University of Alberta

Effects of Trans Fats, Obesity, and Type 2 Diabetes on the Immune System

by

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To the three strongest,
most beautiful women in my life:
Dorota, Margaret, and Victoria

ABSTRACT

Obesity is a major risk factor for the development of type 2 diabetes (T2D). While altered systemic inflammation is associated with the development T2D, the effects of obesity on immune function are not well known. It is not known why some obese individuals develop T2D, and some remain healthy. Dietary components may result in inflammation; relationships between industrially-produced trans fatty acids and inflammation have been established but there is a lack of research focusing on the effects of ruminant-derived trans fatty acids (conjugated linoleic acid (CLA) and vaccenic acid (VA)). The purpose of this study is to compare peripheral immune cell types and function between healthy obese and diabetic obese subjects, and to examine the effect of ruminant-derived trans fats on cytokine expression in adipose tissue of obese rats.

Peripheral blood was obtained from healthy obese (n=10) and diabetic obese (n=9) subjects. Immune cell phenotypes were determined by flow cytometry. T cell proportions were not different between healthy obese and diabetic obese subject; however, diabetic obese subjects had a significantly higher (p<0.05) percentage of CD71-expressing T cells compared with healthy obese subjects. Diabetic subjects also had a significantly higher percentage of regulatory and naïve T cells. After stimulating with the polyclonal T cell mitogen phytohemagglutinin, cells from diabetic obese subjects produced less (P<0.05) IL-6 and TNF-α; IL-2 did not differ between the groups.

Obese JCR:LA-*cp* rats (n=20) were fed a control, VA or CLA diet for 8 weeks. Perirenal fat pads were extracted and cytokine expression measured by

qRT-PCR. Obese rats had higher expression (p<0.05) of TGF-β (1.51 fold), IL-6 (3.7 fold), and IL-12 (2.7 fold) in adipose tissue compared to lean rats. Obese rats fed CLA had higher IL-6 expression in adipose tissue compared with rats fed the VA diet.

These results suggest that when T2D accompanies obesity, T cell and neutrophil dysfunction occurs. Furthermore, naturally occurring trans fatty acids do not appear to exacerbate the production of cytokines in obese rats.

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ALA – alpha-linolenic acid

ANOVA – analysis of variance

BMI – body mass index

BSA – bovine serum albumin

Ca – calcium

CCM – complete culture media

CD – cluster of differentiation

CLA – conjugated linoleic acid

ConA – concanavalin A

CRP – c-reactive protein

CRTH - chemoattractant receptor-homologous molecule expressed on Th2

CVD – cardiovascular disease

DHR - dihydrorhodamine

DIO – diet-induced obesity

DXA – dual-energy x-ray absorptiometry

EOSS – Edmonton obesity staging system

FcR – fragment crystallisable region

FCS – fetal calf serum

FFA – free fatty acid

FITC – fluorescein isothiocyanate

IFN – interferon

IBD – inflammatory bowel disease

Ig – immunoglobulin

IKKβ – inhibitor of nuclear factor kappa-B kinase subunit beta

IL – interleukin

IRS-1 – insulin receptor substrate 1

JNK – c-Jun N-terminal kinase

HDL – high-density lipoprotein

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HFD – high fat diet

LA – linoleic acid

LPS – lipopolysaccharide

MCP – monocyte chemoattractant protein

MHC - major histocompatibility complex

MLN – mesenteric lymph node

MUFA – monounsaturated fatty acids

mRNA – messenger ribonucleic acid

NCD – normal chow diet

ND – not detected

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NK – natural killer cell

OA – oleic acid

PAI – plasminogen activator inhibitor

PAMP - pathogen-associated molecular pattern

PMA – phorbol myristate acetate

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffer saline

PE – phycoerythrin

PerCP - peridinin chlorphyll protein

PGD_2 – prostaglandin D_2

PGF – prostaglandin F

PHA – phytohemaglutinen

PKC – protein kinase C

PMAI - phorbol 12-myristate 13-acetate ionomycin

PPAR – peroxisome proliferator-activated receptor

PRR – pattern recognition receptors

PUFA – polyunsaturated fatty acids

PWM – pokeweed mitogen

ROS – reactive oxygen species

SEM – standard error of the mean

SFA – saturated fatty acids

TCR – T cell receptor

TGF – transforming growth factor

Th1 – type 1 helper T cells

Th2 – type 2 helper T cells

Th3 – type 3 helper T cells

TLR – toll-like receptor

TNF – tumour necrosis factor

Tregs – regulatory T cells

VA – vaccenic acid

WAT – white adipose tissue

WBC – white blood cell

WHR – waist-to-hip ratio

WHO – world health organization

1 INTRODUCTION AND LITERATURE REVIEW

1.1 IMMUNE SYSTEM

The human immune system is a complex and highly integrated dynamic system that is found throughout the body. The overall purpose of the immune system is to protect the body from foreign antigens and abnormal cells that were once self-replicating such as transformed (i.e. in cancer) or auto reactive cells (i.e. in autoimmune diseases). The cells and compounds in the immune system are often classified into two distinct but interacting arms: innate immunity and acquired immunity (1).

1.1.1 THE INNATE IMMUNE SYSTEM

The cells and components of the immune system are illustrated in Table 1.1. The innate immune system has many mechanisms of defense, including external barriers, phagocytosis, and the coating of antigens with toxic substances. The presence of these toxins can result in cellular growth inhibition, loss of cellular integrity or cell death (apoptosis) (2).

The non-specific, innate immune response reacts to a foreign particle in a manner in which a signature antigen is not required. A major form of defence is the presence of external barriers which include the skin and mucus secreted by membranes (1). Internally, the innate system has evolved into a group of various cells and pattern recognition receptors (PRRs) that are able to recognizing specific patterns expressed by pathogens (PAMPs) (1). The main types of cells are monocytes/macrophages and granulocytes which include neutrophils, eosinophils and basophils. One group of receptors that are classified as PRRs are the toll-like receptors (TLRs) (3). These receptors are found on various cells and can recognize PAMPs such as lipopolysaccharides, heat shock proteins and flagellin. In humans, 10 different TLRs have been identified to date (3) and can be found on the extracellular surfaces of macrophages and dendritic cells or in intracellular compartments such as endosomes or Golgi apparatuses.

During an inflammatory response, the main types of cells at the site of infection are neutrophils and macrophages (4). Macrophages, which originate from bone marrow promonocytes, eventually migrate into tissues (1).

Table 1.1 Immune cell composition of peripheral blood in humans (5)

	Basophils and mast cells	Neutrophils	Eosinophils	Monocytes and Macrophages	Lymphocytes and plasma cells	Dendritic cells
% of WBCs in blood	Rare	50-70%	1-3%	1-6%	20-35%	NA
Subtypes and nicknames		Called "polys" or "segs" Immature forms called "bands" or "stabs"		Called the mononuclear phagocyte system	B lymphocytes Plasma cells T lymphocytes Cytotoxic T cells Helper T cells NK cells Memory Cells	Also called Langerhans cells, veiled cells
Primary function (s)	Release chemicals that mediate inflammation and allergic responses	Ingests and destroy invaders	Destroy invaders, particularly antibody-coated parasites	Ingests and destroys invaders Antigen presentation	Specific responses to invaders, including antibody production	Recognize pathogens and activate other immune cells by antigen presentation

Classifications

●———————————— Phagocytes ————————————●

●———————— Granulocytes ————————●

●— Cytotoxic cells —●   ● Cytotoxic cells —●
(some types)

●————————— Antigen-presenting cells —————————●

Neutrophils are the most abundant leukocyte found in the blood, making up approximately 50%-60% of the cells in blood; with a half-life of 6-8 hours while in circulation (6; 7). A key feature of neutrophils is their rapid response to areas of infection, which is considered a fundamentally important feature of the innate system. During an infection, neutrophils as well as macrophages (known as monocytes in circulation) travel to the site of infection/injury where they either produce reactive oxygen intermediates or phagocytose the antigens. The migration of neutrophils to the site of infection is influenced by chemoattractants, which are produced by the host and the pathogen (8). One of the most potent chemoattractants known for neutrophils is IL-8 (9). For phagocytosis to be initiated, TLRs need to bind to PAMPs. Neutrophils contain TLRs 1, 2, 4-10 as well as peptidoglycan recognition receptors (10). The initiation of the phagocytic process also results in the release of NFκB, which in the presence of interferon regulates transcription factors and thus the release of inflammatory cytokines (1).

Once PAMPs bind to the surface of the neutrophils, activation of actin-myosin occurs resulting in the extension of pseudopods around the antigen. When the antigens are enclosed in the neutrophil, specific proteins classified as primary, secondary or tertiary granules are used digest the microbes (11). Lysozyme is classified as a primary granule and is active against gram-positive bacteria. Defensins are classified as a secondary granule and is active against bacteria/fungi and gelatinase is classified as a tertiary granule and is active against organisms expressing gelatin (12). To prevent damage to the neutrophil, the digestion occurs in lysosomes.

Another innate immune response involves a class of lymphocytes called natural killer cells (NK cells) (5). These cells are known to play an important role in innate immunity but are also involved functionally in the acquired immune system. NK cells have surface receptors that recognize MHC I molecules (13). NK cells can be classified as having 3 main functional roles: 1) They are cytotoxic cells, capable of releasing cytolytic granules, such as perforin, into

virally infected cells and/or tumor cells (14) 2) They secret cytokines such as IFN-γ, TNF-α, IL-5 and IL-13 which are important in mediating other cells. 3) They express co-stimulatory ligands which interact with T and B cells (14).

1.1.2 THE ACQUIRED IMMUNE SYSTEM
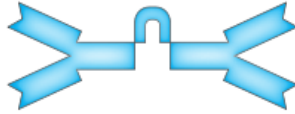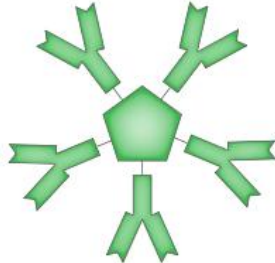
Several days after exposure to a new antigen (pathogen), the acquired immune system responds by increasing production of the specific antibody for the new antigen. The acquired immune system uses antigen-specific recognition and immunological memory as part of its immune response (15). This acquired response is the result of somatic rearrangements by genes for the antigen receptor region (16). This genetic recombination of genes is the premise of the acquired immune system to recognize and remember specific pathogens. Lymphocytes can be broken down into two subgroups: B lymphocytes and T lymphocytes. One of the hallmark features of the acquired immune system is its ability to respond more quickly and with more abundant antibody production when a subsequent infection of the same pathogen occurs.

1.1.2.1 B CELLS

B lymphocytes, also known as B cells, are white blood cells that secrete antibodies. Antibodies, also known as immunoglobulins, can be classified into five groups: immunoglobulin A (IgA), IgD, IgE, IgG and IgM (Table 1.2). Antibodies have two main functions: they are cell-surface receptors that induce cell signalling and activation, and they serve as effector molecules, binding the antigens and neutralizing them (17). Each group of antibodies differs in their structure, specifically in there fragment crystalline region (1; 17). Each B cell has a membrane-bound antibody that binds to only one antigen. During development, B cells complete most of development in the bone marrow, unlike T lymphocytes, which develop in the thymus (18). During this initial stage of development, an antigen is not required by the B cells, thus this stage is called antigen-independent development (18). Once the antigen-independent stage is complete, B cells exit the bone marrow as immature cells that migrate to the spleen for further

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development into mature B cells (18). Mature B cells can be divided into two sub-groups: long-

Table 1.2 Antibody isotypes (1)

Name	Description	Structure
IgA	• Exist in three soluble forms • Monomeric and small amounts of dimeric are found in the serum • In serum they help link pathogens to effector cells • Secretory IgA helps protect the mucosal surfaces of the body	
IgD	• Found primarily on the surface of B cells with IgM • Involved in lymphocyte activation and suppression	
IgE	• Monomeric structure • Low concentrations in serum • Most IgE is likely bound to mast cells • Triggers acute inflammatory reaction and allergic response	
IgG	• Monomeric structure • Major antibody in serum and non-mucosal tissue • It inactivates pathogens directly and through interaction with effector triggering molecules	
IgM	• Pentameric structure • Found in serum • Involved in complement triggering • Monomeric form of IgM is the major antibody used by B cells to recognize antigens	

lived follicular B cells and marginal-zone B cells. Long-lived follicular cells are found circulating to splenic follicles, lymph nodes and to bone marrow until they encounter an antigen or they die. Marginal-zone B cells consist of approximately 10% of all mature B cells and are found only in the spleen.

When a B cell recognizes an antigen, it binds to the antigen and becomes activated. This activation of the B cell initiates the replication and differentiation of the B cell into either memory cells or effector cells/plasma cells. The memory cells are long lasting cells that express the same antibody that was bound to the original antigen and the effector cells are cells that produce large numbers of the same antibody for the newly discovered antigen. The process of forming into effector cells and producing the appropriate antibody at a large scale takes time, thus the delayed response during an infection (4). The activation of the original B cell with the specific antibody leads to the production of only that specific antibody during the immune response.

The majority of B cells are found in the lymphatic system with approximately 5% located in the blood. This 5% constitutes approximately 2.7-4.7% of all white blood cells (5). In general, B cells circulate in blood and lymphoid tissue where the primary goal is to detect antigens. To distinguish B cells from other lymphocytes, the marker CD19 can be used (19). It is involved in the regulation of B cell development, activation and differentiation through signal transduction of Ca^{2+} (20). In normal, healthy populations approximately 3-11% of the peripheral mononuclear blood cells (PMBC) express CD19 (19). B cells can be classified as either naïve/resting or activated cells. Measuring the expression of CD45 and CD80 on B cells can determine if a B cell is resting or activated. CD45 expression reflects the activation of the cells, likely through the activation of tyrosine phosphatase (19). CD45RA is found more often on naïve/resting cells while CD45RO is found on memory/activated cells. The expression percent of CD45RA and CD45RO in peripheral human blood is approximately 71-73% and 34% respectively (21). Activated B cells can also express CD80, with an approximate percentage expression of 10-20% in peripheral blood from a human population (22).

1.1.2.2 T CELLS

T cells, like B cells, originate from the bone marrow, but migrate to the thymus to complete maturation (4). Once T cells have matured, they migrate to the peripheral lymphoid organs through the blood stream where antigens are encountered. Unlike B cells, T cells respond to antigen presenting cells (i.e. dendritic cells, macrophages or B cells) that display antigens with major histocompatibility complexes (MHC). A T cell receptor (TCR) is a heterodimeric antigen receptor that is structurally similar to antibodies found in B cells, except a TCR cannot directly interact with the antigen (1). TCRs recognize peptide fragments from antigens by MHCs. An MHC is a molecule that presents antigens to T cells which in turn results in the activation of the T cell (1). When T cell is activated, genes related to cell division and cytokine production are up-regulated which in turn creates various immune responses (1). There are two classes of TCRs, both being identified with two disulphide-link chains where one class consists of a αβ domains and the other consists of γδ domains (1). Ninety-five percent of T cells circulating in the blood express the αβ domain complex (1).

T cells also express co-receptors, either CD4 or CD8, that also interact with the MHC complex. CD4 is expressed on helper T cells and interact with MHC II molecules while CD8 is expressed on cytotoxic T cells and interact with MHC I molecules. When helper T cells are bound to MHC II (activated), they differentiate into memory helper T cells and effector helper T cells. Effector helper T cells produce cytokines and are involved in activating B cells. Helper T cells can be divided into sub-types: helper 1 T cells (Th1), helper 2 T cells (Th2) and helper 3 T cells (Th3)/T-regulatory cells (Tregs). The division of the T-helper cell into these subtypes depends on the cytokine interactions that occur during proliferation. Cytokines IFN-γ and IL-12 lead to Th1 differentiation while IL-4 stimulates Th2 differentiation (23). Th1 cells activate macrophages by producing IFN-γ and TNF-α (23). They also inhibit the proliferation of Th2 cells. Th2 cells activate B cells by secreting IL-4 and IL-5 and inhibit IFN-γ and TNF-α

production by secreting IL-10. IL-4 is also known as a growth factor for CD4+ T cells and mast cells, while IL-5 is also a chemotactic factor for eosinophils (23). The proportion of T cells that are helpers ranges from 28-59%, with the median being approximately 46%. Th3/Tregs are produced from naïve T cells that have interacted with an antigen presented by dendritic cells (1). Th3/Tregs cells are involved in regulating/supressing the immune system, and are believed to play a major role in preventing autoimmune diseases. Th3/Tregs suppress the proliferation of CD4+ and CD8+ T cells, but the mechanism by which this occurs is unknown . It has been suggested that Tregs mediate this suppression through CTLA-4 and TGF-β but this hypothesis remains controversial (24).

Cytotoxic T cells express the co-receptor CD8 and these T cells interact with MHC 1 molecules. When cytotoxic T cells are activated, they begin to differentiate into memory and effector cells. These effector cells, when bound to MHC I with the antigen, release cytotoxic proteins that initiate a cascade of intracellular events that lead to cell death (apoptosis). When cytotoxic T cells bind to the MHC I complex, the TCR enhances the connection and up regulates the formation of various adhesion molecules (25). Once the granules have been released to the target cell, down regulation of the adhesion molecules occurs to allow the cytotoxic T cell to bind to another target cell.

When T cells are activated they produce cytokines. Cytokines are proteins produced by immune and other cell-types that are involved in transmitting various signals throughout the immune system (1). Depending on the type of helper T cells (Th1 or Th2), the production of cytokines can vary. Th1 cells produce interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-2, IL-3 and IL-10. Th2 cells produce IL-3, IL-4, IL-5, IL-9, IL-10, and IL-13 (1). The role of each cytokine is discussed in Table 1.3.

Table 1.3 Principal producing cell for cytokines and their roles (16)(1)

Cytokine	Type of Cell Producing	Role
IFN-γ	Th1	• activates phagocytes, NK cells, and vascular endothelial cells • promote T and B cell differentiation • increase respiratory burst in neutrohpils
TNF-α	Th1	• activates macrophages, granulocytes, and cytotoxic T cells
TGF-β	Th3/Treg	• promotes apoptosis • inhibits the activation of lymphocytes and monocytes
IL-2	Th1	• promotes growth, differentiation and survival in T cells and b cells • stimulates NK cell growth
IL-3	Th2/ Th1	• increases growth of hematopoietic cells into myeloid progenitor cells
IL-4	Th2	• promotes growth and differentiation in B cells
IL-5	Th2	• stimulates eosinophil growth, differentiation and degranulation • promotes growth and differentiation in B cells
IL-9	Th2	• promotes T cell survival and mast cell activation
IL-10	Th2/ Th1/Th3/Treg	• inhibits IL-2 secretion, Th1 cells • down regulates MHC II and cytokine production by monocytes, macrophages and dendritic cells
IL-13	Th2	• inhibits activation of MCH II and cytokine secretion by macrophages • co-activates B cell proliferation

TH1, Type 1 Helper T cells; TH2, Type 2 Helper T cells; TH3, Type 3 Helper T cells; Treg, Regulatory T cells

As the immune response subsides, so do the effector immune cells that were activated during this process. The memory cells remain after the immune response is resolved; whenever the same immune response re-occurs these memory cells are able to respond more quickly and produce more antibodies, allowing for a quicker, more efficient response (1).

1.1.2.3 CHEMOATTRACTANT RECEPTOR-HOMOLOGOUS MOLECULE EXPRESSED ON TH2

Prostaglandin D_2 (PGD_2) is synthesized from arachidonic acid and is considered a major lipid mediator . PGD_2 is produced by mast cells and increases

in production in patients suffering from allergic diseases, such as asthma, atopic dermatitis, allergic rhinitis and allergic conjunctivitis (26-28). In asthmatic patients, PGD_2 is involved in modulating the airways by causing bronchoconstriction, vasodilation, increases capillary permeability and mucous production (26; 29-31). Chemoattractant receptor-homologous molecule expressed on Th2 (CRTH2) has been identified as one of the receptors that bind to PGD_2. Studies have identified the expression of CRTH2 on inflammatory cells, such as eosinophils, basophils and lymphocytes (26). In humans, CRTH2 expression on lymphocytes is found on cytotoxic T cells and helper T cells (type 2) but in rodents Th1 and Th2 cells express CRTH2 mRNA (32-34). In vitro studies have indicated that CRTH2 worsens the allergic response. It has also been established that as PGD_2 increases, so does the expression of CRTH2 on the inflammatory cells (26; 35). While there is a relationship between allergic inflammation and CRTH2 expression but no research has been conducted on the expression of CRTH2 in an obese population, specifically healthy obese people (classified as obese but do not exhibit symptoms of hypertension, dyslipidemia, glucose abnormalities or any functional limitations, common factors associated with obesity) versus obese people with type 2 diabetes mellitus.

Associations between allergic diseases and obesity have been established in obese children and adults compared to lean individuals (36; 37). It was found that as BMI increased, the number of children with asthma increased. It was also found that the severity of asthma in children increased as BMI increased. Obesity has also been associated with higher IgE circulation when compared to normal weighted children (38). The main cell types to respond to IgE are mast cells (1). Mast cells play an important role in inflammation and allergic response (5). White adipose tissue (WAT) in obese individuals were found to have higher number of mast cell recruitment when compared to lean individuals (39). It was also found that diet induced obese mice fed a western diet had higher number of mast cells in WAT compared to lean mice fed a control diet (39). Genetically altered mice

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($Kit^{W\text{-}sh/W\text{-}sh}$), deficient in mature mast cells, were fed a western diet and found to gain significantly less weight and have improvement in glucose homeostasis when compared to wild type mice fed the same diet(39). This suggests that mast cells are involved in the development of obesity and type 2 diabetes.

1.2 OBESITY

Obesity is an increase in adipose tissue creating excess body fat, where it begins to result in negative health effects (40). Measuring specifically the body fat accurately can become difficult, thus other methods can be used to help classify obesity. The measurement of one's Body Mass Index (BMI), which is calculated by dividing weight by height squared, is a practical and easy method (41). By using BMI values, the World Health Organization (WHO) has created a classification system for obesity (Table 1.4)(41; 42); Health Canada's classification system contains fewer categories (43). Waist circumference and waist-to-hip ratio (WHR) are other methods that are also used to help determine a persons' risk of developing obesity-related illnesses.

Table 1.4 WHO (left) and Canadian (right) classification of adults according to BMI

Classification (WHO)	BMI (kg/m^2)	Classification (Canadian)	BMI (kg/m^2)
Normal Range	18.5 – 24.9	Underweight	≤ 18.5
Overweight	≥ 25.0	Normal Weight	18.5 – 24.9
Preobesity	25.0 – 29.9	Overweight	25.0 – 29.9
Obesity Class I	30.0 – 34.9	Obese	≥ 30.0
Obesity Class II	35 – 39.9		
Obesity Class III	≥ 40.0		

BMI can indicate the risk that a larger body mass incurs for chronic diseases, but it does not measure body composition. Currently there are various techniques available to assess body fat; these techniques vary in accuracy and detail. Dual-energy X-ray absorptiometry (DXA) is one method of measurement that provides whole body and regional estimates of lean and fat mass as well as bone minerals (44). It uses a low-current X-ray tube that generates two sources of energy and the difference in attenuation determines the body composition (44).

