DOCOSAHEXAENOIC ACID-DERIVED METABOLITES TARGET THE B-CELL DRIVEN IMMUNE RESPONSE IN OBESE MICE

by

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Obesity is associated with an impaired humoral immune response. As B cells mediate the humoral immune response, they are major cellular targets in diet induced obesity models. Several studies have established that B cell function is impaired in obesity potentially due to decreased AID levels and impaired leptin signaling. Studies have also found that B cells from obese individuals secrete pro-inflammatory cytokines and that B cells seem to have a pathological role in the visceral adipose tissue in obesity. We have also established that obese mice have decreased numbers of total B cells and B cell subsets in the bone marrow compared to their control counterparts. Despite these findings, mechanisms elucidating how the B cell response is impaired are not well established. We and others have shown that specialized pro-resolving lipid mediator (SPM) precursors and SPMs are decreased in various tissues in obese humans and mice. As SPMs promote tissue homeostasis, prevent chronic inflammation, and can regulate B cell antibody production, we investigated whether deficiencies in the levels of SPM precursors and SPMs could be contributing to the impairments in B cell subsets and B cell function in obesity. We administered a cocktail of 14-HDHA/17-

HDHA/PDX to obese mice for four consecutive days as these DHA-derived metabolites were decreased in the spleens of obese mice. Overall, we found that these metabolites regulate B cell numbers in various tissues in obese mice. In particular, administration of 14-HDHA/17-HDHA/PDX to obese mice rescued decrements in total B cell and B cell subset numbers in the bone marrow. Furthermore, we found that these metabolites also decreased the number of B cells in the visceral adipose tissue, which were elevated in obese mice. We hypothesize that these DHA-derived metabolites are limiting B cell recruitment to the adipose by regulating chemokine receptor interactions with their corresponding ligands as obese mice receiving the DHA-derived metabolites had decreased transcript expression of various chemokine receptors that are upregulated in obesity. Obese mice receiving the DHA-derived metabolites also had decreased levels of pathogenic IgG2c in circulation as well as decreased IgM and IgG levels in the VAT. In addition, we established that supplementation of the parent fatty acid, DHA in the diet can increase antibody production in obese mice infected with influenza. When 14-HDHA, 17-HDHA, and PDX were administered individually to lean mice infected with influenza, 14-HDHA enhanced antibody titers and the number of antibody secreting cells in the bone marrow. Interestingly, we found that SPM precursors and SPM levels were not decreased in obese female mice. The obese female mice also did not have major decrements in B cell numbers in the bone marrow and had a decreased inflammatory profile compared to their male counterparts. Overall, findings from this dissertation propose that administration of DHA-derived metabolites that are deficient in obese mice can regulate B cell numbers across various tissues, potentially modulate

the pro-inflammatory B cell phenotype in the adipose tissue, and enhance antibody production in the context of influenza.

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DEDICATION

I dedicate this dissertation to my late father, Michael Crouch. Without his constant love and encouragement, I would not be the person I am today. This is for you dad. Love and miss you so much.

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I would like to first start off by expressing my gratitude for the opportunity to pursue a PhD. Completing a PhD has been one of the most challenging, yet rewarding times of my life. I am grateful for the knowledge gained, skills acquired, and the relationships that I have formed with others throughout this process. East Carolina University has given me so much during my time here, and I hope that I can give back to this great university in my future endeavors.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AID	Activation induced deaminase
Akt	Protein kinase B
ALA	Alpha linolenic acid
APC	Antigen presenting cell
ASA	Aspirin acetylated
AT	Adipose tissue
AUC	Area under the curve
BAFF	B cell activating factor
BCR	B cell receptor
BLIMP-1	B-lymphocyte-induced maturation protein 1
BMI	Body mass index
CLP	Common lymphoid progenitor
CLS	Crown-like structure
COX	Cyclooxygenase
CSR	Class-switch recombination
CTL	Cytotoxic T lymphocyte
CYP	Cytochrome P-450
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DGLA	Dihomo-gamma linolenic acid
DHA	Docosahexaenoic acid

DIO	Diet-induced obesity
DSB	Double stranded break
EBF	Early B cell factor
ECM	Extracellular matrix
EDA	Eicosadienoic acid
EET	Epoxyeicosatrienoic
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAFH	Fatty acid esters of hydroxy fatty acids
FDC	Follicular dendritic cell
FO	Follicular
GC	Germinal center
GDP	Gross domestic product
GPCR	G protein coupled receptor
GTT	Glucose tolerance test
HAI	Hemagglutination inhibition
HETE	Hydroxyeicosatetraenoic
HFD	High fat diet
HMGB-1	High mobility group box 1 protein
HSC	Hematopoietic stem cell
HSP	Heat shock protein
Ig	Immunoglobulin
IFNs	Interferons

ILI	Influenza-like illness
IRS	Insulin receptor substrate
JAK/STAT5	Janus kinase/signal transducer and activator of transcription 5
LA	Linoleic acid
LLPC	Long-lived plasma cell
LOX	Lipoxygenase
LT	Leukotriene
LX	Lipoxin
LXA_4	Lipoxin A4
MaR1	Maresin 1
MCP-1	Monocyte chemoattractant protein 1
MDSC	Myeloid-derived suppressor cell
MET	Metformin
MHC Class I	Major histocompatibility class I
MUFA	Monounsaturated fatty acid
MZ	Marginal zone
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NOD-like	Nucleotide-binding oligomerization domain-like
OVA	Ovalbumin
PAMPs	Pathogen associated molecular patterns
PDX	Protectin DX
PG	Prostaglandin

PGE_2	Prostaglandin E2
PLA_2	Phospholipase A_2 enzyme
PUFA	Polyunsaturated fatty acid
RIG-I	Retinoic acid-inducible gene-I protein
RvD1	Resolvin D1
SAT	Subcutaneous adipose tissue
SFA	Saturated fatty acid
SHM	Somatic hypermutation
SLC	Surrogate light chain
SPM	Specialized pro-resolving lipid mediator
SVF	Stromal vascular fraction
TCR	T-cell receptor
T_{FH}	T follicular helper
TLR	Toll-like receptor
TX	Thromboxane
T2DM	Type 2 diabetes mellitus
VAT	Visceral adipose tissue
WD	Western diet
XBP1	X-box binding protein 1

CHAPTER 1: INTRODUCTION

Overview

Obesity is one of the most concerning public health issues worldwide. In 2016, it was estimated that more than 2.1 billion adults were overweight, defined as body mass index (BMI) greater than or equal to 25 kg/m^2. Of these individuals, over 650 million or 13% of the worlds' adult population were classified as obese, defined by BMI greater than or equal to 30 kg/m^2 (1,2). Originally thought to be a major problem in developed countries, obesity rates have risen dramatically in low- and middle-income countries. Alarmingly, it is estimated that by 2030, 50% of the global population will be classified as either overweight or obese (1). In the United States, approximately 18% of children and adolescents (2 to 19 years old) and 35% of adult individuals are obese. Obesity rates have increased most rapidly in children, predisposing young individuals to a lifetime risk of various diseases and early mortality (3–5). Furthermore, 300,000 people in the United States die each year from various co-morbidities associated with obesity (6).

Obesity contributes to many pathologies such as cardiovascular disease, type 2 diabetes mellitus (T2DM), cancers, hypertension, non-alcoholic fatty liver disease, psoriasis, and inflammatory bowel disease (7–10). As a result, the economic burden placed on nations, families, and individuals to treat the co-morbidities associated with obesity cannot be overlooked. In 2014, the global economic impact of obesity in the United States was $2 trillion or 2.8% of the global gross domestic product (GDP) (2,11). Potential economic growth is also hindered by obesity due to lower productivity of obese individuals at work, increased mortality, and permanent disability (12). Compared to their non-overweight counterparts, severely obese men experienced 84% higher health care

costs ($6,192). Similarly, severely obese women had 88% higher costs ($5,618) compared to non-overweight women (12,13). As the prevalence of obesity is predicted to increase worldwide over the next ten years, steps to prevent obesity and reduce overall costs to treat the disease and its associated co-morbidities must be taken immediately.

Obese individuals are more susceptible to infections and have poor responses to vaccinations, an overlooked set of complications associated with obesity. Following the first pandemic influenza outbreak in 2009, obesity was first identified as an independent risk factor for increased mortality and morbidity from infection (14). Furthermore, it was reported that 51% of 534 adult cases of influenza in California occurred in obese individuals, and 61% of influenza-associated mortality cases were in obese individuals [15]. This same study also reported that hospitalization rates for respiratory illness during seasonal influenza were higher in the obese. Recent evidence also indicates that obese individuals may not respond to vaccination the same as individuals of healthy weight. To exemplify, obesity has been associated with a poor response to Hepatitis B vaccination (16,17). In addition, overweight children have been linked to lower anti-tetanus IgG antibodies in response to tetanus vaccination compared to healthy-weight children (18). While there are various mechanisms that could explain how obesity increases susceptibility to infection, an underlying theme is evident: obesity impairs the humoral immune response. Therefore, the basics of innate and adaptive immunity will be covered followed by a discussion of potential mechanisms by which obesity may impair humoral immunity.

Classical immune response

Innate immunity

An effective immune response to foreign pathogens involves a coordinated effort between the innate and adaptive immune systems. The innate immune response serves as the initial immune defense against pathogens and other dangerous material (19). Innate immunity entails three broad strategies to recognize foreign pathogens. The first strategy is mediated by germline encoded receptors that recognize “microbial non-self” or conserved pathogen associated molecular patterns (PAMPs) found on microorganisms (20). These receptors include toll-like receptors (TLRs), retinoic acid inducible gene-I protein (RIG-I), C-type lectin receptors, and nucleotide-binding oligomerization domain-like receptors (NOD-like) that recognize and respond to microbial conserved components that are essential for the viability and virulence of the foreign pathogen. In particular, TLRs induce an inflammatory cascade that results in the activation of nuclear factor kappa-B (NF-κB), subsequent release of interferons (IFNs), production of pro-inflammatory cytokines (TNFα, IL-6, IL-1β), and maturation of dendritic cells (DCs) (21–23).

The second strategy is carried out by damage-associated molecular patterns (DAMPs), which detect immunological “danger” in the form of common metabolic consequences of infection and inflammation (19,20). DAMPs, which can be recognized by TLRs include endogenous alarmins, heat-shock proteins (HSPs), uric acid, and high mobility group box 1 protein (HMGB-1). These molecules are released during cell lysis and tissue damage that occurs in an infection and during sterile inflammation (19). In the third strategy, major histocompatibility class I- specific (MHC class I) inhibitory receptors detect “missing-self” or molecules that are expressed by healthy cells rather than infected

cells. This is demonstrated by NK cells that express inhibitory receptors specific for both MHC class I and non-MHC class I molecules. These receptors ensure natural killer (NK) cells attack other infected cells that have low or no expression of MHC class I proteins (24). Recognition of these signals prevent activation of the immune response against host tissues (19) .

In addition, various innate immune cells are critical in the fight against infection and foreign antigens (25,26). Airway epithelial cells are the first target of viral pathogens. These cells produce various antiviral and chemotactic molecules that elicit an innate immune response due to rapid recruitment of neutrophils, NK cells, and monocytes. Secretion of TNFα and IL-1β induce migration of innate immune cells such as macrophages, blood borne dendritic cells (DCs), and NK cells to the site of infection. Alveolar macrophages limit viral spread through phagocytosis of the infected cells, whereas DCs constantly monitor for invading pathogens and serve as the bridge to the adaptive immune response (27,28).

Adaptive immunity

The adaptive immune response is the response of antigen-specific lymphocytes to antigen, which results in recognition of a wide array of foreign pathogens. There are two main components of the adaptive immune response: T-cell mediated cellular immunity and B-cell mediated humoral immunity. A T-cell mediated adaptive immune response is initiated when immature DCs in infected tissues take up pathogens to become an activated antigen presenting cell (APC) (29). Naïve T cells enter the lymph node and recognize a specific antigen that is presented by activated DCs (30). T cells bind stably

to DCs, resulting in activation of the T-cell receptor (TCR) and subsequent production of armed effector T cells, most commonly known as $CD8^+$ T and $CD4^+$ T cells. Upon activation by DCs, $CD8^+$ T cells, which recognize antigens presented by major histocompatibility class I (MHC I) differentiate into cytotoxic T lymphocytes (CTLs). CTLs produce cytokines (IFNs) and other effector molecules such as perforin and granzymes A and B, which limit viral replication and kill virus-infected cells (25,31). CD4 or T-helper cells recognize peptides presented by the major histocompatibility complex II (MHC II) protein, which is expressed on antigen presenting cells (APCs) such as DCs, macrophages, and B cells (32). During the course of an infection, $CD4^+$ T cells target epithelial cells through MHC class II and differentiate into Th1 cells upon activation resulting in the production of IFN-γ and IL-2 (33,34). These cytokines regulate $CD8^+$ T cell differentiation resulting in the eradication of intracellular pathogens and viral-infected cells (35,36). On the other hand, $CD4^+$ T cells can differentiate into Th2 cells, Th17, Treg, and T follicular helper (T_{FH}) cells. Th2 cells recognize foreign peptides bound to MHC class II proteins and produce the cytokines IL-4 and IL-13, which promote B cell activation (25).

Humoral immune response

The other arm of the adaptive immune response, humoral immunity, functions against extracellular pathogens and toxins through the actions of the B cell. One of the primary functions of the humoral immune response is to ensure the production of long-lived memory B cells and plasma cells that produce high-affinity, isotype-switched antibodies essential for host defense after infection or vaccination. Alterations or mutations that affect signals necessary for B cell development or their overall

microenvironment results in antibody deficiencies, which can lead to increased susceptibility to infection and decreased responses to vaccination (37–39). Therefore, it is crucial to understand how B cells develop, undergo activation, and eventually differentiate to become an antibody-secreting or memory B cell in order to understand possible mechanisms to how the B cell response is impaired in obese individuals.

Immunoglobulins

Antibodies, also known as immunoglobulins, are glycosylated protein molecules that are present on the surface of B cells in the form of a cell surface immunoglobulin known as the B cell receptor (BCR) or can be secreted in to extracellular space to neutralize antigens (40). An antibody is a Y-shaped structure that consists of two immunoglobulin (Ig) heavy (H) chains and two Ig light (L) chains that are linked together through disulfide bonds. Each component chain contains one variable (V) domain and one or more constant (C) domains. The *IgL* chain, which consists of a κ and λ chain contains one constant domain, whereas *IgH* chains can contain three or four (C) domains (41). Within the variable region, three hypervariable regions known as variable (V), diversity (D), and joining (J) determine antigen recognition through a somatic process termed *V(D)J* recombination in which these gene segments are combined (42,43). *V(D)J* recombination occurs at various stages during B cell development yielding an abundant repertoire of Ig receptors and antibodies. However, in order to produce high-affinity antibodies, B cells must undergo strenuous and tightly regulated stages of development, self-tolerance checkpoints, and recombination events that are dependent upon

interactions with other cell types, signals received from cytokines and transcription factors, as well as the nature of the antigen presented and recognized by the B cell.

B cell development

B cells originate from a pool of hematopoietic stem cells (HSCs) in bone marrow niches known as microenvironments. These microenvironments are home to various cell types such as stromal cells, osteoblasts, reticular cells, and other immune cell precursors (43,44). Commitment to the common lymphoid progenitor (CLP) pathway and subsequent B-cell lineage requires the coordinated action between networks of cytokines and transcription factors that are secreted by stromal cells. In particular, the transcription factor, IL-7, induces IL-7R-expressing lymphoid and myeloid progenitors to enter the CLP pathway to become pre-pro B cells (Figure 1.1). The expression of various transcription factors (Pax5, E2A, and EBF), the formation of several components of the surrogate light chain (SLC), and the expression of the surface markers B220 and CD19 commit the pro B cell to the B-cell lineage (43,45). Upon expression of the heavy (*IgH*) chain, the SLC forms and the result is the pre-B cell receptor (BCR), which is found on the surface of pre-B cells (46). Pre-B cells undergo rearrangement of their *V(D)J* gene segments, producing a primary repertoire of Ig receptors (47). The end result is the expression of an IgM mature BCR on immature B cells that can bind antigen (40,48). Immature B cells that do not bind self-antigen and pass self-tolerance checkpoints exit the bone marrow and migrate to the spleen or other secondary lymphoid organs to undergo further development and maturation.

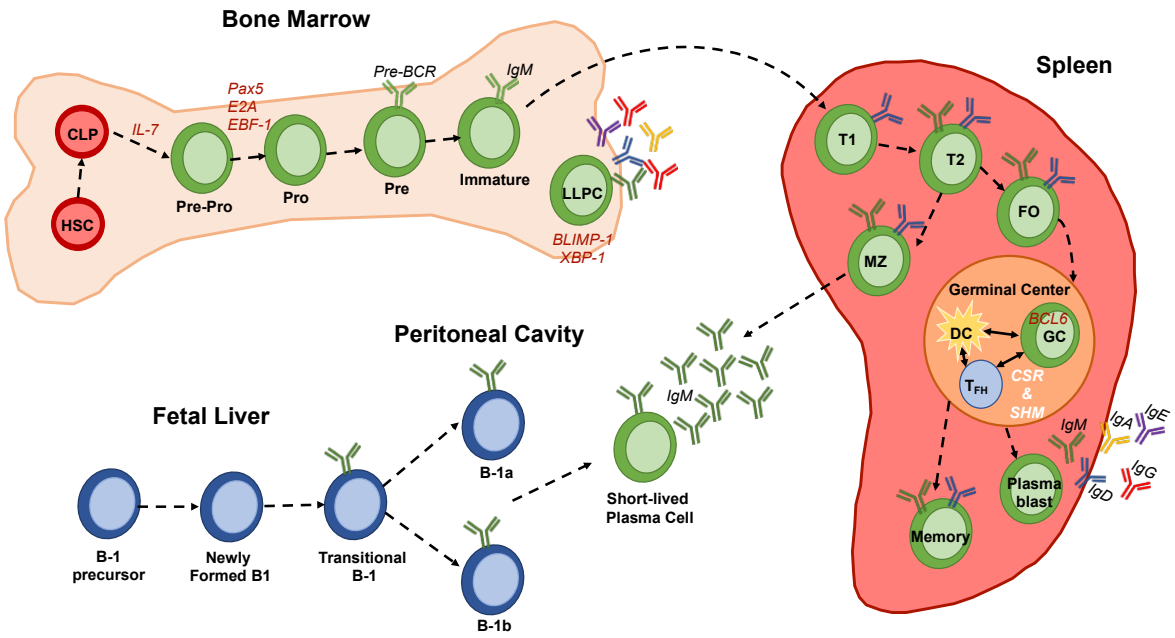

Figure 1.1. B cell development and maturation. Hematopoietic stem cells (HSC) located in the bone marrow give rise to a common lymphoid progenitor (CLP) via the coordination of transcription factor networks. IL-7 signals the CLP to differentiate into the B cell lineage as a pre-pro B cell. Upon the expression of Pax5, the pre-pro B cell becomes a pro-B cell and eventually a pre-B cell that has the pre-BCR present on its surface. Once the mature BCR is expressed on the surface, the immature B cell undergoes several self-tolerance checkpoints that it must pass in order to migrate to the spleen to undergo further development and maturation. Once in the spleen or other secondary lymphoid organ, the naïve transitional 1 (T1) B cell differentiates into a T2 B cell. The T2 B cell can become a marginal zone (MZ) B cell or a follicular (FO) B cell. MZ B cells produce an abundant amount of natural IgM, whereas FO B cells enter the germinal center (GC). Once in the GC, FO B cells interact with dendritic cells (DCs) and T follicular helper (T_{FH}) cells to undergo class switching recombination (CSR) and somatic hypermutation (SHM) to become a plasma cell that gives rise to high affinity and class-switched antibodies or a memory cell. In order to maintain long-term memory, cells differentiate into the memory pool or plasma pool. Each pool upregulates specific transcription factors and signals for formation and maintains signals and niches for survival. Plasma cells upregulate the transcription factors, BLIMP1 and XBP1 to become long-lived plasma cells (LLPCs). LLPCs recirculate back to the bone marrow where they maintain serologic memory. On the other hand, B1 cells arise from a B-1 progenitor in the fetal liver and migrate to the peritoneal cavities to become a mature B-1a or B-1b cell. B-1a and B-1b cells also produce large amounts of short-lived plasma cells that secrete IgM into the periphery.

Maturation of B cells

Mature B cells, which co-express IgM and IgD have the opportunity to become either an antibody-producing plasma cell or a memory B cell, all of which is entirely dependent upon antigen selection. Mature B cells are comprised of two major subsets: B1 cells and B2 cells. B1 cells, which arise from B1 progenitors in the fetal liver are most abundant in peritoneal and pleural cavities and can be subdivided into B-1a ($CD5^+$) and B-1b ($CD5^-$) cells (49–52). These cells are self-renewing and require little help from the bone marrow for development. B2 cells consist of follicular (FO) and marginal zone (MZ) B cells, which arise from transitional 1 and 2 B cells in the spleen (48,53,54). B1 cells and MZ B cells express toll-like receptors (TLRs) in their nascent state and can recognize T-independent antigens without engagement of their BCR. Therefore, B1 and MZ B cells respond like innate cells generating a rapid IgM antibody response within the first 1-3 days of infection before the emergence of FO B cell-derived IgG antibodies usually 7 days post-infection. (40). B1 and MZ B cells can also differentiate rapidly into isotype-switched short-lived plasmablasts and memory B cells in extrafollicular areas without having to enter the germinal center (55). Unlike B1 cells, MZ B cells can generate T-dependent responses due to their ability to interact with natural killer T cells (NKT), neutrophils, and DCs that aid in germinal center (GC) reactions (55). They can also generate isotype-switched IgG antibodies in the extrafollicular areas without entering the GC (54,56).

FO B cells are the conventional B lymphocytes of the adaptive immune response and are primarily responsible for the generation of long-lasting, high-affinity antibodies. Upon antigen recognition by the BCR, FO B cells migrate to the boundary of the follicle adjacent to the T-cell zone also known as the T-B border. Through the help of T follicular

helper cells (T_{FH}), B cells receive costimulatory ligands such as CD40L and cytokines such as IL-4, IL-21, and IFN-γ that promote B cell proliferation and differentiation (40). B cells that do not upregulate the transcriptional repressor, B cell lymphoma 6 (BCL6), can develop into extrafollicular plasmablasts and early memory B cells without entering the follicles and subsequent germinal centers. B cells that do upregulate BCL6 return to the B-cell follicles of secondary lymphoid organs and receive co-stimulatory and clustering signals from T_{FH} cells, follicular dendritic cells (FDCs), and sphingosine-1-phosphate receptor 2 ($S1P_2$) to eventually form GCs, which support immunoglobulin class-switch recombination (CSR) and somatic hypermutation (SHM) (57–60). Within the GC, T_{FH} cells secrete B cell activating factor (BAFF), a pro-survival cytokine that promotes the survival of B cells in the GC that have acquired high-affinity mutations and induces gene expression programs essential for CSR and SHM to generate high-affinity class-switched memory and plasma B cells (61,62).

Class switch recombination and somatic hypermutation

Both CSR and SHM enhance the efficacy of the antibody response against specific antigens, occur at the same stage of B cell differentiation, and depend on the actions of the enzyme, activation induced deaminase (AID). However, these somatic processes are very independent of one another in terms of mechanism and timing. For example, CSR can occur in GCs or outside of GCs as evidenced by the actions of B1 and MZ B cells, whereas SHM occurs in the germinal center (63–66). Upon activation via infection or immunization, CSR allows B cells to switch from expressing IgM and IgD on their surface to IgG (IgG1, IgG2, IgG3, and IgG4), IgE, or IgA. During this somatic process, AID

deaminates cytosine residues in long, non-coding switch (S) regions that lie upstream of each set of C_H exons (67). The deaminated products at the (S) regions are processed as DNA double-stranded breaks (DSBs) and point mutations (67). CSR is completed when AID-initiated DSBs in two (S) regions are fused to delete $C\mu$ and $C\Sigma$ exons and replace them with one of the downstream C_H exons ($C\gamma$, $C\varepsilon$, or $C\alpha$ exons).

This switch generates high-affinity antibody isotypes that are specialized for certain pathogen-elimination functions. Each Ig class differs in size, FcR binding, complement fixation, and effector functions (68). For example, IgG promotes phagocytosis of antibody-coated particles (63,69), and its subclasses exhibit different functional activities as well. During the secondary antibody response, IgG1 and IgG3 antibodies are induced in response to protein antigens whereas IgG2 and IgG4 are associated with polysaccharide antigens (41). Furthermore, IgE triggers mast cell degranulation, and IgA is critical at protecting mucosal surfaces from toxins, virus, and bacteria (41,70,71). Thus, CSR alters an antibody's effector function to one that is most suitable to eliminate a specific pathogen while maintaining the same variable (V) region and antibody-binding specificity (63).

SHM occurs after CSR and can only take place in the germinal centers of B cells. During SHM, AID deaminates cytosine residues at the exons of *IgH* and *IgL* variable regions and alters the affinity of the BCR for the antigen (72). The deamination products are processed through specific repair pathways into predominantly point mutations, as well as a low frequency of small insertions and deletions (72,73). This generates a panel of mature B cells that are selected (based on their BCR affinity) to proliferate and differentiate into antibody-secreting plasma cells and memory B cells (60,74). Overall, this drives the increase in the overall affinity of serum antibodies during the primary

immune response and upon re-immunization or re-infection (75). Once selected into the memory and plasma B cell pools, expression of the transcription factors B lymphocyte-induced maturation protein 1 (BLIMP1) and X-box binding protein 1 (XBP1) commits plasma cells to differentiate into long-lived plasma cells (LLPCs) (76,77). LLPCs recirculate back to the bone marrow where they produce antibodies that maintain serologic memory independent of further antigen exposure (78). Memory B cells can recirculate to secondary lymphoid organs to form extrafollicular aggregates where they can differentiate into plasmablasts or re-enter the GC upon antigen re-challenge, which results in a further diversified secondary antibody response (79–81). Therefore, memory B cells and plasma cells lead to the production of high-affinity and immunoglobulin class-switched antibodies which is the main goal of long-lived humoral immunity.

Obesity impairs humoral immunity

Recent evidence suggests that obesity impairs both arms of the adaptive immune response, predisposing obese individuals to infections and other diseases (26,82). An important role for various subsets of T cells in obese individuals during the course of an influenza infection has been well established in both humans and mice in recent years. For example, studies using mice infected with influenza in diet-induced obesity (DIO) models have demonstrated increased numbers of cytotoxic $CD8^+$ T cells, decreased numbers of Treg cells, and greater lung inflammation compared to their lean counterparts (39,83). Furthermore, obese mice infected with H1N1 influenza virus had decreased memory $CD8^+$ T cell production of IFN-γ, which is a cytokine critical for influenza clearance (84). Likewise, PBMC's from obese individuals stimulated with H1N1 influenza

virus *in vitro* had decreased $CD4^+$ and $CD8^+$ T cells as well as fewer functional and activation markers compared to healthy adults (38,85). While sufficient evidence suggests that obesity impairs T cells during an influenza infection, many questions are left unanswered in relation to other the other key player needed for an effective immune response during infection: The B cell.

Several studies have suggested that obesity impairs the B cell response during infection. Milner et al. reported that obese mice had diminished concentration of influenza-specific antibodies 35 days post-infection (39). Furthermore, obese mice had lower influenza-specific antibody production compared to lean mice despite increased production of neutralizing and non-neutralizing influenza-specific antibodies upon adjuvant vaccination (37). In addition, Frasca et al. recently demonstrated that both young and elderly individuals had decreased antibody production to the influenza vaccine (86). Interestingly, vaccines may fail to provide optimal protection in obese individuals despite sufficient production of neutralizing antibodies. Work from Schultz-Cherry's lab demonstrated that mice receiving an adjuvanted vaccine had a fourfold increase in neutralizing antibody levels that were considered "protective". However, despite improved seroconversion with the use of the adjuvant, the mice still succumbed to influenza infection (37). Similarly, an HAI titer of 40 or higher was not seroprotective against influenza in obese individuals and influenza-like illness (ILI) (87). These studies suggest that supplementing vaccines with adjuvant and boosting antibody levels fails to diminish an obese individual's susceptibility to infection,

Current knowledge of how obesity impairs the B cell-mediated humoral immune response in the context of influenza infection is limited. Until recently, the role of B cells

in the pathogenicity of obesity was largely underappreciated. It is now accepted that B cells not only play a major role in obesity and insulin resistance, but can also regulate systemic and local adipose tissue inflammation through a wide array of mechanisms (88). B cells also undergo phenotypic and functional changes that contribute to their overall pathogenic effect on diet-induced obesity related insulin resistance (89). Therefore, these findings provide new insight and possible mechanisms contributing to the impaired B cell response in obese individuals in the context of infections such as influenza.

