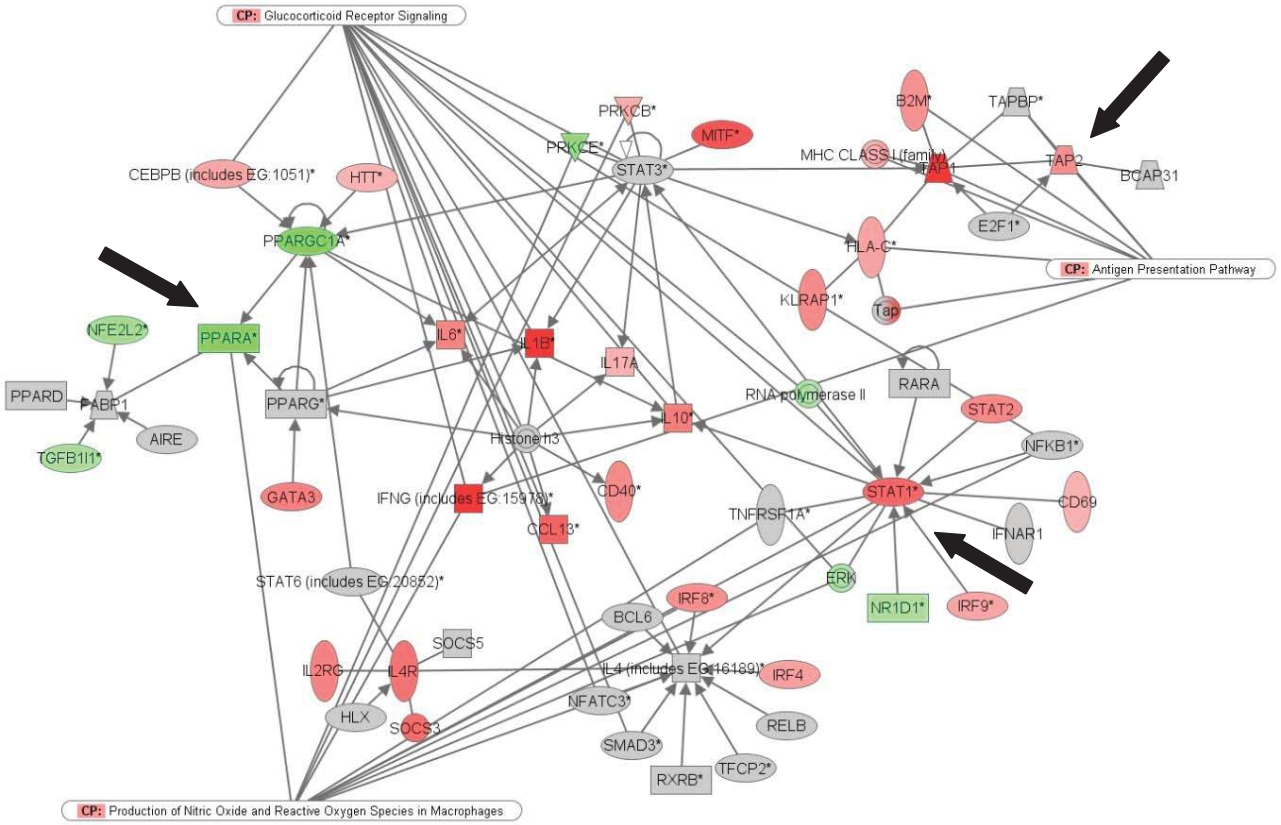


Figure 4.3 Network diagram of the *Ppara*, *Stat1* and *Tap2* genes, showing their expression values in *Il10*$^{-/-}$ mice at 12 weeks of age versus C57BL/6J mice at 12 weeks of age, with canonical pathways overlaid. The canonical pathways shown are those containing the highest number of molecules from the pathways that include each of the three genes of interest (indicated by the arrows). Molecules coloured in green represent a gene with decreased expression in *Il10*$^{-/-}$ mice at 12 weeks of age relative to C57BL/6J mice at 12 weeks of age, while red indicates an increase in expression (see Chapter 2 section 2.3.7.1 for more detail about interpretation of network diagrams).

In this study, a reduction in methylation levels in the intronic CpG sites of the *Stat1* gene was associated with increased *Stat1* mRNA levels in 12 week old *Il10*$^{-/-}$ mice (Chapter 3). Alteration of the methylation status of the *Stat1* gene may have a role in regulating the expression of this gene in IBD. While there was no change in the methylation levels of the *Stat1* gene within the sites assessed in the promoter, there was a change in the methylation levels within an intron. While the promoter region of genes was initially the focus of DNA methylation studies due to its importance in the regulation of transcription, recent studies have shown that changes in methylation status within the intronic regions of genes can also be associated with changes in gene expression [364-366]. More research is required to determine whether there is a causal relationship between a change in the intron methylation level and increased gene expression level of *Stat1* in IBD. Understanding these mechanisms could inform the development of novel therapies to reduce inflammation by reducing STAT1 gene or protein expression.

Consistent with the result presented here, other studies have demonstrated that genetic and epigenetic aberrances in particular genes interact and these interactions are associated with altered gene expression in IBD [367, 368]. A recent study of mucosal genome-wide methylation changes in rectal biopsies from UC and CD patients found consistent differences in DNA methylation between IBD cases and controls at regulatory sites within a number of genes, including genes which have been implicated in IBD susceptibility through genome-wide association analyses [367]. The mRNA levels of a subset of these genes were analysed by qPCR and their expression difference between cases and controls was correlated with DNA methylation [367]. Similarly, another recent study found that reduced methylation of the *Stat4* gene was correlated with increased *Stat4* gene expression, and in addition, there was a correlation between risk alleles and methylation status in the promoter region [368].

While methylation changes were found for the intron region of *Stat1* in this study, no change in methylation levels was found for *Ppara* or the promoter region of *Stat1*. However, it was not possible to assess methylation at all CpG sites within these genes and, in addition, technical limitations did not enable all targeted sites to be measured successfully, particularly for the *Tap2* gene. Thus, a role of DNA methylation in *Ppara* or *Tap2* regulation in colitis cannot be ruled out. The PCR step is considered to

introduce more variability than the bisulfite treatment or the mass-spectrometry in the method used here, and in future experiments using this method, melt-curve analysis or sequencing of the PCR product prior to mass-spectrometry could be used to determine whether measurement of methylation of the target genes is likely to be successful [369]. Other methods of assessing DNA methylation, such as bisulfite conversion or methyl-DNA immunoprecipitation followed by high throughput sequencing [370] may be more successful at measuring the methylation levels of these genes. These analyses provide greater coverage of each gene and may avoid the problem encountered in this study if the PCR step is not responsible for the measurement failure [370].

Epigenetic mechanisms act upstream of transcription and identifying their involvement in the inflammatory process and its regulation is an important step in gaining further insight into the pathogenesis of IBD. Many studies have described gene expression profiles in IBD-affected tissues and mouse models of IBD. Further investigation of the changes in epigenetic mechanisms in these tissues may improve understanding of how these pathways are involved in the development of IBD. Additionally, environmental influences such as diet are known to modulate epigenetic mechanisms, and interact with genetic variation and gene/protein expression profiles to alter host susceptibility to inflammation. Understanding the interaction of epigenetic and genetic factors in IBD could be a more comprehensive strategy for developing dietary intervention to modulate inflammatory responses.

In the following chapter, the results of a dietary intervention study examining the effects of milk and soy-based diets in the *Il10*$^{-/-}$ mouse model are described. The methods used in this chapter may be applicable to the dietary intervention experiment, as DNA methylation changes in the colon mucosa may be associated with changes in gene expression in response to dietary intervention.

5 Milk- and soy-based diets have differential effects on colitis and colon gene expression in the *Il10*$^{-/-}$ mouse

5.1 Abstract

Milk and soy are reported to contain molecules with antibacterial and immune-modulatory actions, which may be beneficial to people with IBD. The aim of this study was to determine whether diets containing ruminant milk or soy solids reduce colon inflammation in *Il10*$^{-/-}$ mice. Male *Il10*$^{-/-}$ mice and C57BL/6J mice were either fed diets containing 40% (*w/w*) sheep, goat, or cow whole milk powder, 40% (*w/w*) soy solids, or one of two control diets (casein-free modified-AIN-76A or standard AIN-76A) from 4 to 11 weeks of age. Diets were based on AIN-76A, which was included to enable comparison with other dietary intervention studies using this model). For all diets except AIN-76A, total protein, fat, carbohydrate and energy were kept as similar as possible. Weight and food intake were measured three times weekly throughout the experiment, and intestinal tissue was taken at 11 weeks of age for histopathology evaluation of inflammation and analysis of gene expression. Analysis of mouse weight and feed intake both showed a significant strain-diet interaction: *Il10*$^{-/-}$ mice fed the cow and goat milk diets ate less and gained less weight than all the other diet groups. This diet effect was not evident for the C57BL/6J mice. *Il10*$^{-/-}$ mice on the cow and goat milk diets had reduced colon histological injury scores relative to those on the other diets. *Il10*$^{-/-}$ mice on the cow and goat milk diets also had decreased expression levels of many immune/inflammatory-related genes and altered expression levels of immune-related pathways, processes and gene sets.

5.2 Introduction

A better understanding of nutrients and how they interact with the gastrointestinal tract is an important element in finding approaches to support the increased nutritional requirements arising from intestinal dysfunction [371]. Milk is a high-quality, highly digestible source of nutrition and immunomodulatory factors that may promote intestinal and wider well-being for IBD patients [372]. Studies using animal models of IBD have shown that CLA and oligosaccharides that are found in milk, when added to the diet, reduced mRNA abundance of colonic genes involved in inflammation [68, 84]. These findings suggest that some nutrients present in milk may interact with and influence target genes, and that enriching diets with these nutrients may have beneficial outcomes for the inflamed colon [68, 84].

There are few studies investigating effects of dietary whole milk solids on the function of the intestinal tract, either in a healthy state, or during a perturbation such as inflammation. After resection of 50% of the distal small intestine, a goat milk solid-based diet increased the apparent digestibility of iron and copper in rats [205]. Nutritional deficiencies including mineral and vitamin deficiencies often occur in IBD due to malabsorption, particularly of iron [15], so these studies suggest that goat milk may have nutritional benefits via improved uptake of minerals. In DSS-induced colonic inflammation in rats, dietary sheep milk reduced histological injury scores in the colon and increased caecal concentrations of short-chain fatty acids, possibly due to alterations in the caecal microbiota composition and function [170].

A food frequency questionnaire completed by IBD patients showed that consumption of goat and sheep milk was perceived to be more beneficial and have less severe adverse effects on the health of IBD patients than cow milk [217]. Soy-based "milk", a common substitute for dairy milks in the diet, was perceived to be even more beneficial than any of the ruminant milks [217]. Some research suggests that soy may have anti-inflammatory properties, but the evidence is inconclusive [231, 236, 373]. Details of which products were consumed, such as types of soy-based "milk" or type of goat and sheep milk (e.g. brand, preparation such as fresh or pasteurised) were not provided [217]. The study was perception-based and therefore did not provide objective evidence of any changes in intestinal function or inflammatory activity in response to the foods.

Based on these studies, IBD patients have different perceptions about the health benefits of ruminant milks, but there is limited data about the health effects of whole ruminant milk when consumed in human IBD or in animal models of IBD. Gene expression profiles in both clinical samples from human IBD patients, and from mouse models of IBD (including the *Il10*$^{-/-}$ mouse model) have shown alterations in the expression of genes coding for proteins associated with epithelial barrier function [58] and cytokine mRNA levels [98] as well as other inflammation-associated genes [110, 115]. Gene expression profiles in the colon may provide some clues as to which cellular processes are altered in *Il10*$^{-/-}$ mice in response to milk diets and provide insight into the mechanisms by which these diets affect colitis.

5.3 Aims and hypothesis

The hypothesis of the study presented here was that diets containing ruminant sheep and goat milk solids, or a milk substitute (soy "milk" solids) would reduce inflammation and associated molecular pathways in the colon mucosa in a mouse model of IBD when compared to a diet containing cow milk solids. Controlled amounts of ruminant species milk (goat, sheep and cow) or soy were fed as part of the diet to *Il10*$^{-/-}$ mice, a commonly used IBD model that shows symptoms and gene expression profiles similar to human IBD. Changes in growth performance, intestinal morphology and immune infiltration (histopathology) and function (whole genome mRNA expression) were measured at 11 weeks of age to determine how the different milk solids interacted with the intestinal mucosa. To gain further insight into the changes in molecular pathways in response to milk diets, in-depth analysis of global gene expression data was performed using IPA, GO analysis and GSEA. Further analyses of colon epithelial or other cell types using LMD and further gene expression profiling and DNA methylation changes in the colon mucosa will be performed if specific anti-inflammatory gene expression pathways are found, in order to further investigate the cellular source and/or mechanism of these changes.

5.4 Methods

The mouse experiment was carried out according to Section 2.2, the microarray analysis was performed according to Section 2.3, and the qPCR validation was performed according to Section 2.5.

5.5 Results

5.5.1 Diet analysis

Formulated diets were analysed in order to confirm that the macronutrient composition was as calculated, and to measure the lactose concentration because high levels of lactose may be responsible for milk intolerances (Table 5.1). The levels of fat were higher than anticipated for the AIN-76A and soy diets (by 0.9-1.1 g per 100 g diet), and lower for the remaining diets (by 0.2-3.4 g). Carbohydrate levels were close to the anticipated amounts for most of the diets. The modified-AIN-76A and soy diets had

Table 5.1 Nutrient composition of diets as tested at the end of the experiment. Expected values from the diet formulation are shown in brackets.

Per 100 g of diet	**AIN-76A**	**Modified AIN-76A**	**Soy**	**Sheep**	**Cow**	**Goat**
Energy (kJ)	1650.0 (1630)	1610.0 (1810)	1570.0 (1660)	1700.0 (1910)	1660.0 (1850)	1620.0 (1800)
Protein (g)	16.9 (20)	17.6 (22)	19.3 (21)	19.6 (22)	18.9 (22)	18.5 (21)
Fat (g)	5.9 (5)	13.5 (14)	14.1 (13)	13.6 (16)	11.6 (15)	11.8 (12)
Moisture (g)	4.2	9.4	8.4	7.7	7.4	7.4
Ash (g)	2.7	3.8	6.3	5.1	5.4	5.8
Fibre (g)	5.5 (5.0)	15.6 (5.6)	18.6 (9.7)	6.1 (5.5)	6.6 (5.4)	10.6 (5.2)
Carbohydrate (g)	64.8 (66)	40.1 (54)	33.3 (50)	47.9 (50)	50.1 (51)	45.9 (53)
Total sugars (g)	59.1	19.7	19.3	26.7	31.8	29.3
Lactose (g)	<0.1	<0.1	<0.1	8.4	13.1	12.5

approximately 10 and 20% less carbohydrate than anticipated, respectively, while the protein content of the other diets was within 3 g of the anticipated values. The levels of protein in AIN-76A and modified-AIN-76A diets were approximately 3% lower than the anticipated amount. Fibre results were close to the anticipated 5 g/100g for half of the diets, but approximately 300% higher than anticipated for the goat milk, modified-AIN-76A and soy diets. As expected, lactose levels were undetectable in AIN-76A, modified-AIN-76A and soy diets. Approximately 8-13% lactose was present when whole milk solids were added. Overall, fat, protein and lactose were close to the anticipated amounts based on the formulation for all diets, carbohydrate levels were lower than anticipated for modified-AIN-76A and soy diets, and fibre levels were higher than anticipated for modified-AIN-76A, soy and goat milk diets.

5.5.2 Growth performance

At the beginning of the experiment, bodyweight was the same for all groups ($P > 0.50$). Repeated measurement analysis of the bodyweight data showed that there was a significant interaction between mouse strain and diet ($P < 0.001$) for average bodyweight during the experiment. As expected, the C57BL/6J mice gained more weight than *Il10*$^{-/-}$ mice ($P < 0.001$). *Il10*$^{-/-}$ mice fed the AIN-76A diet grew more than those fed the modified-AIN-76A diet ($P < 0.05$), and the *Il10*$^{-/-}$ mice fed each milk diet (excluding soy) grew less than *Il10*$^{-/-}$ mice on both control diets ($P < 0.05$). The difference in weight gain was greater for *Il10*$^{-/-}$ mice fed the cow and goat milk diets (Figure 5.1 and Table 5.2). C57BL/6J mice fed soy, cow and goat milk-based diets grew less than C57BL/6J mice fed AIN-76A or modified-AIN-76A ($P < 0.05$) whereas there was no difference in growth between C57BL/6J mice fed sheep milk and C57BL/6J mice fed AIN-76A or modified-AIN-76A ($P > 0.05$).

The sharp decrease in growth of the *Il10*$^{-/-}$ mice fed the three milk diets between days 10 and 15, followed by varying degrees of recovery, is of interest, possibly suggesting a slower adaptation to these diets. At the end of the experiment, only *Il10*$^{-/-}$ mice fed the goat and cow milk diets had lower bodyweight than those fed the modified-AIN-76A diet (Table 5.2).

Figure 5.1 Bodyweight of (A) C57BL/6J and (B) *Il10*$^{-/-}$ mice throughout the experiment. Bodyweight is shown from commencement of treatment diets until the day prior to sampling. * denotes a final mean weight that is different from the modified-AIN-76A diet. The 5% LSD bar from the repeated measurements analysis of body weight over the whole experiment is also shown. The legend for both graphs is shown on the graph for the C57BL/6J mice.