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The advantages to using this device are that it is fast, precise and accurate (45). Disadvantages include the use of radiation and increased error estimation as torso thickness increases (46).

Bioelectric impedance analysis uses the relationship between the volume and length of the human body and the amount of resistance that occurs whenever an electrical current travels through it. By determining conductivity, total body weight and fat-free mass can be determined (47). Advantages of the method are that it is inexpensive, portable and safe. Disadvantages include that it is population-specific and has poor accuracy in specific groups and individuals (i.e. sex, age, and ethnicity).

Magnetic resonance imaging and computed tomography are the most accurate methods for assessing body composition (46). These techniques quantify the distribution of adipose tissue and estimate skeletal muscle, tissues and other organs (48). They have high accuracy but with high operating costs are unable to accommodate overweight people.

Using BMI and the previously mentioned body composition assessment techniques can be useful in assessing the health risk of a population, however, for individual assessments they lack the specificity required in a clinical setting. This includes a lack of detail regarding a subject's functionality, quality of life or other clinical risk factors that a clinician would require (49). A clinical staging system for obesity has been proposed (Table 1.5), known as the Edmonton Obesity Staging Score (EOSS), that allows clinicians to classify subjects with respect to the severity of risk factors, functionality and comorbidities (49). This staging system allows clinicians to go beyond using simple anthropometric measurements without having to carry out complex body composition analysis to classify health risk associated with obesity. The system has several limitations that include a reliance on the definition of risk or comorbid conditions, which are subject to change (49). It also fails to classify people that have weight-related complications but have lower body weights.

The WHO estimated that in 2005 there were approximately 400 million obese people and project that by 2015 this number will double (50). Due to this projection and our current situation of increasing obesity, the WHO regards obesity as an epidemic in the 21st Century (42). In Canada, three different surveys were conducted between 1970 and 1992 that demonstrated the increasing trend in overweight and obesity in the population. These surveys include the Nutritional Canada Survey (1970–72), the Canada Health Survey (1978–79) and the Canadian Heart Health Surveys (1988–92)(51). More recently, Canadian studies indicate that 40.2% and 19.0% of adult males are overweight and obese, respectively, and 27.2% and 16.7% of adult females are overweight and obese, respectively (52)(52).

As the proportion of overweight and obese individuals increase in a country, so does the cost of health care. An increase in weight has been shown to elevate the risk of various diseases and health complications. A cohort study, the Framingham Study, followed 5209 individuals for up to 24 years and reported a high association between obesity and the risk of developing cardiovascular disease (53). Increased BMI was also associated with a larger risk of developing cancer, for all types (54). Consistently, studies have reported that the probability of mortality increases as BMI increases (55). Adult males and females at the age of 40 with BMIs higher than 30 were found to lose 5.8 and 7.1 years of life, respectively, when compared to adults with normal BMIs (55). Respiratory diseases also constitute an important health complication for obese people. Obstructive sleep apnea and obesity hypoventilation syndrome are the most common respiratory diseases for obese people (42). It has been estimated that 40% of people who are obese have obstructive sleep apnea (56). Obesity is associated with increased risk of developing other health complications, including dyslipidemia, gallbladder disease, diabetes, kidney disease, and hyperuricemia.

Table 1.5 Staging system for obesity (49)

Stage	Description	Management
0	No apparent obesity-related risk factors (e.g. blood pressure serum lipids, fasting glucose, etc. within normal range), no physical symptoms, no psychopathology, no functional limitations and/or impairment of well being	Identification of factors contributing to increased body weight. Counseling to prevent further weight gain through lifestyle measures including healthy eating and increased physical activity.
1	Presence of obesity-related subclinical risk factors (e.g., borderline hypertension, impaired fasting glucose, elevated liver enzymes, etc.), mild physical symptoms (e.g., dyspnea on moderate exertion, occasional aches and pains, fatigue, etc.), mild psychopathology, mild functional limitations and/or mild impairment of well being	Investigation for other (non-weight related) contributors to risk factors. More intense lifestyle interventions, including diet and exercise to prevent further weight gain. Monitoring of risk factors and health status.
2	Presence of established obesity-related chronic disease (e.g., hypertension, type 2 diabetes, sleep apnea, osteoarthritis, reflux disease, polycystic ovary syndrome, anxiety disorder, etc.), moderate limitations in activities of daily living and/or well being	Initiation of obesity treatments including considerations of all behavioral, pharmacological and surgical treatment options. Close monitoring and management of comorbidities as indicated.
3	Established end-organ damage such as myocardial infarction, heart failure, diabetic complications, incapacitating osteoarthritis, significant psychopathology, significant functional limitations and/or impairment of well being	More intensive obesity treatment including consideration of all behavioral, pharmacological and surgical treatment options. Aggressive management of comorbidities as indicated.
4	Severe (potentially end-stage) disabilities from obesity-related chronic diseases, severe disabling psychopathology, severe functional limitations and/or severe impairment of well being	Aggressive obesity management as deemed feasible. Palliative measures including pain management, occupational therapy and psychosocial support.

The economic cost associated with obesity on the health care system is often not considered. In the United States, approximately 5-7% of the annual health care budget has been attributed to obesity (57), equivalent to roughly $100 billion. It is believed that this figure may be as large as twice the estimated value (57). This considerable estimation of cost is due to multiple factors that result from medical expenditures as well as decreased employee productivity, insurance costs, and shorter life expectancies. This situation creates fundamental questions that need to be addressed ranging from the production of higher quality food to improved social infrastructure in order to support obese people.

1.3 TYPE 2 DIABETES

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycaemia (58). The classification of diabetes mellitus is presented in Table 1.6. The hyperglycaemia is due to impaired insulin secretion and/or insulin action. There are 3 main types of diabetes: type 1 diabetes, type 2 diabetes and gestational diabetes (Table 1.6). In Canada it has been estimated that there are more than 3 million people who have been diagnosed with some form of diabetes (2010) (59). Alberta has approximately 130 000 adults diagnosed with diabetes (2007) (60). Worldwide, more than 220 million people are diagnosed with diabetes (61). Approximately 10% of diabetics are type 1 diabetics while 90% are type 2 diabetics. Type 1 diabetes is a condition that is usually diagnosed in children and young adolescents, while type 2 diabetes is usually diagnosed in adults but has become increasingly more common in children (59). Gestational diabetes occurs in approximately 2 – 4 % of pregnancies and can increase the risk of the mother and child developing diabetes (type 2 and rarely type 1) (52; 59).

Table 1.6 Three major diabetes descriptions (62)

Type	Description	Associated Health Risks
Type 1 Diabetes	Beta cells unable to produce insulin	Hypoglycaemia/Hyperglycaemia, Cardiovascular Disease, Diabetic Ketoacidosis, Microvascular complications Hypoglycemia
Type 2 Diabetes	Insufficient amount of insulin produced and muscle/adipose cells fail to properly utilize it (insulin resistance)	Blindness, Kidney Failure, Nonketotic hyperosmolar coma, Hyperglycemia, Cardiovascular Disease, Microvascular complications
Gestational Diabetes	Pregnant women who have never been diagnosed with diabetes but have high blood glucose levels during pregnancy	Hyperglycemia, Postpartum Diabetes, Stillbirth, obesity, impaired glucose tolerance, Preterm delivery, hypertensive disorder

Type 1 diabetes is a condition of deficient insulin production by the pancreas (61) due to the destruction of pancreatic β-cells (63). This destruction is facilitated by the abnormal activation of the hosts immune cells. The presence of CD4, CD8 T-lymphocytes, B-lymphocytes and macrophages in pancreatic biopsies of Type 1 diabetics has been reported (64). Type 2 diabetes is classified by insulin resistance and insulin deficiency (65). Insulin resistance, hyperinsulinemia, dyslipidemia and obesity precede diabetes in 75-85% of type 2 diabetic cases (66).

1.3.1 OBESITY AND ITS RELATIONSHIP TO TYPE 2 DIABETES

There is a relationship between obesity and the risk of developing type 2 diabetes. In the 1970s, Ethan A. Sims (67) coined the term 'diabesity', based on his observation of the association between people who were obese and those who developed diabetes. Between 60 and 90% of patients that are diagnosed with type 2 diabetes, are or were at one point obese (68). In Europe overweight and obesity account for an estimated 80-95% of type 2 diabetic cases (69). Epidemiological studies have reported that females and males classified as obese are 10 and 11.2

times more likely to develop type 2 diabetes, respectively when compared to lean individuals (70).

Aging, obesity and lack of physical activity are considered the largest contributors to the development of insulin resistance (71). Several mechanisms are believed to contribute to the association between obesity and insulin resistance. High circulating concentrations of free fatty acids (FFAs) originating from adipose tissue, have been found to contribute to insulin resistance in obese subjects (72). Obesity results in an increase in FFA delivery to liver, muscle, pancreas and brain. The resulting increase in FFA oxidation is associated with insulin resistance in liver and skeletal muscle (72). As FFA oxidation increases, glucose oxidation decreases (inhibited) which is mediated by GLUT-4 defects. An increase in FFA in plasma was also reported to contribute to reactive oxygen species (ROS) which activate protein kinase C (PKC). PKC, when activated, decreases phosphorylated insulin receptor-1 which decreases insulin GLUT-4activity (73). FFAs are also known to affect gene expression. FFAs bind to peroxisome proliferator-activated receptors (PPARs), which in turn promotes gluconeogenesis enzymes and inhibits glycolysis enzymes. Glycogen synthase total protein was found to decrease in the progression from a lean non-diabetic subject to an obese diabetic subject (74). This decrease in glycogen synthase protein contributes to impaired insulin efficacy and a 50% decrease in non-oxidative glucose disposal (74).

As insulin sensitivity decreases, it is important for β-cells to compensate for this reduction with an increase in insulin secretion. When β-cells are unable to compensate for this factor, the development of diabetes becomes an increasing risk. As a result, β-cells dysfunction causes an inability of the cell to release insulin quickly in response to glucose and non-glucose secretagogues, decreased efficiency of converting pro-insulin to insulin and reduced secretion of amylin (75; 76). Type 2 diabetes is a progressive disease and one of the main factors responsible for this is the decrease in β-cell function over time (75; 77). Blood

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glucose elevation in diabetics is thought to contribute to further β-cell dysfunction due to the glucotoxic effects (e.g., production of free radicals) on the cell.

During the development of obesity, adipocytes can undergo hyperplasia and hypertrophy in response to the increased need for storage of triglycerides in states of energy excess. Adipocyte size has been found to be predictive of developing diabetes. For example, a positive relationship between mean subcutaneous abdominal adipocyte size and body fat percent was reported in adult humans (78). A positive correlation between adipocytes size and body fat percentage was also found in subjects that had impaired glucose tolerance (IGT) and diabetes. Subjects that had either of the two conditions (hyperplasia and hypertrophy) had on average a significantly higher adipocyte size when compared to normal glucose tolerant subjects. The rate of insulin-stimulated glucose disposal also decreased as the size of the adipocytes increased.

As the size of the adipocyte changes, so does the risk of developing type 2 diabetes. A longitudinal analysis found that an 8% decrease in the rate of insulin-stimulated glucose disposal was associated with a 14% increase in adipocyte size (78). A cross-sectional study of non-diabetic subjects determined a negative correlation between subcutaneous fat cell size and insulin sensitivity (79). Physiologically, the role of the adipocyte changes in the transition from normal weight to an obese state. Hotamisligil, et al (1993) were the first to establish a difference in adipose tissue between lean and obese models using mice (80). They demonstrated that TNF-α mRNA was five to ten times more elevated in obese mice as compared to the lean mice.

A prospective study in Britain of 6916 males aged 40-59 studied the relationship between obesity and diabetes. In that study, if weight gain was greater than 10%, the relative risk of developing diabetes was 1.61(81). Numerous confounding variables were accounted for including age, initial BMI, smoking status, coronary heart disease and physical activity (81). Ford, E.S. et. al. (1997) looked at an adult population in the U.S. and found a positive relationship

between BMI and diabetes incidence (82). For every kilogram increase in body weight, the risk of diabetes increased by 4.5%. A prospective cohort study of 2000 women and men aged 25-74 investigated the relationship between weight and diabetes risk (83). Subjects with BMI less than 29 had a 9.6% increased risk for diabetes while subjects with BMI greater than 37 had a 26.2% increased risk. Subjects who gained one kg per year for 10 years had a 49% increased risk of developing diabetes compared to subjects who maintained their weight. Odds ratio was adjusted for age, BMI, sex, race, skinfold ratio and systolic blood pressure.

In Canada, Jiang et. al (2008) investigated the contribution of weight gain to the prevalence of diabetes in 72627 subjects aged 20-64. The percentage of overweight (BMI=25-29) was 40.4% and 26.5% for men and women respectively (84). For obesity (BMI $\geq$30) it was 16.6% and 14.9% respectively. The prevalence of diabetes for men was 3.4% and 2.9% for women. Significant associations were found between overweight/obesity and diabetes risk. For men, overweight was associated with a 2.1 fold increased risk of developing diabetes while for women it was 2.6. With respect to obesity, the risk of developing diabetes rose 4.3 and 7.7 fold for men and women respectively.

1.4 INSULIN RESISTANCE/OBESITY, TYPE II DIABETES IMMUNE FUNCTION

In North America, the incidence of obesity is increasing. With obesity come various complications such as insulin resistance, dyslipidemia, high blood pressure, sleep apnea, and the increased risk of cancer, diabetes, and cardiovascular disease. These metabolic complications can create a burden on multiple metabolic pathways and make it difficult for the body to maintain homeostasis. Chronic inflammation is associated with obesity and the pathological changes that accompany it (85).

It is well established that there is an association between insulin resistance, obesity, and low grade chronic inflammation. It is believed that adipose tissue

dysfunction plays an important role in the development of insulin resistance and inflammation in obese people. The expression in adipose tissue and concentration in plasma of many inflammatory markers is elevated in overweight and obese individuals. Some of the inflammatory markers that have been found to be elevated include C-reactive Protein (CRP)(86-88), IL-6 (86; 89), TNF-α (88; 89) and IL-1β (89). All of these elevations were detected in plasma from peripheral blood. Furthermore, obese mice lacking the ability to produce the following molecules: TNF-α, IL-6, MCP-1, and IL-1 have shown improvement in insulin sensitivity, therefore indicating a physiological relationship between the two (90).

1.4.1 IMMUNE CHANGES IN OBESITY

Adipose tissue is comprised of adipocytes, stromal and vascular cells. Previously, adipose tissue was perceived as simply an area to store energy as fat, but more recently it has been determined that the tissue has important autocrine, paracrine and endocrine functions, which in turn can affect immune function (91).

Various adipokines (proteins secreted by adipose tissue) have been identified that either exert pro-inflammatory or anti-inflammatory effects. Leptin, which regulates feeding behaviour, has been found to be positively correlated in plasma with body fat size (92). Leptin has pro-inflammatory effects, increasing TNF-α and IL-6 as its production increases (93). A study in 1999 examined the effects of leptin on circulating monocytes and it was found that leptin induced the production of two inflammatory cytokines, TNF-α and IL-6 (94). Activation markers were also assessed and an increase in expression of CD25, HLA-DR, CD38, CD71, CD11b and CD11c were observed after incubation with leptin (94). In vitro, leptin increased the mRNA expression of CC-chemokine ligands in murine macrophages, which are involved in the regulation of pro-inflammatory cytokines and chemotaxis of immune cells such as macrophages and T cells (95).

TNF-α, IL-6, IL-18 and monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) can also be classified as adipokines produced by adipose tissue. They have been identified to have pro-inflammatory effects. TNF-α, which is

mainly produced by monocytes and macrophages, was the first noted immune change in obese models of rats (fa/fa) and mice (db/db) (96). Obese animals had 5 to 10 fold elevation of TNF-α in adipose tissue when compared to lean animals. Studies have shown that as adipose tissue increases, TNF-α levels increase and whenever there is a reduction in body fat percentage, the expression of TNF-α decreases (97).

Plasma concentration of IL-6 has been found to be associated with obesity-related insulin resistance (93). Serum IL-6 concentrations are significantly higher in obese subjects compared to lean subjects (89). It has been estimated that one-third of the total circulating IL-6 originates from adipose tissue (98). IL-18 is also produced by adipose tissue (99). A higher concentration of IL-18 in serum was associated with increases in proteins VCAM-1 and ICAM-1 in the aorta of male Wistar rats suggesting a role in macrophage infiltration in the aorta (100). A positive correlation between MCP-1 and adipose tissue mass (epididymal fat pads) has been reported (101). High levels of mRNA MCP-1 have been associated with macrophage recruitment and inflammation in adipose tissue (101). The MCP-1 signal cascade involves the binding to a G-protein which activates intracellular signalling cascades and promotes monocyte migration to the source (site of intracellular signalling) (102). A relationship between central fat distribution and inflammation has also been demonstrated in obese individuals (103). An increase in central fat obesity was associated with a 53% higher increase in CRP protein, 30% higher TNF-α, 17% higher white blood cell count and 42% higher IL-6 expressions from serum samples.

Epidemiological evidence has indicated that people who are obese have higher and more severe incidences of infection compared to lean people (104; 105). The overall composition and function of immune cells from blood in obese groups (BMI = 33) was found to vary when compared to lean groups (106). Elevated leukocytes (31%), neutrophils (53%), lymphocytes (17%) and monocytes (24%) were found in obese subjects. T cells were found to be 18% and

B cells were found to 32% higher in the obese groups when compared to the lean groups. Increased T cell numbers is attributed by the higher helper T cell numbers (18%) in the obese group. Peripheral blood monocytes and granulocytes were found to have higher phagocytosis and oxidative burst activity. A complete understanding of the effects of obesity on the immune system is still to be determined but it is clear that there are some obesity-related influential factors that affect the equilibrium of the immune system.

1.4.2 INNATE IMMUNE SYSTEM (INFLAMMATION) AND OBESITY

Altered immune function in obesity was first reported in an animal model of obesity in 1993 (96). Male Zucker rats and lean controls were injected with a specific strain of bacterial yeast, *Candida albicans*, and the degree of colonization was measured. Obese rats were found to have significantly higher colonization compared to lean rats, suggesting reduced resistance. This reduced resistance was attributed to altered innate immunity as macrophages isolated from the obese rats did not phagocytose yeast as effectively as macrophages from lean rats. (107). Another study of obese fa/fa Zucker rats demonstrated a higher proportion of macrophages (CD11b/c) in spleen compared to lean rats (108). It was suggested that this increase in macrophages may be responsible for the higher production of inflammatory cytokines found in the obese rodents (108).

C-reactive protein (CRP) is an important acute phase protein responsible for early infection responses, and is involved in activating the complement pathway. A study of obese children determined that CRP concentration in serum was 3.7 to 5.1 times more likely to be elevated in obese boys compared to lean boys and 2.9 to 3.2 times more likely to be elevated in obese girls compared to lean girls (109). A cross-sectional survey in 2001 and 2005 reported that compared to subjects with normal body fat distribution, obese subjects had a 53% higher CRP concentration (87; 103). Body fat distribution has been found to be a contributing factor to CRP concentrations in blood, with central adiposity being associated with higher CRP compared to normal body fat distribution.

Orosomucoid, another acute phase protein, and CRP were both positively correlated with waist circumference and BMI in an adult population in 2003 (110). A study in 2006 of overweight children demonstrated that obese children had higher leukocyte counts than the normal weight children (111). Granulocytes cells, neutrophils and eosinophils, were also found to be higher in overweight children, other important components of the innate system (111).

Toll-like receptors (TLRs), which recognize PAMPs, were found to be elevated in the presence of LPS in obese subjects compared to lean non-diabetic subjects; specifically in adipose tissue (112). Abdominal subcutaneous adipocytes treated with LPS expressed more TLR-2 in the obese subjects (112). Circulating levels of LPS were also found to be higher in the obese subjects, suggesting that gut-derived bacteria or bacterial products may explain the higher expression of TLRs in circulation (112).

Natural killer cells (NK cells) are lymphocytes that play an important role in the innate immune system. Several cytokines, such as IL-2, IL-12 and IL-15, have been shown to have either stimulatory or inhibitory effects on NK cells (113). A study investigating NK cell activity in obese (BMI=36) and non-obese individuals did not report a difference between the two groups (113). As predicted, in vitro incubation of NK cells with IL-2 stimulated NK cells while cortisol inhibited the cells. Interestingly, the macronutrient content of the diet was associated with the response to stimulation/inhibition ex vivo by these two molecules. NK cell activity after in vitro IL-2 incubation increased as the percentage of energy intake from fat increased (obtained from dietary analysis) and decreased as the percentage of energy intake from carbohydrates increased (113). A study in 2009 found obese subjects (BMI = 47) had a significantly lower proportion of NK cells in blood compared to the lean subjects (9.1% vs. 12.3%) (114).

IL-17 and IL-23 are cytokines involved in the surveillance of cancers and infections but have also been implicated in the immune pathology of autoimmune

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diseases (115; 116). IL-23 has been established to be a recruiter/activator of monocyte and dendritic cells and these cells in turn are known to induce the production of IFN-γ and IL-17 (116). One study compared 26 obese women (BMI = 35.2) to 20 healthy lean women and investigated cytokine and hormone concentration in plasma (115). Plasma concentrations for leptin, macrophage migration inhibitory factor (MIF), IL-17 and IL-23 were all significantly higher in obese subjects compared to lean subjects (115). A positive correlation was also observed between IL-17 and IL-23 concentrations in plasma but interestingly there was no correlation between BMI, waist circumference and these two cytokines.

1.4.3 ACQUIRED IMMUNE SYSTEM AND OBESITY

    The acquired system has also been found to be altered in the obese state. A study in 2008 looked at the differences in immune function in lean and obese Zucker rats that were both fed a control diet (108). Overall results indicated that there was altered immune function in the obese fa/fa zucker rats compared to the lean control rats. Obese rats had higher production of TNF-α, IL-1β, and IL-6 by splenocytes when stimulated with mitogens ex vivo. IL-2 production was lower in the obese rats after concanavalin A (ConA) stimulation indicating a dysfunction in T cells. Immune phenotype analysis showed a decrease in total T cells(CD3+), helper T cells(CD3+CD4+) and activated NK cells (CD8+CD25+) in the spleens of obese rats when compared to lean rats.Immune dysfunction was also observed in another study of fa/fa Zucker rats over a 38 week period. Throughout the study obese rats maintained a hyperinsulinemic state without developing type 2 diabetes. At 8 weeks of age, there was significant reduction in the concentration of T cells in blood in obese rats compared to lean rats (117). CD3+ (T cells), helper T cells, (CD4+) and cytotoxic T cells (CD8+), were all found to be reduced in concentration from isolated splenocytes. At age 20 weeks and 38 weeks, splenocyte proliferation in response to ConA and

phytohemagglutinin (PHA) were reduced significantly, showing deterioration in immune response with age in obese rats.

