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Nadeem K. Mohammed, Student

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Dr. Howard Glauert, Director of Graduate Studies

DIET, BACTERIA AND INFLAMMATION: THE INTESTINAL MUCOSA AND METABOLIC SYNDROME

THESIS

A thesis is submitted in partial fulfillment of the
requirements for the degree of Doctorate of Philosophy in the
College of Nutritional Sciences
at the University of Kentucky

By

Nadeem K. Mohammed

Lexington, Kentucky

Co-Directors: Dr. Erik Eckhardt, Professor of Nutritional Sciences
and Dr. Deneys van der Westhuyzen, Professor of Nutritional Sciences

Lexington, Kentucky

2012

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ABSTRACT OF THESIS

DIET, BACTERIA AND INFLAMMATION:
THE INTESTINAL MUCOSA AND METABOLIC SYNDROME

Long term consumption of a high fat diet (HFD) increases the risk of developing Metabolic Syndrome and type 2 diabetes. This led us to hypothesize that long term HFD consumption impairs immune tolerance to the intestinal bacteria. Our studies had two goals. First, we characterized the effect of long term HFD consumption on the systemic immune response by comparing C57BL6 mice fed a HFD and low fat diet (LFD). Plasma immunoglobulin G (IgG) against *Escherichia coli* (LF-82), *E. coli* (Nissle 1917), *Bacteroides thetaiotaomicron* and *Lactobacillus acidophilus* were measured by a lab-developed ELISA. Fasting blood glucose and inflammation were measured in LFD mice and HFD mice. To test whether our findings were clinically relevant, anti-bacterial IgG and TNF-α were measured in plasma samples from lean healthy individuals, obese non-diabetics and obese diabetics. Our second aim was to investigate the relationship between HFD consumption and intestinal immunity. The effect of HFD consumption on immune responses in the GI tract was assessed by measuring fecal IgA levels in HFD mice and LFD mice. HFD mice had higher plasma IgG against the LF82 strain of *Escherichia coli* as well as higher plasma TNF-α, neutrophil percentage and fasting blood glucose levels. Obese diabetics had higher plasma IgG against the LF82 strain of *E. coli* than lean healthy controls. Studies on the effect of HFD on intestinal immunity revealed that HFD mice had lower fecal IgA than LFD mice. Our findings are novel in that they show an association between long term HFD consumption, systemic inflammatory immune responses to pathogenic intestinal bacteria and insulin resistance. These studies also showed that HFD consumption may impair intestinal immunity.

KEYWORDS: Diabetes, Intestinal Bacteria, Inflammation, GI Tract, Diet

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10 / 8 / 2012

DIET, BACTERIA AND INFLAMMATION:
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CHAPTER 1 INTRODUCTION

Metabolic syndrome: a global public health threat.

The drastic increase in the prevalence of type 2 diabetes is a worldwide problem, with an estimated six percent of the world's adults categorized as diabetic. The main impact of type 2 diabetes in the past has been in the developed world. However this is rapidly changing as a result of the increased affluence of developing nations. By the year 2025 it is projected that 300 million individuals will be diagnosed with diabetes worldwide with most new cases being from developing countries [1]. The diagnostic criteria for type 2 diabetes is a fasting plasma glucose level >126 mg dl^{-1} [2]. Untreated diabetes may lead to a variety of debilitating complications. These include coronary artery disease, peripheral vascular disease, blindness and amputations [3]. Therefore it is no surprise that the rise in prevalence of type 2 diabetes has been accompanied by an increased financial burden via increased medical expenditure as well as lost productivity [4]. As a consequence, investigators are interested in fully characterizing the underlying causes of diabetes to develop new strategies to prevent the development of this condition. The dramatic surge in the number of diabetics has been attributed to the increase in the number of individuals who are overweight and obese. According to the guidelines set by the World Health Organization a body mass index (BMI) between twenty five and thirty qualifies an adult as being overweight. Obesity is defined as a BMI of thirty or higher [5]. There has been a dramatic increase in the prevalence of obesity in all segments of American society in the past four decades [6]. It is estimated that a third of the

adults in the United States are obese [7]. Furthermore, seven in ten American adults can be categorized as being overweight and obese [8]. The increase in the prevalence of overweight and obese individuals has been accompanied by the increased prevalence of type 2 diabetes. As a result, excess body weight is an established risk factor for developing type 2 diabetes [9]. While lifestyle choices are a major cause for the rise in the number of diabetics it is worth noting that **not all diabetics are obese.** Additionally not all obese individuals develop diabetes, since a third of adults are obese but the prevalence of type 2 diabetes in the total population is 8.3% [10]. There are other risk factors for developing diabetes. These include genetic defects of beta-cell function and genetic defects in insulin action [11].

Increased body weight results when the intake of calories exceeds the expenditure of calories. Surplus dietary calories are converted into triglyceride and stored. There are two major forces that have contributed to increased average bodyweight. The first is an increase in dietary intake as larger portions of food can be obtained at a cheaper cost. The second factor is a more sedentary lifestyle due to automation and limited physical activity. High caloric intake and sedentary lifestyle can predispose an individual to developing a variety of metabolic abnormalities including elevated triglyceride levels, reduced high density lipoprotein cholesterol levels and increased blood pressure values that may occur simultaneously with increased body weight. This is referred to as metabolic syndrome.

Metabolic syndrome (MetS), also referred to as metabolic syndrome X, syndrome X or insulin resistance syndrome, refers to a combination of risk factors that increases the risk of developing type 2 diabetes and cardiovascular disease [12]. It has been estimated that metabolic syndrome affects almost one quarter of US adults [13]. The risk factors for metabolic syndrome utilized by the National Cholesterol Education Program (NCEP) are any three of the following five risk factors: a waist circumference ≥ 102 centimeters (cm) in men or 88 cm in women, triglyceride levels ≥150mg/dL, high density lipoprotein cholesterol levels (≤50 in women, ≤40 in men), fasting blood glucose ≥100mg/dL) and systolic BP ≥130mmHg and diastolic BP ≥85mmHg [14]. The International Diabetes Federation (IDF) criteria is waist circumference values ≥ 94 centimeters (cm) in men, ≥ 80 cm in women and any two of the other aforementioned risk factors.

Introduction to the immune system.

Since these studies involve characterization of immune responses, it is essential first of all to introduce some key immunology concepts. The immune system exists to protect the host from infection by potentially harmful microorganisms. The human body is under the constant threat of infection by microorganisms. Microorganisms such as bacteria, viruses, fungi and parasites are ubiquitous. Although the majority of microorganisms are harmless, some species have the potential to cause disease and death. Furthermore, there is also the threat of aberrant cellular replication which may lead to tumor development. To eliminate these threats the body has devised elaborate and diverse mechanisms that are highly specialized.

The entrance of microorganisms into the tissues and systemic circulation is termed infection. There are generalized mechanisms which protect against infection. The skin acts as a physical barrier which prevents infection. Chemical mechanisms also act against a broad range of infectious microbes. For example, lysozyme cleaves bacterial cell wall components. Lysozyme is present in nasal secretions, tears, on skin and in other bodily secretions [15]. A variety of immune cells eliminate infectious microorganisms. These include natural killer (NK) cells, macrophages and neutrophils [16-18]. These components of the host response are the first lines of defense against the establishment of infection. **Inflammation** also eliminates certain infections and it involves leukocytes and plasma proteins. The generalized mechanisms of immunity that eliminate microbes are collectively referred to as innate immunity.

There are receptors which recognize molecular structures resulting from the presence of pathogens referred to as pattern recognition receptors (PRRs). There are membrane bound PRRs and cytoplasmic PRRs. The membrane bound PRRs include the C-type lectin receptors and the toll like receptors (TLRs). The C-type lectin receptors (CLRs) are present on dendritic cells and detect carbohydrate structures on pathogens [19]. CLRs play a vital role in the elimination of pathogens [20]. This is because detection by CLRs results in endocytosis and degradation of pathogens [21]. This facilitates the presentation of antigens for detection by the B and T lymphocytes. Another group of PRRs are the toll like receptors (TLRs). TLRs detect microbial compounds present on the surface of microbes such as lipopolysaccharide (LPS), peptidoglycan, flagellin

and lipotechoic acid. They also detect the genetic material of foreign microorganisms such as single stranded RNA, double stranded RNA as well as CpG-containing DNA. Detection of molecular structures found on microorganisms by TLRs triggers signaling cascades that culminate in NF-κB translocation into the nucleus and subsequent expression of genes encoding inflammatory products [22, 23].

There are also cytosolic sensors to detect microbial products. These include the NOD-like receptors (NLRs) [24-26]. NLRs act by eliciting the production of inflammatory cytokines, antimicrobial peptides and interferon-β [27]. Alternatively, NLR activation results in procaspase-1 activation and the production of the inflammatory cytokines interleukin-1β (IL-1β) and IL-18 [28, 29]

However, many pathogenic microorganisms have devised mechanisms that have enabled them to avoid the mechanisms of innate immunity. This has resulted in the development of elaborate and specific mechanisms of host immunity. These specific mechanisms of immunity which combat infections by microorganisms are characterized as the **adaptive immune response**. In addition to pathogen-specific effector mechanisms, another hallmark of the adaptive response is immunological memory. Subsequent infection by the same pathogen does result in disease since the immune system can mount a more rapid response. This prevents the reoccurrence of disease. The key components of the adaptive immune response are cells called lymphocytes that mature from lymphoid progenitors. Lymphocytes are categorized based on the location of

their maturation and their effector functions. There are two kinds of lymphocytes: B lymphocytes and T lymphocytes.

B lymphocytes refer to lymphocytes which mature in the bone marrow and possess immunoglobulin receptors [30]. They detect antigens in soluble form via immunoglobulin receptors. They respond to infection by secreting antibodies. The binding of antibodies to antigens lead to their destruction via a variety of specialized immune pathways and cells. The next group of lymphocytes is the T-lymphocytes. They possess T-cell receptors and mature in the thymus. The T-lymphocytes can further be subdivided on the basis of surface markers and effector mechanisms [31]. Those that express the CD4-surface glycoprotein and respond to exogenous peptides are the CD4 T lymphocytes. Exogenous peptides are derived from phagocytosed antigens, e.g., extracellular protozoan parasites and bacteria. Those that bear the CD8 glycoprotein and respond to endogenous peptides are the CD8 T lymphocytes. Endogenous peptides are derived from proteins synthesized within the cell cytosol. These include the proteins synthesized in the cytosol by viruses or intracellular bacteria that have infected host cells.

Inflammation is associated with Metabolic Syndrome.

Some of the hallmarks of metabolic syndrome including central obesity, elevated blood glucose and hypertension have been shown to be associated with inflammation. Inflammation is a complex host defense mechanism that results from both internal and external stimuli [32]. Bacterial infection is a potent trigger of inflammation. Under normal circumstances the processes involved in

inflammation are self-limiting. However disease may arise if inflammation becomes continuous and chronic. A variety of disease states are associated with chronic inflammation. These include rheumatoid arthritis, Crohn's Disease and systemic lupus erythematosus [33-35]. Chronic unwanted inflammation is also associated with impaired glucose tolerance [36]. It has been reported that when tumor necrosis factor alpha (TNF-α), an inflammatory marker is administered to cultured cells insulin action is impaired [37]. Furthermore, enhanced insulin sensitivity has been observed in obese mice deficient in functional TNF-α receptors [38]. These findings suggested that the insulin signaling pathway may be altered by TNF-α. Insulin is produced by the beta cells of the pancreas and it is essential to metabolic pathways of carbohydrate and fat [39]. Insulin interacts with cell surface receptors in adipose and muscle tissue. This results in autophosphorylation and phosphorylation of the insulin receptor substrate (IRS) family, initiating the insulin signaling pathway which culminates with glucose uptake. TNF-α and fatty acids have been shown to inhibit the phosphorylation of IRS-1, which in turn impairs insulin action [40, 41]. As a result deciphering the underlying causes of chronic systemic inflammation has become an important priority.

High fat diet consumption and inflammation.

Consumption of high levels of dietary fat are associated with the development of the obesity, insulin resistance, elevated blood pressure and dyslipidemia. Therefore, high fat diet consumption increases the risk of developing metabolic syndrome [42]. There has been an appreciable increase in

the fat content of food. The most extreme cases can be found at some fast food restaurants where a single serving contains more than twice the recommended daily intake of saturated fat.

Dietary fat itself has been implicated as the culprit that promotes inflammation. This is not surprising given the association between high fat diet consumption and inflammatory disorders. Furthermore, various studies have implicated high fat intake to the development of insulin resistance. Saturated fats and monounsaturated fatty acids have been shown to be detrimental to insulin signaling, while omega-3 fatty acids and polyunsaturated fatty acids do not negatively affect insulin signaling [43, 44]. Furthermore, the inhibition of insulin signaling has been attributed to increased free fatty acid levels [36]. As a consequence, it was imperative to investigate the role of fatty acids in promoting inflammation. Free fatty acids activated toll like receptor 4 signaling in adipocytes and macrophages [45]. Furthermore, myristic acid, palmitic acid and oleic acid induced interlukin-6 (IL-6) messenger RNA expression in the RAW264.7 macrophage cell line.

However, the role of fatty acids in promoting inflammation is controversial. It seems counterintuitive that the host would mount inflammatory immune responses against fatty acids. This is because fatty acids are integral in cell membrane structure and serve a variety of physiologic roles. Furthermore, a subsequent study showed that the bovine serum albumin (BSA) that had been complexed with fatty acids in the previous study were contaminated by lipopolysaccharide (LPS), the ligand of the toll like receptor 4 (TLR 4) [46]. Fatty

acids complexed with BSA elicted TLR dependent signaling. However, fatty acids by themselves were unable to elicit TLR signaling. The findings of this study cast serious doubt on the role of dietary fat as the causative factor for inflammation.

Adipose tissue inflammation and insulin resistance.

Adipose tissue was initially thought to be an inert triglyceride storage depot. However, the association between obesity and conditions such as type 2 diabetes and cardiovascular disease prompted researchers to take a closer look at adipose tissue. It was observed that use of BMI values by itself was not an accurate means of predicting the risk of developing type 2 diabetes [47]. Visceral adipose tissue accumulation is associated with an increased risk of insulin resistance, whereas the subcutaneous adipose tissue depot is not associated with an increased risk of developing insulin resistance [48-51]. A variety of mechanisms have been proposed to explain how visceral adipose tissue accumulation results in the development of insulin resistance.

One proposed mechanism by which visceral fat promotes insulin resistance is the secretion of adipokines which impair insulin signaling in the liver and muscle tissues [52]. Excess lipid accumulation also promotes the development of insulin resistance. Saturated fatty acids may increase the biosynthesis of ceramide which may precede insulin resistance [53]. The conversion of triacylglycerols to diacylglyerols by adipose triglyceride lipase (ATGL) may also result in intracellular diacylgyercerol accumulation. ATGL activity has been shown to promote the development of insulin resistance [54].

Macrophage accumulation in visceral adipose tissue is another proposed mechanism by which adipose tissue may promote insulin resistance. The macrophages that accumulate in the adipose tissue release inflammatory cytokines, which can impair insulin sensitivity. It has been estimated that in lean mice and humans the percentage of macrophages is less than ten percent. This increases to forty percent in obese individuals [55]. Additionally, it was shown that macrophages present in the adipose tissue secrete a variety of inflammatory markers including TNF-α, iNOS and IL-6 [56-62]. It should also be noted that the adaptive immune response is involved in the inflammation that results from long term HFD consumption. CD4-T lymphocytes are present in the adipose tissue inflammation observed in mice fed a HFD [63]. Evidence suggests that the T lymphocytes may contribute to inflammation in the visceral adipose tissue prior to the recruitment of macrophages. Considering all these experimental findings, it is not surprising that inflammation associated with high fat diet intake was thought to be the result of excess adipose tissue accumulation.

Linking intestinal bacteria, dietary fat and inflammation.

Germ free mice (GF mice) were crucial in linking the intestinal bacteria to the obesity, inflammation and insulin resistance. Germ free mice do not harbor microorganisms [64]. This is because they are reared in sterile isolators which prevent them from being colonized by bacteria, viruses and eukaryotic parasites. The intestinal bacteria have been linked to the development of obesity [65]. Three groups of adult B6 male mice were analyzed; GF mice, mice colonized by intestinal bacteria from birth to adulthood (conventionally raised, CONV-R) and

GF mice colonized with bacteria from the cecum (conventionalized, CONV-D). GF mice had significantly lower body fat percentage compared to CONV-R and CONV-D mice. Furthermore, the epididymal fat pads of GF mice weighed less than CONV-R and CONV-D mice. These findings were intriguing since the GF mice consumed more food and had lower metabolic rates than the CONV-R and CONV-D mice. These findings were confirmed in a subsequent study when it was observed that GF mice are less prone to becoming obese after long term consumption of a high-fat, high-carbohydrate Western Diet [66]. These studies indicated that the intestinal bacteria may play a role in the development of obesity.

However, it was a study by Rabot *et al* that showed the full extent of the effects of the intestinal bacteria. GF C57BL/6J mice and conventionally raised (conv) C57BL/6J mice were fed a high fat diet (60% of energy from fat). The GF mice had improved metabolic parameters compared to conventionally raised (conv) controls [67]. These included lower fasting blood glucose levels compared to the conventional controls on the high fat diet (conv/HF) as well as lower plasma insulin concentrations and showed improved response to an oral glucose challenge. GF/HF mice also had significantly lower plasma TNF-α, serum amyloid A (SAA), leptin and IFN-γ levels. Collectively these studies implicated the intestinal bacteria in the development of obesity and accumulation of adipose tissue. Furthermore, they showed that the intestinal bacteria play a role in the development of systemic inflammation and insulin resistance. However, these studies failed to identify the point of origin of the systemic inflammation.

The growing body of evidence linking the intestinal bacteria to the development of obesity and inflammation suggests that inflammation is not solely due to adipose tissue depots. Studies had also shown that the gut microbiota differed between obese and lean mice, as well as lean and obese human subjects [68]. These findings suggested that the GI tract may be the source of inflammation. This is not an unreasonable proposal. There are situations where intestinal inflammation occurs, giving rise to a variety of debilitating conditions, including Crohn's Disease and Ulcerative Colitis [69].

The GI tract as the source of inflammation.

A study performed by Ding *et al* was the first to investigate whether high fat diet consumption in combination with the intestinal bacteria can result in pro-inflammatory changes within the GI tract [70]. Conventional specific pathogen free (SPF) C57BL/6 mice and GF mice were fed a HFD (45% kcal from fat) or a LFD (10% kcal from fat). Their findings were in accordance to previous studies using GF mice. GF mice on a HFD gained less weight than CONV controls, were more responsive to insulin and had lower levels of inflammatory plasma cytokines. TNF-α levels in the ileum of CONV/HFD was significantly increased compared to CONV/LFD mice whereas there was no increase in ileal TNF-α levels in the GF/HFD mice. This provides evidence that the GI tract is the point of origin of inflammation. The GI tract contains large numbers of immune cells which regulate the intestinal bacteria in a non-inflammatory manner. Inflammation of the GI tract can result in severe gastroenterological disorders, collectively referred to as inflammatory bowel disease (IBD). A variety of factors

are associated with IBD. These include certain pathogenic bacteria as well as defects in the immune system. For example certain strains of *E. coli* are associated with Crohn's Disease [71, 72]. With regards to the immune system, defective sensors for molecular structures located on pathogenic microbes result in chronic inflammation [73, 74]. These include mutations in constituents of the inflammasomes [75]. Inflammasomes by definition are protein complexes capable of eliciting immune responses to a variety of stimuli including bacteria [76]. NLRP6 deficiency leads to reduced IL-18 and outgrowth of the Bacteroidetes. This promoted the development of colitis (inflammation of the colon).

The intestinal bacteria are a source of inflammatory molecules.

An estimated one hundred trillion (10^{14}) microorganisms reside in the gastrointestinal tract [77]. To put things in perspective the number of microorganisms in the GI tract is so immense that intestinal microbes outnumber the total number of cells in the host by a factor of ten. The overwhelming majority of microorganisms that inhabit the GI tract are bacteria. Smaller numbers of archea and eukarya are also present [78]. Characterization of the intestinal bacteria remains challenging. There is considerable variability in the bacterial composition between different individuals. Furthermore, most of the intestinal bacterial species are anaerobic and difficult to culture using conventional methods. Advances in molecular techniques have facilitated characterization of the intestinal bacteria. Sequencing of the 16S ribosomal RNA gene (rRNA) led

researchers to identify the dominant intestinal bacterial divisions as the Bacteroidetes and the Firmicutes [79].

Pyrosequencing, which involves the detection of chemiluminescent signals subsequent to DNA synthesis has led researchers to identify in excess of 5,000 bacterial taxa [80]. The majority of the intestinal bacterial species belong to the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* [81]. The large and diverse bacterial population of the gastrointestinal (GI) tract is a rich source of inflammatory molecules. Lipopolysaccharide (LPS) is present in the outer cell membranes of the gram negative intestinal bacteria [82]. LPS is a potent inducer of inflammation, triggering the release of pro-inflammatory cytokines such as TNF-alpha, IL-6, and IL-1 [83]. It should be noted that gram positive bacteria residing in the GI tract can also trigger inflammation. Bacterial DNA from gram positive bacteria induces inflammation [84]. Furthermore, long term HFD consumption is associated with increased plasma LPS. Long term HFD consumption increases LPS containing bacteria residing in the GI tract [85]. It was observed that LPS infusion results in insulin resistance, thereby establishing a crucial link between the contents of the GI tract, inflammation and insulin resistance.

Regulation and confinement of the intestinal bacteria.

Considering the sheer quantity of inflammatory molecules within the GI tract, it is not surprising that a variety of mechanisms exist to prevent intestinal bacteria from entering the systemic circulation. The intestinal epithelial cells (IECs) form a physical barrier referred to as the intestinal epithelial-cell barrier

[86]. The IECs also possess a brush border, which is an actin rich microvilli covered surface on the apical surface of the IECs. This prevents the intestinal bacteria from adhering to the surface, thereby minimizing bacterial invasion of the epithelial layer. Furthermore, the proteins occludin and claudin form tight physical seals at the apical epithelial surface [87, 88]. However, it should be noted that these so called tight junctions are not fully impermeable since the GI tract has to absorb a variety of nutrients and ions. Goblet cells also play a role in preventing the intestinal bacteria from adhering to the epithelial layer. Goblet cells are differentiated epithelial cells which secrete mucus. The secretion of mucus forms a viscous impermeable glyocalyx which prevents bacterial adhesion to the epithelium of the GI tract [89].

The IECs do not only function as a physical barrier. They also act by secreting a variety of host defense peptides that kill bacteria. These include defensins and cathelicidins which have broad spectrum antibiotic activity [90, 91]. Defensins and cathelicidins are cationic proteins. These antimicrobial proteins function by forming pores in bacterial cells walls. IECs are not the only cells that secrete these antimicrobial peptides. Paneth cells, which are specialized epithelial cells, secrete antimicrobial peptides [92]. In additions to defensins and cathelicidins other anti-microbial compounds have been identified including phospholipases, lysozyme, and Reg III-gamma [93-97].

Intestinal epithelial serum amyloid A (SAA) also plays a role in regulating the intestinal bacteria population. SAA regulates Gram-negative bacteria present in the lumen of the GI tract, including *E. coli* [98]. SAA binds to the outer

membrane protein A (OmpA) of *E. coli,* which leads to its elimination by neutrophils [99]. SAA also promotes homeostasis with the intestinal bacteria, since SAA deficient mice are more susceptible to dextran sodium sulfate (DSS)-induced colitis [100] . The production of such a diverse array of antimicrobial compounds emphasizes the importance of regulating the immense population of bacteria residing within the lumen of the GI tract.