B cell development is impaired in obesity

Proper B cell development, maturation, and differentiation into long-lived plasma and memory B cells are critical in generating a diverse antibody repertoire that can elicit a strong immune response. Detrimental changes in B cell development can negatively impact humoral immunity. Currently, there is conflicting evidence as to whether obesity enhances or compromises B cell development in the bone marrow. For example, Trottier et al. found that C57BL/6 mice fed a 45% kcal high fat diet (HFD) for 180 days had a sustained increase in the total number of B cells after 90 days on the HFD. Furthermore, this study showed that bone marrow cells isolated from mice on the HFD for 157 days had increased numbers of pre, immature, and mature B cells compared to their control counterparts (90). On the contrary, 7-week old C57BL/6 mice that were fed a 60% kcal HFD for 2 days, 1 week, and 6 weeks showed reductions in B cell numbers beginning at week 1 of HFD feeding. B cell development markers such as IL-7, EBF1, and Pax5 were notably reduced in mice receiving the high fat diet compared to the control mice (91). Similarly, long-term HFD feeding for 7 months reduced B cell numbers in the bone marrow

by 52% and in the blood by 36% compared to control mice (92). Our laboratory has demonstrated that the number of B cells in the bone marrow are lowered in obese male mice. Conflicting results from these studies could potentially be attributed to different diet compositions used, the duration of the feeding protocol, and methods used to determine B cell numbers in the bone marrow.

Phenotypic and functional changes of B cell subsets in obesity

In response to excess caloric intake as seen in diet-induced obesity, the adipose tissue (AT) undergoes drastic remodeling that is characterized by rapid and massive expansion, changes in extracellular matrix components (ECM), secretion of adipokines and hormones, blood vasculature, and infiltration of various immune cells that produce cytokines and chemokines upon activation, which contributes to chronic inflammation (93–96). B cells traffic to and infiltrate the visceral adipose tissue (VAT) from other tissues early during the course of diet-induced obesity. Duffaut et al. reported that B cells accumulated in the VAT as early as 3 weeks after initiation of a high fat diet (HFD) with those numbers remaining constant over the course of the 12 week study (97). Furthermore, B cells were found to infiltrate the VAT before T cell infiltration, which occurred after 6 weeks of HFD feeding (97). Despite their early recruitment, B cells are not the first immune cells to infiltrate the VAT. Neutrophils have been observed to directly adhere to adipocytes in the VAT of mice 3 days after initiation of HFD (98). Furthermore, a subpopulation of macrophages ($F4/80^+$ $CD11b^+$ $CD11c^+$) were detected in the VAT after just 1 week of HFD feeding (99). In addition to their early recruitment to the VAT, B cells can also surround stressed or dying adipocytes in crown-like structures (CLSs) along with

other immune cells such as macrophages and T cells (89). Within these CLS, B cells can modulate macrophage and T cell function as well as sample antigen (89). Likewise, McDonnell et al. used immunohistochemistry to identify B cells within crown-like structures (CLS) of subcutaneous adipose tissue (SAT) and in the perivascular space in both male and female obese subjects. In over 50% of the subjects used in the study, B cells in the sCLS were relatively more numerous than T cells (100).

During the onset of diet-induced obesity total B cell numbers are increased in the VAT of obese mice relative to their control counterparts (89). This same study also reported that obese mice had increased numbers of follicular B cells, B1a, and B2 cells in the VAT after 6-12 weeks of HFD (89). Frasca et al. reported that obese individuals had decreased percentages of anti-inflammatory B cell subsets (transitional B cells) and increased percentages of proinflammatory late/exhausted memory B cells (101). Similarly, Mooney et al. demonstrated that obese/T2D mice infected with *S. aureus* had reduced numbers of germinal centers in the popliteal lymph nodes compared to control mice infected with *S. aureus*. Despite reduced GCs, obese mice had increased total number of B cells at day 0 and day 14 post-infection. Obese mice also had decreased numbers of activated B cells on day 7 and day 14 post-infection as well as reduced GC B cells by day 14 post-infection (102).

In addition to phenotypic changes, B cells undergo drastic functional changes upon the initiation of HFD. B cells from obese mice have increased class switching to IgG^+ cells, especially to pro-inflammatory IgG2c in the VAT as a consequence of increased fat mass, hypoxia, and apoptosis (89). Furthermore, total B cells from both young and elderly individuals with obesity showed higher immune activation as measured by increased

intracellular TNFα before stimulation. Upon stimulation with CpG, total B cells from these obese individuals had decreased levels of AID, a functional measure of CSR. In addition, culture supernatants collected from challenged B cells had increased secretion of pro-inflammatory cytokines (IL-6) and decreased secretion of the anti-inflammatory cytokine, IL-10 in obesity, compared to lean individuals (101). Interestingly, obese/T2D mice challenged with *S. aureus* had reduced levels of AID accompanied with decreased IgG and IgE antibody production compared to their control counterparts further demonstrating obese mice have impaired B cell function. (102). Our lab found that obese humans had decreased B-cell IL-6 secretion and elevated IgM levels but not IgG upon *ex-vivo* challenge with anti-BCR/TLR9 stimulation. This result supports that notion that hyperstimulated B cells in obese individuals function sub-optimally (103). Therefore, these phenotypic and functional changes in B cells are likely contributors to the impaired immune response to various infections and diseases in the obese population and warrant further studies.

Adipokines modulate B cells

Adiponectin and leptin are fat-derived adipokines that undergo drastic changes during the onset of obesity (104). Adiponectin exerts anti-inflammatory effects and increases insulin sensitivity via effective disposal of glucose from circulation. Adiponectin is decreased in obesity and circulating levels are inversely correlated with body fat percentage, increasing after weight reduction. Several studies suggest that this inverse relationship between adiponectin and adiposity phenotypes is solely dependent upon the development of insulin resistance (105–107). Various studies also support the idea that

adiponectin plays a protective role during the development and early stages of obesity and energy balance. In particular, C57BL/6J mice on a high fat diet have increased circulating adiponectin concentration during the initial stages of adiposity development before decreasing after 18-weeks of high fat feeding (108).

Despite its well-established role in obesity, adiponectin's effect on B cell activation, maturation, and expansion in the context of obesity is poorly understood. It has been suggested that adiponectin promotes myelopoiesis and inhibits B lymphopoiesis in long-term bone marrow cultures when early lymphoid progenitors are in contact with stromal cells. This study also reported that prostaglandin E2 (PGE_2) had a direct inhibitory influence on purified hematopoietic cells, suggesting that adiponectin can negatively and selectively influence lymphopoiesis through the induction of PGs (109). Recently, Obeid et al. demonstrated that adiponectin knock-out mice ($APN^{-/-}$) receiving dextran sulfate sodium exhibited severe colitis, increased B cell infiltration in the colon, and upregulated B cell activation, which resulted in the secretion of pro-inflammatory cytokines (110). Therefore, these findings provide strong evidence that adiponectin can directly modulate B cell function. However, this relationship has not been established in the context of diet-induced obesity.

Conversely, leptin is a pro-inflammatory hormone that is elevated in obesity. Leptin inhibits hunger by physiologically regulating energy balance and is secreted primarily by adipocytes. Circulating levels of leptin are directly correlated with the amount of fat in the body (111). Leptin signals through the JAK/STAT signaling pathways resulting in translocation of STAT3 into the nucleus and subsequent transcription of leptin-induced genes. Leptin targets the medio-basal nucleus of the hypothalamus, where it inhibits

appetite. As a result, leptin deficiency or resistance leads to an energy imbalance, hyperinsulinemia, IR, uncontrolled food intake, obesity, and T2DM (112).

Leptin resistance in obese individuals regulates both the innate and adaptive immune response and contributes to the exacerbated inflammatory environment in obesity (113). Leptin equally affects immune cells from the innate and adaptive immune system. Almost all immune cells, including B cells, express Ob-R, an isoform of the leptin receptor that is required for leptin signaling (114–116). Leptin can directly target and modulate B cell responses. For example, leptin signaling promotes B cell survival by inhibiting apoptosis and inducing cell cycle entry through activation of Bcl-2 and cyclin D1 (117). Furthermore, leptin can induce peripheral blood B cells to secrete various cytokines such as IL-6, IL-10, and TNFα (118). Leptin also modulates B cell development. Fasted mice, characterized by low leptin levels, displayed decreased numbers of pro, pre, and immature B cells and increased numbers of mature B cells in the bone marrow (119). Continuous leptin administration prevented the B cell reductions observed in the bone marrow of fasted mice, indicating an important role for leptin in B-cell lymphopoiesis (119,120). In addition, Frasca *et al.* proposed that leptin may be responsible for B cell intrinsic inflammation. In this study, B cells from lean individuals treated *in vitro* with leptin had increased STAT3 phosphorylation (crucial for TNFα production) and decreased phosphorylation of the anti-inflammatory AMPK pathway, which helps mediate an antibody response in B cells and improves insulin resistance in obesity (86). As a result, increasing leptin levels, which are associated with obesity could be a potential mechanism responsible for reduced B cell function in obese individuals.

Obesity-induced insulin resistance negatively affects B cell function

Obesity is a critical risk factor in the development of insulin resistance (121). Initial molecular signaling for insulin action involves the activation of the insulin receptor tyrosine kinase, which results in the phosphorylation of insulin receptor substrates (IRSs) on multiple tyrosine residues. However, in an insulin resistant state, the phosphorylation of IRSs (IRS-1) is decreased, receptor phosphorylation and tyrosine kinase activation are reduced, and insulin binding to its receptor is mitigated (122,123). Insulin resistance is defined as resistance to the effects of insulin on glucose uptake, metabolism, or storage. In obesity, IR is exacerbated by decreased insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscle and by impaired suppression of hepatic glucose output (124,125). As a result, IR induces hypertriglyceridemia and results in defective glucose transport via GLUT4. Changes associated with insulin resistance and metabolic homeostasis leads to alterations in the distribution of leukocyte populations, lymphocyte activity, and overall immune defenses (126,127).

B cells have emerged as mediators of insulin resistance in DIO models. B cells provoke insulin resistance via several mechanisms including modulation of cytokine production, T cells, and antibody production. To elaborate, IL-10 appears to have a protective role in insulin resistance by reducing macrophage and cytokine responses in the skeletal muscle of obese mice (128). IL-10 secretion by B cells is decreased in the blood of patients with T2D as well as upon challenge with TLR agonists, which could predispose obese individuals to insulin resistance (129,130). Furthermore, in DIO models, B cells can interact with T cells to induce IFN-γ expression, which contributes to local and

systemic inflammation and IR (89). Likewise, the VAT of B cell-deficient mice compared to controls displayed reduced T-cell IFNγ and IL-17 (131).

Interestingly, metformin (MET), which has been shown to reduce chronic inflammation through lowering of IR, hyperglycemia, and atherogenic dyslipidemia in obesity has a strong immunoenhancing effect in B cells. Frasca et al. showed B cells from obese patients taking MET had increased transcript levels of AID in response to stimulation with CpG. Similarly, B cells from obese patients treated *in vitro* with MET had increased AID levels compared to those that were not treated with MET. Obese individuals taking MET had increased percentages of circulating switched memory B cells, decreased percentages of pro-inflammatory exhausted memory B cells, and reduced B-cell intrinsic inflammation via upregulation of AMPK. Notably, obese individuals on MET had an increased *in vitro* AID response to the influenza vaccine compared to obese individuals not taking MET (132).

In addition, insulin resistance impacts antibody production and isotype. Winer et al. demonstrated that total splenic B cells from mice on a high fat diet have increased IgG secretion and reduced IgM production (89). Furthermore, overweight children that are associated with an unfavorable metabolic phenotype have increased circulating IgG and IgA, and DIO mice have increased class-switched, pro-inflammatory IgG2c antibody (89,133). Interestingly, oral feeding of ovalbumin (OVA) antigen to obese mice induced the production of IgG2c antibody, whereas in lean mice IgG1 was the predominant antibody isotype produced (134). While obesity impacts antibody production and isotype, it also appears that the antibody isotype has a direct effect on insulin resistance in obesity. Several studies have demonstrated the transfer of IgG antibodies from insulin-resistant

mice on a high fat diet to mice on a normal chow diet increases the polarization of classically activated pro-inflammatory macrophages, increases TNFα production, and enhances targeting of gut-derived antigens (134–136). Therefore, it is plausible that the net pathogenic effect of B cells on DIO-mediated insulin resistance could contribute to the impaired B cell response in obese individuals

The role of PUFA-derived lipid mediators on the humoral immune response

PUFAs are precursors to bioactive lipid mediators

Obesity is associated with an increase in free fatty acid levels, which promotes insulin resistance and the development of systemic hyperglycemia (137–140). In European populations, 28-42% of the total energy consumed in the diet is fatty acids, representing a considerable increase compared to past ancestral populations (141–143). Humans consume over 20 different fatty acids, which can be classified as saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) (144). Of these three, PUFAs cannot be made by humans and must be consumed through the diet, rendering them essential fatty acids (145,146). PUFAs (n-3 and n-6) are characterized based on the location of the first double bond in relation to the methyl terminal group in the fatty acid backbone (1,145,146). PUFAs are released from membrane phospholipids into the cytosol primarily by the action of the phospholipase A_2 enzyme (PLA_2) and are quickly converted into n-3 and n-6-derived lipid mediator derivatives, fatty acid esters of hydroxy fatty acids (FAFH), nitroalkenes, and endocannabinoids (147,148).

Western diets consumed today are rich in saturated fats and n-6 PUFAs but are lacking in appropriate n-3 PUFA levels (141,142,149). It is estimated that ratios of n-6 PUFAs/n-3 PUFAs present in Western diets range from 10:1 to 20:1 (141,142). As a result, the excessive intake of n-6 PUFAs results in the increased production of n-6 PUFA-derived lipid mediators compared to those derived from n-3 PUFAs (148). N-6-derived lipid mediators are generally considered to be pro-inflammatory and pro-thrombotic, whereas n-3-derived lipid mediators display dual anti-inflammatory and pro-resolving actions (150,151). Thus, diets characterized by lower intake of n-3 PUFAs and higher n-6 PUFA consumption increase the risk of individuals developing various inflammatory, thrombotic, carbohydrate, and lipid disorders (148). It is important to note, however, that various studies have suggested that higher intake of n-6 PUFAs can actually be cardioprotective and are associated with lower risk of CVD (152). As a result, not all n-6 PUFAs lead to negative effects in all disease models. As lipid mediators derived from n-3 and n-6 PUFAs directly influence the immune response in obesity, it is essential to understand their biosynthetic and signaling pathways, which will be discussed below.

Pro-inflammatory lipid mediator biosynthesis

Arachidonic acid (AA, 20:4) is the precursor and parent compound of various bioactive signaling lipid mediators that are generated through the actions of cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P-450 (CYP) enzymes (153,154). The COX pathway results in the production of prostaglandins (PGs) and thromboxanes (TXs), whereas the LOX pathway catalyzes the formation of leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs) (Figure 1.2) (148,155,156). AA can

also generate bioactive lipid mediators through the cytochrome P-450 (CYP) epoxygenase (157). CYP epoxygenases add an oxygen across one of the double bonds of AA to generate epoxyeicosatrienoic acids (EETs) (148,157), which regulate vascular tone, inflammation, and organ and tissue regeneration (158). AA also has the ability to produce the lipoxins (LXs) through the actions of the LOX enzymes. LXs have both anti-inflammatory and pro-resolving activities and are classified as specialized pro-resolving lipid mediators (SPMs) (159–161).

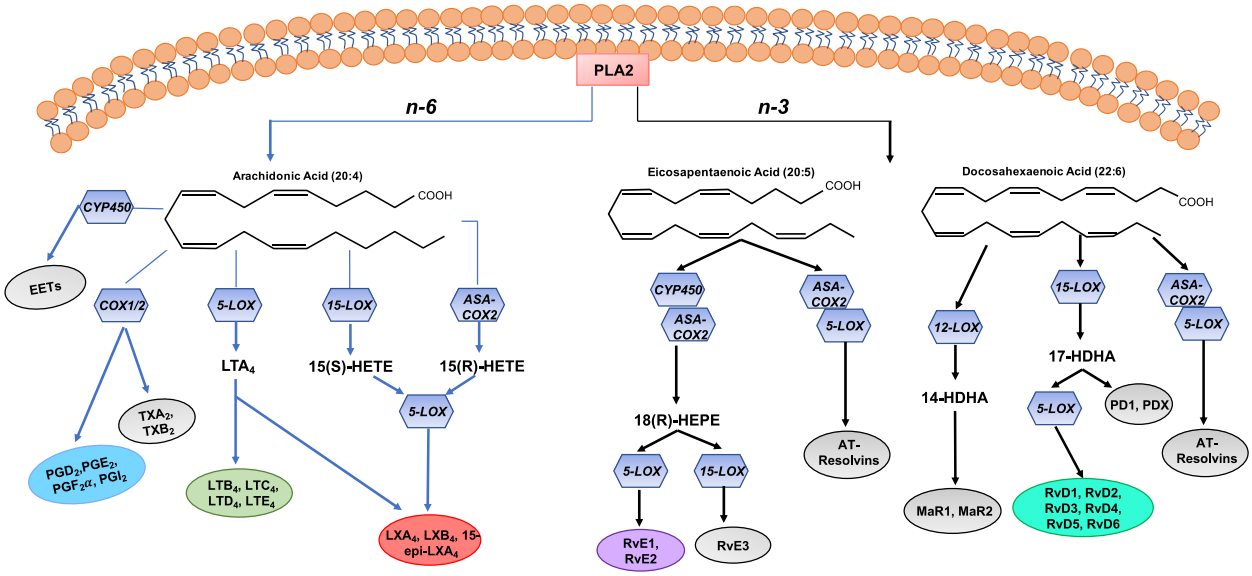

Figure 1.2. Derivation and biosynthesis of PUFA-derived lipid mediators. PUFAs embedded in membrane phospholipids are released into the cytosol primarily by phospholipase A_2 (PLA_2) activity. AA, an n-6 PUFA can serve as a substrate for the cytochrome P-450 (CYP) enzyme to generate the epoxyeicosatrienoic acids (EETs). AA can also serve as the substrate for the cyclooxygenases (COX1/2) to produce prostaglandins or thromboxanes. Alternatively, AA can be converted by 5-LOX to the leukotrienes (LTA_4), which can be converted downstream to other LT products such as LTB_4. Alternatively, LTA_4 can be converted to the lipoxins (LXA_4) by 12-LOX. LXA_4 can also be generated through the coordinated actions of 15-LOX and 5-LOX or by the actions of aspirin (ASA)-acetylated COX2 and 5-LOX. On the other hand, the n-3 PUFA, eicosapentaenoic acid (EPA), generates 18(R)-hydroxy-EPE (18(R)-HEPE) through the actions of both CYP-450 and ASA-COX2. 18(R)-HEPE is then converted to the resolvins

(RvE1, RvE2) by 5-LOX. 18(R)-HEPE can be converted by 15-LOX to RvE3. The other n-3 PUFA, docosahexaenoic acid (DHA), is converted to 17(S)-hydroxy-DHA, which is subsequently converted to the resolvins by 5-LOX. On the contrary, 17(S)-HDHA can generate the protectins via an epoxidation hydrolysis reaction. Through the coordinated actions of ASA-COX2 and 5-LOX, DHA can generate the aspirin-triggered (AT)-resolvins, and the macrophage-derived maresins (MaR1, MaR2) through the actions of the 12-LOX. For simplicity, the downstream lipid mediators of DPA are not shown.

While AA-derived lipid mediators are primarily known to be pro-inflammatory, they also have important homeostatic roles in the acute inflammatory response. The acute inflammatory response is divided into two phases: initiation and resolution. During the initiation phase of the acute inflammatory response, leukocytes traffic from circulation to the damaged tissue or site of injury to form inflammatory exudates or “battlefields” (151). In addition, PUFAs, primarily AA, are released from membrane phospholipids or delivered to sites of inflammation by tissue oedema and undergo conversion within minutes to specific lipid mediator families via the actions of LOX and COX enzymes (162). 5-LOX-derived lipid mediator families such as the LTs and HETEs promote bronchoconstriction, regulate vascular permeability and leukocyte recruitment to the sites of tissue damage by promoting transmigration along chemotactic gradients (151,153,162–165).

The actions of COX-derived PGs and TXs elicit the cardinal signs of inflammation including heat, swelling, redness, pain, and loss of function (166). TXs produced by platelets and other cell types induce the platelet response to injury and also have a homeostatic role in platelet aggregation (153). Prostaglandin E_2 (PGE2) and PGI_2 inhibit platelet aggregation and promote vasodilation in endothelial cells (153,162). Prostaglandins and leukotrienes (LTB4) along with other chemokines, cytokines, and complement components (C3b and C5a) stimulate chemotaxis of polymorphonuclear neutrophils (PMNs) into the damaged tissue to phagocytize and neutralize invading

pathogens (151,167,168). These lipid mediator families can bind to and interact with their G-protein coupled receptors (GPCRs) to modulate functions of target tissues and cells (Figure 1.3). GPCRs can serve as good targets for drug therapies (169). PGs and TXs bind to ten types and subtypes of prostanoid receptors (148,170). On the other hand, LTB4 binds BLT-1 and BLT-2, while LTC4 and LTD4 binds Cys-LT1 and Cys-LT respectively (171). Ultimately, the actions of these AA-derived lipid mediators are necessary to clear the site of infection and promote wound healing, which occurs during the second phase of the acute inflammatory response: resolution.

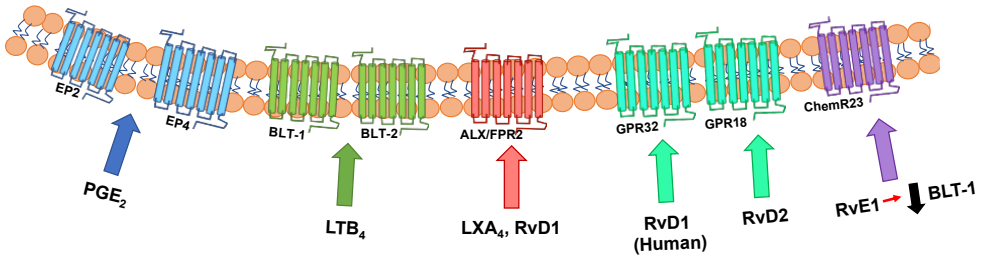

Figure 1.3. PUFA-derived lipid mediators exert actions by binding to GPCRs. Lipid mediators produced from n-6 and n-3 PUFAs exert their bioactions through high affinity binding to one or more GPCRs located on the plasma membrane. Prostaglandin (PGE_2) can bind to the prostanoid receptors, EP2 and EP4. The LT, LTB_4 binds to BLT-1 and BLT-2, whereas LXA_4 and RvD1 bind with high affinity to the formyl peptide receptor 2 (ALX/FPR2). In humans, RvD1 binds to GPR32, whereas RvD1 binds to GPR18 in both mice and humans. RvE1, derived from EPA, binds to ChemR23 in both mice and humans, while simultaneously downregulating BLT-1.

Pro-inflammatory lipid mediators and the humoral immune response

AA-derived lipid mediators not only play a large role in regulating the immune response, but they can also directly impact B cell development, activation, and function. Some of the first evidence that lipid mediators could potentially be involved in B cell function was the evidence that PGE_2 suppressed splenic B cell colony formation due to its ability to directly influence the growth and differentiation of human B cells (172,173). Phipps et al. have extensively shown that PGs, particularly PGE_2, is not only produced and secreted by B cells, but can also influence B cell antibody production, cytokine secretion, and class-switching, providing further evidence that lipid mediators can directly target B cells (174–176). In contrast to prostaglandins, leukotriene synthesis is restricted to a few cell types, predominantly myeloid cells and B cells (177). Interestingly, the p38 mitogen-activated protein kinase seems to be directly involved in LT synthesis in B cells (178). The immunomodulator, LTB_4, is expressed on B cells and can enhance B cell activation, antibody production, and proliferation in tonsillar B cells (179,180).

Furthermore, Yang et al. demonstrated that COX-1 is an essential regulator of B cell development in particular from the pro-B to pre-B cell stage, which is dependent upon the activation of the Janus kinase/signal transducer and activator of transcription 5 (JAK/STAT5) signaling (181). Notably, administration of a TXA_2 agonist rescued deficits in B cell development seen in COX-1 deficient mice alluding to the idea that TXA_2 plays a crucial role in early B cell development through regulation of JAK/STAT5 signaling (181). It would be interesting to see how these pro-inflammatory lipid mediators affect specific B cells subsets and B cell function in the context of disease models such as diet-induced obesity.

Specialized pro-resolving lipid mediators (SPMs) biosynthesis

Resolution of the acute inflammatory response is an active process that inhibits leukocyte infiltration to the inflammatory site, increases apoptosis of PMNs, and promotes clearance of debris and apoptotic cells by macrophages (182–184). These actions are carried out by bioactive mediators known as the lipoxins, resolvins, protectins, and maresins, which were identified and coined as specialized pro-resolving mediators (SPMs) by Charles Serhan and his laboratory (182,185,186). SPMs are derived from PUFAs in an enzyme-dependent manner and are active in the picogram to nanogram range (162). The first resolution signals and SPM biosynthesis are initiated by lipid mediators of the initiation phase (168,182). For example, PGE_2 and PGD_2 initiate a temporal lipid mediator class switch, which results in the biosynthesis of lipoxin A_4 (LXA_4) (162). LXA_4 serves as the "stop" signal of the acute inflammatory response resulting in decreased production of pro-inflammatory AA-derived lipid mediators and decreased PMN infiltration.

Lipoxins, which are derived from AA are formed by transcellular biosynthesis. For example, lipoxins can be formed via the actions of 5-LOX in activated leukocytes and platelet 12-LOX to form LXA_4 and LXB_4 (148,187) (Figure 1.2). The second route of synthesis of the lipoxins involves 15-LOX expressing endothelial and epithelial cells interacting with 5-LOX-containing leukocytes (188). Finally, lipoxins can be generated through aspirin-mediated acetylation of COX2, which results in the conversion of AA to 15(R)-hydroxyeicosatetraenoic acid (15(R)-HETE). 15(R)-HETE serves as the substrate for 5-LOX, which results in the production of aspiring-triggered (AT) lipoxins (189–191).

It is important to note that the formation of AT-lipoxins is specific for aspirin treatment (148).

In addition to the AA-derived lipoxins, SPMs are enzymatically derived from the n-3 PUFAs, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). EPA can be converted by aspirin-acetylated COX-2 and CYP450 into 18R-hydroxy-EPE (18(R)-HEPE). 18(R)-HEPE is subsequently transformed into resolvin E1 (RvE1) and resolvin E2 (RvE2) by leukocyte 5-LOX (Figure 1.2) (185,192). 18(R)-HEPE can also serve as the substrate for 15-LOX to generate RvE3 (148). Similarly, DHA can generate resolvins (RvD1-RvD6) and their AT isomers (AT-RvD1-RvD6) (193). DHA can be converted into the SPM precursor, 17(S)-HDHA via the actions of 15-LOX. 17(S)-HDHA is subsequently transformed into RvD1-RvD6 by 5-LOX or converted to protectin D1(PD1) and its isomer, PDX by 15-LOX (Figure 1.2) (148,194). Furthermore, DHA is unique in that it generates the maresin SPM family in macrophages. DHA is readily converted by 12-LOX into the SPM precursor, 14(S)-HDHA, which undergoes further lipoxygenation to give rise to maresins 1 and 2 (MaR1 and MaR2) in macrophages (162,195). Maresins stimulate the switch of classically activated, pro-inflammatory macrophages to alternatively activated, anti-inflammatory macrophages (151).

The resolution machinery includes other key players besides PUFA-derived SPMs. Microparticles from self-resolving exudates enhance efferocytosis and transmit pro-resolving signals in mice and human macrophages (196). The Annexin A1 protein, cytokines such as TGFβ and IL-10, carbon monoxide, and microRNAs also display pro-resolving capabilities (197–200). Similar to the pro-inflammatory lipid mediators, SPMs exert their bioactions through the action of one or more GPCRs. For example, RvE1 binds

to ChemR23, which enhances macrophage phagocytosis through phosphorylation signaling (Figure 1.3) (192). In addition, RvE1 simultaneously binds to BLT-1 and blocks the binding of LTB4 to the receptor while promoting neutrophil apoptosis and clearance by macrophages (201). RvD1 binds to the human receptor, GPR32 and to the LXA_4 receptor, ALX-FPR2 (202). Of note, ALX-FPR2 can be activated by various ligands such as Annexin A1 and pro-inflammatory peptides (203). RvD1-GPR32 resolution involves the upregulation of microRNAs (miR-208) and anti-inflammatory IL-10, and downregulation of miR-219, which decreases LTB_4 levels (204). AT-RvD1, RvD2, and RvD5 can bind to an activated GPR32 with high affinity in various cell types, which alludes to the fact that SPMs can target various cell types in different tissue locations (199,202,205).

Imbalance of SPMs in obesity

Obesity is associated with a low-grade chronic inflammatory state that results in the overproduction of various pro-inflammatory cytokines and AA-derived lipid mediators. Several studies have reported that levels of the lipid mediator, LTB_4 are increased in the VAT, skeletal muscle, and liver of mice consuming a HFD compared to mice consuming a control diet (206–208). Increased levels of BLT-1, the receptor for LTB_4, also play a negative role in the adipose of obese mice. This was evident in a recent study, which showed that deleting BLT-1 in obese mice protected them from systemic insulin resistance (209). Furthermore, studies have found that n-3-derived SPM precursors and SPMs are significantly reduced in the adipose of obese mice. Neuhofer et al. reported that mice consuming a HFD had decreased levels of 17-HDHA and PD1 in the gonadal

adipose tissue after only 4 and 14 days of high fat feeding (210). In addition, Claria et al. found that RvD1, PD1, 18-HEPE, 14-HDHA, and 17-HDHA were decreased in the adipose tissue of mice consuming a HFD for 18 weeks compared to their control counterparts (159).