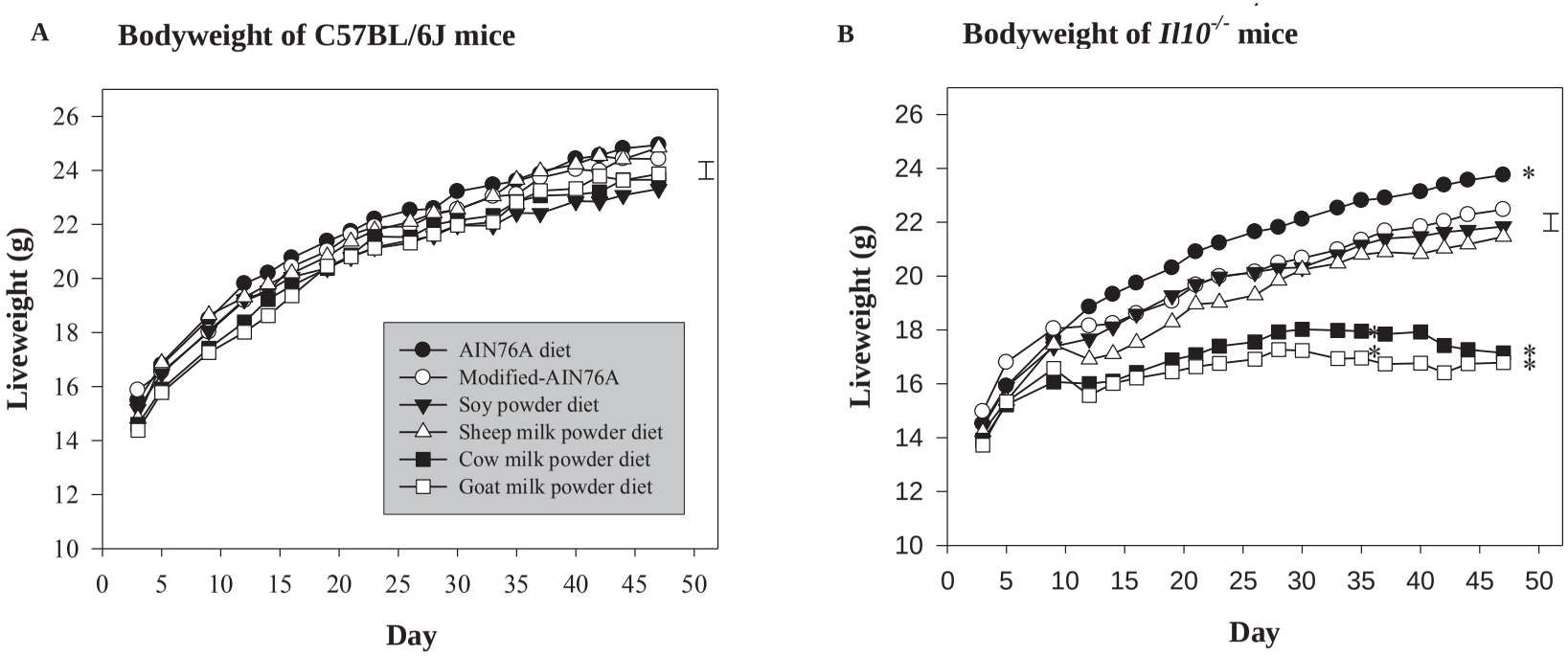


Table 5.2 Predicted means for body weight (g) for each treatment over the whole experiment and at the end. *denotes a mean that is significantly different to modified-AIN-76A or AIN-76A for the same strain (using maximum LSD at 5% for all comparisons of 0.64 for the repeated measurements analysis of weight over the length of the experiment, and 1.14 for the final weight).

	Diet					
Genotype	**AIN-76A**	**Modified AIN-76A**	**Soy**	**Sheep**	**Cow**	**Goat**
Average weight over whole experiment						
C57BL/6J	21.3	21.0	20.3*	21.0	20.3*	20.2*
Il10$^{-/-}$	20.3	19.5	19.2	18.6*	16.8*	16.3*
Final weight						
C57BL/6J	24.9	24.4	23.3	24.8	23.7	23.9
Il10$^{-/-}$	23.8	22.5	21.8	21.5	17.1*	16.8*

5.5.3 Food intake

Intake (grams per mouse per day) was averaged over the experiment for analysis because it was consistent over time. It was more appropriate to compare intake between the mouse strains for each diet, rather than comparing between diets, because the diets had different energy density. Overall, *Il10*$^{-/-}$ mice ate more than the C57BL/6J mice per gram of bodyweight ($P < 0.05$). There was a trend for the difference in intake between strains of mice to be smaller for some diets (AIN-76A) and greater for others (goat and soy) ($P = 0.06$). The *Il10*$^{-/-}$ mice fed the soy diet ate approximately 3% more than C57BL/6J mice fed the same diet (3.22 *vs.* 3.11 g/day; $P < 0.05$), whereas *Il10*$^{-/-}$ mice fed the cow and goat milk diets ate approximately 10% less than C57BL/6J mice fed the same diets (2.66 *vs.* 2.99 g/day, and 2.79 *vs.* 3.11 g/day, respectively; $P < 0.05$) (Table 5.3). Analysis of the final intake for each treatment also showed that the *Il10*$^{-/-}$ mice on goat and cow milk ate less than other groups ($P = 0.008$ for the diet x strain interaction). There was a significant interaction between diet and strain ($P < 0.001$) for the average intake over the experiment.

Reduced food intake did not seem to be directly responsible for the reduced growth of *Il10*$^{-/-}$ mice fed the milk-based diets. Average daily food intake in terms of energy is shown in Table 5.3. *Il10*$^{-/-}$ mice on cow and goat milk diets ate only about 2 kJ per day less than those on AIN-76A. Per unit bodyweight (Table 5.3), *Il10*$^{-/-}$ and C57BL/6J mice on soy, cow and goat milk diets ate more than mice on either of the control diets. While *Il10*$^{-/-}$ mice fed the goat and cow milk-based diets ate less in terms of total energy, they ate more per unit of bodyweight. Examination of graphs of average daily intake in g/day (not shown) and weight gain (Figure 5.1) suggested that average daily intake became consistently lower for the *Il10*$^{-/-}$ mice fed the goat and cow milk-based diets in the last half of the experiment, whereas the weight gain of the *Il10*$^{-/-}$ mice fed the goat and cow milk-based diets appeared to become consistently lower than that of the *Il10*$^{-/-}$ mice fed the other diets by around nine days after the beginning of the experiment. This may suggest that the lower overall intake by weight of food for the *Il10*$^{-/-}$ mice fed the goat and cow milk-based diets was partly due to their lower body weight.

Table 5.3 Predicted means for average daily food intake per mouse (g) for each treatment over the whole experiment, from repeated measurements analysis. * denotes a mean that is significantly different from the mean for AIN-76A or modified-AIN-76A for mice of the same strain (maximum LSD at 5% for all comparisons for intake in grams = 0.08; for intake per unit bodyweight = 0.004).

Genotype	AIN-76A	Modified-AIN-76A	Soy	Sheep	Cow	Goat
Food intake per day (g)						
C57BL/6J	3.18	2.99	3.11	3.01	2.99	3.11
Il10$^{-/-}$	3.18	3.02	3.22	2.94	2.66*	2.79*
Per unit of bodyweight (g/g)						
C57BL/6J	0.149	0.142	0.153*	0.144	0.148*	0.154*
Il10$^{-/-}$	0.156	0.154	0.167*	0.157	0.159*	0.170*
Energy intake using formulated energy per gram values of each diet (kJ)						
C57BL/6J	51.9	54.1	51.7	57.7	55.5	55.9
Il10$^{-/-}$	51.9	54.7	53.6	56.3	49.4	50.2
Energy intake using measured energy per gram values of each diet (kJ)						
C57BL/6J	52.5	48.1	48.8	51.2	49.6	50.4
Il10$^{-/-}$	52.5	48.6	50.6	50.0	44.2	45.2

5.5.4 Clinical signs of intestinal dysfunction

Il10$^{-/-}$ mice fed the milk diets, especially the cow and goat milk diets, developed diarrhoea (soft faeces) from one week after the initial feeding of the treatment diets (Table 5.4) and a decline in growth within the following week (Figure 5.1B). The weight gain recovered to varying degrees depending on the diet (Figure 5.1B), suggesting this may represent a period of adaptation to the diets. At the end of the experiment, the *Il10*$^{-/-}$ mice fed cow and goat milk diets had reduced growth relative to those on the other diets (Figure 5.1B).

Two *Il10*$^{-/-}$ mice fed the goat milk diet were sampled two weeks earlier than intended due to intestinal bleeding and rectal prolapse development, and another *Il10*$^{-/-}$ mouse on the goat milk diet was sampled three weeks early due to falling below the GHS score of 3. When dissected, this mouse was found to have a blocked rectum. Each of these mice had no histological abnormalities in the intestine, suggesting that colitis was not the cause of death. Three additional *Il10*$^{-/-}$ mice on the goat milk diet died during the experiment but had no histological intestinal abnormalities on necropsy, with no cause of death established. These 6 mice were not used for any other data analysis for the experiment.

Throughout the experiment, *Il10*$^{-/-}$ mice fed the soy and modified-AIN-76A diets had a small amount of hair loss around the anus, but this typically grew back by the end of the experiment. Those fed the sheep milk-based diet had more severe hair loss, but this was also resolved for many mice before the end of the experiment. *Il10*$^{-/-}$ mice fed the cow and goat milk-based diets on the other hand continued to have softened faeces and hair loss for the whole experiment, and also had a tendency to aggravate the area by cleaning and chewing it, including around the base of the tail. Hair loss and skin irritation were likely exacerbated in the mice fed the milk diets because *Il10*$^{-/-}$ mice are susceptible to skin irritants, and faeces that sticks to the skin acts as an irritant [98].

During sampling, faecal consistency, amount and colour was recorded for all mice on the same day. These observations confirm that many *Il10*$^{-/-}$ mice fed the cow and goat milk diets had soft faeces for the duration of the experiment, as did some fed sheep, soy and modified-AIN-76A diets. Not all animals in these groups were affected to the same degree, as some mice had normal faeces.

Table 5.4 Health scores and diarrhea observations. Table shows the percentage of *Il10*$^{-/-}$ mice fed each diet that were observed to have diarrhoea or a reduced health score at day 27-28 (mid-way throughout the experiment) and day 46-47 (the end of the experiment).

	Day 27-28		Day 46-47	
Diet	**%[#] GHS<1**	**%[#] diarrhoea**	**%[#] GHS<1**	**%[#] diarrhoea**
AIN-76A	0	0	0	7*
Modified-AIN-76A	40	47	13	40
Soy	0	0	0	40
Sheep	33	0	20	73
Goat	58	33	67	78
Cow	67	20	67	93

A GHS of 1 denotes a mouse that appears healthy in terms of appearance and behaviour (maximum score is 5 which denotes a dead or dying mouse – mice were euthanased in this study once they reached a GHS of 3).

[#] Percentages are for the total number of mice in the experiment at each time point for each treatment (three *Il10*$^{-/-}$ mice (goat milk diet) and one C57BL/6J mouse (cow milk diet) died before days 27-28, while three *Il10*$^{-/-}$ mice (goat milk diet) died after days 27-28).

***** Percentage accounted for by only one mouse, and this observation was unusual (only one other *Il10*$^{-/-}$ mouse on the modified-AIN-76A was ever observed to have diarrhoea, on day 30).

Only one C57BL/6J mouse was observed to have diarrhoea (modified-AIN-76A diet, day 7) throughout the experiment.

At day 7, only one *Il10*$^{-/-}$ mouse had diarrhoea (modified-AIN-76A), and no mice had lowered GHS.

One *Il10*$^{-/-}$ mouse fed the AIN-76A diet had a small degree of faecal softening on the day prior to sampling but this was unusual as these mice typically had firm and dry faeces throughout the experiment. The faeces of the C57BL/6J mice were pale and only slightly modified by the diet colour, whereas faeces of the *Il10*$^{-/-}$ mice tended to be much darker and brighter and modified by diet colour.

Faecal bleeding could only be observed in *Il10*$^{-/-}$ mice by examining faeces for the appearance of blood, and was rarely observed. However, this method is subjective and the more sensitive Hemoccult Fecal Occult Blood Test (Beckman Coulter, Inc., Brea, California, USA) [374] could provide more accurate assessment of faecal bleeding in future experiments.

5.5.5 Histological injury scores

5.5.5.1 Small intestine

In jejunum and ileum intestinal tissue, there was no significant diet x strain interaction ($P > 0.10$). However, there was some indication of a diet x strain effect in the duodenum ($P = 0.03$), with *Il10*$^{-/-}$ mice on the cow milk diet having 38% higher duodenum HIS than those on the modified-AIN-76A diet and those on sheep and soy having 22-25% lower duodenum HIS than the AIN-76A diet. However, using the maximum 5% LSD there were no significant differences between the HIS of mice on any of the diets compared to either control diet (Table 5.5).

5.5.5.2 Colon and caecum

A significant strain x diet effect was observed for total colon HIS, and the subtotals for the presence of inflammatory cells and changes in tissue architecture ($P < 0.01$). The *Il10*$^{-/-}$ mice had higher colon HIS scores than C57BL/6J mice, and there were no differences in colon HIS between diets for the C57BL/6J mice.

Il10$^{-/-}$ mice fed cow and goat milk diets had lower colon HIS than those fed the other diets, with colon HIS for the goat milk *Il10*$^{-/-}$ mice being lower than those fed the cow milk diet (Table 5.5). This pattern was the same regardless of whether inflammatory cell infiltration, tissue architecture, or total features were analysed (data not shown). Colon

Table 5.5 Predicted mean histological injury scores for colon, caecum and duodenum for all treatments. *denotes a mean that is significantly different from the mean for modified-AIN-76A for mice of the same strain, using maximum 5% LSDs for each tissue (duodenum (square-root transformed) 0.5; jejunum 1.3; ileum 1.3; caecum 5.4; colon (square-root transformed) 0.6). Predicted means for duodenum and colon have not been back-transformed.

	C57BL/6J						***Il10*$^{-/-}$**					
Tissue	**AIN-76A**	**Modified-AIN-76A**	**Soy**	**Sheep**	**Cow**	**Goat**	**AIN-76A**	**Modified-AIN-76A**	**Soy**	**Sheep**	**Cow**	**Goat**
Duodenum	1.7	1.7	1.7	1.6	1.6	1.5	2.3	2.1	2.0	1.9	2.4	2.4
Jejunum	2.7	3.1	2.6	2.3	2.2	2.4	3.9	4.3	4.1	3.6	3.5	3.8
Ileum	2.3	2.1	2.6	2.6	2.4	2.7	3.6	3.1	2.9	2.9	2.7	3.1
Caecum	3.6	3.8	3.7	4.4	6.6	5.4	16.7	17.1	12.4	16.5	14.0	11.7
Colon	1.8	2.2	1.8	1.9	2.2	2.1	5.3	5.0	5.0	5.1	4.1*	3.3*

HIS for each mouse is shown in Figure 5.2 to demonstrate the range of scores in each group. Using exact 5% LSD values, *Il10*$^{-/-}$ mice fed the soy, goat milk and cow milk diets had lower caecum HIS compared to those fed the modified-AIN-76A diet ($P < 0.05$). However, using the maximum 5% LSD values as criteria for significance, as described in Section 2.2.8, there were no differences in caecum HIS between groups of *Il10*$^{-/-}$ mice.

5.5.6 Colon gene expression

The microarray data discussed in this chapter are accessible through GEO Series accession number GSE39830.

5.5.6.1 Quality control analysis of slide effects

Lists of differentially expressed genes for the *Il10*$^{-/-}$ versus C57BL/6J mice comparisons within the AIN-76A diet were analysed and compared between the time-course experiment and the milk diet experiment to confirm the presence of similar gene expression profiles between the *Il10*$^{-/-}$ and C57BL/6J mice between experiments. The gene lists from both experiments were also examined using IPA and EASE, both before and after REML analysis to remove day and array effects from the gene expression data. The gene lists for each experiment were similar, with similar top EASE categories (Table 5.6). The number of total significant EASE categories was 162 for the time - course experiment and 164 for the milk diet experiment. The Pearson correlation coefficient for differentially expressed genes between the *Il10*$^{-/-}$ versus C57BL/6J mice on AIN-76A at 12 weeks of age in both mouse experiments was 0.98, indicating a 98% similarity between the gene lists. These data indicate that colon microarray data was reproducible and consistent between experiments using this mouse model.

The corrected gene list was similar to the uncorrected gene list in terms of the genes with the most significant FDR, and the degree of change in expression in these genes was also similar between corrected and uncorrected gene lists (Table 5.7). The uncorrected gene expression data was therefore used for the remaining analyses.

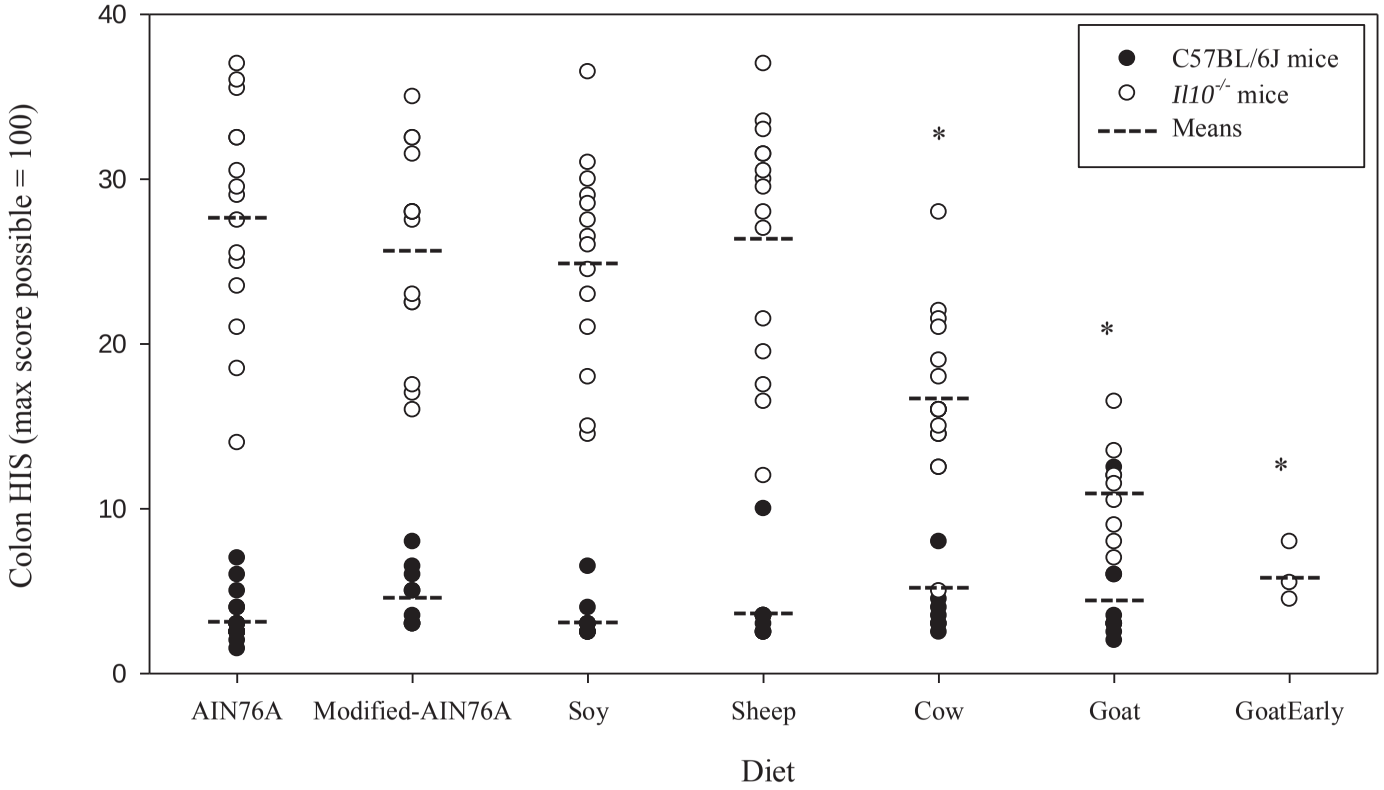


Figure 5.2 Colon histological injury scores (HIS). Individual value plot of colon HIS for all mice sampled during the experiment. Means shown were calculated from non-transformed data. *denotes a mean that is significantly different from the mean for modified-AIN-76A for *Il10*$^{-/-}$ mice, using maximum 5% LSD.