Adaptations of intestinal immunity.

It has been previously stated that the immense antigenic load of the GI tract is a vast reservoir of inflammatory molecules. As a consequence a number of mechanisms exist to prevent the huge bacterial population of the lumen of the GI tract from entering the systemic circulation. Entrance of the immense antigenic load into the systemic circulation has the potential to result in catastrophic consequences including systemic shock and sepsis. Furthermore, the GI tract is also exposed to a variety of pathogens that are ingested by the host. The large surface area of the GI tract (approximately $300m^2$) makes it especially susceptible to infection. In order to prevent bacteria from the lumen of the GI tract from undergoing uncontrolled proliferation and entering the circulation, the GI tract contains a vast and diverse population of immune cells. The GI tract contains B and T lymphocytes, dendritic cells as well as the largest reservoir of macrophages (Mψ) in the body [101]. The immune cells are located in specialized structures including the Peyer's Patches as well as in isolated lymphoid follicles.

However, a paradox exists. On one hand, the presence of immune defenses is required to prevent the intestinal bacteria from entering the systemic circulation. However, it is crucial that the intestinal bacteria be regulated without inflammation. Failure to engage bacteria without inflammation can have catastrophic consequences that are usually observed in patients with inflammatory bowel diseases such as ulcerative colitis (UC) and Crohn's Disease (CD) [102]. Furthermore it is essential that the immune system tolerate the intestinal bacteria since some bacterial species perform vitamin K and biotin synthesis [103-105]. Additionally, the intestinal bacteria may play a role in the overall health of the digestive system [106]. Germ-free animals are more susceptible to infection [107].

Although intestinal macrophages are abundant, they possess a variety of modifications that render them inert against the bacteria in the lumen of the GI tract. These include a lack of receptors that may activate inflammatory pathways such as CD14, complement receptors (CR) and the triggering receptor expressed on myeloid cells (TREM-1) [108, 109]. It was initially proposed that the intestinal macrophages did not express toll like receptors (TLRs) [110]. However, further analysis revealed that intestinal macrophages do in fact express toll like receptors [111]. The reason for these macrophages existing in an anergic state is a lack of downstream signaling molecules including MyD88, IRAK and TRAF6 [112-115]. The presence of molecules that inhibit TLR pathways such as IRAK-M also play a role in rendering intestinal macrophages hyporesponsive [116]. Impaired TLR signaling prevents the transcription factor NF-κB from translocating

into the nucleus. This prevents the expression of inflammatory genes and inflammatory cytokines.

Another mechanism of intestinal immune tolerance is the inhibition of inflammatory cytokine production by antigen presenting cells (APCs). APCs are cells which express major histocompatibility complex (MHC) molecules which bind and present antigenic peptides for recognition by the T lymphocytes [117]. Dendritic cells (DCs) are specialized APCs which can induce cell mediated immune responses by the T lymphocytes [118]. However, the DCs are also capable of inducing tolerance to certain antigens, including those of the intestinal bacteria. The intestinal epithelial cells (IECs) secrete certain factors which limit inflammatory cytokine production by DCs. The most prominent of these IEC derived factors are thymic stromal lymphopoietin (TSLP), transforming growth factor-β (TGF-β), Interleukin-10 (IL-10) and prostaglandin E_2 [119-122]. These IEC derived factors create a microenvironment that promotes the tolerogenic phenotype of dendritic cells.

The adaptive immune response to the intestinal bacteria.

In addition to the aforementioned generalized mechanisms, specialized immune responses occur to prevent the intestinal bacteria from entering the systemic circulation. Since the 1960's, researchers have observed that the vast majority of immunoglobulins in the external secretions belong to the immunoglobulin A (IgA) isotype [123]. IgA exists in a monomeric form or a polymeric form. The predominant form of secreted polymeric IgA (pIgA) is dimeric IgA, which is comprised of two IgA subunits bridged by the J chain

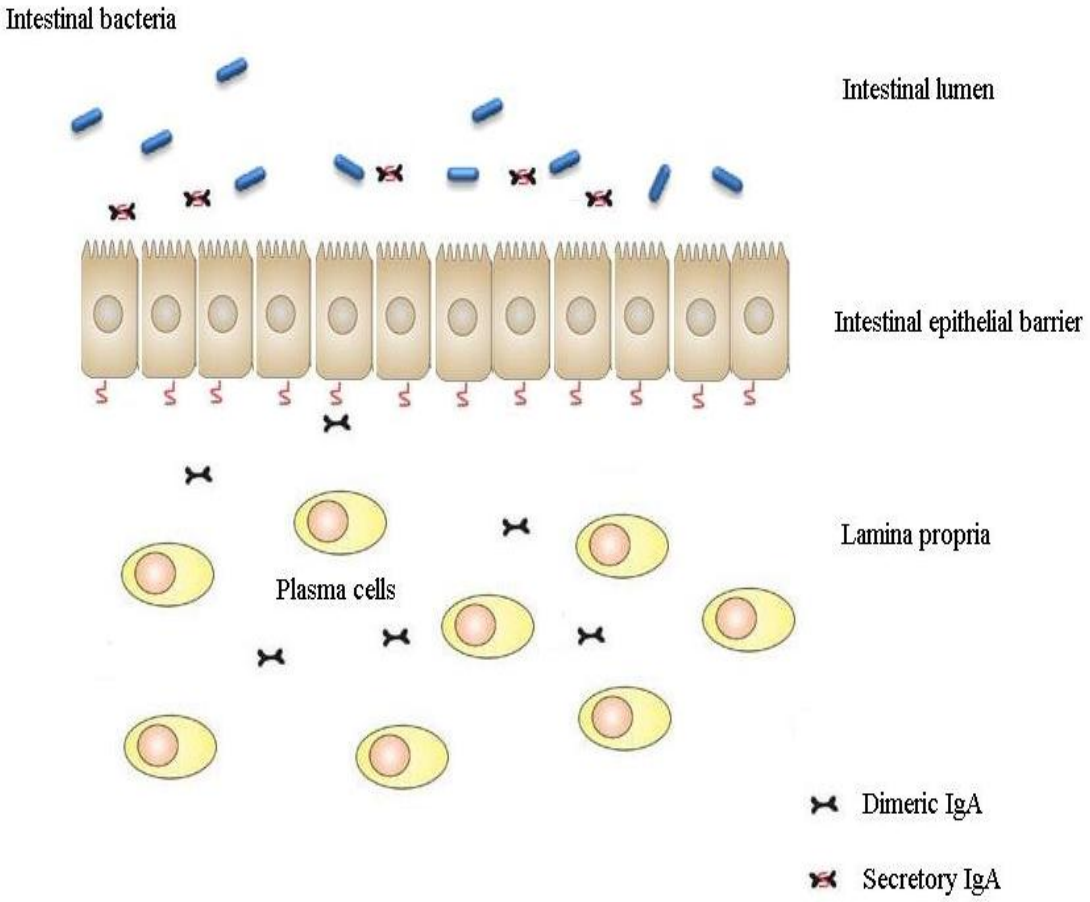
polypeptide [124]. Plasma cells present in the lamina propria secrete dimeric IgA [125].

The polymeric immunoglobulin receptor (pIgR) is responsible for transepithelial transport of polymeric IgA and IgM [126]. pIgR binds and internalizes polymeric immunoglobulin at the basolateral surface of the intestinal epithelial cells. The bound immunoglobulin is shuttled through the cell to the apical surface where it is secreted. It is secreted with the extracellular ligand-binding fragment of pIgR as secretory IgA (sIgA) [127]. Adherence and invasion of the intestinal epithelium by bacteria is minimized by secretory IgA (sIgA) and secretory IgM (sIgM) [128-130]. IgA is the predominant immunoglobulin subclass produced in the GI tract [131]. The IgA subclass accounts for four fifths of the immunoglobulins produced in the duodenum and jejunum. IgA comprises ninety percent of the immunoglobulins produced in the colon.

In addition to its established role in preventing the bacteria present in the lumen of the GI tract from entering the systemic circulation, sIgA is essential to preventing inflammatory responses. sIgA minimizes inflammatory immune responses against the commensal bacteria by downregulating the expression of the inflammatory cytokines TNF-α, IL-6, Cox-2 and IFN-γ [132]. The presence of intestinal bacteria stimulates the production of intestinal IgA [133, 134]. Although the vast majority of IgA is produced at the intestinal mucosa, there is also IgA present in the plasma. Plasma IgA production is independent of intestinal IgA production [135]. Plasma IgA is primarily monomeric and is produced by B

lymphocytes resident in the bone marrow [136]. Small quantities of monomeric serum IgA enter intestinal secretions by diffusion [137].

Figure 1.1 IgA secretion



. Within the lamina propria plasma cells secrete dimeric IgA. The polymeric immunoglobulin receptor (pIgR) present on the basolateral end of intestinal epithelial cells binds IgA dimers. The IgA dimers are transported to the apical end and secreted into the lumen of the GI tract with a portion of pIgR.

pIgR knockout mice: defining the role of active IgA secretion.

Dimeric IgA and pentameric IgM exported by the polymeric immunoglobulin receptor are not the only immunoglobulins present in the intestinal muocsa. Serum IgA and IgG also enter intestinal mucosal secretions by paracellular diffusion. Mice deficient in the polymeric immunoglobulin receptor (pIgR KO mice) were generated to characterize the role of active immunoglobulin secretion by insertion of a targeting vector into the third exon of the polymeric Ig receptor locus (PIGR). Immunofluorescence staining of small intestine sections revealed that pIgR KO mice have significantly reduced IgA at the epithelial surface compared to wild type controls. However, pIgR KO mice have increased interstitial IgA indicating that the lack of IgA at the epithelium is due to impaired active transport, not to a defect in IgA synthesis. Comparison of serum, whole saliva, small intestinal secretions and fecal extracts revealed a number of major differences between pIgR KO mice with wild type controls. pIgR KO mice had elevated serum IgA. This was the direct result of impaired IgA secretion since Western Blot analysis revealed that the majority of this IgA was polymeric IgA. pIgR KO mice had increased serum anti-*E. coli* IgG. However, there was no difference in anti-*Lactobacillus* IgG between pIgR KO mice and wild type controls. Therefore, reduced active secretion of IgA by pIgR resulted plasma IgG being produced selectively against *E*. *coli.* IgG was higher in the small intestinal secretions of pIgR KO mice than in the wild type controls. Fecal IgG was also significantly higher in pIgR KO mice than in the wild type controls. This shows

that impairment of the mucosal barrier results in the bulk transport of IgG into the mucosa.

Studies have been performed using pIgR KO mice to determine the role of active immunoglobulin secretion in protection against disease. pIgR KO mice are more susceptible to *Mycobacterium bovis bacillus* Calmette-Guérin (BCG) infections than wild-type mice [138]. pIgR KO mice had higher bacterial loads in their lungs compared to the wild-type controls. The capacity of pIgR KO mice to produce IFN-γ and TNF-α was also significantly reduced compared to wild type controls. pIgR KO mice are also more susceptible to nasal colonization by *Streptococcus pneumonia* [139] . Wild type C57BL/6 mice had significantly less serum IgG against intestinal bacterial antigens than pIgR KO mice. pIgR KO mice also had increased numbers of bacteria in their mesenteric lymph nodes than wild type controls. These findings provide evidence that active IgA secretion is essential in the containment of bacteria to the lumen of the GI tract [140].

Immune responses to intestinal bacteria are localized.

As previously stated, immune responses are mounted against the intestinal bacteria. These immune responses involve the generation of IgA exclusively within the GI tract [141]. A study by Konrad *et al* characterized systemic immune responses against select intestinal bacterial antigens [142]. ELISA assays revealed that the serum immunoglobulin G (IgG) response to these antigens was negligible in C3H/HeJ mice. Furthermore, there were no specific CD4+ T-cell responses to bacterial antigens. However, although these mice did not have systemic immune responses against bacterial protein antigens

they produced intestinal IgA to the same antigens. This provides strong evidence of a very tight compartmentalization of immunity.

Mesenteric lymph nodes are crucial for oral tolerance.

There is an abundance of antigens in the GI tract. These include bacterial antigens as well as numerous food antigens. The oral administration of antigens significantly impairs systemic immune responses to these same antigens if they are administered intravenously. This phenomenon is referred to as oral tolerance [141]. Mesenteric lymphadenectomy (the removal of mesenteric lymph nodes) abolishes oral tolerance [143]. This provides evidence that the mesenteric lymph nodes (MLNs) are the sites of induction of tolerance to harmless antigens present in the GI tact. The MLNs act as barriers, preventing the dendritic cells carrying commensal bacteria from gaining access to the systemic circulation [144]. The inability of dendritic cells carrying commensal bacteria to enter the systemic circulation is a major contributing factor in preventing systemic immune responses to the intestinal bacteria.

The MLNs are situated along the route of chylomicron transport [145]. Chylomicrons are lipoprotein particles secreted by the enterocytes subsequent to consuming a meal containing triglycerides [146]. Triacylglyceride (TAG) is the major constituent of chylomicrons. In addition to TAGs chylomicrons contain phospholipids, cholesterol as well as proteins [147]. Chylomicrons are comprised of a hydrophobic core containing TAG and cholesterol esters. The hydrophilic surface is a phospholipid monolayer in addition to cholesterol and proteins. It is therefore possible that the long term consumption of high levels of dietary fat can

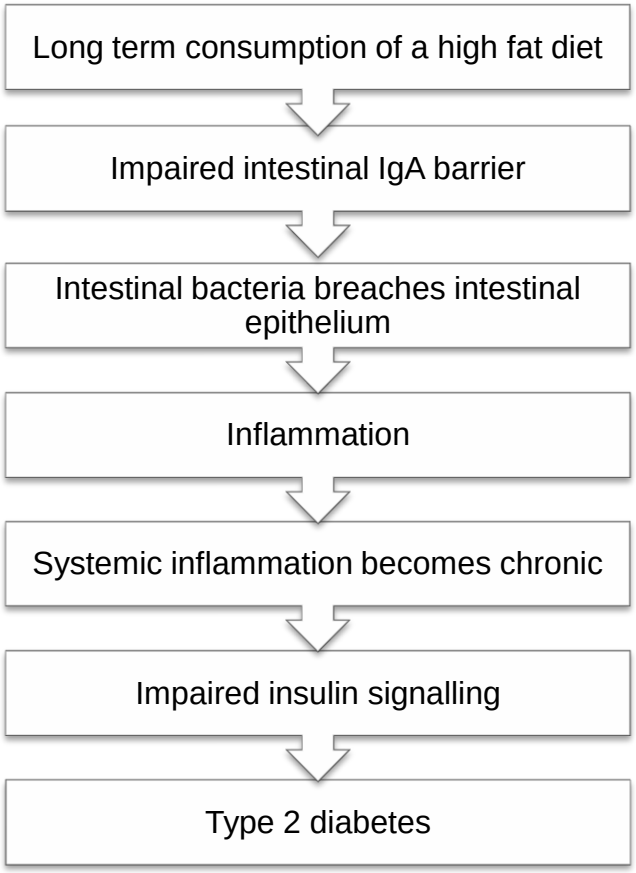
affect the functioning of the mesenteric lymph nodes. Atrophy of the mesenteric lymph nodes has been observed in obese mice along with a reduction in the numbers of regulatory T lymphocytes. Long term consumption of a high fat diet is associated with apoptosis of the regulatory T lymphocytes within the MLNs [148].

The regulatory T cells (Tregs) downregulate immune responses, preventing uncontrolled immune responses [149]. A reduction in Tregs due to long term consumption of a HFD may result in a switch from tightly regulated immune responses to the intestinal bacteria to uncontrolled systemic inflammation. These findings were exciting since they demonstrated that the implications of long term HFD consumption may also extend to impairment of immune tolerance. If this occurs then a switch from the tightly controlled non-inflammatory responses against the intestinal bacteria to systemic inflammatory responses against the intestinal bacteria could result. Loss of tolerance to the intestinal bacteria being linked to metabolic syndrome was a novel concept that we sought to investigate in a series of studies.

Proposed hypothesis.

Based on our preliminary findings and the published literature we propose that long term consumption of a high fat diet impairs the immune tolerance to the intestinal bacteria resulting in increased systemic inflammation. This systemic inflammation may become chronic and lead to impaired insulin signaling and the development of type 2 diabetes.

Figure 1. 2 Experimental Model



Experimental approach.

In previous studies, HFD fed mice harboring gut bacteria had higher fasting blood glucose levels and elevated plasma inflammatory cytokines than to HFD fed GF mice. As a result, our first experimental goal was to determine whether long term HFD consumption resulted in triggering of systemic immune response against intestinal bacteria. We assessed the effect of long term HFD consumption on plasma IgG against different intestinal bacterial strains. Preliminary analyses revealed that long term HFD consumption resulted in increased plasma IgG against the LF-82 strain of *Escherichia coli*, an invasive intestinal bacterial strain.sIgA in the GI tract plays a major role in preventing adherence of bacteria to the intestinal epithelium. This minimizes intestinal bacterial translocation. As a consequence, our second experimental goal was to determine whether long term HFD consumption affected IgA secretion into the lumen of the GI tract. This was done by comparing fecal IgA in C57BL6 mice fed a low fat diet (LFD) as well as HFD mice. Our third goal was to characterize the role of the polymeric immunoglobulin receptor (pIgR) in shaping the bacterial composition of the GI tract. The polymeric immunoglobulin receptor (pIgR) is a membrane glycoprotein is responsible for the active secretion of polymeric immunoglobulins into the lumen of the GI tract. Mice lacking the pIgR (pIgR KO mice) and wild-type C57BL6 littermates were placed on a high-fat, high-carbohydrate Western Diet and a variety of metabolic parameters were evaluated.

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CHAPTER 2 THE EFFECT OF HIGH FAT DIET ON SYSTEMIC IMMUNITY.

INTRODUCTION.

Studies showing improved metabolic parameters in GF mice prompted an investigation as to whether the bacteria residing in the GI tract are responsible for the development of inflammation and insulin resistance. As previously mentioned, immune responses against the bacteria in the GI tract are generated at the intestinal mucosal surface. Theoretically the systemic immune cells are oblivious to the presence of intestinal bacteria. Serum immunoglobulin G (IgG) and $CD4^+$ T-cell specific responses against intestinal bacterial antigens are negligible.

To gain a more comprehensive understanding of the effects of long term HFD consumption, pilot studies were performed using the plasma from BALBc mice fed a high fat diet (Research Diets Inc. D12492 Rodent Diet with 60% kCal fat) and mice fed a low fat diet (Open Source Diets D12450B Rodent Diet with 10 kcal% fat) for 10 weeks. The pilot analyses involved measuring plasma IgG against the LF82 strain of *E. coli*, an intestinal bacterial strain. Plasma IgG was evaluated by Western Blot analysis, as well as by ELISA analysis. After performing the preliminary studies in mice, a 10 week feeding study was performed on C57BL6 mice fed a HFD and a LFD. Plasma IgG against protein extracts from *E. coli* LF82, *E. coli* Nissle 1917 (EcN), *Bacteroides thetaiotaomicron (B. thetaiotaomicron)* and *Lactobacilus acidophilus (L. acidophilus)* were measured by a lab-developed ELISA. Plasma TNF-α, blood

cell composition and fasting blood glucose levels were measured in order to determine whether a link existed between plasma IgG against intestinal bacteria, systemic inflammation and elevated fasting blood glucose.

To determine whether the findings in the animal studies were clinically relevant, plasma samples from human subjects were also analyzed. Plasma IgG against *E. coli* LF82, *E. coli* Nissle 1917 (EcN), *Bacteroides thetaiotaomicron (B. thetaiotaomicron)* and *Lactobacilus acidophilus (L. acidophilus)* was measured in lean healthy controls as well as obese non-diabetics and obese diabetics. Plasma TNF-α was also measured in human plasma samples to determine whether a relationship existed between plasma IgG against intestinal bacteria and inflammation. The findings of this study can be found in the journal **Metabolism: Clinical and Experimental.** The title of the paper is ***Elevated IgG levels against specific bacterial antigens in obese patients with diabetes and in mice with diet-induced obesity and glucose intolerance.*** The article is available online and it is currently still in press. Permission to reproduce this data was obtained.

METHODS.

PRELIMINARY ANIMAL STUDIES

The preliminary studies involved the analysis of plasma from eight BALB/c mice. Four of the mice had been placed on a high fat diet (Research Diets Inc. D12492 Rodent Diet with 60% kCal fat) and the other four had been placed on a low fat diet (Open Source Diets D12450B Rodent Diet with 10 kcal% fat) for ten

weeks. Western blots were performed to determine whether long term HFD consumption was associated with increased plasma IgG against the intestinal bacteria.

Bacterial cultures.

The LF82 strain of the gut microbe *Eschericha coli* was cultured overnight in Luria-Bertani Medium (LB Medium) at 36.9°C in an Isotemp Incubator (Fisher Scientific).

Preparation of bacterial proteins.

In order to obtain bacterial proteins, approximately 5 ml of bacterial cultures were centrifuged at 10,000 rpm for ten minutes. The supernatant was discarded and the bacterial pellet obtained was re-suspended in two hundred and fifty microliters (250µl) 4X SDS and seven hundred and fifty microliters (750µl) water. The re-suspended bacterial pellet was then boiled for ten minutes.

Western Blot Analysis of anti-*E. coli* LF82 IgG.

The *E. coli* proteins were separated on NuPAGE® 4-12% Bis Tris Gels and then transferred by an Invitrogen iBlot® Gel Transfer Device to PVDF membranes. The membranes were blocked with non-animal protein (NAP) blocking solution diluted 1:2 with 1X femto-TBST. The PVDF membranes were cut into individual strips. Each membrane strip was incubated overnight in plasma from an individual mouse that had been diluted 1:10 in the blocking solution. The membrane strips were then washed with 1X Tris-Buffered Saline Tween-20 (TBST). Anti-Mouse IgG (Fc specific)-Peroxidase antibody was diluted

1:5000 to detect the presence of anti-*E.coli* IgG. The membrane strips were then washed again with TBST and then simultaneously exposed to ECL Western Blotting Substrate. The western blots were then visualized on the Kodak Image Station 440.

Western Blot Analysis of plasma anti-bacterial IgG.

The intestinal bacteria are poorly characterized. To determine whether long term HFD consumption resulted in increased systemic immune responses to the intestinal bacteria, the contents of the cecum were isolated. The contents of the cecums were removed from each of the LFD mice, pooled and added to 750µl of water and 250µl 4X SDS loading buffer. The mixture was boiled for approximately ten minutes. The mixture was then centrifuged at 10,000 rpm for ten minutes. The pellet was discarded while the supernatant containing the cecal antigens was kept for analysis. The cecal antigens were separated on NuPAGE® 4-12% Bis Tris Gels and then transferred by an Invitrogen iBlot® Gel Transfer Device to PVDF membranes.

Each membrane was cut into four individual strips. Each individual membrane strip was placed in non-animal protein (NAP) blocking solution diluted 1:2 with 1X femto-TBST. Each membrane strip was incubated overnight in plasma from an individual mouse that had been diluted ten times in the blocking solution. The membrane strips were then washed with Tris-Buffered Saline Tween-20 (TBST) [1X]. Anti-Mouse IgG (Fc specific)-Peroxidase antibody was diluted 1:5000 to detect the presence of anti-*E.coli* IgG. The membrane strips were then washed again with TBST and then simultaneously exposed to ECL

Western Blotting Substrate. The western blots were then visualized on the Kodak Image Station 440.

Development of a novel ELISA technique to quantify anti-bacterial IgG.