These findings were confirmed in humans as the VAT from obese patients showed an upregulation of both pro-inflammatory lipid mediators (LTs and PGs) as well as the SPMs (RvD1 and RvD5). The ratio between SPM production with respect to LT and PG production was significantly reduced in the VAT from obese patients (160). Deficits in SPMs in the WAT could be the result of decreased intake of n-3 PUFAs, which drives diminished levels in various tissues (211,212). Alternatively, obese WAT could be facilitating the conversion of SPMs to inactive oxidized metabolites (oxo-resolvins) or PUFAs into inactive diols, which would not allow for SPM conversion (159,213). However, recent studies by Claria et al. suggest that deficits in SPM production could be a result of impaired LOX activity, particularly decreased 15-LOX production rather than altered cellular uptake of DHA (214). In this study, leukocytes from obese patients had reduced levels of 17-HDHA and unbalanced formation of resolvins derived from DHA accompanied by increased production of the pro-inflammatory lipid mediator, LTB_4 (214). When the leukocytes where incubated with 17-HDHA in culture, the formation of the resolvins was completely rescued. As DHA serves as the substrate for 15-LOX to yield the production of the SPM precursor, 17-HDHA, these findings highlight 15-LOX as a key player in efficient SPM production at the cellular level and as a potential therapeutic target in diet-induced obesity models.

SPMs modulate B cell function

Recent research has revealed that SPM treatment alters B cell function, antibody class switching, and differentially affects B cell subsets. Studies by Ramon et al. demonstrated that RvD1 and 17-HDHA but not PD1 increase B cell antibody production and B cell differentiation (215). Treatment of human B cells with 17-HDHA without antigen stimulation did not increase antibody production. However, when these B cells were challenged with CpG plus αIgM, 17-HDHA increased antibody production without enhancing cell death or proliferation (215). Challenged B cells treated with 17-HDHA also increased the number of IgM and IgG producing, $CD27^{+}CD38^{+}$ plasma cells, and increased IL-10 production (215). Collectively, these results suggest that the SPM precursor, 17-HDHA could promote B cell differentiation towards an antibody-secreting phenotype, which has important implications for humoral immunity (215). This same group also reported that 17-HDHA enhanced the antigen-specific antibody response in a pre-clinical influenza immunization mouse model. Mice vaccinated with HA plus 17-HDHA had a 2-fold increase in the number of bone marrow $CD138^{+}$ B cells and HA-specific antibody secreting cells, which was confirmed by an increase in the transcript levels of BLIMP-1 (216). Upon influenza infection, the mice vaccinated with HA plus 17-HDHA had increased survival and higher antibody titers. These results suggest that 17-HDHA might be a useful adjuvant to improve vaccine efficiency (216).

In addition to antibody production, SPMs can differentially affect B cell subsets and class-switching. Ramon et al. demonstrated that treatment of human memory B cells with LXA_4 decreased antibody production and cell proliferation via an ALX/FPR2-dependent mechanism. Interestingly, they reported that human memory B cells had increased

expression of ALX/FPR2 compared to human naïve B cells (217). SPMs seem to differentially affect Ig classes. To elaborate, 17-HDHA and RvD1 promote the differentiation of IgG-secreting B cells but inhibit IgE production in human B cells. This inhibition is due to a decrease in the expression of the epsilon germline transcript (εGLT), which is essential for IgE class switching (218). Overall, this further supports the idea that SPMs can differentially affect B cell subsets and potentially alter B cell class switching by targeting specific Ig classes.

Can SPM deficiencies impair the B cell response in diet-induced obesity?

A missing gap in the literature is how SPM precursors and SPMs modulate B cell responses in the context of diet-induced obesity. Many clues suggest that there is a connection between the two. For example, obesity is associated with increased levels of pro-inflammatory lipid mediators and decreased levels of SPMs, the B cell response is impaired in obesity, and SPMs can directly target B cells. Furthermore, evidence from our lab reveals that the parent PUFAs of SPMs, EPA and DHA can differentially increase B cell subset numbers and antibody production in DIO models (219–221). Interestingly, Ying et al. demonstrated that recruitment and activation of adipose tissue B2 cells, which induce insulin resistance was mediated by signaling through the pro-inflammatory lipid mediator, LTB4 and its receptor, BLT-1 in mice consuming a HFD (206). Furthermore, leukocytes isolated from obese subjects had decreased production of SPM precursors and SPMs. These studies provide increasing evidence that deficiencies in SPM production and levels can affect B cells in the context of diet-induced obesity.

Despite this evidence, the role of DHA-derived SPMs on humoral immunity in the context of obesity is not known. My dissertation aims to address this gap by determining if SPM precursors and SPMs can directly target and enhance the number of B cells subsets and B cell function in DIO mouse model systems. My research not only addresses this missing piece of information in the literature, but also provides key evidence that SPMs can improve the B cell response in obesity. My research will have a broad scope as I seek to answer whether SPMs can modulate B cells in different tissues where they have key roles or induce inflammation in the context of diet-induced obesity including the bone marrow, spleen, and adipose. My research also has a sex-specific component to determine if the role of SPMs in the B cell immune response is equivalent in female mice consuming a HFD. These sex-specific studies will give necessary insight as to whether obese females have the same SPM deficiencies that have been seen in obese males thus far. Collectively, my research has several clinical implications. As obese individuals have increased susceptibility to influenza infections despite robust serological responses, my research can potentially identify SPMs as a future therapeutic target to enhance the humoral immune response in the obese population. In addition, I hope to establish that SPM deficiencies in obesity affect B cells in several tissues and identify a potential link between B cell recruitment and maturation in these tissues. My research will also highlight the idea that administration of SPMs rather than the parent PUFAs might be a more effective way of improving chronic inflammatory conditions such as obesity. Finally, my work will emphasize significant sex-specific differences in SPM levels present, which will give clinically relevant information as to why males and females do not respond the same to various infections and vaccinations.

SPECIFIC AIMS

Obesity is associated with an increased risk of infections and a poor response to vaccinations, which poses a significant threat to public health in the U.S. and worldwide. One potential explanation for this phenomenon could be due to decreased dietary intake of omega-3 fatty acids, which have a critical role in regulating inflammation and adaptive immunity. Omega-3 fatty acids serve as substrates for lipoxygenase (LOX) enzymes that mediate the production of specialized pro-resolving mediators (SPM). SPMs maintain homeostasis of the tissue by resolving low-grade chronic inflammation that is associated with obesity and its various co-morbidities. However, much less is known about how SPMs regulate adaptive immunity. B cells are key players in the adaptive humoral immune response as they produce antibodies that are necessary for the elimination of infection and proper vaccination. Our lab has recently generated preliminary data to show omega-3 fatty acids can increase murine antibody production in obese mice, potentially through production of SPMs. However, the mechanism of how obesity impairs the B cell-driven humoral immune response remains unclear, in addition to how omega-3 fatty acids may improve humoral immunity. Preliminary data from our lab suggests that B cells from obese mice have reduced levels of lipoxygenase enzymes, which mediate the production of SPMs. This raises the exciting possibility that lowered omega-3 levels in obesity could be dampening the ability of the B cells to produce SPMs due to decreased lipoxygenase activity.

The **long-term goal** of this project is to determine how obesity and omega-3 fatty acids influence B cell-driven humoral immunity by targeting the enzyme that is responsible for SPM production. *We hypothesize that low levels of omega-3 fatty acids*

and their downstream metabolites in obesity result in impairment of the B cell-driven humoral immune response, which is due to reduced activity of lipoxygenase enzymes involved in SPM production. *Furthermore, we hypothesize omega-3 fatty acids improve immunity by compensating for the loss of SPMs.* Our approach will use dietary (Aim 1) and genetic knockout (Aim 2) approaches to address the following aims:

Aim 1: *To test the hypothesis that obesity impairs the humoral immune response, which is improved with the supplementation of omega-3 fatty acids and administration of SPM precursors and SPM.*

A. Determine how a high fat diet low in omega-3 fatty acids and SPMs impairs B cell development in the bone marrow and splenic B cell function.
B. Determine how the modulation of a high fat diet with omega-3 fatty acids and SPMs improves B cell development and antibody production.
C. Determine if the outcomes of diet-induced obesity on B cell development and function are sex specific.

Expected Outcomes: We predict that obesity will impair B cell development leading to a dysregulated antibody response. These impairments will be rescued with the addition of omega-3 fatty acids to the diet, which will increase SPM levels. We will establish if these effects are sex-dependent.

Aim 2: *To test the hypothesis that B cell-specific 5-lipoxygenase regulates the humoral immune response in diet-induced obesity.*

A. Establish that 5-lipoxygenase levels and corresponding SPM products are lowered in diet-induced obesity.

B. Determine the role of the 5-lipoxygenase enzyme, which drives omega-3 fatty acid SPM production, in the B cell-driven humoral immune response in lean mice by the use of a genetic knockout mouse model.

C. Determine if the adoptive transfer of wild type (CD45.1) B cells or the addition of SPMs to lean Alox5$^{-/-}$ mice enhances the humoral immune response upon antigen exposure.

Expected Outcomes: We predict that the absence of the 5-lipoxygenase enzyme will impair humoral immunity resulting in a diminished B cell response. Furthermore, the adoptive transfer of wild type B cells and the addition of SPMs to Alox5$^{-/-}$ mice will enhance the B cell-driven response upon antigen challenge due to increases in SPM levels and pro-resolving activity.

Results from this proposal's aims will give new insights into how the B cell-driven humoral immune response contributes to increased infection and poor vaccination response in obese individuals. Furthermore, these results will provide a greater understanding for the role of omega-3 fatty acids in improving immunity. Finally, these results will reveal the 5-lipoxygenase enzyme as a new and exciting target in regulating the humoral immune response in obesity.

CHAPTER 2: A REDUCTION IN DHA-DERIVED MEDIATORS IN MALE OBESITY CONTRIBUTES TOWARD DEFECTS IN SELECT B CELL SUBSETS AND CIRCULATING ANTIBODY[1]

INTRODUCTION

Diet-induced obesity negatively impacts innate and adaptive immunity (127,222–224), which contributes toward a wide range of secondary complications (222,225–228). Several studies show that B cells are one major cellular target of high fat diets (101,222,229,230). In humans and mice, B cell cytokine secretion and antibody production are dysregulated, which contributes toward increased susceptibility to infections and poor responses to vaccinations (89,131,229,231). At the cellular level, impairments in B cell responses of obese mice are associated with a loss of differing B cell populations that control humoral immunity (91,103).

Strong modifications in lipid metabolism are one potential mechanism by which the number of B cells could become modified upon exposure to high fat diets (219,232–235). In particular, specialized pro-resolving lipid mediators (SPMs), which provide resolution signals that regulate adaptive immunity, could be impaired in secondary lymphoid organs in obesity (192,236–240). In support of this hypothesis, specific SPM pathway metabolites are lowered in adipose tissue of obese mice and humans, which prevents the resolution

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[1] This research was originally published in the Journal of Leukocyte Biology: Crouch, MJ, Kosaraju, R, Guesdon, W, Armstong, M, Reisdorph, N, Jain, R, Fenton, J, Shaikh, SR. A reduction in DHA-derived mediators in male obesity contributes toward defects in select B cell subsets and circulating antibody. *Journal of Leukocyte Biology*. 2018; 1– 17.

of adipose tissue inflammation (159, 210). Furthermore, deficiencies in the levels of the n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), which are parent molecules of SPMs, could impair B cell development and maturation. Indeed, there are some data demonstrating that EPA and DHA levels are lowered in circulation of obese humans, although their relationship with B cells are unexplored (241–243).

There is emerging evidence from our laboratory and others that select SPM precursors and SPMs influence B cell differentiation. To exemplify, the SPM precursors 14-HDHA and 17-HDHA, which are generated through the action of the 12/15 lipoxygenase using DHA as a substrate, promote B cell differentiation toward $CD138^{+}$ plasma cells (103,216). DHA-derived SPMs also suppress B cell differentiation into IgE producing cells (218) and the arachidonic acid-derived SPM, lipoxin A4 (LXA4), inhibits antibody production from memory B cells (244). There is also speculation that resolvins could promote the development of germinal center reactions in specific lymphoid tissues (245). Therefore, SPMs are emerging to have a critical role in regulating B cell phenotypes but their role in obesity is completely unknown.

The first objective of this study was to use metabololipidomics to quantify the levels of various PUFA-derived metabolites in bone marrow and spleen followed by studies in which obese male mice were administered SPM precursors to test their ability to rescue deficits in B cell populations and circulating antibody levels. Mechanistically, we used a combination of cell culture studies, lipidomics, and $ALOX5^{-/-}$ mice to determine if obesity promoted impairments at the level of the B cell or if extrinsic factors such as a loss of dietary DHA were responsible for the observed reduction in SPM precursors. Subsequent

studies were conducted with female mice. The rationale was that major differences in immunity between sexes are prevalent but relatively little is known about B cell responses with females, particularly in the context of lipid metabolism and obesity (246). For example, premenopausal obese women display increased class switching from IgM to IgG and increased B cell numbers, which is hypothesized to enhance humoral immunity in women (247). Finally, we assayed for B cell populations and functional outcomes with B cells isolated from obese female mice and compared the results with humans.

MATERIALS AND METHODS

Mice. All murine experiments fulfilled the guidelines established by East Carolina University and The University of North Carolina at Chapel Hill for euthanasia and humane treatment. Male and female C57BL/6J mice of approximately 5-6 weeks of age were purchased from Jackson Laboratories. Mice were fed control (10% of total kcal from lard) or high fat (60% of total kcal from lard) diets (Research Diets, New Brunswick, NJ) for 15 weeks. ALOX5$^{-/-}$ mice were purchased from Jackson laboratories at 4-6 weeks of age and aged up to ~20 weeks prior to conducting experiments.

Metabolic studies. Echo-MRI for fat and lean mass was conducted as previously described (248). Mice were fasted for 6 hours followed by the establishment of baseline glucose values with a glucometer. 2.5g of dextrose (Hospira Inc., Lake Forest, IL) per kg lean mass was delivered to each animal via an intraperitoneal injection (i.p.). Glucose measurements were made throughout a 90-minute time interval from the tail vein. Fasting

insulin levels were assayed with an insulin ELISA kit (Abcam) followed by the calculation of the HOMA-IR index (249).

Metabololipidomics. All standards and internal standards used for LC/MS/MS analysis of arachidonic acid, docosahexaenoic acid and linoleic acid derived lipid mediators were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). All solvents and extraction solvents were HPLC grade or better.

B cells were pretreated for solid phase extraction (SPE) as follows. Briefly, 400μl of ice-cold methanol was added along with 10μl of 10pg/μl internal standard solution (100pg total/each of 5(S)-HETE-d_8, 8-iso-PGF2a-d_4, 9(S)-HODE-d_4, LTB4-d_4, LTD4-d_5, LTE4-d_5, PGE2-d_4, PGF2a-d_9 and RvD2-d_5 in ethanol) to isolated B cells that were previously frozen in 1.5mL microcentrifuge tubes. The samples were allowed to incubate for 15 minutes on ice, and then centrifuged at 14,000 RPM for 10 minutes at 4°C. The supernatant was removed and placed into a new microcentrifuge tube. Another 200μl of ice-cold methanol was added to the B cell pellet, vortexed and then centrifuged at 14,000 RPM for 10 minutes at 4°C. The supernatant was removed and then combined with the previously obtained supernatant. The combined supernatants were dried in a vacuum, centrifuged, and then reconstituted with 1.0 mL of 10% methanol prior to solid phase extraction.

Spleen samples were also pretreated for solid phase extraction. Spleen samples were pre-weighed and transferred into a dry ice chilled, pre-weighed TissueLyser tube (Qiagen, Hilden, Germany) with a 5mm stainless steel ball. 1.0mL of -20°C chilled methanol and 10μl of internal standard solution was added and the samples were

homogenized at 50 Hz for 2 minutes. The sample was then centrifuged at 14,000 RPM for 10 minutes at 4°C. The supernatant was removed and transferred to a new 1.5mL microcentrifuge tube and dried in a speedvac until completely dry. The dried sample was then reconstituted with 1.0 mL of 10% methanol prior to solid phase extraction.

SPMs from B cells and spleen samples were isolated and purified using SPE. The reconstituted extracts were loaded on a Strata-X 33um 30mg/1mL SPE column (Phenomenex, Torrance, California, USA) preconditioned with 1.0mL of methanol followed by 1.0mL of water. The SPE column was then washed with 10% methanol and then eluted directly into a reduced surface activity/maximum recovery glass autosampler vial with 1.0mL of methyl formate. The methyl formate was evaporated completely from the vial with a stream of nitrogen and then the SPE cartridge was then eluted with 1.0mL of methanol directly into the same autosampler vial. The methanol was evaporated to dryness with a stream of nitrogen and then the sample was reconstituted with 20uL of ethanol. Samples were analyzed immediately or frozen at -70°C until analysis. Liquid chromatography-mass spectrometry of SPMs was performed as previously described (103).

Fatty acid extraction and GC/MS lipidomics. Fatty acids were extracted from spleen samples using microwave assisted extraction (250). Fatty acids were extracted using a 4:1 (v/v) ethyl acetate:methanol solution in a Mars 6 microwave system (CEM Corporation, Matthews, NC) with the following heating profile: 1 minute ramp to 40°C, 12 min hold with max power of 400W. Fatty acyl methyl esters were synthesized using the HCl method (251). A 680/600S GC-MS (Perkin Elmer, Waltham, MA) equipped with

Agilent Technologies (Santa Clara, CA, USA) DB-23 30-m column was used for data acquisition. The GC parameters were as follows: the initial temperature was set to 100°C for 30 seconds, ramp was set from 7.0°C/min to 245°C, and a hold for 2 minutes. Standard curves were created using Supelco 37 Component FAME mix (Sigma). A 22:5n-3 curve was created using a standard from Cayman Chemical. Seven-point curves were created for all standards. Data analysis was conducted using TargetLynx software. Concentrations were normalized based on the weights of the spleens.

Analysis of B cell SPM precursor production. $4x10^6$ B cells (purity >95%) from C57BL/6J mice were resuspended at a concentration of $1x10^6$ cells/mL and were placed into 15mL conical tubes for 0 or 4 hours post activation. Cells were treated with or without 0.5μM DHA complexed to BSA followed by activation with 1μg/mL CPG 1826 plus 2μg/mL anti-IgM. Cell pellets were washed twice with 1X PBS and stored in -80°C until samples were analyzed via mass spectrometry as described above.

Flow cytometry. Flow cytometry analyses were conducted using C57BL/6J and $ALOX5^{-/-}$ mice as previously described (103). Briefly, 2.5×10^5 splenocytes and 5×10^5 bone marrow cells were plated on 96-well flat bottom plates followed by one wash in PBS. FcR blocker (Miltenyi Biotech) was added according to manufacturer recommendations. Cells were washed once with cold 1X PBS and stained with fluorescently labeled antibodies for 25 minutes and subsequently washed 3 times. A BDLSRII flow cytometer was used for data acquisition.

The fluorescently labeled antibodies used in the experiment were obtained from Biolegend and included: CD19 (PerCP-Cy5.5, Catalog 152406), CD43 (APC, Catalog 143208), CD24 (Pacific Blue, Catalog 101820), IgM (PE, Catalog 406508), IgD (APC, Catalog 405714), CD138 (APC, Catalog 142506), $B220^{+}$ (FITC, Catalog 103206), CD21/35 (PB, Catalog 123414, CD23 (APC, Catalog 101620), GL7 (AF-647, Catalog 144605), CD80 (APC, Catalog 104714), CD1d (APC, Catalog 123522) and Zombie NIR (Catalog, 423106). All cells were gated off of a Live/Dead cell indicator (Zombie NIR). The following B cell subsets in the bone marrow were analyzed: $CD19^{+}CD43^{+}CD24^{+}IgM^{-}$ (pro), $CD19^{+}CD43^{-}CD24^{+}IgM^{-}$ (pre), $CD19^{+}CD24^{+}IgM^{+}IgD^{-}$ (immature), $CD19^{+}IgM^{+}IgD^{+}$ (mature), $CD19^{+}CD138^{+}$ (long lived plasma cells). The following B cell subsets in the spleen were analyzed: $B220^{+}IgM^{+}CD21^{low}CD23^{-}$ (T1), $B220^{+}IgM^{+}CD21^{-}CD23^{+}$ (T2), $B220^{+}IgM^{+}CD21^{high}CD23^{+}$ (T2-MZ), $B220^{+}IgM^{low}CD21^{int}CD23^{+}$ (Follicular), $B220^{+}IgM^{high}CD21^{+}CD1d^{high}$ (Marginal), $B220^{+}CD19^{+}IgM^{+}GL7^{+}$ (Germinal), $B220^{+}IgM^{+}CD35^{+}CD80^{+}$ (IgM^{+} Memory), $B220^{low}IgM^{+}CD138^{+}$ (Plasmablasts). Data were analyzed by FlowJo V.10.4.

<u>B cell activation.</u> Mouse splenic B cells were isolated using a murine B cell isolation kit (Miltenyi Biotech) and resuspended at a concentration of $1x10^{6}$ cells/mL. $2x10^{5}$ cells were plated in a 96-well U-bottom plate and activated with: 1) 1μg/mL CPG 1826 (Novus Biologicals, Littleton, CO) plus 2μg/mL anti-IgM (Jackson Immunoresearch, West Balt Pike, PA); or 2) 1μg/mL LPS (Sigma Aldrich, St. Louis, MO).

ELISAs. Serum collected from control and experimental groups was used to assay IgG2a, IgG2b, IgG2c, and IgG3 levels via an ELISA (eBioscience). Supernatants from splenic B cells were collected 2 days post-activation and cytokine levels were measured using an ELISA (Biolegend). Leptin levels were measured using an ELISA from Abcam (Cambridge, MA). Supernatants from activated splenic B cells were collected 3 days post-activation to measure IgM and IgG levels using ELISAs from eBioscience (San Diego, CA).

qRT-PCR. Total RNA was isolated from murine splenocytes and total splenic B cells using the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA). 250ng of RNA was reverse transcribed and amplified by using a One-Step SYBR green PCR Mix (Biorad). The fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. GAPDH and β-actin were the housekeeping genes used in all PCR experiments, as previously described (103). The primer sets used in the study are presented in Supplemental Table 2.1.

Rescue studies. SPM precursors and SPMs were injected as previously described at a concentration that leads to elevated levels of 14-HDHA, 17-HDHA and PDX (252). Briefly, 14(S)-HDHA (0.1mg/mL), 17R-HDHA (0.1mg/mL), and Protectin DX (0.1mg/mL) were purchased from Cayman Chemicals (Ann Arbor, MI). SPMs were prepared in ethanol in the dark and kept on ice at all times. The final cocktail concentration was 900ng per mouse (300ng of each SPM precursor and SPM) in PBS or vehicle control (ethanol in PBS) and was administered i.p. to lean and obese mice for 4 consecutive days followed by euthanasia on day 5. Control mice were administered a vehicle control.

PBMC and B cell functional analyses in humans. Human blood samples for B cell studies were procured after obtaining informed consent and were in accordance by the East Carolina University Institutional Review Board. Female subjects were classified as non-obese (BMI<25kg/m^2) or obese (BMI>30kg/m^2) (Supplemental Table 2.2). Subjects were free of chronic/autoimmune disease, not taking n-3 PUFA supplements, free of infection for at least one month, and were non-smokers.

Peripheral blood was collected in vacutainer tubes and diluted in PBS (1:1) followed by separation of the PBMCs by the use of Ficoll Paque (GE Healthcare, Washington, NC) gradient centrifugation. Flow cytometry was used to analyze cell populations from isolated PBMCs. All fluorescently labeled antibodies were purchased from Biolegend (San Diego, CA) or Miltenyi Biotech (San Diego, CA) and were the following: Zombie NIR (Catalog, 423106), CD19 (APC, Catalog 982406), CD14 (FITC, Catalog 367116), CD8 (PE-Cy5, Catalog 300910), CD4 (FITC, Catalog 357406), CD2 (Pacific Blue, Catalog 309216), CD45 (PE, Catalog 368510), CD27 (Pacific Blue, Catalog 302822), CD38 (FITC, Catalog 980304), and IgD (PE-Cy7, Catalog 348210). The following populations were analyzed using a BDLSRII flow cytometer: $CD45^+CD3^+CD4^+$ (helper T cells), $CD45^+CD3^+CD8^+$ (cytotoxic T cells), $CD45^+CD3^-CD14^+$ (monocytes), $CD45^+CD14^-CD19^+$ (B cells), $CD19^+IgD^+CD27^+$ (memory B cells), and $CD19^+CD38^+CD27^+IgD^-$ (plasma B cells).

B cells were isolated from PBMCs using a Human B cell isolation kit (Miltenyi Biotech San Diego, CA) with a resulting purity of >99% as previously described ([103]). Briefly, B cells were cultured in RPMI 1640 with 5% FBS, 2mM L-Glutamine, $5x10^{-5}$M 2-Mercaptoethanol, 10mM HEPES, and 50μg/μL gentamicin at a concentration of $3x10^6$

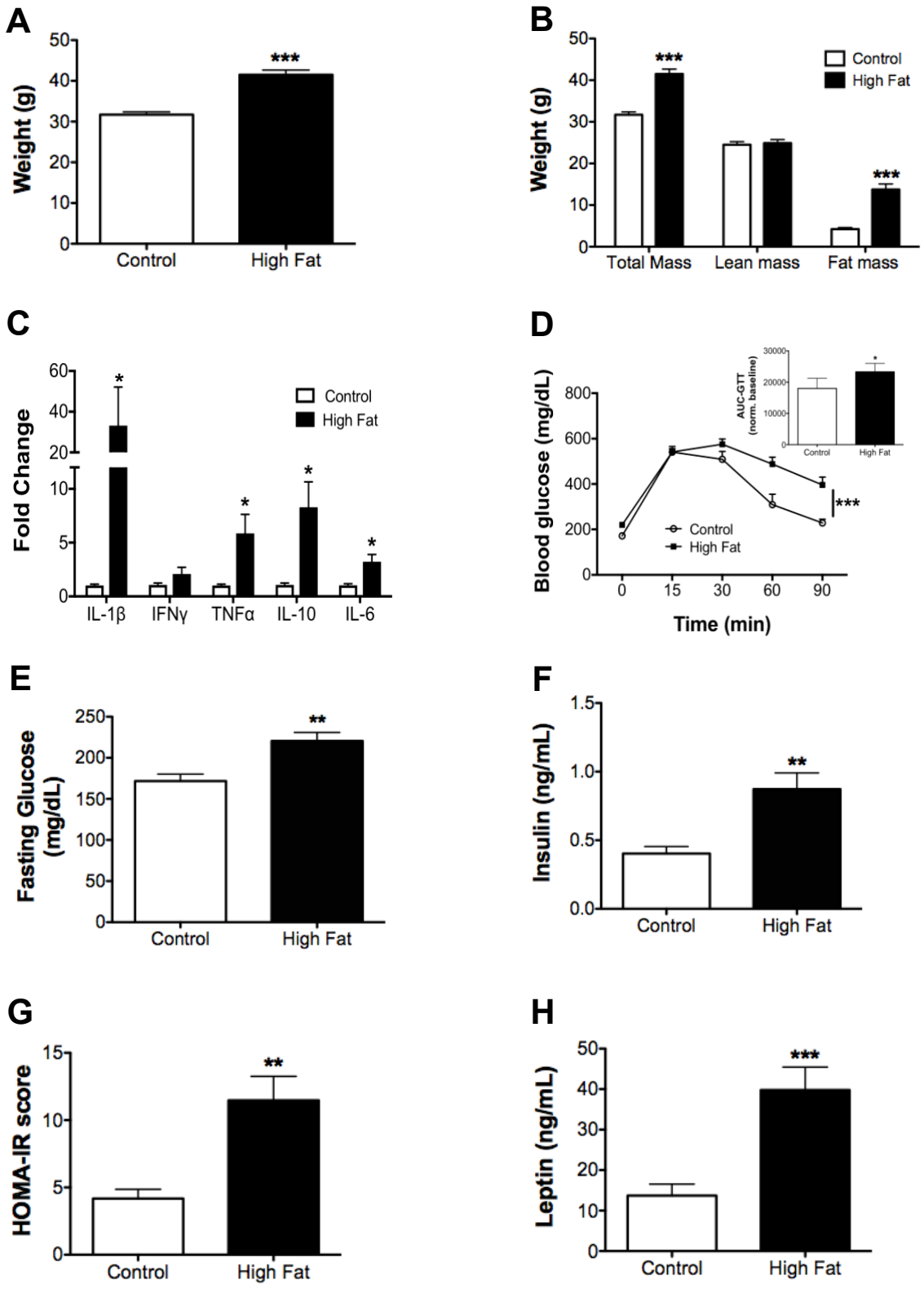
cells/mL. B cells were stimulated with: 1) CPG oligodeoxynucleotides (ODN) 2395 (TLR9 agonist) at 1μg/mL plus BCR stimulation (rabbit anti-human IgM Ab fragment) at 2μg/mL: 2) PAM3CSK4 (TLR1/2 agonist) at a concentration of 10μg/mL. $2x10^5$ B cells were plated in round bottom inert grade 96-well plates and cultured at different time points upon activation. Supernatant was collected 2 days post activation to measure IL-6, IL-10, and TNFα levels via Luminex Assay Kits (Thermo Fisher Scientific, Waltham, MA). Supernatant was also collected 3 days post activation to assay IgM and IgG levels via an ELISA (Abcam Cambridge, MA).