The JCR:LA-*cp* rat is a rat that exhibits obesity, insulin resistance, hyperlipidemia, vasculopathy and atherosclerosis whenever they are homozygous for the autosomal recessive cp phenotype (118). A study in 2009 of JCR:LA-*cp* rats fed either a control, low fish oil or a high fish oil diet (119) determined that splenocytes isolated from obese insulin-resistant rats had a higher proportion of helper T cells but a lower proportion of non B cells expressing MHC class II molecule (CD11b/c+OX6+) as compared to lean animals fed the same diet. With a lower proportion of cells expressing MHC class II molecules, detection of cells with foreign particles may be impeded. It was determined that the phospholipid composition in the splenocytes did differ between the obese and lean rats fed the same control diet. Obese rats had a higher proportion of 16:0, 18:1n-9, 20:3n-3, 22:5n-3, 22:6n-3 and total MUFA and n-3 PUFA and lower proportion of 18:0, 24:1n-9 and n-6:n-3 PUFA, suggesting that obesity can alter the fatty acid composition of cell membranes which in turn can alter the immune response. The difference in phospholipid composition was hypothesized to contribute to the lower production of inflammatory cytokines (108). Studies have indicated that dietary lipids can influence T cell function through their incorporation into the cellular membrane (120; 121).

Immune dysfunction has also been reported in diet-induced obesity (DIO) animal models. Although diet may have contributed to the immune dysfunction, the findings of immune dysfunction in genetic models of obesity (108; 119; 122; 123) suggest that the obese state contributes to some extent to the changes in immune function. Female C57BL/6 and BALB/c mice were fed a high fat/calorie diet supplemented with sucrose and oleic acid and a mitogen (2,4,6-trinitrochlorobenzene) was administered to the ears and the subsequent swelling (immune response) assessed (123). The DIO mice had a lower degree of swelling

(induration) when compared to the lean mice when challenged a second time with this mitogen.

DIO has also been reported to affect lymphocyte numbers in adipose tissue (124). In mice, DIO resulted in a significantly higher proportion of CD3+CD8+ T cells, and lower CD3+CD4+ T cells and regulatory T cells when compared to the lean mice fed the control diet (124). The majority of the cytotoxic T cells that infiltrated the adipose tissue expressed CD62L⁻ and CD44+, indicating that they were activated effector cells. A time course trial was also conducted to determine the time necessary for DIO to alter the lymphocyte population in adipose tissue. After 2 weeks of DIO, significant increases of cytotoxic T cells were observed in the stroma, compared to control mice and this trend continued for 11 weeks. Interestingly, the reduction in helper and regulatory T cells as well as the infiltration of macrophages occurred after the accumulation of cytotoxic T cells, indicating that cytotoxic T cells play an important role in the inflammation cascade in adipose tissue (124).

Differences have also been observed in lymphocyte populations of C57BL/6 (B6) mice fed a high fat diet (HFD) compared to a normal chow diet (NCD) (125). After 14 to 18 weeks, mice fed the HFD had a trend of higher CD8 to CD4 ratios compared to the NCD in epididymal visceral adipose tissue (VAT). The HFD mice also had a 70% lower proportion of regulatory T cells compared to the NCD mice, which is believed to contribute to the proinflammatory state of the VAT (125). Rag1-null mice were then used to determine the effects of T cells on obesity. Rag1-null mice lack lymphocytes, gain more weight and have more VAT than the wild type mice. The Rag1-null mice fed a HFD had impaired fasting glucose, hyperinsulinemia and low insulin sensitivity when compared to mice fed a NCD. Mice were subsequently transplanted with either helper T cells or cytotoxic T cells the helper T cells were found to reduce the rate of weight gain in both diet groups. Also, when compared to cytotoxic T cells, helper T cells lowered serum concentrations of leptin, resistin and MCP-1, which are all obesity-

related adipokines (125). Helper T cells were also found to lower fasting insulin and glucose concentrations and increase insulin sensitivity.

Human studies have also reported immune cell dysfunction associated with obesity. A cross-sectional study in 1999 compared obese (BMI=33) and non-obese (BMI=21) subjects and found that total T and B cell count in blood were higher in the obese group (106). Of the T cell population, helper T cells were higher while cytotoxic T cells remained the same. Following ex vivo stimulation of lymphocytes with PHA, ConA and pokeweed mitogen (PWM), 25% lower lymphocyte proliferation was observed in obese individuals; suggesting that there was also a functional difference in T/B cells in obese subjects compared to lean subjects. Another study in 2005 of both lean and obese subjects investigated immune cell phenotypes in peripheral blood samples (126). A positive correlation was reported between helper T cells and BMI and negative correlations between concentrations of cytotoxic T cells and BMI. A negative correlation between BMI and the expression of CD95, a marker for apoptosis, on cytotoxic T cells was also found. This reduction in T cell function is one hypothesis as to why obese individuals have higher infection rates than the normal population (126). The overall literature indicates that there is a reduction T cell function but the mechanism still needs to be understood.

A 2009 study of NK cells in obesity (114), also compared cytotoxic T cells between obese and lean individuals and found that there were significantly less cytotoxic T cells in the obese individuals. Within the obese population, healthy obese individuals had significantly more cytotoxic T cells compared to the unhealthy obese population. The healthy obese were also found to have significantly more NK cells compared to the unhealthy obese which indicates that obesity-related comorbidities alter the overall inflammatory state beyond uncomplicated obesity (114).

The literature has shown that with obesity there are changes in the helper T cell population but little research has looked at which sub-population is most

effected (124; 127). As mentioned previously helper T cells can be divided into type 1 cells and type 2 cells. A study in 2012 showed that obese individuals compared to lean had a higher circulation of type 2 compared to type 1 cells (128) Surendar et al found that cytokine production (IL-12, IFN-γ, IL-4, IL-5 and IL-13) was higher in both sub-populations in individuals with metabolic syndrome when compared to individuals without indicating that both populations are effected (129). Type 1 to type 2 cell ratios have been shown to be higher in visceral adipose tissue from both DIO mice and humans (125). Ratios were found to increase but absolute numbers of type 2 cells were found to be the same indicating a shift to a pro-inflammatory response.

Obesity is no longer believed to be simply a state of excess body fat but rather a disease that has many important implications with respect to overall immune health. Studies investigating obesity in both humans and animals have shown a clear indication that systemic inflammation occurs (106; 119). Little is known how systemic inflammation is physiologically occurring but it has been reported that adipose tissue plays an important role in the secretion of various inflammatory/anti-inflammatory cytokines (93; 96). Animal studies have shown an increased production of inflammatory cytokines, such as TNF-α, IL-6 and IL-18, in adipose tissue of obese rodent models (94; 96). Human studies have also shown alteration in the innate system, with higher concentrations of circulating acute phase proteins and granulocytes in blood (103; 111; 115). NK cells numbers and activity was also found to be altered when compared between lean and obese states (113; 114). It was found in one study that neutrophil cell numbers did differ in the obese subjects (146), but functions were not studied in this or other studies of immune changes in obesity in the literature. Adipose tissue is not the only factor believed to be involved in systemic inflammation; T cells also play an important role in both regulating and potentiating inflammation. There are suggestions that this aspect of the acquired immune system may be altered in obese animals and possibly humans. In obese rodents, T cell phenotypes were

found to be altered with decreases in total T cell numbers and helper T cells from spleens when compared to lean rodents (108). In humans, helper T cell populations were found to be positively associated with BMI while cytotoxic T cells were found to be negatively associated with BMI (126). Altered immune states are well documented in animal models but less is known for humans. It is also unclear if these immune changes are related to the development of insulin resistance. Overall there is a clear indication that as you go from a lean state to an obese state, there is an alteration in the immune system but further research is required to understand physiologically why this is occurring.

1.5 DETECTING IMMUNE MARKERS FOR OBESITY
1.5.1 CYTOKINES AND IMMUNOGLOBULINS

Various techniques have been developed that can be used to detect important immune proteins, such as immunoglobulins and antibodies. One of the first assays to be commonly used throughout the scientific community was the immunometric assays also known as sandwich assays (6). Enzyme-linked immunounosorbent assays (ELISAs) are more commonly used today. This type of assay can be classified into either a competitive assay or a non-competitive assay. In a competitive assay, unlabelled antigens are added to wells with labelled antigens and the displacement of labelled antibodies is measured. A non-competitive assay, also known as sandwich assay, measures the fluorescence of labelled antibodies without the presence of unlabelled antigen, thus no competitive displacement takes place. ELISAs use two different antibodies (Fig. 1.1). The first antibody is bound to the plate which is used to keep the antigen adhered to the well so it does not dissipate during the washing cycles. Once the wells have been washed to remove any excess antigens that have accumulated a second antibody is added to the well (1). This antibody is labelled with an enzyme that is fluorescent when activated. Once the enzyme is activated, the intensity of fluorescence is used to determine to determine the relative concentration of the antigen in question.

1.5.2 IMMUNE CELL FUNCTION

One approach for functional assessment of T cells and B cells is through cell stimulation using various mitogens. A mitogen is a chemical compound that is used to induce cell division, also known as mitosis. Phytohemagglutinin (PHA) is a plant mitogen derived from lectins in plants and it is commonly used to stimulate T cells (6). Lipopolysaccharide (LPS) is another mitogen, consisting of polysaccharides and lipids that can be found on the outer membrane of gram negative bacteria (130). This type of mitogen stimulates B cells. Stimulation of lymphocytes can be used to determine the relative production of cytokines in T cells or immunoglobulins in B cells. ELISAs can then be used to determine the concentration of the protein of interest.
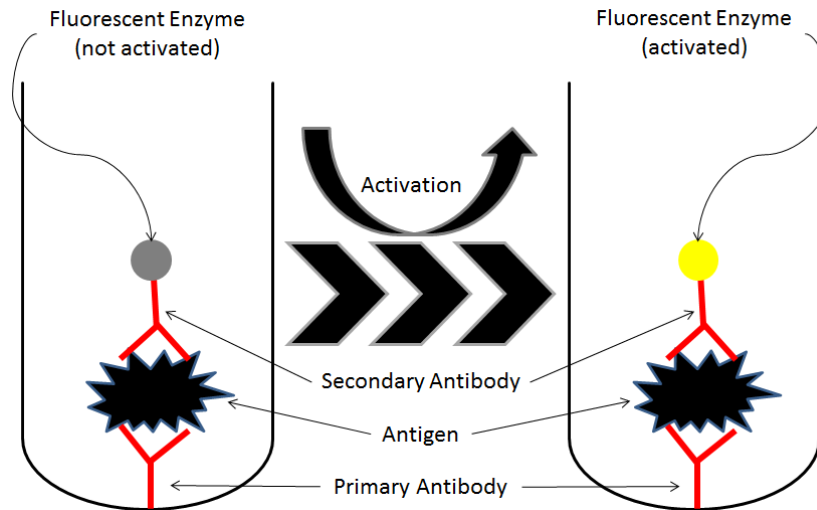
Figure 1.1 The make-up of an ELISA where the left side is the inactivated form and the right side is the activated form (1).

1.5.3 IDENTIFYING THE DIFFERENT TYPES OF IMMUNE CELLS PRESENT IN BLOOD

A standard tool in the evaluation of immune cells is a technique called flow cytometry (6). This technique can be used to characterize extracellular as well as intracellular conditions, achieved by tagging the cells with immunofluorescent markers. Monoclonal antibodies are conjugated with a

fluorochrome, a component which absorbs and emits light at a specific wavelength (131). The three most common fluorochromes used are fluorescein isothiocynate (FITC) which emits a green colour, phycoerthrin (PE) which emits an orange colour and peridin chlorophyll protein (PerCP) which emits a red colour (6).

The fluorescent antibodies are attached to the desired cells which are suspended in a fluid environment. Acquisition of the cells by the flow cytometer occurs by passing the cells through a laser in a single file fashion (Fig. 1.2). As the cells pass through the laser, two non-fluorescent characteristics besides the fluorescent characteristics are obtained which include forward scatter (fsc) and side scatter (ssc) (Fig. 1.2). Forward scatter refers to the size of the cell, while side scatter refers to the granularity of the cell (130)(118)(48)(47)(48).

The use of flow cytometry has many advantages which include the ability to analyze multiple colours simultaneously. Also, time-wise, the use of flow cytometry is less time consuming. Approximately 1000-2000 cells per second can be analyzed which allows for large amounts of information in a short period of time.
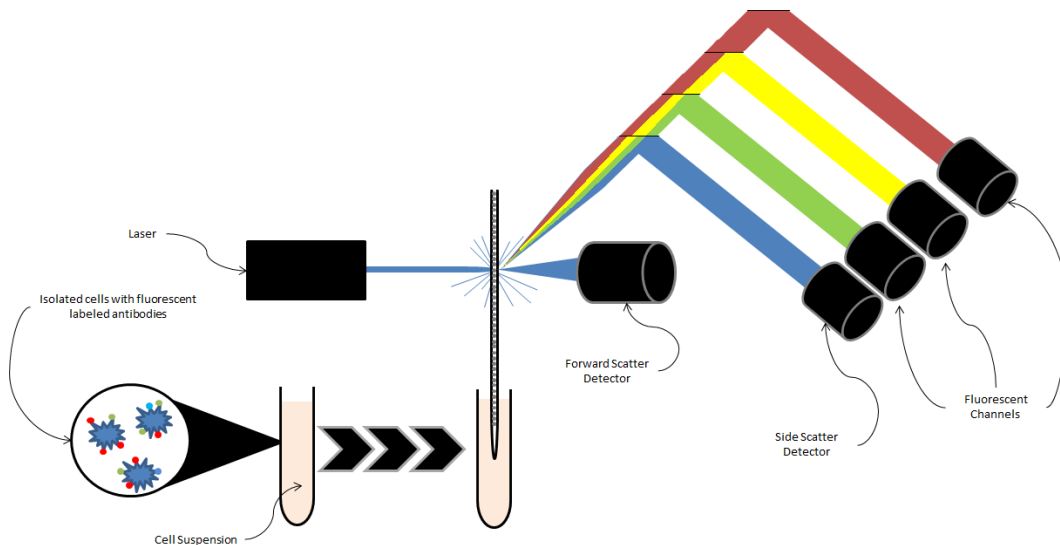
Figure 1.2 Technique used for analyzing cells using flow cytometry (132).

1.6 DIETARY TRANS FATS - VA AND CLA REVIEW

The links between obesity, adipose tissue and inflammation are prominent in the literature. Diet contributes to the development of obesity but little is known as to how this affects adipose tissue production and secretion of cytokines. Elaidic acid is one of the major trans-fats that is consumed in the human diet and is mainly derived from hydrogenated vegetable oils. This trans-fat in particular can impact health negatively, and is associated with the development of type 2 diabetes and dyslipidemia, both of which are major risk factor for cardiovascular disease (133; 134). The average consumption of industrially-produced trans-fats, a least in 2006, was estimated to be 2-3 percent of total calories, with the major sources being deep-fried foods, pastries and packaged foods (135). Clinical trials have demonstrated that consumption of trans-fats can increase low-density lipoprotein (LDL) cholesterol, decrease high-density lipoprotein (HDL) cholesterol, increase triglycerides in blood and reduce LDL particle size, which are all considered risk factors for cardiovascular disease (136).

Associations between industrially-produced trans-fats and negative health outcomes have been well-established; however, consumption of naturally occurring trans-fats has not been demonstrated to result in negative health outcomes. Trans vaccenic acid (VA) and conjugated linoleic acid (CLA) are two types of trans fats that are found in products of ruminant fat (i.e., Beef and dairy) (137). VA is classified as a positional and geometric isomer of oleic acid (138). The bovine rumen contains an extensive amount of bacteria (10^{10} to 10^{11} of per a millilitre of rumen) and protozoa (139). CLA is predominantly synthesized by the bacteria *Butyrivibrio*fibrosolvens but is also known to be synthesized by bacteria from the genus *Propionibacter*(140). *Propionibacter* is also involved in the conversion of polyunsaturated fatty acids to VA by the incomplete biohydrogenation of linoleic and linolenic acid in the rumen (141).

Once VA is absorbed into the body, this fatty acid has the potential to be converted to CLA in ruminants, rodents and humans (139; 142; 143). CLA is classified as a heterogeneous group of positional and geometrical isomers of linoleic acid (144). The most common isomer for CLA in the diet is the cis-9,trans-11 isomer which represents 80% of what is found in food and is the end product of VA conversion (140). Other isomer forms include trans-7,cis-9, cis-11,trans-13, cis-8,trans-10, and trans-10,cis-12 which are predominantly synthesized for neutraceutical preparations of CLA. Trans fats have had the stigma of having detrimental health effects when consumed, but these naturally occurring fats, VA and CLA (specifically cis-9, trans-11), have been shown to have various beneficial health effects including anti-carcinogenic, anti-atherogenic, anti-diabetogenic, anti-inflammatory effects, as well as enhanced immune function as well as anti-inflammatory (145).

Cardiovascular disease risk has been associated with the consumption of industrially-produced trans-fats (135) but more recently, VA and CLA have been shown to have either beneficial or no association with CVD risk (146-148). Moderate consumption of VA (4.03g/2500 kcal) in a double-blinded study resulted in neutral effects on plasma lipids and other risk factors for cardiovascular disease when compared to industrially consumed trans-fats (149). Compared to the industrial trans-fats diet group, waist girth, LDL, and apolipoprotein B all decreased in the moderate VA diet group. Another study investigated the consumption of partially hydrogenated vegetable oils and VA and found that VA decreased plasma total: HDL-cholesterol and non HDL:HDL-cholesterol ratios, which in turn could reduce coronary heart disease (150).

1.6.1 RELATIONSHIP BETWEEN TRANS-FATS AND IMMUNE FUNCTION

A study in 2005 explored the relationship between trans fats and biomarkers for inflammation (151) and reported that the most abundant trans-fat consumed was elaidic acid. It was also reported that there was increasing plasma

concentrations of CRP and IL-6 in higher quintiles of trans fat consumption (151). It was determined that CRP and IL-6 increased by 73% and 17% respectively when compared to the highest quintile of trans fat consumption to the lowest quintile. Results from this study suggest a strong positive correlation between the consumption of trans-fats (specifically industrially made) and concentrations of two key inflammatory markers in circulation. In another study, total trans-fats were also found to be positively correlated with TNF-α concentrations in women aged 25-42, suggesting that a higher intake of trans-fats was associated with a higher inflammatory state, but the study did not discriminate between the effects of industrial versus ruminant trans fats (152). The immune effects of ruminant trans fat appears to be different.

A study in 2009 looked at the impact of VA on the JCR:LA-*cp* rat, a rodent model for metabolic syndrome, and determined that VA consumption had no negative effects on the function of splenic and mesenteric lymph node (MLN) cells (153). More specifically when MLN cells were stimulated it was found that the cells from VA-fed rats produced a higher concentration of IL-10, a regulatory/anti-inflammatory cytokine (153). Another study in 2008 looked at the effects of CLA on an obese model of rats (108) and reported that when obese rats were fed CLA they produced less TNF-α, and IL-1β compared to obese rats fed a control diet, which suggests there is less macrophage infiltration occurring in the CLA fed rats.

A human study in 2005 assessed the effect of consuming CLA on the immune system (154). Two different isomers of CLA, c9, t11 and t10, c12 were utilized. A total of 42 healthy and moderately overweight people were recruited for the study. For 13 weeks, subjects were separated into 3 groups, one group consumed 3 g of a placebo dairy-like drink, another group consumed 3 g of enriched 9c, t11 and the final group consumed 3 g of enrich t10, c12. Overall it was found that there were no effects of daily consumption of the different dietary treatments on ex vivo cytokine production by isolated peripheral blood

mononuclear (PBMC) cells or by PBMC stimulated with LPS. A study in 2006 of subjects fed either 115 g fat/d (3.6 g VA) of butter or a control butter low in VA observed the impact of VA on serum CRP, oxidative stress and blood lipids (155). Intake of VA resulted in a decrease in total and HDL-cholesterol by 6% and 9% respectively. No differences were observed in serum CRP or oxidative stress between diets. Kuhnt et al. (2007) studied the effects of VA supplementation in human subjects on immune cells. In this study, the concentrations of IL-8, IL-1β, IL-6, IL-10, TNF-α, immunophenotyping in blood and phagocytosis were examined in whole blood. No differences were found between the control diet and VA diet for immune cell phenotypes, phagocytic function, plasma cytokines and adipokines and CRP.

Dendritic cells from BALB/c mice were isolated, treated with c9, t11, stimulated with LPS and cytokine production was measured (156). It was found that cells treated with c9, t11 had a higher production of IL-10, compared to cells treated with DMSO (vehicle control). This demonstrates the ability of CLA to inhibit the inflammatory response in dendritic cells, which play important roles in both innate and acquired immunity (156). Another study also looked at LPS stimulation of epithelial cell lines (157). Decreases in mRNA levels for TNF-α, IL-12 and IL-6 were observed in cells treated with CLA.

VA and CLA diets have also been shown to improve allergic inflammation in an animal model (158). Nine week old mice were fed either a control diet or an enriched milk fat diet with CLA (c9, t11) and VA. Allergen challenge administered to both groups led to suppressed airway inflammation in the enriched diet group compared to the control group. Feeding VA and CLA resulted in a reduced number of eosinophils and lymphocytes in bronchial fluid compared to normal milk fat and control diet.

Results from human studies have shown that when consuming the ruminant trans fat versus the control diet, no differences were found in the cytokine production, immunophenotyping or adipokines (159). Phagocytosis of

leukocytes, the migration, ingestion and oxidative burst, were also found not to be different between the diets indicating no negative effects on the innate immunity. Mitogen stimulation also showed that there was no difference in the cytokine production from LPS, which stimulates B-cells. As mentioned previously, adipose tissue has been shown to be involved in inflammatory production of cytokines which leads to a low grade chronic inflammatory state in obesity (96; 160).

In conclusion, the majority of studies have shown that VA and CLA improve immune function in lean models as well as obese mice and rats. More specifically these improvements resulted from higher production of regulatory/anti-inflammatory cytokines such as IL-10 (153). Higher production of IL-10 is consistent with increased production of regulatory T cells which play an important role in maintaining the homeostatic balance of immune cells. Currently there is no literature available pertaining to the relationship between the effects of diet on adipose tissue inflammation. More specifically, investigating whether naturally occurring trans-fats (VA and CLA) alter potential inflammatory mediators/cytokines in adipose tissue in the obese state is required.