We wanted to assess whether long term HFD consumption resulted in quantitative differences in IgG against bacterial proteins. To do this we developed a novel enzyme-linked immunosorbent assay (ELISA) to measure IgG against the bacteria used in these studies: *E. coli* LF82, *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus.* Soluble proteins from these bacteria were extracted using B-PER Bacterial Protein Extraction Reagents (ThermoSCIENTIFIC). Bacteria cultures were centrifuged at 5000 × g for 10 minutes.

The supernatant was discarded and the remaining pellet was weighed. B-PER Reagent was added at a ratio of 4ml/gram of cell pellet. Lysozyme and DNase I were added at a ratio of 2µL/mL of B-PER Reagent in order to obtain a higher concentration of soluble proteins. The suspension was incubated at room temperature for 15 minutes and then centrifuged at 15,000 × g for 5 minutes. The supernatant containing soluble bacterial proteins was used for preparing the ELISA plates. Bacterial proteins were quantified using the BCA™ Protein Assay (Thermo Scientific) and dissolved in a carbonate coating buffer of pH 9.6 to give a final concentration of 100µg/ml. 96 well flat-bottom ELISA plates (BD-Falcon) were coated with 10µg of bacterial protein per well and incubating the plate overnight at 4ºC. Details of how the ELISAs were performed are given below.

Long term feeding study on C57BL6 mice.

Twelve six week old male C57BL6 mice were ordered at 5 weeks of age from Jackson Laboratories. The mice were housed three per cage and maintained in a 12hour light/dark cycle. We allowed the mice to become acclimated to their environment. We then marked them for identification using tail markings. We measured the body weight and fasting blood glucose values prior to switching these mice from regular chow diets to special diets.

The special diets of the mice were open formula, open source purified ingredients. The mice were divided into two equal groups (n = 6). One group was fed a high fat diet (Rodent Diets with 60 kcal% Fat D12492 from Research Diets Incorporated). The other group was fed a low fat diet (Rodent Diets with 10 kcal% Fat D12450B from Research Diets Incorporated). Details of these diets are listed in table 2.1. The mice were placed on these diets at six weeks of age and euthanized after 10 weeks on the special diets.

Measurement of body weights and fasting blood glucose

The mice were weighed weekly to monitor changes in bodyweights. To assess fasting glucose levels, mice were fasted for 4 hours. Blood was collected from the tail tips of the mice. Blood glucose levels were assessed using the TRUEtrack® glucose meter (Home Diagnostics Inc.).

Measurement of plasma anti-bacterial IgG.

The ELISA developed by this laboratory was used to measure plasma IgG against intestinal bacteria. 96 well flat-bottom ELISA plates (BD-Falcon) were coated with 10µg of bacterial protein per well. Plates were incubated overnight at 4ºC. The wells were washed five times with 1X Tris-Buffered Saline Tween-20 (TBST). Each washing step was performed for duration of five minutes. The plates were then blocked for one hour at room temperature by adding 250µl of NAP Blocking reagent diluted 1:2 in 1X TBST to each well. 100µl of plasma diluted 1:100 in blocking solution was then added to each well and incubated for 2 hours at room temperature. The plates were then washed five times with 1X Tris-Buffered Saline Tween-20 (TBST). Bound IgG was detected by adding 100µl/well of alkaline phosphatase-conjugated anti-mouse IgG (Fc specific) from Sigma-Aldrich diluted 1:5000 in blocking buffer for 1 hour. The plates were then washed five times with 1X Tris-Buffered Saline Tween-20 (TBST). 50µl/well of p-nitrophenyl phosphate (pNPP) from Sigma-Aldrich) was added, and the color reaction was stopped with 2M sulfuric acid. Absorbance values were read at optical density 405nm (A405) in a Bio-Rad microplate reader.

Processing of samples.

Mice were humanely euthanized using carbon dioxide followed by cervical dislocation. Blood was immediately collected by cardiac puncture and placed into EDTA containing eppendorf tubes that had been kept on ice. The majority of blood obtained was immediately centrifuged at 8,000 rpm for 10 minutes. The

plasma was collected and stored at -82°C. The remaining whole blood fractions were used to characterize the blood cell types present by hematological analysis.

Plasma TNF-α measurements.

To assess the levels of circulating TNF-α we utilized the MILLIPLEX® Mouse Cytokine Kit (from Millipore Catalog # MPXMYCTO-70K). The data was read in a Bio-Plex® 200 system (BIO-RAD).

Hematological analysis.

Subsequent to euthanasia approximately 50µl of whole blood was collected from the mice by cardiac puncture. The tubes containing the whole blood was briefly rocked. The whole blood was then analyzed using a HEMAVET® 950 FS Multispecies Hematology Systems (Drew Scientific Inc.). This is a device which gives comprehensive hematology profiles using 20 µL of whole blood from different animals including mice. The hematology profiles given are for both leukocytes and erythrocytes.

The leukocyte parameters given are white blood cell count, absolute number and percentage of neutrophils, absolute number and percentage of lymphocyte, absolute number and percentage of monocyte, absolute number and percentage of eosinophil, and absolute number and percentage of basophil. The erythrocyte parameters given are red blood cell count, hemoglobin, hematocrit, mean cell volume, mean corpuscular hemoglobin concentration and red cell distribution width. Additionally thrombocyte parameters including platelet counts and mean platelet volume are given.

Measuring plasma IgG against intestinal bacteria.

Cultures of *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus* were kindly provided by Dr. Charlotte Kaetzel (Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky). These bacteria had been obtained from the American Type Culture Collection (ATCC). Proteins were extracted from *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus* as well as the LF82 strain of *E. coli*. The ELISA developed by this laboratory was used to measure plasma IgG against these intestinal bacteria. Ninety six well flat-bottom ELISA plates (BD-Falcon) were coated overnight with 100µl of the bacterial protein extracts. Plasma from the LFD mice and the HFD mice was diluted 1:100 to quantify IgG against *E. coli* LF82, *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus.*

To determine if inflammation originates in the GI tract.

RNA was extracted from ileum and colon using using the E.Z.N.A® Total RNA Kit. We used the Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Scientific) to measure RNA concentration. The qScript™ cDNA Synthesis Kit was used to generate cDNA. We analyzed the intestinal samples for expression of inflammatory genes. The genes we examined were Chemokine (C-C motif) ligand 5 (CCL5 also known as RANTES), NOD-like receptor family pyrin domain containing 6 (NLRP6), IL-18 and thymic stromal lymphopoietin (TSLP) expression using a BIORAD CFX96™ Real Time PCR detection system. Gene expression was normalized to Glyceraldehyde 3-phosphate

dehydrogenase (GAPDH). The reason for examining NLRP6, IL-8 and CCL5 was due to their association with inflammatory responses against intestinal bacteria. TSLP was examined since TSLP secreted by the intestinal epithelial cells promote tolerance to the intestinal bacteria. The primer sequences used for RT-PCR analysis of these genes are listed in table 2.4.

HUMAN STUDIES.

Human plasma samples from a Centers of Biomedical Research Excellence (COBRE) pilot study “The influence of SAA and CETP activity on HDL remodeling during active weight loss” were kindly provided by Dr. Anisa Jahangiri. All of the plasma samples obtained from this study were from obese patients (BMI ≥ 30). One group of the obese patients had not been diagnosed with type 2 diabetics (obese non-diabetics). The other group of obese patients had been diagnosed with type 2 diabetes (fasting plasma glucose ≥126 mg/dl). Plasma samples from non-diabetic individuals with BMI values in the healthy range (BMI ≥ 20 and ≤25) were obtained commercially from Biospecialty Corp. (Colmar, PA, USA) for experimental controls. The study was approved by the Institutional Review Board (IRB) at the University of Kentucky. Plasma samples were stored at -82°C and not subjected to freeze thaw cycles. Details of the human plasma donors are given in table 2.2.

Western Blot Analysis of human plasma.

Proteins were then extracted from *Escherichia coli* and separated on NuPAGE® 4-12% Bis Tris Gels as previously described. After the proteins were transferred to PVDF membranes, the membranes were placed in non-animal protein (NAP) blocking solution diluted 1:2 with 1X femto-TBST. The PVDF membranes were sliced into individual strips and incubated with plasma samples from ten healthy control individuals as well as six obese non-diabetics and six obese diabetics.

The plasma samples were diluted 1:400 in the blocking solution and incubated overnight at 4°C. The strips were then washed 5 times in 1X TBST. The membrane strips were incubated in anti-human IgG (Fc specific)-Peroxidase antibody diluted 1:5000 in the blocking solution for one hour. The membrane strips were washed five times in 1X TBST. ECL Western Blotting Substrate was simultaneously added to each of the membrane strips and imaging was performed on the Kodak Image Station 440.

Measurement of anti-bacterial IgG.

IgG against *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus* and *E. coli* LF82 was measured in plasma samples from the lean healthy controls, obese non-diabetics and obese diabetics by lab developed ELISA. Human plasma samples were diluted 1:400 in blocking solution.

Bound IgG was detected by adding 100µl/well of alkaline phosphatase-conjugated anti-human IgG (Fc specific) from Sigma-Aldrich diluted 1:5000 in blocking buffer for 1 hour. 50µl/well of p-nitrophenyl phosphate (pNPP) from Sigma-Aldrich) was added, and the color reaction was stopped with 2M sulfuric acid. Absorbance values were read at optical density 405nm (A405) in a Bio-Rad microplate reader.

Measurement of TNF-α

Plasma TNF-α was measured in the obese non-diabetics and the obese-diabetics using the Human TNF-alpha High Sensitivity ELISA from eBioscience (BMS223HS; sensitivity 0.13 pg/mL). 50 µl of human plasma was added in duplicate. The human plasma samples were diluted in 50 µl of sample diluent.

Statistical analyses.

Results were expressed as mean ± S.E.M and were analyzed with GraphPad Prism v5.04. Groups were compared with unpaired Student's t-tests and ANOVA and Bonferroni's post-hoc analysis. Statistical significance was assumed when $p<0.05$.

LIST OF TABLES.

Table 2.1: Details of the rodent diets.

Diet	% kcal Protein	% kcal Carbohydrate	% kcal Fat
Teklad Global 18% Protein Rodent Diet 2918	24	58	18
Research Diets Inc. Low fat diet (LFD) D12450B	20	70	10
Research Diets Inc. High fat diet (HFD) D12492	20	20	60
Research Diets Inc. Western Diet D12079B	17	43	41

Table 2.2: Human plasma sample details.

Patient group	Sample size (n)	BMI	Gender ratio
Lean, healthy	10	24.8 ± 3.0	1♀, 9♂
Obese non-diabetics	16	41.1 ± 10.4	7♀, 9♂
Obese diabetics	16	40.6 ± 7.1	7♀, 9♂

Table 2.3: List of primer sequences used for genotyping.

Primer	Sequence
Pigr WT A	GAACTCTTGTCTTTTGTCTCC
Pigr WT B	CTCGCCTGAATACTCCTT
Pigr KO A	GAACTCTTGTCTTTTGTCTCC
Pigr KO B	TCCAGACTGCCTTGGGAAA

Table 2.4: List of real time PCR primer sequences.

Primer	Sequence
mGAPDH-F	CCAGGTTGTCTCCTGCGACTT
mGAPDH-R	CCTGTTGCTGTAGCCGTATTCA
mTNFa-F	CCCTCACACTCAGATCATCTTCT
mTNFa-R	GCTACGACGTGGGCTACAG
mIL-6-F	AAGAGCCGGAAATCCACGAAA
mIL-6-R	TCTTGGCGTTACAGAGGATCA
mNLRP6f1	TCTCTCCGTGTCAGCGTTCA
mNLRP6r1	CGGAAGAGCCGATTAAAAGTGT
mIL-18-F	GACTCTTGCGTCAACTTCAAGG
mIL-18-R	CAGGCTGTCTTTTGTCAACGA
mCCL5-F	TTTGCCTACCTCTCCCTCG
mCCL5-R	CGACTGCAAGATTGGAGCACT
mTSLP-F	ACGGATGGGGCTAACTTACAA
mTSLP- R	ACTCCTCGATTTGCTCGAACT

RESULTS

Preliminary mouse studies.

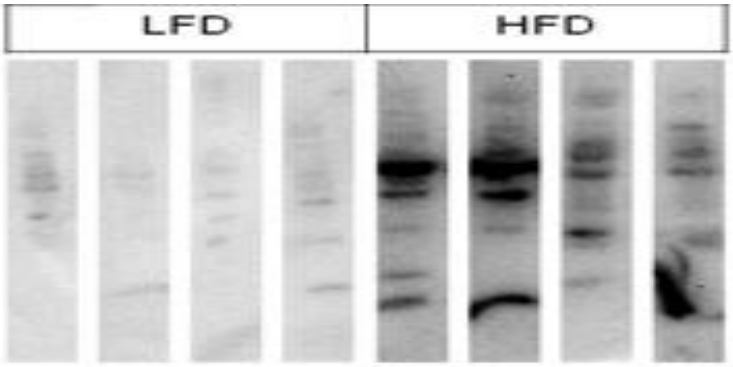
HFD mice have higher plasma IgG against invasive intestinal bacteria.

To investigate whether a high fat diet is associated with systemic immune responses against the intestinal bacteria, we performed Western Blots to determine whether there is an association between long term (10 weeks) HFD consumption and immunoglobulin G (IgG) against invasive intestinal bacteria. Our data showed that the plasma of the mice on the HFD had higher levels of IgG against antigens from invasive intestinal bacteria than the mice on the LFD (Figure 2.1).

HFD mice have higher anti-bacterial IgG.

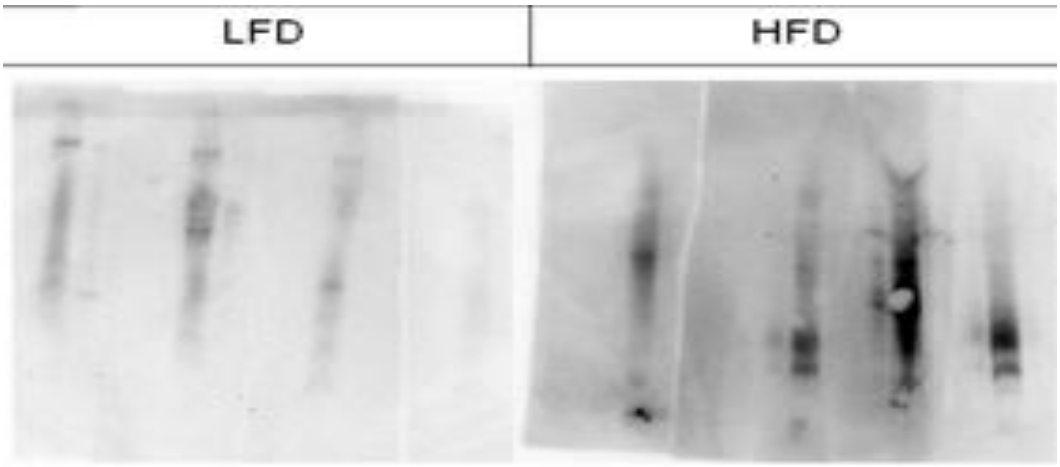
Western blot analysis was then performed to compare plasma IgG against the antigens from the cecums of LFD mice. Plasma IgG against the antigens present in the cecum were low in each of the LFD mice (left panel Figure 2.2). Plasma IgG against the antigens present in the cecum was high in each of the individual HFD mice.

Figure 2.1 Anti-*E. coli* LF82 IgG



PVDF membranes with *E. coli* LF82 protein extracts were cut into individual strips. Each strip was incubated in plasma from an individual mouse on a LFD (left) and individual mouse on a HFD (right). Plasma was diluted 1:10 in blocking reagent.

Figure 2.2 Anti-bacterial IgG.



Membrane strips were incubated in plasma from individual mice on a LFD (left) and individual mice on a HFD (right). Plasma was diluted 1:10 in blocking reagent.

LONG TERM FEEDING STUDY IN C57BL6 MICE.

Increased weight gain and fasting blood glucose in HFD mice.

As expected, HFD fed mice subsequently gained significantly more weight over the duration of the study than the mice on the LFD. Six weeks after commencing the special diets we observed a statistically significant difference between the bodyweights of LFD and HFD mice (Figure 2.3 A). Fasting blood glucose was measured using the TRUEtrack® glucose meter. After 10 weeks on the special diets, HFD mice had significantly higher fasting blood glucose levels (Figure 2.3 B, $p<0.05$).

Long term HFD consumption leads to increased plasma TNF-α in mice.

Plasma obtained from mice by cardiac puncture after the 10 week feeding regimen was analyzed for total TNF-α by the MILLIPLEX® Mouse Cytokine Kit. HFD mice had higher plasma TNF-α than LFD mice. The difference between the plasma TNF-α in the HFD mice and the LFD mice was significant (Figure 2.3 C, $p<0.05$).

Plasma IgG against invasive intestinal bacteria is increased in HFD mice.

HFD mice had significantly higher plasma IgG against protein extracts from the LF82 strain of *E. coli* (Figure 2.4 A). However statistical analysis revealed that there were no significant differences between LFD and HFD mice

in plasma IgG against *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus* (Figures 2.4 B, 2.4 C and 2.4 D).

Neutrophil percentage is increased in HFD mice.

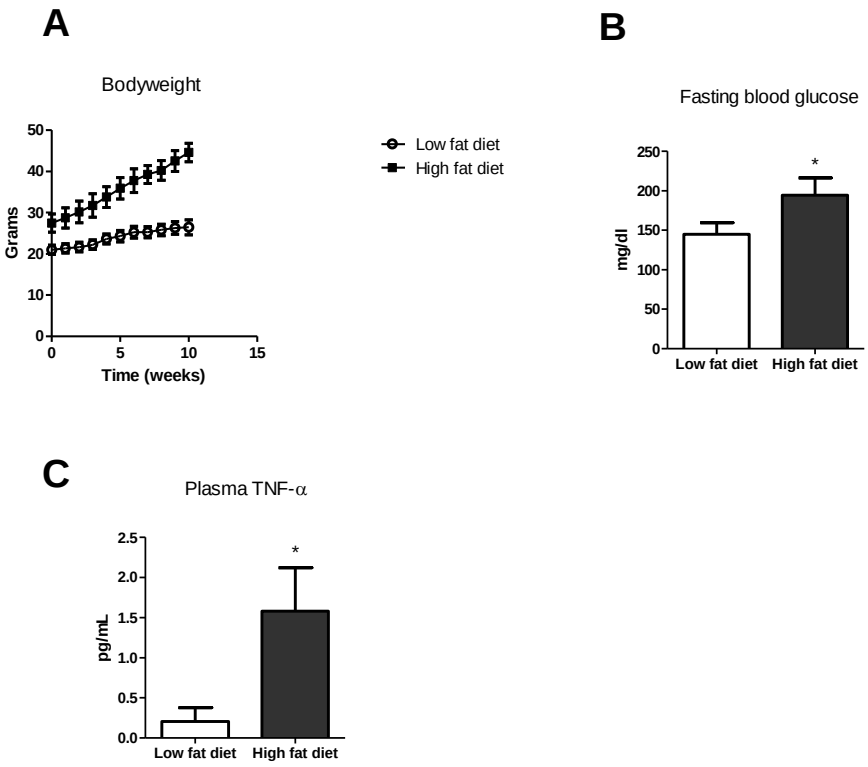
The HEMAVET® 950 FS Multispecies Hematology System was used in order to measure the inflammatory immune cell subsets. It was observed that the HFD mice had a higher percentage of neutrophils compared to LFD mice. Statistical analysis revealed that the difference between both groups was significant (Figure 2.5 A, $p < 0.05$). Lymphocyte percentage in the HFD mice was significantly lower than in the LFD mice (Figure 2.5 B, $p < 0.05$). No differences were observed between the LFD mice and the HFD mice in the percentages of the monocytes, eosinophils and basophils.

Long term HFD consumption promotes intestinal inflammation.

Real-time quantitative PCR was performed in order to determine whether inflammation was present in the GI tract of HFD mice. A variety of pro-inflammatory genes were analyzed. These included interleukin-18 (IL-18), chemokine (C-C motif) ligand 5 (CCL5) and the NOD-like receptor family pyrin domain containing 6 (NLRP6). These genes were chosen since they encode constituents associated with specialized inflammatory complexes against intestinal bacteria referred to as inflammosomes. The ileum and colon sections were of special interest since they harbor the highest densities of intestinal bacteria. There were no differences in IL-18 expression in the colon (Figure 2.6 A). However a trend towards increased CCL5 expression in the ileum was observed (Figure 2.6 B, $p = 0.19$). There was also a trend towards increased

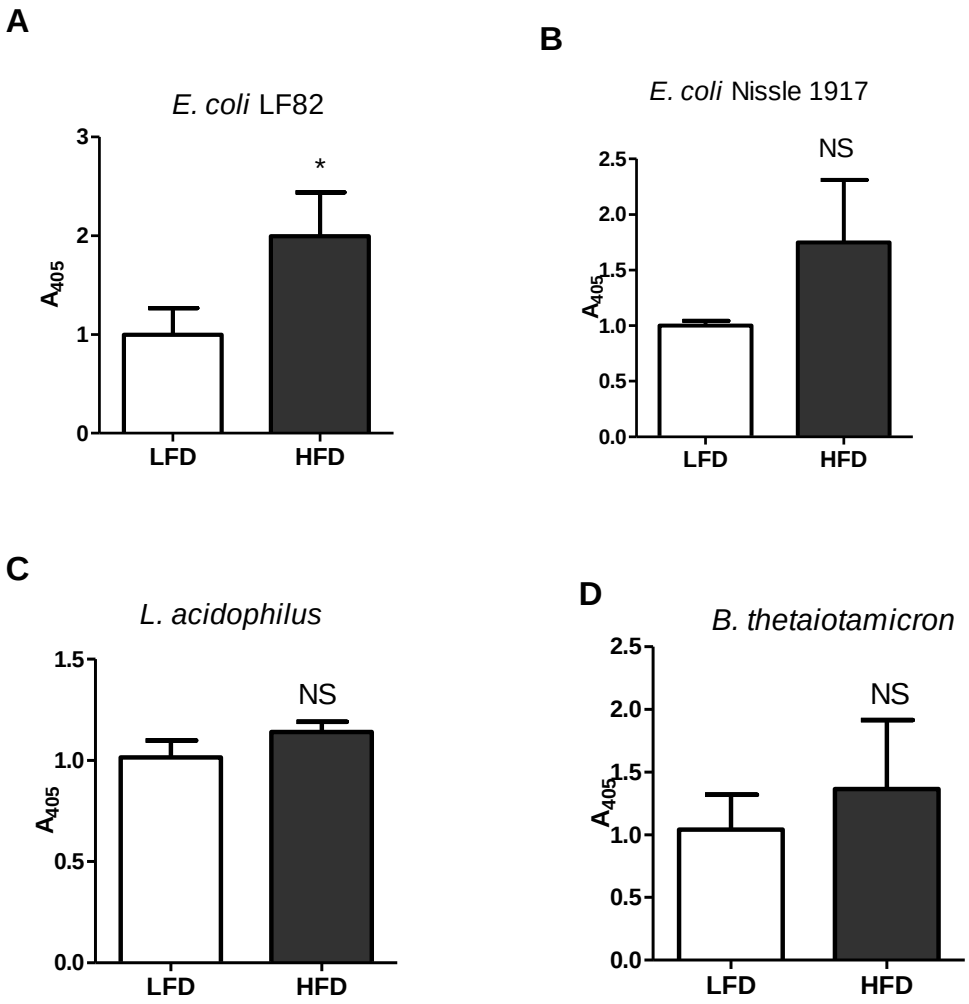
NLRP6 expression in the ileum of HFD mice (Figure 2.6 C, p = 0.1154). Thymic stromal lymphopoietin (TSLP) expression was measured to determine whether tolerance to the intestinal bacteria was impaired by long term HFD consumption. TSLP is secreted by the intestinal epithelial cells (IECs) to prevent the dendritic cells from promoting inflammatory responses. A trend towards decreased TSLP expression in the colon of HFD mice was observed (Figure 2.6 D, p = 0.1857). Collectively the trend towards increased inflammation and decreased tolerance provides evidence that the systemic inflammation observed in HFD mice originates in the GI tract.