Statistics. All data from murine experiments are from multiple cohorts of mice except SPM analyses in female mice, which are from a single cohort. Due to logistics, studies of B cell populations with male and female were not conducted simultaneously. Data were analyzed using Graph Pad Prism Version 7.0. Statistical significance for female human studies, *in vitro* experiments, and experiments comparing control vs. obese mice were analyzed with a two-tailed Student's unpaired t-test. Statistical significance for lipidomic analysis in the bone marrow comparing control vs. obese mice was determined using a paired t-test. This test was used to account for differences in the number of B cells between cohorts of mice. Murine experiments involving SPM injections were analyzed using a one-way ANOVA followed by a post-hoc Bonferroni multiple comparisons test. Most data sets displayed normalized distribution as determined by a Kolmogorov-Smirnov test. Those sets that did not display normal distributions were analyzed with a Kruskal-Wallis test followed by a Dunn's multiple comparison test. Metabolic studies as a function

of time were analyzed with a two-way ANOVA. For all analyses, $p<0.05$ was considered statistically significant.

RESULTS

Metabolic phenotype of obese male mice. The metabolic profile for obese male mice was first established. Obese mice showed a significant increase in body weight after a 15-week feeding period compared to controls (Figure 2.1A). The increase in total body mass was driven by elevated fat mass (Figure 2.1B). qRT-PCR analysis revealed that obese mice had strong upregulation of IL-1β, TNFα, IL-6, and IL-10 in white adipose tissue (Figure 2.1C). The increase in IL-10 secretion was consistent with a counterregulatory effect of obesity (253). Glucose clearance (Figure 2.1D) was also impaired, as quantified by the area under the curve (Figure 2.1D inset). Fasting glucose levels (Figure 2.1E) were modestly increased and fasting insulin was increased 2-fold with the high fat diet relative to the lean controls (Figure 2.1F). Obese male mice displayed a 2-fold increase in the HOMA-IR score, a measure of insulin sensitivity, compared to controls (Figure 2.1G). Finally, leptin levels were increased by ~4-fold with obese mice relative to lean animals (Figure 2.1H).


A
Weight (g)
50
40
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Control
High Fat
B
Weight (g)
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Control
High Fat
Total Mass
Lean mass
Fat mass
C
Fold Change
60
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Control
High Fat
IL-1β
IFNγ
TNFα
IL-10
IL-6
D
Blood glucose (mg/dL)
800
600
400
200
0
0
15
30
60
90
Time (min)
Control
High Fat
***
AUC-GTT (norm. baseline)
30000
20000
10000
0
*
Control
High Fat
E
Fasting Glucose (mg/dL)
250
200
150
100
50
0
**
Control
High Fat
F
Insulin (ng/mL)
1.5
1.0
0.5
0.0
**
Control
High Fat
G
HOMA-IR score
15
10
5
0
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Control
High Fat
H
Leptin (ng/mL)
50
40
30
20
10
0
***
Control
High Fat

Figure 2.1: Metabolic profile of male C57BL/6J mice consuming a high fat diet. (A) Body weights of male mice at the completion of the study. (B) Body composition assayed by Echo-MRI after 15 weeks of consuming experimental diets. (C) qRT-PCR analysis of inflammatory cytokines in the adipose tissue of control and obese mice. (D) Glucose tolerance test (GTT), performed by intraperitoneal injection of glucose after a 6 hour fast. Inset shows the area under the curve (AUC), calculated by integration of the glucose curves shown in D, normalized to baseline values. (E) Fasting blood glucose and (F) insulin levels determined after a 6 hour fast. (G) HOMA-IR scores. (H) Fasting leptin levels. N=9-10 mice per diet (A-B, D), N=8 mice per diet (C), N=6-8 mice per diet (E-H). Data are average ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 by a Student's unpaired t-test (A-C E-H) or a two-way ANOVA analysis followed by a post-hoc t-test (D).

SPM precursor levels are lowered in obese male C57BL/6J mice. We investigated if diet-induced obesity lowered splenic SPM precursors and SPMs that may regulate B cell populations, in addition to analyses of other key PUFA-derived metabolites (160,210,215,254). 14-HDHA and 17-HDHA were lowered by 2-2.5-fold relative to controls in the spleen (Figure 2.2A). Protectin DX (PDX) trended (p=0.06) to be lowered in obese mice compared to controls whereas resolvin D2 (RvD2) did not show a significant effect. In addition, RvD1 was at the detection limit (Figure 2.2A). Other SPMs (except LXA4) were not detectable in the spleen. We also measured mediators synthesized from arachidonic and linoleic acids. For simplicity, we present key molecules. Obesity lowered 12-HETE by 4-fold (Figure 2.2B) and increased LTB4 by 2.8-fold (Figure 2.2C) but had no influence on thromboxanes (Figure 2.2D). Comparative analysis of total bone marrow cells showed that 14-HDHA was modestly decreased in male obese mice by 1.5-fold with no change in 17-HDHA or PDX between the two groups (Figure 2.2E). 12-HETE was modestly lowered with obese mice by 1.4-fold (Figure 2.2F). There was no effect of obesity on LTB4 (Figure 2.2G) and TXB2 was slightly decreased by 1.3-fold relative to the lean control (Figure 2.2H).

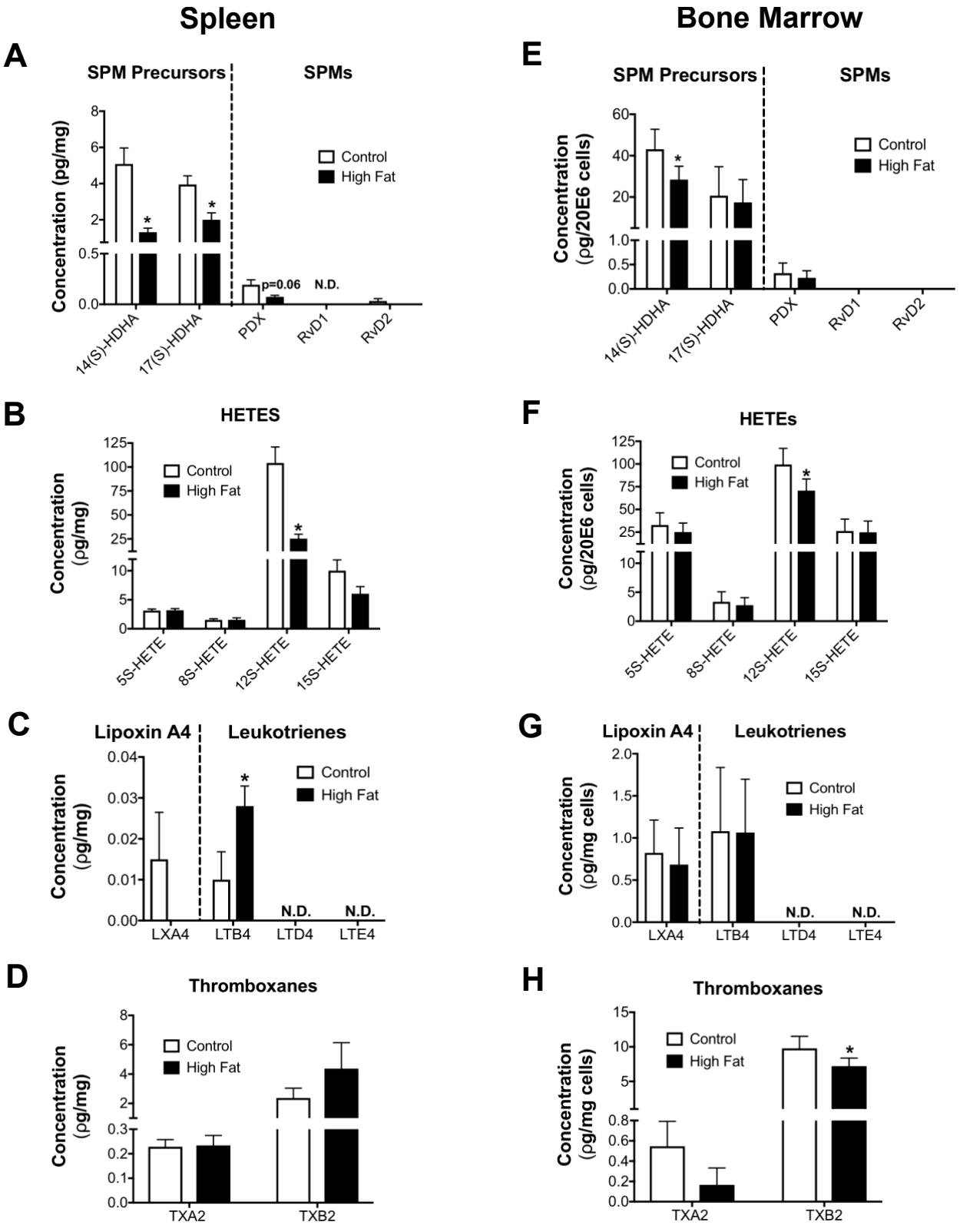


Figure 2.2: DHA-derived SPM precursors and SPMs are lowered in obese male mice. Metabololipidomic analyses of (A) DHA-derived SPM precursors and SPMs, (B)

AA-derived HETES, (C) AA-derived LXA4 and leukotrienes, and (D) AA-derived thromboxanes in the spleens of mice consuming a control or high fat diet. Metabololipidomic analyses of (E) DHA-derived SPM precursors and SPMs, (F) AA-derived HETEs, (G) AA-derived LXA4 and leukotrienes, and (H) AA-derived thromboxanes in the bone marrow of control and high fat mice. N.D. indicates not detectable. Mice consumed experimental diets for 15 weeks. N=6 mice per diet (A-H). Data are average ± S.E.M. *P<0.05 by an unpaired Student's t-test.

Administration of 14-HDHA/17-HDHA/PDX modestly increases germinal center B cells in obese mice accompanied by a rescue of pro-inflammatory IgG2c. Next, we tested if obesity lowered select B cell subsets and if 4 days of simultaneous administration of 14-HDHA, 17-HDHA, and PDX could influence the number of key B cell populations. The rationale for 4 days of administration was based on a previous report to show this time range is adequate to increase SPM levels in obese mice (255). We confirmed that our protocol of injecting 14-HDHA, 17-HDHA, and PDX increased splenic levels of these mediators (Supplemental Table 2.3).

Obese mice receiving SPMs had no change in the number of total $B220^+$ cells in the spleen (data not shown). Splenic B cell populations were analyzed by flow cytometry for mice consuming the control, high fat, and high fat diet + 14-HDHA/17-HDHA/PDX (Supplemental Figure 2.1, Figure 2.3A). A modest 1.65-fold decrease was observed in the percentage of $B220^+IgM^+CD19^+GL7^+$ germinal center B cells in male obese mice compared to the lean controls, which was increased with 14-HDHA/17-HDHA/PDX (Figure 2.3B). The number of germinal center B cells were also modestly elevated with 14-HDHA/17-HDHA/PDX administration relative to the mice consuming the high fat diet (Figure 2.3C).

We determined if mice receiving the 14-HDHA/17-HDHA/PDX had modifications to their serum IgG antibody subclasses (Figure 2.3D). Mice consuming the high fat diet

that received 14-HDHA/17-HDHA/PDX had elevated IgG2b and IgG3 (p=0.06) compared to their control and high fat counterparts (Figure. 2.3D). Notably, administration of 14-HDHA/17-HDHA/PDX to obese mice rescued the high fat diet-induced elevation in IgG2c, which is pathogenic in obesity (Figure 2.3D).

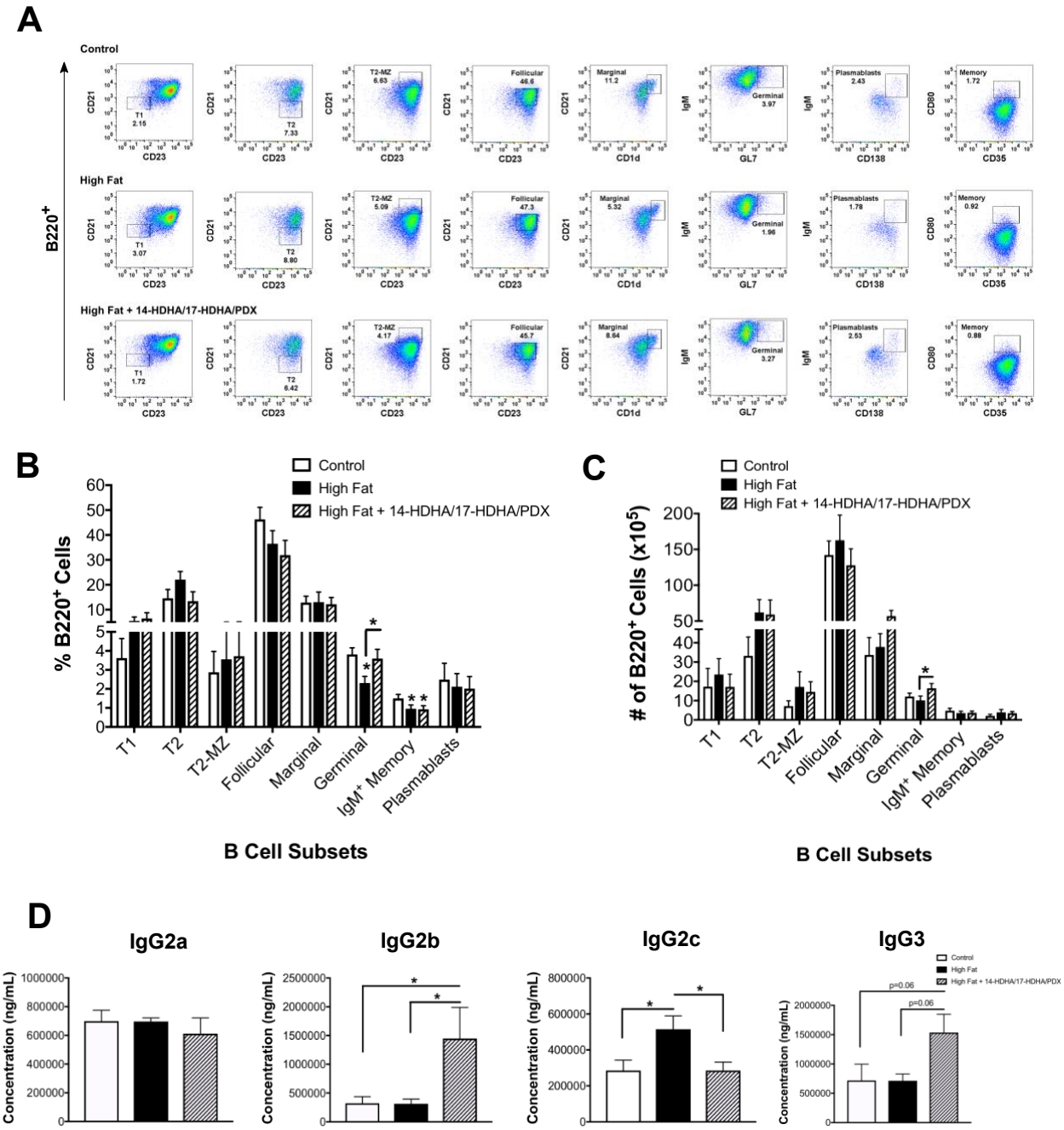


Figure 2.3: DHA-derived SPM precursors modestly increase germinal center B cells of male obese mice accompanied by increased IgG2b and a rescue of IgG2c. (A) Sample flow cytometry plots of splenic B cell subsets for mice consuming a control, high fat, or high fat + 14-HDHA/17-HDHA/PDX including: $B220^{+}IgM^{+}CD21^{low}CD23^{-}$ (T1), $B220^{+}IgM^{+}CD21^{-}CD23^{+}$ (T2), $B220^{+}IgM^{+}CD21^{high}CD23^{+}$ (T2-MZ), $B220^{+}IgM^{low}CD21^{int}CD23^{+}$ (Follicular), $B220^{+}IgM^{high}CD21^{+}CD1d^{high}$ (Marginal), $B220^{+}CD19^{+}IgM^{+}GL7^{+}$ (Germinal), $B220^{+}IgM^{+}CD35^{+}CD80^{+}$ (IgM^{+} Memory), $B220^{low}IgM^{+}CD138^{+}$ (Plasmablasts). (B) Percentage and (C) number of splenic B cell subsets. (D) Serum concentrations of IgG2a, IgG2b, IgG2c, and IgG3. Mice consumed experimental diets for 15 weeks. Mice received either an injection of vehicle control or a cocktail (900ng/mouse) consisting of the SPM precursors 14-HDHA, 17-HDHA, and the SPM PDX once every day for 4 consecutive days. N=6-10 mice per condition. Data are average ± S.E.M. *P<0.05 by a one-way ANOVA followed by a Bonferroni post-test (B, C).

Administration of 14-HDHA/17-HDHA/PDX rescues the number of bone marrow B cells in obese mice. We also evaluated the effects of the SPM precursors and SPM on bone marrow B cells. The total $CD19^{+}$ B cell population (Figure 2.4A) in the bone marrow was decreased by ~2.3-fold when calculated as the percentage (Figure 2.4B) and total number (Figure 2.4C) with obese mice compared to controls. The male mice receiving 14-HDHA/17-HDHA/PDX had a 2-2.5-fold rescue in the percentage (Figure 2.4B) and number (Figure 2.4C) of $CD19^{+}$ cells in the bone marrow compared to obese mice. Flow cytometry analysis of the B cell subsets in the bone marrow (Figure 2.4D) revealed that the DHA-derived mediators rescued the percentage of pre B cells (Figure 2.4E). Administration of 14-HDHA/17-HDHA/PDX to obese mice strongly rescued the reductions in the number of pre, immature, mature, and $CD138^{+}$ long-lived plasma cells in the bone marrow (Figure 2.4F). Collectively, these results establish that DHA-derived mediators are not just targeting plasma cells, as previously reported, and can influence several B cells subsets, notably in the bone marrow (103,215,217).

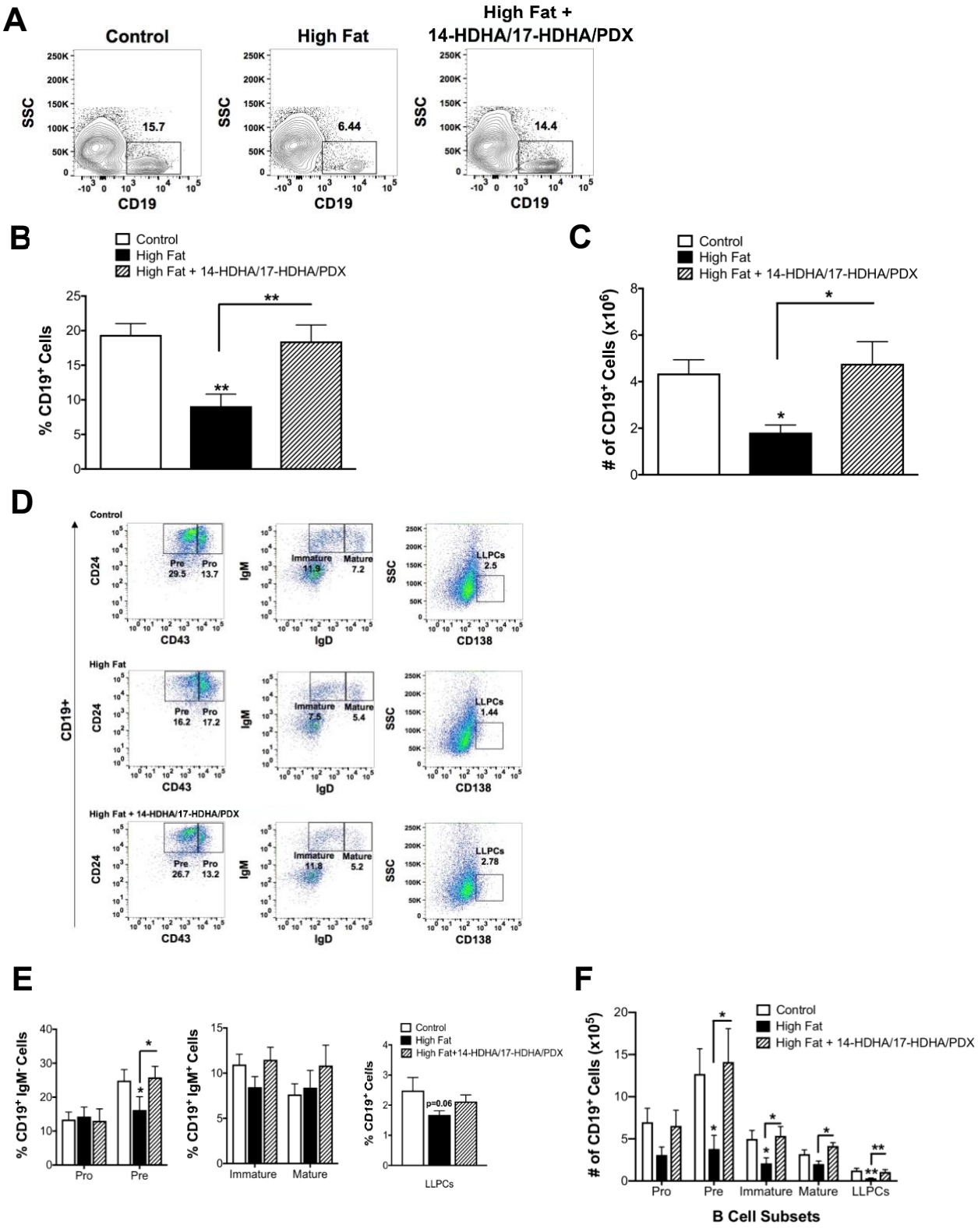
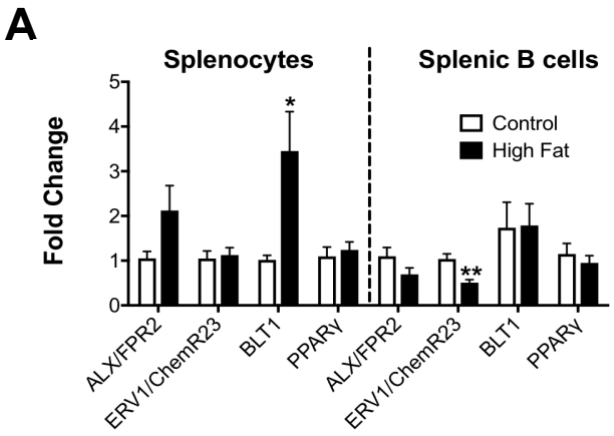


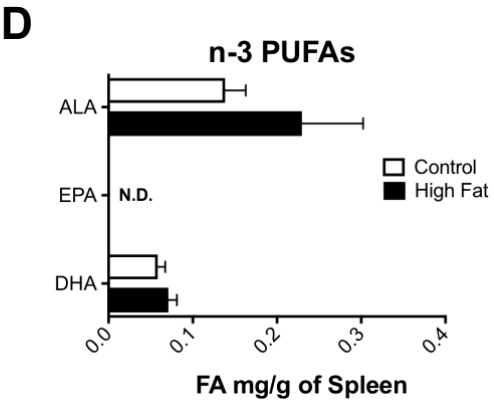
Figure 2.4: DHA-derived SPM precursors and SPM rescue the decrease in the number of bone marrow B cell subsets of obese male mice. (A) Sample flow

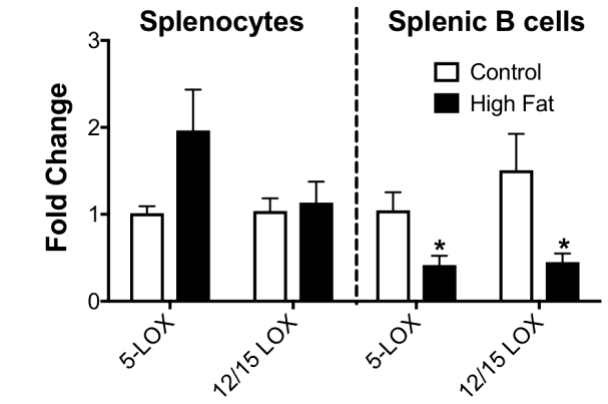
cytometry plots for total $CD19^+$ B cells in the bone marrow of mice consuming a control, high fat, and high fat diet +14-HDHA/17-HDHA/PDX. (B) Percentage and (C) number of $CD19^+$ cells in the bone marrow. (D) Sample flow cytometry plots for B cell subsets in the bone marrow including $CD19^+CD43^+CD24^+IgM^-$ (pro), $CD19^+CD43^-CD24^+IgM^-$ (pre), $CD19^+CD24^+IgM^+IgD^-$ (immature), $CD19^+IgM^+IgD^+$ (mature), and $CD19^+CD138^+$ (long-lived plasma cells, LLPCs). (E) Percentage and (F) number of B cell subsets in the bone marrow. Mice received either an injection of vehicle control or an SPM cocktail (900ng/mouse) consisting of 14-HDHA, 17-HDHA, and PDX once every day for 4 consecutive days. N=7-10 mice per condition. Data are average ± S.E.M. *P<0.05, **P<0.01 by a one-way ANOVA followed by a Bonferroni post-test.

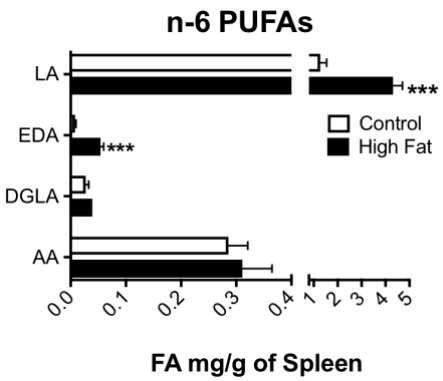
Splenic B-cell LOX levels are lowered in obese mice but do not account for the loss of SPM precursors or impairments in B cell populations. The next set of experiments addressed if the reduction in splenic SPM precursors with obesity was due to a deficiency in the transcript levels of SPM receptors of splenic B cells compared to splenocytes (Figure 2.5A). The specific receptors for the SPM precursors 14-HDHA and 17-HDHA are unknown; thus, we conducted a general analysis of major known lipid mediator receptors. qRT-PCR analysis revealed a 3-fold increase in the transcript of BLT1 (a receptor for the leukotriene LTB4) in splenocytes, which may reflect changes in immune cell populations (Figure 2.5A, left panel). ERV1/ChemR23, a receptor for RvE1, was decreased in splenic B cells of obese mice by 2-fold compared to their lean counterparts (Figure 2.5A, right panel). Transcripts of ALX/FPR2, a receptor for RvD1 and LXA4, were identical between control and obese mice in splenocytes and splenic B cells (Figure 2.5A). There were no differences in transcript expression of the RvD2 receptor, DRV2/GPR18, in splenic B cells between control and obese mice (data not shown). SPMs can potentially mediate their effects via PPARγ, which serves as a natural ligand for parent n-3 PUFAs (256). However, there was no effect of obesity on splenocyte or B cell PPARγ transcripts between control and obese mice (Figure 2.5A).

We investigated if a defect in lipoxygenase (LOX) enzymes, which are responsible for the production of DHA-derived mediators could be driving SPM precursor deficiencies in obesity (189,208,257,258). Analysis of total splenocytes revealed no difference in the transcript levels of 5-LOX and 12/15-LOX in lean and obese male mice (Figure 2.5B, left panel). Obese mice showed a 2-2.5-fold decrease in the transcript levels of 5- and 12/15-LOX in total splenic B cells (Figure 2.5B, right panel).









C

	14-HDHA (pg)	17-HDHA (pg)
Total Spleen	553.7 ± 95.7	430.6 ± 51.1
B cells – DHA	30.7 ± 2.5	3.5 ± 0.4
B cells + DHA	45.9 ± 1.7	94.5 ± 4.4

Figure 2.5: The reduction in SPM precursor production of male mice is not driven by a defect in dietary DHA or B cell production of 14-HDHA and 17-HDHA. (A) qRT-PCR analysis of SPM receptors in splenocytes and splenic B cells. (B) qRT-PCR analysis of 5-LOX and 12/15-LOX in splenocytes and splenic B cells. (C) Concentration of B cell SPM precursor production with or without substrate (DHA) relative to the whole spleen. (D) GC/MS analyses of splenic ALA (18:3n-3), EPA (20:5n-3), and DHA (22:6n-3). (E) Fatty acid analysis of the major splenic n-6 polyunsaturated fatty acids including linoleic acid (LA) (18:2n-6), eicosadienoic acid (EDA) (20:2n-6), dihomo-gamma-linolenic acid

(DGLA) (20:3n-6), and arachidonic acid (AA) (20:4n-6). Fatty acid analyses show milligrams of fatty acid (FA) per gram of spleen. Mice consumed experimental diets for 15 weeks. N=7 mice per diet (A-B), N=3 mice per condition (C) and N=10 mice per diet (D-E). Data are average ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 by an unpaired Student's t-test.