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2 STUDY RATIONALE

2.1 RATIONALE

Currently in Canada, 34.2 % of the population is overweight and 18.1% are obese (1). Obesity, which leads to low-grade chronic inflammation, is considered a major risk factor for the development of type 2 diabetes (2-4). In 2010, 6.4% of Canadians aged 12 or older were diagnosed with type 1 or type 2 diabetes (5). Approximately 90% of people diagnosed with diabetes have type 2 as opposed to type 1.

The obese state is characterized by increases in circulating inflammatory cytokines (i.e. TNF-α, IL-6), leptin, free fatty acids, decreased β-cell function, and adiponectin, which are all believed to be involved in the increased risk of developing type 2 diabetes (2; 6; 7). Using BMI has been useful in assessing the health risk of a population, but for individual assessments they lack the specificity required in a clinical setting. This includes a lack of detail regarding a subject's functionality, quality of life or other clinical risk factors that a clinician would require (8). The EOSS system allows clinicians to classify subjects with respect to the severity of risk factors, functionality and comorbidities (8). This staging system allows clinicians to go beyond using simple anthropometric measurements without having to carry out complex body composition analysis to classify health risk associated with obesity. Currently a pro-inflammatory profile with the associative stages is lacking in the literature.

Studies have shown associations between high-fat diets and increase risk of obesity (9-11). The consumption of higher fat diets has been associated with a higher trans-fat intake, more specifically elaidic acid. This trans-fat is an industrially-produced fat that has been associated with increased risk of developing type 2 diabetes, cardiovascular disease and systemic inflammation (12-15).

Clinical trials have shown that trans-fat intake result in higher levels of LDL, very-low-density lipoprotein, triglycerides and lipoprotein A, which all

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increase the risk of developing CVD (16-19). This finding has led to legislation directed at reducing the intake of trans-fat in the population. Although reduction in total trans fat intake has become a public health goal, naturally occurring trans-fats, such as VA and CLA, have been shown to exert no negative health impacts and may even possess beneficial health effects (20-22). Animal studies have shown that VA consumption had no negative function on splenic or MLN cells when compared to control fed rats (23). Another study that fed rats CLA found the CLA fed rats produced less TNF-α and IL-1β compared to rats fed the control diet (24).

The risk of developing CVD has not only been associated with trans-fats but also with systemic inflammation (25). Malik et al. showed that increased CRP levels are associated with increased risk for CVD (26). Risk of developing CVD increased 2 fold with increased CRP levels. Inflammatory cytokines IL-6 and TNF-α have also been linked to CVD (27). Study participants classified as having high levels of IL-6 (3.24 pg/mL) and TNF-α (4.42 pg/mL) were found to have high risks of developing CVD by 27% and 22% respectively (27).

Studies have suggested that inflammatory state of obesity may be mediated by the adipose tissue (28). Altered systemic inflammation is often associated with altered T cell function (24). In support of this, alteration in T cell phenotype and function have also been noted to be altered in states of obesity and these changes may contribute to both adipose tissue and systemic inflammation (24; 29). Not all obese individuals have the associated health risks (healthy obese). Currently there is little information on the differences in immune phenotypes and function in healthy obese populations compared to obese populations with metabolic complications such as type 2 diabetes. Altered immune cell function might contribute to the systemic inflammation and increased risk of infections in individuals with type 2 diabetes.

Therefore the overall goal of this thesis is to firstly establish the contribution of a pro-inflammatory status between obesity and type 2 diabetes. Secondly to

explore whether increased intake of natural trans fats , that have been shown to benefit pro-inflammatory profile, will in fact modulate adipocyte contribution to improve this status.

2.2 HYPOTHESES AND OBJECTIVES

Hypothesis 1: That obese human subjects with type 2 diabetes will have a greater pro-inflammatory profile compared to the 'healthy' obese. More specifically that:

i)   Obese human subjects with type 2 diabetes will have higher leukocyte circulation and higher proportion of T cells and/or B cells compared to 'healthy' obese subjects.

ii)   Obese human subjects with type 2 diabetes will have impaired T cell/ B cell function and greater inflammatory cytokine response compared to 'healthy' obese subjects when stimulated with mitogens in vitro.

iii)   Obese human subjects with type 2 diabetes will have neutrophils that produce higher number of free radicals compared to 'healthy' obese subjects when stimulated with a mitogen in vitro.

Objective 1: To identify and compare immune profile and function of circulating immune cells between 'healthy' obese human subjects, and those who are obese with type 2 diabetes. More specifically,

i)   To identify differences in T cell and B cell phenotypes

ii)   To identify differences in the ability of isolated mononuclear cells to produce cytokines when stimulated with mitogens in vitro.

iii)   To identify differences in the ability of neutrophils to produce free radicals when stimulated with a mitogen in vitro.

Hypothesis 2: That an increased dietary intake of natural trans fats will have a positive effect on pro-inflammatory status in an obese rat model relative to control. More specifically,

i)   The deposition of fat in adipose tissue will contribute to the pro-inflammatory status of adipocytes in an obese rat model relative to control.

ii)   An increased dietary intake of conjugated linoleic acid and vaccenic acid in obese rats will result in an improvement in the pro-inflammatory status of adipocytes in obese rat models.

Objective 2: To determine the effects of increased dietary intake of natural trans fats on the cytokine expression in adipose tissue in an obese rat model. More specifically,

i)   To determine the modulation of cytokine expression in adipose tissue in obese rat model relative to control.

ii)   To determine if an increased dietary intake of conjugated linoleic acid and vaccenic acid will modulate cytokine expression in adipose tissue in an obese rat model.

2.3 CHAPTER FORMAT

Chapter 3 contains the experiment that identified and compared the immune profile and function of circulating immune cells between the 'healthy' obese and those who are obese with type 2 diabetes. This chapter addresses objective 2.

Chapter 4 contains the experiment that examined the effects of consuming natural trans-fats on the expression of cytokines in adipose tissue from obese rats. This chapter addresses objective 1.

Chapter 5 summarizes the findings for both experiments and provides an overall general discussion.

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**3 IDENTIFYING AND COMPARING IMMUNE PROFILE AND
FUNCTION OF CIRCULATING IMMUNE CELLS BETWEEN
'HEALTHY' OBESE AND THOSE WHO ARE OBESE WITH TYPE 2
DIABETES**
3.1 INTRODUCTION

Currently in Canada, the incidence of overweight and obesity in the population is 34.2% and 18.1%, respectively (1). With obesity, various complications such as dyslipidemia, high blood pressure, increased risk of cancer and sleep apnea have been associated (2; 3). More recently links between obesity, insulin resistance and altered immune states have been established (4). Immune dysfunction also appears to be a characteristic of obesity. Altered immune function in obesity was first reported in an animal model of obesity in 1993 (5). Furthermore, epidemiological evidence has indicated that people who are obese have higher and more severe incidences of infection when compared to lean people (6; 7). Immune dysfunction associated with the obese state has been reported for both functions of the innate as well as the acquired immune system.

In the innate system, animal studies have shown that obese rats have a higher proportion of macrophages (CD11b/c) in spleen when compared to the lean rats (8). It was suggested that the higher proportion of macrophages (i.e. the formation of crown-like structures) is one of the reasons as to why a higher production of inflammatory cytokines were found in the obese rodents (8) and why there may be chronic systemic inflammation (9). Immune dysfunction was also observed in another study on *fa/fa* Zucker rats, where they found significantly lower concentration of T cells in blood in the obese rats when compared to the lean rats (10). Obese rats also had higher production of TNF-α, IL-1β, and IL-6 by splenocytes when stimulated with mitogens ex vivo. T cells (CD3+), and the subset of T cells, helper T cells (CD4+) and cytotoxic T cells (CD8+), were also reported to be reduced in the spleen (10).

Individuals classified as obese are usually at a high risk of developing cardiovascular disease, type 2 diabetes and cancer (11), but not all obese individuals have the associated health risks (12; 13). Currently there is little

information on the differences in immune phenotypes and function in healthy obese populations compared to obese populations with metabolic complications such as type 2 diabetes. One study found that obese individuals (BMI = 30.9±5.2) with ≥ 2 MS components or diabetes had significantly higher circulating inflammatory markers of CRP, IL-6, TNF-α and PAI-1 compared to obese individuals with ≤ 1 MS component (13). Altered immune cell function might contribute to the systemic inflammation and increased risk of infections in individuals with type 2 diabetes.

Altered inflammatory status is well documented in both animal models (8) and in humans (14) with obesity. Studies have shown that these immune changes are related to the development of insulin resistance (15-17). T cell phenotype (CD3+, CD3+CD4+ and CD3+CD8+) and T cell cytokine secretion have been noted to be altered in states of obesity and these changes have been suggested to contribute to both adipose tissue and systemic inflammation (8; 18). However not all obese individuals have the associated health risks (19; 20). Evidence is mounting that not all obese individuals have the pathologies and risks that have been associated with obesity. These individuals are sometimes considered 'metabolically benign' or 'healthy obese' (13). They are classified as obese but do not exhibit symptoms of hypertension, dyslipidemia, glucose abnormalities or any functional limitations, common factors associated with obesity (13; 19). Using the Edmonton obesity staging system (EOSS) score, these individuals would be classified as stage 0 individuals (19). The EOSS score is a clinical staging system for obesity that allows clinicians to classify subjects with respect to the severity of risk factors, functionality and comorbidities (19).

Currently there is little information on the differences in immune phenotypes and function in healthy obese populations compared to obese populations with metabolic complications such as type 2 diabetes. Altered immune cell function does contribute to the overall systemic inflammation and increased risk of infections in individuals with type 2 diabetes (21; 22). The

current literature suggests that lean individuals have a difference in immune function than those that are obese. Therefore the purpose of this study is to identify and compare the immune profile and function of circulating immune cells between 'healthy' obese and those who are obese with type 2 diabetes.

3.2 MATERIALS AND METHODS

3.2.1 SUBJECTS

All procedures received ethical approval from the University of Alberta research ethics board. Subjects also gave informed consent. Peripheral blood samples were obtained from 19 obese subjects (5 male, 14 female; BMI≥35). Edmonton obesity staging classification system was used to classify subjects as either stage 0 or stage 2 (Table 1.5) (19). A total of 10 subjects were recruited for stage 0 and 9 subjects for stage 2. Criteria for inclusion were having a BMI≥35 and having no known infection at the time of blood draw (i.e. viral, bacterial, fungal). The stage 2 subjects also had to be diagnosed with type 2 diabetes. Subjects were excluded from the study if they had any surgery (major or minor) in the past 3 months, were diagnosed with any inflammatory condition (i.e. IBD, lupus, asthma, psoriasis, etc.), were currently taking anti-inflammatory medication or had recent fluctuation in body weight (i.e. their body weight changed by more than 5% in the last 3 months). Age, gender, height, weight, BMI, and medication records were obtained from the subjects' Alberta health care chart.

3.2.2 PERIPHERAL BLOOD SAMPLES

Subjects were asked whether they were in a fasted state at the time of blood sampling. A total of 18 mL of peripheral blood was collected into 4 heparinized vacutainer tubes (2 x 3 mL tubes and 2 x 6 mL tubes). Tubes were shaken thoroughly to prevent coagulation and kept on ice, except for one 3 mL tube which was kept at room temperature.

3.2.3 LYMPHOCYTE ISOLATION AND STIMULATION

Whole blood was centrifuged for 10 minutes at 1250 x g (Thermo Scientific Sorvall RT1 Centrifuge). Once centrifugation was completed, the

plasma (top layer) was removed and stored in 1 mL aliquots at -80°C. The remaining solution, buffy coat layer (which contains the platelets and leukocytes) and the red blood cell layer, was diluted and re-suspended with 1% bovine serum albumin (BSA; Sigma-Aldrich, Oakville, ON, Canada) in phosphate buffer saline (PBS). Four mL of re-suspended solution was then gently layered over 4 mL of histopaque 1077 solution (Sigma-Aldrich, St. Louis, MO, US). Tubes were centrifuged as before with no brake to prevent mixture of the gradient. The purpose of the histopaque solution is to separate the blood cell types in the re-suspended solution (Fig 3.1). Separation is established by the density gradient in the histopaque, allowing for the heavier cells (i.e. red blood cells) to migrate lower during the centrifugation process.
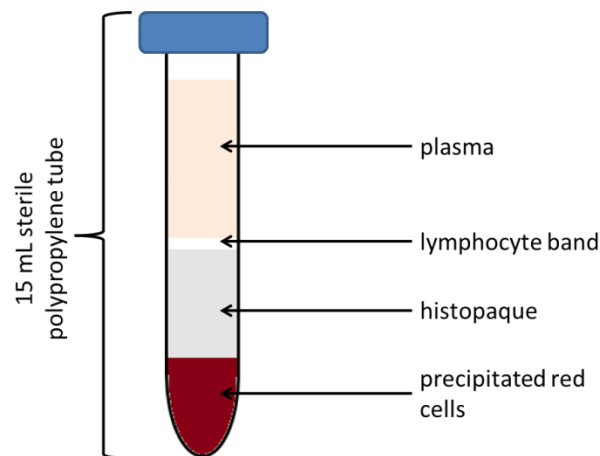
Figure 3.1 Cellular gradient after centrifuging with histopaque

The lymphocyte band at the gradient interface is removed using a transfer pipette and transferred to a 15 mL sterile polypropylene tube. BSA (1% w/v) in PBS was then added to a total volume of 14 mL and centrifuged at 750 x g for 10 mins at 4°C to remove the histopaque. Supernatant was then discarded and 2 mL of 5% complete culture media (CCM) (RPMI 1640 with NaHCO$_3$, fetal calf serum (FCS; Invitrogen), HEPES buffer, 2-mercaptoethanol and antibiotics) was added to re-suspend the cells. Twenty μL of 0.4% trypan blue and 20 μL of the

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lymphocyte sample are mixed together and loaded onto a hemocytometer to count the number of lymphocytes in the solution. Cells were counted in five different square grids and the total number was added. The value 3 was then divided by this number and multiplied by 10000 to give a volume in μL. Three 4 mL tubes were prepared with 3 mL of 5% CCM each. The volume amount determined by the above equation was then removed from the 4 mL tubes and replaced with the same amount of volume of re-suspended cells, therefore the total volume was still 3 mL. This adjustment converts the cell concentration to $1x10^6$ cells/mL.

Ex. $13 + 10 + 14 + 10 + 16 = 63 \rightarrow {}^3/_{63} = 0.0476 \times 10000 = 476\ \mu L$

To the three 4 mL tubes containing $3x10^6$ number of cells, 300 μL of either 5% CCM, phytohaemagglutinin (PHA, Sigma-Aldrich; Oakville, ON, CA)(10 μg/mL), or lipopolysaccharide (LPS, Sigma-Aldrich; Oakville, ON, CA)(25 μg/mL) was added and tubes were incubated at 37^oC for 2 days. After incubation tubes were centrifuged at 750 x g for 5 mins and the supernatant and pellet were aliquoted into 1.5 mL micro centrifuge tubes and stored at -80^oC until further analysis.

3.2.4 IDENTIFICATION OF IMMUNE PHENOTYPES

Whole blood was centrifuged (as described above) and the supernatant (plasma) was removed and stored at -80^oC until cytokine assays were performed. The red blood cells were treated with lysis buffer and centrifuged at 750 x g for 5 mins. After discarding the supernatant, the cells were washed with 4% FCS in PBS and centrifuged again. The supernatant was removed and 2 mLs of 4% FCS in PBS was added to re-suspend the cells. 100 μL of the re-suspended cells were added to V well plates (Corning, Lowell, MA, US) pre-conditioned with 4% FCS in PBS for 30 mins. To each well 20 μL of diluted antibodies were added according to the template in Table 3.1 and 20 μL of 4% FCS in PBS to the unstained well. The following pre-labelled monoclonal anti-bodies (mAb) were used: CD3, CD4, CD19, CD25 and β7 (FITC-labelled); CD8, CD25, CD71, CD80 and CD278 (PE-labelled); CD8, CD45RA and CD45RO (PECy5-labelled); and

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CD3, CD4 and CD20 (APC-labelled). All mAb were purchased from *e-bioscience* (San Diego, CA, US) except for CD25FITC which was purchased from *BioLegend* (San Diego, CA, US). Table 3.2 identifies the cell surface protein that is identified by each of the antibodies used.

Table 3.1 Antibody template

Well	Antibody
A1	4% FCS in PBS
A2	CD3FITC/CD56PE/CD8PECy.5/CD4APC
A3	CD25PE/CD8PECy.5/CD4APC
A4	CD19FITC/CD80PE/CD3APC
A5	CD19FITC/CD45RAPECy5/CD3APC
A6	CD3FITC/CD71PE/CD8PECy.5/CD4APC
A7	CD20APC
A8	CD25FITC/CD80PE/CD20APC
A9	β7FITC/CD25PE/CD45ROPECy.5/CD4APC
A10	CD4FITC/CD278PE/CD3APC
B1	CD3FITC
B2	CD8PE
B3	CD8PECy.5
B4	CD4APC

FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; PerCP, Peridinin chlorphyll protein; PerCP-Cy5.5, Peridinin chlorphyll-cyanine 5.5; PECy.5, Phycoerythrin cyanine 5

After the appropriate antibodies were added to each well, the plate was incubated at 4°C for 30 minutes. To wash the cells, 180 μL of 4% FCS in PBS was then added and centrifuged for 3 minutes at 150 x g. The excess fluid was removed by aspiration and the plate was vortexed to break up the pellet. To fix the cells, 200 μL of 1% paraformaldehyde was then added and after fixation the cells were transferred to falcon tubes to be analyzed by the flow cytometer (FACSCanto II, BD Bioscience, San Jose, CA, US). The percent of cells staining positive for the antibody were determined by counting the number of cells staining positive for each marker using Cell-Quest software (Becton Dickinson, San Jose, CA, US).

Table 3.2 Antibody description

Antibody	Cells Identified
CD3	T cells - identifies the TCR complex
CD4	Helper T cells - MHC class II coreceptor
CD8	Cytotoxic T cell - MHC class I coreceptor
CD3-/CD56+	NK cells
CD3+/CD4+/CD71+	Activated Helper T cells – Transferrin receptor on Helper T cells
CD3+/CD8+/CD71+	Activated Cytotoxic T cells – Transferrin receptor on Cytotoxic T cells
CD4/CD25/Foxp3	Regulatory T cells
CD19	B-cells
CD20	B-cells
CD19+/CD80+	Activated B-cells – Protein B7-1
CD25	IL-2 receptor α chain
CD45RA	Expressed on naïve cells – Protein tyrosine phosphatase, receptor type C isoform RA
CD45RO	Expressed on memory and activated cells - Protein tyrosine phosphatase, receptor type C isoform RO
CD278	Inducible T cell costimulator

The staining of lymphocytes with the antibody Foxp3 requires a modified procedure as this protein is intracellular. The 100 µL of re-suspended cells in the falcon tube was stained with 15 µL of an antibody cocktail containing CD4 and CD25 and incubated for 30 minutes in the dark at 4^oC. After incubation, 2 mL of flow staining buffer (FSB)(buffered saline solution containing fetal bovine serum and sodium azide (0.09%)) was added and centrifuged for 5 minutes at 750 x g. Supernatant was then aspirated and cells were re-suspended in 1 mL of freshly prepared Foxp3 LWB fixation/permeabilization (1:3) (for intracellular staining) working solution, vortex and incubated for 60 minutes at 4^oC in the dark. After incubation cells were washed with FSB, followed by centrifuging at 750 x g for 5 minutes. Supernatant was then aspirated and 20 µL of the Foxp3 antibody was added, vortex and incubated at 4^oC in the dark overnight. The following day the cells are washed with 2 mL of FSB, and centrifuged for 5 minutes at 750 x g. The supernatant was then aspirated and cells were re-suspended in 200 µL of 1% paraformaldehyde. After fixation the cells were analyzed using the flow cytometer

(FACSCanto II, BD Bioscience). The percent of cells staining positive for the antibody were determined by counting the number of cells staining positive for each marker using Cell-Quest software (Becton Dickinson, San Jose, CA, US).

3.2.5 IDENTIFICATION OF CRTH2

The identification of CRTH2+ cells required an additional assay. A total of 10 falcon tubes (5 mL) were required per sample, with 5 of the tubes containing the CRTH2 antibody and 5 for the negative control (Table 3.3). Each tube contained 100 µL of whole blood and were each mixed with 2 µL of fragment crystallisable region (FcR) blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany), 16.7 µL of mouse IgG (Invitrogen, Carlsbad, CA, US) and 16.7 µL of rat IgG blockers (Invitrogen, as above) to prevent non-specific binding. After the blockers were added, the samples were incubated in the dark at room temperature for 30 minutes. After the incubation, 12 µL of a CRTH2-biotin complex (Miltenyi Biotec, as above) was added to the positive tubes and 13.2 µL of IgG2a-biotin (AbD Serotec, Raleigh, NC, US) was added to the negative controls. Tubes were mixed well and incubated for 30 minutes at room temperature. After incubation 2 mL of lysing buffer was added to each tube, vortexed and incubated for 15 minutes in the dark at room temperature. After incubation, tubes were centrifuged for 5 minutes at 750 x g (Jouan, Perkin Elmer, Woodbridge, ON, Canada) and the supernatant was removed by aspiration. Each tube was then washed with 2 mL of PBS-FACS and centrifuged again with the same settings. The supernatant was removed by aspiration and 10 µL of streptavidin APC was added to each tube. To each tube the specific monoclonal antibodies were added (Table 3.3)(CD3FITC, CD3PerCP, CD8PE, CD193PE, γδPE, IgG1PE, IgG2aFITC, and IgG2bPE were purchased from *BDbioscience*, Mississauga, ON, CA; CD4FITC, IgG1FITC, and IgG2abiotin were purchased from *Abd Serotec*, Raleigh, NC, US; CD16FITC, CD14PerCP-Cy5.5, IgG1FITC, and IgG1PerCP-Cy5.5 were purchased from *ebioscience*, San Diego, CA, US; CD203cPE, and IgG1PE were purchased from *Beckman Coulter*, Mississauga,

ON, CA; CD294biotin was purchased from *Miltenyi Biotec*, Auburn, CA, US).

Refer to Table 3.4 regarding the cells each antibody identifies.