Figure 2.3 Comparison of LFD mice and HFD mice during a long time feeding study



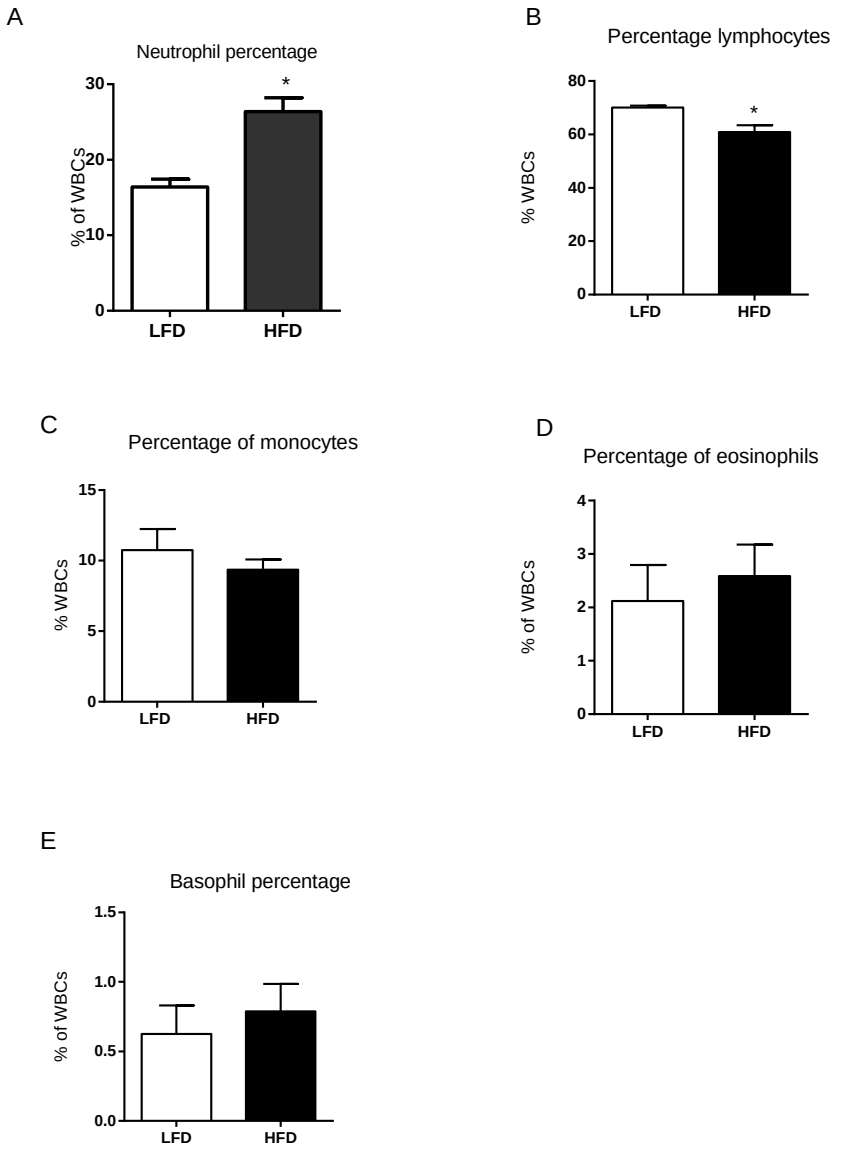
(A) HFD mice gained significantly more weight than LFD mice. (B) After ten weeks the fasting blood glucose levels in HFD mice was significantly higher than in LFD mice. (C) Plasma TNF-α is higher in HFD mice than in LFD mice. * indicates that $p < 0.05$

Figure 2.4*:* The effect of long term HFD consumption on plasma IgG against different intestinal bacteria in C57BL6 mice.



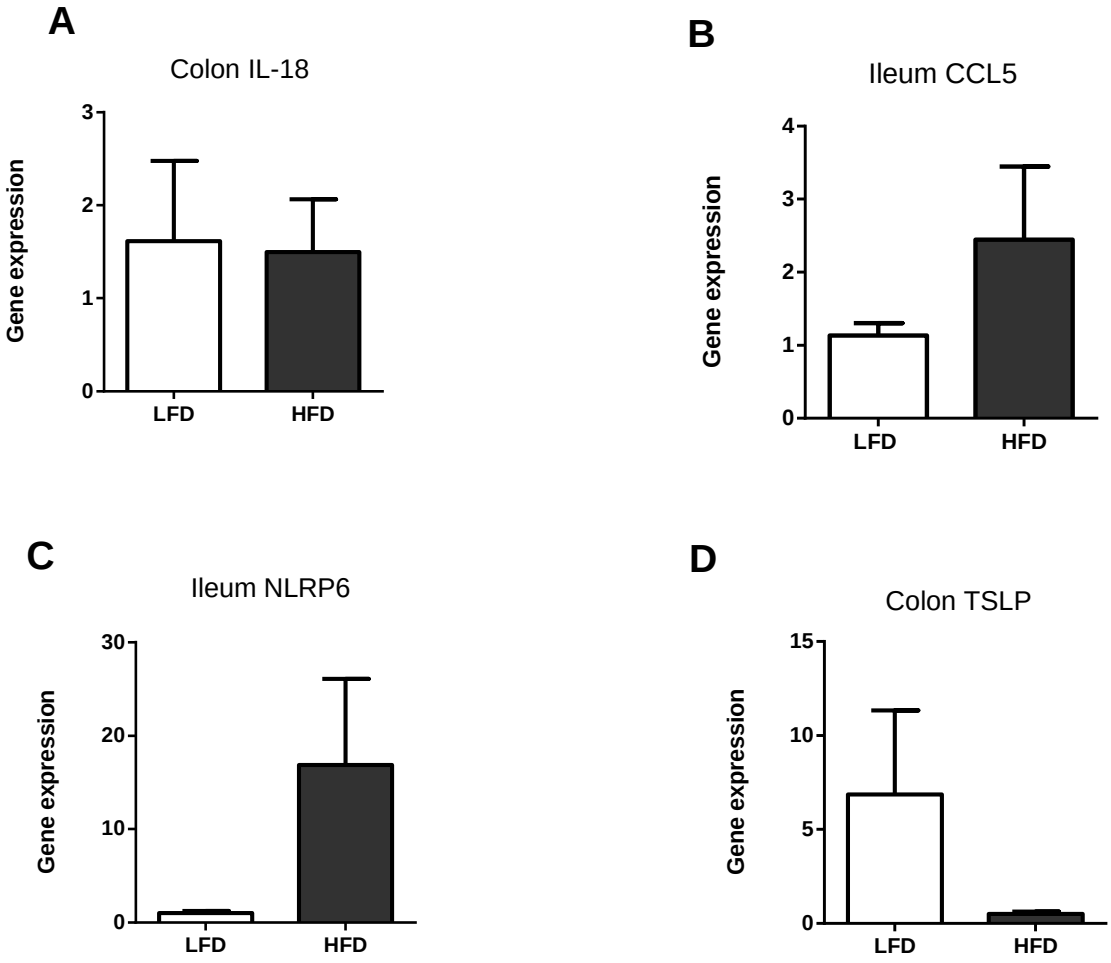
(A) HFD mice have increased plasma anti-*E. coli* LF82 IgG (B) There is no difference between HFD mice and LFD mice in plasma IgG against protein extracts from EcN (C) There is no difference between HFD mice and LFD mice in plasma IgG against *L. acidophilus* (D) No effect of HFD on plasma IgG against *B. thetaiotaomicron* * Indicates $p < 0.05$. For both groups $n = 6$. Plasma dilutions were 1:100.

Figure 2.5. Hematological analysis of LFD mice and HFD mice



(A) Neutrophil percentage is increased in HFD mice compared to LFD mice. (B) Lymphocyte percentage is decreased in HFD mice. (C) No differences in monocyte percentage. (D) No differences in eosinophil percentage (E) No differences in basophil percentage * indicates that $p < 0.05$, significant.

Figure 2.6 RT-PCR analysis of the ileums and colons of LFD mice and HFD mice.



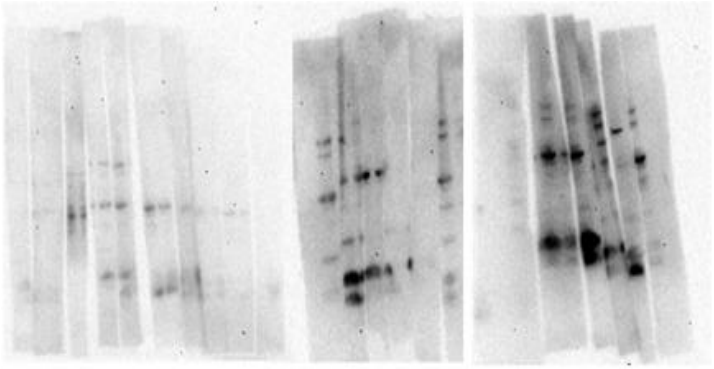
(A) There was no difference in IL-18 expression in the colon of LFD mice and HFD mice. (B) There was a trend towards increased CCL5 expression in the ileum of HFD mice compared to LFD mice (p =0.19) (C) There was a trend towards increased NLRP6 expression in the ileum in HFD mice (p = 0.1154). (D) There was a trend towards decreased TSLP expression in the colon in HFD mice (p = 0.1857)

HUMAN STUDIES

Obesity is associated with increased anti-*E. coli* LF82 IgG.

Plasma IgG against protein extracts from the LF82 strain of *E. coli* was low in all of the lean healthy controls. Plasma anti-*E. coli* IgG was high in all of the obese subjects. Obese diabetics had higher plasma IgG against protein extracts from the LF82 strain of *E. coli* IgG than the obese non-diabetics (Figure 2.7).

Figure 2.7: Western Blot Analysis of plasma from human test subjects



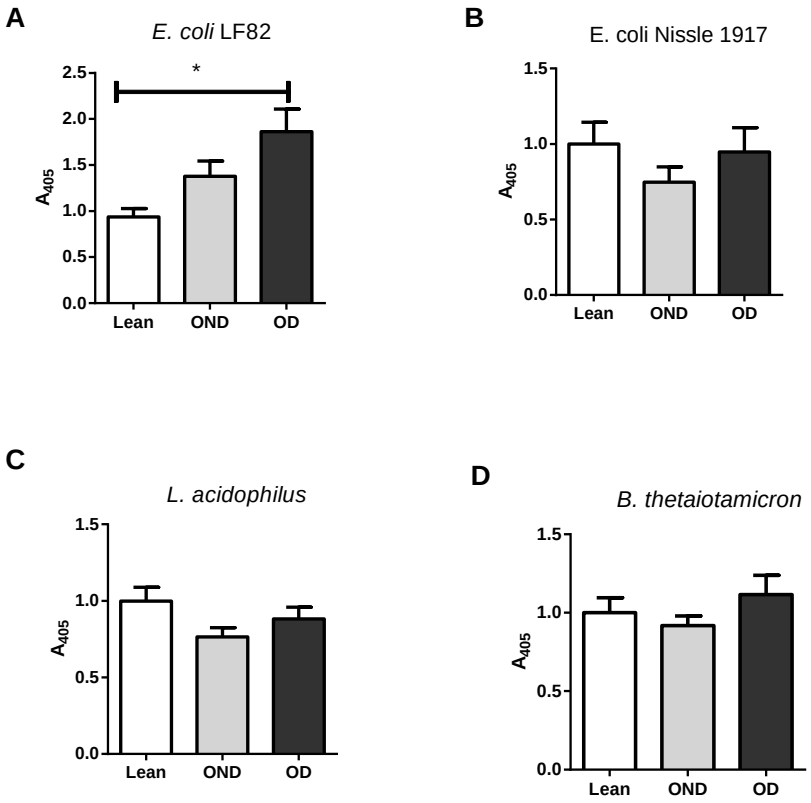
Membranes were cut into individual strips and incubated in 1:400 dilutions of human plasma. Left: strips blotted in plasma samples taken from lean health controls. Center: strips blotted in plasma from obese non-diabetics. Right: strips blotted in plasma obtained from obese diabetics.

IgG against *E. coli* LF82 extracts is increased in obese diabetics.

Obese diabetics had significantly higher plasma IgG against protein extracts from the LF82 strain of *E. coli* than the lean healthy controls (Figure 2.8 A). However, there were no differences in plasma IgG against *E. coli* Nissle

1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus* between the lean healthy controls, the obese non-diabetics and the obese-diabetics (Figures 2.8 B, 2.8 C and 2.8 D).

Figure 2.8 Plasma IgG against different intestinal bacteria human subjects



For each obese group n =16, healthy lean controls n=10 (A) Diabetics have significantly increased anti-*E. coli* LF82 IgG compared to lean controls (B) No differences in IgG against EcN (C) No differences in IgG against *L. acidophilus* (D) No difference in plasma IgG against *B. thetaiotaomicron* * indicates $p < 0.05$. Plasma dilutions of 1:400 were used.

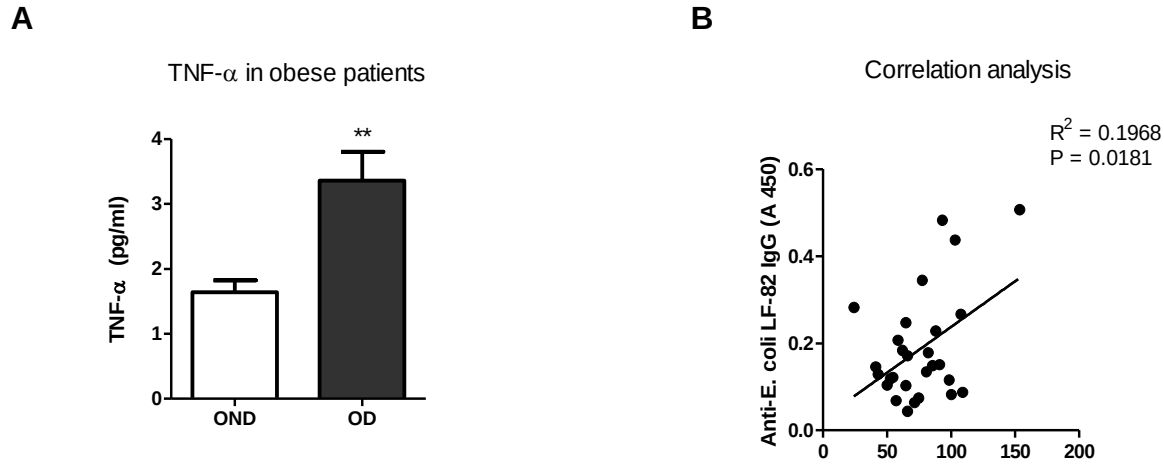
Obese diabetics have higher plasma TNF-α than obese non-diabetics.

We observed that plasma TNF-α was twofold higher in obese diabetics than obese non-diabetics. Statistical analysis revealed that the difference in plasma TNF-α in the obese diabetics and the obese non-diabetics was highly significant (Figure 2.9 A, $p<0.01$).

Human plasma TNF-α correlates with IgG against the LF82 strain of *E. coli.*

To further investigate the relationship between IgG against *E. coli* LF82 and plasma TNF-α, we investigated whether a correlation existed between both parameters. We observed that there was a statistically significant correlation between TNF-α levels and plasma IgG against the protein extracts from the *E. coli* LF-82 strain (Figure 2.9 B).

Figure 2.9 Analysis of plasma TNF-α in obese human subjects



For each group *n* =16 (A) Obese diabetics had significantly higher plasma TNF-α than the obese non-diabetics (B) There is a statistically significant correlation between TNF-α and plasma anti-*E. coli* LF82 IgG. ** indicates $p < 0.001$. Plasma dilution 1:2.

DISCUSSION

PRELIMINARY MOUSE STUDIES: HFD mice have higher plasma IgG against invasive intestinal bacteria.

These studies were commenced in March 2010. By that time there was accumulating evidence that the intestinal bacteria in conjunction with long term consumption of a high fat diet played a role in the development of systemic inflammation and insulin resistance. Our research group hypothesized that long term consumption of a high fat diet might cause a loss of systemic immune

ignorance to the intestinal bacteria. We postulated that long term HFD consumption would result in increased systemic immune responses to the intestinal bacteria. To do this we decided to look at systemic immune responses against bacteria found exclusively in the GI tract.

The intestinal bacteria are poorly characterized. We chose to measure plasma IgG against the LF82 strain of *E*. *coli* which was isolated from an ileal lesion of a Crohn's Disease patient [150]. *E. coli* LF82 is designated as an **a**dherent **i**nvasive ***E. c****oli* strain (AIEC strain). Invasive strains are characterized by their ability to enter epithelial cells and subsequently survive and replicate [151, 152]. *E. coli* LF82 invades a number of epithelial cell lines including Caco-2, Intestine-407, and HCT-8 cells [153]. *E. coli* LF82 induces altered cytoskeletal arrangements in epithelial cells which leads to its uptake by endocytic vacuoles. This bacterial strain is more prevalent in the lesions of Crohn's disease patients than in control patients without inflammatory bowel disease [154]. It should be emphasized that this bacterial strain is not present in the GI tract of mice. However, since it is an adherent and invasive strain, there may be antigens that are shared between this bacterial strain and uncharacterized invasive bacterial strains.

Our pilot western blot analysis of the plasma of LFD and HFD mice revealed drastic differences between the two groups. The plasma from HFD mice had higher levels of IgG against invasive intestinal bacteria than the mice on the LFD. Plasma IgG against invasive intestinal bacteria very low in all four individual LFD mice (Figure 3). The systemic immune system is oblivious to the presence

of the immense bacterial load of the GI tract. This is essential since the intestinal bacteria play important roles in vitamin synthesis and contribute to the overall health of the host individual.

The higher levels of plasma IgG against invasive intestinal bacteria in HFD mice suggested that these mice had lost systemic ‘ignorance’ to the bacteria in the GI tract. IgG is produced in response to the presence of antigens in the systemic circulation. This may lead to triggering of the systemic immune response against these intestinal bacterial pathogens, resulting in increased IgG the amount of antigenic material present in the systemic circulation. Therefore the higher plasma IgG against intestinal bacteria in HFD mice is indicative of a loss of systemic immune ignorance to invasive intestinal bacteria.

HFD mice have higher anti-bacterial IgG

As it has been previously mentioned, the GI tract contains at least 500 bacterial species. Although our finding that higher levels of anti- *E. coli* IgG in the plasma of HFD mice suggested that these mice had lost systemic ‘ignorance’ to the bacteria in the GI tract, we needed to establish whether there were higher levels of IgG against other bacterial species in the plasma of HFD mice.

However, this aim was complicated by two major factors. The first was the poor characterization of the composition of the intestinal bacteria. Additionally, most intestinal bacterial species are anaerobic. This makes the culturing of intestinal bacteria challenging. To overcome these problems cecal extracts were prepared. The cecum is a pouch shaped structure situated between the ileum

and the colon. It is situated in a region of the GI tract where bacterial population density is extremely high. It is therefore a reservoir of intestinal bacteria.

HFD mice had higher levels of plasma IgG against the antigenic contents of the cecum compared to the LFD control group (Figure 2.2). This further confirmed that HFD consumption is associated with a loss of systemic immune ignorance to the intestinal bacteria. This is because the systemic immune system is theoretically supposed to be oblivious to the contents of the cecum. Furthermore these findings strengthened our hypothesis since IgG against the intestinal bacteria of mice was measured. On the other hand, the LFD controls had low levels of IgG against the cecal contents. This may be due to the fact that these mice have not experienced any significant translocation of the intestinal bacteria into the systemic circulation. As a result, the levels of IgG against the antigenic components of the cecum are significantly much lower in the LFD controls than the HFD fed mice.

Results from long term feeding study in C57BL6 mice

The preliminary studies resulted in the observation that mice fed a high fat diet (Rodent Diets with 60 kcal% Fat D12492 from Research Diets Incorporated) for 10 weeks had elevated plasma anti-*E. coli* LF82 IgG. Although these findings were novel, they offered only a narrow range of interpretation. This is because the only parameter that had been examined thus far was plasma anti-*E. coli* LF82 IgG in relation to high fat diet consumption. To gain greater insight into the effects of long term HFD consumption on the systemic immune system a long term (ten week) feeding study was performed using C57BL6 mice.

Two parameters were used to define the end point of the study (i.e. the point at which the mice would be euthanized). These were body weight and fasting blood glucose. As expected, C57BL6 mice fed a HFD (60% calories from fat) gained significantly more weight than the mice fed a LFD (10% calories from fat), with bodyweights diverging significantly six weeks into the study (Figure 2.3 A). However, weight gain by itself is not an accurate indicator of the development of elevated fasting blood glucose. The mice were euthanized once a significant difference in fasting blood glucose levels was observed. Based on the proposed hypothesis a significant elevation in fasting blood glucose would be observed subsequent to systemic inflammation and bacterial translocation.

After 10 weeks the fasting blood glucose levels of the HFD mice were higher than the LFD mice (Figure 2.3 B). Statistical analysis revealed that these differences were significant ($p<0.05$) and it was at this point the study was terminated. The mice were euthanized and we were able to collect samples to evaluate a wide range of parameters.

Long term HFD consumption leads to increased plasma TNF-α in mice.

HFD mice had elevated plasma IgG against invasive intestinal bacteria. Previous studies have linked the IgG response to certain bacterial species to disease severity [155]. This is because cross-linking of FcγRs leads to inflammation [156, 157]. However, plasma IgG levels alone cannot be used to determine whether there is increased systemic inflammation. TNF-α is a widely used marker of inflammation. Since the proposed hypothesis states that the

immune responses to the intestinal bacteria lead to systemic inflammation, TNF-α was measured in LFD mice and HFD mice. Statistical analysis revealed that the plasma TNF-α in the HFD mice was higher than in the LFD mice (Figure 2.3 C; p <0.05). These findings strengthened the hypothesis that long term HFD consumption leads to systemic inflammatory immune responses being mounted against the bacterial antigens present in the lumen of the GI tract.

A possible explanation for the increased plasma TNF-α could be the increase in intestinal bacteria crossing the intestinal epithelial barrier. This could lead to immune responses being mounted by the B lymphocytes. The B lymphocytes produce IgG which bind to the antigens present on the surface of the intestinal bacteria. It is possible that the increase in systemic IgG against intestinal bacteria results in increased FcγR crosslinking. This in turn leads to the release of TNF-α.

Plasma IgG against invasive intestinal bacteria is increased in HFD mice.

HFD mice had higher plasma IgG against invasive intestinal bacteria than LFD mice (Figure 2.4 A). However, there were no significant differences in plasma IgG against protein extracts from *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus* in HFD and LFD mice (Figures 2.4 B, 2.4 C and 2.4 D). As it has been previously stated the systemic immune cells are naïve to the presence of the intestinal bacteria. This feeding study resulted in increased IgG against invasive intestinal bacteria in the HFD mice. These results further support the proposal that long term HFD consumption is associated with

increased IgG against antigens that are usually confined to the lumen of the GI tract. IgG is produced as a consequence of the B lymphocytes detecting the presence of a particular antigen. Thus the elevated IgG against invasive intestinal bacteria in HFD mice was possibly due to the fact that that the B lymphocytes were no longer oblivious to these antigens. A possible explanation for this is that long term HFD consumption promotes entry of the bacteria present in the lumen of the GI tract into the systemic circulation. This triggers systemic immune responses including IgG production.