To determine if a reduction in B cell SPM production could account for the defect in splenic 14-HDHA, 17-HDHA, and PDX levels, we calculated SPM precursor production from B cells relative to the whole spleen. Compared to the concentration in the whole spleen, 30.7 pg of the total concentration of 14-HDHA was attributable to B cells, which was increased to 45.9 pg upon addition of the LOX substrate DHA (Figure 2.5C). B cells treated without or with DHA did not produce any detectable amount of PDX (data not shown). B cells in the absence of DHA accounted for 3.5 pg of the total splenic 17-HDHA. Upon addition of DHA, B cells accounted for 94.5 pg of the total splenic 17-HDHA (Figure 2.5C). These data demonstrate for the first time that B cells can generate SPM precursors although the concentration of 14-HDHA and 17-HDHA produced by splenic B cells was a minor proportion of the total concentration in the whole spleen (Figure 2.5C).

Experiments were also conducted with ALOX5$^{-/-}$ mice to test if this enzyme is key for generation of splenic and bone marrow B cells. Studies with ALOX12/15$^{-/-}$ mice were not pursued since B cell subsets are not decreased in this model (259). Analyses of key B cell subsets from ALOX5$^{-/-}$ mice did not reveal a decrease in the percentage (Supplemental Figure 2.2A) or number (Supplemental Figure 2.2B) of B cell subsets in a manner similar to obese mice. Thus, these results suggest that the obesity-driven defect in SPM precursor levels and B cell populations was not at the level of the B cell or driven by 5-LOX, despite the ability of B cells to produce SPM precursors and a reduction in B cell 5-LOX levels.

Male obese mice have elevated levels of splenic n-6 PUFAs that have a high affinity for LOX. We hypothesized that the levels of dietary DHA, the parent molecule for 14-HDHA and 17-DHA, were reduced in obese mice to lower 14-HDHA and 17-HDHA levels. Strikingly, GC/MS analyses revealed DHA and its parent molecule alpha-linolenic acid (ALA), were maintained in obese male mice (Figure 2.5D). Alternatively, n-6 PUFAs could be elevated and thus simply outcompete n-3 PUFAs for key enzymes that synthesize SPM precursors. Indeed, obese mice had a 4.5-fold increase in the total amount of the parent n-6 PUFA, linoleic acid (LA) (Figure 2.5E), and a 4.5-fold increase in its elongation product eicosadienoic acid (EDA) (Figure 2.5E). There was no difference in the total amount of dihomo-γ-linoleic acid (DGLA) (Figure 2.5E) or arachidonic acid (AA) (Figure 2.5E) in the spleens of lean versus obese mice. These results suggest that male obese mice have elevated levels of linoleic acid that are abundant in the western diet and known to bind with a higher affinity to LOX enzymes than DHA when present in excessive amounts (141,260–262).

SPM precursors levels, antibody concentration, and B cell subsets are generally not lowered in female obese mice. The next set of studies focused on female mice. The metabolic phenotype of the female mice was first established. Female mice showed a significant increase in body weight after consuming experimental diets (Figure 2.6A). The increase in total body mass was driven by elevated lean and fat mass (Figure 2.6B). There were no changes in adipose tissue inflammatory transcripts between the two groups (Figure 2.6C). These mice had impaired glucose clearance (Figure 2.6D), as quantified by the area under the curve (Figure 2.6D inset). Fasting glucose levels (Figure

2.6E) were modestly increased and fasting insulin was not influenced by the obese female mice (Figure 2.6F). Obesity did not influence the HOMA-IR score compared to controls (Figure 2.6G). Finally, leptin levels were increased by 3-fold with obese female mice relative to controls (Figure 2.6H).

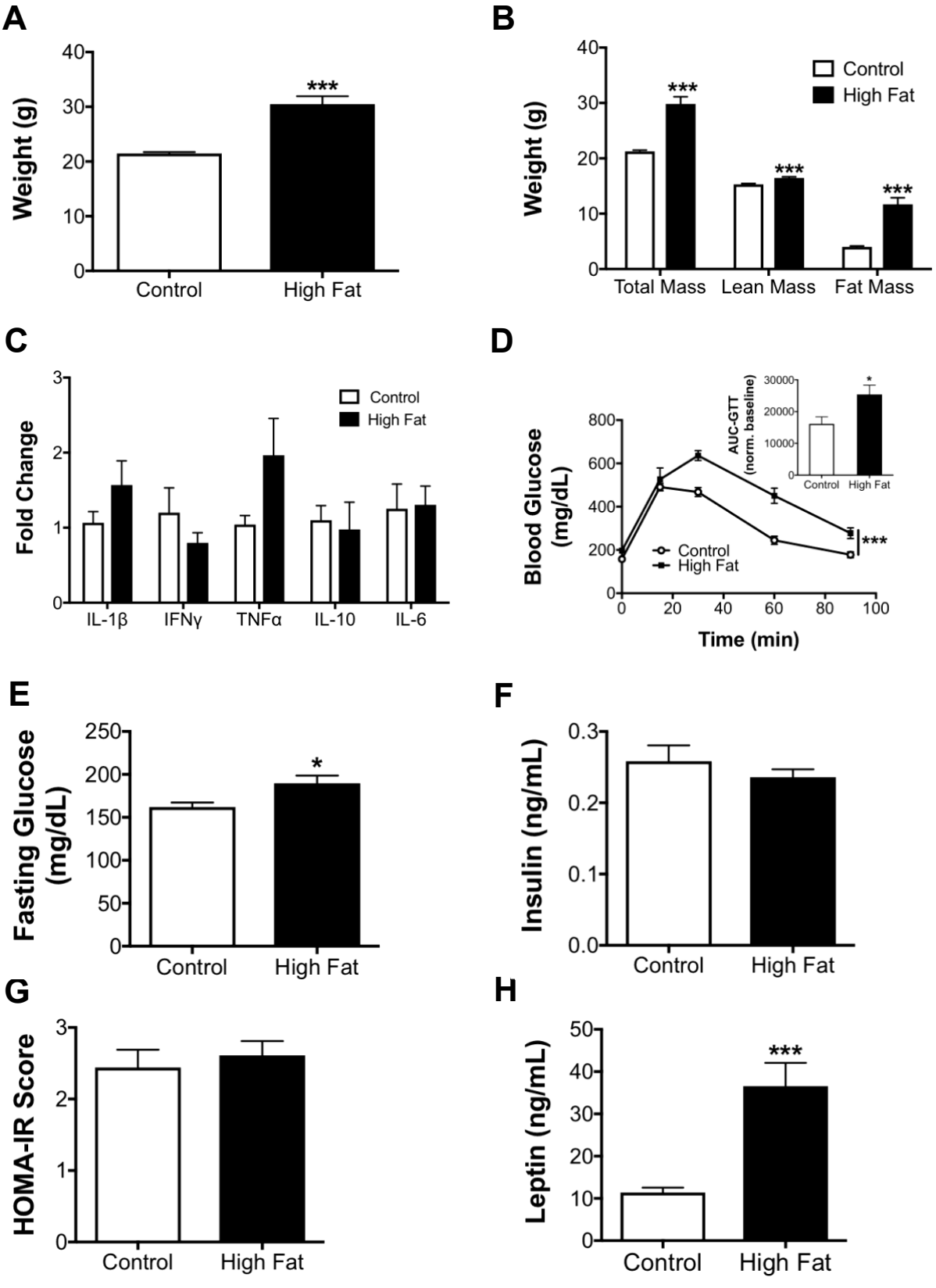

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Figure 2.6: Metabolic profile of female mice consuming a high fat diet. (A) Body weights of female mice at the completion of the 15-week study. (B) Body composition assayed by Echo-MRI. (C) qRT-PCR analysis of inflammatory markers from white adipose tissue of control and obese mice. (D) Glucose tolerance test (GTT), performed by intraperitoneal injection of glucose after a 6 hour fast. (inset) Area under the curve (AUC), calculated by integration of the curve normalized to baseline values. (E) Fasting blood glucose levels determined after a 6 hour fast. (F) Fasting insulin levels determined after a 6 hour fast. (G) HOMA-IR scores. (H) Fasting leptin levels. N=8-10 mice per diet (A-D), N=6-8 mice per diet (E-H). Data are average ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 by a Student's unpaired t-test (A-C, E-H) or a two-way ANOVA analysis followed by a post-hoc t-test (D).

Metabololipidomics revealed no difference in the levels of 14-HDHA or 17-HDHA between control and obese female mice (Supplemental Figure 2.3A). The levels of PDX, RvD1, and RvD5 were maintained in obese female mice (Supplemental Figure 2.3B). Experiments were also conducted to determine if antibody production and cytokine levels were impaired in response to obesity as previously reported in male mice (103). There was no change in murine *ex vivo* IgM or IgG production with the high fat diet upon stimulation with either CpG-ODN+anti-IgM or LPS (Supplemental Figure 2.3C). Furthermore, the splenic B cells of control and obese female mice showed no differences in the expression of inflammatory transcripts in the absence of activation (Supplemental Figure 2.3D).

Subsequent experiments tested if obesity impaired the percentage and number of select B cell subsets in the spleen and bone marrow of female C57BL/6J mice. Flow cytometry analysis revealed a modest decrease in the percentage of splenic IgM^+ memory B cells (Figure 2.7A) by 1.65-fold in obesity with no change in the number of splenic B cell subsets (Figure 2.7B). In the bone marrow, the percentage of $CD19^+$ cells was statistically lowered by 3.6% (Figure 2.7C); however, the number of $CD19^+$ cells (Figure 2.7D) was not influenced with obesity. Flow cytometry analysis of bone marrow B

cell populations revealed that female obese mice showed no differences in the percentages of B cell subsets in the bone marrow (Figure 2.7E). Female obese mice displayed a 1.62-fold decrease in the number of long-lived plasma cells in the bone marrow (Figure 2.7F).

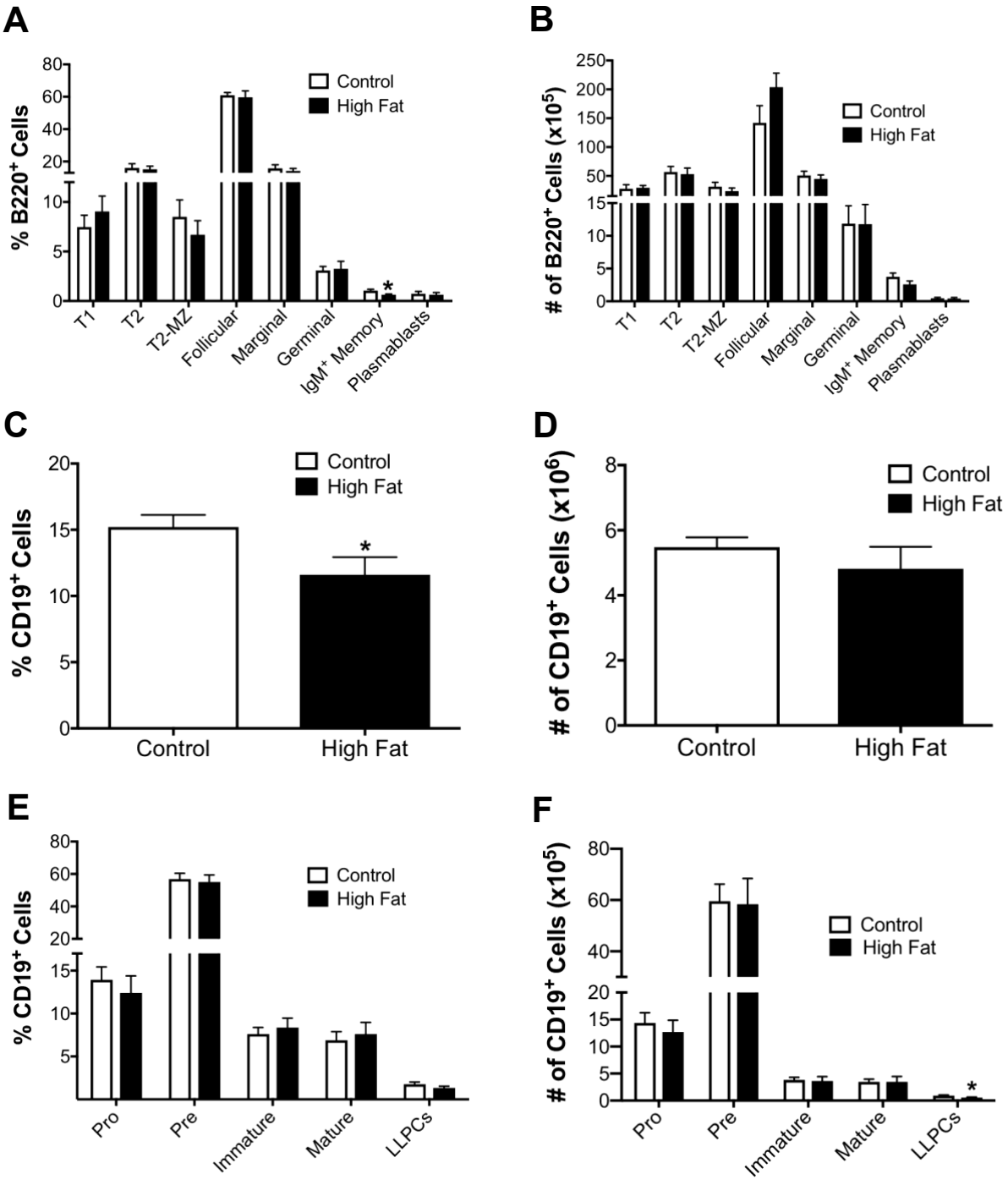

Figure 2.7: Obese female mice display small changes in splenic and bone marrow B cell subsets. (A) Percentages and (B) numbers of B cell subsets in the spleen of control and high fat female C57BL/6J mice. (C) Percentage and (D) number of total $CD19^+$ B cells in the bone marrow. (E) Percentages and (F) number of B cell subsets in the bone marrow. Mice were fed lean control or high fat diets for 15 weeks. N=7-8 mice

per diet (A, B) and N=9-10 for (C-F). Data are average ± S.E.M. *P<0.05 by a Student's unpaired t-test.

Obese female humans have increased percentage of $CD38^{+}IgD^{-}CD27^{+}$ B cells and elevated B cell cytokine secretion. We finally determined if female obese subjects, relative to non-obese controls, displayed impairments in circulating B cell populations as we have previously measured in males (103). Flow cytometry analysis of PBMC populations (Figure 2.8A) revealed no significant changes in the percentages of monocytes, helper T, or cytotoxic T cells (Figure 2.8B). In addition, percentages of total B cells were not influenced by obesity. Flow cytometry analysis (Figure 2.8C) of B cell subsets in obese females revealed a 5-fold increase in the percentage of plasma $CD19^{+}CD38^{+}IgD^{-}CD27^{+}$ B cells, but no changes in naïve or memory B cells, compared to non-obese subjects (Figure 2.8D).

B cells from female humans were also challenged *ex vivo* with CPG-ODN+anti-IgM and Pam3CSK4 (TLR1/2 agonist). Obese females had increased *ex vivo* B-cell TNFα and IL-10 secretion by 2.8-fold and 2.5-fold, respectively, when challenged with CPG-ODN+anti-IgM but not when challenged with PAM3CSK4 (Figure 2.8E). Activation of B cells with CPG-ODN+anti-IgM did not reveal any difference with IgM or IgG levels between obese and non-obese subjects (Figure 2.8F).

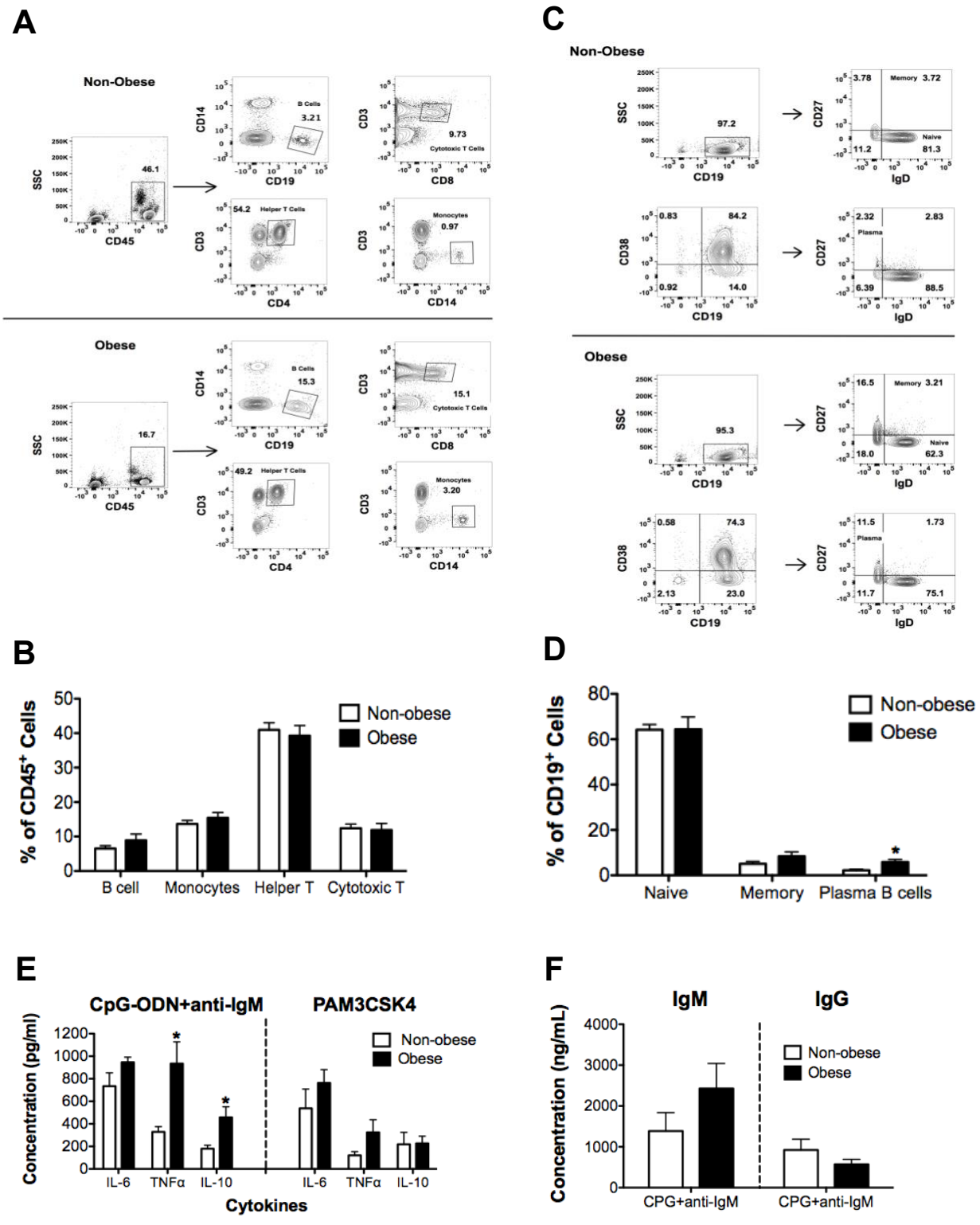

Figure 2.8: Obese females have increased levels of plasma B cells relative to non-obese females and elevated levels of select cytokines. (A) Sample flow cytometry data showing the percentage of $CD45^+CD14^-CD19^+$ (B cells), $CD45^+CD3^+CD8^+$ (cytotoxic T cells), $CD45^+CD3^+CD4^+$ (helper T cells), and $CD45^+CD3^-CD14^+$ (monocytes) in

peripheral blood mononuclear cells. (B) Quantification of the percentages of B cells, monocytes, helper T cells, and cytotoxic T cells. (C) Sample flow cytometry plots of memory ($CD19^{+}IgD^{+}CD27^{+}$) and plasma B cells ($CD19^{+}CD38^{+}IgD^{-}CD27^{+}$). (D) Quantification of the percentages of naïve, memory and plasma B cells in peripheral blood mononuclear cells. I *Ex vivo* B-cell cytokine secretion upon CpG-ODN + anti-IgM or PAM3CSK4 stimulation. (F) *Ex vivo* IgM and IgG concentrations upon stimulation of B cells with CpG-ODN+anti-IgM for non-obese and obese females. N=10 female human subjects per group for (A-D). N=9 female human subjects per group for (E-F). Data are average ± S.E.M. *P<0.05 by unpaired Student's t test.

DISCUSSION

The data from this study advance the fields of B cell immunology, lipid metabolism, and obesity by demonstrating that: 1) 14-HDHA, 17-HDHA, and PDX are decreased in male obese mice and that administration of these molecules rescues some of the reductions in differing B cell subsets and IgG2c, which is pathogenic in obesity; 2) the underlying reduction in 17-HDHA and 14-HDHA levels and thereby dysregulated B cell subsets are not due to a defect at the level of the B cell or driven by a loss of dietary DHA; 3) B cells can generate SPM precursors; 4) female obese mice have no change in levels of SPMs and their precursors accompanied by small to modest impairments in splenic and bone marrow B cell populations, and 5) obese female mice and humans show no defects in *ex vivo* antibody production although humans have elevated plasma cells and some elevated B cell cytokines upon BCR/TLR9 stimulation.

The results suggest a complex model by which several cellular and molecular factors regulate SPM precursor levels and thereby B cell populations in the spleen and bone marrow of male mice. Strikingly, DHA and its precursor ALA, which regulate metabolic responses, were not lowered in obese male mice (262). This demonstrated that a reduction in dietary DHA or ALA levels is not driving the reduction in murine SPM precursors. This may not hold true in humans given that essential and conditionally-essential fatty acid deficiencies exist with obese subjects, which could lead to impaired SPM pathway metabolites (241–243).

DHA in membrane phospholipids is liberated and subsequently hydroxylated by LOX enzymes to generate SPM precursors. B cell 12/15- and 5-LOX transcripts were lowered in obese male mice suggesting a potential defect at the level of the B cell.

However, we found no evidence that B cell production of 17-HDHA or 14-HDHA could account for the loss of SPMs. Furthermore, experiments with ALOX5$^{-/-}$ mice showed that a defect in this enzyme was not responsible for the reduction in splenic and bone marrow B cell subsets. We did not pursue experiments with ALOX12/15$^{-/-}$ mice since it is reported that some B cell populations actually increase upon the loss of B cell 12/15-LOX (259).

Future studies will need to focus on how other key cells in the spleen such as macrophages, monocytes, neutrophils, and T cells are contributing toward lowering of DHA-derived mediators (238,258,263). In the bone marrow, mesenchymal stromal cells can produce SPMs, which could contribute toward reduced levels of SPM precursors (264). It is also possible that reduced levels of SPMs in obese mice are limiting leukocyte trafficking and signaling to primary and secondary organs such as the bone marrow and spleen, respectively (265–267). The discovery that SPM precursors were decreased in the spleen and bone marrow of obese mice was highly consistent with data demonstrating diminished SPMs in mouse and human adipose tissue depots (210,255).

Select splenic n-6 PUFAs were elevated several fold compared to DHA, which modeled how humans consume considerably larger quantities of n-6 to n-3 fatty acids (268). Linoleic acid was dramatically increased relative to DHA in obese male but not female mice. This was driven by the high levels of linoleic acid present in the mouse diet. LOX prefers n-3 PUFAs if n-6 PUFA abundance is relatively low; however, linoleic acid is more likely to bind 12/15-LOX when it is in excess (141,260). As a result, DHA likely did not serve as the primary substrate for the LOX with differing cell types, resulting in less hydroxylation and subsequent generation of 14-HDHA and 17-HDHA. In addition, the consistently high levels of n-6 PUFAs could prevent lipid mediator class-switching to n-3-

derived SPMs in obesity, which is needed to promote resolution of the inflammatory response.

The finding that SPM precursors increased B cell population numbers in the bone marrow and $B220^{+}IgM^{+}CD19^{+}GL7^{+}$ germinal center B cells of obese mice strongly support the emerging notion that SPMs are critical for humoral immunity. For instance, germinal center B cells are an essential source for the development of plasma and memory B cells, which both provide protection upon exposure to pathogens (80,269). In addition, it was recently reported that splenic B cells from obese/type 2 diabetic mice administered *S. aureus* displayed inadequate class switching (102). Therefore, it is possible that SPMs are aiding the process of somatic hypermutation in the germinal center of obese mice, which produces highly specialized plasma and memory B cells (215). We did not measure a change in the production of plasma and memory B cells, or in the number of IgG^{+} memory B cells. Subsequent experiments will need to address this using different antigens that will stimulate differing B cell subsets including germinal center, plasma, and IgG^{+} memory B cells. Furthermore, we will need to further investigate the flow cytometry data with immunohistochemistry analyses of the spleen. We also did not determine if the increase in B cell populations with the cocktail was due to increased trafficking of B cells into differing tissues such as the adipose or driven by enhanced proliferation. Future studies will also need to dissect which lipid mediator is rescuing each B cell subset. It is plausible that each lipid mediator has unique effects on each B cell subset.

A notable finding was that administration of 14-HDHA/17-HDHA/PDX rescued the obesity-induced elevation in IgG2c. The increase in IgG2c was highly consistent with

previous work by Winer et al., to show that pro-inflammatory IgG2c is elevated in obesity (89). The ability of the DHA-derived mediators to rescue this was notable as these molecules are highly potent immunoresolvants (238). The DHA-derived mediators also increased other IgG subclasses, providing evidence that SPMs regulate antibody production (216). These results open the door to investigating the role of DHA and its metabolites on B cell class switching.

Females generally exhibit a stronger humoral and cellular immune response after infection as well as improved vaccination compared to males during premenopausal years (247,270). Our findings, compared to our previous work, significantly expand on this notion by establishing that female obese mice did not display large reductions in B cell populations of the bone marrow and spleen (103). Similarly, obese women had increased numbers of plasma B cells relative to their controls, differing from obese men (103). Females are known to have elevated B cell numbers compared to males (271). Therefore, obese females could have increased B cell pools in the bone marrow and spleen resulting in a constant flux of B cells, which maintains adequate B cell numbers.

There are likely several mechanistic factors that could explain why female mice did not show the same results as the males. The females displayed no deficiencies in 14-HDHA, 17-HDHA, PDX, or RvD1 with obesity. It is reported that overall synthesis of DHA is higher in women than men and is independent of dietary differences, offsetting the increased flux of n-6 PUFAs present in a high fat diet (272–275). Furthermore, the capacity to generate DHA from the parent n-3 PUFA, ALA, is higher in women than men suggesting that DHA could be converted to downstream mediators more efficiently in obese females than obese males. The results open a new area study; that is, investigating

differences in fatty acid and SPM metabolism between sexes in the context of obesity, which could have strong implications for the use of drugs that target PUFA metabolic pathways. For instance, Pace et al., recently demonstrated that females are more responsive to leukotriene biosynthesis inhibitors than males due to differences in androgens between sexes (276). Similarly, there are data to show that female humans are protected from inflammation induced endothelial impairments due to elevated levels of specific SPMs (277).

The males and females had different metabolic profiles, which would influence the levels of SPM precursors. Notably, male mice tended to weigh more than the females after administering the diets for the same period of time although the amount of fat mass was comparable between sexes. Furthermore, the obese males had increased insulin levels and elevated adipose tissue inflammatory gene expression compared to females, which would then influence glucose uptake. Finally, obese females may have protective B cell responses due to the effects of estrogen and the increased production of IL-10. For instance, estrogen increases somatic hypermutation and class switching recombination resulting in high affinity-Ig producing cells in females (246,278,279).

Collectively, the data establish that a reduction in SPM pathway metabolites synthesized from DHA contribute toward some of the impairments in the proportion of B cell subsets and antibody production of obese male, but not female mice. Mechanistically, the defect is not at the level of the B cell or due to a decrease in the levels of DHA and may be due extrinsic factors such elevated levels of n-6 fatty acids. The results set the foundation for future studies on underlying metabolic factors that may drive abnormal B

cell responses in obesity through changes in DHA metabolism in male obese mice (271)

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CHAPTER 3: DHA-DERIVED METABOLITES LOWER B CELL ACCUMULATION IN VISCERAL ADIPOSE TISSUE OF OBESE MALE MICE AND DECREASE ANTIBODY LEVELS

INTRODUCTION

Obesity negatively impacts B cell-driven humoral immunity (26,84,101,222,229). For instance, B cells from obese individuals have impaired function due to decreased AID levels in response to stimulation with antigen (101). Furthermore, B cell cytokine secretion is impaired in both obese mice and humans as these B cells secrete more pro-inflammatory cytokines (IL-6) and fewer anti-inflammatory cytokines (IL-10) (101,131,280). In addition, B cells have emerged as major contributors to obesity-induced inflammation and insulin resistance in the visceral adipose tissue (88). B cells from mice consuming high fat diets infiltrate the VAT in higher numbers compared to lean animals, secrete pro-inflammatory cytokines that can directly regulate macrophages and T-cells in the VAT, and produce increased amounts of IgG antibody that contribute to obesity-associated insulin resistance (IR) (88,89). Thus, establishing mechanisms that are driving impaired B cell phenotypes in tissues such as the adipose are limited.