Table 3.3 CRTH2 antibody template

Positive Antibody Tubes	Negative Control Tubes
CD4FITC/CD3PerCP/CRTH2-biotin-strep-APC	IgG1FITC/CD3PerCP/Negative-biotin-strep-APC
CD8PE/CD3PerCP/CRTH2-biotin-strep-APC	IgG1PE/CD3PerCP/Negative-biotin-strep-APC
CD16FITC/CD193PE/CRTH2-biotin-strep-APC	IgG1FITC, K/IgG2bPE, K/Negative-biotin-strep-APC
CD203cPE/CD14PerCP-Cy5.5/CRTH2-biotin-strep-APC	IgG1PE/IgG1PerCP-Cy5.5, k/Negative-biotin-strep-APC
CD3FITC/γδPE/CRTH2-biotin-strep-APC	IgG1FITC/IgG1PE/Negative-biotin-strep-APC

FITC, Fluorescein isothiocyanate; PE, Phycoerythrin; PerCP, Peridinin chlorphyll protein; biotin-strep-APC, Bitoin-streptavidin-allophycocyanin; PerCP-Cy5.5, Peridinin chlorphyll-cyanine 5.5

Table 3.4 Antibody description

Antibody	Cells Identified
CD3	T cells
CD4	Helper T cells, MHC class II coreceptor
CD8	Cytotoxic T cell, MHC class I coreceptor
CD16	Neutrophils
CD193	Eosinphils
CD203c	Basophils
CD14	Monocytes
γδ	γδ T cells

After the antibodies were added to the tubes, they were incubated for 30 minutes at room temperature. After the incubation, 2 mL of PBS-FACS was added to each tube to wash any excess antibodies, centrifuged and the supernatant was removed by aspiration. Cells were then re-suspended in 250 μL of PBS-FACS and 250 μL of 4% paraformaldehyde in PBS and incubated for 30 minutes in the dark at room temperature. After incubation, 1 mL of PBS-FACS was added to each tube and centrifuged. The supernatant was removed by aspiration and the

cells were re-suspended in 500 µL of PBS-FACS and stored in the fridge (2-8°C) until analysis. Cell analysis was done by flow cytometry (FACSCantoII). Positive cells were determined by the relative fluorescence intensity from each marker using Cell-Quest software (Becton Dickinson, San Jose, CA, US). Two methods of analysis were used in this procedure which allowed for better distinction between cell populations. The first method used was gating the cell population of interest for one of the following antibodies: CD4, CD8, CCR3, CD203, and CD14 (low granular cells were gated with low SSC-H and high granular cells were gated with high SSC-H).The cells that were found to be positive for the desired antibody were then gated for CRTH2+ staining as shown in Fig. 3.2. The second method used was by gating the cell population that was CRTH2+ (low granular cells were gated with low SSC-H and high granular cells were gated with high SSC-H) and then gate for the specific phenotype (CD4, CD8, CD203, and CD14) (Fig. 3.3).
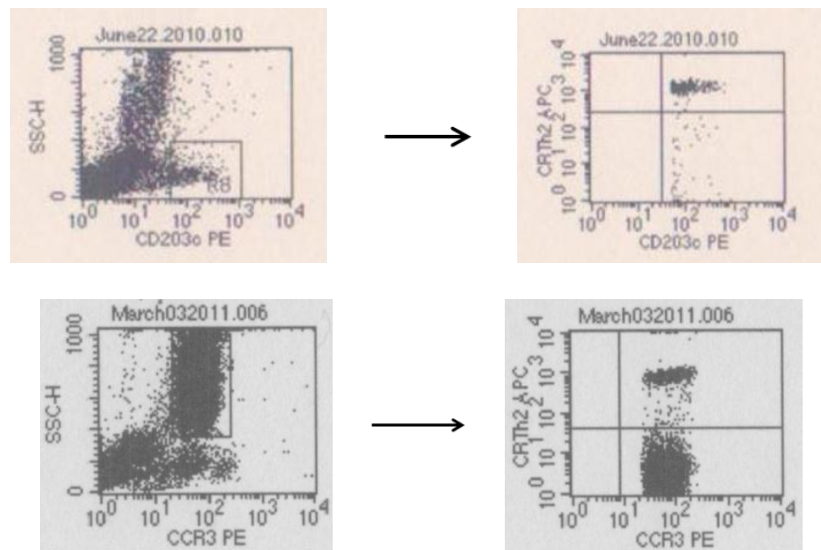
Figure 3.2 Analysis of the cell population using flow cytometry by first gating for the positive antibody CD4, CD8, CCR3, CD203 or CD14 (ex. CD203 for low SSC-H and CCR3 for high SSC-H) then gating the positive cells for CRTH2.
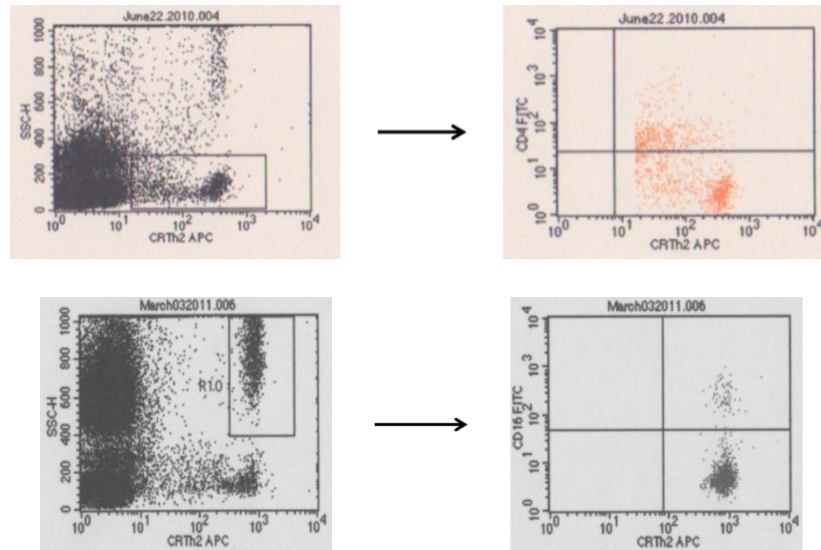
Figure 3.3 Analysis of the cell population using flow cytometry by first gating for the positive CRTH2 cell population and then gating for the antibody of choice (ex. CD4 for low SSC-H and CD16 for high SSC-H).

3.2.6 ANALYSIS OF CYTOKINE CONCENTRATIONS

Commercial ELISA kits IL-6, and CRP (R & D Systems; Minneapolis, MN, US) were used to quantitate these cytokines in plasma, following the manufacturer instructions. Concentrations of the standard curves for cytokines measured in plasma were ng/mL for CRP (0 – 50) and pg/mL for IL-6 (0 – 300). Commercial ELISA kits IFN-γ, IL-6, TNF-α, IL-10, IL-2, IL-1β (eBioscience, San Diego, US), and IgG (Bethyl Laboratories, Montgomery, US) were used to quantify these proteins in culture medium after stimulation, following the manufacturer instructions. Concentration of the standard curves for the cytokines was pg/mL except for IgG which was ng/mL. IFN-γ (0 – 2000), TNF-α (0 – 4000), IL-6 (0 – 2000), IL-10 (0 – 4000) and IgG (0 – 1000) cytokines were measured in unstimulated, PHA and LPS stimulated cells. IL-2 (0 – 2000 ng/ml) was measured in unstimulated and PHA stimulated cells. IL-1β (0 – 2000 ng/ml) was measured in unstimulated and LPS stimulated cells.

3.2.7 NEUTROPHIL FUNCTION ASSAY

Prior to aliquoting the whole blood, lysis buffer was warmed in a water bath (37°C) for 10 minutes. A total of 400 µL of the freshly drawn whole blood (performed assay within 2 hrs of acquiring blood) was aliquoted into a falcon tube and 4 mLs of warm lysis buffer (37°C) was added. The cells were then incubated for 10 minutes in a water bath at 37°C. After incubation the cells were centrifuged for 5 minutes at 750 x g and the supernatant was removed by aspiration. If the cells included too many red blood cells, the addition of the lysis buffer step would be repeated. Cells were then re-suspended in 4 mL of wash buffer, centrifuged at 750 x g for 5 minutes and the supernatant was removed by aspiration. Cells were then re-suspended in 500 µL of wash buffer. After the cells were re-suspended, 1.8 µL of dihydrorhodamine 123 (DHR) (Invitrogen, Burlington, ON, CA) was added to the cells and then incubated for 5 minutes in a water bath (37°C). After incubation, 100 µL of the sample was removed and placed in a falcon tube as a baseline measure and put on ice. 100 µL of phorbol myristate acetate (PMA) (Sigma-Aldrich, Oakville, ON, CA) was then added to the sample and put back into the water bath for another 5 minutes. After 5 minutes, 100 µL of the sample was removed and put in a falcon tube and put on ice. The remaining sample was then put back in the water bath and repeated two more times, resulting in 4 different time points (0, 5, 10 and 15 mins post-stimuation with PMA). After the cells were incubated, analysis by flow cytometry (FACScan-BSL2) was used to estimate cell granularity, size and free radical production. When the PMA was added to the sample, neutrophils were stimulated and the conversion of DHR (non-fluorescent) to rhodamine 123 (R123) (green fluorescent) occurs. This conversion occurs through the activation of NADPH oxidase which catalyzes the reduction of O_2 to O_2^- which dismutates to H_2O_2 (23). The production of hydrogen peroxide is what drives the conversion of DHR to R123, which in turn is what is measured by the flow cytometer. Positive cells were determined by the relative fluorescence intensity (concentration of the fluorescently labelled probes that bind

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to the cell), forward-scatter (cellular size) and side-scatter (cellular granularity) using Cell-Quest software (Becton Dickinson, San Jose, CA, US).

3.2.8 STATISTICAL ANALYSIS

All results are expressed as Means ± SD. All statistical analyses were conducted using PASW statistical software (version 18; IMB Inc., Armonk, NY, USA). Data was tested for normal distribution using kurtosis and skew and if it was not normally distributed, a non-parametric Mann-Whitney U test was done. The differences between stage 0 and stage 2 groups were analyzed using two sample equal variance T test with two-tailed distribution. For all results, P<0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 SUBJECT CHARACTERISTICS

A total of 19 individuals were recruited for the study. Ten Subjects, all female, were recruited for the category stage 0 and 9 subjects, 5 males and 4 females, were recruited for the category stage 2 (Table 3.5). All stage 0 subjects and 2 of the stage 2 subjects were in a fasting state. There were no significant differences in weight or BMI between the two groups (Table 3.5).The average ages for the stage 0 and 2 subjects were 42.8±11.7 and 55.8±7.8 yrs (p<0.05) respectively. The average height for the stage 0 and 2 subjects were 164.9±4.3 and 172.5±6.0 cm (p<0.05).

3.3.2 WHOLE BLOOD ANALYSIS

There was no difference in the concentration of white blood cells (Table 3.6) between the subjects. Group means were all within in the reference ranges as indicated in Table 3.6. The stage 0 subjects had significantly higher number of platelets compared to the stage 2 subjects (p<0.05).

Table 3.5 Anthropometric and hemoglobin A1c (HbA1c) measurements of study participants.

	Stage 0	Stage 2
Sex	10 females	5 males/4 females
Age	42.8±11.7[a]	55.8±7.8[b]
Height (cm)	164.9±4.3[a]	172.5±6.0[b]
Weight (kg)	123.2±24.04	118.2±11.0
BMI (kg/m^2)	45.3±8.6	39.1±5.0
HbA1c (%)	Not reported	7.29±1.29

Values represent mean ± SD. Values not sharing a common letter are significantly different ($p<0.05$).

Table 3.6 Whole blood analysis.

	Reference Values	Stage 0	Stage 2
RBC (10^{12}/L)	4.00 – 5.60	4.39 ± 0.31	4.49 ± 0.49
WBC (10^9/L)	4.00 – 11.00	6.57 ± 1.39	7.52 ± 2.60
HGB (g/L)	120 – 160	131.00 ± 9.93	136.40 ± 10.98
HCT (L/L)	0.36 – 0.48	0.40 ± 0.03	0.41 ± 0.04
MCV (fL)	82 – 100	90.40 ± 2.95	91.50 ± 4.23
MCH (pg)	26 – 34	29.85 ± 1.13	30.13 ± 2.42
MCHC (g/L)	320 – 360	330.70 ± 6.77	328.83 ± 19.23
RDW (%)	11 – 16	11.51 ± 0.60	10.90 ± 0.82
PLT (10^9/L)	150 – 400	277.20 ± 48.41[a]	214.20 ± 65.95[b]
MPV (fL)	5.9 – 9.8	7.90 ± 0.61	8.20 ± 0.96
Neutrophils (%)	50 – 70	58.68 ± 7.87	58.30 ± 8.20
Lymphocytes (%)	20 – 35	29.96 ± 6.64	29.09 ± 6.91
Monocytes (%)	1 – 6	7.12 ± 0.83	8.05 ± 1.82
Eosinophils (%)	1 – 3	3.38 ± 1.55	3.47 ± 2.01
Basophils (%)	0 – 1	0.85 ± 0.34	0.80 ± 0.32
Neutrophils (10^9/L)	2.00 – 9.00	3.83 ± 1.24	4.48 ± 1.98
Lymphocytes (10^9/L)	0.5 – 3.30	1.89 ± 0.48	2.14 ± 0.79
Monocytes (10^9/L)	0.00 – 1.00	0.45 ± 0.08	0.60 ± 0.22
Eosinophils (10^9/L)	0.00 – 0.70	0.20 ± 0.06	0.24 ± 0.10
Basophils (10^9/L)	0.00 – 0.20	0.07 ± 0.05	0.14 ± 0.26

Values presented as indicated in the brackets and represent mean ± SD; n=10 for stage 0 subjects and n=9 for stage 2 subjects. Values not sharing a common letter are significantly different ($p<0.05$). RBC, Red Blood count; WBC, White Blood Count; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, Red Cell Distribution Width; PLT, Platelet; MPV, Mean Platelet Volume

3.3.3 NEUTROPHIL FUNCTION

At time 0 and at every measured time point post-stimulation neutrophils from the stage 2 subjects produced more free radicals (as determined by a significantly higher mean fluorescence)(Fig. 3.4), were larger (Fig. 3.5) and more granular (Fig. 3.6), compared to the stage 0 subjects. The stimulation index was significantly higher at 10 min post-stimulation for neutrophils from stage 2 subjects (Fig. 3.7).
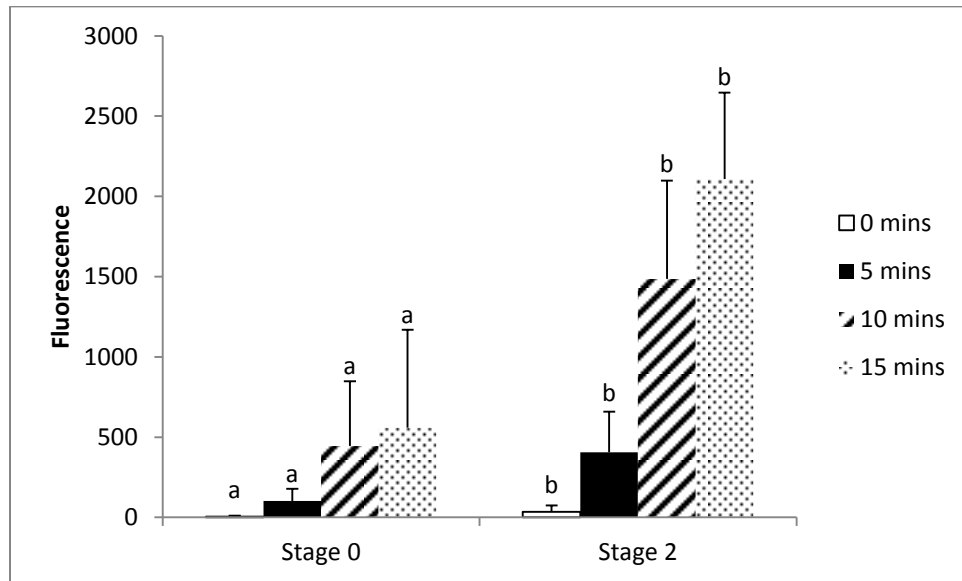
Figure 3.4 Fluorescence intensity for neutrophil cells at 4 different time points for stage 0 and stage 2 subjects. Bars represent mean ± SD (n=10 for stage 0 and n = 9 for stage 2). Bars at a time point that do not share a common letter are significantly different (p<0.05).
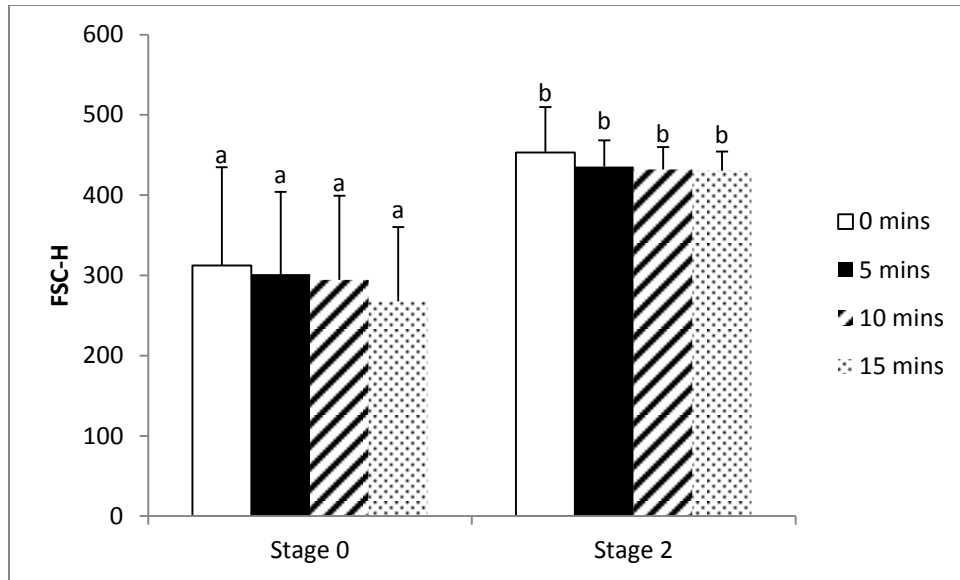
Figure 3.5 Forward scatter (FSC-H) intensity for neutrophil cells at 4 different time points for stage 0 and stage 2 subjects. Bars represent mean ± SD (n=10 for stage 0 and n = 9 for stage 2). Bars at a time point that do not share a common letter are significantly different (p<0.05).
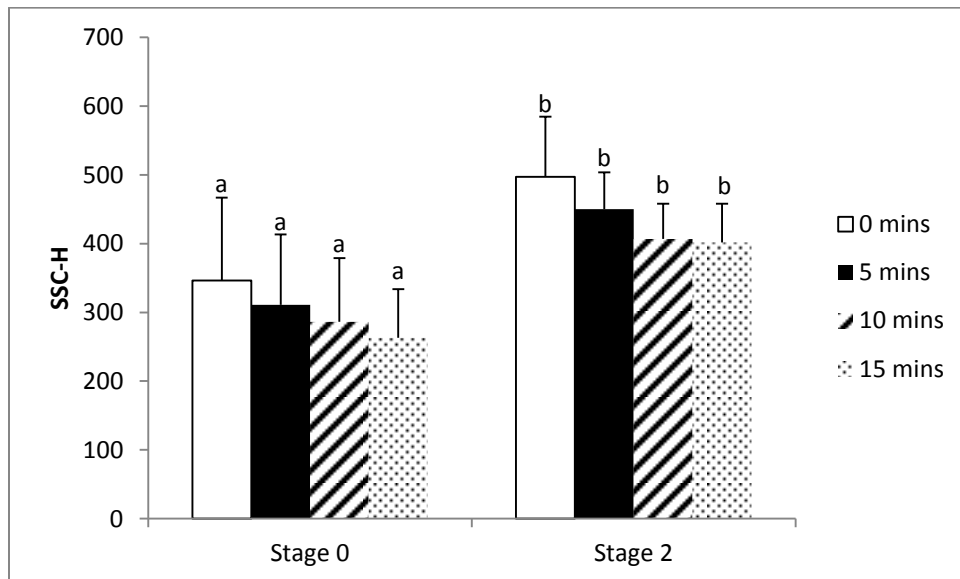
Figure 3.6 Side scatter (SSC-H) intensity for neutrophil cells at 4 different time points for stage 0 and stage 2 subjects. Bars represent mean ± SD (n=10 for stage 0 and n = 9 for stage 2). Bars at a time point that do not share a common letter are significantly different (p<0.05).
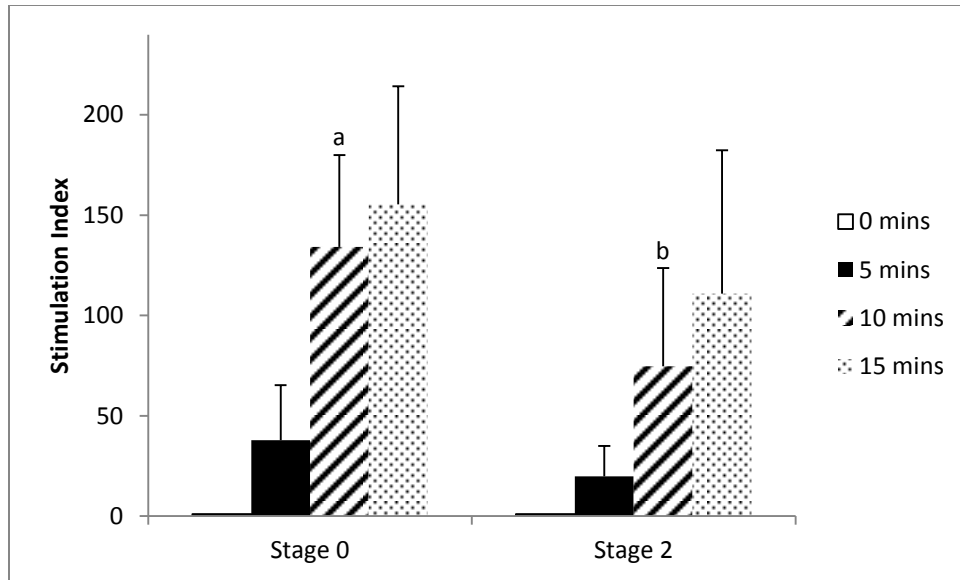
Figure 3.7 Stimulation index for neutrophil cells at 4 different time points for stage 0 and stage 2 subjects. Stimulation index refers to the ratio of fluorescence for cells exposed to the mitogen in relation to cells prior to exposure. Bars represent mean ± SD (n=10 for stage 0 and n = 9 for stage 2). Bars at a time point that do not share a common letter are significantly different (p<0.05).

3.3.4 IMMUNE PHENOTYPE

There was no difference in total proportion of CD3+, CD3+CD4+, CD3+CD8+ lymphocytes (Table 3.7), CD25+ or cells expressing CRTH2+ (Table 3.8, Table 3.9). Stage 2 subjects had a higher proportion of CD3+CD4+CD71+ and CD3+CD8+CD71+ lymphocytes compared to the stage 0 subjects (Table 3.6; Fig. 3.8, Fig. 3.9). Stage 2 subjects had a higher proportion of CD4+CD278+ (Table 3.7), CD3+CD45RA (naïve T cells) (Table 3.7) and CD4+CD25+Foxp3+ (Regulatory T cells) (Fig. 3.10) when compared to the stage 0 subjects.