Table 5.6 Consistency of gene expression data between mouse experiments. Top ten EASE categories for both mouse experiments for the comparison between *Il10*$^{-/-}$ mice and C57BL/6J mice at 11-12 weeks of age fed the AIN-76A diet.

	Top 10 gene categories ranked by ascending EASE score	**EASE score**
Milk diet experiment	defense response	<0.001
	response to biotic stimulus	<0.001
	immune response	<0.001
	response to pest/pathogen/parasite	<0.001
	response to external stimulus	<0.001
	signal transducer activity	<0.001
	response to wounding	<0.001
	humoral immune response	<0.001
	cell communication	<0.001
	extracellular	<0.001
Time-course experiment	response to biotic stimulus	<0.001
	defense response	<0.001
	immune response	<0.001
	response to external stimulus	<0.001
	response to pest/pathogen/parasite	<0.001
	response to wounding	<0.001
	humoral immune response	<0.001
	signal transducer activity	<0.001
	response to stress	<0.001
	extracellular	<0.001

Table 5.7 Correction of microarray data for slide effects. Fold changes (FC) and false discovery rates (FDR) for the top 10 genes for *Il10*$^{-/-}$ mice vs. C57BL/6J mice fed the AIN-76A diet (milk diet experiment) before and after correction for slide effects using REML analysis. Genes are listed in order of descending FC.

GeneName	Original FC	Original FDR	Adjusted FC	Adjusted FDR
Reg3g	63.81	<0.001	62.47	<0.001
Pap	57.17	<0.001	56.77	<0.001
S100a9	43.11	<0.001	42.13	<0.001
Iigp1	24.70	<0.001	24.57	<0.001
Cxcl5	18.92	<0.001	18.48	<0.001
S100a8	18.71	<0.001	18.28	<0.001
Indo	18.56	<0.001	18.20	<0.001
Ubd	18.22	<0.001	17.73	<0.001
Cxcl9	17.91	<0.001	17.71	<0.001
Tgtp	17.12	<0.001	16.98	<0.001
Cxcl10	15.90	<0.001	15.63	<0.001
NAP065157-1	15.45	<0.001	15.39	<0.001
1810030J14Rik	-15.32	<0.001	-15.20	<0.001
Slc28a3	14.81	<0.001	14.55	<0.001
Igh-1a	14.68	<0.001	13.61	<0.001
Clec7a	13.46	<0.001	13.23	<0.001
Ighg	13.41	<0.001	13.20	<0.001
Prlpm	13.37	<0.001	13.08	<0.001
Cyp4b1	-13.21	<0.001	-12.85	<0.001
Irg1	12.87	<0.001	12.61	<0.001

5.5.6.2 Relationship between colon histological injury score and gene expression

Gene expression data within each treatment was analysed to determine whether the expression levels of any genes correlated with HIS. Mice were grouped into high and low-scoring groups according to colon HIS within each diet and strain, and gene lists of differential expression between the three mice with the highest scores and the three mice with the lowest scores were generated for each treatment. Three treatments, the sheep milk diet, modified-AIN-76A and cow milk diets fed to *Il10*$^{-/-}$ mice, had some differentially expressed genes between the mice with the highest and the mice with the lowest histology scores (Table 5.8). The numbers of significantly differentially expressed genes were between 1 and 48, less than 5% of the total number of genes, therefore the difference in expression of these genes could have been significant merely by chance for a probability cutoff of 0.05. Gene expression data was therefore pooled for all mice within each treatment for further analysis.

5.5.6.3 Comparison of gene expression results between AIN-76A and modified-AIN-76A diets

Only one gene was differentially expressed between the AIN-76A and modified-AIN-76A diets in *Il10*$^{-/-}$ mice, whereas 199 genes were differentially expressed between the AIN-76A and modified-AIN-76A diets in C57BL/6J mice. One gene set (apoptosis) and 17 GO biological processes (mostly related to apoptosis and development, see Table 5.9) were enriched with differentially expressed genes between the AIN-76A and modified-AIN-76A diets in C57BL/6J mice. Although only one gene was differentially expressed, four gene sets were enriched between the AIN-76A and modified-AIN-76A diets in *Il10*$^{-/-}$ mice (Parkinson's disease, pyruvate metabolism, gap junction, and cardiac muscle contraction). Overall, however, the differences in gene expression profiles between the modified-AIN-76A and AIN-76A diets were minimal. The modified-AIN-76A diet, which matched to the macronutrient composition of the milk and soy diets, contained no milk or soy, and was considered the control diet of choice when assessing differentially expressed genes between treatments in the following sections.

Table 5.8 Gene expression differences between high and low histological injury score (HIS). Table shows numbers of differentially expressed genes within each treatment group comparing high and low HIS-scoring mice.

Treatment	Number of significant genes for treatment between the high and low-HIS-scoring mice
C57 Modified-AIN-76A	0
C57 AIN-76A	0
C57 Cow	0
C57 Goat	0
C57 Sheep	0
C57 Soy	0
IL10 Modified-AIN-76A	48
IL10 AIN-76A	0
IL10 Cow	1
IL10 Goat	0
IL10 Sheep	46
IL10 Soy	0

C57 = C57BL/6J mice
IL10 = *Il10*$^{-/-}$ mice

Table 5.9 Over-represented gene ontology processes for AIN-76A *vs.* modified-AIN-76A diet in C57BL/6J mice.

System	Gene category	EASE score
GO Cellular Component	actin filament	<0.001
GO Molecular Function	molecular function unknown	0.012
GO Biological Process	development	0.013
GO Biological Process	morphogenesis	0.014
GO Biological Process	organogenesis	0.015
GO Molecular Function	phosphodiesterase I activity	0.015
GO Cellular Component	cornified envelope	0.015
GO Molecular Function	hormone activity	0.018
GO Biological Process	response to external stimulus	0.019
GO Cellular Component	plasma membrane	0.022
GO Molecular Function	nucleotide diphosphatase activity	0.023
GO Biological Process	positive regulation of programmed cell death	0.035
GO Biological Process	induction of apoptosis	0.035
GO Biological Process	positive regulation of apoptosis	0.035
GO Biological Process	induction of programmed cell death	0.035
GO Biological Process	regulation of programmed cell death	0.035
GO Biological Process	keratinocyte differentiation	0.037

5.5.6.4 Interaction between mouse genotype and diet

5.5.6.4.1 Differentially expressed genes

The numbers of differentially expressed genes for each diet relative to modified-AIN-76A for each strain of mice are shown in Table 5.10. The numbers of differentially expressed genes in each strain and diet show a distinct diet x genotype interaction. In general, where there were changes in gene expression levels in one strain, there were few or none in the other strain, suggesting these diets had different effects on colon gene expression in each strain. Overall, the highest number of differentially expressed genes occurred in the C57BL/6J mice, comparing AIN-76A to modified-AIN-76A. Only 2-30 genes were differentially expressed between the other diets and modified-AIN-76A in C57BL/6J mice. In *Il10*$^{-/-}$ mice, only cow and goat milk diets had a large number of differentially expressed genes relative to modified-AIN-76A (674 and 994 respectively). Many of the genes in these two lists overlap (Figure 5.3).

5.5.6.4.2 Pathway, gene ontology and gene set analyses

A heatmap of over-represented KEGG pathways identified using GSEA demonstrated that the colon gene expression profiles of the mice in this experiment cluster primarily by strain, as expected, and in the *Il10*$^{-/-}$ mice, those fed the goat and cow milk-based diets cluster together while the *Il10*$^{-/-}$ mice fed the other diets cluster together (Figure 5.4).

There were 37 enriched GO categories for the soy diet (Table 5.11), and nine for the sheep milk-based diet (Table 5.12) in C57BL/6J mice. Gene lists for both strains of mice fed soy and sheep milk diets were enriched with 10 or fewer gene sets (Table 5.13), which were related to a wide range of processes (such as oocyte meiosis, Parkinson's disease and gastric acid secretion) which have little apparent relationship to each other. Overall, the effects of soy and sheep milk diets on colon gene expression levels were minimal for both C57BL/6J and *Il10*$^{-/-}$ mice.

There was a decrease in expression levels of many immune-related genes in *Il10*$^{-/-}$ mice fed the cow and goat milk relative to the *Il10*$^{-/-}$ mice fed the modified-AIN-76A diet. Analysis of enriched gene ontology (GO) categories in these gene lists using EASE also

Table 5.10 Numbers of differentially expressed genes for each diet relative to the modified-AIN-76A diet.

Diet	**Number of differentially expressed genes in C57BL/6J mice**	**Number of differentially expressed genes in *Il10*$^{-/-}$ mice**
AIN-76A	199	1
Soy	30	0
Sheep milk	12	0
Cow milk	18	994
Goat milk	2	674

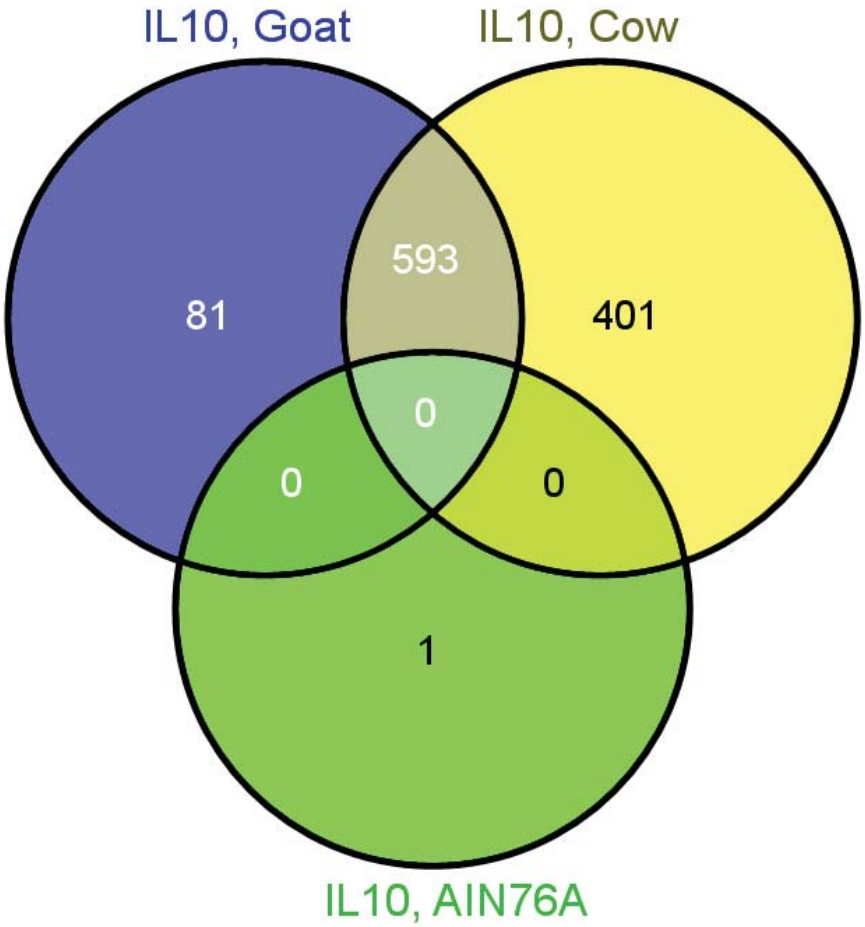


Figure 5.3 Venn diagram showing numbers of differentially expressed genes unique to each diet or shared between diets relative to the modified-AIN-76A diet for *Il10*$^{-/-}$ mice (represented by IL10 in the diagram).

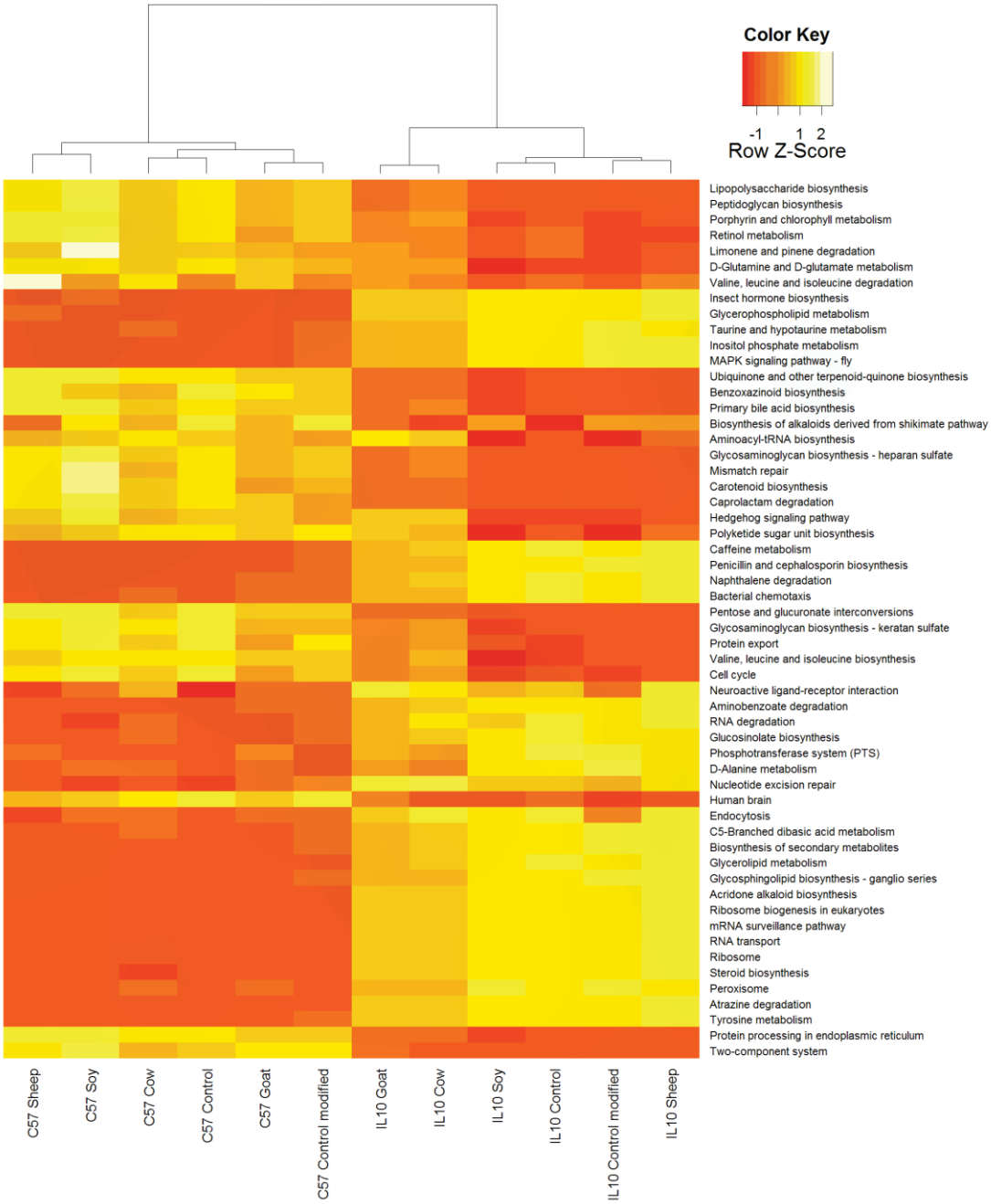


Figure 5.4 Heatmap of KEGG pathways for all treatments. Only the pathways with the top 25% of variation across the treatment groups are shown. The dendrogram above the heatmap is a tree diagram that illustrates the arrangement of the clusters produced by hierarchical clustering of treatment groups in columns, with higher similarity represented by closer relationship in the tree. C57 = C57BL/6J mice, IL10 = *Il10*$^{-/-}$ mice, "Control" = AIN-76A diet, and "Control modified" = modified-AIN-76A diet.

Table 5.11 Over-represented gene ontology processes for the soy diet in C57BL/6J mice (compared to the modified-AIN-76A diet).