Recent studies from our lab and others suggest insufficient levels and production of specialized pro-resolving lipid mediators could be a potential mechanism driving impaired B cell phenotypes in various tissues (281,214). Specialized pro-resolving lipid mediators (SPMs) are bioactive metabolites that are primarily produced from polyunsaturated fatty acids such as arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (162). SPMs prevent the vicious cycle of chronic

inflammation by exerting dual anti-inflammatory and pro-resolution properties to achieve timely resolution of the immune response (265). Several tissues that B cells home to display decreased levels of SPM precursors and SPMs (103,159,210,211,281,282). Our lab showed that mice consuming a high fat diet for 15 weeks had reduced levels of the SPM precursors, 14-HDHA and 17-HDHA along with the SPM, PDX in the spleen compared to their lean counterparts (281). Furthermore, our studies revealed 14-HDHA was decreased in the bone marrow of obese mice (281). Several studies have also shown that the VAT from obese mice and humans has decreased levels of SPM precursors and SPMs and elevated levels of pro-inflammatory lipid mediators such as the AA-derived prostaglandins (PGs), LTB4, and its receptor BLT-1 (206,208,207,209,159,210,214). Therefore, decreased levels of SPMs and increased levels of pro-inflammatory mediators are likely contributing to the underlying low-grade inflammatory tone in the obese adipose tissue and the inability to actively resolve inflammation.

SPMs directly alter B cell function, antibody class switching, and differentially affect B cell subsets in various tissues and disease models (283). The DHA-derived SPM precursor, 17-HDHA increases IL-10 production, the number of plasma cells, and overall antibody production in B cells from lean mice challenged with CpG plus α-IgM (284). In addition, 17-HDHA enhanced the antigen-specific antibody response in a pre-clinical influenza immunization mouse model. SPMs can also differentially affect B cell subsets and class switching (284). For example, treatment of human memory B cells with LXA4 decreased antibody production, whereas 17-HDHA and RvD1 promote the differentiation of IgG-secreting B cells but inhibit IgE production in human B cells (217,218). Our lab has recently shown that the SPM cocktail 14-HDHA/17-HDHA/PDX can rescue reduced B cell

numbers in the bone marrow of obese mice and lower IgG2c levels in circulation (281). In addition, we showed that administration of a single injection of 14-HDHA in parallel with influenza in lean mice can boost HAI titers as well as the percentage of long-lived plasma cells in the bone marrow (103).

Despite this evidence, whether SPMs can target B cell recruitment and modulate B cell function in the obese adipose tissue is not known. The objective of this study was to determine if administration of select DHA-derived SPM precursors and SPMs that are reduced in obesity could modulate B cell recruitment and B cell subset numbers in the adipose tissue. Overall our study provides the first evidence that DHA-derived metabolites decrease B cell recruitment and antibody levels in the obese VAT. As B cells have pathogenic roles in the VAT, we propose that DHA-derived metabolites can modulate this phenotype and boost B cell function in the obese population (88,89,131,206,285).

MATERIALS AND METHODS

Mice. All murine experiments fulfilled the guidelines established by The University of North Carolina at Chapel Hill for euthanasia and humane treatment. Male C57BL/6J mice of approximately 17-19 weeks of age were purchased from Jackson Laboratories. Mice were fed control (10% of total kcal from lard) or high fat (60% of total kcal from lard) diets (Research Diets, New Brunswick, NJ) at Jackson Laboratories and were fed the same diet upon delivery for 1-3 weeks. Mice were sacrificed between 19-21 weeks of age.

Administration of SPMs. SPM precursors and SPMs were injected as previously described in Chapter 2. Briefly, 14(S)-HDHA (0.1mg/mL), 17(R)-HDHA (0.1mg/mL), and

Protectin DX (0.1mg/mL) were purchased from Cayman Chemicals (Ann Arbor, MI). SPMs were prepared in ethanol in the dark and kept on ice at all times. The final cocktail concentration was 900ng per mouse (300ng of each SPM precursor and SPM) in PBS or vehicle control (ethanol in PBS) and was administered i.p. to lean and obese mice for 4 consecutive days followed by euthanasia on day 5. Control mice were administered a vehicle control.

Metabolic studies. Echo-MRI for fat and lean mass was conducted as previously described (281). Briefly, mice were fasted for 6 hours. Baseline glucose values were then established with a glucometer. Each animal received an intraperitoneal injection (i.p.) of 2.5g of dextrose (Hospira Inc., Lake Forest, IL) per kg lean mass. Throughout a 90-minute interval, glucose measurements were collected from the tail vein of each mouse. An insulin ELISA kit (Abcam) was used to calculate the fasting insulin levels using blood collected from the tail vein of each mouse. Finally, HOMA-IR index was calculated as previously described (249).

VAT Digestion and SVF Isolation. Approximately 1g of epididymal adipose tissue was isolated from each mouse. For the control mice, 2-3 mice were pooled to achieve 1g of tissue, whereas only one mouse was used for the high fat mice. Upon extraction from the mouse, adipose tissue was placed in digestion buffer (0.5% BSA in 1x PBS). Tissue was blotted and then weighed. Tissue was placed in the weigh boat with 2 mL of digestion buffer. The tissue was minced into small pieces using scissors. This mixture was poured into a 15 mL falcon tube and the weigh boat was rinsed with 1-2 mL of

digestion buffer to reach a total of 5 mL volume. If the VAT weighed more than 2g, the mixture was split into two 15mL falcon tubes with approximately 5mL volume in each tube. 0.5 mL of Collagenase Type IV (Gibco) at a stock concentration of 10mg/mL was added to the mixture to achieve a final concentration of 1mg/mL. The mixture was then incubated for 60 minutes at 37*C accompanied by shaking at 150 RPM. The mixture was vortexed every 10 minutes throughout this incubation period. Following the incubation period, 100 μL of 0.5M EDTA (final concentration of 10 mM) and 5mL of cold RPMI complete media (with FBS) was added to the tube. The mixture was filtered through a 70 μM filter into a new 50 mL canonical tube and rinsed with 3 mL of cold 2% FBS in 1x PBS. The tube was centrifuged at 1400 RPM for 5 minutes. Using a P1000 pipet tip with the end cut off, the fat layer at the top was slowly aspirated leaving 2-3 mL of the mixture. The remaining supernatant was poured off. 1mL of ACK lysis buffer was added next to the tube followed by a 1-minute incubation period on ice and in the dark. 2% FBS in 1x PBS was added up to 10 mL for a wash step. The adipose tissue mixture was centrifuged at 1400 RPM for 5 minutes. The supernatant was poured off and resuspended in 10 mL of complete media. The cells were then counted using a hemocytometer.

Isolation of bone marrow and spleen. The right leg of the mouse was used for bone marrow isolation and the same protocol used in Chapter 2 was followed here. The spleen was isolated as previously described in Chapter 2.

Flow cytometry for adipose, bone marrow, and spleen. Flow cytometry on the bone marrow and spleen was performed as described previously in Chapter 2. For the adipose, 3×10^5 stromal vascular cells were plated in a 96-well U-bottom plate. Cells were washed once with PBS followed with a 10-minute incubation in the dark with FcR blocker in 0.5% BSA + 1x PBS. Cells were washed thoroughly once. Following the wash, the cocktails for all three tissues were added to each well. 0.4 μg of each antibody was added to the appropriate cocktail. Cells were incubated for 25 minutes on ice and in the dark. Cells were washed 3 times with 1X PBS and were resuspended in a final volume of 200 μL of 1X PBS. The plates were run immediately on a LSRII flow cytometer. The total number of B cells in the spleen were analyzed on the flow cytometer by gating on B220+ cells. The antibody, B220 (FITC) was purchased from Biolegend. The following subsets were analyzed in the bone marrow: Pro – $CD19^+$ IgM^- $CD24^+$ $CD43^-$, Pre – $CD19^+$ IgM^- $CD24^+$ $CD43^+$, Immature – $CD19^+$ IgM^+ IgD^-, Mature – $CD19^+$ IgM^+ IgD^+, CD138 LLPCs – $CD19^+$ $CD138^+$. All antibodies used were the same as those described in Chapter 2.

The following subsets were analyzed in the adipose: B-1a – $CD19^+$ IgM^+ IgD^- $CD5^-$, B-1b – $CD19^+$ IgM^+ IgD^- $CD5^+$, B-2 – $CD19^+$ $B220^{high}$ $CD5^-$, classically activated (M1) macrophages – $CD11b^+$ $CD11c^+$ $Ly6C^{high}$, alternatively activated (M2) macrophages – $CD11b^+$ $CD11c^-$ CD206. The following antibodies were used in the study: CD19 (PerCP-Cy5.5), IgM (PE), IgD (Pacific Blue), B220 (FITC), CD5 (APC), CD206 (FITC), CD11b (APC), CD11c (PE), Ly6C (Pacific Blue), Zombie (NIR). All antibodies were purchased from BioLegend with the exception of CD19 and IgD, which were purchased from BD eBiosciences.

VAT ELISAs. 0.07g of frozen epididymal visceral adipose tissue was homogenized in 1 mL of 1X RIPA buffer supplemented with 10 μL of Phosphatase/Protease inhibitor. Samples were placed on ice at all times when they were not being homogenized. Samples were then sonicated for approximately 90 seconds in microcentrifuge tubes (5, 10 second pulses with 10 second rests in between on ice, pulse at 20%). The homogenized samples were centrifuged at maximum speed for 10 minutes at 4*C. The supernatants were collected, transferred to another tube, and used immediately or put in the -80 for storage. Samples were then used for ELISAs for the following immunoglobulins: IgM, IgG, IgG2b, IgG2c, IgG3, and IgA (eBiosciences).

Lipidomics. All standards and techniques used for metabololipidomics are as previously described in Chapter 2. However, epididymal visceral adipose tissue was used for sample extraction.

qRT-PCR. Total RNA was isolated from epididymal visceral adipose tissue from mice in control, obese, and obese mice injected with SPM precursors and SPM using the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA). RNA was also extracted from sorted $B220^+$ splenic B cells and $CD19^+B220^+$ bone marrow B cells. RNA was reverse transcribed and amplified by using a One-Step SYBR green PCR Mix (Biorad). The fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. β-actin was the housekeeping genes used in all PCR experiments. The primer sets used in the study are presented in Supplemental Table 3.1.

Statistics. All data from murine experiments are from multiple cohorts of mice. Data were analyzed using Graph Pad Prism Version 7.0. Murine experiments involving SPM injections were analyzed using a one-way ANOVA followed by a post-hoc Bonferroni multiple comparisons test. Most data sets displayed normalized distribution as determined by a Kolmogorov-Smirnov test. Those sets that did not display normal distributions were analyzed with a Kruskal-Wallis test followed by a Dunn's multiple comparison test. Metabolic studies as a function of time were analyzed with a two-way ANOVA. For all analyses, $p<0.05$ was considered statistically significant.

RESULTS

Metabolic profile of lean, obese, and SPM-administered mice. We first established the metabolic phenotype of mice for each experimental group. Obese mice injected with and without 14-HDHA/17-HDHA/PDX had elevated fat mass relative to the control group (Figure 3.1A). Furthermore, administration of 14-HDHA/17-HDHA/PDX to obese mice did not improve glucose tolerance as both obese groups had impaired glucose clearance (Figure 3.1B) as quantified by the area under the curve (Figure 3.1B, inset). In addition, DHA-derived metabolites did not improve fasting glucose levels as both obese groups had elevated fasting glucose relative to the control group (Figure 3.1C). However, the administration of 14-HDHA/17-HDHA/PDX greatly affected the insulin response. This was evident as obese mice had a 2.5-fold increase in fasting insulin levels, which was significantly reduced in obese mice receiving the SPM precursors and SPM cocktail (Figure 3.1D). Obese mice receiving the cocktail had a 2-fold decrease in the HOMA-IR score, a measurement of insulin sensitivity (Figure 3.1E).

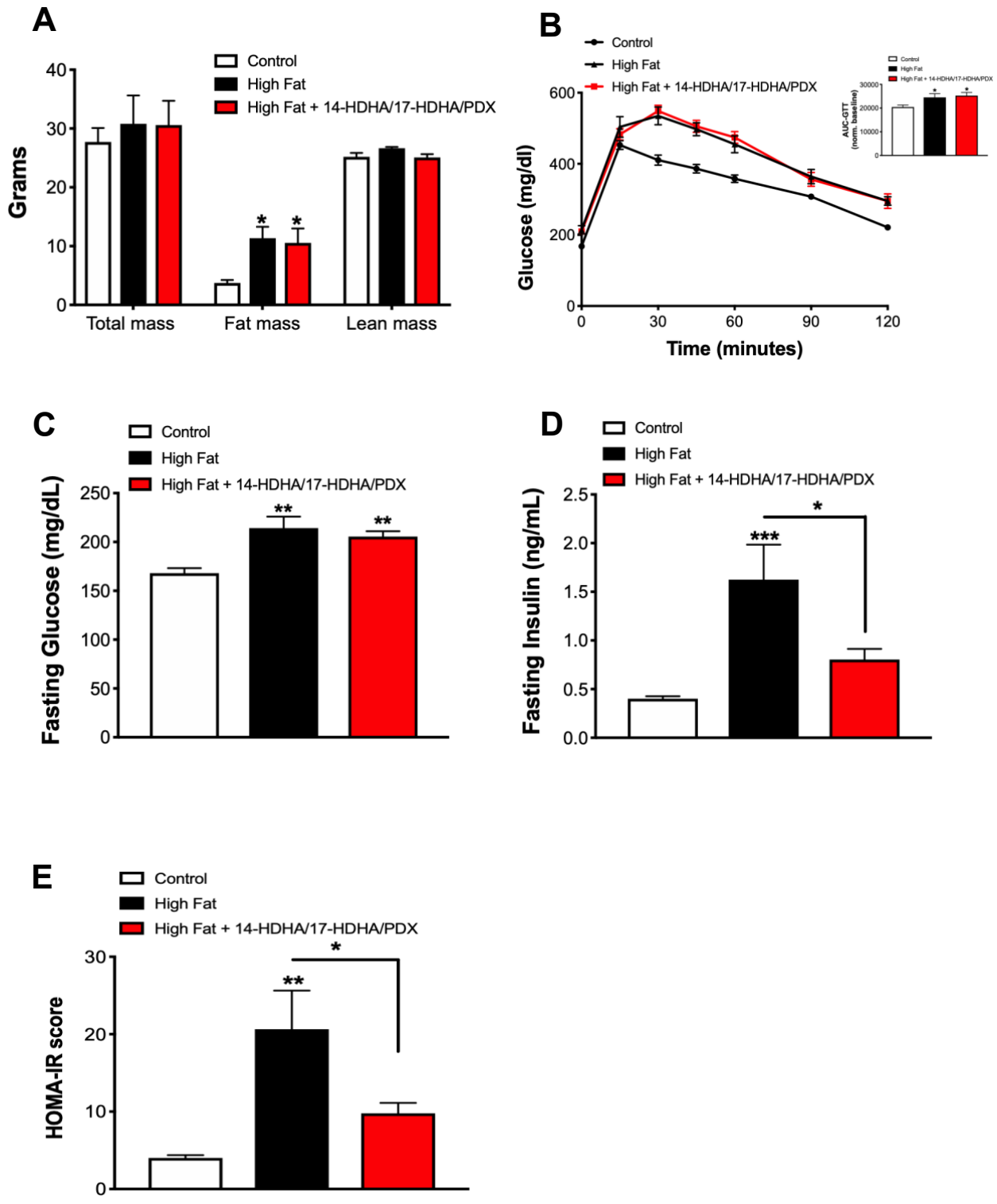

Figure 3.1: Metabolic profile of lean, obese, and SPM-administered mice. (A) Body composition of mice in each group assayed by Echo-MRI. (B) Glucose tolerance test (GTT), performed by intraperitoneal injection of glucose following a 6-hour fast (inset) Area under the curve (AUC), calculated by integration of the curve normalized to baseline values. (C) Fasting blood glucose levels after a 6 hour fast. (D) Fasting insulin levels after

a 6-hour fast. (E) HOMA-IR scores of mice in each group. N=5-6 mice per diet (A-E). Control and one obese group received vehicle control injections, whereas the other obese group received a 14-HDHA/17-HDHA/PDX injection once a day for four consecutive days. Data are average ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 by a two-way ANOVA followed by a Bonferroni post-test.

DHA-derived metabolites decrease the number of pro-inflammatory macrophages ($CD11b^+$ $CD11c^+$ $Ly6C^{high}$) and increase IL-10 expression in the adipose. SPMs have emerged as a major regulator of macrophages during the immune response. Several studies have reported that SPMs promote an alternatively activated "M2" phenotype (286–288). Therefore, we wanted to determine if our SPM precursors and SPM cocktail could promote a M2 phenotype ($CD11b^+$ $CD11c^-$ $CD206^+$) in obese male mice. Flow cytometric analysis of the macrophages in the SVF of the adipose tissue (Figure 3.2A) revealed a trending increase in the percentage of pro-inflammatory, classically activated macrophages ($CD11b^+$ $CD11c^+$ $Ly6C^{high}$) in both obese groups compared to the control group (Figure 3.2B). In addition, there was a trending decrease in the percentage of anti-inflammatory, alternatively activated macrophages ($CD11b^+$ $CD11c^-$ $CD206^+$) in the obese group that did not receive the SPM precursors and SPM cocktail. Interestingly, obese mice had a 2.5-fold increase in the number of pro-inflammatory, classically activated macrophages ($CD11b^+$ $CD11c^+$ $Ly6C^{high}$), which was significantly decreased in obese mice that received the SPM precursors and SPM cocktail for 4 consecutive days (Figure 3.2C). There were no significant changes in the number of alternatively activated macrophages ($CD11b^+$ $CD11c^-$ $CD206^+$) among the three groups (Figure 3.2C).

We also examined the transcript expression of pro-inflammatory cytokines and anti-inflammatory cytokines that are secreted by macrophages and other immune cell

types in the adipose tissue. Obese mice had a 2.5-fold increase in the levels of TGF-β, which was significantly reduced in obese mice receiving the 14-HDHA/17-HDHA/PDX cocktail (Figure 3.2D). Obese mice administered the SPM cocktail had trending increases in TNF-α, but no significant differences in the transcript expression of IL-13. Obese mice administered the DHA-derived metabolites displayed a 10-fold increase in IL-10 transcript expression relative to the control group (Figure 3.2D). Thus, our results suggest that administration of 14-HDHA, 17-HDHA, and PDX reduces the number or pro-inflammatory macrophages ($CD11b^{+}$ $CD11c^{+}$ $Ly6C^{high}$) in the VAT and increases IL-10 production.

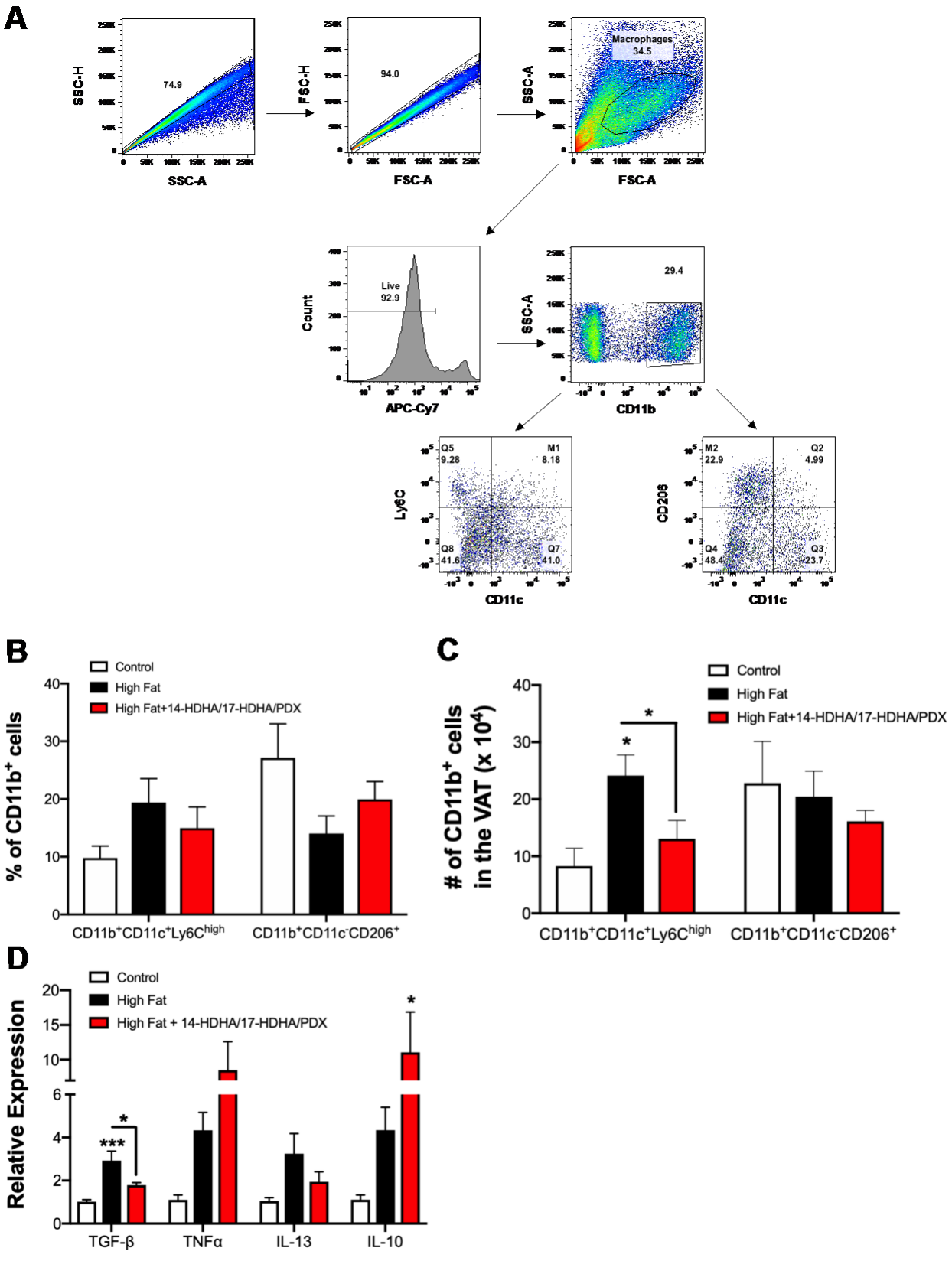
A
SSC-H
SSC-A
74.9
FSC-H
FSC-A
94.0
SSC-A
FSC-A
Macrophages
34.5
Count
APC-Cy7
Live
92.9
SSC-A
CD11b
29.4
Ly6C
CD11c
Q5
9.28
M1
8.18
Q8
41.6
Q7
41.0
CD206
CD11c
M2
22.9
Q2
4.99
Q4
48.4
Q3
23.7
B
% of CD11b+ cells
Control
High Fat
High Fat+14-HDHA/17-HDHA/PDX
CD11b+CD11c+Ly6Chigh
CD11b+CD11c-CD206+
C
# of CD11b+ cells in the VAT (x 10^4)
Control
High Fat
High Fat+14-HDHA/17-HDHA/PDX
CD11b+CD11c+Ly6Chigh
CD11b+CD11c-CD206+
D
Relative Expression
Control
High Fat
High Fat + 14-HDHA/17-HDHA/PDX
TGF-β
TNFα
IL-13
IL-10

Figure 3.2: DHA-derived metabolites decrease the number of pro-inflammatory, classically activated macrophages (CD11b^{+} CD11c^{+} Lys6C^{high}) in the adipose of obese mice. (A) Flow cytometry gating strategy of both classically activated M1 macrophages (CD11b^{+} CD11c^{+} Ly6C^{high}) and alternatively activated M2 macrophages (CD11b^{+} CD11c^{-} CD206^{+}) in the visceral adipose tissue. Percentage (B) and number (C) of both classically and alternatively activated macrophages in the visceral adipose tissue of control, obese, and obese mice receiving the SPM precursor and SPM cocktail. (D) qRT-PCR analysis of select inflammatory genes in the VAT of mice in all three groups. Animals were fed a control or high diet for 15-17 weeks. Mice consuming a high fat diet received 14-HDHA/17-HDHA/PDX injections once a day for four consecutive days. Control and the obese group without SPMs received vehicle control injections once a day for four consecutive days. N=5-7 (B-D). Data are average ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 by a one-way ANOVA followed by a Bonferroni post-test.

Administration of 14-HDHA/17-HDHA/PDX limits B cell recruitment and decreases antibody levels in the VAT. As administration of DHA-derived metabolites decreased the number of pro-inflammatory macrophages in the obese adipose tissue, we wanted to determine if they could also alter B cell numbers in the obese adipose tissue. Several labs have shown that the number of B cells are increased in the adipose tissue of obese mice (89,289). In addition, obese adipose tissue has increased immune cell infiltration compared to learn mice (88,290). Our data revealed that mice consuming a HFD had increased numbers of immune cells in the SVF compared to mice consuming a control diet (Figure 3.4A). Obese mice receiving the SPM precursors and SPM cocktail had a 1.5-fold reduction in the total number of immune cells in the SVF relative to the obese group (Figure 3.4A). Flow cytometry analysis of the CD19^{+} B cell population in the SVF (Figure 3.4B) revealed that obese mice had very robust increases in the percentage (Figure 3.4C) and number (Figure 3.4D) of B cells, which were decreased to that of the control group in obese mice administered the 14-HDHA/17-HDHA/PDX cocktail (Figure 3.4B-C). Flow cytometric analysis (Figure 3.3A) of B cell subsets in the adipose tissue revealed that obese mice had a 3-fold increase in the percentage of B2 cells in the VAT,

compared to the control group (Figure 3.4E). Obese mice that received that 14-HDHA/17-HDHA/PDX cocktail had a 2-fold decrease in the number of B2 cells in the VAT relative to the obese group that received vehicle control (Figure 3.4F).

We have previously shown that splenic B cells from mice consuming a high fat diet were hyperstimulated upon no antigen challenge. In particular, supernatants from these obese mice displayed increased levels of IgM and IgG compared to their control counterparts. As a result, we wanted to determine if obese mice had increased IgM and IgG levels in the whole adipose and whether administration of SPM precursors and SPM to the obese mice could lower these elevated antibody levels. Our results revealed that obese mice had significantly elevated levels of IgM and IgG relative to the control group in the whole adipose (Figure 3.4G). Obese mice that received that 14-HDHA/17-HDHA/PDX cocktail had significantly reduced concentrations of IgM and IgG relative to the obese group (Figure 3.4G).

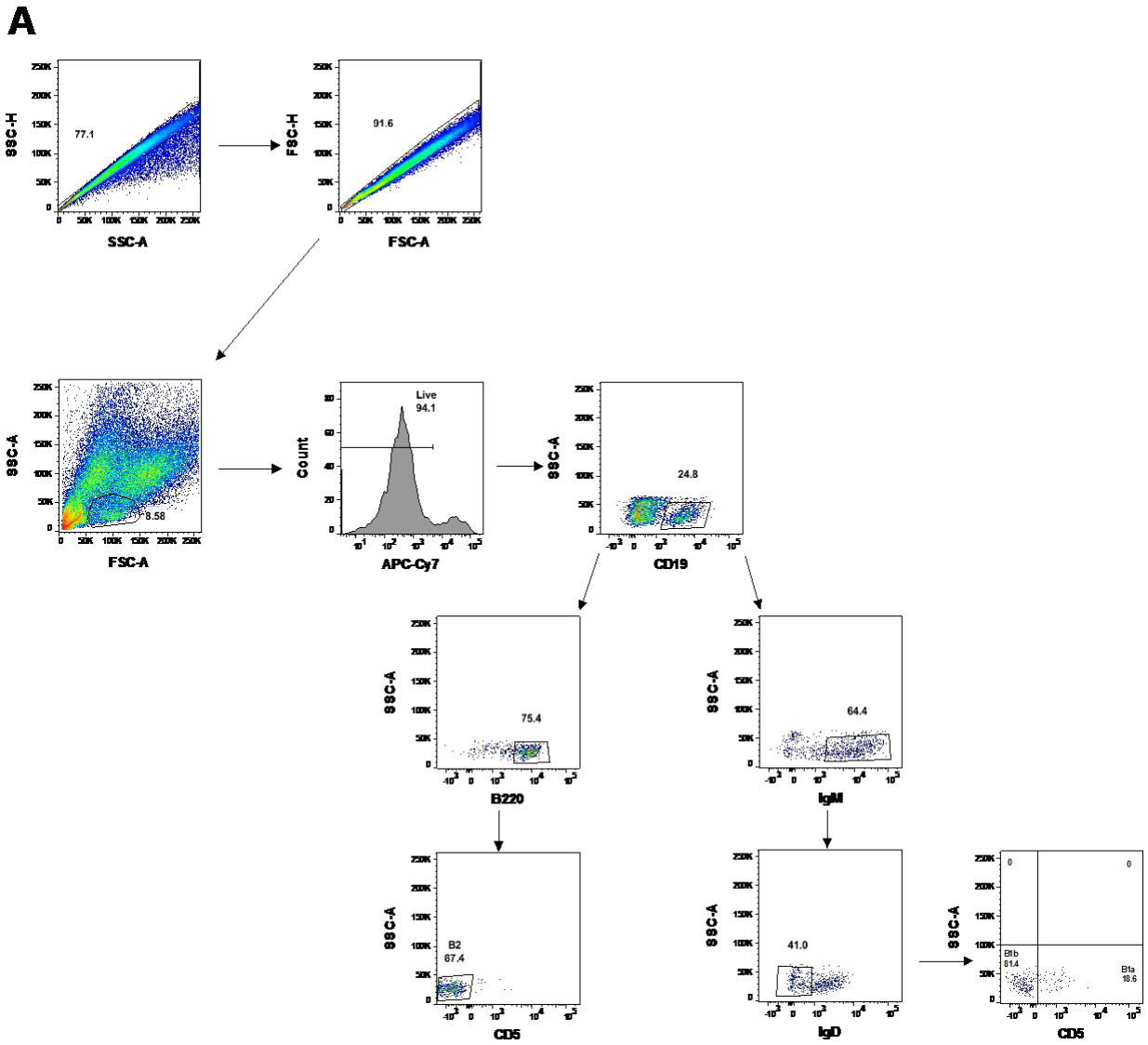

Figure 3.3: Gating strategy for B cell populations in the visceral adipose tissue. (A) Flow cytometry gating strategy for B-1a, B-1b, and B2 cells in the visceral adipose tissue.