Table 3.7 Lymphocyte phenotypes of stage 0 and stage 2 subjects.

Phenotype	Stage 0	Stage 2
Total CD3	58 ± 20	67 ± 7
CD3+CD4+	41 ± 20	33 ± 13
CD4+CD25+	3 ± 3	2 ± 2
CD3+CD8+	15 ± 4	20 ± 9
CD8+CD25+	0 ± 0	0 ± 1
CD3+CD4+CD71+	3 ± 2[a]	8 ± 7[b]
CD3+CD8+CD71+	4 ± 2[a]	10 ± 7[b]
CD3+CD45RA+	22 ± 11[a]	41 ± 17[b]
CD4+CD25+Foxp3+	3 ± 2[a]	13 ± 10[b]
CD20+	8 ± 5	11 ± 6
CD19+	7 ± 3	7 ± 6
CD45RO+CD4-	5 ± 5[a]	10 ± 4[b]
CD45RO+CD4+	6 ± 5	8 ± 6
CD4+CD278-	34 ± 20	15 ± 15
CD4+CD278+	0[a]	10 ± 7[b]

Data represent mean ± SD; n=10 for stage 0 subjects and n=9 for stage 2 subjects. Values are a proportion of total gated cells as determined by immunofluorescence. Values not sharing a common letter are significantly different (p<0.05).
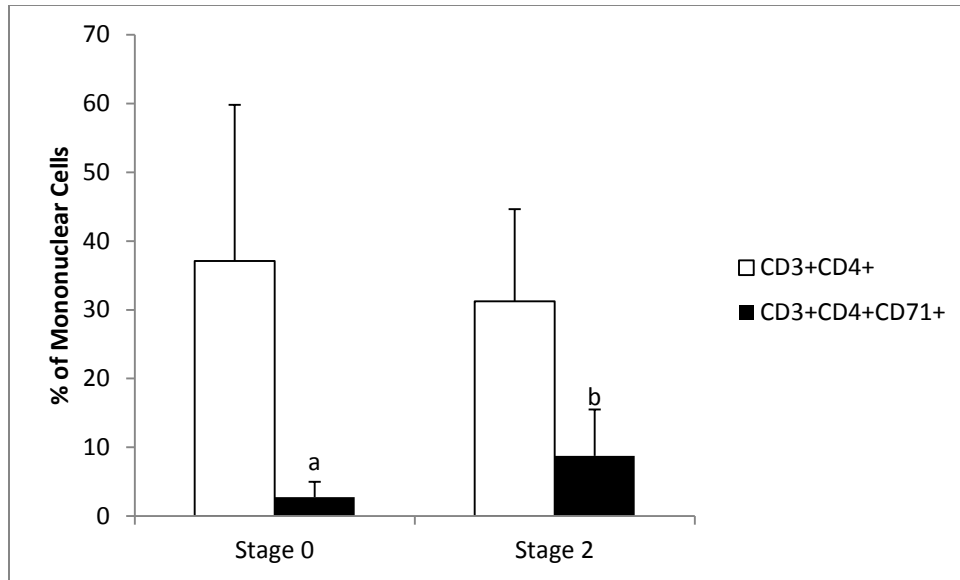
Figure 3.8 Lymphocyte phenotypes for CD3+CD4+ and CD3+CD4+CD71+ of stage 0 and stage 2 subjects. Data represent mean ± SD; n=10 for stage 0 subjects and n=9 for stage 2 subjects. Values are a proportion of total gated cells as determined by immunofluorescence. Bars not sharing a common letter are significantly different (p<0.05).
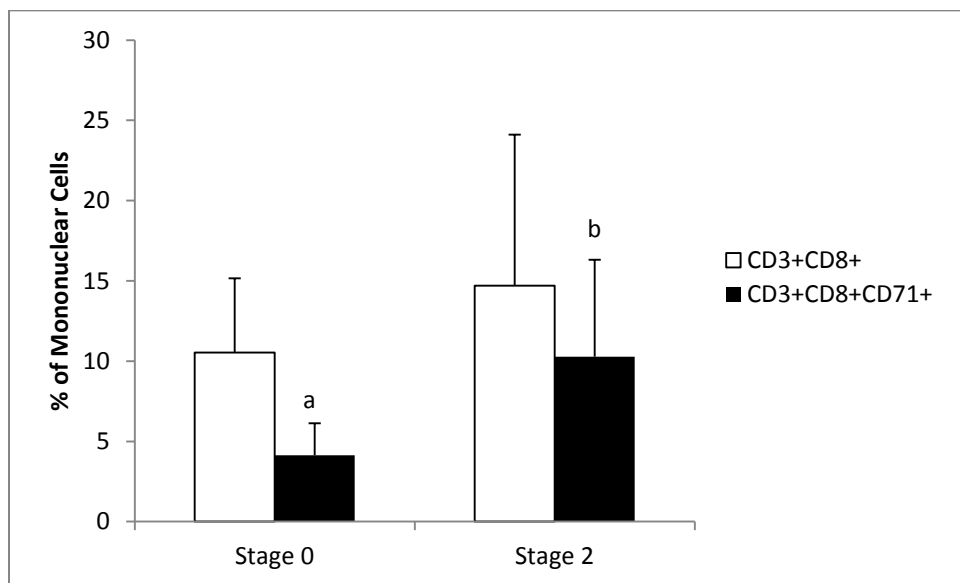
Figure 3.9 Lymphocyte phenotypes for CD3+CD8+ and CD3+CD8+CD71+ of stage 0 and stage 2 subjects. Data represent mean ± SD; n=10 for stage 0 subjects and n=9 for stage 2 subjects. Values are a proportion of total gated cells as determined by immunofluorescence. Bars not sharing a common letter are significantly different (p<0.05).
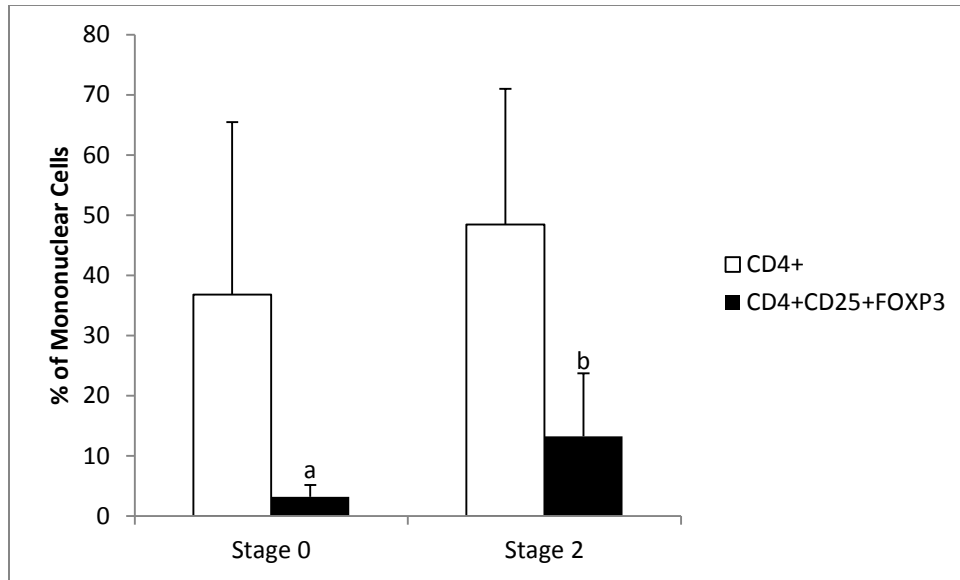
Figure 3.10 Lymphocyte phenotypes for CD4+ and CD4+CD25+Foxp3 of stage 0 and stage 2 subjects. Data represent mean ± SD; n=10 for stage 0 subjects and n=9 for stage 2 subjects. Values are a proportion of total gated cells as determined by immunofluorescence. Bars not sharing a common letter are significantly different (p<0.05).

Table 3.8 Leukocyte phenotypes of stage 0 and stage 2 subjects.

	% of Cells	
Phenotype	Stage 0	Stage 2
CD4+CRTH2+	2 ± 1	2 ± 2
CD8+CRTH2+	5 ± 8	6 ± 8
CD203+CRTH2+	71 ± 29	79 ± 8
CD14+CRTH2+	2 ± 2	1 ± 1
CCR3+CRTH2+	94± 10	96 ± 10

Data represent mean ± SD; n=10 for stage 0 subjects and n=9 for stage 2 subjects. Values are a proportion of cells positive for CD4+/CD8+/CCR3+/CD203+/CD14+ as determined by immunofluorescence. Values not sharing a common letter are significantly different (p<0.05).

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Table 3.9 Leukocyte phenotypes of stage 0 and stage 2 subjects.

Phenotype	% of Cells Positive for CRTH2	
	Stage 0	Stage 2
CRTH2+CD4+	20±8	24±11
CRTH2+CD8+	9±9	21±25
CRTH2+CD203+	47±11	41±17
CRTH2+CD14+	5±5	7±3
CRTH2+CCR3+	99±1	99±1
CRTH2+CD16+	7±4	8±5

Data represent mean ± SD; n=10 for stage 0 subjects and n=9 for stage 2 subjects. Values are a proportion of cells positive for CRTH2 as determined by immunofluorescence. Values not sharing a common letter are significantly different (p<0.05).

3.3.5 CYTOKINE PRODUCTION

Lymphocytes from stage 2 subjects produced significantly less IL-6 and TNF-α after PHA stimulation when compared to the stage 0 subjects (Fig. 3.11). Although not reaching significance there seemed to be a trend for cells from stage 2 subjects to produced less of each of the cytokines than stage 0 subjects after mitogens stimulation (Table 3.10)

Table 3.10 Cytokine production of lymphocytes stimulated with mitogens PHA and LPS for 48 hrs.

	Stage 0	Stage 2
IFN-γ (LPS)	184 ± 204	352 ± 316
IFN-γ (PHA)	6144 ± 6094	5781 ± 3493
IL-6 (LPS)	11463 ± 4132	8419 ± 2241
IL-6 (PHA)	2994 ± 2260[a]	979 ± 358[b]
TNF-α (LPS)	2380 ± 3788	1137 ± 439
TNF-α (PHA)	2144 ± 736[a]	1353 ± 660[b]
IL-10 (LPS)	624 ± 374	415 ± 245
IL-10 (PHA)	561 ± 265	560 ± 137
IL-2 (PHA)	4141 ± 2079	2333 ± 2346
IL-1β (LPS)	317 ± 155	371 ± 183
IgG (LPS)	807 ± 290	874 ± 248
IgG (UNS)	757 ± 127	618 ± 102
IgG (Plasma) (x1000)	7771 ± 11541	6514 ± 1397
IL-6 (Plasma)	11 ± 18	4 ± 2
CRP (Plasma)	3655 ± 3218	5244 ± 3477

All cytokine values are in pg/mL except for IgG and CRP which are in ng/mL. Values represent mean ± SD (n=10 for stage 0 and n = 9 for stage 2). Values not sharing a common letter are significantly different ($p < 0.05$).
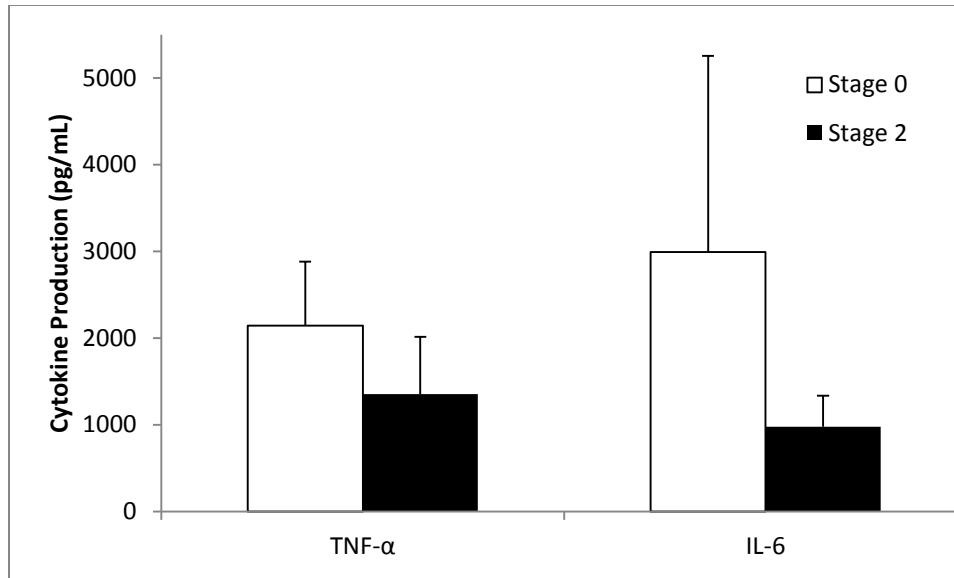
Figure 3.11 PHA-stimulated lymphocyte cytokine production of stage 0 and stage 2 subjects. Bars represent mean ± SD (n=10 for stage 0 and n=9 for stage 2). Both cytokines were found to be significantly different (p<0.05).

3.4 DISCUSSION

3.4.1 GENERAL DISCUSSION

The results from this study suggest that there is a difference in the immune system between subjects who are considered obese without type 2 diabetes and those who are obese with type 2 diabetes (refer to Figures 3.4-3.10). Differences between the groups were predominately found in what could be considered a qualitative manner as opposed to a quantitative one; in that the concentration and proportion of major cell phenotypes did not differ significantly between the groups. However markers that suggest functional differences of these cells did.

In the literature, differences in leukocyte counts have been observed when comparing a lean state to an obese state (24; 25). However leukocyte proportions and concentrations in blood were found not to be different between the stage 0 and stage 2 subjects. Our results showed that eosinphils and monocytes proportions in blood for both groups were slightly higher when compared to a specific set of reference ranges (Table 3.6). Technically there are no distinctive references ranges and can actually vary from 2-8% for monocytes and 1-6% for

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eosinophils (26-28). It should also be noted that the proportions used for the two cell types may have been found to be higher but the concentrations were still within the said reference ranges. No relationship between BMI and proportion of monocytes and eosinophils were found (not presented). Eosinophils and basophils are two main types of cells involved in allergic inflammation and because they were found in the upper end of the normal range, this could be an indication that they were in an allergic state (not measured). Platelet concentrations were found to be significantly higher in the stage 0 subjects compared to the stage 2 subjects but were still found to be within the reference range. Differences between the groups can be attributed to the differences in the sexes. Males have been shown to have lower platelets distribution compared to females and the stage 2 individuals included 5 males compared to the stage 0 having none (29).

In our study, the two groups were matched for obesity suggesting that the presence or absence of obesity may have accounted for the differences reported in these previously published studies. It has also been reported that total and differential blood cell counts were found to be higher as the number of components of metabolic syndrome increased (MS) (30). Our results suggest that reasonably well controlled (HbA1c=7.29±1.29) type 2 diabetes is not associated with these changes.

There was no difference between the B cell and the T cell proportion in blood, as well as the proportion of helper T cell, cytotoxic T cells, and cells expressing CRTH2, between groups. In the present study, differences between the two obese populations were found in the functionality of the neutrophils, production of cytokines by T cells and markers of maturation and activation of specific immune phenotypes (within a cell population) (Fig. 3.4-3.10). Interestingly the stage 2 group was found to have a higher expression of activated helper T cell (CD3+CD4+CD71+), cytotoxic T cells (CD3+CD8+CD71+), regulatory T cells (CD4+CD25+Foxp3+) and naïve T cells (CD3+CD45RA+) when compared to the stage 0 subjects (Table 3.7) (31). Stage 2 subjects produced

significantly less IL-6 and TNF-α compared to the stage 0 subjects when stimulated with PHA, a polyclonal T cell mitogen (Table 3.10).

Activated T cells are known to express surface makers CD25+, CD45RO (also expressed on memory T cells), and CD71+ (32; 33). These markers have been shown to be up-regulated on stimulated T cells, with similar proportions in proliferating cells (32). Both CD25 and CD45RO were used as activation markers but there were large discrepancies between the subjects therefore making them unreliable to determine cell phenotypes. CD71+ is a receptor for transferrin and once transferrin is bound to the receptor, it is taken up by the cell where it begins to release iron intracellularly (32). Iron is important in up-regulating enzymes involved in DNA replication of an activated cell (32). CD71+ has been shown to be higher when stimulated with leptin (34). Concentration of circulating leptin has been demonstrated to be higher in obese individuals when compared to lean individuals (35). Interestingly the same study showed that obese individuals with type 2 diabetes had higher concentrations of circulating leptin when compared to the obese individuals without type 2 diabetes (35). This is an indication that the stage 2 subjects could have a higher concentration of circulating leptin (leptin was not measured in our study) compared to the stage 0 subjects which could be why the stage 2 subjects had higher expression of CD71+ on T cells.

The literature for regulatory T cells in obesity and type 2 diabetes is conflicting (36; 37). One study suggests that with obesity, there is a higher expression of regulatory T cells in subcutaneous adipose tissue (36). Another study looked at regulatory T cells in adipose tissue from obese mice and found lower expression compared to the lean controls (37). Both these studies looked at mRNA expression in visceral adipose tissue, where as our study looked at circulating cells. With respect to regulatory T cells and diabetes, no effects have been observed in models of non-obese diabetic mice (type 1 model) (38). It was also shown that long-term effects of hyperglycemia had no effects on proportion of regulatory T cells when compared non diabetic mice (21). Further investigation

is required to look into the relationship between regulatory T cells and obesity/type 2 diabetes.

The higher proportion of activated naïve and/or regulatory T cells was likely not due to higher circulation of inflammatory cytokines (39) because circulating IL-6 was measured between the groups did not differ significantly (Table 3.10). Although CRP did not differ significantly, the mean value for CRP was higher in the stage 2 subjects, suggesting that there may have been a slightly higher inflammatory state (Table 3.10). The rate of new T cell generation is determined by differentiation of existing T cells, therefore with a higher proportion of activated T cells in the stage 2 subjects, there is an up-regulation of new T cells to replace the depleted ones (40), explaining the higher proportion of naïve T cells in the stage 2 subjects. Although the concentration of circulating monocytes were not found to be different between the two groups, the differences in immune cell types could be due to inflammation from activated macrophages that have infiltrated into adipose tissue (41). It has also been shown that this infiltration is preceded by activated cytotoxic T cells which could be why the stage 2 subjects had higher proportion of activated cytotoxic T cells (42). In obesity the macrophages typically found to infiltrate the adipose tissue are referred to as classically activated macrophages (M1) which are known for their pro-inflammatory cytokine production (IL-1β, IL-6, and TNF-α) (43). Besides the cytokines that are secreted by M1 macrophages, generally macrophages are also known to secret IL-8, which is considered a chemokine and an important cytokine in the activation of neutrophils (44). It has also been observed that plasma concentrations of IL-8 were higher in obese individuals when compared to lean subjects (45). The macrophages in adipose were not measured in the current study, but as the previous statement suggests there could be a difference between the groups, suggesting further investigation is required.

3.4.2 PERIPHERAL BLOOD MONONUCLEAR CELL CYTOKINE PRODUCTION

PBMC stimulation overall showed a trend of a lower cytokine response in the stage 2 subjects compared to the stage 0 subjects (Table 3.10). Cells stimulated with PHA produced significantly less IL-6 and TNF-α in the stage 2 subjects compared to the stage 0 subjects (Table 3.10). The lower production in cytokines could be an indication of an impaired T cell response. Another study has also shown that obese subjects compared to lean subjects have lower mitogen-stimulated PBMC proliferation (46). Even though the stage 2 subjects have a higher proportion of CD71+ T cells, their ability to produce and respond to cytokine production appears to be impaired which in turn could be a compensatory effect (i.e. more T cells are activated in an attempt to maintain cytokine production similar to the of stage 0 subjects).

IL-2 is considered an important cytokine involved in T cell function and one study found that with obesity, there is a lower serum IL-2 reported in obese subjects compared to lean controls (47). Our results also showed a trend for decreasing production of IL-2 in the stage 2 subjects when compared to the stage 0 subjects which furthermore suggests a possibly impaired T cell response.

No differences were found when cells were stimulated with LPS. LPS is expressed by all gram-negative bacteria and is used to stimulate B cells (48). Our results showed no differences in the proportion of B cells between the two stages (Table 3.10) but studies have shown higher proportions of B cells in obese subjects when compared to lean subjects (14). In the same study, it was also found that B cells from the obese subjects had lower induced mitogen proliferation when compared to the lean subjects. No studies have looked at the ability of B cells to respond to LPS ex vivo in individuals with type 2 diabetes.

3.4.3 NEUTROPHIL FUNCTION

Neutrophil fluorescence (free radical production) was found to be higher in the stage 2 subjects compared to the stage 0 subjects (Fig. 3.4). They were also

found to be larger in size and have more granules prior to and after stimulation (Fig. 3.5-3.6). Consistent with a higher initial activation stage (TIME 0), the stimulation index was lower (reaching significance at the 10 min point) for neutrophils from stage 2 subjects compared to the stage 0 subjects (Fig. 3.7). This higher activation state could be due to the IL-8 secretions from the macrophages residing in adipose tissue. Obese subjects compared to lean subjects have been shown to have higher number of macrophage in adipose tissue when compared to lean individuals (49). IL-8 is considered an important cytokine in neutrophil recruitment and function (50). Higher concentrations of IL-8 have been shown to increase the release of superoxides (51). Being in a higher stimulatory state, the neutrophils would have a reduced the ability to be further stimulated with PMA. There are very few studies looking at neutrophil function in obesity however one study reported improved phagocytic capacity of neutrophils in obese subjects that underwent a small bowel shunt operation (which is followed by weight loss) (52). Compared to the neutrophils pre-operative, there was a normalization of bactericidal capacity similar to the controls (52). This could be an indication that even though the stage 2 subjects have a higher basal production of free radicals, their ability to effectively eliminate foreign invaders may be inferior. Studies have shown subjects with type 2 diabetes have a higher number of neutrophils when compared to non-diabetic subjects, suggesting the possibility of a low grade infection (53). In this same study these type 2 diabetic subjects also had higher number of neutrophils expressing activation markers (CD66B+) when compared to the non-diabetic subjects (53). Consistent with our results, another study showed that there was significantly higher superoxide production from unstimulated neutrophils in type 2 diabetics compared to non-diabetic subjects indicating exhaustion of neutrophils in a unstimulated state (54). In relation to activated T cells, it has been shown that activated neutrophils can induce T cell proliferation and activation, which helps explain why our study showed stage 2 subjects having higher proportion of CD3+(CD4+/CD8+)CD71+ cells (55).

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3.4.5 CONCLUSION

In conclusion, our results suggest that people who are obese with type 2 diabetes (stage 2) have a change in function of T cells from whole blood compared to obese individuals without type 2 diabetes (stage 0). In addition to having a lower production of cytokines after stimulating with a T cell mitogen (PHA), the stage 2 subjects had a higher proportion of CD71+ helper T and cytotoxic T cells and a lower proportion of T regulatory cells. Further research is required to understand the implications these differences have on immune function and the pathologies associated with type 2 diabetes.