Neutrophil percentage is increased in HFD mice.

A variety of immune cells participate in inflammatory reactions. Since the sample number for the mouse studies (n = 6 for each group) was relatively small, further evidence was needed that long term HFD consumption results in inflammatory immune responses. The HEMAVET® 950 FS Multispecies Hematology Systems is useful in characterizing the various blood cell types present. After the ten week feeding study was completed, and the mice were euthanized we characterized the various immune cells present in whole blood fractions. The neutrophil percentage in the blood of the HFD mice was significantly increased compared to the LFD mice (Figure 2.5 A, p <0.0001). The increase in neutrophil percentage in HFD mice provides further evidence that long term HFD consumption leads to increased systemic inflammatory immune responses against the intestinal bacteria. Neutrophils are present in the initial phase of inflammation [158]. They ingest infectious microbes and eliminate them. The intracellular destruction of microbes by neutrophils is mediated by their

cytoplasmic granules via superoxide production as well as the proteases elastase and cathepsin G [159].

A possible explanation for the increase in neutrophil percentage may be the increased plasma IgG against invasive intestinal bacteria in the HFD mice. Since FcγRs are expressed on the surface of neutrophils, there may be increased cross-linking of FcγRs with the Fc domains of the IgG against the intestinal bacterial antigens [160]. No significant differences were noted in monocyte percentage, basophil percentage or eosinophil percentage in the blood of HFD mice and LFD mice (Figures 2.5 C, 2.5 D and 2.5 E). This was not surprising since basophils are usually produced in response to infections by helminthes [161]. They are also usually associated with allergens and are associated with allergic reactions. Eosinophil percentage did not differ between the LFD and HFD mice. Eosinophils protect against parasitic infections [162]. Our discovery that long term consumption of a high fat diet specifically increases the percentage of neutrophils was significant. The increase in neutrophil percentage was observed along with increased plasma IgG against invasive intestinal bacteria, providing further evidence that the systemic immune responses against intestinal bacteria are inflammatory.

Long term HFD consumption promotes intestinal inflammation.

Real time PCR (RT-PCR) analysis was performed in order to determine whether the systemic inflammation we observed originated in the GI tract. It has been traditionally thought that the higher levels of inflammatory cytokines associated with high fat diet consumption originate in the adipose tissue [163].

However, there is a growing body of evidence which suggests that inflammation resulting from long term HFD consumption may originate in the gastrointestinal (GI) tract [65]. For example, it has been reported that long term HFD consumption may alter the bacterial composition of the GI tract [68]. Furthermore Ding *et al* observed that conventionally raised (CONV) mice consuming a high fat diet experienced intestinal inflammation [70]. By contrast GF mice fed a high fat diet did not exhibit intestinal inflammation.

Therefore it was crucial to determine whether the HFD mice experienced intestinal inflammation. The ileum and colon sections of the GI tract were analyzed for intestinal inflammation. This is because these regions of the GI tract harbor the largest bacterial populations. Different pro-inflammatory markers were measured. These were the NOD-like receptor family pyrin domain containing 6 (NLRP6), Chemokine (C-C motif) ligand 5 (CCL5 also known as RANTES) and IL-18. RT-PCR revealed a trend towards increased NLRP6 expression in the ileum HFD mice (LFD 1.000 ± 0.2285, HFD 16.90 ± 9.220, $p = 0.1154$). NLRP6 is thought to play a role in inflammation via the activation of NF-κB [164]. Although the difference between the LFD mice and the HFD mice was not statistically significant, this was probably due to the relatively small sample size (where n = 6) for each group. Although intestinal IL-18 did not differ between LFD and HFD mice, there was a trend towards increased CCL5 in the ileum of the HFD mice (LFD 1.136 ± 0.1660, HFD 2.444 ± 1.001, $p = 0.1900$). CCL5 plays a role in inflammation by recruiting macrophages [165]. Thus the trend towards increased

CCL5 expression in the GI tract supports the idea that the systemic inflammation observed originates from the GI tract.

Thymic stromal lymphopoietin (TSLP) expression was also measured. TSLP is constitutively secreted by the intestinal epithelial cells (IECs). The IECs secrete TSLP which prevents the dendritic cells (DCs) from promoting inflammatory immune responses. The DCs sample antigens and present them to T lymphocytes. RT-PCR showed a trend towards reduction of TSLP expression in the colon of HFD mice compared to the LFD controls (LFD 6.867 ± 4.469, HFD 0.5141 ± 0.1141, p = 0.1857). Since TSLP is crucial to preventing intestinal inflammation, the trend towards decreased TSLP expression provides further evidence that the systemic inflammation may have originated in the GI tract.

HUMAN STUDIES

We decided to investigate whether long term consumption of a high fat diet is associated with increased plasma IgG against invasive intestinal bacteria in humans. We made the assumption that humans who had consumed a high fat diet over a long time period would have body mass index values in the overweight and obese range (≥ 25). When we performed Western Blot analysis we observed what appeared to be striking differences between the three groups. Plasma IgG against protein extracts from the LF82 strain of *E. coli* was lowest in the lean healthy control group. Plasma anti-*E. coli* LF82 IgG was higher in the plasma of the obese individuals. Notably there appeared to be a difference in plasma anti-*E. coli* LF82 IgG between the non-diabetic individuals and diabetic

patients. The obese diabetics had higher plasma anti-*E. coli* LF82 IgG than their non-diabetic counterparts.

These findings provided further evidence that long term HFD consumption is associated with a loss of systemic immune ignorance to the intestinal bacteria. As it has been previously stated, the LF82 strain of *E*. *coli* is only known to reside in the GI tract. The low levels of plasma anti-*E. coli* LF82 IgG in the lean healthy controls was not surprising. The systemic immune system is 'ignorant' to the existence of the immense bacterial load of the GI tract. This is important for minimizing potentially harmful inflammatory immune responses against the immense reservoir of antigenic material in the GI tract. However, what was particularly striking was the difference in anti-*E. coli* LF82 IgG in obese non-diabetics and obese diabetics. Anti-*E. coli* LF82 IgG was higher in obese diabetics than in obese non-diabetics. This finding suggested that the systemic immune responses against intestinal bacterial pathogens may be linked to insulin resistance and the development of type 2 diabetes.

IgG against invasive intestinal bacteria is increased in obese diabetics.

Quantifying plasma IgG against the intestinal bacteria was crucial in determining whether the apparent differences observed in our Western Blot analyses had statistical significance. Furthermore, in order to determine whether any quantitative relationships existed between IgG against the intestinal bacteria and inflammatory cytokine levels we needed a reliable quantitative assay. As a consequence, we developed our own laboratory made ELISA by coating 96 well

flat-bottom ELISA plates with soluble protein extracts from *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus* and *E. coli* LF82 dissolved in a carbonate coating buffer. The difference between the plasma anti-*E. coli* LF82 IgG in lean controls and obese diabetics was significant (p = 0.0107). The lean controls had the lowest levels of plasma IgG against invasive intestinal bacteria. The low plasma anti- *E. coli* LF82 IgG in lean healthy controls is probably due to the fact that the immune system these individuals maintain a state of ignorance to the intestinal bacteria.

What was particularly interesting was that while obese diabetics had higher plasma anti- *E. coli* LF82 IgG than the lean controls, there was no difference in plasma anti-*E. coli* LF82 in obese non-diabetic individuals compared to the lean healthy controls. The obese non-diabetics had body mass index values that were statistically equivalent to the obese diabetic patients. This led us to hypothesize that plasma IgG against the intestinal bacteria may be related to the transition from a non-diabetic state to a diabetic state.

Immunoglobulin G is the major immunoglobulin subclass produced in response to the presence of antigens in the systemic circulation. The IgG response to certain bacterial species has also been correlated with disease severity [166]. IgG is comprised of two identical heavy chains and two identical light chains, arranged in a Y shaped configuration. The IgG molecule is comprised of an antigen binding fragment (Fab) domain and a crystalizeable fragment (Fc) domain. Receptors for the Fc domain of IgG (FcγRs) are located on the surface of immune cells. Inflammation results from the cross-linking of

FcγRs. It is possible that the increased IgG against intestinal bacteria could have resulted in increased FcγR cross-linking. This in turn could have led to increased systemic inflammation. Uncontrolled systemic inflammation can adversely affect insulin signaling pathways, leading to insulin resistance and the development of type 2 diabetes.

Human plasma TNF-α correlates with IgG against the LF82 strain of *E. coli.*

Statistical analysis was performed to determine whether there was a correlation between anti-*E. coli* LF82 IgG and TNF-α. This was done to establish a more definitive link between the immune responses against the intestinal bacteria and inflammatory markers. The sample size in the animal study (n=6 for each group) was insufficient to perform a correlation analysis.

The correlation analysis was performed using the human plasma samples. This is because the sample size for human subjects was larger (n=16 for each group, 32 in total). There were two groups of obese subjects: obese non-diabetics and obese diabetics. Both groups had mean BMI values that were statistically equivalent (obese non-diabetics mean BMI 41.1 ± 10.4 versus obese diabetics mean BMI 40.6 ± 7.1). As a consequence, a discrepancy in adiposity could be ruled out. It was observed that there was a statistical correlation between anti-*E. coli* LF82 IgG and plasma TNF-α in the human subjects. The correlation between anti-*E. coli* LF82 IgG and TNF-α is further evidence that the systemic immune responses to the intestinal bacterial antigens are associated with inflammation. It is possible that the increase in IgG against intestinal

antigens promotes cross-linking of FcγRs, which in turn causes the release of pro-inflammatory markers such as TNF-α [167].

The correlation between anti-*E. coli* LF82 IgG and plasma TNF-α linked inflammation to the immune responses against intestinal antigens. If systemic inflammation was linked to the development of type 2 diabetes, then theoretically the obese diabetics would have higher levels of plasma TNF-α. To determine whether this was the case, TNF-α in the human plasma samples was measured by ELISA. It was observed that there was a difference in plasma TNF-α between both groups of obese subjects. TNF-α in the obese diabetics was significantly higher than in the obese non-diabetics. This finding provides further evidence that the inflammation resulting from intestinal bacterial antigens may lead to the development of type 2 diabetes. It is possible that insulin signaling could have been adversely affected by the presence of high levels of plasma TNF-α.

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CHAPTER 3 HIGH FAT DIET CONSUMPTION AND INTESTINAL IMMUNITY.

INTRODUCTION

Immunoglobulin A (IgA) actively secreted into the GI tract minimizes the adherence of bacteria to the intestinal epithelium. In our initial studies we observed an association between long term consumption of a HFD and elevated plasma anti-*E. coli* LF82 IgG. As a consequence, we investigated whether long term HFD consumption impairs IgA secretion into the lumen of the GI tract. To do this we measured fecal IgA in mice fed a LFD (10% calories from fat) and mice fed a HFD (60% calories from fat). Plasma IgA levels were measured in LFD and HFD mice. IgA is the second most abundant immunoglobulin in the plasma. Plasma IgA neutralizes antigens and suppresses inflammatory effects of antibody dependent cellular toxicity [168]. Furthermore, small quantities of plasma IgA enter the intestinal mucosal secretions by paracellular diffusion [131].

A goal of this study was to assess whether the absence of the active secretion of IgA into the lumen of the GI tract by the pIgR is a predisposing factor for development of metabolic syndrome. The polymeric immunoglobulin receptor (pIgR) actively secretes polymeric immunoglobulins into the lumen of the GI tract. An estimated 3 grams of IgA are secreted daily. Since this involves considerable energy expenditure, we reasoned that this specialized secretory mechanism plays a role in preventing adherence of the commensal bacteria to the intestinal epithelial cells.

. Our previous studies had shown that long term HFD consumption was associated with increased IgG against adherent invasive intestinal bacteria. We postulated that perhaps mice lacking pIgR (pIgR KO mice) might be more susceptible to intestinal epithelial cell adherence and invasion. This could result in inflammatory immune responses being mounted against the resident gut bacteria. To determine whether this was the case, pIgR knockout mice and C57BL6 wild type littermate controls were placed on a Western Diet (Rodent Diets with 41 kcal% Fat, 43 kcal% Carbohydrate and 17 kcal% Protein). A Western Diet feeding regimen was chosen since the Western Diet is representative of the diets that are associated with metabolic disorders in humans.

The human plasma samples were also analyzed by total IgA ELISA. This was done in order to determine whether plasma IgA levels differed between the lean healthy controls, obese non-diabetics and obese diabetics. It was observed that plasma IgA differed between the three groups of human subjects. Lean controls had the highest levels of plasma IgA. Obese non-diabetics had significantly reduced plasma IgA compared to the lean controls. Obese diabetics have significantly reduced plasma IgA compared to both obese non-diabetics and lean controls.

METHODS

Determining the effect of long term HFD consumption on fecal IgA

C57BL6 mice aged 6 weeks were divided into two equal groups (n = 6). One group was fed a high fat diet (Rodent Diets with 60 kcal% Fat D12492 from Research Diets Incorporated). The other group was fed a low fat diet (Rodent Diets with 10 kcal% Fat D12450B from Research Diets Incorporated). Details of these diets are listed in table 2.1. The mice were placed on the special diets for 10 weeks.

Fecal IgA extraction

Fecal IgA was extracted utilizing a protocol previously employed by Ferguson e*t al* [169]. Fecal IgA extraction buffer was prepared utilizing phosphate-buffered saline (PBS, pH 7.4), 0.5% Tween® 20 and complete, EDTA-free protease inhibitor cocktail. The extraction buffer was added to each tube at a ratio of 1 ml of buffer to 0.10 g of feces. The samples were manually homogenized and the resulting mixtures were vortexed. The resulting fecal suspensions were centrifuged at 14,000 rpm for 20 minutes and the supernatants were collected for further use.

Total fecal IgA measurements

Total fecal IgA was quantified using an ELISA for Mouse IgA from MABTECH. 96 well flat-bottom ELISA plates (BD-Falcon) were coated overnight with monoclonal anti-IgA antibody diluted 2µg/ml in PBS (pH 7.4). Blocking was performed for one hour at room temperature using incubation buffer (PBS with

0.05% Tween 20 and 0.1% BSA). Subsequent to blocking fecal IgA extracts were diluted 100X in incubation buffer and added in triplicate. Total IgA was detected using anti-IgA-ALP diluted 1:500 in incubation buffer. P-nitrophenyl phosphate was then added and the optical density was measured at 405nm using a Bio-Rad microplate reader. IgA concentration was divided by the mass of fecal pellets to give the quantity of IgA present per gram of feces (IgA/g feces).

Quantifying total fecal output and food intake.

To gain an accurate estimate of total fecal IgA production per day total daily fecal output was measured in a separate group of C57BL6 mice. C57BL6 mice were divided into two groups. One group was fed a high fat diet (Rodent Diets with 60 kcal% Fat D12492 from Research Diets Incorporated). The other group was fed a low fat diet (Rodent Diets with 10 kcal% Fat D12450B from Research Diets Incorporated).

Total fecal output**:** Mice were placed in a cage with clean bedding, with free access to food and water. Fecal pellets were collected at intervals of 24 hours and weighed. It should be noted that metabolic cages were available from the **COCVD PHYSIOLOGIC RESEARCH CORES** at the University of Kentucky. However, the Eckhardt Laboratory did not have the required protocol approvals to measure fecal output in mice using these facilities.

Quantifying food intake**:** Mice were given free access to food and water. Food was weighed at 24 hour intervals. The weight of food was subtracted from the previous weight to give total consumption. This was then divided by the

number of mice in the cage to give an estimate of the amount of food consumed by each mouse.

Effect of HFD consumption on plasma IgA.

The effect of HFD consumption on plasma IgA was assessed in HFD mice and LFD mice. Plasma IgA was measured using the ELISA for Mouse IgA. 96 well flat-bottom ELISA plates (BD-Falcon) were coated overnight with monoclonal anti-IgA antibody diluted 2µg/ml in PBS (pH 7.4). Blocking was performed for one hour at room temperature using incubation buffer (PBS with 0.05% Tween 20 containing 0.1% BSA). Subsequent to blocking plasma diluted 100X in incubation buffer was added in triplicate. Total IgA was detected using anti-IgA-ALP diluted 1:500 in incubation buffer. P-nitrophenyl phosphate was then added and the optical density was measured at 405nm using a Bio-Rad microplate reader.

To evaluate whether impaired IgA secretion increases the risk of developing metabolic syndrome.

Mice: Six pIgR knockout mice (pIgR KO mice) on a C57BL/6 background were kindly donated by Dr. Charlotte Kaetzel (Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky). pIgR KO mice are unable to actively secrete polymeric IgA into the lumen of the GI tract. This is because the third exon of the PIGR locus has been disrupted by the use of a targeting vector [170]. For controls we utilized wild type C57BL/6 littermates (n=7). Mice were housed three per cage and maintained in a 12h light / dark cycle. We allowed the mice to become acclimated to their new environment.

When the mice were obtained they were being fed the Teklad Global 18% Protein Rodent Diet (hereafter referred to as regular chow diet). Both groups of mice were maintained on the regular chow diet for one week after receiving them. The reason for doing this was to measure a variety of baseline metabolic parameters. The mice were then switched to a high fat, high carbohydrate Western Diet (Rodent Diets with 41 kcal% Fat, 43 kcal% Carbohydrate, 17 kcal% Protein). Details of the diets are listed in table 2.1. The mice were fed the Western Diet for 16 weeks.

Processing of samples for analysis.

Mice were euthanized using carbon dioxide. Blood was immediately collected by cardiac puncture and placed into EDTA containing eppendorf tubes that had been kept on ice. The majority of blood obtained was immediately centrifuged at 8,000 rpm for 10 minutes. The plasma was collected and stored at -82°C. The remaining blood (whole blood fraction) was used for hematological analysis. After mice were euthanized the ileums and colons were harvested. Intestinal mucosal scrapings were collected and frozen immediately in liquid nitrogen.

Identification of mice.

After the mice were obtained they were implanted with IPTT-300 transponders (Vendor BMDS) for identification. After the mice were euthanized, they were identified and tail snips were collected from individual mice. DNA was extracted from the tail snips using the Qiagen DNeasy Blood and Tissue kit (QIAGEN Catalog# 69504). Polymerase chain reaction (PCR) was then

performed using a Perkin Elmer GeneAmp PCR System 9600. The primer sequences to detect the Pigr mutant gene (Pigr KO) and the wild type Pigr gene (Pigr WT) are listed in table 2.3.

Body weight and body fat analyses

pIgR KO mice and the C57BL/6 controls were weighed prior to commencing the Western Diet feeding regimen. Once the feeding study was started the mice were weighed weekly. EchoMRI™ analysis was performed in order to determine whether there were differences in body fat percentage.

Plasma cytokine analysis

To assess the levels of circulating inflammatory cytokines we utilized MILLIPLEX® Mouse Cytokine Kits (from Millipore). The first kit (Catalog # MPXMYCTO-70K) quantified the cytokines IL-1b, IL-6, IL-10 and TNF-α, IL-12, IL-7, IL-5 and IL-13 prior to commencing the Western Diet, as well as two and sixteen weeks after commencing the Western Diet feeding regimen. The second kit used (Catalog # MADPK-71K) measured insulin, resistin, leptin, PAI-1 and TNF-α eight and sixteen weeks after commencing the Western Diet feeding regimen.

Hematological analysis.

50µl of whole blood fractions collected from the mice after euthanasia was used. The tubes containing the whole blood was placed on a rocker for 2 minutes. Samples were then analyzed using a HEMAVET® 950 FS Multispecies Hematology Systems (Drew Scientific Inc.).

Oral glucose tolerance test (OGTT)

To assess oral glucose tolerance, mice were fasted for 6 hours. Blood was collected from the tail tips of mice and blood glucose levels were recorded using the TrueTrack glucose meter (Home Diagnostics Inc). The mice were gavaged with a dose of 2.0g/kg glucose. Blood glucose measurements were recorded at 15, 30, 60, 90 and 120 minutes post-gavage. We performed the OGTT prior to commencing the Western Diet feeding and two weeks, ten weeks and sixteen weeks after commencing the Western Diet feeding regimen.

Real time PCR analysis

We compared the levels of inflammatory markers in the intestinal tissue of pIgR KO mice and C57BL6 controls. After mice were euthanized the small intestine and colon were harvested. RNA was extracted from intestinal tissues by the E.Z.N.A® Total RNA Kit. The RNA was converted into cDNA using the qScript™ cDNA Synthesis Kit. We analyzed the samples for expression of TNF-α and IL-6 using a BIORAD CFX96™ Real Time PCR detection system.

Statistical analyses.

Results were expressed as mean ± S.E.M and were analyzed with GraphPad Prism v5.04. Groups were compared with unpaired Student's t-tests and ANOVA and Bonferroni's post-hoc analysis. Statistical significance was assumed when $p<0.05$. Area under the curve analyses were performed using SigmaPlot.

ANALYSIS OF HUMAN PLASMA IgA.

We also measured plasma IgA in humans. Details of the human plasma donors are listed in table 2.2. Total plasma IgA in the humans was assessed with a Human IgA ELISA kit from MABTECH (3860-1AD-6). 96 well flat-bottom ELISA plates (BD-Falcon) were coated overnight with MT57 antibody diluted 2µg/ml in PBS (pH 7.4). Blocking was performed with incubation buffer and human plasma was diluted 400X in incubation buffer and added in triplicate. Total IgA present in human plasma was detected by adding a 1000 fold dilution of MT20-ALP. P-nitrophenyl phosphate was then added and the optical density was measured at 405nm using a Bio-Rad microplate reader.

RESULTS

HFD mice have reduced fecal IgA.

The effect of diet on secretion of IgA into the lumen of the GI tract was studied in C57BL6 mice fed a HFD and a LFD. During the first three weeks of the study statistical analysis revealed that there were no differences between the fecal IgA levels in the LFD mice or the HFD mice. However, by the fourth week of the study fecal IgA levels between LFD and HFD mice had diverged significantly. The LFD mice had significantly higher fecal IgA than the HFD mice. Higher fecal IgA in the LFD mice compared to the HFD mice was observed throughout the duration of the feeding study (Figure 3.1 A). Analysis of the fecal IgA levels in the LFD mice and HFD mice revealed that there was an increase in fecal IgA over time in the LFD mice. On the other hand, the levels of fecal IgA in the HFD mice remained relatively unchanged. Thus the differences noted between fecal

IgA in LFD and HFD mice were a consequence of increased fecal IgA in LFD mice relative to fecal IgA in HFD mice.