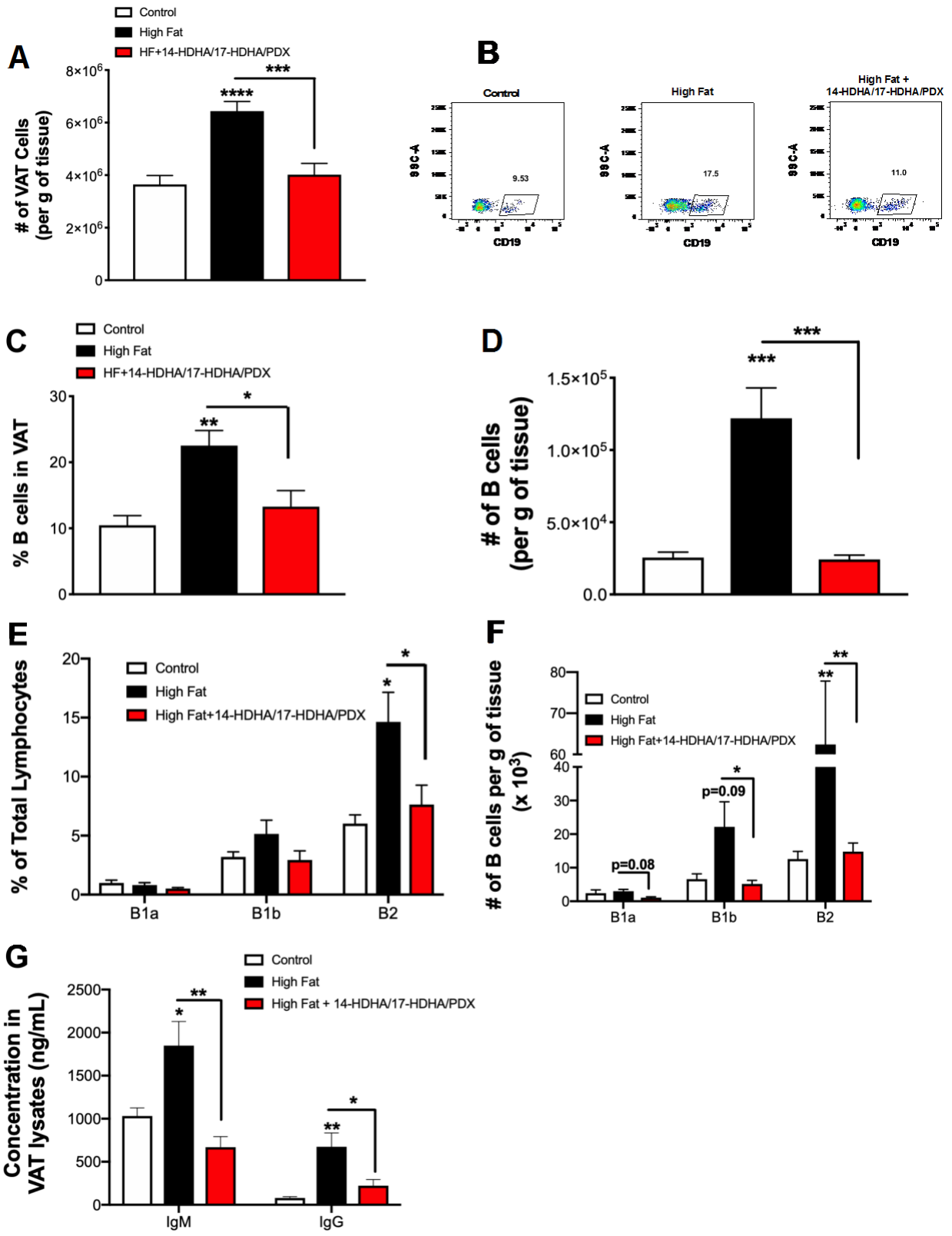

Figure 3.4: Administration of 14-HDHA/17-HDHA/PDX decreases the number of total B cells, B-1b, and B2 cells in the adipose tissue, which are elevated in obese

mice. (A) Total number of cells in the stromal vascular fraction across all three groups. (B) Raw flow cytometry plots for the $CD19^{+}$ population in the VAT. Percent (C) and number (D) of total B cells in the VAT. (E) Percentage of B cell subsets in the lymphocyte pool in control, obese, and obese mice receiving the SPM precursors and SPM injection. Number (F) of B cell subsets in the VAT calculated per g of tissue homogenized and used in the study. VAT lysate concentrations (D) of IgM and IgG. ELISAs were performed on VAT lysates that were extracted from 0.07g of frozen adipose tissue. Animals were fed a control or high diet for 15-17 weeks. Mice consuming a high fat diet received 14-HDHA/17-HDHA/PDX injections once a day for four consecutive days. Control and the other obese group received vehicle control injections once a day for four consecutive days. N=7-9 (A-D), N=5-6 (E-F), N=7-14 (G). Data are average ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 by a one-way ANOVA followed by a Bonferroni post-test.

Administration of DHA-derived metabolites decreases transcript expression of chemokine receptors involved in B cell recruitment to the adipose tissue.

Obesity increases the expression of chemokines and their receptors, which are involved in immune cell recruitment to the adipose tissue (291,292). Therefore, we tested whether administration of DHA-derived metabolites influences the expression of various chemokines and their receptors that are involved in the recruitment of immune cells including B cells to the VAT. Our results showed that obese mice in both groups had elevated transcript levels of MCP-1 and CCL5, which exacerbate pro-inflammatory conditions and mediate insulin resistance in obesity (Figure 3.5A) (293–296). Interestingly, both obese groups had decreased transcript expression of MCP-2 in the adipose tissue (Figure 3.5A). While CXCL10 (IP-10) has been shown to be elevated in obesity, our obese mice had a 2.5-fold decrease in transcript levels of CXCL10, which was rescued in obese mice administered the SPM precursors and SPM cocktail (Figure 3.5A). Obese mice had increased expression of all corresponding chemokine receptors (Figure 3.3B). Strikingly, obese mice receiving the SPM precursors and SPM injection

had significantly reduced transcript levels of CCR2 (receptor for MCP-1), CCR3 (receptor for MCP-2 and CCL5), and CXCR3 (receptor for CXCL10) in the VAT (Figure 3.5B).

We then wanted to verify that B cells in the bone marrow and spleen could express these chemokines and their corresponding receptors as this is not known to our knowledge. qRT-PCR analysis of sorted splenic and BM cells revealed that B cells in both tissues have high expression of MCP-1, MCP-2, CXCL10, and their corresponding receptors, which was evident by low Ct values (Figure 3.5C). This further suggests that 14-HDHA, 17-HDHA, and PDX could be limiting B cell recruitment to the adipose tissue by targeting chemokine receptors that are expressed on B cells.

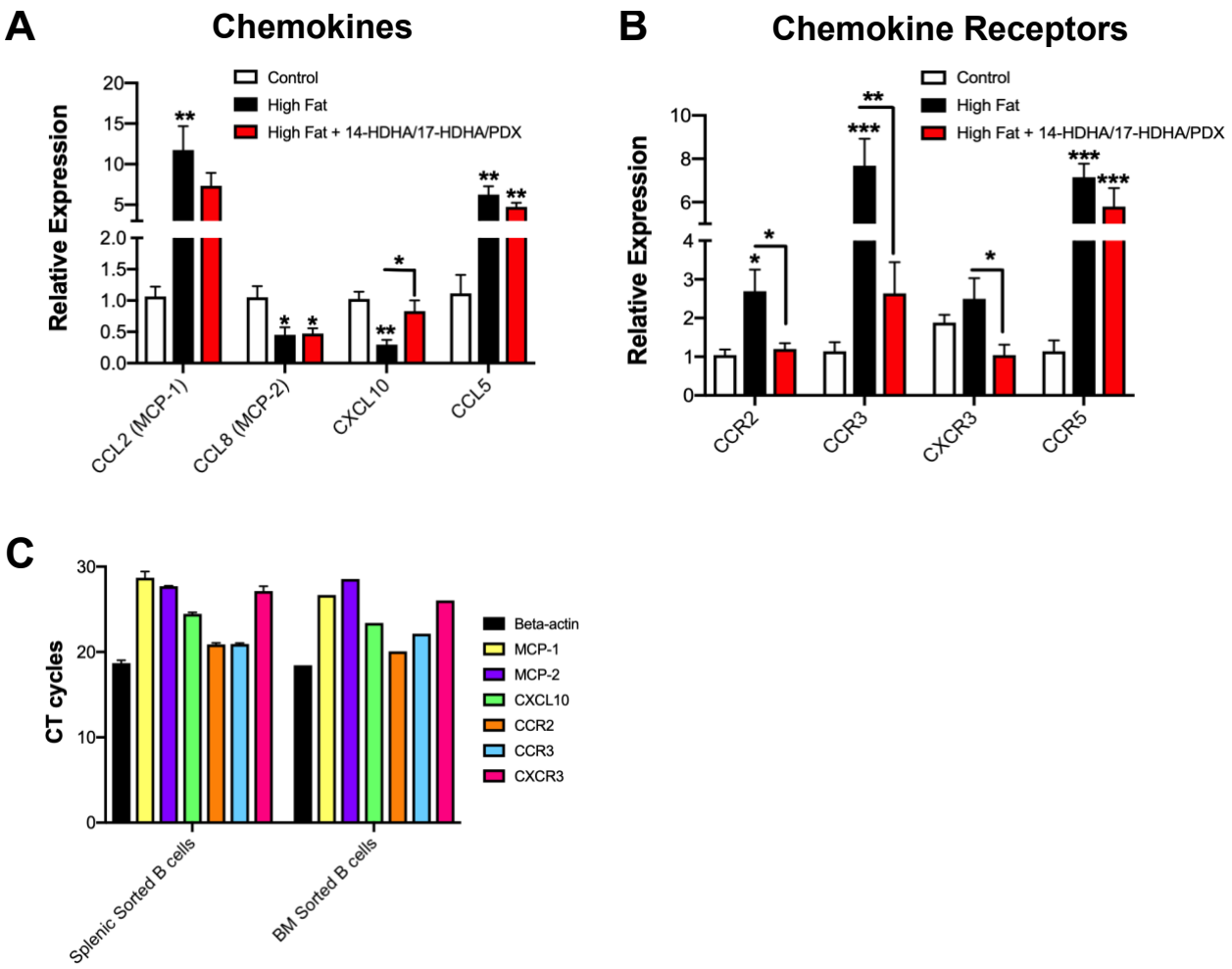

Figure 3.5: Obese mice have increased expression of chemokine receptors, which are reduced by DHA-derived metabolites. qRT-PCR analysis in the VAT of chemokines (A) and their corresponding receptors (B) involved in B cell recruitment to the adipose tissue. (C) Ct values of chemokines and their corresponding receptors in sorted splenic and bone marrow B cells from mice fed a control diet for 15-17 weeks. Mice received an injection of vehicle control or 14-HDHA/17-HDHA/PDX once a day for four consecutive days. N=5-6 (A-B), N=1-2 (C). Data are average ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 by a one-way ANOVA followed by a Bonferroni post-test.

Administration of DHA-derived metabolites does not enhance B cell development markers in the bone marrow of obese mice. We have previously reported that mice consuming a high fat diet have decreased numbers of B cells in the bone marrow as well as reduced numbers of B cell subsets (103,281). PCR data revealed that bone marrow cells isolated from these obese mice had reduced transcript expression of various transcription factors

necessary for proper B cell development in the bone marrow, including Pax5 (103). When we administered 14-HDHA/17-HDHA/PDX to these obese mice, we found that B cell numbers were rescued along with the numbers of B cells subsets in the bone marrow (281). Therefore, we wanted to test whether these DHA-derived metabolites were enhancing B cell development markers in the bone marrow. Before answering this question, we first reproduced our results from our previous study and found that B cell numbers are decreased in the bone marrow obese mice and rescued upon administration of 14-HDHA/17-HDHA/PDX (Figure 3.6A). To our surprise, obese mice administered 14-HDHA/17-HDHA/PDX had significantly decreased transcript expression of IL7-Rα, IL-7, Pax5, STAT5, and BLIMP-1 suggesting these metabolites may not be targeting B cell development to increase B cell numbers in the obese mice, at least in the absence of antigen stimulation (Figure 3.6B). As the numbers of B cells were elevated in the adipose tissue, we looked at the total number of B cells in the spleen. Compared to the control group, there was a trending increase in the total number of splenic B cells from obese mice suggesting that B cells in obese mice are being shuttled to the adipose and secondary lymphoid organs such as the spleen (Figure 3.6C).

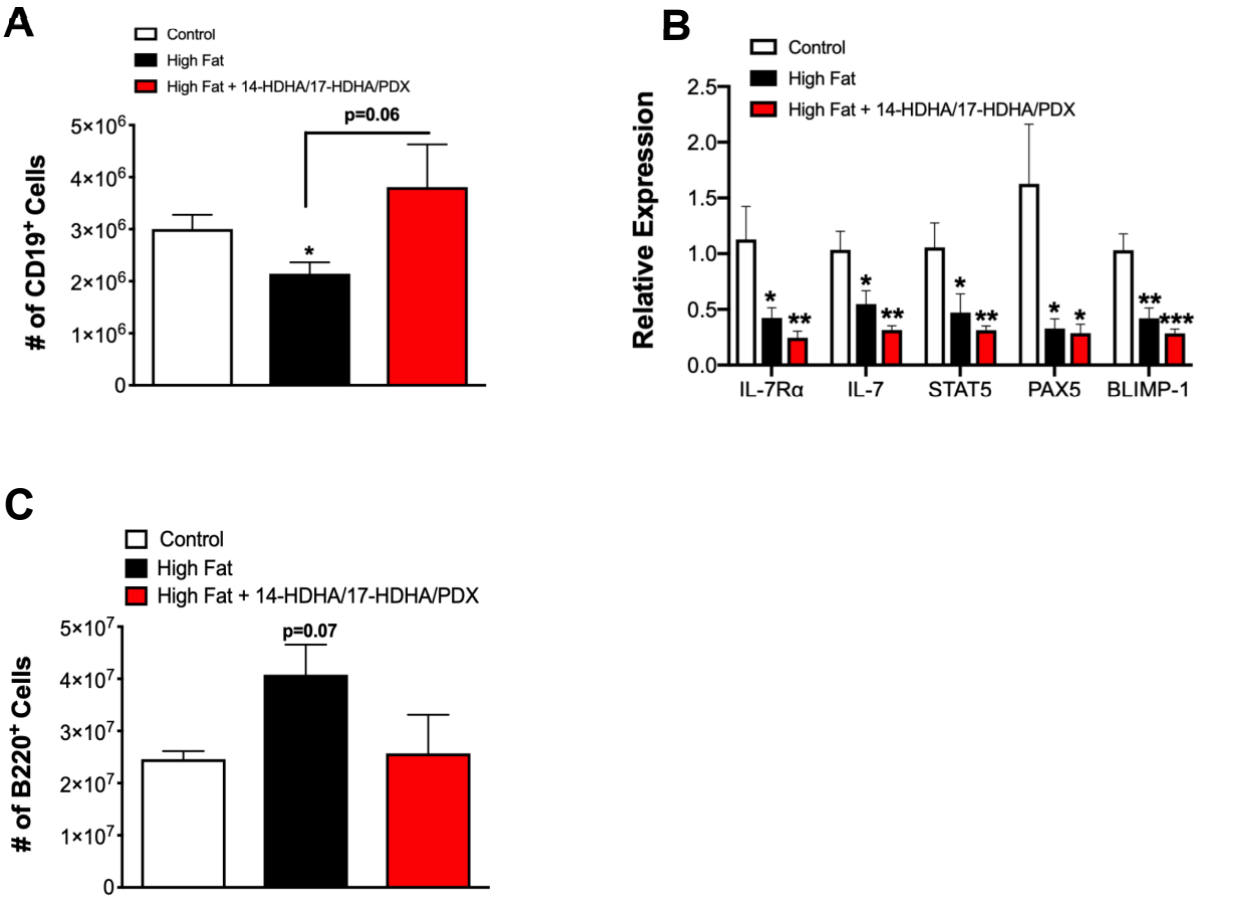

Figure 3.6: Administration of 14-HDHA/17-HDHA/PDX does not increase B cell development markers in the bone marrow of obese mice. (A) Total number of B cells in the bone marrow as determined by flow cytometry analysis. (B) qRT-PCR analysis of B cell development markers in total bone marrow cells. (C) Total number of B cells in the spleen as determined by flow cytometry analysis. Animals were fed a control or high diet for 15-17 weeks. Mice consuming a high fat diet received 14-HDHA/17-HDHA/PDX injections once a day for four consecutive days. The control group and the other obese group received vehicle control injections once a day for four consecutive days. N=6-7 (A), N=5 (B), and N=4-5 (C). Data are average ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 by a one-way ANOVA followed by a Bonferroni post-test.

DISCUSSION

In this study, we reveal that administration of a 14-HDHA/17-HDHA/PDX cocktail can directly target B cell recruitment and B cell subset numbers in the visceral adipose tissue of obese mice. First, we show that obese mice receiving the 14-HDHA/17-HDHA/PDX cocktail have reduced fasting insulin levels and decreased HOMA-IR scores. Second, we confirmed that total B cell numbers and select B cell subsets that induce insulin resistance in the VAT are increased in mice consuming a high fat diet. Interestingly, administration of DHA-derived metabolites limits the recruitment of B cells to the obese VAT resulting in decreased numbers of total B cells and B2 cells, which are responsible for the production of pathogenic IgG antibody (297). Third, obese mice administered the DHA-derived metabolites have decreased antibody concentrations in the VAT lysate compared to obese mice. Fourth, DHA-derived metabolites alter transcript levels of select chemokines and their receptors in the obese VAT. Fifth, we show that splenic and bone marrow B cells can express various chemokines and their corresponding receptors, which are both involved in B cell recruitment to the adipose tissue. Finally, we show that obesity decreases the number of B cells and the transcript levels of transcription factors imperative in B cell development. However, administration of 14-HDHA/17-HDHA/PDX does not enhance the transcript levels of B cell development markers in the bone marrow.

Several studies have reported that SPMs can improve glucose tolerance and insulin sensitivity (161,210,286,298). As a result, we established if DHA-derived metabolites could improve glucose tolerance and insulin sensitivity in obese mice. We found that obese mice receiving the 14-HDHA/17-HDHA/PDX cocktail had elevated

fasting glucose levels comparable to the obese group and were still glucose intolerant. However, obese mice receiving SPM precursors and SPMs had decreased fasting insulin levels and improved HOMA-IR scores. Interestingly, other studies have also found that administration of SPMs only improves insulin sensitivity or has no effect on the metabolic profile (299–301). For example, administration of RvE1 to mice consuming a high fat diet twice a week for 4 consecutive weeks did not improve metabolic parameters (299). On the other hand, diet-induced obesity (DIO) mice receiving daily injections of MaR1 (maresin 1) for 10 consecutive days had increased protein kinase B (Akt) phosphorylation, improved insulin tolerance tests, increased adiponectin gene expression, and increased AMPK phosphorylation (301). A potential explanation for SPMs not improving glucose tolerance could be that the dosing of the SPMs is insufficient to reverse the glucose intolerant phenotype of these obese mice (300). Therefore, higher and more frequent doses may be needed to improve glucose tolerance in DIO models. As a result, we hypothesize that SPMs could be regulating insulin secretion from the pancreas, but not glucose clearance. Furthermore, the effects of SPMs may be more pronounced as a prevention rather than a treatment modality in the context of murine obesity.

Our results suggest a complex model by which DHA-derived metabolites are regulating B cell recruitment to various tissues including the bone marrow, spleen, and adipose tissue. We have established that administration of 14-HDHA/17-HDHA/PDX to obese mice can rescue decrements in B cell numbers in the bone marrow. As obese mice have reduced transcript levels of transcription factors necessary for B cell development including Pax5, we wanted to determine if B cells were decreased in other tissues that B

cells migrate to during the onset of obesity. In the spleen, we found that obese mice had slightly increased number of B cells compared to the control group and the obese group receiving the 14-HDHA/17-HDHA/PDX cocktail suggesting that B cells are being shuttled to the spleen in adequate amounts. One potential explanation for the slight increase in the number of splenic B cells in the obese group could be driven by the low-grade chronic inflammatory tone that is stimulating B cells without the need of additional antigen stimulation (302,303). We have previously found that splenic B cell supernatants from obese mice have increased IgM and IgG upon no antigen stimulation suggesting that obese B cells are in fact hyperstimulated in the spleen (103). We have not looked at the supernatants of splenic B cells collected from obese mice receiving the 14-HDHA/17-HDHA/PDX cocktail to determine if these B cells are hyperstimulated but future studies will address this.

It is well established that diet-induced obesity drives increased infiltration of immune cells to the VAT including B cells (206,88). These B cells produce pathogenic IgG antibody that induces insulin resistance and can directly modulate macrophage and T cell function (89). We hypothesized that a potential explanation for the decrease in bone marrow B cells in the obese mice could be due to increased B cell recruitment to the adipose tissue. We found in our study that obese mice receiving DHA-derived metabolites had decreased immune cell infiltration to the VAT and decreased percentages and numbers of B cells compared to the obese group that received vehicle control. B2 cells are the main supplier of IgG antibody in the VAT and mediate B-cell induced inflammation in the adipose (297). Obese mice receiving the SPM precursors and SPM cocktail had decreased numbers of B2 cells in the VAT suggesting that Ig antibody levels could be

modulated by these metabolites. As the adipose tissue is very hypoxic, toxic intermediates generated during cell death can induce pro-inflammatory responses and activate B cells to produce autoantibodies (303). In addition, Frasca et al. found that ongoing cell death in the subcutaneous adipose tissue (SAT) releases “self” antigens that are responsible for the chronic stimulation of B cells without the need for additional stimulation such as CpG (303). Therefore, we analyzed antibody concentrations in the VAT lysates. Our results revealed that obese mice had significantly elevated levels of IgM and IgG further suggesting that B cells in the adipose tissue are hyperstimulated or there are simply a greater number of B cells in the adipose in response to a high fat diet. Administration of 14-HDHA/17-HDHA/PDX to obese mice resulted in the reduction of IgM and IgG levels back down to those of the control group. Therefore, the DHA-derived metabolites may be resolving the chronic hyperstimulation of B cells in the adipose or more likely, they are simply lowering antibody levels by decreasing the number of B cells in the adipose.

Many questions arise from these findings including where the B cells are being recruited from and how the SPMs are limiting B cell recruitment to the adipose tissue. Several studies have suggested that a bone marrow-adipose axis exists where bone marrow hematopoietic stems cells (HSCs) can migrate from the bone marrow to the adipose (304). This axis also serves as a “cross-talk” mechanism between the metabolic status of pathophysiological AT and function of the bone marrow (BM) environment (304). Whether these HSCs can develop in the adipose tissue is currently not known at least to our knowledge. However, a recent study by Frasca et al. identified germinal center B cells in the subcutaneous adipose tissue (SAT), cytokines that promote class switching, and

found expression of BLIMP-1, the transcription factor involved in plasma cell differentiation (303). Therefore, these results imply that the adipose has the capacity to generate plasma and memory cells further suggesting that HSCs or immature B cells from the bone marrow could potentially undergo development and maturation in the adipose. However, further studies will need to confirm this hypothesis. B1 cells trafficking from the peritoneal cavity as well as B2 cells from the spleen and other secondary lymphoid organs could also be the source of B cells in the adipose tissue (285). However, our results are limited to make any of these conclusions and more in-depth studies on B cell trafficking will need to be performed to determine exactly what tissues the B cells are being recruited from and what tissues SPMs are targeting to limit this recruitment to the visceral adipose tissue.

Another question that arises from our findings is the mechanism by which DHA-derived metabolites are limiting B cell recruitment to the adipose tissue. Our findings suggest a role for chemokines and their corresponding receptors. Chemokines along with their chemokine receptors are incremental in the recruitment of leukocytes including B cells (292). Chemokines are classified as constitutive or inducible (305). Constitutive or "homeostatic" chemokines are believed to be involved in specific migration control as they modulate the homing of naive lymphocytes and dendritic cells to various secondary lymphoid organs (292). Inducible or "inflammatory" chemokines are highly involved in inflammatory diseases such as obesity (305). Inflammatory chemokines attract leukocytes to areas of inflammation and are critical in leukocyte activation (292). Therefore, we wanted to determine if DHA-metabolites were modulating inflammatory chemokine expression in the adipose tissue, which could potentially explain why obese

mice administered DHA-derived metabolites had decreased numbers of total immune cells and B cells to the adipose tissue. While there were no significant decreases in the transcript expression of the chemokines in the VAT of obese mice who were administered DHA-derived metabolites, we did find that the transcript levels of the chemokine receptors, CCR2, CCR3, and CXCR3 were significantly reduced compared to the obese group. These receptors are significantly increased in obesity and are positively correlated with insulin resistance (291,306,307).

More studies must be conducted to confirm that SPM precursors and SPMs are modulating chemokine receptors to limit B cell recruitment to the adipose tissue. We acknowledge that decreased transcript expression of these chemokine receptors could be due to decreased number of B cells that express these receptors in the adipose tissue. However, as many of the chemokines elevated in obesity were also elevated in obese mice receiving 14-HDHA/17-HDHA/PDX, we suspect that these metabolites are in fact influencing migration of B cells to the adipose tissue. Therefore, chemotaxis studies involving adipocytes, B cells that have been treated with 14-HDHA/17-HDHA/PDX, and various inflammatory chemokines that we analyzed in this study must be performed. This will give us a clear indication if DHA-metabolites are limiting recruitment through modulation of chemokine receptor interaction with their respective chemokine ligands. Furthermore, future studies could also involve the widely available $CCR2^{-/-}$ and $CCR5^{-/-}$ mice to analyze B cell recruitment and migration to the adipose tissue.

An additional mechanism by which DHA-metabolites are limiting B cell recruitment to the adipose tissue could be mediated through the chemoattractant, LTB4. LTB4 is a proinflammatory lipid mediator produced from arachidonic acid through the actions of the

5-lipoygenase enzyme (5-LOX) and is highly expressed in the obese adipose tissue (207, 308). LTB4 binds with high affinity to its receptor, BLT-1, which promotes leukocyte infiltration into various tissues and regulates pro-inflammatory cytokine production (309,310). LTB4 can also induce macrophage-mediated inflammation and induce insulin resistance in obese hepatocytes and myocytes (308). A recent study revealed that depletion of BLT-1 can prevent the recruitment of B2 cells to the adipose tissue, which mitigated obesity-induced adipose tissue inflammation and insulin resistance (206). Furthermore, when fluorescently labeled B2 cells from BLT-1 knockout animals were injected into obese animals, migration of these cells to the adipose tissue and peritoneal cavity was markedly impaired compared to WT animals (206). Therefore, we hypothesize that administration of 14-HDHA/17-HDHA/PDX to obese mice could serve as a local damper of LTB4/BLT-1 signaling by binding to BLT-1 or by driving decreased levels of LTB4. As RvE1 binds to BLT-1 as a partial agonist and reduces BLT-1 mediated insulin resistance and inflammation in obesity, it is plausible that 14-HDHA/17-HDHA/PDX could also be binding to BLT-1 (311). However, receptors for these DHA-derived metabolites have not been identified to our knowledge and more in-depth studies are needed to determine this.

Another important finding from our study was that administration of 14-HDHA/17-HDHA/PDX to obese mice did not increase the expression of transcription factors involved in B cell development and antibody production in the bone marrow, specifically Pax5 and BLIMP-1. This was surprising to us as we expected transcript levels of B cell development markers to increase in obese mice receiving the DHA-derived metabolites since B cell numbers are increased in the bone marrow and there is data to show that 17-

HDHA increases BLIMP-1 levels in plasma B cells (216). Based on our findings in the bone marrow, it seems that B cells are still developing and migrating to various tissues in both the obese group and the obese mice receiving 14-HDHA/17-HDHA/PDX as there is not a dramatic reduction of B cells in the spleen between the two. Whether these DHA-derived metabolites can improve B cell function in obese mice is an area that will have to be explored in future studies as it is well known that obesity impairs B cell function.

A potential explanation for our PCR results in the bone marrow could involve the transcription factor, EBF. EBF is an overlooked transcription factor that is essential for the earlier stages of B lymphocyte development (312). While EBF induces the expression of Pax5, Pongubala et al. found that sustained expression of EBF in $Pax5^{-/-}$ hematopoietic progenitor cells repressed alternative lineage genes for myeloid and T lineage progenitors and promoted B cell commitment independently of Pax5 (313). Therefore, obese mice from both groups could be upregulating transcription factors such as EBF to compensate for the loss of Pax5 in B-cell development. This further supports our hypothesis that B cells from obese mice are being shuttled and recruited to other tissues resulting in depletion of B cells in the bone marrow. Another limitation to our studies is that we performed PCR on total bone marrow cells rather than sorted B cells from the bone marrow. Thus, future studies will need to perform PCR analysis on these transcription factors in sorted B cells in mice from all three groups to reach a conclusion as to whether the SPMs are impacting B cell trafficking.