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4 EFFECTS OF CONSUMING NATURAL TRANS-FATS ON EXPRESSION OF CYTOKINES IN ADIPOSE TISSUE IN OBESE LEPTIN RECEPTOR DEFICIENT JCR:LA-*CP* RATS

4.1 INTRODUCTION

In obesity, adipose tissue plays an important role in low grade chronic inflammation. Adipose tissue has been demonstrated to have higher gene expression and secretion of pro-inflammatory cytokines in obese rats and humans (1; 2). In obese/diabetic mice models adipose tissue was found to have higher macrophage infiltration (3; 4). The specific macrophages that are present, classically activated macrophages, are known to secret pro-inflammatory cytokines (4).

Elaidic acid is one of the most commonly consumed industrially produced trans-fats coming mainly from sources of hydrogenated vegetable oil. The consumption of this particular acid has been associated with an increased risk of developing type 2 diabetes and cardiovascular disease as reported in animal and human studies (5; 6). Contrary to the evidence for industrially produced trans fats such as elaidic acid, naturally occurring trans-fatty acids such as ruminant fats, have not been reported to be associated with these negative health outcomes reported for industrially produced trans-fatty acids. The two types of ruminant fats include trans vaccenic acid (VA) and conjugated linoleic acid (CLA) (7).

The JCR:LA-*cp* rat is genetic model of metabolic syndrome that exhibits characteristics of obesity, dyslipidemia, insulin resistance and the development of atherosclerosis (8-10). For these characteristics to manifest, the rat has to be homozygous recessive for the corpulent phenotype (*cp/cp*) (10). Homozygous recessive for the *cp* phenotype, results in the inability of leptin to bind to the receptor. As a result, these rats can still be fed what is considered a healthy diet and still develop these metabolic and pathophysiological characteristics (8). Dysfunction in the leptin receptor allows the JCR:LA-*cp* rat to be an ideal model for metabolic syndrome that is exhibited in humans. Using these characteristics

and criteria, the JCR:LA-cp rat could be categorized under stage 2 from the EOSS system (11).

Feeding VA to insulin resistant obese JCR:LA-*cp* rats did not negatively influence cardiovascular risk factors (12), lipid synthesis (12), inflammation (13) or the function of immune cells isolated from spleen or mesenteric lymph nodes (13). Similarly, feeding the c9,t11 isomers of CLA to *fa/fa* zucker rats resulted in a lower production of TNF-α and IL-1β in splenocytes after stimulation compared to rats fed diet of similar fat content that was not supplemented with CLA (13). Additionally, there was no negative effect of feeding VA to obese rats on immune cell phenotype proportions of T cells, cytotoxic T cells, B cell and macrophages in spleen and MLN as compared to obese rats fed a control diet that did not contain trans fats (13).

The majority of human studies have confirmed that there are no detrimental effects of consuming diets containing VA and CLA on various parameters of cardiovascular and cancer health but more recently associations with VA and increased CVD risk have been shown (14-17). The consumption of CLA by healthy overweight individuals did not alter ex vivo cytokine production in unstimulated and stimulated PBMC (18). Consumption of VA by normal weight subjects did not alter oxidative stress, by measuring 8-iso-PGF$_{2\alpha,}$ an indicator of oxidant injury, in urine samples or the concentration of serum CRP (19). Kuhnt et al. (2007) looked at the effects of VA supplementation in human subjects on immune cells. In this study, the concentration of IL-8, IL-1β, IL-6, IL-10, TNF-α, immune phenotypes (T cells, helper T cells, cytotoxic T cells and B cells) in blood and a measure of phagocytosis by flow cytometry were examined using whole blood. No differences were found between the control diet and VA diet for immune cell phenotypes, phagocytic function, plasma cytokines and adipokines and CRP, supporting the conclusion that consuming VA does not negatively influence immune function.

Contrary to the studies in humans, feeding VA and CLA have been shown to have beneficial effects on CVD risk factors and immune function in animal models. Tyburczy et. al (2009) looked at partially hydrogenated vegetable oils and VA and found that feeding VA decreased plasma total: HDL-cholesterol and non HDL:HDL-cholesterol ratios, which suggest that this trans fat might be beneficial in reducing coronary heart disease risk (20). A study in 2008 looked at the effects of CLA in obese rats (*fa/fa* zucker) (21) and reported that when obese rats were fed CLA, isolated splenocytes after stimulation produced less TNF-α, and IL-1β compared to obese rats fed control diet which is consistent with a less reactive inflammatory response. In obese JCR:LA-*cp* rats fed VA, plasma triglycerides, total cholesterol and serum haptoglobin were all lower when compared to the obese controls, indicating significant improvements in dyslipidemia (12; 22). In the same model of rats, VA was also shown to normalize the production of mesenteric lymph node cytokine IL-2 and TNF-α, indicating that VA reduces the pro-inflammatory response when stimulated *ex vivo* (13).

The purpose of this study is to follow up on the beneficial effects that consuming ruminant trans fats have on immune function, inflammation and cardiovascular risk in an important tissue that is associated with inflammation in obesity. More specifically the purpose was to determine the effects of consuming natural trans-fatty acids on expression of cytokines in adipose tissue from obese insulin resistant rats. Adipose tissue expression of proinflammatory cytokines in obese rats and humans has been shown to be higher when compared to lean individuals (23; 24). Low grade chronic inflammatory state in obesity has been shown to be contributed to by adipose tissue adipokines and/or cytokines (25; 26). However, it is not known what the effects of consuming a diet high in the trans fats, VA and CLA, on adipose tissue expression of inflammatory cytokines. Characterizing the effects of diet on cytokine expression in adipose tissue may contribute to our understanding as to how ruminant fat consumption has beneficial effects on immune function and inflammatory state in obese rats.

4.2 MATERIALS AND METHODS

Procedures were reviewed and approved by the Committee of Animal Policy and Welfare of the Faculty of Agricultural, Life and Environmental Sciences at the University of Alberta and conducted in accordance with the Canadian Council on Animal Care guidelines. Nineteen male obese (*cp/cp*) and 8 lean (+/+ or +/cp) JCR:LA-*cp* rats were raised in an established breeding colony at the University of Alberta. At 3 wk of age, rats were removed from the breeding colony area and transferred to individually ventilated cages (TecniplastTM, Exton PA, USA) and had access to a standard rat chow diet (5001, PMI Nutrition International). At 8 wk of age, the lean rats were assigned a control diet and the obese rats (all groups n=8 except for obese controls n=3) were randomized and assigned to one of three nutritionally complete semi-purified diets (Table 4.2) for 16 wk (control, Vaccenic acid and CLA-c9, t11 diet). The fat content of the diet was 15% (wt:wt) and the fatty acid composition is reported in Table 4.3. The VA (1% w/w VA) and CLA (1% w/w CLA) diet was prepared weekly, formed into pellets, dried at room temperature, and stored at 4ºC in air-tight containers (27). VA was produced using chemical alkali isomerization from linoleic acid-rich vegetable oil (28). CLA was obtained from Lipid Nutrition which contained 59.8% of cis-9,trans-11 CLA and 14.4% of trans-10, cis-12 CLA (29). Following the treatment period of 16 weeks, rats were fasted overnight and euthanized using isofluorane anesthesia. Perirenal adipose tissue was removed, weighed and snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

Approximately 0.095 g of adipose tissue was used to analyze mRNA expression (Qiagen RNeasy Lipid Tissue Mini Kit) for cytokines TNF-α, TGF-β, IFN-γ, IL-1, IL-10 and IL-12. The mRNA was analyzed using probe and primer sets (to bracket the sequence gene of interest) from Applied Biosystems (Foster City, CA, US) by a 7900 HT Fast Real-Time PCR system for 40 cycles (Applied Biosystems, Carlsbad, US). One cycle consisted of samples being held at 95°C to denature and 60°C for annealing and extension. The number of cycles required for

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the fluorescents to meet the cycle threshold (C_t – the number of cycles the PCR system requires to begin detecting the increase in the fluorescent signal) was measured (Table 4.1). A comparative C_t method was used to quantify the results. Firstly, ΔC_t was determined by subtracting the C_t value of the gene of interest from the C_t value of the housekeeping gene (18S) ($\Delta C_t = C_{t(gene\ of\ interest)} - C_{t(housekeeping\ gene)}$). ΔC_t value was then used to subtract from a 100 and final results were expressed as 100 - ΔC_t.

Table 4.1 Average C_t values for cytokines

Cytokines	Average C_t for cytokines
18s	16.25±0.11
TNF-α	29.60±0.12
TGF-β	22.71±0.16
IFN-γ	32.01±0.15
IL-1	28.84±0.19
IL-6	32.71±0.35
IL-10	32.93±0.22
IL-12	33.21±0.33

Table 4.2. Diet composition (g/kg)

	Control Diet	CLA Diet	VA Diet
Casein	266.7	266.7	266.7
L-Methionine	2.4	2.4	2.4
Dextrose, monohydrate	231.3	231.3	231.3
Corn Starch	221.8	221.8	221.8
Cellulose	49.4	49.4	49.4
Sodium selenite	0.4	0.4	0.4
Manganese sulphate ($MnSO_4 \cdot H_2O$)	0.3	0.3	0.3
Mineral mix, Bemhart-Tomarelli (170750)	50.2	50.2	50.2
Vitamin mix, A.O.A.C. (no. 40055)	9.9	9.9	9.9
Inositol	6.2	6.2	6.2
Choline chloride	1.3	1.3	1.3
Cholesterol	10.0	10.0	10.0
Sunflower oil	65.0	65.7	65.7
Flaxseed oil	6.1	6.4	6.4
Soy tallow	58.6	62.9	62.9
Olive oil	20.3	0.0	0.0
VA	0.0	0.0	15.0
CLA	0.0	15.0	0.0

Table 4.3 Fatty acid composition (% of total fatty acids) of diets

	Control Diet	Vaccenic Acid Diet	CLA Diet
16:0	9.1	8.9	9.1
18:0	47.3	47.1	44.3
19:0	0.2	0.3	0.2
20:0	0.4	0.3	0.4
21:0	0.1	ND	0.1
22:0	0.4	0.6	0.1
24:0	ND	0.1	ND
16:1cis	0.1	0.1	0.1
18:1 t-11 (VA)	ND	6.0	5.6
18:1 n7	ND	0.6	ND
18:1 c-9 (OA)	17.3	9.0	10.5
18:1 c-11	ND	ND	0.5
18:2 n6 (LA)	23.3	24.7	20.4
18:3 n3 (ALA)	1.6	1.9	1.6
CLA c-9, t-11	0	0	3.9
CLA t-10, c-12	ND	0	0.8
other CLA	ND	0.1	0.3
20:2 n6	0.1	ND	1.6
22:4 n6	ND	0.6	ND
Summary			
$\sum$ total SFA	57.6	57.3	54.2
$\sum$ cis MUFA	17.4	16.1	11.1
$\sum$ PUFA	25.0	26.7	22.0
$\sum$ n-6 PUFA	23.4	25.4	22.0
$\sum$ n-3 PUFA	1.6	1.9	1.6
P/S ratio	0.4	13.4	0.4
$\sum$ CLA	0.0	0.0	5.0

VA, Vaccenic Acid; OA, Oleic Acid; LA, Linoleic Acid; ALA, alpha-Linolenic Acid; CLA, Conjugated Linoleic Acid; SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids. ND indicates not detected

4.2.1 STATISTICS

All results are expressed as Means ± SEM. All statistical analyses were conducted using SAS statistical software (version 9.1; SAS Institute Inc., Cary, NC, USA). Data was tested for normal distribution and if it was not normally distributed, groups were compared using the Kruskal-Wallis analysis (indicated with [†]). An ANOVA was used to determine the effect of diet on cytokine concentrations. Differences between the groups were identified using least square means. For all results, $P < 0.05$ was considered statistically significant.

<center>**4.3 RESULTS**</center>

4.3.1 BODY AND FAT WEIGHT

Obese rats fed the control, VA and CLA diet all had a higher final body weight (650±26 g, 660±33 g, 706±51 g vs 382±18 g, p<0.05) than the lean control rats (JCR:LA-*cp* strain)(Table 4.4). Fat pads from perirenal, inguinal and epididymal weighed significantly more in obese rats than the lean control-fed rats (Table 4.4). Diet did not significantly influence body or fat pad weight of the obese rats.

Table 4.4 Effects of diet on body and fat weight in lean and JCR:LA-*cp* rats.

Characteristics	Diet Treatment			
	Lean Control	Obese Control	VA	CLA
Body Weight (g)	382±18[a]	650±26[b]	660±33[b]	706±51[b]
Perirenal Fat (g)	0.68±0.19[a]	3.72±0.70[b]	3.57±0.63[b]	4.30±1.29[b]
Inguinal Fat (g)	0.70±0.24[a]	8.94±1.66[b]	9.30±1.59[b]	9.23±1.62[b]
Epididymal Fat (g)	1.02±0.35[a]	5.75±0.54[b]	5.95±0.63[b]	6.52±0.91[b]

Values represent mean ± SEM. Values not sharing a common letter are significantly different (p<0.05).
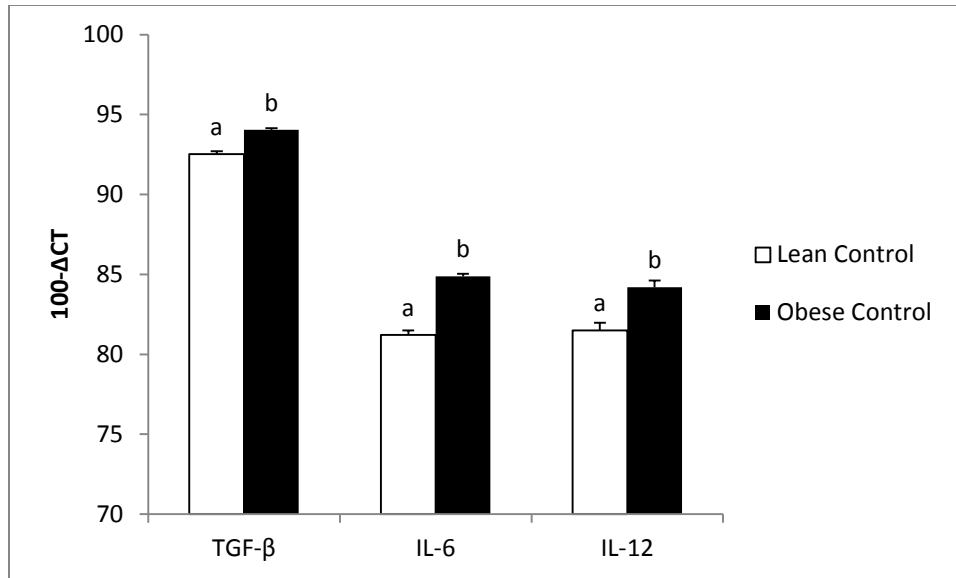
4.3.2 ADIPOSE TISSUE CYTOKINE MRNA EXPRESSION

Obese vs Lean (control diet): Fat pads from obese rats had a significantly higher expression of TGF-β, IL-6 and IL-12 (Table 4.5). The expression of TNF-α, IFN-γ, IL-1 and IL-10 in adipose tissue did not differ between lean and obese rats.

Effect of diet in obese rats: Obese rats fed CLA had a significantly higher expression of IL-6 than those fed VA (Table 4.5; Fig. 4.2). The expression of IL-6 and TNF-α in adipose tissue from CLA-fed rats was significantly higher than that of the lean controls (P<0.05) (Table 4.5; Fig. 4.3). The expression of IL-12 in adipose tissue from obese rats fed the control or VA diet, but not the CLA-diet were significantly higher than that in adipose tissue from lean control fed rats (Table 4.5; Fig. 4.4).

Table 4.5 mRNA expression in perirenal adipose tissue from lean and obese rats.

Cytokines (100-ΔCT)	Diet Treatment			
	Lean Control	Obese Control	VA	CLA
TNF-α	86.10 ± 0.18^{a}	86.92 ± 0.18^{ab}	86.58 ± 0.23^{ab}	87.41 ± 0.32^{b}
TGF-β	92.53 ± 0.18^{a}	94.04 ± 0.10^{b}	93.77 ± 0.22^{b}	94.54 ± 0.16^{b}
IFN-γ	83.99 ± 0.20^{a}	84.55 ± 0.34^{a}	83.90 ± 0.26^{a}	84.83 ± 0.28^{a}
IL-1†	86.82 ± 0.24^{a}	87.58 ± 0.08^{a}	87.13 ± 0.19^{a}	88.44 ± 0.44^{a}
IL-6	81.21 ± 0.43^{a}	84.88 ± 0.15^{bc}	84.42 ± 0.51^{b}	85.34 ± 0.14^{c}
IL-10	82.93 ± 0.52^{a}	83.61 ± 0.27^{a}	83.26 ± 0.31^{a}	83.80 ± 0.24^{a}
IL-12	81.49 ± 0.48^{a}	84.20 ± 0.42^{b}	83.74 ± 0.41^{b}	83.34 ± 0.50^{ab}

Values represent mean ± SEM (n=8 for lean controls and n = 3 for obese controls). Values not sharing a common letter are significantly different (p<0.05). †Statistical test Kruskal-Wallis was done due to not being normally distributed.

Figure 4.1 mRNA expression in perirenal adipose tissue from lean and obese rats. Bars represent mean ± SEM (n=8 for lean controls and n = 3 for obese controls). Bars not sharing a common letter are significantly different (p<0.05).
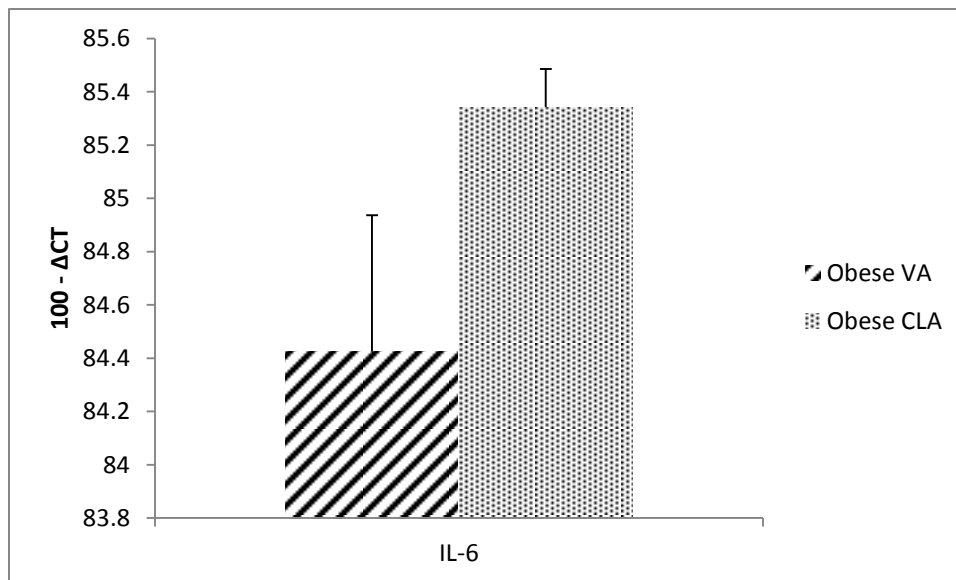
Figure 4.2 mRNA expression in perirenal adipose tissue from obese rats fed a CLA diet and obese rats fed a VA diet. Bars represent mean ± SEM (n=8 for obese CLA and obese VA). Bars were found to be significantly different (p<0.05).
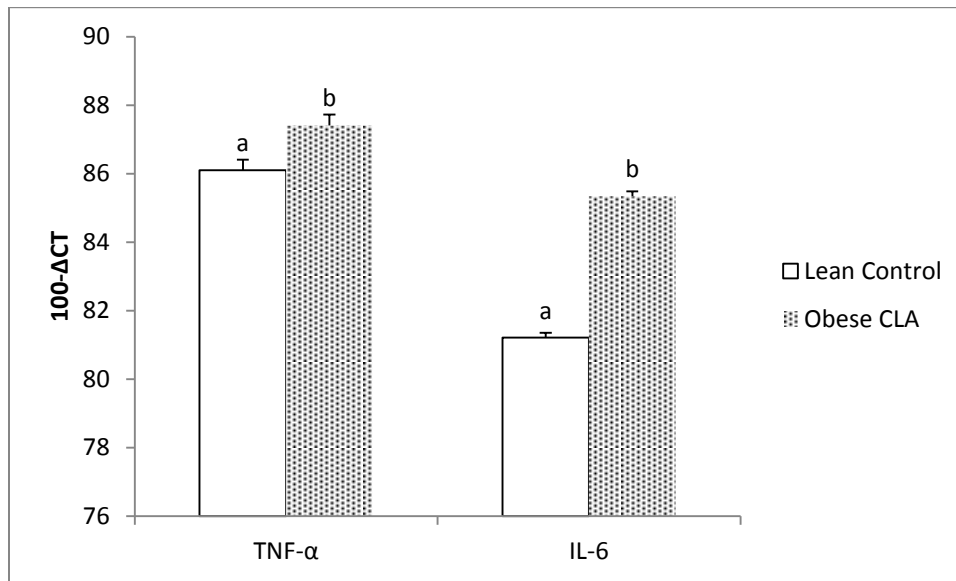
Figure 4.3 mRNA expression in perirenal adipose tissue from lean rats fed a control diet and obese rats fed a CLA diet. Bars represent mean ± SEM (n=8 for lean controls and obese CLA). Bars not sharing a common letter are significantly different (p<0.05).
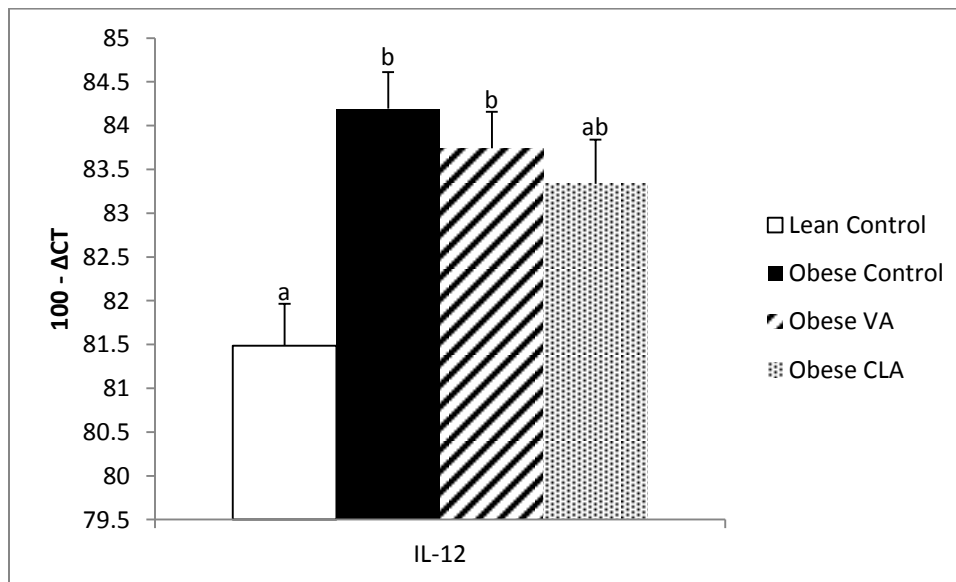
Figure 4.4 mRNA expression in perirenal adipose tissue from lean rats fed a control diet, obese rats fed a control diet, obese rats fed a VA diet and obese rats fed a CLA diet. Bars represent mean ± SEM (n=8 for lean controls, obese CLA obese VA and n=3 for obese control). Bars not sharing a common letter are significantly different (p<0.05).