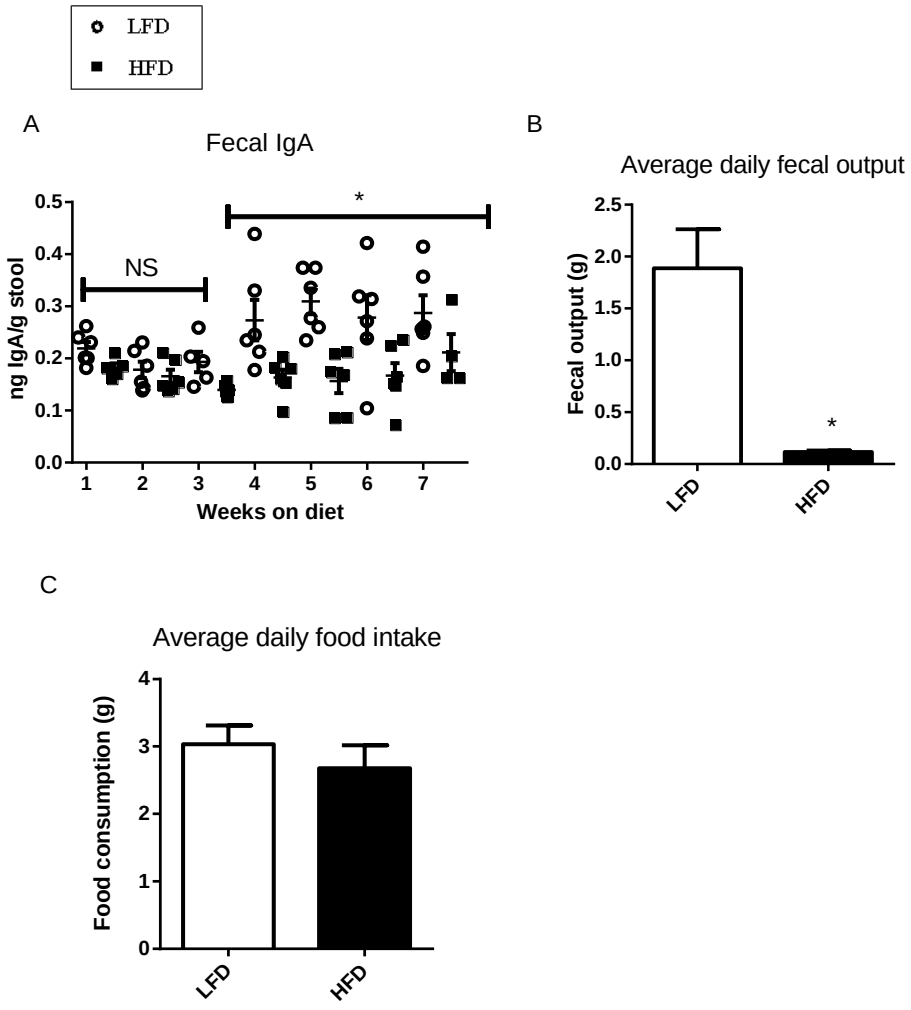
HFD consumption reduces fecal output.

In order to obtain a more accurate estimate of fecal IgA output, total fecal output was measured. HFD consumption reduced the fecal output in C57BL6 mice compared to LFD controls. Statistical analysis revealed that this reduction was highly significant (Figure 3.1 B, $p<0.01$).

Diet does not alter food intake.

Since the C57BL6 mice were fed different special diets, we wanted to investigate whether this led to any changes in food intake. Statistical analysis revealed that there were no significant differences in food intake between the LFD mice and the HFD mice (Figure 3.1C).

Figure 3.1 Effect of diet on fecal IgA.

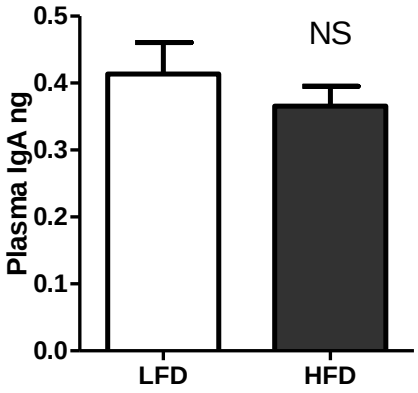


(A) Fecal IgA change over time. No differences in fecal IgA for the first three weeks of the study (NS). From week four until the end of the study fecal IgA in LFD mice was higher than HFD mice. (B) Fecal output was significantly reduced in C57BL6 mice fed a HFD compared to C57BL6 mice fed a LFD (C) No difference in food intake in C57BL6 mice fed a HFD compared to C57BL6 mice fed a LFD. * indicates $p < 0.05$.

No effect of HFD consumption on plasma IgA.

Plasma IgA from C57BL6 mice fed special diets (LFD and HFD) for 10 weeks of was measured by total IgA ELISA. There was no significant difference in plasma IgA between HFD mice and LFD mice (Figure 3.2).

Figure 3.2 The effect of HFD consumption on plasma IgA in C57BL6 mice.



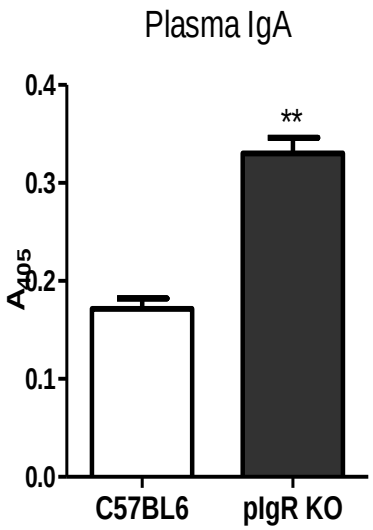
The effect of HFD consumption on plasma IgA in C57BL6 mice. Total plasma IgA was measured in HFD mice (n=6) and LFD mice (n=6). Statistical analysis revealed that plasma IgA did not differ between LFD mice and HFD mice.

To assess whether impaired IgA secretion into the lumen of the GI tract is a predisposing factor for development of metabolic syndrome.

pIgR KO mice have higher plasma IgA.

To investigate whether long term consumption of a high-fat, high-carbohydrate Western Diet would affect plasma IgA in pIgR KO mice a total IgA ELISA was performed. Plasma IgA in the pIgR KO mice was higher than in the wild type C57BL6 controls. Statistical analysis revealed that this difference was highly significant (Figure 3.3; $p < 0.01$).

Figure 3.3 Plasma IgA in pIgR KO mice and wild type C57BL6 controls.



The pIgR KO mice had higher plasma IgA compared to the C57BL6 WT controls. ** indicates $p < 0.001$. Plasma dilution 1:100.

Impaired IgA secretion does not affect weight gain or fat accumulation in Western Diet fed mice.

Previous studies have linked the intestinal bacteria to increased weight gain and body-fat accumulation [67, 171]. Considering the magnitude of IgA transport by the pIgR, it is plausible that impaired IgA secretion could lead to changes in the composition of the intestinal bacteria. This could result in changes in bodyweight and fat accumulation. The bodyweights of pIgR KO mice and wild type C57BL6 controls were measured prior to commencing the Western Diet feeding regimen.

Both groups of mice showed similar patterns of weight gain throughout the duration of the feeding study. At no point in the study did the bodyweights differ significantly between the C57BL6 mice and the pIgR KO mice (Figures 3.4A and 3.4B). EchoMRI™ analysis gave similar body fat percentage values for the C57BL6 controls and the pIgR KO mice (Figure 3.4C). These results suggest that the absence of the polymeric immunoglobulin receptor does not significantly alter the accumulation of body fat or weight gain.

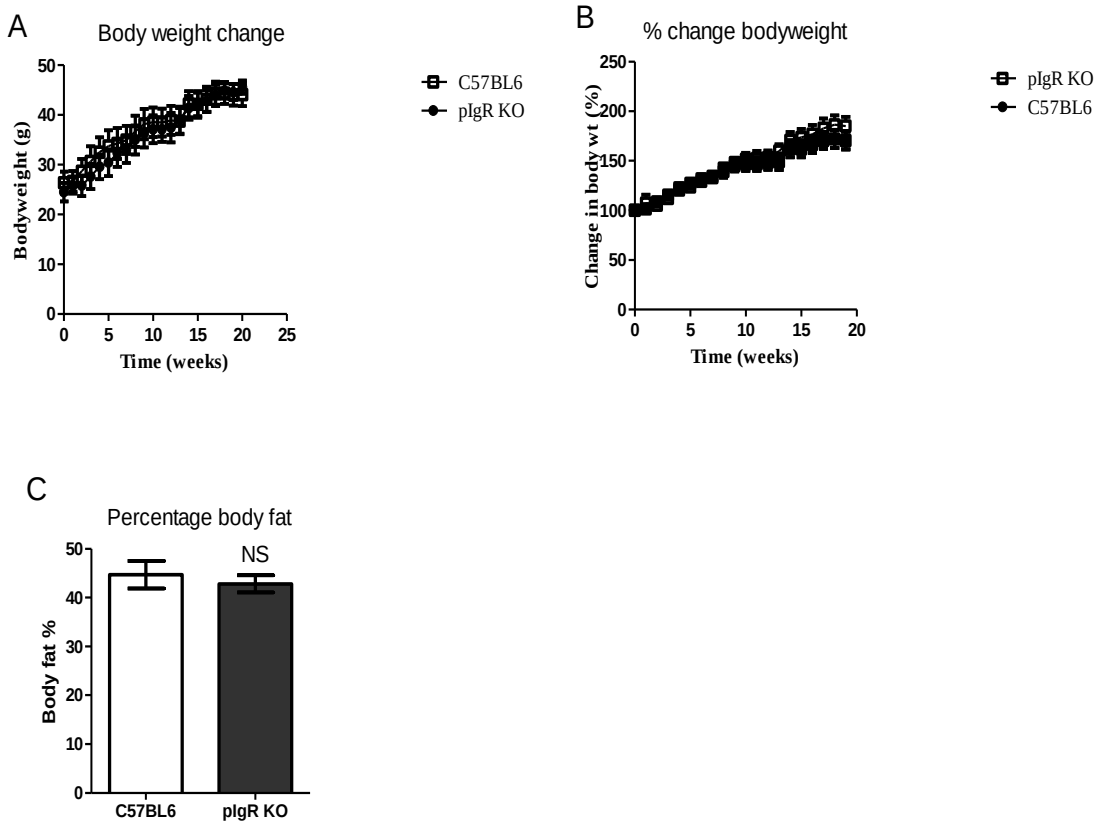
Absence of pIgR does not increase plasma inflammatory markers in Western Diet fed mice.

Plasma from the C57BL6 wild type controls and the pIgR KO mice was analyzed by MILLIPLEX® Mouse Cytokine Kits to see if consumption of a high-fat, high-carbohydrate Western Diet in conjunction with impaired IgA secretion would promote increased systemic inflammation. The plasma from the C57BL6 controls and the pIgR KO mice was analyzed prior to commencing the Western

Diet as well as two and sixteen weeks after commencing the Western Diet feeding regimen. No differences were observed between the C57BL6 controls and the pIgR KO mice in plasma IL-1b, IL-6, IL-10 and TNF-α at any time point (Figures 3.5 A, 3.5 B, 3.5 C and 3.5 D). No differences were observed in plasma IL-13, IL-12, IL-5 and IL-7 (Figures 3.6 A, 3.6 B, 3.6 C and 3.6 D). These results indicate that there are no baseline differences in plasma inflammatory cytokines between C57BL6 controls and pIgR KO mice prior or subsequent to consuming a high-fat, high-carbohydrate Western Diet.

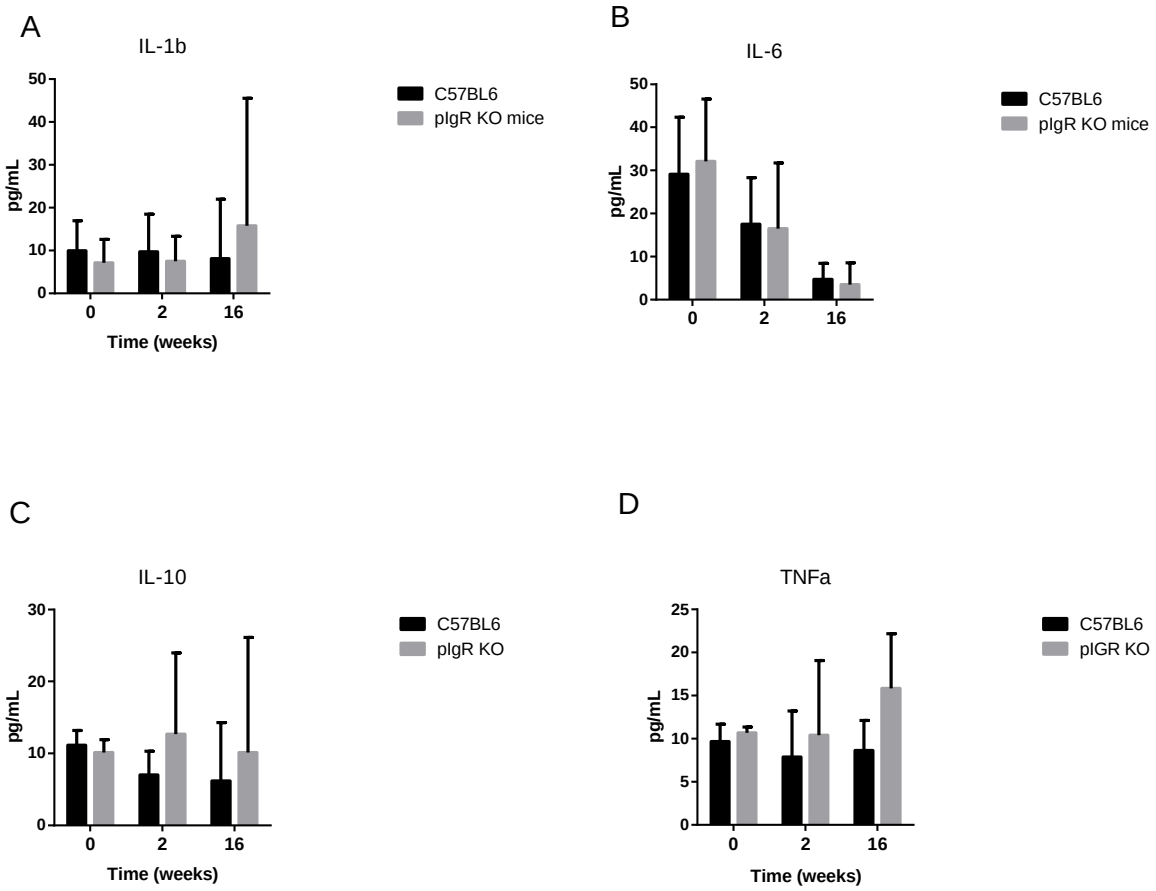
Plasma adipokine levels were also analyzed subsequent to commencing the Western Diet feeding regimen. Notably, the pIgR KO had reduced plasma PAI-1 compared to the C57BL6 controls. Statistical analysis revealed that this difference was significant (Figure 3.7 B). There were no differences between the C57BL6 controls and the pIgR KO mice in plasma insulin or resistin (Figures 3.7 A and 3.7 C). Plasma IL-6 and TNF-α were below the detectable range in the C57BL6 controls and the pIgR KO mice. These results provide evidence that the absence of the polymeric immunoglobulin receptor does not promote increased systemic inflammation either in the presence or absence of high-fat diet consumption. It should be noted that plasma adipokine levels were not measured in these mice prior to commencing the Western Die and as a result there is no baseline date for the plasma adipokine values.

Figure 3.4. **Bodyweight and body fat analyses**



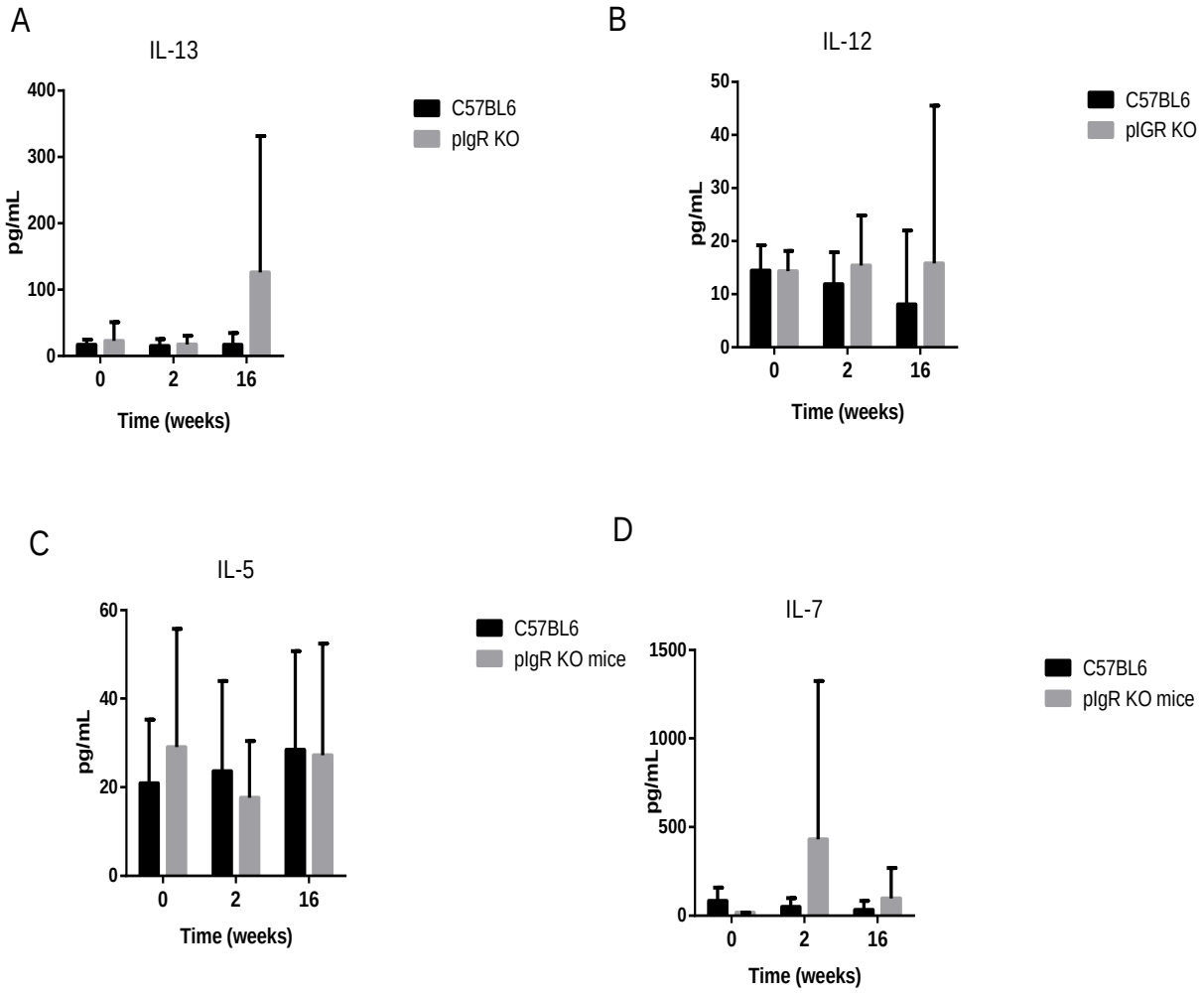
(A) C57BL6 controls and pIgR KO mice had similar bodyweights throughout the duration of the study (B) Percentage of bodyweight relative to the initial bodyweight was calculated. There was no difference in percentage weight gain. (C) There was no significant difference in percentage body fat between C57BL6 mice and pIgR KO mice.

Figure 3.5 Plasma cytokine levels.



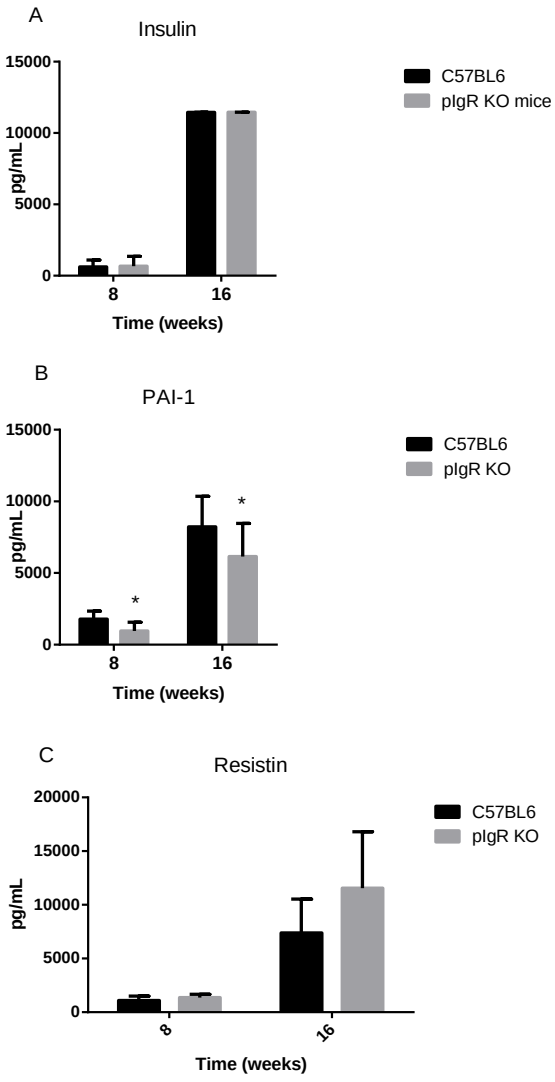
Analysis of plasma from the C57BL6 wild type controls (n = 7) and the pIgR KO mice (n = 6) by the MILLIPLEX® Mouse Cytokine Kit (Millipore Catalog # MPXMYCTO-70K) after consumption of Western Diet at different time points. There were no differences between pIgR KO mice and C57BL6 controls in (A) Plasma IL-1b (B) Plasma IL-6. (C) Plasma IL-10 (D) Plasma TNF-α.

Figure 3.6 Plasma cytokine levels.



Analysis of plasma from the C57BL6 wild type controls (n = 7) and the pIgR KO mice (n = 6) by the MILLIPLEX® Mouse Cytokine Kit (Millipore Catalog # MPXMYCTO-70K) after consumption of Western Diet at different time points. There were no differences between pIgR KO mice and C57BL6 controls in (A) Plasma IL-13. (B) Plasma IL-12 (C) Plasma IL-5. (D) Plasma IL-7.

Figure 3.7 Plasma adipokines

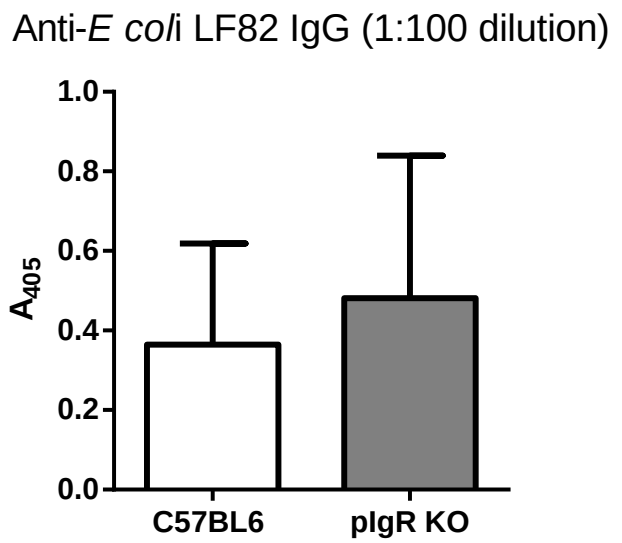


Analysis of plasma from the C57BL6 wild type controls (n = 7) and the pIgR KO mice (n = 6) by the MILLIPLEX® Mouse Cytokine Kit (Millipore Catalog # MADPK-71K) after consumption of Western Diet at different time points (A). There were no differences in plasma insulin (B) Plasma PAI-1 was higher in C57BL6 wild type controls than pIgR KO mice. (C) No difference in plasma resistin. * indicates $p < 0.05$

Absence of pIgR does not result in increased plasma IgG against intestinal bacteria.

Plasma anti-*E. coli* LF82 IgG was measured to determine whether the absence of the polymeric immunoglobulin receptor leads to enhanced epithelial invasion by invasive intestinal bacteria. There were no differences between the C57BL6 wild type controls and the pIgR KO mice in plasma IgG against invasive intestinal bacteria (Figure 3.8).