System	Gene category	EASE score
GO Biological Process	nuclear division	<0.001
GO Biological Process	M phase	<0.001
GO Biological Process	amino acid metabolism	<0.001
GO Biological Process	mitosis	<0.001
GO Biological Process	amino acid and derivative metabolism	<0.001
GO Biological Process	M phase of mitotic cell cycle	<0.001
GO Molecular Function	ATP binding	<0.001
GO Molecular Function	adenyl nucleotide binding	<0.001
GO Biological Process	cell cycle checkpoint	<0.001
GO Biological Process	amine metabolism	<0.001
GO Molecular Function	purine nucleotide binding	0.001
GO Molecular Function	nucleotide binding	0.001
GO Biological Process	mitotic cell cycle	0.002
GO Molecular Function	catalytic activity	0.002
GO Biological Process	mitotic checkpoint	0.003
GO Biological Process	carboxylic acid metabolism	0.004
GO Biological Process	organic acid metabolism	0.004
GO Biological Process	cell cycle	0.008
GO Biological Process	physiological process	0.008
GO Molecular Function	water transporter activity	0.012
GO Molecular Function	shikimate kinase activity	0.012
GO Biological Process	DNA replication and chromosome cycle	0.013
GO Biological Process	regulation of mitosis	0.013
GO Biological Process	cell proliferation	0.013
GO Cellular Component	mitochondrial outer membrane	0.015
GO Biological Process	cytokinesis	0.016
GO Molecular Function	transporter activity	0.016
GO Biological Process	cell growth and/or maintenance	0.017
GO Molecular Function	L-cystine transporter activity	0.024
GO Molecular Function	sulfur amino acid transporter activity	0.024
GO Cellular Component	outer membrane	0.024
GO Biological Process	mitotic chromosome segregation	0.031
GO Biological Process	phosphate transport	0.037
GO Cellular Component	membrane fraction	0.042
GO Cellular Component	mitochondrial membrane	0.043
GO Biological Process	transport	0.045
GO Molecular Function	lipid transporter activity	0.046

Table 5.12 Over-represented gene ontology processes for the sheep milk diet in C57BL/6J mice (compared to the modified-AIN-76A diet).

System	Gene category	EASE score
GO Molecular Function	ATP binding	<0.001
GO Molecular Function	adenyl nucleotide binding	<0.001
GO Molecular Function	purine nucleotide binding	<0.001
GO Molecular Function	nucleotide binding	<0.001
GO Molecular Function	catalytic activity	0.030
GO Biological Process	lipid transport	0.038
GO Molecular Function	ATP-binding cassette transporter activity	0.042
GO Molecular Function	binding	0.043
GO Cellular Component	cytoplasm	0.045

Table 5.13 Enriched gene sets for *Il10*$^{-/-}$ and C57BL/6J mice fed the soy and sheep milk diets compared to the modified-AIN-76A diet.

Strain	Diet	Pathway ID	Pathway name	P value
Il10$^{-/-}$	Soy	mmu04540	Gap junction	0.013
		mmu05012	Parkinson's disease	0.015
		mmu04730	Long-term depression	0.028
		mmu00760	Nicotinate and nicotinamide metabolism	0.030
		mmu04270	Vascular smooth muscle contraction	0.039
		mmu04720	Long-term potentiation	0.040
		mmu04971	Gastric acid secretion	0.041
		mmu04070	Phosphatidylinositol signaling system	0.042
		mmu04970	Salivary secretion	0.046
		mmu05100	Bacterial invasion of epithelial cells	0.049
	Sheep milk	mmu04114	Oocyte meiosis	0.028
		mmu04914	Progesterone-mediated oocyte maturation	0.049
C57BL/6J	Soy	mmu04120	Ubiquitin mediated proteolysis	0.004
	Sheep milk	mmu04740	Olfactory transduction	0.022
		mmu04145	Phagosome	0.045

highlighted many immune processes, as well as cell death and cell proliferation, defence, external stimulus, damage, wounding, pest, pathogen, and parasite stress.

Few enriched GO categories for these comparisons were noted for the C57BL/6J mice fed the cow or goat milk-based diets. No GO processes were identified for the C57BL/6J mice fed the goat milk-based diet. Three enriched GO categories were noted for the C57BL/6J mice fed the cow milk-based diet: (1) ATP-binding cassette (ABC) transporter activity, (2) ATPase activity, coupled to transmembrane movement of substances and (3) hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances.

Regardless of the method used to categorise the differentially expressed genes in response to goat and cow milk diets, the changes were immune-related. Many of the differentially expressed genes in the *Il10*$^{-/-}$ mice fed the cow and goat milk diets were enriched with immune-related GO categories and pathways. A similar set of Ingenuity canonical pathways were enriched with differentially expressed genes for the cow and goat milk-fed *Il10*$^{-/-}$ mice, as shown in Table 5.14 which lists the top 20 canonical pathways for *Il10*$^{-/-}$ mice fed the cow milk diet versus the modified-AIN-76A diet, and Table 5.15, which lists the top 20 canonical pathways for *Il10*$^{-/-}$ mice fed the goat milk diet versus the modified-AIN-76A diet. Both groups share the same top four pathways (all immune-related), with the fifth being natural killer cell signalling for the cow milk diet and altered T and B cell signalling in rheumatoid arthritis for the goat milk diet. The top network identified in IPA was the same for both goat and cow milk diets: cell-to-cell signalling and interaction, haematological system development and function, and immune cell trafficking.

This network is shown in Figure 5.5 with the expression data overlaid for the comparison of *Il10*$^{-/-}$ mice *vs*. C57BL/6J mice on the modified-AIN-76A diet, in Figure 5.6 with the expression data overlaid for the goat milk-based diet, and in Figure 5.7, with the expression data overlaid for the cow milk-based diet. The networks show a general decrease in expression levels of many immune-related genes in *Il10*$^{-/-}$ mice fed the goat and cow milk-based diets, including *Stat1*, Lymphotoxin β (*Ltb*), and Tumour necrosis factor (*Tnf*). *Stat1*, interleukin-18 (IL18) and interleukin-1 (IL1) appear as network hubs and also have altered expression levels themselves, suggesting their

Table 5.14 Top 20 canonical pathways for *Il10*$^{-/-}$ mice fed the cow milk diet *vs.* the modified-AIN-76A diet. There were 49 canonical pathways with $P < 0.05$ and ratios greater than 10%. Pathways are listed by ascending probability value.

Canonical pathway	P value	Ratio (%)
Primary Immunodeficiency Signaling	7.94×10^{-13}	27
Natural Killer Cell Signaling	1.58×10^{-11}	22
T Helper Cell Differentiation	1.12×10^{-10}	26
Systemic Lupus Erythematosus Signaling	1.23×10^{-10}	15
Communication between Innate and Adaptive Immune Cells	1.55×10^{-10}	17
Dendritic Cell Maturation	1.78×10^{-09}	14
B Cell Development	7.41×10^{-09}	30
iCOS-iCOSL Signaling in T Helper Cells	1.32×10^{-08}	17
T Cell Receptor Signaling	2.04×10^{-07}	17
CD28 Signaling in T Helper Cells	3.72×10^{-07}	15
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	5.89×10^{-07}	17
Leukocyte Extravasation Signaling	1.12×10^{-06}	13
Role of NFAT in Regulation of the Immune Response	1.12×10^{-06}	12
PKCθ Signaling in T Lymphocytes	1.91×10^{-06}	13
Crosstalk between Dendritic Cells and Natural Killer Cells	2.63×10^{-06}	16
CTLA4 Signaling in Cytotoxic T Lymphocytes	3.55×10^{-06}	16
OX40 Signaling Pathway	5.50×10^{-06}	13
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	5.62×10^{-05}	12
Graft-versus-Host Disease Signaling	5.89×10^{-05}	18
Calcium-induced T Lymphocyte Apoptosis	5.89×10^{-05}	16

Table 5.15 Top 20 canonical pathways for *Il10*$^{-/-}$ mice fed the goat milk diet *vs*. the modified-AIN-76A diet. There were 26 canonical pathways with $P < 0.05$ and ratios greater than 10%. Pathways are listed by ascending probability value.

Canonical pathway	P value	Ratio (%)
Communication between Innate and Adaptive Immune Cells	2.00×10^{-14}	18
T Helper Cell Differentiation	2.00×10^{-12}	25
Primary Immunodeficiency Signaling	1.58×10^{-11}	22
Systemic Lupus Erythematosus Signaling	3.16×10^{-11}	13
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	5.01×10^{-11}	20
Crosstalk between Dendritic Cells and Natural Killer Cells	3.39×10^{-10}	18
Natural Killer Cell Signaling	2.24×10^{-09}	16
Dendritic Cell Maturation	5.62×10^{-09}	11
iCOS-iCOSL Signaling in T Helper Cells	4.17×10^{-08}	14
B Cell Development	6.31×10^{-08}	24
Graft-versus-Host Disease Signaling	2.95×10^{-07}	20
CD28 Signaling in T Helper Cells	4.90×10^{-06}	11
FXR/RXR Activation	5.25×10^{-06}	13
OX40 Signaling Pathway	7.76×10^{-06}	11
T Cell Receptor Signaling	2.40×10^{-05}	12
CTLA4 Signaling in Cytotoxic T Lymphocytes	3.16×10^{-05}	12
Autoimmune Thyroid Disease Signaling	1.23×10^{-04}	12
Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	2.88×10^{-04}	14
B Cell Activating Factor Signaling	4.37×10^{-04}	16
Calcium-induced T Lymphocyte Apoptosis	4.90×10^{-04}	11

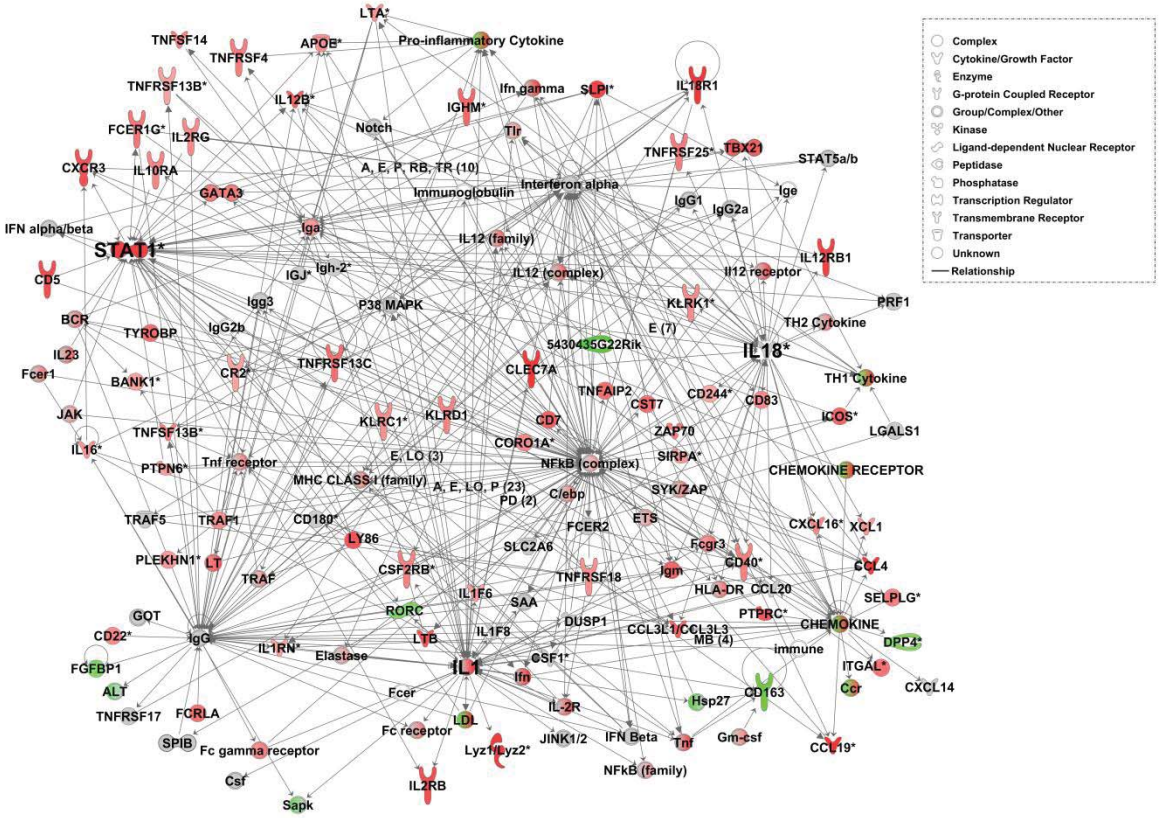


Figure 5.5 Network diagram showing gene expression levels for the *Il10*$^{-/-}$ *vs.* C57BL/6J mice on the modified-AIN-76A diet. Network built using all genes in the top network identified using IPA from both the goat and cow milk diet comparisons in *Il10*$^{-/-}$ mice (the "cell-to-cell signaling and interaction, haematological system development and function, immune cell trafficking" network), overlaid with expression data from the comparison of *Il10*$^{-/-}$ mice vs. C57BL/6J mice on the modified-AIN-76A diet. Molecules coloured in green represent a gene with decreased expression relative to the modified-AIN-76A diet, while red indicates an increase in expression (See section 2.3.7.1 for more detail about interpretation of network diagrams).

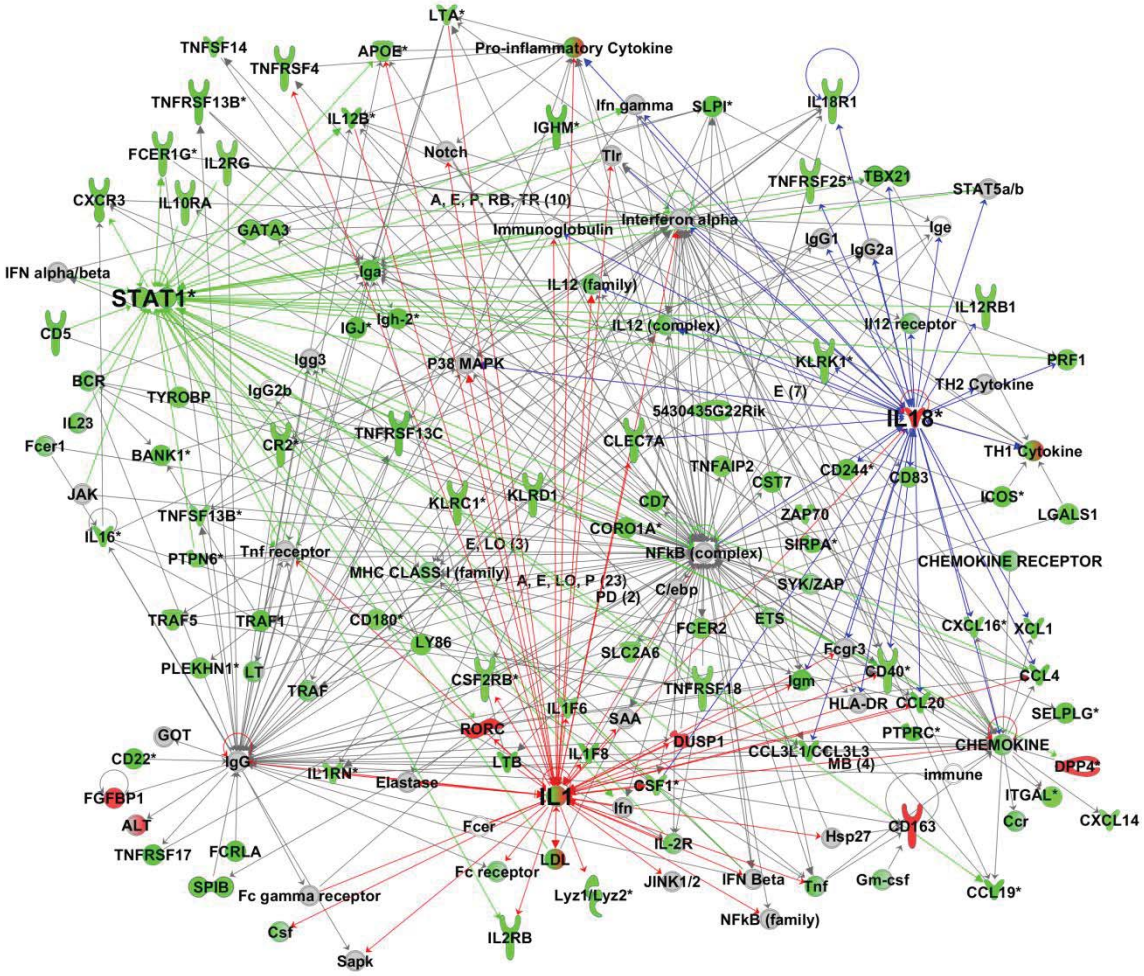


Figure 5.6 Network diagram showing gene expression levels for *Il10*$^{-/-}$ mice fed the goat milk diet *vs*. modified-AIN-76A diet. Network described in the legend to Figure 5.5 overlaid with the expression data from the goat milk diet. Red lines indicate relationships between the IL1 molecule and others; blue lines indicate relationships between the IL18 molecule and others; green lines indicate relationships between the STAT1 molecule and others.