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<center>**4.4 DISCUSSION**</center>

4.4.1 CYTOKINE EXPRESSION IN LEAN VS OBESE RATS

Obese rats fed the control diet were found to have a higher TGF-β, IL-6 and IL-12 mRNA expression when compared to the lean control rats in adipose tissue (Table 4.5). These differences suggest that there is an altered immune state in adipose tissue of obese rats. Differences in inflammatory cytokine expression in adipose tissue was first reported in obese rats, where a five to tenfold expression of TNF-α was observed in obese mice (26). TNF-α expression in the current study was not found to be different between the lean and obese control groups. This could be due to the fact that the Hotamisligil study used a db/db mouse model. This specific model of mice develops, hyperglycemia (30), which is not a characteristic of the JCR:LA-*cp* obese rat. Studies have observed higher TNF-α levels with hyperglycemia (31). Associations have also been observed with circulating cytokines IL-6, IL-10 and TNF-α, all reported to increase as adiposity increases (32-34). Within in the Field lab group, it was shown that obese rats had higher circulating haptoglobin and a decrease in IL-10 compared to the lean control rats indicating an higher inflammatory state (12; 27).

In the current study there was a higher expression of IL-6 in adipose tissue from obese rats compared to lean. No human studies have reported higher mRNA expression of IL-6 in adipose tissue from obese subjects, only circulating IL-6. It has also been noted that obese rats had a higher production of IL-6 in the spleen when stimulated with ConA (T cell mitogen) compared to lean rats (13). Higher production of IL-6 has been shown to be involved in an activation loop, causing cells to further secret IL-6 (35). This could also be a contributing factor to the higher expression of IL-6 mRNA in adipose tissue. Although not measured in the current study, the higher IL-6 expression in the obese rats might be due to the accumulation of macrophages in adipose tissue (36). Studies have shown that in obesity, macrophages will typically infiltrate the adipose tissue and form crown like structures (37). This increase in macrophage infiltration in adipose tissue

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creates a positive feedback loop, leading to higher production of inflammatory cytokines (4). One study suggests that cytotoxic T cells may also play an essential role in the initiation of macrophage infiltration (38). Another study found that DIO C57BL/6J mice had an increase presence of cytotoxic T cells in adipose tissue when compared to the lean group (39). IFN-γ, a common cytokine secreted by cytotoxic T cells, was higher in the obese rats fed CLA but not significantly (40).

Our results have also shown high concentrations of mRNA expression of IL-12 and TGF-β in the obese rats as compared to the lean rats. This is also consistent with the literature, which reports that TGF-β mRNA expression is higher in adipose tissue of obese rats and humans (41; 42). It has been shown that TGF-β is an important cytokine in regulating growth and differentiation of various cell types (43). In obesity and insulin resistance, TGF-β has been shown to be an important inducer of plasminogen activator inhibitor-1 (PAI-1), which is an inhibitor of plasminogen activator and urokinase-like plasminogen activator (42; 44). Higher PAI-1 has been observed in obese humans compared to lean humans (45). It has also been shown in mice that are deficient in producing PAI-1 are protected against diet induced obesity and have increased insulin sensitivity compared to the wild type mice (44). The administration of TGF-β to mice was found to increase active PAI-1 in plasma by 60-fold and increase expression of mRNA in adipose tissue (42). The same study was also able to show that TNF-α induces TGF-β mRNA in adipose tissue of normal-weight CB6 mice. There have also been relationships observed between TGF-β and IL-6 in PBMCs (46). Treatment of PBMC cells with TGF-β was found to lead to the higher production of IL-6 (46). Expression of IL-6 mRNA was also found to increase indicating the TGF-β is involved in mediating IL-6 expression and production (46), possibly explaining the higher expression of both of these cytokines in the adipose tissue from obese rats.

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In obesity and diabetes, IL-12 has be shown to be synthesized in adipocytes when stimulated with resistin (47). Obesity studies have shown elevated levels of serum resistin in *ob/ob*, *db/db* mice and obese humans but no studies have looked at resistin in the JCR:LA-*cp* model (48; 49). The administration of resistin to macrophages from rats and humans stimulated the macrophages to secrete more IL-12 and TNF-α through the NF-κB-dependent pathway (47). Therefore the increased macrophage infiltration in adipose tissue, as well as the higher resistin circulating that has been found in the literature (47; 48), might have contributed to the higher expression of IL-12 in adipose tissue.

4.4.2 EFFECT OF FEEDING TRANS FATS TO OBESE RATS

Cytokine mRNA adipose tissue expression in obese rats fed VA and CLA did not differ significantly from obese rats fed the control diet (Table 4.5). This suggests that feeding naturally occurring trans fats does not exacerbate the production of cytokines when rats are already in an obese state. Although there are no studies that have been done on cytokine expression in adipose tissue of obese rats fed VA or CLA, there are some done on splenocytes and mesenteric lymph nodes (13; 21). One study found that feeding CLA to lean rats had no effect on the production of TNF-α, IL-1β or IL-6 (21). However another study found that feeding VA to obese rats decreased TNF-α, IL-10 and IL-2 production when stimulated (13). Ruth et al (2008) has shown that splenocyte IL-2 production is normalized in the obese male *fa/fa* zucker rats fed CLA compared to the lean rats fed CLA, suggesting that mRNA expression may be decreased in the adipose tissue of the CLA fed obese rats (21). IL-2 is important in T cell function such that normalization of IL-2 production could be an indication of increased T cell function. Feeding VA to obese rats normalized the production of IL-2 and TNF-α in the direction of that of lean rats (13). In the present study we did not measure IL-2 mRNA expression in adipose tissue but since the other cytokines were not altered by diet, we do not predict that IL-2 would be.

There were a few differences observed in the expression of cytokines in the obese rats fed the different trans fat diets. Obese rats fed CLA had a higher expression of IL-6 mRNA when compared to obese rats fed VA. It was also found that the obese rats fed the CLA diet had a higher expression of TNF-α when compared to the lean control rats, but not to VA fed rats. No differences were found between the remaining cytokines and the obese fed rats.

Interestingly although TNF-α mRNA did not differ between diets in the obese rats, there was a significantly higher expression in adipose tissue from the obese rats fed CLA, but not VA, compared to the lean rats (Table 4.5). Higher TNF-α production has been observed in stimulated splenocytes from *fa/fa* Zucker rats (21). CLA fed obese rats were found to have higher production TNF-α when stimulated with ConA, PMAI, PWM and LPS when compared to the lean control and CLA fed groups. It has been suggested that this increase is partially due to the higher proportion of macrophages and activated antigen-presenting cells (21). The differences observed in the CLA fed group could also be due to diet composition. Diet has been shown to influence the immune system/inflammatory response which could also contribute to the differences found in mRNA production (50; 51). Furthermore it was found that there was less incorporation of CLA into the splenocytes in the obese rats when compared to the lean rats suggesting less of a dampening effect on the cytokine production (21).

CLA fed rats were also found to have significantly higher expression of IL-6 in adipose tissue when compared to the VA fed obese rats. Like TNF-α, IL-6 in CLA fed obese rats were found to have higher production when stimulated with ConA, PMAI, PWM and LPS when compared to the lean control and CLA fed groups (21). In this study, obese rats had lower incorporation of CLA in the splenocytes when compared to the lean rats, indicating that obesity may impair their incorporation into the splenocytes (21). If incorporation into the membrane is important then a lower CLA incorporation occurring in the obese rats might result in a difference in cytokine production. The higher expression of IL-6 could

also be due to the breed of the rat. As mentioned before, JCR:LA-*cp* rats exhibit symptoms of obesity, insulin resistance and hypertriglyceridemia (8; 9). One study showed when C57BL/6J mice were fed CLA over a 28 day period; there was an increase of 3 to 4 times in glucose-stimulated insulin secretion (i.e. trigger hyperinsulinaemia). Studies have also shown correlations between the circulation of inflammatory cytokines and the development of type 2 diabetes (52). Thus the combination of exhibiting insulin resistance and feeding CLA may have led the increase expression of IL-6 in the adipose tissue. The consumption of VA has been shown to have no effect on insulin sensitivity when fed to JCR:LA-*cp* rats which further suggests the higher expression of IL-6 in the CLA fed rats compared to the VA fed rats (53).

Although our results showed no difference between the trans diets and the control diets for the obese rats, previous research has shown differences in immune function from isolated spleen and MLN cells (21; 54). However, studies have shown that adipocyte cell lines treated with CLA (specifically the *cis*-9,*trans*-11 isomer) did not affect lipid accumulation or the expression of adipogenic genes when compared to control (vehicle BSA) treated cells (55-57). This could be an indication that changes in adipocytes are less affected by dietary CLA than other tissues, which would be consistent with the result of a lack of effect on cytokine mRNA expression, compared to the control diet. Even though no differences were found in the expression of the cytokines, posttranslational modification could be occurring, including phosphorylation, glycosylation, or changes in amino acid sequence, therefore the protein functionality could be altered despite the absence of changes in mRNA expression (58).

The results from this study demonstrate that JCR:LA-*cp* rats have differences in cytokine mRNA expression in adipose tissue when compared to lean rats, and furthermore the inclusion of ruminant trans fats, VA and CLA, have no detrimental outcomes to cytokine production when consumed, although

differences in cytokine expression was observed between these two diets. Further research is required to look into the physiological importance of these outcomes.

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5 GENERAL SUMMARY AND DISCUSSION
5.1 SUMMARY OF RESULTS

The overall goal for this thesis was to firstly establish the contribution of a pro-inflammatory status between obesity and type 2 diabetes. Secondly to explore whether increased intake of natural trans fats , that have been shown to benefit pro-inflammatory profile, will in fact modulate adipocyte contribution to improve this status.

1. **The first objective of this thesis was to identify and compare immune profile and function of circulating immune cells between 'healthy' obese human subjects, and those who are obese with type 2 diabetes. This objective was tested with the following hypotheses:**

    i.) **Obese human subjects with type 2 diabetes will have higher leukocyte circulation and higher proportion of T cells and/or B cells compared to 'healthy' obese subjects.**

The results from chapter 3 partly support this hypothesis. The results presented in this chapter found that stage 2 subjects had significantly higher proportions of activated (expressing the transferrin receptor) cytotoxic (CD3+CD8+CD71+), helper T (CD3+CD4+CD71+) and regulatory (CD4+CD25+Foxp3+) T cells. Furthermore, the stage 2 subjects had significantly higher proportions of naïve T cells, and T cells expressing inducible T cell costimulator (CD4+CD278+). There was no difference in total proportion of CD3+, CD3+CD4+, CD3+CD8+, CRTH2+ lymphocytes or in the concentration of white blood cells. The higher proportion of activated T cells in the stage 2 group suggests that they have been activated *in vivo*, therefore were in a more altered immune state when compared to the stage 0 group.

    ii.) **Obese human subjects with type 2 diabetes will have impaired T cell/ B cell function and greater inflammatory cytokine response compared to 'healthy' obese subjects when stimulated with mitogens in vitro.**

The results from chapter 3 partly support this hypothesis. The results reported in this chapter found stage 2 subjects to produce significantly less IL-6 and TNF-α when stimulated with PHA when compared to the stage 0 subjects. The remaining cytokines (IFN-γ, IL-10, IL-2, IL-1β), and IgG were not found to be different with either mitogen when compared between the two stages. This decrease in production could be an indication of an impaired T cell response which in turn may reduce their ability to respond to further stimulation *ex vivo* which could also explain why the stage 2 subjects had a higher proportion of CD71+ T cells. No differences were found for LPS (B cell mitogen) stimulation between the two groups. This suggests that there is no effect on the B cell response, which also complements the fact that no differences were found in the proportion or activation state of B cells between the two groups.

iii.) Obese human subjects with type 2 diabetes will have neutrophils that produce higher number of free radicals compared to 'healthy' obese subjects when stimulated with a mitogen in vitro.

The results from chapter 3 support this hypothesis. The results reported that the stage 2 subjects had significantly higher fluorescence intensity (which means higher activation of NADPH oxidase) when compared to the stage 0 subjects. Neutrophils from the stage 2 subjects were also found to be larger in size and more granular when compare to the stage 0 subjects. The stimulation index for the stage 2 subjects at the 10 min time point was significantly lower when compared to the stage 0 at the same time point. This suggests neutrophils from stage 2 subjects are in a higher activated state in vivo when compared to the stage 0 neutrophils. Consistent with the higher initial activation stage (TIME 0), the stimulation index was lower (reaching significance at the 10 min point) for neutrophils from stage 2 subjects compared to the stage 0 subjects (Fig. 3.7). This suggests that the neutrophils for the stage 2 subjects are already in an exhausted state which in turn could impede their ability during an immune response.

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2. **The second objective of this thesis was to determine the effects of increased dietary intake of natural trans fats on the cytokine expression in adipose tissue in an obese rat model. This objective was tested with the following hypotheses:**

   i.) **The deposition of fat in adipose tissue will contribute to the pro-inflammatory status of adipocytes in an obese rat model relative to control.**

   The results from chapter 4 support this hypothesis. The results reported that obese JCR:LA-*cp* rats had higher mRNA expression of TGF-β, IL-6 and IL-12 in adipose tissue. No differences were found for IL-1, IL-10 or IFN-γ between the lean and obese rats fed the control diet. Our results suggest that the obese rats had higher expression of inflammatory cytokines which could have been due to the reported macrophages, cytotoxic T cell infiltration into adipose tissue, a higher circulation of resistin, which are all found to be related to the development of metabolic syndrome in these obese rats.

   ii.) **An increased dietary intake of conjugated linoleic acid and vaccenic acid in obese rats will result in an improvement in the pro-inflammatory status of adipocytes in obese rat models.**

   The results from chapter 4 do not support this hypothesis. Obese JCR:LA-*cp* rats fed CLA had higher expression of IL-6 in adipose tissue when compared to rats fed the VA diet. The remaining cytokines did not differ between the VA and CLA fed rats. Compared to the control diet, VA and CLA fed rats did not differ in cytokine mRNA expression. These results indicate that naturally occurring trans fats do no exacerbate the production of cytokines when rats are already in an obese state, suggesting that VA and CLA have no detrimental outcomes to cytokine production. Comparing CLA to the VA diet suggests that CLA has less of an effect when none of the diets were significantly different from the controls.

5.2 GENERAL DISCUSSION

Previous research has shown that there is an increased risk of developing type 2 diabetes when an individual is currently or has been obese at one point in their lifetime (1). Metabolic syndrome, which by definition includes obesity, has been reported to have links to altered immune states which have been shown in obese models of rats and humans (2; 3). The results from our human study demonstrate that people who are obese with type 2 diabetes (stage 2) have a change in function of T cells in peripheral circulation compared to obese individuals without type 2 diabetes (stage 0). Whether the immune parameters found for the obese subjects are different from lean individuals was not assessed in the current study. However the direction of the changes, suggests, immune dysfunction in stage 2 subjects.

The etiology of obesity has been suggested to involve genetic, metabolic, environmental and behavioural factors (4). From behavioural factors, dietary macronutrients have been identified as possible contributors, including the type of fat (5; 6). Industrially produced trans fats, commonly found in hydrogenated oil, have been found to be associated with metabolic syndrome while ruminant trans fats have shown to have positive health effects (7; 8). The results from our animal study demonstrate that JCR:LA-*cp* rats have differences in cytokine mRNA expression in adipose tissue when compared to lean rats, and furthermore the inclusion of ruminant trans fats, VA and CLA, have no detrimental outcomes to cytokine production when consumed. There was higher expression of IL-6 mRNA in obese rats fed CLA when compared to the obese VA fed rats suggesting that CLA has less of an effect on reducing the inflammatory state found in obese models. The next step would be to look at the immune cell make-up of the adipose tissue to determine which cells are effected/affecting this change in the obese rats.

The study comparing immune profile in humans used circulating inflammatory parameters from blood and the study looking at the effects of

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ruminant trans fats in rats used mRNA expression from adipose tissue. Even though two different methods were used to look at the differences with respect to the immune system, they are both synopsis with alterations in the immune system. Higher circulating immune cells/proteins have been correlated with obesity and disease status (9; 10). Higher cytokine expression in adipose tissue has also been indicative of increased obesity (11). It has been suggested that because adipose tissue produces cytokines, the change in circulating cytokines could be an indication of alterations in adipose tissue production (11; 12). It should be noted that circulating cytokines are not only produced from adipose tissue, but from all areas of the body, therefore total changes found in circulating immune cells/proteins should not only be associated with changes in adipose tissue.

Although the study comparing the immune profile between stage 0 and stage 2 individuals was a pilot study one of the limitations for the study include the fact that only 19 subjects were recruited to participate, the stage 2 subjects were significantly older and taller than the stage 0 subjects and the sex make-up of the two groups varied. BMI, which factors in both height and weight, was used and no differences were found between the groups when using this measurement. Also, to my knowledge, no studies have shown that height has any effect on immune parameters. Regarding the age of the subjects, the likely reason that we recruited older subjects was due to the fact that the prevalence of type 2 diabetes in Alberta is the highest in the 55 - 59 years category (13). The stage 0 subjects were recruited because of their obesity staging which uses disease related/functional factors (not including age) (14). Future studies looking at obesity should include the EOSS score, allowing for a better understanding of the differences within the obese population. Also with age comes immunosenescence (15). Studies have shown decreases in neutrophil respiratory burst, phagocytosis and naïve T cells (16; 17). Increases in helper T cell type 2 response (IL-4, IL-5 and IL-10) as well as IL-6 serum concentrations have also been observed (16)(16)(15). Even though the ages were found to be significantly different,

studies have shown these difference between the two groups does not affect the immune cell make up, with people under 30 and people over 70 showing significant differences in T cells (18). Thus the overall difference between our age groups can be considered minimal due to being from the same upper age category. Therefore the results in our study suggest that type 2 diabetes is the main contributing factor and not age. No other study has shown the differences we found between our groups in the older population. Ideally it would have been preferential to have both groups with either the same number of each sex or simply just one of the sexes seeing as sex hormones can have an effect on the immune system (19).

Due to the fact that we observed a difference between the stage 0 and stage 2 subjects, the direction of the changes in the type 2 subjects, suggests a healthier immune function in the stage 0 subjects. It will be imperative in future studies to include an age-matched healthy body weight group. By adding this additional group, changes between the healthy weight and the 'healthy' obese individuals can be determined. The literature indicates that obese individuals have an impaired immune response as well as higher circulation of inflammatory cytokines when compared to lean individuals (3; 20; 21), however there was no indication as to the type of comorbidities that were present in these studies (i.e. unknown if these individuals are a stage 0 or stage 4 on the EOSS score) in the obese group. The results in this study are novel, suggesting that not all obesity are associated with the same immune profile/parameters.

Immune abnormalities have been studied between non-diabetic and diabetic individuals but little has been looked at the pre-diabetes (stage 1) stage to see if there are any differences (22). Another study could be to do a meal challenge (high fat meal) on 3 groups: lean control, stage 0 and stage 1subjects and examine the immune abnormalities over an 8 hour time period. This would allow for a better understanding of the immune abnormalities that occur in the pre-diabetic state during a physiological challenge. In our animal study we looked

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at JCR:LA-*cp* obese rats and compared them to lean rats. As mentioned previously, the JCR:LA-*cp* rat exhibits symptoms of metabolic syndrome which include obesity, insulin resistance, hypertension as well as developing complications related to cardiovascular disease (23). Using these characteristics and the criteria from the EOSS score, the JCR:LA-*cp* rat could be categorized under stage 2 (14). Even though these rats could be classified as stage 2 individuals they would not fit the criteria that was set for the stage 2 individuals in the human study.

With the JCR:LA-*cp* rats, we were studying a pre-diabetic stage and comparing it to lean non-insulin resistant rats. Thus a human study could be done to look at a stage 1 group immune profile and compare it to the healthy lean, stage 0 and stage 2 individuals. This would, again like the meal challenge study, give a better understanding into the progressive change that occurs from when an individual goes from a non-diabetic to a pre-diabetic to a diabetic stage. It would also allow for more insight into the differences in the varying effects that metabolic syndrome has on the immune system. Also during this study, adipose tissue biopsies could be done to look at the mRNA expression of the same cytokines looked at in our animal study. This could provide more information on the changes in mRNA expression in obese models, specifically in humans and help explain the changes observed in our human study.

The use of animal models to help further understand human obesity have been used since the 1940s (24). Animal models have contributed a large part to understanding the physiological and genetic complications that are associated with obesity (24). It should be noted there have been discrepancies when comparing animals to human studies (2; 10). It should also be noted that this is true for all forms of animal studies, not simply obesity (25). Approximately 1/3 of animal studies have been translated to human studies and only 1/10 of those have gone on to be used in patient treatments (25). Using either genetic or DIO animal models will always have similarities and differences (26). Important factors to

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consider are the methodologies of the studies, which would include factors such as study model, diet, cell/tissue of interest, experiments as well as how they were performed. All of these factors can influence the overall result of the study which is in turn can lead to conflicting results which leads to an important factor as to why human studies should also be done, not just animal studies. An important point to consider is not what individual studies conclude, but what the overall consensus of the literature shows (25) and the overall literature has shown impaired immunity in both animal and human studies.

In conclusion, in our animal study, our results demonstrate that in an animal model of metabolic syndrome (the JCR:LA-*cp* rat) there was a higher expression of inflammatory cytokines in perirenal adipose tissue when compared to lean rats (mix of heterozygous +/*cp* and homozygous +/+). In addition, it was also shown that the inclusion of ruminant trans fats to the diet does not exacerbate the inflammatory state of the rats. This is consistent with other studies that have shown VA and CLA to have either no effect or positive effects on the immune system. In our human study, our results demonstrate that the stage 2 subjects are in a more altered immune state when compared to the stage 0 subjects. The stage 2 subjects were found to have a higher proportion of CD71+ T cells, impairment in neutrophil function and lower cytokine production after stimulation with a T cell mitogen. This suggests that the immune system is altered in obese people with type 2 diabetes when compared to obese people without type 2 diabetes.

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