Figure 3.8 Anti-*E coli* LF82 IgG in plasma



Measurement of anti-*E. coli* LF82 IgG in plasma from the C57BL6 wild type controls (n = 7) and the pIgR KO mice (n = 6). There was no difference in plasma anti-*E. coli* LF82 IgG between both groups. Plasma dilution 1:100.

Absence of pIgR does not alter the percentages of immune cell subsets after long term Western Diet consumption.

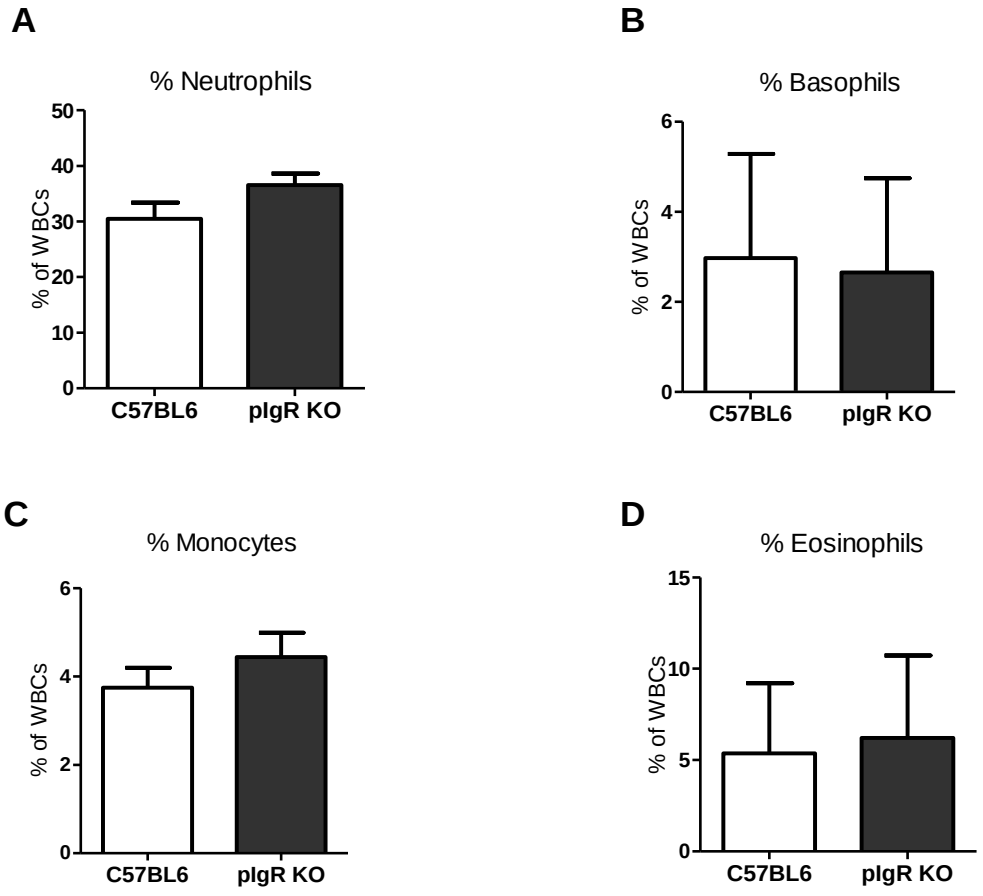
In order to determine whether the absence of the polymeric immunoglobulin receptor leads to increased inflammation after consuming a high-fat Western Diet, inflammatory immune cells were measured by the HEMAVET® 950 FS Multispecies Hematology System. Hematological analysis of whole blood revealed that there were no differences between the C57BL6 wild type controls and the pIgR KO mice in neutrophil percentage, basophil percentage, monocyte percentage and eosinophil percentage (Figures 3.9 A, 3.9 B, 3.9 C, and 3.9 D).

Absence of pIgR does not promote increased intestinal inflammation after long term Western Diet consumption.

Real time PCR (RT-PCR) was performed in order to investigate whether the absence of pIgR in conjunction with long term consumption of a high-fat Western Diet can promote intestinal inflammation. TNF-α and IL-6 expression was measured in the ileum and the colon. These regions of the GI tract were selected for analysis since they harbor the largest bacterial populations. After sixteen weeks of consuming the Western Diet, no differences were observed between the C57BL6 controls and the pIgR KO mice in IL-6 expression in the ileum, and colon (Figures 3.10 A and 3.10 B). There were no significant differences in TNF-α expression in the ileum and colon (Figures 3.10 C and 3.10 D). These findings suggest that impaired IgA secretion by pIgR does not lead to

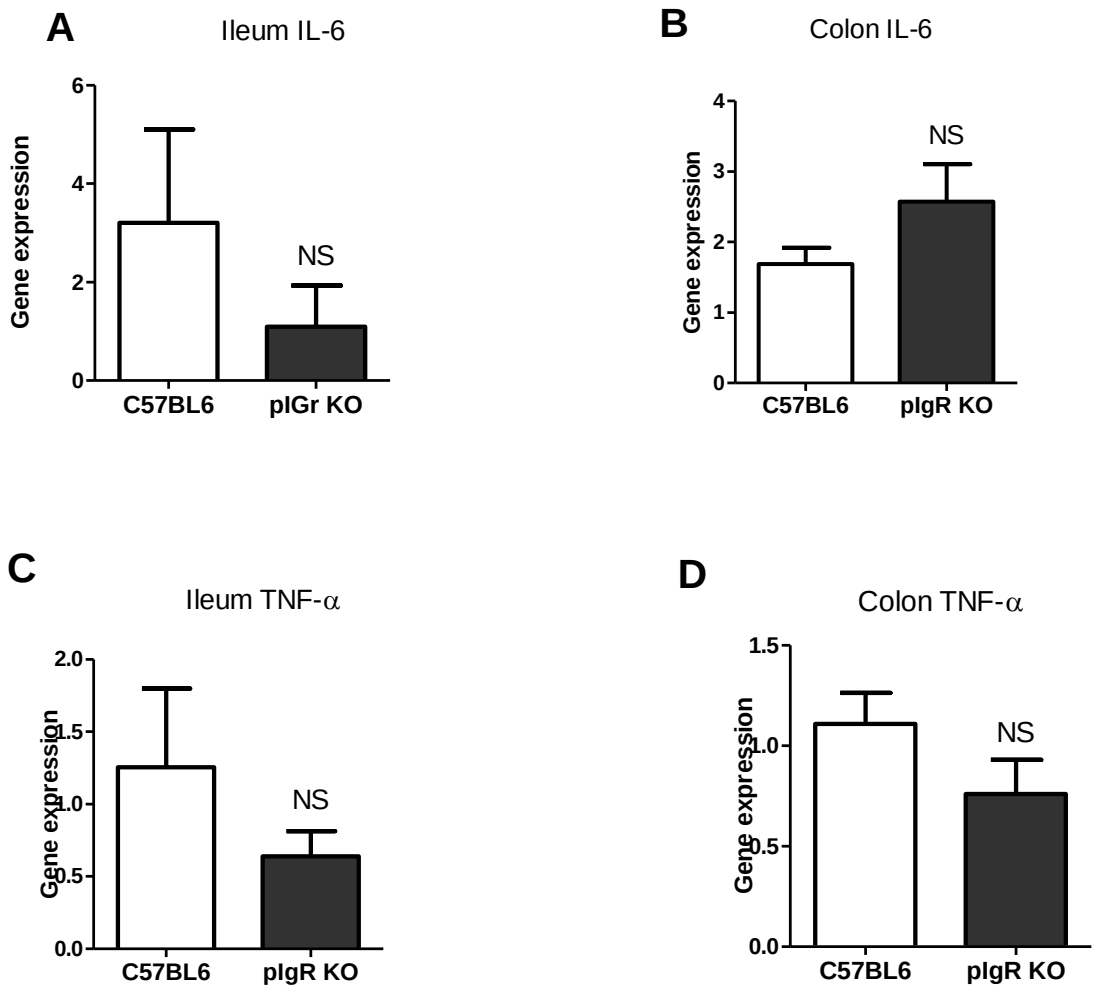
pro-inflammatory changes in the GI tract after long term consumption of a high-fat Western Diet.

Figure 3.9 Hematological analysis



Comparison of blood cell subsets in C57BL6 wild type controls (n = 7) and the pIgR KO mice (n = 6) after sixteen weeks of Western Diet consumption. There were no differences between pIgR KO mice and C57BL6 controls in (A) Neutrophil percentage (B) Basophil percentage (C) Monocyte percentage (D) Eosinophil percentage. Percentages of white blood cells are given.

Figure 3.10 Intestinal inflammatory markers



Intestinal inflammatory markers in C57BL6 wild type controls (n = 7) and the pIgR KO mice (n = 6) after sixteen weeks of Western Diet consumption. There were no differences between pIgR KO mice and C57BL6 controls in (A) Ileum IL-6 (B) IL-6 in the colon (C) Ileum TNF-α expression (D) TNF-α in the colon.

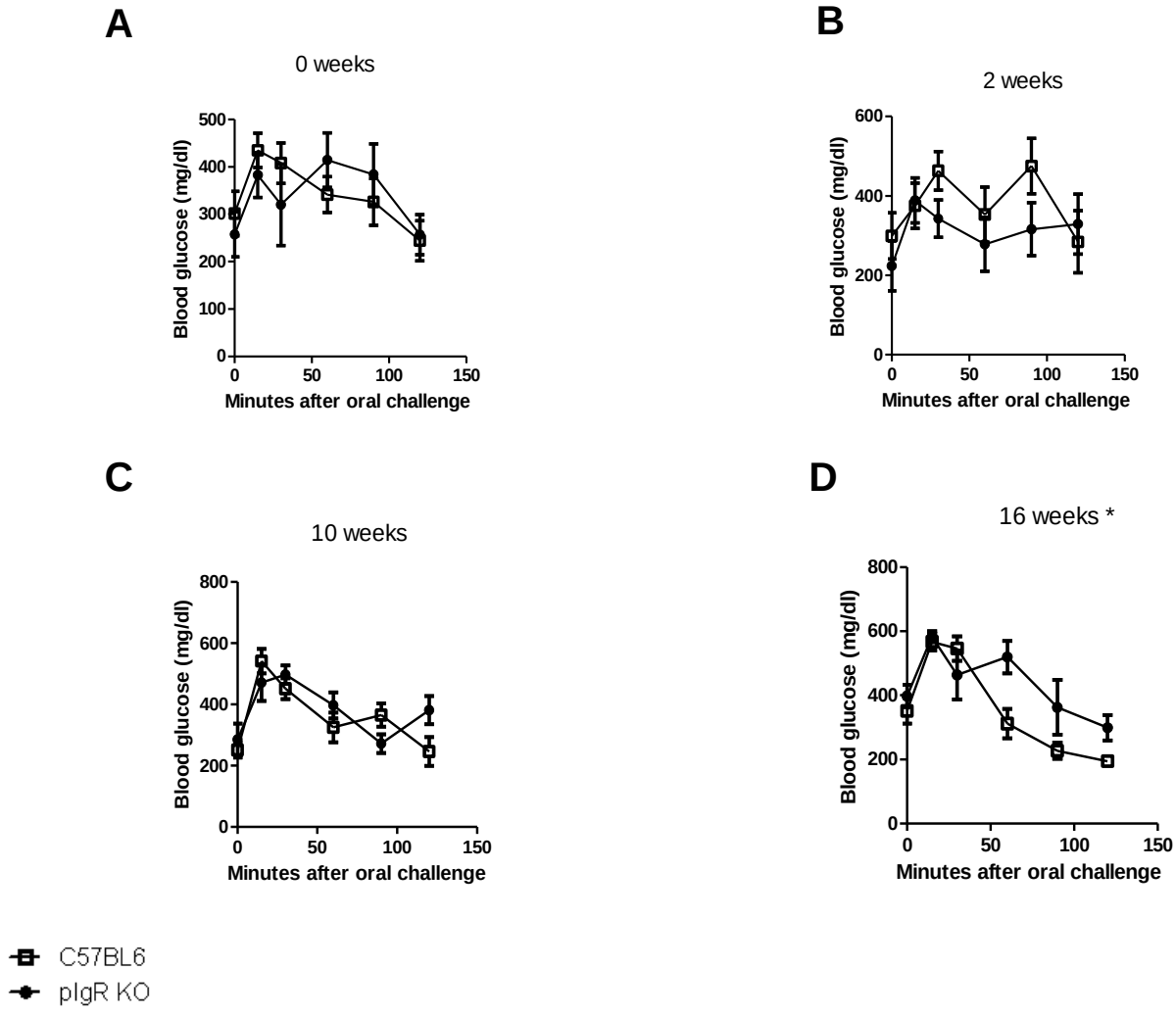
Absence of pIgR impairs insulin sensitivity after long term Western Diet consumption.

Oral glucose tolerance tests (OGTTs) were performed in order to determine whether the absence of pIgR altered the ability to respond to an oral glucose challenge. OGTTs were conducted prior to commencing the Western Diet feeding regimen as well as at various time points throughout the feeding study. Area under the curve (AUC) analysis revealed that the ability to respond to an oral glucose challenge was similar in the C57BL/6 wild type controls and the pIgR KO mice prior to commencing the Western Diet (Figure 3.11 A).

OGTT tests were subsequently performed at two, ten and sixteen weeks after the C57BL6 controls and pIgR KO mice were placed on the Western Diet feeding regimen. Area under the curve (AUC) analysis did not reveal any significant difference between the C57BL/6 controls and the pIgR KO mice in responding to an oral glucose challenge after two weeks on the Western Diet and ten weeks on the Western Diet (Figures 3.11 B and 3.11 C).

However at the sixteenth week of the study the pIgR KO mice had an impaired ability to respond to an oral glucose challenge compared to the C57BL6 controls (Figure 3.11 D). These results suggest that the absence of pIgR together with the consumption of a high-fat, high carbohydrate diet may negatively affect the ability to respond to an oral glucose challenge.

Figure 3.11 Oral glucose tolerance tests (OGTTs)



C57BL6 wild type controls (n=7) and pIgR KO mice (n=6). Area under the curve (AUC) analysis was performed. No differences between the AUC for pIgR KO mice and C57BL6 controls (A) Before commencing the Western Diet. (B) After two weeks on the Western Diet (C) After ten weeks on the Western Diet (D) After sixteen weeks on the Western Diet the difference between AUC for the C57BL6 controls and pIgR KO mice was statistically significant. * indicates $p < 0.05$.

Decreased plasma IgA in obese diabetics.

Analysis of the human plasma samples by total IgA ELISA revealed significant differences between the three groups of human subjects. The lean healthy controls had the highest levels of plasma IgA. Obese non-diabetics had significantly reduced plasma IgA compared to the lean controls ($p < 0.05$). There were also differences between plasma IgA between both groups of obese individuals. The obese diabetic group had plasma IgA that was significantly reduced compared to the obese non-diabetic group as well as the lean, healthy controls (Figure 3.12, $p < 0.05$).

Figure 3.12: Human plasma IgA.

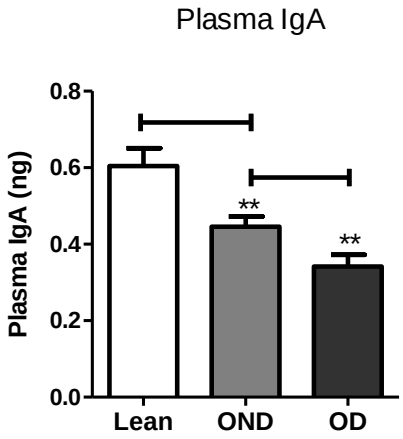


Figure 3.12: Human plasma IgA was assessed by total IgA ELISA in three groups of subjects. Lean healthy controls (Lean) had significantly higher levels of plasma IgA than obese non-diabetics (OND). Obese diabetics (OD) had significantly lower plasma IgA than obese non-diabetics. Sample sizes for Lean (n=10), OND (n =16) and OD (n =16).

DISCUSSION

Long term HFD consumption decreases fecal IgA.

Immunoglobulin A (IgA) secreted into the lumen of the GI tract minimizes adherence of intestinal bacteria to the intestinal epithelium. Since long term HFD consumption is linked to elevated plasma IgG against invasive intestinal bacteria, it is possible that IgA secretion into the lumen of the GI tract could be impaired. To determine whether this was the case, fecal IgA levels were measured in C57BL6 mice fed a HFD and a LFD diet on a weekly basis. It was expected that prior to commencing the feeding study that the fecal IgA levels would be similar. With time, fecal IgA levels in the HFD mice would decrease relative to the LFD controls. This study is not the first to investigate dietary intervention on fecal IgA production. The consumption of probiotic containing foods may improve overall health [172]. These improved indicators of health have been correlated with increased fecal sIgA levels.

Prior to switching the mice to the special diets, both groups of mice had similar fecal IgA levels. However, by the fourth week of the feeding study there was a significant difference between the LFD mice and HFD mice. After four weeks HFD mice had significantly lower fecal IgA levels compared to LFD controls (Figur*e* 3.1). However, fecal IgA/g feces did not decrease in the HFD mice. Instead what was observed was an increase in LFD mice relative to the HFD mice. When the mice were purchased they were five weeks old. The feeding study was commenced when they were six weeks old.

Secretory IgA production increases with age [173]. It is possible that in this feeding study an age effect was observed. B-1 cells play a major role in IgA production in the gut [174]. Furthermore, B-1 cells are adversely affected by consumption of high levels of dietary fat [175]. Therefore it is possible the differences between fecal IgA in LFD and HFD mice are a consequence of dietary fat inhibiting development of a normal immune repertoire in the HFD mice.

The effect of long term HFD consumption on plasma IgA

Long term HFD consumption did not appear to have an effect on plasma IgA in mice. After the ten week feeding study, both groups of mice had similar levels of plasma IgA (Figure 3.2). Considering the relatively small sample size in the study (n=6 for each group), it is likely that further studies should be performed using larger sample sizes.

Absence of pIgR does not exacerbate inflammation.

A major component of our study was to investigate whether the absence of active IgA secretion into the lumen of the GI tract by the polymeric immunoglobulin receptor (pIgR) is a predisposing factor for development of metabolic syndrome. As it has been previously mentioned, pIgR actively secretes polymeric immunoglobulins into the lumen of the GI tract. The quantity of IgA secreted into the lumen of the GI tract by pIgR is immense (an estimated three grams per day) [168, 176]. What is even more intriguing is that the pIgR traversing the IECs are never recycled. pIgR not bound to IgA is also secreted into the lumen of the GI tract as secretory component (SC). This raises the

question as to whether such an elaborate transport mechanism requiring a considerable input of energy serves a crucial purpose. We observed that long term HFD consumption led to increased plasma IgG against an invasive intestinal bacterial strain.

In addition to our experimental findings, we were intrigued by the study performed by Gewirtz *et al.* That study utilized mice deficient in the Toll-like receptor 5 (TLR5) [171]. TLR5 binds flagellin, a constituent of intestinal bacteria [177]. TLR5 KO mice developed features of the metabolic syndrome. These include increased weight gain, adiposity and insulin resistance. Since TLR5 KO mice are unable to respond to flagellin containing bacteria, it is probable that that these mice have an altered intestinal bacterial population from wild type control mice.

Since pIgR KO mice lack a specialized method of transporting pIgA, it was reasonable to hypothesize that a significant reduction in the amount of stable secretory IgA could result in a drastically altered intestinal bacterial composition. It was expected that a combination of an intestinal bacterial population enriched in pathogenic strains and long term consumption of a high-fat, high-carbohydrate Western Diet would lead to metabolic dysregulation as well as increased systemic inflammation.

A wide range of parameters were evaluated prior to commencing the Western Diet feeding regimen. These included body weight and body fat, plasma inflammatory cytokines and the ability to respond to an oral glucose challenge.

Considering the vast quantity of IgA secreted into the lumen of the GI tract, we reasoned that pIgR KO mice would be more susceptible to epithelial invasion by invasive intestinal bacteria. Instead it was observed that plasma IgG levels against invasive intestinal bacteria was similar in both pIgR KO mice and the C57BL6 littermate controls.

No significant differences were observed in these parameters between the pIgR KO mice and the C57BL6 littermate wild type controls prior to commencing the Western Diet feeding regimen. The mice used were relatively young (age six weeks). It is likely that during the nursing stage both groups of mice received ample amounts of IgA from the breast milk thereby negating any potential differences in intestinal bacteria due to the deficiency in pIgR. Despite the obvious difference in IgA secreting capacity, the lack of differences in metabolic parameters prior to consuming the Western Diet feeding regimen was not unexpected.

Body weight, body fat percentage, plasma inflammatory cytokines, immune cell subsets, intestinal inflammation and oral glucose tolerance were evaluated prior to commencing the Western Diet feeding regimen, as well as at various points subsequent to commencing the Western Diet. There were no differences in plasma inflammatory cytokines between pIgR KO mice and C57BL6 wild type controls either prior to commencing the Western Diet or at any point during the feeding study. With the exception of PAI-1, plasma adipokine levels did not differ at 8 or 16 weeks after commencing the Western Diet feeding regimen.

There were no baseline differences in the ability to respond to an oral glucose challenge between the pIgR KO mice and C57BL6 wild type controls. Two and ten weeks after commencing Western Diet feeding there were no differences in responding to an oral glucose challenge between pIgR KO mice and C57BL6 wild type controls. However after sixteen weeks on the Western Diet area under the curve (AUC) analysis revealed that pIgR KO mice had impaired responses to an oral glucose challenge compared to C57BL6 controls.

After performing euthanasia on both groups of mice, intestinal inflammation was measured. There were no differences in any of these parameters between pIgR KO mice and C57BL6 controls. This was surprising given the fact that a large quantity of IgA is secreted into the lumen of the GI tract by pIgR. However, there are a few possible explanations as to why no differences in intestinal inflammation were observed between the C57BL6 and the pIgR KO mice. First of all, this study lasted for a shorter duration than the study using TLR5 KO mice. Whereas this study was performed for a total of 16 weeks, the study performed by Gewirtz *et al* was a twenty week feeding study. It is possible that the effects of pIgR deficiency had not yet become apparent.

Another possible reason no differences were observed between both groups of mice is because pIgR KO mice are not completely deficient in intestinal IgA. A study performed by Johansen *et al*, compared fecal IgA levels in pIgR KO mice and wild type controls [170]. Fecal IgA was measured in pIgR KO mice and C57BL/6 controls by ELISA. Statistical analysis revealed that fecal IgA in pIgR KO mice was less than in the C57BL/6 controls. However, IgA was still

detectable in the feces of pIgR KO mice despite their inability to actively secrete IgA. This is because plasma IgA diffuses into the intestinal mucosa by paracellular diffusion. Furthermore, pIgR KO mice have significantly elevated fecal IgG which may confer protection against the intestinal bacteria.

We observed impaired responses to an oral glucose challenge at the end of the study in the pIgR KO mice compared to the wild type C57BL6 mice. However this did not correspond to an increase in plasma and intestinal inflammatory cytokines. It is possible that the MILLIPLEX analysis was not the best method of evaluating systemic inflammation. Furthermore, in the intestinal tissue only a limited number of inflammatory markers were examined. Perhaps, a wider range of inflammatory markers need to be examined. The study of long term HFD consumption on pIgR KO mice is inconclusive and should be repeated with pIgR KO mice housed separately from wild-type controls.

Reduced plasma IgA in obese diabetics.