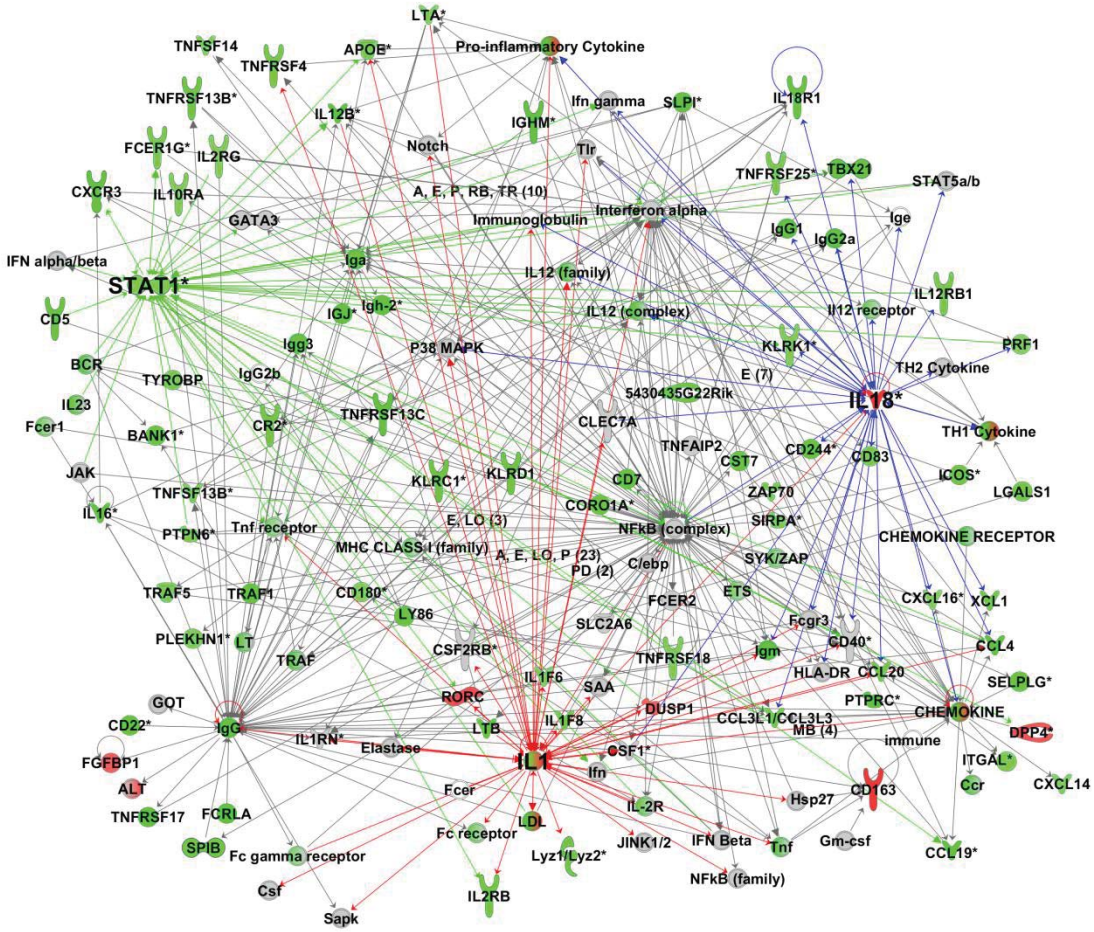


Figure 5.7 Network diagram showing gene expression levels for *Il10*$^{-/-}$ mice fed the cow milk diet *vs*. modified-AIN-76A diet. Network described in the legend to Figure 5.5 overlaid with the expression data from the cow milk diet. Colours of relationship lines are as described in the legend for Figure 5.6.

importance in regulating inflammation in response to dietary intervention. Many genes that had decreased expression in response to the cow and goat milk-based diets had increased expression in *Il10*$^{-/-}$ mice on the modified-AIN-76A diet, suggesting that the goat and cow milk-based diets acted to reverse the gene expression changes seen in the *Il10*$^{-/-}$ mice.

The enriched GO processes were similar between the *Il10*$^{-/-}$ mice fed the cow and goat milk diets, and as with the Ingenuity canonical pathways, were immune-related. For the goat milk diet (Table 5.16), two GO processes out of the top 30 were associated with cytokines, four with immune cell activation, 12 with immune or defense responses. For the cow milk diet (Table 5.17), four GO processes out of the top 30 were associated with cytokines, chemokines or chemotaxis, four with immune cell activation, and 12 with immune or defense responses. Many of the same processes appear for each gene list.

The enriched gene sets were also similar between the goat and cow milk diets. Among the top 30 enriched gene sets for the goat milk diet (Table 5.18), two gene sets were associated with cytokines, chemokines or chemotaxis, nine with immune cells or antibodies, five with cancer, four with autoimmune or allergic disease, and two with immune or defense responses. Some gene sets were involved with reproduction. Among the top 30 enriched gene sets for the cow milk diet (Table 5.19), two gene sets were associated with cytokines, chemokines or chemotaxis, nine with immune cells or antibodies, four with cancer, nine with autoimmune or allergic disease, and two with immune or defense responses not already counted in previous categories. Overall, both gene lists are enriched with many immune-related gene sets and categories.

In the C57BL/6J mice, there were a small number of enriched gene sets for the goat milk diet (4) and the cow milk diet (11) (Table 5.20). For the goat milk diet, they were metabolism-related, whereas for the cow milk diet they were a mixture of a number of immune and inflammation-related gene sets and some metabolism-related gene sets. Out of both gene lists there was only one enriched gene set that had a probability value of below 0.01, so the significance of the enriched gene sets was much lower for the C57BL/6J mice than the *Il10*$^{-/-}$ mice. Enriched gene sets were also analysed for the comparison between the goat and cow milk diets in *Il10*$^{-/-}$ mice (Table 5.21). There were 52 enriched gene sets, the majority of which were metabolism-related, although

Table 5.16 Top 30 over-represented gene ontology processes for *Il10*$^{-/-}$ mice fed the goat milk diet. There were 83 processes with EASE score (P) < 0.05. * indicates a process that is not listed in the top 30 processes for the *Il10*$^{-/-}$ mice fed the cow milk diet, but is significant. Processes without an asterisk are also present in the top 30 processes for *Il10*$^{-/-}$ mice fed the cow milk diet.

System	Gene category	EASE score
GO Biological Process	defense response	<0.001
GO Biological Process	response to biotic stimulus	<0.001
GO Biological Process	immune response	<0.001
GO Biological Process	response to external stimulus	<0.001
GO Biological Process	response to pest/pathogen/parasite	<0.001
GO Biological Process	humoral immune response	<0.001
GO Molecular Function	signal transducer activity	<0.001
GO Biological Process	response to stress	<0.001
GO Biological Process	humoral defense mechanism	<0.001
GO Biological Process	cell communication	<0.001
GO Biological Process	response to wounding	<0.001
GO Biological Process	antimicrobial humoral response	<0.001
GO Biological Process	antimicrobial humoral response	<0.001
GO Cellular Component	plasma membrane	<0.001
GO Biological Process	signal transduction	<0.001
GO Molecular Function	receptor activity	<0.001
GO Cellular Component	integral to membrane	<0.001
GO Biological Process	cellular defense response	<0.001
GO Cellular Component	integral to plasma membrane	<0.001
GO Molecular Function	cytokine binding	<0.001
GO Cellular Component	membrane	<0.001*
GO Biological Process	lymphocyte activation	<0.001
GO Biological Process	T-cell activation	<0.001
GO Biological Process	cell activation	<0.001
GO Biological Process	immune cell activation	<0.001
GO Molecular Function	transmembrane receptor activity	<0.001
GO Molecular Function	receptor binding	<0.001*
GO Molecular Function	cytokine activity	<0.001*
GO Biological Process	cellular process	<0.001*
GO Cellular Component	extracellular	<0.001*

Table 5.17 Top 30 over-represented gene ontology processes for the *Il10*$^{-/-}$ mice fed the cow milk diet. There were 96 processes with EASE score (P) < 0.05. * indicates a process that is not listed in the top 30 processes for the *Il10*$^{-/-}$ mice fed the goat milk diet, but is significant. Processes without an asterisk are also present in the top 30 processes for *Il10*$^{-/-}$ mice fed the goat milk diet.

System	Gene category	EASE score
GO Biological Process	response to biotic stimulus	<0.001
GO Biological Process	immune response	<0.001
GO Biological Process	defense response	<0.001
GO Biological Process	response to external stimulus	<0.001
GO Biological Process	response to pest/pathogen/parasite	<0.001
GO Biological Process	humoral immune response	<0.001
GO Biological Process	response to stress	<0.001
GO Biological Process	humoral defense mechanism	<0.001
GO Molecular Function	signal transducer activity	<0.001
GO Biological Process	antimicrobial humoral response	<0.001
GO Biological Process	antimicrobial humoral response	<0.001
GO Biological Process	cell communication	<0.001
GO Biological Process	signal transduction	<0.001
GO Biological Process	response to wounding	<0.001
GO Biological Process	cellular defense response	<0.001
GO Molecular Function	cytokine binding	<0.001
GO Molecular Function	receptor activity	<0.001
GO Cellular Component	plasma membrane	<0.001
GO Biological Process	taxis	<0.001*
GO Biological Process	chemotaxis	<0.001*
GO Cellular Component	integral to plasma membrane	<0.001
GO Biological Process	lymphocyte activation	<0.001
GO Biological Process	T-cell activation	<0.001
GO Biological Process	cell activation	<0.001
GO Biological Process	immune cell activation	<0.001
GO Cellular Component	integral to membrane	<0.001
GO Molecular Function	transmembrane receptor activity	<0.001
GO Biological Process	cell adhesion	<0.001*
GO Biological Process	cell surface receptor linked signal transduction	<0.001*
GO Molecular Function	chemokine receptor activity	<0.001*

Table 5.18 Top 30 enriched gene sets for *Il10*$^{-/-}$ mice fed the goat milk diet. There were 50 gene sets with P < 0.05. * indicates a gene set that is not listed in the top 30 processes for the *Il10*$^{-/-}$ mice fed the cow milk diet, but is significant. ** indicates a gene set that is not significant for the *Il10*$^{-/-}$ mice fed the cow milk diet. Processes without an asterisk are also present in the top 30 processes for *Il10*$^{-/-}$ mice fed the cow milk diet.

Pathway ID	Pathway name	P value
mmu04060	Cytokine-cytokine receptor interaction	<0.001
mmu04062	Chemokine signaling pathway	<0.001
mmu04070	Phosphatidylinositol signaling system	<0.001*
mmu04142	Lysosome	<0.001*
mmu04145	Phagosome	<0.001
mmu04370	VEGF signaling pathway	<0.001
mmu04514	Cell adhesion molecules	<0.001
mmu04630	Jak-STAT signaling pathway	<0.001
mmu04640	Hematopoietic cell lineage	<0.001
mmu04650	Natural killer cell mediated cytotoxicity	<0.001
mmu04660	T cell receptor signaling pathway	<0.001
mmu04662	B cell receptor signaling pathway	<0.001
mmu04664	Fc epsilon RI signaling pathway	<0.001
mmu04666	Fc gamma R-mediated phagocytosis	<0.001
mmu04670	Leukocyte transendothelial migration	<0.001
mmu04672	Intestinal immune network for IgA production	<0.001
mmu05140	Leishmaniasis	<0.001
mmu05146	Amoebiasis	<0.001
mmu05210	Colorectal cancer	<0.001
mmu05211	Renal cell carcinoma	<0.001**
mmu05212	Pancreatic cancer	<0.001
mmu05214	Glioma	<0.001
mmu05223	Non-small cell lung cancer	<0.001
mmu05310	Asthma	<0.001
mmu05322	Systemic lupus erythematosus	<0.001
mmu05340	Primary immunodeficiency	<0.001
mmu00562	Inositol phosphate metabolism	<0.001*
mmu04912	GnRH signaling pathway	<0.001*
mmu04114	Oocyte meiosis	<0.001*
mmu04914	Progesterone-mediated oocyte maturation	<0.001*

Table 5.19 Top 30 enriched gene sets for *Il10*$^{-/-}$ mice fed the cow milk diet. There were 37 gene sets with P < 0.05. * indicates a process that is not listed in the top 30 processes for the *Il10*$^{-/-}$ mice fed the goat milk diet, but is significant. Processes without an asterisk are also present in the top 30 processes for *Il10*$^{-/-}$ mice fed the goat milk diet.

Pathway ID	Pathway name	P value
mmu04060	Cytokine-cytokine receptor interaction	<0.001
mmu04062	Chemokine signaling pathway	<0.001
mmu04145	Phagosome	<0.001
mmu04370	VEGF signaling pathway	<0.001
mmu04514	Cell adhesion molecules	<0.001
mmu04612	Antigen processing and presentation	<0.001*
mmu04620	Toll-like receptor signaling pathway	<0.001*
mmu04630	Jak-STAT signaling pathway	<0.001
mmu04640	Hematopoietic cell lineage	<0.001
mmu04650	Natural killer cell mediated cytotoxicity	<0.001
mmu04660	T cell receptor signaling pathway	<0.001
mmu04662	B cell receptor signaling pathway	<0.001
mmu04664	Fc epsilon RI signaling pathway	<0.001
mmu04666	Fc gamma R-mediated phagocytosis	<0.001
mmu04670	Leukocyte transendothelial migration	<0.001
mmu04672	Intestinal immune network for IgA production	<0.001
mmu04722	Neurotrophin signaling pathway	<0.001*
mmu04912	GnRH signaling pathway	<0.001
mmu04940	Type I diabetes mellitus	<0.001*
mmu05140	Leishmaniasis	<0.001
mmu05142	Chagas disease	<0.001*
mmu05146	Amoebiasis	<0.001
mmu05210	Colorectal cancer	<0.001
mmu05212	Pancreatic cancer	<0.001
mmu05214	Glioma	<0.001
mmu05223	Non-small cell lung cancer	<0.001
mmu05310	Asthma	<0.001
mmu05322	Systemic lupus erythematosus	<0.001
mmu05330	Allograft rejection	<0.001*
mmu05340	Primary immunodeficiency	<0.001*

Table 5.20 Enriched gene sets for C57BL/6J mice fed the goat and cow milk diets.

Path ID	Pathway name	P value
Goat milk diet		
mmu00590	Arachidonic acid metabolism	0.021
mmu00591	Linoleic acid metabolism	0.024
mmu04914	Progesterone-mediated oocyte maturation	0.031
mmu00140	Steroid hormone biosynthesis	0.038
Cow milk diet		
mmu04120	Ubiquitin mediated proteolysis	0.006
mmu05416	Viral myocarditis	0.013
mmu04141	Protein processing in endoplasmic reticulum	0.016
mmu04612	Antigen processing and presentation	0.019
mmu05330	Allograft rejection	0.023
mmu00510	N-Glycan biosynthesis	0.027
mmu05320	Autoimmune thyroid disease	0.028
mmu04142	Lysosome	0.031
mmu04940	Type I diabetes mellitus	0.031
mmu04914	Progesterone-mediated oocyte maturation	0.036
mmu04622	RIG-I-like receptor signaling pathway	0.039

Table 5.21 Enriched gene sets for *Il10*$^{-/-}$ mice fed the cow milk diet *vs.* those fed the goat milk diet.

Path ID	Pathway name	P value
mmu00010	Glycolysis / Gluconeogenesis	<0.001
mmu00071	Fatty acid metabolism	<0.001
mmu00140	Steroid hormone biosynthesis	<0.001
mmu00230	Purine metabolism	<0.001
mmu00240	Pyrimidine metabolism	<0.001
mmu00260	Glycine, serine and threonine metabolism	<0.001
mmu00280	Valine, leucine and isoleucine degradation	<0.001
mmu00330	Arginine and proline metabolism	<0.001
mmu00350	Tyrosine metabolism	<0.001
mmu00380	Tryptophan metabolism	<0.001
mmu00480	Glutathione metabolism	<0.001
mmu00561	Glycerolipid metabolism	<0.001
mmu00590	Arachidonic acid metabolism	<0.001
mmu00620	Pyruvate metabolism	<0.001
mmu00830	Retinol metabolism	<0.001
mmu00980	Metabolism of xenobiotics by cytochrome P450	<0.001
mmu00982	Drug metabolism - cytochrome P450	<0.001
mmu00983	Drug metabolism - other enzymes	<0.001
mmu01100	Metabolic pathways	<0.001
mmu02010	ABC transporters	<0.001
mmu03320	PPAR signaling pathway	<0.001
mmu04080	Neuroactive ligand-receptor interaction	<0.001
mmu04144	Endocytosis	<0.001
mmu04146	Peroxisome	<0.001
mmu04260	Cardiac muscle contraction	<0.001
mmu04270	Vascular smooth muscle contraction	<0.001
mmu04340	Hedgehog signaling pathway	<0.001
mmu04360	Axon guidance	<0.001
mmu04510	Focal adhesion	<0.001
mmu04512	ECM-receptor interaction	<0.001
mmu04530	Tight junction	<0.001
mmu04540	Gap junction	<0.001
mmu04916	Melanogenesis	<0.001
mmu04960	Aldosterone-regulated sodium reabsorption	<0.001
mmu04970	Salivary secretion	<0.001
mmu04971	Gastric acid secretion	<0.001
mmu05012	Parkinson's disease	<0.001
mmu05100	Bacterial invasion of epithelial cells	<0.001
mmu05217	Basal cell carcinoma	<0.001
mmu05410	Hypertrophic cardiomyopathy (HCM)	<0.001
mmu05412	Arrhythmogenic right ventricular cardiomyopathy	<0.001
mmu05414	Dilated cardiomyopathy	<0.001

mmu04020	Calcium signaling pathway	0.002
mmu05014	Amyotrophic lateral sclerosis (ALS)	0.002
mmu05010	Alzheimer's disease	0.006
mmu00591	Linoleic acid metabolism	0.006
mmu05016	Huntington's disease	0.007
mmu04920	Adipocytokine signaling pathway	0.008
mmu04070	Phosphatidylinositol signaling system	0.008
mmu00500	Starch and sucrose metabolism	0.015
mmu04310	Wnt signaling pathway	0.022
mmu04720	Long-term potentiation	0.028

five were related to cardiovascular disease and four were related to epithelial cell junctions, transporters and bacterial invasion. There were differences in gene expression between the cow and goat milk diets in *Il10*$^{-/-}$ mice, despite the similarities between each gene list. The only enriched gene set for the C57BL/6J mice for the goat milk diet versus the cow milk diet was the RIG-I-like receptor signalling pathway ($P = 0.04$), which is an innate antiviral response pathway and was also one of the enriched gene sets in the C57BL/6J mice fed the cow milk diet.

5.5.6.4.3 Expression of genes encoding membrane proteins in the colon in response to milk-based diets

To investigate whether there were differences in the expression of genes encoding membrane proteins between the *Il10*$^{-/-}$ mice fed the milk and modified-AIN-76A diets, which might suggest changes in epithelial transporter or receptor activity, a heatmap showing the expression of all genes encoding membrane-associated genes was generated (Figure 5.8). Two *Il10*$^{-/-}$ mice on the sheep milk diet clustered with the *Il10*$^{-/-}$ mice fed the goat and cow milk diets, while the remaining four clustered with the modified-AIN-76A diet. The *Il10*$^{-/-}$ mice on the goat and cow milk diets clustered together and had different gene expression profiles to the *Il10*$^{-/-}$ mice on the modified-AIN-76A diet. Relative to the modified-AIN-76A diet, genes encoding membrane-associated genes were not differentially expressed for *Il10*$^{-/-}$ mice on the sheep milk-based diet ($P > 0.4$). In contrast, genes encoding membrane-associated genes were differentially expressed for *Il10*$^{-/-}$ mice on the cow and goat milk-based diets ($P < 0.001$).

In *Il10*$^{-/-}$ mice on the goat milk-based diet, 19% of genes encoding membrane-associated genes contributing to significance had decreased expression ($P = 0.01$) relative to *Il10*$^{-/-}$ mice on the modified-AIN-76A diet. This shows that the *Il10*$^{-/-}$ mice on the cow and goat milk-based diets had altered gene expression of membrane-associated proteins in the colon, which may be involved in barrier function, absorption and secretion and possibly have a functional link to the mechanism of diarrhoea and/or growth reduction occurring in these mice. However, there was a lack of differential expression in the *Il10*$^{-/-}$ mice on the sheep milk-based diet that also had altered faecal consistency.

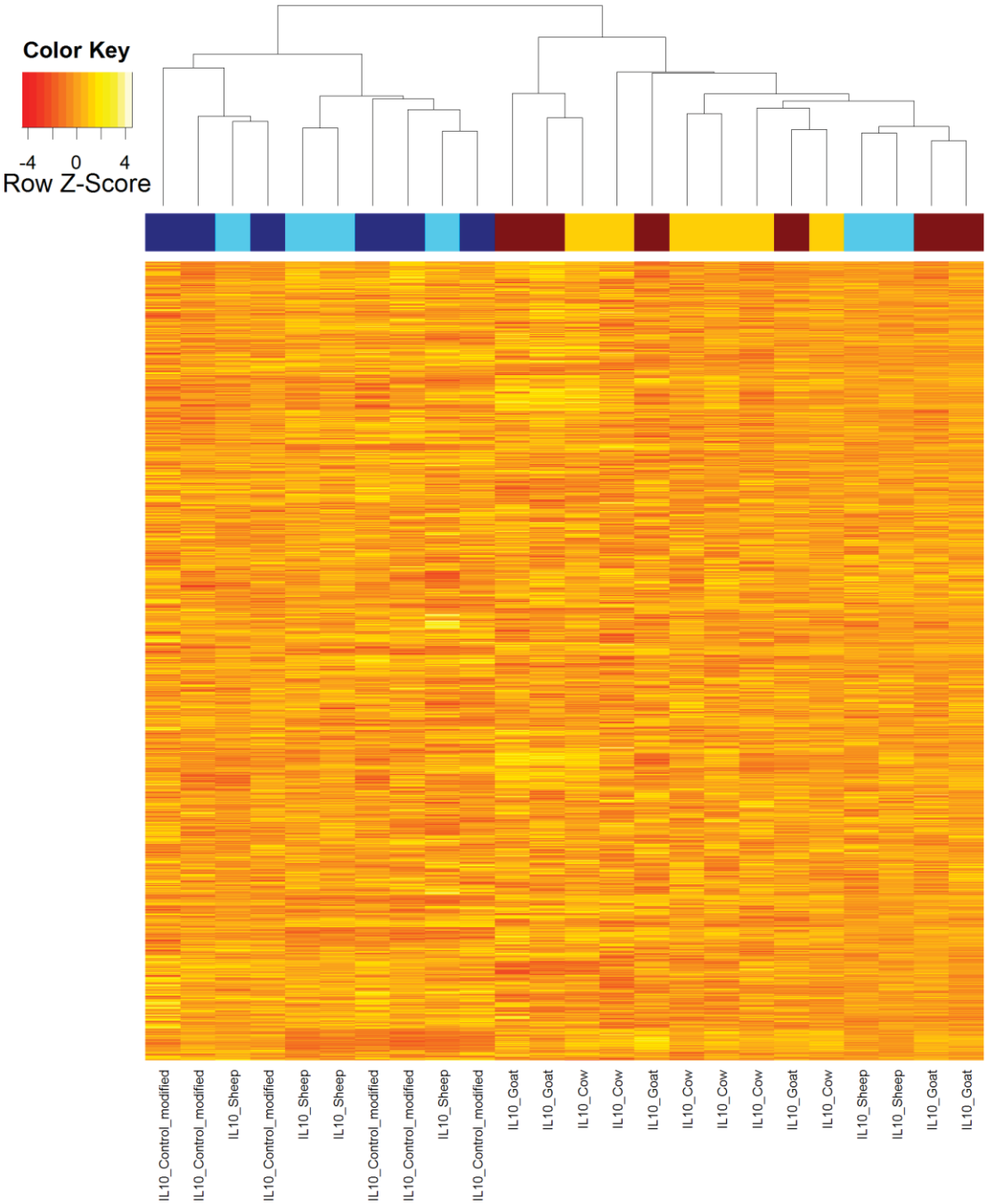


Figure 5.8 Heatmap of the expression of genes encoding membrane proteins for *Il10*$^{-/-}$ mice fed milk diets and the modified-AIN-76A (control) diet. Individual genes are represented by rows with samples in columns. The dendrogram above the heatmap is a tree diagram that illustrates the arrangement of the clusters produced by hierarchical clustering of treatment groups in columns, with higher similarity represented by closer relationship in the tree. Colour blocks beneath the dendrogram represent treatment groups as follows: dark blue = *Il10*$^{-/-}$ mice on modified-AIN-76A; light blue = *Il10*$^{-/-}$ mice on sheep milk-based diet; red = *Il10*$^{-/-}$ mice on goat milk-based diet; yellow = *Il10*$^{-/-}$ mice on cow milk-based diet.

5.5.6.5 qPCR validation of microarray results

The qPCR results confirmed the microarray FC and FDR/P values for the six genes measured (Table 5.22). One gene was differentially expressed in the qPCR results but not in the microarray results (*Stat1*, cow milk diet in *Il10*$^{-/-}$ mice). This was expected to occur for some genes due to the increased sensitivity of the qPCR method of measuring gene expression, and the direction of fold change was consistent between both methods.

5.6 Discussion

In this experiment, the *Il10*$^{-/-}$ mouse model performed as expected in terms of inflammation, and their gene expression profiles were similar to other experiments using this model [109, 110, 118, 282] and to the first experiment described (Chapter 3).

The findings of this study showed that there was an interaction between diets containing 40% milk solids and the pathophysiology of the *Il10*$^{-/-}$ mouse intestine, in particular the diets incorporating goat and cow milk solids. These findings point to a differential effect of diet according to mouse strain (an interaction between strain and diet) which was expected based on the hypothesis for this study. The soy diet was expected to have more beneficial effects on growth performance than the milk-based diets [236], which was the case. However, the soy, goat milk and sheep milk-based diets were expected to have greater anti-inflammatory effects [68, 170, 217, 231] relative to the cow milk-based diet, and while the goat milk-based diet did have a greater anti-inflammatory effect than the soy and sheep milk-based diets, the soy and sheep milk-based diets did not have a greater anti-inflammatory effect than the cow milk-based diet.

5.6.1 Relationship between diet composition and growth performance

Diet composition affected the growth of the *Il10*$^{-/-}$ mice to a certain extent, because the *Il10*$^{-/-}$ mice fed any of the diets with modified composition (including modified-AIN-76A) did not grow as much as those on standard AIN-76A. However, differences in diet composition did not account for all of the growth reduction because the *Il10*$^{-/-}$ mice fed the milk-based diets did not grow as well as those fed modified-AIN-76A.

Lactose concentrations in the milk diets were similar and thus did not explain why growth was reduced to varying degrees for the *Il10*$^{-/-}$ mice fed these diets. In another

Table 5.22 qPCR validation of microarray results. Table shows fold changes (FC) and P values (for qPCR data) or false discovery rates (FDR; for microarray data) for the six genes validated using qPCR for three treatment comparisons. All genes that were differentially expressed in the microarrays were differentially expressed according to qPCR and all fold changes were in the same direction in both qPCR and microarray. * denotes genes that were significantly differentially expressed according to qPCR but not microarray.

Gene	qPCR FC	qPCR P value	Microarray FC	Microarray FDR
***Il10*$^{-/-}$ mice, modified-AIN-76A diet *vs.* C57BL/6J mice, modified-AIN-76A diet**				
Mmp3	3.157	<0.001	4.507	0.001
Il18	-1.232	0.480	-1.278	0.561
Ccl19	4.443	<0.001	6.515	<0.001
Gcg	-1.684	0.072	-1.819	0.079
S100g	1.425	0.392	1.528	0.605
Stat1	2.624	0.003	3.269	<0.001
***Il10*$^{-/-}$ mice, goat milk diet *vs.* *Il10*$^{-/-}$ mice, modified-AIN-76A diet**				
Mmp3	-1.245	0.278	-1.453	0.620
Il18	1.505	0.076	1.996	0.060
Ccl19	-3.257	<0.001	-3.865	<0.001
Gcg	1.644	0.049	2.335	0.025
S100g	-8.000	<0.001	-7.821	0.001
Stat1	-1.580	0.051	-1.472	0.206
***Il10*$^{-/-}$ mice, cow milk diet *vs.* *Il10*$^{-/-}$ mice, modified-AIN-76A diet**				
Mmp3	1.140	0.492	1.025	0.984
Il18	1.682	0.024	2.060	0.032
Ccl19	-2.762	0.001	-3.624	<0.001
Gcg	2.156	0.002	2.958	0.048
S100g	-2.762	<0.001	-6.704	0.002
Stat1	-2.762	0.047*	-1.606	0.133

Note: *Stat1* appears as significantly differentially expressed in the IPA network diagram for the cow milk diet (Figure 5.7), but not in the microarray results in this table, because duplicate probes were resolved using median values in IPA, and mean values in this table. For some probes, FDR values were high (~0.60) but the majority were < 0.05.

study where goat and cow milk-based diets were fed to *Il10*$^{-/-}$ mice, the lactose content of all diets was equalised, and the milk-based diets still caused growth reduction and diarrhoea relative to the soy-based control diet [236]. Casein, egg white, total fat and vitamin/mineral content seem unlikely to explain the growth differences between diets for the *Il10*$^{-/-}$ mice. The AIN-76A diet, which contains bovine casein, did not reduce weight gain and induce diarrhoea in *Il10*$^{-/-}$ mice. The modified-AIN-76A diet had egg white as the sole protein source, and the *Il10*$^{-/-}$ mice on this diet did not exhibit chronic diarrhoea and severe reduction in growth. Total fat was similar for the milk-based diets, and all diets contained the minimum AIN-76A vitamin and mineral mix to meet growth requirements.

Reduced growth performance and altered faecal consistency were observed in *Il10*$^{-/-}$ mice fed the cow milk-based diet. The goat milk-based diet caused a more severe reduction in growth performance than the cow milk-based diet. The cow milk was sourced from cows that had the A1 β-casein genotype and did not contain A2 β-casein. Goat milk does not contain a β-casein variant homologous to the bovine A1 β-casein. A1 β-casein has (controversially) been proposed to have deleterious effects on intestinal function, especially in people with a compromised intestinal barrier, such as those with IBD [254, 255, 375]. The cow milk diet was thus expected to have a more negative effect on intestinal function (in terms of faecal consistency) in *Il10*$^{-/-}$ mice than the goat milk diet. However, this was not the case, providing some evidence that A1 β-casein was not a factor explaining how the inflamed colon reacted to cow and goat milk diets.

5.6.2 Relationship between colitis severity, faecal consistency and growth performance

Colon inflammation was reduced, but diarrhoea symptoms increased, for the *Il10*$^{-/-}$ mice fed the cow and goat milk diets. Growth retardation and diarrhoea are typical in *Il10*$^{-/-}$ mice, although on the C57BL/6J background they are usually less severe than *Il10*$^{-/-}$ mice on other backgrounds [81, 98]. An increase in severity of clinical signs usually associated with enterocolitis in *Il10*$^{-/-}$ mice (diarrhoea, growth retardation, rectal prolapse, and faecal bleeding) would be expected to coincide with increased colon HIS, but the opposite occurred in this study. Whether or not the reduction in colon inflammation was mechanistically associated with the increased growth retardation and diarrhoea remains unknown.

Causes of the diarrhoea observed in the milk-fed *Il10*$^{-/-}$ mice may include effects of milk components on osmosis, active secretion, or exudation due to alterations in ion transport or epithelial barrier function, intestinal motility, and fat or bile acid malabosorption. Generally, driving forces for diarrhoea are: osmosis, active secretion, exudation, and altered motility. Fat and/or bile acid malabsorption in the small intestine (specifically, ileum, in the case of bile acids) can lead to diarrhoea due to the hydroxylation of fatty acids by colonic bacteria, producing amphiphilic molecules that induce secretion [376]. Conditions characterised by intense local or systemic inflammatory responses are associated with abnormalities in ion transport, which in the small or large intestine can lead to diarrhoea [377]. When the intestinal barrier is disturbed, diarrhoea can occur [378].

The sharp decrease in weight gain that occurred a few days after the mice were initially fed the milk diets, and the subsequent increase in weight gain for some of the *Il10*$^{-/-}$ mice fed the milk diets, suggest that adaptation of the microbiota to the intestinal environment created by the milk diets, or adaptation of the intestine itself may have occurred in the first two weeks of the experiment. The growth effects of the milk diets could have been caused by milk components affecting digestion and absorption in the small intestine. In another study where milk-based diets were fed to *Il10*$^{-/-}$ mice, the mice also had reduced growth and diarrhoea, however, skimmed milk powders were used to formulate the diets [236] in contrast to whole milk. This suggests that milk fat was not the cause of the reduced growth. Milk protein or carbohydrate components (other than lactose) may therefore be likely dietary candidates for the causative agent of the reduction in growth seen in the *Il10*$^{-/-}$ mice fed milk-based diets.

The reduction in colitis in the *Il10*$^{-/-}$ mice fed the cow and goat-milk based diets indicated that the changes in growth performance were not caused by the presence of high levels of inflammation in the colon. Typically, growth in children and adolescents with IBD is compromised when severe inflammation is present, due to reduced absorptive capacity of the jejunum and ileum as well as reduced intake due to nausea [15]. Diarrhoea in IBD is related to inflammatory activity, but also to underlying barrier function changes that impact both active and passive transport mechanisms in the small and large intestine as well as increased release of pro-inflammatory cytokines [379]. It is plausible that some component of the goat and cow milk-based diets, and to a lesser

extent the sheep milk-based diet, exacerbated the underlying barrier dysfunction of the small intestine. Some GO processes identified for the *Il10*$^{-/-}$ mice fed the cow and goat-milk based diets were related to membrane proteins, some of which may be electrolyte transporters, the activity of which can be altered in some types of diarrhoea.

One possible explanation for the effects of the milk diets on faecal consistency, growth and colitis that were observed in this experiment could be that some component of the milk-based diets, such as some peptides released from digestion of milk proteins, altered the microbiota composition and function via altered intestinal motility, thereby inducing a decrease in inflammatory activity. Indomethacin-induced colitis in rats is characterised by a chronic oscillation of active and quiescent phases of inflammation associated with phases of hypo and hypermotility [380]. In active phases of inflammation, generalised intestinal hypomotility and bacterial overgrowth occurs, while in quiescent phases, increased motor activity and a normal bacterial load are present [380]. Increased intestinal permeability is present in both active and quiescent phases of inflammation, but bacterial translocation coincides with the active phases, which have increased transcellular permeability, reduced motor activity and increased bacterial overgrowth [28]. It remains to be seen whether or not the reverse could occur, where an increase in intestinal motility above normal could reduce bacterial load to such an extent that colitis severity was reduced.

Faecal bleeding in the euthanased *Il10*$^{-/-}$ mice fed the goat milk-based diet could be due to severe constipation, which could be due to a reduction in intestinal motility. Damage to the distal large intestine could have been missed in the histopathology analysis due to the rectum not being examined. In support of this idea, one of these mice was observed to have a blocked rectum upon sampling. However, the presence of constipation suggests that the goat milk-based diet caused a reduction in motility in *Il10*$^{-/-}$ mice, rather than an increase, unless a reduction in intestinal motility occurred in some mice as a physiological response to counteract an increase in motility. Some mice may have responded to the goat and cow milk-based diets differently, resulting in the presence of both constipation and diarrhoea in the same group of mice.

While milk components are mostly absorbed in the small intestine and the majority does not reach the large intestine to have direct effects on the colon mucosa, some components do reach the large intestine. For example, milk oligosaccharides have

beneficial prebiotic effects in models of colitis, via modifying bacterial colonisation [381]. Components that do not reach the large intestine may be incorporated into mucosal cells of the colon from the blood supply after being digested and absorbed, and exert their effects within the mucosa.

5.6.3 Colon gene expression in response to milk-based diets

The effects of the milk-based diets were related to growth and faecal consistency, and in the case of the goat and cow milk diets, reduced colonic expression levels of immune-related genes and degree of inflammation also. None of these effects were observed in the C57BL/6J mice. There were similar levels of colitis and minimal colon gene expression changes for either strain of mice fed the sheep milk-based diet, soy diet, AIN-76A or modified-AIN-76A. The *Il10*$^{-/-}$ mice fed the sheep milk-based diet had similar, albeit less severe symptoms (reduced weight gain, transiently looser faeces), as those fed the goat and cow milk diets, but these changes were not associated with gene expression changes in the colon.

The study presented here showed that the levels of immune-related gene expression were decreased in response to diets containing 40% whole goat and cow milk solids in *Il10*$^{-/-}$ mice that have reduced colitis severity. No other published studies have demonstrated reduced abundance of inflammatory gene transcripts in the colon in response to the consumption of whole milk solids. In a study where milk-based diets were fed to *Il10*$^{-/-}$ mice, abundance of inflammatory gene transcripts in the colon was increased in response to cow and goat milk-based diets, and more so for the cow milk-based diet [236]. This is in contrast with the results of this experiment, where abundance of inflammatory gene transcripts was reduced in *Il10*$^{-/-}$ mice fed the cow and goat milk-based diets. The main difference between the studies was that milk solids used were skimmed prior to drying, whereas in the study presented here, whole milk solids were used. This suggests that the milk fat components included in the diets, or metabolites derived from milk fat components via digestion and absorption, may have had an anti-inflammatory effect in this study.