Analysis of the human plasma samples revealed differences between the three groups of human subjects. The lean controls had the highest levels of plasma IgA. Obese non-diabetics had significantly reduced plasma IgA compared to the lean controls. Obese diabetics had significantly reduced plasma IgA compared to both obese non-diabetics and lean controls (Figure 3.12). Relatively little is known about the precise role of plasma IgA. This has been attributed to difficulties in purification of plasma IgA. Plasma IgA can neutralize antigens. However, it has a poor ability to promote opsonization. There are a number of effector functions possessed by plasma IgA. These include

enhancement of antibacterial effect of lactoperoxidase and suppression of inflammatory effects of immune lysis, NK cell activity and antibody dependent cellular toxicity.

Reduced serum IgA is more prevalent in individuals who have lost tolerance to normal food antigens [178]. Serum IgA neutralizes normally 'harmless' antigenic material such as food antigens and autoantigens. Serum IgA clears these antigens from the systemic circulation in a non-inflammatory manner. It is has been suggested that IgA clearance of antigens prevents inappropriate responses to these antigens [179]. The higher prevalence of autoimmune disorders in IgA deficient individuals has been attributed inappropriate immune destruction of these antigens [180-182]. Thus the significant decrease in plasma IgA in obese-diabetics may have led to inflammatory immune responses that in turn led to insulin resistance.

However, further analysis is needed on plasma IgA levels with regards to type 2 diabetes. A previous study by Gill *et al* noted that type 2 diabetics had higher plasma IgA levels than healthy controls [183]. This was attributed to the fact that some of the diabetic individuals in that study had acute or chronic bacterial infections. Furthermore, subclinical infections could not be ruled out in the diabetics who did not have bacterial infections. A possible reason for the reduced plasma IgA in obese diabetics is a defect in IgA synthesis.

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CHAPTER 4 DISCUSSION

EVALUATION OF PROPOSED HYPOTHESIS.

Our preliminary findings led us to propose that long term consumption of a high fat diet could impair intestinal IgA production, facilitating adherence and invasion of the intestinal epithelium by the commensal bacteria. Invasion of the intestinal epithelium by the commensal bacteria could trigger inflammatory responses, which in turn could impair insulin signaling, culminating in the development of type 2 diabetes.

Decreased IgA production by long term HFD consumption: Over the course of a long term (10 week) feeding study C57BL6 mice fed a low fat diet (10% kCal from fat) experienced an increase in fecal IgA production relative to mice fed a high fat diet (60% kCal from fat). These findings provided evidence that consumption of high levels of dietary fat may impair the mucosal IgA barrier.

Revision to proposed model-Invasion of the intestinal epithelium by invasive bacteria: Initially we proposed that long term consumption of a high fat diet could result in the commensal bacteria being able to breach the intestinal epithelium and elicit inflammatory responses. However, a major limitation of our preliminary studies was the exclusive use of *E. coli* LF82, an invasive intestinal bacterial strain. To address this concern non-invasive intestinal bacteria were analyzed. IgG against *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus* was measured in the plasma of HFD and LFD mice. Plasma IgG against these bacterial strains was measured using the lab based

ELISA. There were no significant differences in IgG against these bacteria in LFD mice or HFD mice (Figures 2.4 B, 2.4 C and 2.4 D).

The studies cited earlier using GF mice suggested that the presence of intestinal bacteria is required for the negative effects of long term HFD consumption. However, none of those studies examined the role of specific intestinal bacterial species. The findings of our studies provide evidence that invasive intestinal bacterial strains are associated with inflammation and insulin resistance.

The other intestinal bacterial strains examined are beneficial to the host. *E. coli* Nissle 1917 (EcN) is a widely studied probiotic. Probiotics are viable microbes which may confer health benefits when consumed [184]. The ability of EcN to confer health benefits has been known for almost a century since the First World War when it was isolated from a lone soldier who did not succumb to enterocolitis. Investigations on the effects of EcN supplementation show promising results for treating colitis [185, 186]. An interesting coincidence is that EcN has been utilized to counteract the effects of the LF82 strain of *E. coli* [187]. Reports have also provided evidence that *Bacteroides thetaiotaomicron* may prevent rotavirus infections [188]. It has been reported that *Lactobacilus acidophilus* has the ability to diminish inflammation caused by *Helicobacter pylori* [189]. By contrast, the LF-82 strain of *E. coli* has been described as being invasive in nature [151]. *E. coli* LF82 was isolated from an ileal lesion of a Crohn's Disease patient [150]. *E. coli* LF82 is designated as an adherent invasive

E. coli strain (AIEC strain). Invasive strains are characterized by their ability to enter epithelial cells and subsequently survive and replicate.

Immune responses against invasive intestinal bacteria are associated with inflammation. In these studies a statistical correlation between anti-*E. coli* LF82 IgG and TNF-α was observed. The correlation analysis was performed using the plasma samples from the obese patients. Both groups had equivalent mean BMI values, ruling out differences in adiposity as a TNF-α source. The correlation between anti-*E. coli* LF82 IgG and TNF-α provided evidence that immune responses to the intestinal bacterial antigens are associated with inflammation. It is possible that the increase in IgG against intestinal antigens promoted cross-linking of FcγRs, which in turn caused the release of TNF-α.

Inflammatory responses resulting from invasion of the intestinal epithelium impairs insulin signaling. Elevated plasma anti-*E. coli* LF82 IgG in the HFD mice provided evidence that long term consumption of high levels of dietary fat is associated with invasion of the intestinal epithelium by invasive bacteria. Increased plasma TNF-α and neutrophil percentage in the blood of HFD mice suggests that a consequence of epithelial penetration by invasive bacteria is inflammation. Inflammation is associated with a variety of disease states.

One of these conditions is type 2 diabetes. The increase in fasting blood glucose levels in HFD mice compared to LFD mice was significant (Figure 5B, $p<0.05$). It is possible that elevated anti-*E. coli* LF82 IgG against intestinal bacteria led to increased cross-linking of FcγRs with the Fc domains. This in turn

may have led to increased TNF-α secretion as well as increased neutrophils in the blood. The chronic inflammation could have in turn impaired insulin signaling pathways, leading to the development of insulin resistance.

Significance of studies.

These studies have significant clinical relevance. Although there is widespread awareness about the risk factors for developing type 2 diabetes, the prevalence of obesity and metabolic syndrome has continued to rise. This has been costly as treatment and the loss of productivity due to various disabling complications of type 2 diabetes continue to burden the healthcare sector. The research into novel mechanisms of treating type 2 diabetes is in itself a tacit admission that it is impossible to expect the tens of millions of overweight individuals to alter their lifestyle radically enough to maintain a healthy bodyweight.

Apart from individuals with Crohn's Disease the LF82 strain of *E. coli* is rare. It is unlikely that a large percentage of individuals harbor this particular strain. However, *E. coli* LF82 may possess antigens that are shared by other intestinal bacterial species. The intestinal bacteria remain poorly characterized. There is a possibility that invasive intestinal bacterial strains that have not yet been identified reside in the GI tract. Long term HFD consumption may enhance the capacity of invasive bacterial strains to cross the IEC barrier. This can elicit inflammation leading to insulin resistance.

Furthermore, our findings may explain why some obese individuals develop type 2 diabetes whereas others do not. Individuals harboring invasive intestinal bacterial strains may be more prone to chronic inflammation and impaired insulin signaling. To more comprehensively define the role of the intestinal bacteria in promoting systemic inflammation, further studies will need to be performed. IgG against protein extracts from invasive intestinal bacterial strains can be measured in diabetics and non-diabetics. Based on our findings we expect that diabetics would have higher IgG against invasive intestinal bacterial strains. Comparison of IgG in a larger group of obese non-diabetics and obese-diabetics would give a better indication of whether long term HFD consumption enhances invasion of the IEC barrier by invasive intestinal bacterial strains. The clinical implications of these studies could be novel methods of treating type 2 diabetes. It is possible that specific anti-microbial compounds could be developed to selectively eliminate invasive intestinal bacterial strains residing in obese individuals. This could spare these individuals from developing type 2 diabetes. It would also reduce the financial burden to the healthcare sector significantly.

Technical contributions.

These studies resulted in technical contributions for assessing immune responses against the intestinal bacteria. ELISA kits to assay systemic immune responses against intestinal bacterial antigens were difficult to obtain. To address the lack of commercial ELISAs we developed our own lab based ELISA. This lab based ELISA is a reliable, reproducible assay for assaying the humoral immune

response against antigens from intestinal bacterial strains. To the best of our knowledge, no one had developed the methods of Western Blotting to analyze the levels of plasma IgG against intestinal bacterial proteins. Researchers hoping to assess the systemic immune response against intestinal bacterial strains in a timely manner can use the information provided in this publication.

EVALUATION OF THESE STUDIES.

Strengths: These studies were the first to investigate the association between specific intestinal bacteria and type 2 diabetes. Previous studies had linked the presence of intestinal bacteria to obesity, inflammation and insulin resistance. However, a key flaw of those studies was that they did not examine which particular bacteria resulted in the development of inflammation and insulin resistance. The findings of previous studies implicated that bacteria residing in the GI tract promoted the development of inflammation. Although there is extensive evidence supporting this proposal, it should be kept in mind that there are hundreds of bacterial species residing in the GI tract. Given the enormous diversity of the intestinal bacteria, it cannot be assumed that all intestinal bacteria are equally capable of promoting systemic inflammation and insulin resistance. As the results from our studies indicate, not all intestinal bacteria possess the same ability to cross the intestinal epithelial cell barrier.

These studies provided a more definitive link between the intestinal bacteria, inflammation and insulin resistance. A critical component lacking in the previous studies in GF mice was showing that the inflammation observed as a consequence of HFD consumption was the result of intestinal bacteria. Although

the studies comparing GF mice to conventionally raised mice measured a variety of parameters, none of these studies performed immunological assays that showed immune responses to the intestinal bacteria. This study quantified plasma IgG against intestinal bacteria in addition to quantifying plasma TNF-α and fasting blood glucose values, linking all three parameters.

Our studies characterized the effect of HFD consumption on the localized immune responses at the mucosal surface of the GI tract. Although previous studies had linked the intestinal bacteria to systemic inflammation and insulin resistance, none of these studies investigated whether this was due to impairment of the localized adaptive intestinal mucosal responses. It should be noted that the effect of impairment of the intestinal immunity on shaping the composition of the gut microbiota was examined by Gewirtz *et al* using TLR5 deficient mice. However, their study looked at the deficiency of innate immune mechanisms in the GI tract. Our studies examined the effect of HFD consumption on the adaptive immune response to the intestinal bacteria. This is an important topic to address given the immense load of IgA secreted into the GI tract as well as the documented protective effects of IgA.

Weaknesses: A major critique of this study is the extensive use of the LF82 strain of *E. coli*. as it has been previously stated, this strain of bacteria is recognized as an adherent invasive bacterial strain. Therefore it is not an ideal strain to use as an experimental model. This experimental model deficiency was addressed by assessing the effect of HFD consumption on the systemic immune response to the non-pathogenic bacteria *E. coli* Nissle 1917, *Bacteroides*

thetaiotaomicron and *Lactobacilus acidophilus.* Despite the fact that HFD consumption did not appear to alter the systemic immune response to these bacteria, Western Blot analysis showed that HFD mice had higher plasma IgG against the antigens present in the cecum of control mice. Higher plasma IgG against the antigens from the cecum in HFD mice strengthened our hypothesis that HFD consumption may lead to translocation of intestinal bacteria into the systemic circulation. However, at this point, the only bacterial strain to which the systemic immune response has shown to be increased as a result of HFD consumption the LF82 strain of *E. coli*.

The findings of these studies point to the possibility that individuals harboring invasive intestinal bacterial strains are at increased risk of developing systemic inflammatory immune responses that may increase their risk of developing type 2 diabetes. However, these studies did not determine whether the composition of the gut microbes changed during the HFD feeding regimen. As a consequence, it is not known if HFD consumption increases the proportion of invasive intestinal bacteria.

There were flaws in the assessment of HFD consumption on the localized immune responses at the mucosal surface of the GI tract. Although fecal IgA was quantified, no other immunoglobulin classes were measured. It is known that IgM plays a role in preventing the intestinal bacteria from adhering to the intestinal epithelium. Fecal IgM was not measured. Thus it is not conclusive as to whether HFD consumption impairs mucosal immunity. Furthermore, a number of mechanisms prevent the commensal bacteria in the lumen of the GI tract from

adhering to the intestinal epithelial cells. These include mucus production by goblet cells as well as the production of defensins, cathelicidins, phospholipases, lysozyme, and Reg III-gamma. These parameters were not examined in LFD mice and HFD mice.

EXPERIMENTAL DESIGN.

Lab developed ELISA: A key difference between the lab developed ELISA and the Western Blot analysis was that the Western Blot showed differences between the three groups of human subjects. Western Blot analysis indicated that plasma anti-*E. coli* LF82 IgG was highest in obese diabetics, followed by obese non-diabetics with the lowest plasma anti-*E. coli* LF82 IgG found in the lean healthy controls. On the other hand, ELISA analysis showed that the obese diabetics had significantly higher plasma anti-*E. coli* LF82 IgG than lean controls with no significant differences between the obese non-diabetics and lean healthy controls. A possible explanation for this discrepancy is that the ELISA was prepared by extracting bacterial proteins using Bacterial Protein Extraction Reagent (B-PER). This reagent specifically extracts soluble proteins. It is possible that during the process of soluble protein extraction, non-soluble proteins that possessed epitopes for immune recognition were lost. Furthermore, the lab-developed ELISA only consisted of soluble bacterial proteins. Non-protein compounds such as lipopolysaccharide (LPS) can also elicit immune reactions. LPS is a constituent of the outer cell membrane of gram negative bacteria including *E. coli* LF82. It is one of the more potent inducers of inflammation and results in the release of the pro-inflammatory cytokines TNF-α,

IL-6, and IL-1 [190-192]. However, despite these limitations we chose to perform the ELISAs since it was the most readily available method to give us quantitative measurements. There was concern that the lab developed ELISA optimization was not performed accurately. These do not negate our results since the Western Blots show distinct differences between LFD mice and HFD mice.

Technical flaws of pIgR KO mouse study: The magnitude of active IgA secretion into the lumen of the GI tract suggests that there is a rationale behind this specialized mechanism of active IgA transport. Furthermore, pIgR KO mice have significantly increased serum IgG against intestinal bacterial antigens than C57BL6 wild type controls. There were a number of design flaws in these studies. The mice were not housed according to genotype. As a consequence, it was possible that differences in the gut microbe composition of both groups of mice were minimized by coprophagy. Consumption of the fecal pellets produced by the wild type C57BL6 mice could result in harmless commensal bacteria being repopulated in the GI tract of the pIgR KO mice. These bacteria could in turn out-compete any invasive strains, thereby preventing the anticipated outgrowth of invasive bacterial strains in the pIgR KO mice. Co-housing of pIgR KO mice with wild type C57BL6 controls also resulted in an additional source of IgA for the pIgR KO mice. Small quantities of IgA reach the lumen of the GI tract in pIgR KO mice via paracellular diffusion. The co-housed pIgR KO mice were provided with an additional source of IgA in the form of fecal pellets. In addition to the IgA reaching the lumen of the GI tract via paracellular diffusion, the pIgR KO mice also obtained IgA by coprophagy. This in turn could have compensated for the

lack of active IgA transport by the pIgR. These technical shortcomings should be addressed in future studies of the effect of HFD consumption on pIgR KO mice.

Alternative reasons for increased plasma anti-*E. coli* LF82 IgG.

A question that was not addressed in these studies is whether live invasive bacteria invade the epithelium. The assays performed in these studies involved quantification of plasma IgG against *E. coli* LF82, *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus.* However, there were no direct methods used to determine whether live bacteria had been able to invade the intestinal epithelium. Given the huge bacterial population in the GI tract it is inevitable that some leakage across the epithelium takes place. The mesenteric lymph nodes act as a barrier between the GI tract and the systemic circulation. Dendritic cells actively sample bacteria from the lumen of the GI tract. The MLNs prevent the dendritic cells carrying commensal bacteria from gaining access to the systemic circulation. HFD consumption results in atrophy of the mesenteric lymph nodes and a reduction in the numbers of regulatory T lymphocytes. It is possible therefore that increased systemic immune responses resulted from defects in the immune repertoire, not increased IEC invasion by the intestinal bacteria.

FUTURE DIRECTION OF STUDIES.

Resolving technical issues of these studies: These studies linked the presence of systemic immune responses against intestinal bacteria to the development of type 2 diabetes. However there are a number of technical issues that need to be resolved. The lab based ELISA assay needs to be optimized by

preparing serial dilutions and plotting standard curves in order to determine the dynamic or linear portion of the standard curves. This would make quantification of plasma IgG more accurate.

Another technical issue was the oral glucose tolerance tests performed in the Western Diet feeding study using the pIgR KO mice. The OGTTs were performed by administering an oral gavage of 2.0g/kg glucose. The blood glucose levels were beyond the detectable limit of the TrueTrack glucose meter at various time points subsequent to the oral gavage. To address this issue, an oral gavage of either 1.5g/kg glucose 1.0g/kg glucose needs to be administered to the mice.

The effect of HFD consumption on the systemic immune response: Although there is now considerable evidence that the bacteria residing within the GI tract may promote systemic inflammation and insulin resistance, there are still some issues that need to be addressed. The first is to provide evidence that intestinal bacteria other than the LF82 strain of *E. coli* are associated with systemic inflammation and insulin resistance. This method may rely on preparing extracts from the cecums of control mice and performing Western Blots to assess IgG levels in the plasma of HFD mice and LFD mice. If HFD consumption is associated with increased plasma IgG against antigens from the cecum, then proteomic analysis could be performed to decipher what antigens are eliciting the systemic immune responses. This would be advantageous because identification of antigens triggering a systemic immune response would aid in identifying specific bacteria capable of penetrating the mucosal layer. Furthermore, these

antigens can be used for developing ELISAs for further quantitative assays of HFD consumption and systemic IgG responses against bacteria resident in the gut.

It is also necessary to determine when the intestinal bacteria associated with systemic inflammatory immune responses are present. On one hand it is possible that some individuals harbor invasive intestinal strains that make them more prone to developing systemic inflammatory immune responses. On the other hand, HFD consumption may alter the composition of the intestinal bacteria resulting in an outgrowth of intestinal bacteria that may promote inflammation. To assess the relationship between HFD consumption and the intestinal bacteria a time course analysis could be performed. Fecal pellets can be collected from mice prior to commencing a HFD feeding regimen. The bacteria present in the feces can be characterized by 16SrRNA sequencing. Fecal pellets can be collected at various time points during feeding study and the bacteria present can be sequenced. These can then be referenced to LFD controls. This would indicate whether HFD consumption alters the intestinal bacteria over time.

Our studies did not detect the presence of intestinal bacteria in the systemic circulation. Instead, we measured systemic immune responses against intestinal bacterial antigens by Western Blots and ELISAs that measured serum immunoglobulin G (IgG) responses to bacterial antigens. Trying to detect the presence of intestinal bacteria would have provided evidence that adherent bacteria breach the intestinal epithelium.

HFD consumption and mucosal immunity: To further characterize the effect of HFD consumption on the immune responses at the mucosal surface of the GI tract, fecal IgM will have to be measured in LFD mice and HFD mice. This would further strengthen the argument that mucosal immunity is impaired by HFD consumption. Additionally, intestinal sections can be immunostained for IgA, IgM and IgG in HFD mice and LFD mice. Comparisons of these IgG classes at the epithelium and the lamina propria may give a more comprehensive indication as to how HFD consumption affects immunity at the mucosal surface.

One proposed experiment was to characterize the role of IgA responses in the GI tract using IgA knockout mice. The proposed study involved measuring and comparing body weight, adiposity, plasma inflammatory cytokines, intestinal inflammation and insulin resistance in mice lacking the gene for IgA production (IgA KO mice) and C57BL6 wild type controls. These parameters were supposed to be measured prior to commencing a high-fat, high-carbohydrate Western Diet and at different time points during the feeding study.

IgA knockout mice are unable to synthesize IgA. This is because these mice lack the gene for the switch and constant regions [179]. IgA is undetectable in the serum and gastrointestinal secretions of IgA KO mice. Since mucosal IgA is important in restricting bacteria to the lumen of the GI tract, it is possible that the mucosal barrier of IgA KO mice may be impaired in comparison to wild type controls. It should be noted that IgA KO mice have significantly increased IgM and IgG levels in intestinal secretions. Elevations of IgM and IgG in the intestinal secretions of IgA KO mice may be compensatory mechanisms for the lack of IgA.

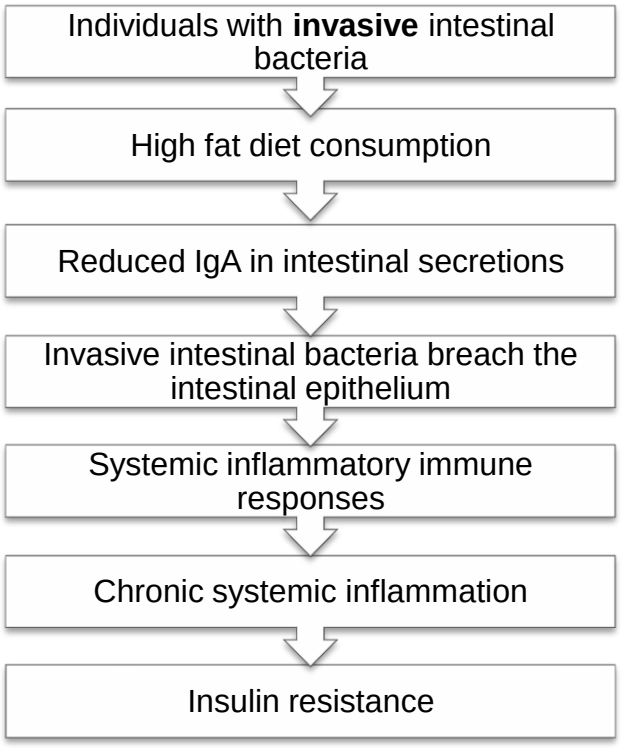
Therefore future evaluation of the effect of HFD consumption should not focus on one specific aspect of mucosal immunity. A more comprehensive array of parameters should be evaluated. Since the commensal bacteria residing in the GI tract do not cause problems in most individuals, millions of years of co-evolution could have resulted in a number of redundant mechanisms to restrict bacteria to the GI tract lumen. As a result future studies should not only focus exclusively on IgA, but IgM as well as the production of mucus and anti-microbial peptides.

Concluding statement:

The results of these studies show that long term HFD consumption in mice is associated with increased plasma IgG against **invasive** intestinal bacterial strains. This is a revision from our proposed experimental model. HFD mice had higher levels of IgG against invasive intestinal bacteria than LFD mice. However, there were no significant differences in plasma IgG against the non-invasive intestinal bacterial strains *E. coli* Nissle 1917, *Bacteroides thetaiotaomicron* and *Lactobacilus acidophilus* in HFD and LFD mice. These findings are clinically relevant since obese-diabetics had significantly higher plasma IgG against the invasive intestinal bacteria than lean healthy controls. Furthermore, a correlation exists between TNF-α and IgG against *E. coli* LF82. Increased anti-*E. coli* LF 82 IgG and plasma TNF-α in obese-diabetics established a link between systemic immune responses against invasive intestinal bacterial strains, systemic inflammation and insulin resistance. The effect of long term HFD consumption on the secretory immune response in the intestinal mucosa was also investigated. It

was observed that fecal IgA levels were lower in HFD mice compared to LFD mice. These findings provide evidence that long term HFD consumption may reduce sIgA present in the lumen of the GI tract. Reduced sIgA may promote the translocation of pathogenic intestinal bacterial strains into the systemic circulation.

Figure 4.1: A proposed model based on our experimental findings of how the intestinal bacteria can promote inflammation and insulin resistance.



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