Fatty acids found in milk such as CLA and n-3 polyunsaturated fatty acids have anti-inflammatory effects in animal models of colitis which are associated with changes in colon gene expression. CLA reduced colon inflammation and altered colonic

peroxisome proliferator-activated receptor (PPAR)-responsive gene expression in a DSS pig model of IBD [84]. Eicosapentaenoic acid (20:5, n-3) reduced the severity of colon inflammation compared to oleic acid (18:1, *cis*-9, n-9) when included in the diet of *Il10*$^{-/-}$ mice and although it did not alter the expression levels of many inflammatory genes, it did reverse the decrease in expression of the *Ppara* gene that is seen in *Il10*$^{-/-}$ mouse colitis [118, 161]. Medium-chain triglycerides, which are found at higher levels in sheep and goat milk, have anti-inflammatory effects in models of colitis [168, 279, 280]. Goat milk fat is comprised of 16% medium-chain fatty acids, with sheep milk fat being comprised of 12% and cow milk of 8% medium-chain fatty acids. In terms of colitis severity in this experiment, goat milk had a stronger anti-inflammatory effect than cow milk, which had a stronger effect than sheep milk. In terms of growth, sheep milk had a more beneficial effect than cow milk, which in turn had a more beneficial effect than goat milk, suggesting that milk fat composition cannot explain the results of this experiment, at least in isolation.

Although some milk-derived fatty acids are considered anti-inflammatory, a recent study in *Il10*$^{-/-}$ mice showed that diets high in milk-derived saturated fat promoted the expansion of a typically low-abundance bacterium, *Bilophila wadsworthia* [382]. Increased colonisation by this species in *Il10*$^{-/-}$ mice was associated with a pro-inflammatory T_H1 immune response and increased incidence of colitis [382]. The milk-derived fat appeared to promote taurine conjugation of hepatic bile acids, increasing the availability of sulfur to sulfite-reducing microorganisms including *B. wadsworthia* [382]. These results support the hypothesis that milk components could alter the microbiota composition of the intestine with subsequent effects on severity of colitis, although in contrast to the study presented here, the study by Devkota *et al.* [382] shows an increase in colitis severity in response to milk components. The milk-fat diet used by Devkota *et al.* was more highly saturated than the non-milk saturated fat diet (65% compared to 39%) so it cannot be ruled out that the saturation level was the most important factor, rather than the fat source. The milk diets used in the study presented here contained a higher percentage of saturated fat (and milk-derived fat) than the AIN-76A or modified-AIN-76A diets, yet none of the milk diets increased the severity of colitis. However, the percentage of total energy contributed by milk fat in these diets was lower at 30% compared to 38% in the study by Devkota *et al.*, and the Devkota *et*

al. study used a 24-week feeding period which was four times longer than that used in the study presented here.

Oligosaccharides and other types of soluble fibre are fermented in the caecum and colon by bacteria resulting in production of short-chain fatty acids (and other metabolites) which have anti-inflammatory effects [383]. Oligosaccharides from goat milk reduced intestinal inflammation in DSS and TNBS-induced rat models of colitis [68, 69]. In TNBS-induced colitis, oral dosing with goat milk oligosaccharides resulted in increased weight gain and improved macroscopic appearance of the intestine in rats, and a lower abundance of the pro-inflammatory inducible nitric oxide synthase and cyclo-oxygenase-2 proteins, and reduced expression of the IL1β gene, demonstrating that the goat oligosaccharides had anti-inflammatory actions in the colon [69]. In DSS colitis, oligosaccharides included in the diet normalised the expression levels of some genes involved in intestinal inflammation, including immune-related genes, growth factors, mucins and alkaline phosphatase (a marker of inflammation) [68]. Based on oligosaccharide content, sheep and cow milk would be expected to have similar effects as they have similar oligosaccharide content, although cow milk contains more than sheep milk. Goat milk has a much higher oligosaccharide content than both cow and sheep milk, so might be expected to have more anti-inflammatory effects (Table 1.5). In terms of colitis severity in this experiment, goat milk did have a stronger anti-inflammatory effect than the other milks.

In studies showing anti-inflammatory effects of milk components, only those on fatty acids and oligosaccharides included analysis of colon gene expression levels. The studies on fatty acids showed alterations in PPAR-related gene expression [84, 118] but in the study presented here, *Ppara* mRNA levels were only increased in the cow milk-based diet. In the studies on oligosaccharides, expression levels of the IL1β gene was reduced in TNBS colitis [69], and in DSS colitis, the expression levels of a number of immune-related genes were normalised [68]. In the study presented here, the expression levels of IL1β were decreased in the *Il10*$^{-/-}$ mice fed the goat milk-based diet. The expression levels of other interleukin genes were decreased, however, and there was a general decrease in expression levels of genes associated with inflammation, which agrees with the study by Lara-Villoslada *et al*. [68].

The increase in expression levels of the *Il18* gene in both cow and goat milk-fed *Il10*$^{-/-}$ mice alongside the decrease in mRNA levels of immune-related genes relative to the modified-AIN-76A diet is counterintuitive. IL18 is generally accepted to be a pro-inflammatory cytokine. Polymorphisms in the *Il18* gene are also associated with susceptibility to IBD [384, 385]. Anti-IL18 antibodies have been proposed as a therapy for colitis [386] and *Il18* mRNA levels are increased in IBD patients [387]. *Il18* gene expression levels were reduced in *Il10*$^{-/-}$ mice at 12 weeks of age compared to 6 weeks of age in the colon epithelium but was not differentially expressed in the intact colon (Chapter 3). It may be that increased IL18 mRNA abundance is not strongly associated with colitis in this mouse model, at least to the extent that it can be identified as differentially expressed using the FDR cutoff applied here, or that analysis of epithelial cell gene expression is more sensitive than intact colon and required to detect changes in this transcript.

Expression of genes encoding membrane-associated proteins were differentially expressed in the colon of *Il10*$^{-/-}$ mice fed diets incorporating goat and cow milk solids. Membrane-associated proteins fulfil a variety of functions unrelated to epithelial barrier function, including cell signalling and adhesion, but also include proteins that act as channels or carriers to facilitate transport of molecules across the cell membrane. Changes in the function of transport proteins may be associated with the imbalance between fluid absorption and secretion that can result in diarrhoea. The differential gene expression of membrane proteins that occurred in the *Il10*$^{-/-}$ mice fed the goat and cow milk-based diets suggests that the function of transporters may have been affected in response to cow and goat milk solids. Membrane-associated proteins may also be involved in the immune response, however, and so differential gene expression of membrane-associated proteins could also reflect the changes in immune-related gene expression that occurred in the *Il10*$^{-/-}$ mice fed the goat and cow milk-based diets.

One important question is why the cow and goat milk diets had an apparent anti-inflammatory effect in the colon according to gene expression changes when the sheep milk diet did not. Sheep and goat milk are very similar in composition, and many of the known anti-inflammatory or immune-modulatory components found in goat milk or cow milk are also found in sheep milk. The changes in gene expression correlated well with the changes in colitis severity, but not with the changes in growth for all milk diets.

However, the immune-related gene expression changes for the *Il10*$^{-/-}$ mice fed the cow and goat milk-based diets suggest that there may not be a direct link between growth performance changes and colon gene expression changes in this experiment. The anti-inflammatory effect of goat and cow milk in the colon may have occurred via a separate mechanism to the changes in growth, although the correlation between increased severity of growth and intestinal function changes and decreased severity of colitis does point to an association of some sort.

Analysis of gene expression in specific cell types and analysis of DNA methylation was not performed using samples from the dietary intervention experiment. Neither method enhanced understanding of molecular changes in the colon mucosa at 12 weeks of age in the *Il10*$^{-/-}$ mouse when inflammation is fully developed, and would be more appropriately applied to studying the changes in early inflammation, for which no samples were collected. Further work needs to be carried out to understand the mechanisms behind the effect of the milk diets on the growth and severity of inflammation before in-depth analysis of the molecular changes occurring in the mucosa are relevant, or are able to be explained as truly anti-inflammatory and beneficial. For example, if indeed altered intestinal motility had a positive effect on colon inflammation via altered microbiota composition, the molecular changes in the colon may be more reflective of an indirect effect rather than a direct anti-inflammatory effect within the colon mucosa. If the molecular changes occurring in the colon were mechanistically directly linked to the apparent anti-inflammatory effect of the milk diets, laser microdissection of the colon mucosa could be particularly useful for unravelling the cellular changes occurring in the earlier stages of inflammation in a repeat mouse experiment where samples were collected over the course of the study.

Gene expression data can provide a useful indication of the molecular pathways underpinning a particular process, such as dietary modulation of the inflammatory process in IBD. However, protein expression is dependent on a number of factors, such as microRNA activity and post-translational processing, not just mRNA levels. Protein was extracted from the same samples as the RNA used for gene expression analysis. These protein samples have been used for global protein expression analysis (proteomics) by our collaborators and will be included in publications (beyond the scope of this thesis). Preliminary analysis of protein samples from this experiment

showed that the protein expression profiles bear a number of similarities to the gene expression profiles for the *Il10*$^{-/-}$ mice fed the goat milk diet compared to those fed the modified-AIN-76A diet. The most striking finding was that a large proportion of the proteins with reduced expression in the *Il10*$^{-/-}$ mice fed the goat milk diet compared to those fed the modified-AIN-76A diet are immune-related, as was the case with the gene expression profile (Chapter 5). These data confirm an anti-inflammatory effect of the goat milk diet in *Il10*$^{-/-}$ mice at both the mRNA transcript and protein levels, however the mechanism is still unknown and requires further investigation.

5.7 Conclusions

Diets enriched with whole milk from different ruminant species affected growth, digestive function, and colon inflammation in the *Il10*$^{-/-}$ mouse model of IBD, via as yet unexplained mechanisms. These affects appear contradictory, in that a reduction in colitis co-occurred with reduced weight gain and increased diarrhoea (and bleeding in some mice) indicating these effects may be separate with respect to mechanism and/or location. An interaction between diet and mouse strain was expected according to the hypothesis, but the co-occurrence of seemingly beneficial and adverse effects that occurred in *Il10*$^{-/-}$ mice in response to the goat and cow milk-based diets was unexpected. The reduction in colitis severity associated with goat and cow milk-based diets, rather than with the soy, goat and sheep milk diets was also unexpected based on results of the IBD patient dietary questionnaire [217].

Significant changes in histological injury score in *Il10*$^{-/-}$ mice in response to milk-based diets were associated with significant changes in colon global gene expression (mainly of immune-related genes), with the largest differences seen in the *Il10*$^{-/-}$ mice fed cow and goat milk diets. There was an interaction between diet and mouse strain which resulted in higher numbers of differentially expressed genes in *Il10*$^{-/-}$ mice when there were fewer changes in the C57BL/6J mice fed the same diet, and vice versa. The differentially expressed genes in C57BL/6J mice fed the soy and sheep milk diets fell into mostly metabolism-related gene ontology categories, yet there were no differentially expressed genes in the *Il10*$^{-/-}$ mice fed these diets.

Overall, changes in levels of mucosal inflammation and immune gene expression were seen in the colon of *Il10*$^{-/-}$ mice in response to goat and cow milk-based diets indicating

an anti-inflammatory effect of goat and cow milk solids. The mechanism(s) behind this effect needs to be studied further in order to determine whether the anti-inflammatory effects of milk can be maximised and benefit people suffering from inflammatory disease.

6 General discussion and future perspectives

6.1 Background

While there has been much research into the pathogenesis of IBD, its aetiology remains poorly understood. Unravelling the complexity of the interactions between the intestinal immune system and microbiota, genetic factors, and environmental factors that prevail in IBD is essential to enable prevention and effective treatment [388, 389]. While many pharmacological and surgical treatments are successful at achieving and maintaining remission for most patients, early intervention or prevention would be the preferred option for alleviating the symptoms of IBD. A dietary approach to prevention in the early stages of inflammation development, or during remission, could provide many benefits to IBD patients, including fewer drug-related side effects and a greater ability to prevent future relapse into active inflammation. However, this requires a thorough understanding of the contributing factors and how they interact to affect molecular processes in the colon mucosa, as well as an in-depth understanding of how dietary components can modify these processes.

As described in Chapter 1, studies in animal models of IBD have demonstrated anti-inflammatory effects of milk components, and food frequency questionnaire studies in IBD patients have suggested that milk consumption has variable effects on gastrointestinal symptoms, being beneficial in some cases and adverse in others. Milk from some species (e.g. goat, sheep) was perceived to be more beneficial than that from other species (e.g. cow), and there was a tendency for higher fat milk (e.g. whole cow milk) to be perceived more negatively than lower fat milk (e.g. skimmed cow milk). However, soy-based milk substitutes were perceived to be more beneficial overall than ruminant milk products, and are a common alternative consumed by IBD patients who cannot tolerate milk or choose to avoid it in the belief that it worsens their symptoms.

There is evidence that dietary consumption of products enriched in milk components (such as oligosaccharides), and perhaps whole milk, has potential for reducing IBD symptoms as described in Chapter 1. However, there are few studies in either humans or animal models investigating the effects of consuming whole milk solids on colitis. Effects may vary depending on the species from which the milk was obtained, however, limited data are currently available to address this issue. Studies of the effects of whole milk solids on the development of inflammation in animal models are required to assist the translation of results obtained from IBD patient food frequency questionnaires into

dietary recommendations for IBD patients. The central hypothesis in this thesis was that diets containing sheep and goat milk solids, or a milk substitute (soy "milk" solids) would reduce the level of colon inflammation and associated molecular pathways in a mouse model of IBD when compared to a diet containing cow milk solids. The research presented in this thesis was performed with the aim of furthering knowledge of the underlying molecular processes in colon inflammation, and understanding how different ruminant species (sheep, goat and cow) milk-based diets and a soy-based diet impact on them, using a well-described immune-mediated mouse model of IBD (*Il10*$^{-/-}$ mice).

6.2 Gene expression profiles in the colon epithelium of *Il10*$^{-/-}$ mice

The experiment described in Chapter 3 showed that intact colon is an appropriate tissue in which to determine gene expression changes in the mucosa of *Il10*$^{-/-}$ mice with established IBD-like symptoms, because it produces similar gene expression profiles (in terms of over-represented pathways, processes and gene sets) to the epithelium alone. The results also indicated that studying the gene expression patterns of colon epithelial cells may be more relevant at earlier stages of inflammation because isolation of specific cell types may improve sensitivity or the ability to detect early changes in cell signalling and function that may lead to the development of inflammation. This appears to be the first reporting of such a finding.

The findings presented in Chapter 3 raise a number of questions, including: (1) which cells in the epithelium had increased immune-related gene expression profiles in the colon of *Il10*$^{-/-}$ mice, and (2) why are these profiles similar in intact colon and colon epithelium in 12-week-old *Il10*$^{-/-}$ mice?

The origin of the immune-related gene expression changes in the epithelium could be determined by isolating specific cell types from the epithelium (such as enterocytes, goblet cells or IELs) in 12-week-old *Il10*$^{-/-}$ mice and comparing their gene expression profiles. The main limitation to this approach would be the time taken to isolate the number of individual cells required for microarray analysis, which if greater than 20-30 minutes would impact on the RNA integrity of the isolated cells. Microarrays can be performed with limited amounts of RNA using two rounds of amplification (Chapter 3). The high sensitivity version of the amplification kit used requires 500 pg total RNA starting material which could typically be obtained from 250 cells (generally 2-5 pg

RNA can be harvested from a single epithelial cell from a 7 μm thick section [31]). The staining and LMD procedure used in Chapter 3 may require further optimisation to ensure that RNA of sufficient integrity and quantity can be isolated. Alternatively, cells from several animals or slides would need to be pooled.

As described in Chapters 1 and 3, enterocytes, Paneth cells, goblet cells and IELs are already known to have different roles in host defence and inflammatory signalling. For example, enterocytes are involved in maintaining the epithelial barrier, Paneth cells (present only in the small intestine) secrete anti-microbial factors, goblet cells maintain the mucus layer protecting the epithelium, and IELs are involved with host immune defence and cross-talk between the lumenal microbiota and mucosa. Examining gene expression profiles of these cells in early compared to established inflammation could provide further clues as to their roles in the development of inflammation.

The gene expression profile of immune cell aggregates present in inflamed colon of *Il10*$^{-/-}$ mice could be examined using tissues obtained in Chapter 3 to further investigate why the intact colon and colon epithelium profiles were similar in 12-week-old *Il10*$^{-/-}$ mice. The immune cell aggregates formed a large part of the tissue section in inflamed colon, indicating that the intact colon and colon epithelium gene expression profiles would differ in the number of differentially expressed immune-related pathways and genes. For example, when mucosal and lymphocytic cells were microdissected out from *Helicobacter*-infected mouse stomach, their gene expression profiles differed and were used to identify the cellular origin of gene expression changes in whole stomach [122].

The experiment presented here showed that the profiles were similar between epithelium and intact colon of *Il10*$^{-/-}$ mice with established colitis. This suggests that the immune cell aggregates present in inflamed colon may have a relatively small contribution to the gene expression profiles of the intact colon in colitis. Comparison of the profiles of immune cell aggregates and epithelium in the colon of 12-week-old *Il10*$^{-/-}$ mice could confirm or disprove this hypothesis. This would involve LMD of immune aggregates using the embedded colon samples from which the epithelial profiles were obtained followed by microarray analyses. This may provide information on how transcriptionally active these cells are in inflamed colon tissue.

Gene expression changes in the intermediate stage of colitis may be relevant to understanding its development. Mueller *et al.* [122] showed that the vast majority of gene expression changes occurred in the early stages of gastric lymphoma, reflecting the marked infiltration of immune cells into the gastric mucosa. In Chapter 3, epithelial profiles were examined before histologically detectable infiltration of the mucosa by immune cells, and at 12 weeks of age when it was expected, based on a previous time-course study, that the majority of immune cell infiltration would have already occurred and the inflammatory process would have reached a plateau [115]. The epithelial and intact colon gene expression profiles may differ more during an intermediate stage, such as 8-10 weeks of age, providing more information on important inflammatory signalling occurring in the epithelium. In support of this, a metabolomic study of urine in the *Il10*$^{-/-}$ mouse found two out of four urinary metabolites identified as being associated with inflammation in the reached their peak concentration at 8 weeks of age, while another peaked at 9-10 weeks of age [390]. Studying epithelial changes during the intermediate stage of inflammation development may provide clues as to which pathways and processes could be targeted to reduce pro-inflammatory signalling.

While gene expression profiles provide an indication of signalling pathways involved in inflammation, not all gene expression changes result in changes in protein expression or activity. Altered protein expression and/or activity leads to a change in phenotype, whereas altered gene expression may not. Findings from gene expression analyses of tissues and cell types in IBD should be followed up by analyses of protein expression to determine whether the processes identified as being potentially important to the development of inflammation are altered at the protein level and therefore affect tissue or cell function. Analysis of the intestinal epithelial cell proteome has been performed in isolated primary intestinal epithelial cells from IBD patients [391], however this method requires freshly isolated tissue, whereas the epithelial cell proteome could be studied in cells isolated from frozen sections using LMD [392]. Due to the fact that protein cannot be amplified, and proteomic techniques such as 2D gel electrophoresis require large sample quantities, the isolation of a large enough number of cells by LMD to perform proteomic analysis is time-consuming but feasible [392]. Further optimisation of proteomic methods for small samples, such as mass spectrometry-based techniques, may allow lower abundance proteins as well as the medium to high abundance proteins to be analysed in cells isolated by LMD [393-398]. In addition, immunohistochemistry

can be performed on sections prepared for LMD analysis, and could enable the study of expression levels of key proteins within specific cell types [117].

6.3 Gene methylation in the colon of *Il10*$^{-/-}$ mice

Global levels of DNA methylation were increased in *Il10*$^{-/-}$ mice compared to C57BL/6J mice at 6 weeks of age (Chapter 4), i.e. during the early stage of inflammation where no histopathological changes indicative of colon inflammation were seen but changes in epithelial cell immune gene expression were present (Chapter 3). However, at 12 weeks of age, global DNA methylation levels were the same in *Il10*$^{-/-}$ and C57BL/6J mice, but DNA methylation levels of specific CpG sites in an intronic region of *Stat1* were reduced in the *Il10*$^{-/-}$ mice. The reduction in methylation levels of the *Stat1* gene was associated with an increase in *Stat1* mRNA levels in the inflamed colon, suggesting that DNA methylation may be one mechanism whereby gene expression is influenced towards the "inflammatory signature". These data indicate that both gene expression and DNA methylation changes occur in the colon mucosa prior to, and after, the development of histologically detectable inflammation.

Methylation changes can occur at the promoter region or throughout a gene, and the effects of alterations at many methylation sites are still unclear. Therefore the study of specific gene methylation may be most relevant when a particular central gene in a particular pathway is of interest. Otherwise, many regions throughout the genome may need to be analysed. The global methylation approach using HPLC measurement of total methylation is too broad to identify where in the genome the changes in methylation are occurring, whereas the specific gene approach is low-throughput and requires the identification of relevant genes prior to methylation measurement. Therefore, neither of these methods enable rapid discovery of novel methylation changes that may be of relevance in inflammation.

A more global approach to the study of DNA methylation of important regulatory genes may provide a more complete picture of the role of DNA methylation in IBD. Further work should include the analysis of methylation changes in specific genes across the genome, by using technologies such as methylated DNA immunoprecipitation (mDIP) combined with microarray hybridisation [399], the use of methylation-sensitive restriction enzymes combine with high-density CpG island microarrays [400], or

bisulfite conversion followed by high throughput sequencing [370]. These methods have been used in recent studies to enable identification of methylation changes in genes associated with IBD susceptibility in genome-wide association studies and to correlate these with changes in gene expression in IBD [367, 368, 401].

Methylation of genes could be studied further in particular cell types isolated using LMD methodologies. Due to the small yield of DNA obtained by LMD, a modified protocol would need to be used, such as methylation sensitive restriction enzyme digestion followed by qPCR [402] or bisulfite sequencing [403]. In these studies, between 0.6 and 3 ng/μL DNA was obtained from breast cancer tissues, and 3 genes were assessed at one gene region [402], or one to three cells were microdissected and one CpG island analysed by bisulfite sequencing [403]. These findings suggest that for such small cell yields, methylation analysis would need to be targeted.

Other epigenetic mechanisms, such as DNA-histone interactions, could be studied using a similar method, chromatin immunoprecipitation (ChIP)-on-chip, another microarray-based technology [404]. Histone modifications change DNA-histone interactions and interact with DNA methylation to alter gene expression, and histone modifications have been associated with colitis [405]. Understanding how histones change during inflammation is also important for unravelling the contribution of epigenetic mechanisms to IBD.

A small number of micro-RNAs have recently been implicated in IBD [406] and micro-RNAs have been shown to be regulated by some dietary components [407]. An example of nutritional modification of epigenetic mechanisms is the discovery that dietary polyphenols regulate both micro-RNA and mRNA expression in the livers of apolipoprotein E deficient mice and counteract the modulation of micro-RNA-induced apolipoprotein E knock-out [407]. Micro-RNA expression can be regulated by DNA methylation [408] and may be an effective method of epigenetically modifying the expression of key inflammatory genes.

Epigenetic mechanisms are complex and interact with each other to affect gene transcription. There are many methods available to measure mechanisms, such as DNA methylation and histone modifications, with higher-throughput methods becoming more reliable, sensitive and affordable. High-throughput genome-wide profiling of epigenetic

changes is required in addition to gene and protein expression profiling to provide a comprehensive understanding of IBD. While this study showed changes in global methylation and specific gene methylation levels of *Stat1* in the *Il10*$^{-/-}$ mouse model, more high-throughput and genome-wide analyses (beyond the scope of this PhD) are required to better understand how epigenetic mechanisms interact and contribute to the gene expression profiles seen in colitis.

6.4 Effects of milk-based diets in *Il10*$^{-/-}$ mice

The research findings presented in Chapter 5 showed that diets containing whole milk solids from different ruminant species affected growth, the occurrence of diarrhoea, and colon inflammation in the *Il10*$^{-/-}$ mouse model, via as yet unexplained mechanisms. A reduction in colitis was observed in mice that also exhibited reduced weight gain and increased diarrhoea (and bleeding in some cases) (*Il10*$^{-/-}$ fed goat and cow milk-based diets). These effects may be separate with respect to mechanism and/or location, or may be mechanistically linked. Goat and cow milk-based diets reduced the expression levels of a number of inflammation-related genes in *Il10*$^{-/-}$ mice. The gene expression analysis data could be combined with the results of colon protein expression analysis, which is underway (beyond the scope of this PhD), to provide further support for the identification of pathways involved in the reduction of inflammation. Further work needs to be carried out to investigate the mechanisms responsible for the reduced weight gain and changes in faecal consistency before conclusions can be drawn about the colon gene and protein expression changes in response to milk-based diets.

The changes in mouse growth and intestinal function could be related to the adaptation of the mucosa or resident microbiota to the different milk diets. Microbiota composition and function could impact the severity of colitis if known beneficial species were present in higher abundance. The intestinal microbiota profile of animal models, including the *Il10*$^{-/-}$ mouse model [65, 409], is known to impact on disease pathogenesis and plays a role in causing variation between individuals in animal experiments [410]. Changes in microbiota composition have been implicated in the exacerbation of colitis by high milk-derived saturated fat diets [382], as well as the reduction in inflammation in response to sheep milk solids [170], in rodent colitis models.

Samples from the milk diet experiment (Chapter 5) could be used to determine whether there were differences in caecal microbiota profiles in the *Il10*$^{-/-}$ mice fed the goat and cow milk-based diets compared to those fed the sheep milk-based diet. The caecal microbiota composition could be analysed using next generation sequencing (GS FLX Titanium sequencing of bar-coded 16S rRNA gene amplicons). Differences in microbiota composition between individual mice may also be of interest, because some mice had higher growth and/or less incidence of diarrhoea within diet groups.

The intestinal microbiota break down complex polysaccharides into short-chain fatty acids (SCFAs) (mostly acetate, proprionate and butyrate), which are used as an energy source by colonocytes, but also affect epithelial barrier integrity, intestinal motility and cytokine and chemokine release from immune cells of the intestinal tract [411, 412]. Measurement of SCFA concentrations in caecal contents could provide an indication of changes in microbiota function that could impact on inflammation via these mechanisms. Changes in concentrations of other bacterial metabolites, such as those formed from the transformation of bile acids, may also be associated with a change in microbiota function or composition [411]. An example of this is the increased abundance of sulfite-reducing bacteria seen in the *Il10*$^{-/-}$ mouse in response to a high milk-derived saturated fat diet [382]. Sulfite-reducing bacteria metabolise bile acids into secondary bile acids and hydrogen sulfide which can then disrupt epithelial barrier integrity [382].

Intestinal motility changes may be another mechanism by which the milk diets altered growth, diarrhoea incidence and severity and/or colitis severity. Peptides released from milk by digestion, such as those with opioid activity [413], can alter intestinal motility. Reduced motility in the small intestine is associated with increased bacterial overgrowth in the caecum and colon, which is linked to increased bacterial translocation into the mucosa and increased severity of colitis [28, 380]. Conversely, increased motility is associated with reduced bacterial overgrowth and reduced bacterial translocation [28, 380]. Increased motility could explain the diarrhoea, and potentially the reduction in colitis severity, observed in the *Il10*$^{-/-}$ mice fed goat and cow milk-based diets (Chapter 5). *Ex vivo* motility assays using excised *Il10*$^{-/-}$ mouse colon tissues [414] could be used to determine whether reconstituted milk powders affect the motility of *Il10*$^{-/-}$ mouse intestinal tissue. Intestinal motility is affected by a number of processes, including

effects on smooth muscle ion channels and contractile elements [415], so determining the mechanisms behind any effects the milk diets had on motility may be challenging. Frozen embedded intestinal sections from this experiment could be used to visually examine the degree of bacterial translocation that occurred in the colon mucosa of the *Il10*$^{-/-}$ mice fed milk-based diets, which may be associated with the degree of bacterial growth, epithelial barrier dysfunction, intestinal motility and colitis severity. Blood levels of lipopolysaccharide (LPS) could be used as a measure of systemic translocation of gram-negative bacteria, although dysfunctional phagocytosis of bacteria also contributes to elevated levels of plasma LPS [416]. Spleen and liver bacterial levels (and in future experiments, mesenteric lymph node levels) could be measured as an additional indicator of bacterial translocation using culture-based methods or qPCR amplification and sequencing of 16S ribosomal RNA from main bacterial groups harboured by mice [416-418].

As previously discussed with respect to the findings of Chapter 3, studying an intermediate stage of inflammation development (for example, at 8-10 weeks of age) may be important for understanding the development of inflammation. This may be particularly relevant when studying the effects of a dietary intervention. The milk diet experiment could be repeated with additional sampling time-points, such as 7 and 9 weeks of age, with only *Il10*$^{-/-}$ mice and a subset of the diets used in Chapter 5, for example, using only the modified-AIN-76A control diet and milk diets. This would enable the in-depth study of disease progression in terms of colitis incidence and severity, incidence of diarrhoea, and microbiota changes. By including extra mice in each treatment, motility could also be measured in excised intestine at these time-points.

Once the effects of ruminant milk on intestinal function are better understood, the techniques used in Chapter 3 to study the molecular mechanisms underpinning inflammation development (LMD and microarray) may be useful to examine how these mechanisms are altered by milk components. Studying the molecular pathways that are altered in response to milk components may then provide clues regarding the mechanism of the anti-inflammatory effect of the cow and goat milk diets. DNA methylation analysis of the *Stat1* gene, as performed in Chapter 4, could confirm that methylation changes in *Stat1* are correlated with diet-induced gene expression changes, as the mRNA levels of *Stat1* were decreased in response to the cow milk-based diet in

Il10$^{-/-}$ mice. However, more comprehensive epigenetic analyses of inflammation in the *Il10*$^{-/-}$ mouse are required to justify further investigation of epigenetic responses to milk-based diets in this model.

Changes in metabolite levels may better reflect the functional status of a cell than changes in gene and protein expression levels because alterations in their levels occur downstream of DNA, RNA, and protein [419]. In addition, changes in metabolites can be measured in samples collected non-invasively, such as blood and urine [420]. Non-invasive methods of studying the effects of dietary intervention may be required in future studies of the effects of milk-based diets in IBD patients. Plasma and urine metabolite profiles have been studied in some mouse models of IBD [421-423] with recent studies identifying differences in metabolite profiles between IBD patients and healthy controls [420, 424].

In addition to metabolites, peripheral blood mononuclear cells (PBMCs) can be harvested and their gene expression profile analysed. PBMCs appear to be appropriate surrogates for hepatic cells in dietary intervention studies investigating metabolic effects of intervention [425], and their gene expression profiles have been used to monitor metabolic conditions such as fasting and fish oil supplementation [426-428]. PBMCs also show modulation of genes related to immunity and inflammation in response to anti-inflammatory and antioxidant dietary intervention [425] and their gene expression profiles have been used to monitor modulation of the inflammatory process [429]. In a Chinese cohort, the levels of chemokine (C-C motif) ligand 20 (*Ccl20*) mRNA transcripts in PBMCs isolated from patients with IBD were significantly higher than that of patients with non-IBD intestinal diseases and healthy controls, and were higher in active IBD than in remission and lower in patients treated with salazosulphapyridine or prednisone [430]. Performing metabolomic and/or PBMC analysis in mouse models of IBD in response to milk-based diets may provide a foundation for future studies in humans.

Identification of specific pathways that counteract the inflammatory process and result in a reduction in tissue damage could enable these pathways to be targeted with dietary intervention prior to the development of acute periods of active inflammation or relapse.

6.5 Future perspectives

There is great potential for food products to be used to alleviate IBD symptoms, particularly in the early stages of disease development, as shown by the use of enteral diets to control the disease prior to pharmacological or surgical treatment becoming necessary [15]. IBD patients also report self-selection of foods in order to prevent worsening of gastrointestinal symptoms; although it is unclear whether the avoided foods affect the inflammatory process itself, or simply irritate the inflamed mucosa or are unable to be processed effectively by the inflamed intestine, leading to intestinal discomfort and other symptoms [217]. Further enabling the use of diet to ameliorate IBD requires that the interaction between particular foods and the inflamed intestine is better understood.

Previous studies in animal models have demonstrated anti-inflammatory effects of food components such as n-3 polyunsaturated fatty acids [118], milk components such as oligosaccharides [69], and polyphenolic compounds such as curcumin [158]. In the case of some food components, such as curcumin, these effects appear to be model-specific [431]. Before applying such "anti-inflammatory" food components to studies in IBD patients, a mechanistic link between feeding of a dietary component and the reduction of colitis needs to be found, such as the one proposed for curcumin of inhibiting IFNγ signalling in colon epithelial cells [432]. Gene and protein expression analysis in the colon can help to identify these mechanisms. For example, gene expression analysis in PPARγ knockout mice has shown that dietary CLA modulates colitis through a PPARγ-dependent mechanism [84, 433].

The experiments described in this thesis provide a foundation for further work to investigate the effects of milk-based diets in colitis. Understanding these effects in terms of both colitis severity and other gastrointestinal symptoms is a necessary step towards unravelling the complex interactions between the inflamed intestinal tract and dairy components. Once the mechanism behind the reduction in colitis in response to goat and cow milk-based diets is elucidated, a study of these effects in a human IBD cohort may be possible. Ultimately, improved dietary guidelines may be able to be issued to IBD patients regarding milk consumption.

With a greater understanding of the molecular changes occurring during early inflammation, and how individual food components can impact on the inflammatory process, the potential impact of whole foods on the development of inflammation will be further elucidated. This will increase the opportunity for nutritional therapies for the early stages of IBD, which may improve or be used alongside current enteral nutritional therapy. Studies have shown, for example, that the type and amount of fat in enteral diets affects their efficacy [222]. Fat content and composition has recently been shown to affect colitis in the *Il10*$^{-/-}$ mouse model via altering the microbiota composition and possibly function [382] and to affect IBD patient response to dairy products [220]. There is a need to better understand the role of fat content and composition of diets in intestinal inflammation.

Studies of isolated components, as often used in dietary intervention in animal models [69, 84, 118, 158], are useful in identifying the effects and mechanism of action of these specific components. However, dietary components interact with each other so the impact of whole foods cannot be predicted from studies of isolated components. The experiment described here investigate the effects of different types of a whole food, milk, which is known to contain components with demonstrated anti-inflammatory effects in animal models (oligosaccharides, CLA, lactoferrin) [69, 84, 169] as well as other nutritionally beneficial ingredients (e.g. MCT) [189]. This is a further step towards unravelling the effects of foods on IBD.

Health challenges such as disease susceptibility, aging or high performance (as with athletes) require specific nutritional requirements in individuals that may differ from those of the general population. One goal of nutrigenomic research is to develop nutritional products or guidelines for people with these specialised requirements to optimise their health, based on an understanding of the influence of dietary factors on gene function. Nutrigenomic research involves the use of "omics" techniques (transcriptomics, proteomics, metabolomics), which is broadly termed "systems biology". While only a transcriptomic approach has been applied in this research, the application of other "omics" techniques in the future may better enable the detection of the subtle effects of foods such as milk diets on the inflammatory process [429]. The application of "omics" techniques to blood and urine samples will enable responses to dietary intervention with dairy products to be studied in IBD patients [434].

Nutrigenomic research can enable the development of dietary recommendations for IBD patients based on the altered molecular pathways responsible for their susceptibility to disease. Genetic association studies have identified a number of gene variants associated with IBD susceptibility, including 71 susceptibility loci for CD as of 2010 [83, 355], suggesting there may be subtypes of the disease based on which pathways are affected. Animal models of colitis are becoming increasingly targeted towards these gene variants [80]. For example, variants of the NOD2 gene are associated with susceptibility, thought to be due to malfunctioning of bacterial recognition, while other susceptibility genes are associated with related pathways such as autophagy [61]. There are now two mouse models associated with the NOD2 gene: the NOD2 variant knock-in mouse (*Nod2*^{2939iC}) and the NOD2 knockout mouse [435]. The relevance of the study presented here in the *Il10*$^{-/-}$ mouse model to pathways involved in human IBD susceptibility is becoming more evident as emerging research demonstrates a link between disease susceptibility and variants of the IL10 gene [95, 97, 436, 437], including a link between NOD2 mutations and suppression of IL10 gene transcription [96]. The research presented in this thesis represents one step on the path towards developing targeted nutrition for IBD using a nutrigenomic approach.

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