In addition, 14-HDHA/17-HDHA/PDX could be enhancing B cell numbers in the bone marrow of obese mice due to changes in the bone marrow microenvironment. One of these changes could involve the interaction of these DHA-derived metabolites with the

chemokine CXCL12 and its receptor, CXCR4. To elaborate, CXCL12 is essential for the generation of pre-pro B cells and pro-B cells in the bone marrow as well as homing of plasma cells to the bone marrow (44). Whether SPM precursors and SPMs can target and enhance this chemokine ligand-receptor interaction in the bone marrow of obese mice is not known. However, a study found that PGE_2, a lipid mediator generated from arachidonic acid was a driving factor for the expression and function of CXCR4 as well as for the production of CXCL12 in cancer-associated MDSCs (314). Therefore, based on our chemokine and chemokine receptor PCR data and the findings of this study, it is possible that these DHA-derived metabolites could be enhancing B cell numbers in the bone marrow of obese mice by upregulating CXCL12/CXCR4 signaling, which is critical for early B cell lymphopoiesis.

In conclusion, our findings establish that DHA-derived metabolites are limiting the recruitment of B cells and B cell subsets capable of inducing insulin resistance (IR) to the visceral adipose tissue of obese mice. Furthermore, these metabolites are rescuing obesity-driven increases of various Ig levels further suggesting that these SPMs are actively resolving the chronic inflammatory state that is associated with the obese VAT. Mechanistically, our results suggest that these DHA-derived metabolites are limiting B cell recruitment to the obese VAT likely through modulation of chemokine-chemokine receptor networks. As B cell transcription factors were not increased in the bone marrow of obese mice receiving 14-HDHA/17-HDHA/PDX injections, we hypothesize that B cells from obese mice are being recruited to various secondary lymphoid organs such as the spleen and the adipose tissue at increased rates. As part of their resolution mechanism, DHA-derived metabolites are limiting the recruitment of

B cells to pathophysiological tissues such as the adipose tissue. Therefore, our studies identify these DHA-derived metabolites as potential therapeutic options that could modulate the pathogenic B cell phenotype in the adipose tissue and boost B cell function in the obese population.

CHAPTER 4: B-CELL ACTIVITY IS IMPAIRED IN HUMAN AND MOUSE OBESITY AND IS RESPONSIVE TO AN ESSENTIAL FATTY ACIDY UPON MURINE INFLUENZA INFECTION[2]

INTRODUCTION

Obesity is associated with an impaired immune response, which contributes toward a wide range of co-morbidities (315–318). Obese populations display compromised innate and adaptive immune responses, which is due to several factors such as hormonal imbalances, oxidative stress, and nutrient overload (319–321). While several studies have identified various cellular and molecular mechanisms in the obese adipose tissue that promote a pro-inflammatory phenotype, far less is known about how obesity influences the humoral immune response (322,323). As obesity is associated with increased susceptibility to infections and poor responses to vaccination, it is essential to address this knowledge gap in order to adequately treat and protect this population from various infections and diseases (222,224,324,325).

Several studies suggest that obesity impairs the humoral immune response. However, this is not completely clear. Several mouse models suggest that obesity diminishes antibody production. For example, obese mice that were infected with

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influenza had lowered hemagglutination inhibition antibody titers (HAI) 7 days post-infection (p.i.) as well as diminished titers 35 days p.i. (39,326). In human studies, obese humans displayed normal HAI titers 30 days p.i. However, these titers were lowered 12 months post-vaccination in obese humans compared to non-obese subjects (222). Another study reported that obese humans could mount an influenza-specific IgM and IgG response 8 weeks post-influenza vaccination. Relative to the obese diabetic cohort, the antibody response was diminished (327). Interestingly, the effects of obesity are not just limited to influenza infection as studies have shown that obese mice have diminished antibody production upon *Staphylococcus aureus* infection (328).

B cells have emerged as a major regulator and contributor to adipose tissue inflammation in obesity (51,101,329,330). IgG2c, which is a mouse-specific, pro-inflammatory Ig subclass is extensively elevated in the adipose tissue of obese mice (89). Furthermore, various B cell subsets in the AT such as B1a, B1b, and B2 cells have been shown to influence insulin resistance and pro-inflammatory condition in the obese AT (89,280,289,297). Despite this knowledge in the adipose tissue, far less is known about the influences of obesity on B-cell antibody production and cytokine secretion in other tissues that are imperative for B cell development and maturation such as the bone marrow, spleen, and lymph nodes (331). Some conflicting studies suggest that B-cell cytokine secretion could be impaired with type II diabetes, a co-morbidity of obesity (101,332). For instance, B cells from newly diagnosed diabetics secrete pro-inflammatory cytokines when stimulated with antigen. However, when these patients were vaccinated with influenza, their antibody production was normal (332,333). Similarly, another study

found that B cells from obese type II diabetic mice secret pro-inflammatory cytokines, which is similar findings to that of diabetic and/or obese patients (101,131).

It is essential to identify factors that modulate B cell activity in obesity. One neglected factor in humoral immunity studies is essential fatty acid status, which includes essential long-chain n-3 polyunsaturated fatty acids (PUFA). PUFAs, which have immunomodulatory properties, could contribute towards impairments in humoral immunity as their levels are lower in obese individuals compared to lean individuals (146,242). Eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are two PUFAs of interest as they exert anti-inflammatory effects (146). Our lab as well as others have recently discovered that n-3 PUFAs, particularly, DHA may improve B-cell responses (219,334). Despite this evidence, more in-depth studies are needed to understand their full influence on B-cell activity.

The objective of this study was to investigate if obesity impairs B cell responses in a diet model system that resembles that of Western societies and whether DHA and its downstream metabolites can modulate antibody levels. We tested this by examining the effects of a murine Western diet (WD) model (that provides moderate levels of fat) in the absence or presence of DHA on the antibody response upon influenza infection. We chose the influenza virus to stimulate the B cells *in vivo* as this virus is a significant burden to the obese population in various aspects (318,335). We then wanted to determine how DHA could potentially improve the B cell response in obese mice upon influenza infection. As a result, we focused our studies on whether downstream DHA-derived specialized pro-resolving lipid mediators (SPMs) could enhance the B cell antibody response as these mediators have immunoenhancing properties in B cells (151,336).

MATERIALS AND METHODS

Obesity mouse models. All murine experiments fulfilled the guidelines for euthanasia and humane treatment established by East Carolina University. Male C57BL/6 mice between the ages of 5-7 weeks old were fed experimental diets for 15 weeks (Envigo, Indianapolis, IN), which included a low-fat control diet, a western diet (WD), or a WD supplemented with DHA (WD+DHA) ethyl esters (Cayman Chemical, Ann Arbor, Michigan, USA). The ethyl ester was of greater than 93% purity as previously reported (220). 45% of total kilocalories in the WD was from a milkfat source. In addition, DHA accounted for 2% of the total energy, which is an amount that is easily achievable in humans through intake of over-the-counter supplements or prescription supplements (337). More details regarding the composition of this diet are presented in Supplemental Table 4.1.

Influenza Infection model. All murine experiments fulfilled guidelines provided by East Carolina University. Briefly, mice were infected with 0.03 HAU of influenza A/PR/8/34 virus, which is a mouse adapted strain. Mice received the virus intranasally. Following the completion of the infection time course, mice were euthanized with CO_2 inhalation followed by cervical dislocation. Tissues were collected following euthanasia and immediately put in liquid nitrogen.

SPM precursor and SPM studies *in vivo*. SPMs were prepared as described before (284). Briefly, SPMs were kept in the dark and on ice throughout handling. Following use, nitrogen was lightly run over the samples to prevent premature oxidation. For the intraperitoneal injections, 1μg of SPM precursors and SPMs derived from DHA was

prepared in PBS. Each mouse received one injection (100uL) of the appropriate metabolite either in conjunction with the 0.03 HAU of influenza A/PR/8/34 virus or 24 hours after administration of the influenza virus. PBS/ethanol alone served as the vehicle control.

Flow cytometry. Flow cytometry was performed on B cell subsets in the bone marrow of infected mice. The right leg of the mouse was used for consistency. Bone marrow was extracted as previously described (220). All antibodies used in experiments were obtained from Biolegend and consisted of: CD19 (PerCP-Cy5.5), CD43 (APC), CD24 (Pacific Blue), IgM (PE), IgD (APC), and CD138 (APC). The following B cell subsets were analyzed in the bone marrow: $CD19^{-}CD43^{+}CD24^{-}IgM^{-}$ (pre-pro), $CD19^{+}CD43^{+}CD24^{+}IgM^{-}$ (pro), $CD19^{+}CD43^{-}CD24^{+}IgM^{-}$ (pre), $CD19^{+}CD24^{+}IgM^{+}IgD^{-}$ (immature), $CD19^{+}IgM^{+}IgD^{+}$ (mature), and $CD19^{+}CD138^{+}$ (plasma cells).

ELISAs, HAI, and microneutralization assays. For the HAI assay, serum collected from each mouse was treated overnight with receptor destroying enzyme (RDE; Denka Seiken Campbell CA), followed by heat inactivation for one hour the following morning. The mixture was then diluted 1:10 in PBS (39). Hemagglutination inhibition (HAI) assays were performed according to WHO guidelines (Global Influenza Surveillance Network Manual for the laboratory diagnosis and virological surveillance of influenza). RDE-treated sera were incubated with 4 HAU influenza virus at room temperature for 15 minutes followed by a 1 hour incubation at $4^{o}C$ with 0.5% turkey red blood cells. Values for HAI were

calculated by taking the reciprocal of the highest dilution of serum that completely inhibited hemagglutination of the turkey red blood cells (39).

For microneutralization assays, RDE-treated sera were diluted at 1:2 in microneutralization media (DMEM, 2 mM glutamine, 1% bovine serum albumin). Sera were then incubated with 100 $TCID_{50}$ virus for 1 h at 37 °C on white polystyrene plates. Following this incubation, $3x10^4$ MDCK cells were added to each well in the appropriate plate(s) and were incubated at 37 °C overnight. Most of the media was aspirated and the plates were frozen for approximately 30 minutes at -80 °C. 25 µl Nano-Glo substrate solution (Promega) was added to each well in accordance to manufacturer's instructions. The plates were placed in a Synergy H1 Hybrid Reader in order to determine luminescence values. Neutralization of the virus was considered to be any well below half the luminescence generated by a well infected with 100 $TCID_{50}$ A/Puerto Rico/8/34 NLuc virus (338).

"Lipidomic sample preparation. All standards and internal standards used for LC/MS/MS analysis were purchased from Cayman Chemical. All solvents were HPLC grade or better. Tissue homogenate samples were pretreated for solid phase extraction as follows. Briefly, a volume of tissue homogenate equivalent to 500µg was brought to a volume of 1 ml in 10% methanol along with 10µl of 10pg/µl internal standard solution (100pg total/each of 5(S)-HETE-d_8, 8-iso-PGF2α-d_4, 9(S)-HODE-d_4, LTB4-d_4, LTD4-d_5, LTE4-d_5, PGE2-d_4, PGF2α-d_9 and RvD2-d_5 in ethanol).

Serum samples were pretreated for solid phase extraction. Proteins were precipitated from 50µl of serum by adding 200µl of ice cold methanol and 10µl of the

internal standard solution, followed by vortexing and then incubating on ice for 15 minutes. The samples were then placed in a microcentrifuge for 10 minutes at 4°C at 14,000 RPM. A 200μl portion of the supernatant was diluted to 10% methanol by adding 1.4ml of water. Lipid mediators were isolated from the pretreated samples by solid phase extraction as described (339). Lipid mediators were extracted using Strata-X 33um 30mg/1ml SPE columns (Phenomenex, Torrance, CA) on a Biotage positive pressure SPE manifold. Columns were washed with 2ml of methanol followed by 2ml of H_2O. After applying the sample, the columns were washed with 1ml of 10% methanol and the lipid mediators were then eluted with 1ml of methanol directly into a reduced surface activity/maximum recovery glass autosampler vial. The methanol solvent was then evaporated to dryness under a steady stream of nitrogen directly on the SPE manifold. The sample was immediately reconstituted with 15-20μl of ethanol and analyzed immediately or stored at -70°C until analysis for no more than 1 week.

Lipid mediators in whole spleen samples were isolated as described by Yang et al (340). Spleen samples were pre-weighed and transferred into a 1.5ml microcentrifuge tube. 1ml of methanol and 10μl of internal standard solution was added and then the sample was vortexed and stored overnight at -20°C. The sample was transferred to a DUALL all glass size 21 tissue homogenizer and ground until completely homogenized. The homogenate was transferred to a 1.5ml centrifuge tube and centrifuged at 14,000 RPM for 10 minutes at 4°C. The supernatant was diluted to 10ml with water adjusted to pH 3.5. The samples were then applied to Hypersep C18 500mg/6mL SPE columns (Thermo-Fisher) that were prewashed with 20ml of methanol followed by 20ml of water. The SPE columns were washed with 10ml of water followed by 10ml of hexane. Lipid

mediators were eluted with 8ml of methyl formate (eicosanoids and docosanoid fraction) followed by 10ml of methanol (cysteinyl leukotriene fraction). Both fractions were dried under a stream of nitrogen and reconstituted with ethanol and combined into a reduced surface activity/maximum recovery glass autosampler vial analyzed immediately or stored at -70^{o}C until analysis for no more than 1 week.

Liquid chromatography-mass spectrometry. Quantitation of lipid mediators was performed using 2-dimensional reverse phase HPLC tandem mass spectrometry (LC/MS/MS). The HPLC system consisted of an Agilent 1260 autosampler (Agilent Technologies, Santa Clara CA), an Agilent 1260 binary loading pump (pump 1), an Agilent 1260 binary analytical pump (pump 2) and a 6 port switching valve. Pump 1 buffers consisted of 0.1% formic acid in water (solvent A) and 9:1 v:v acetonitrile:water with 0.1% formic acid (solvent B). Pump 2 buffers consisted of 0.01% formic acid in water (solvent C) and 1:1 v:v acetonitrile:isopropanol (solvent D).

10μl of extracted sample was injected onto an Agilent SB-C18 2.1X5mm 1.8um trapping column using pump 1 at 2ml/min for 0.5 minutes with a solvent composition of 97% solvent A: 3% solvent B. At 0.51 minutes the switching valve changed the flow to the trapping column from pump 1 to pump 2. The flow was reversed and the trapped lipid mediators were eluted onto an Agilent Eclipse Plus C-18 2.1X150mm 1.8um analytical column using the following gradient at a flow rate of 0.3mls/min: hold at 75% solvent A: 25% solvent D from 0-0.5 minutes, then a linear gradient from 25-75% D over 20 minutes followed by an increase from 75-100% D from 20-21 minutes, then holding at 100% D for 2 minutes. During the analytical gradient pump 1 washed the injection loop with 100% B

for 22.5 minutes at 0.2ml/min. Both the trapping column and the analytical column were re-equilibrated at starting conditions for 5 minutes before the next injection.

Mass spectrometric analysis was performed on an Agilent 6490 triple quadrupole mass spectrometer in negative ionization mode. The drying gas was 250^{o}C at a flow rate of 15ml/min. The sheath gas was 350^{o}C at 12mls/min. The nebulizer pressure was 35psi. The capillary voltage was 3500V. Data for lipid mediators was acquired in dynamic MRM mode using experimentally optimized collision energies obtained by flow injection analysis of authentic standards. Calibration standards for each lipid mediator were analyzed over a range of concentrations from 0.25-250pg on column. Calibration curves for each lipid mediator were constructed using Agilent Masshunter Quantitative Analysis software. Tissue and serum samples were quantitated using the calibration curves to obtain the column concentration, followed by multiplication of the results by the appropriate dilution factor to obtain the concentration in pg/μg of protein (tissue homogenates) or pg/ml (serum)" (103).

Statistics. Multiple cohorts of mice were used in collection of data for the murine results. All data were analyzed with GraphPad Prism version 5.0b. A Kolmogorov-Smirnov test was used to determine normalized distributions. Most data sets displayed a normal distribution. Statistical significance was determined for the Western diet studies by use of one-way ANOVAs followed by a post-hoc Bonferroni multiple comparisons test. P values less than 0.05 were considered significant.

RESULTS

A Western diet results in a diminished antibody response upon murine influenza infection, which is improved with DHA supplementation. DHA levels are known to be decreased in obese mice (241). Therefore, we wanted to determine how the B cell response is influenced in a murine Western diet upon the addition or absence of DHA in the diet in response to PR8 infection. PR8 is a mouse-adapted influenza strain (329). Throughout the course of the study, the diets were monitored to ensure that oxidation did not occur as well as to confirm DHA levels were present in the composition of the WD+DHA diet (data not shown). Prior to infection, the weights of mice in all three groups were recorded. Mice from both WD groups displayed increased body weight compared to the control group (Figure 4.1A). Mice from both WD groups had increased fat mass (Figure 4.1B) and elevated HOMA-IR (Figure 4.1C), which is a measure of insulin sensitivity.

We next assayed the antibody response among the three groups over the course of the infection. Mice consuming a WD had lowered HAI titers 7 days post-infection and 21 days post-infection (Figure 4.1D). The addition of DHA to the diet result in a 4-fold increase in HAI titers relative to the WD (Figure 4.1D). Notably, only 66% of mice consuming a WD had a measurable titer 7 days p.i., which was improved upon the addition of DHA to diet (Figure 4.1E). Mice consuming a WD had 43% lowered HAI titers compared to the control group 21 days p.i. DHA improved the titers by 25% relative to the WD (Figure 4.1D). As this effect was not as pronounced as seen 7 days p.i., we verified these results by use of a microneutralization assay. Results from this assay revealed that the WD lowered the titers by 2.6-fold relative to the control group. The addition of DHA in the WD elevated titers by 3.7-fold compared to those in a WD (Figure 4.1F).

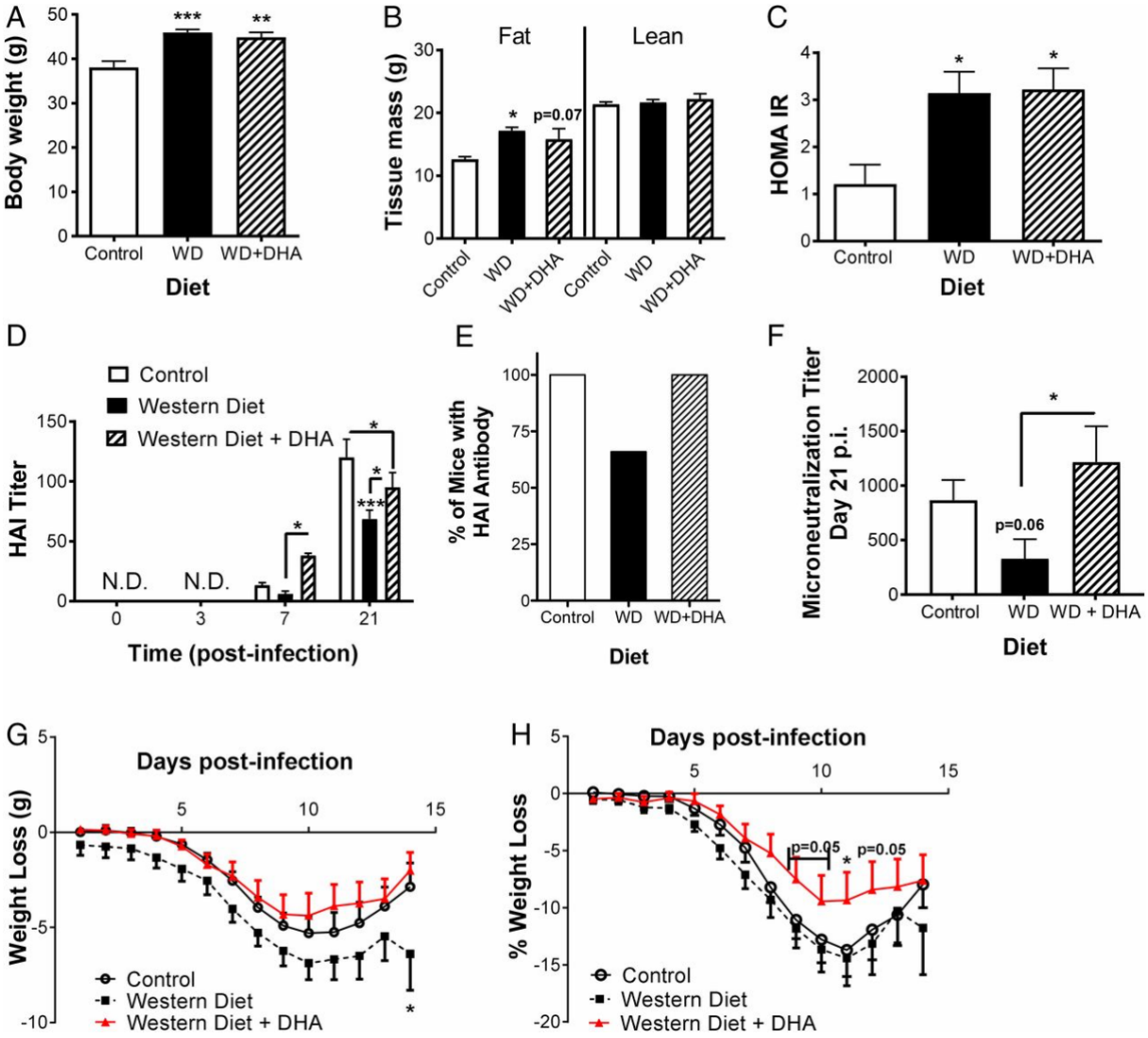


Figure 4.1: The essential fatty acid, DHA, improves suppressed antibody titers in the Western diet. (A) Body weights of mice prior to infection after 15 weeks of dietary intervention. (B) Lean and fat mass as measured by Echo-MRI in control, Western diet, and WD+DHA mice. (C) Mice were fasted 6 hours prior to HOMA-IR index measurements, which were calculated from fasting glucose/insulin. (D) HAI titers for C57BL/6 mice that were fed a control, Western diet, or Western diet + DHA diets for 15 weeks followed by influenza infection. (E) Percentage of mice showing HAI antibodies at day 7 p.i. (F) Microneutralization titers for C57BL/6 mice that were fed special diets for 15 weeks followed by influenza infection for 21 days. (G) Absolute weight loss and (H) percent weight loss as a function of time. Data are average ± SEM. N=4-9 mice per diet

for A-F and 15-20 mice per diet for G-H. *P<0.05, **P<0.01, ***P<0.001 by one-way ANOVA followed by a Bonferroni post-test (A,B,C, F) or a two-way ANOVA followed by a Bonferroni post-test (D,G,H).

Our next objective was to determine if DHA could improve the recovery over the course of the influenza infection. Our results revealed that mice consuming the WD had significantly lower body weights beginning at Day 14 relative to their control and WD+DHA counterparts (Figure 4.1G). When calculated as the percent weight loss, mice consuming the WD+DHA diet had improved recovery relative to mice consuming the WD (Figure 4.1H). Overall, these results reveal that DHA can improve the B cell response, which is diminished in mice consuming a WD.

Addition of DHA to the WD increases the number of $CD138^+$ plasma cells and the levels of downstream DHA-derived metabolites. As the addition of DHA to the WD resulted in elevated antibody titers, we also wanted to determine if DHA could increase the production of $CD19^+CD138^+$ plasma cells in the bone marrow 21 days p.i. Mice consuming a WD had modestly decreased numbers of total $CD19^+$ B cells in the bone marrow (Figure 4.2A). Mice consuming the WD+DHA diet had no significant changes in this number even though there was a slight rescue trend compared to the WD (Figure 4.2A). There were no changes in the percent (Figure 4.2B) or number (Figure 4.2C) of B cell subsets across the groups. Mice consuming the WD had a modest decrease (p=0.07) in the percent and number of $CD19^+CD138^+$ LLPCs compared to the control group, which was rescued by the addition of DHA to the WD (Figure 4.2D).

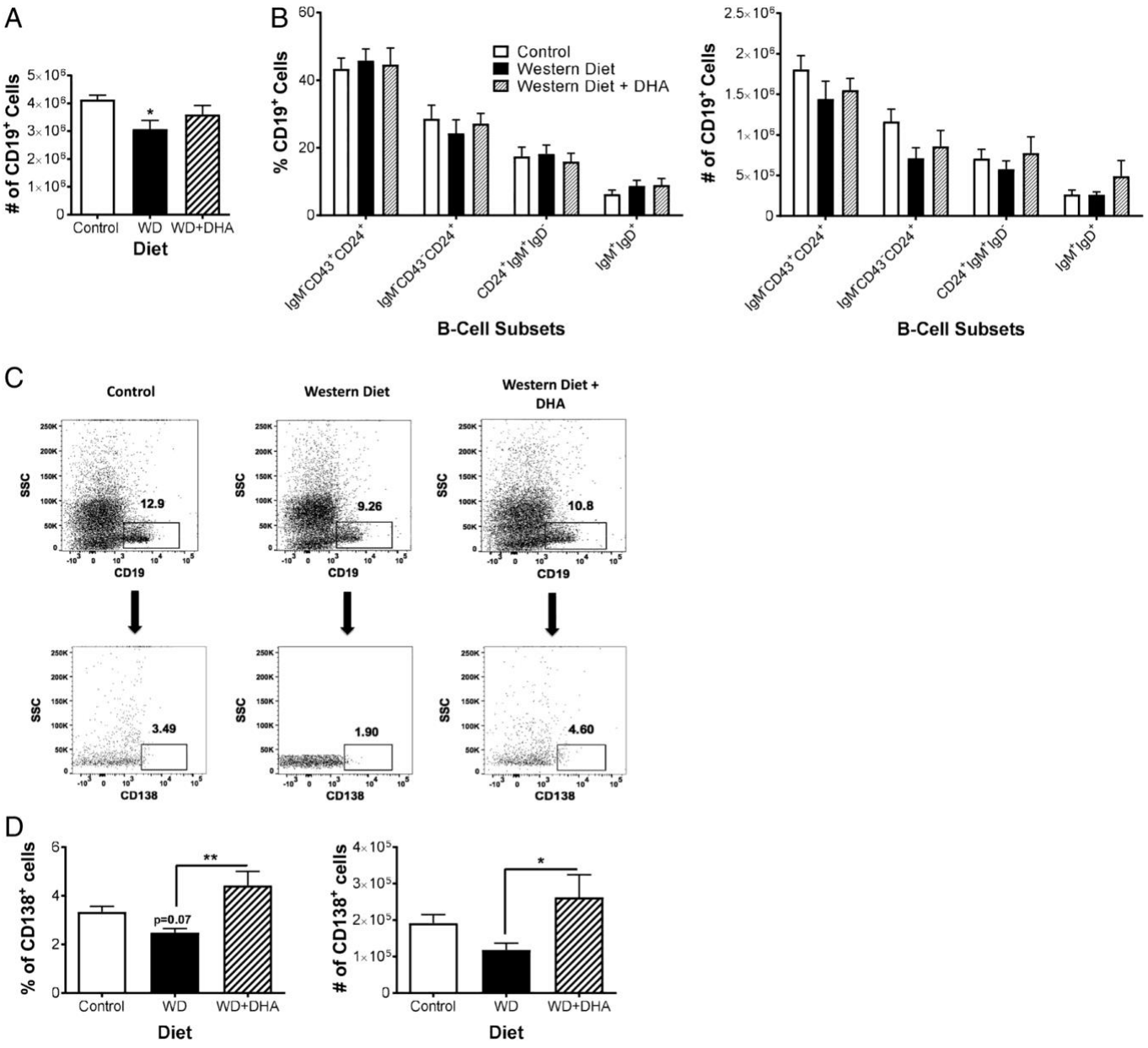


Figure 4.2: Mice consuming a Western diet supplemented with DHA have increased numbers of bone marrow CD138⁺ plasma cells. (A) Number of total B cells in the bone marrow in mice consuming a control, Western diet, or Western diet + DHA for 15 weeks. (B) Percentage and number of B cell subsets in the bone marrow. (C) Sample raw flow cytometry plots of CD138⁺ plasma cells in the bone marrow. The results reported are from mice consuming a control, Western diet, or Western diet + DHA for 15 weeks as well as from 21 days post-infection. DHA increases the frequency of bone marrow CD138⁺ plasma cells in mice consuming a Western diet. Data are average ± S.E.M. N= 7-8 mice per diet. *P<0.05 by one-way ANOVA followed by a Bonferroni post-test.

Ramon et al. demonstrated that 17-HDHA, an immediate downstream metabolite of DHA increased antibody production through increased production of $CD19^{+}CD138^{+}$ plasma cells and through the upregulation of BLIMP-1, a transcription factor necessary for plasma cell differentiation (341,284). Through the use of LC-MS and lipidomics in the sera and spleens of mice across all three groups, we wanted to determine if the addition of DHA to the WD increased the levels of downstream metabolites of DHA (Figure 4.3A-D). Lipidomics data revealed that DHA increased the levels of the SPM precursors, 14-HDHA and 17-HDHA as well as the SPM, protectin DX (PDX) in the spleen (Figure 4.3F) DHA did not increase these levels in the serum (Figure 4.3E). Therefore, these data reveal that DHA can metabolized to it downstream mediators when incorporated in the diet.

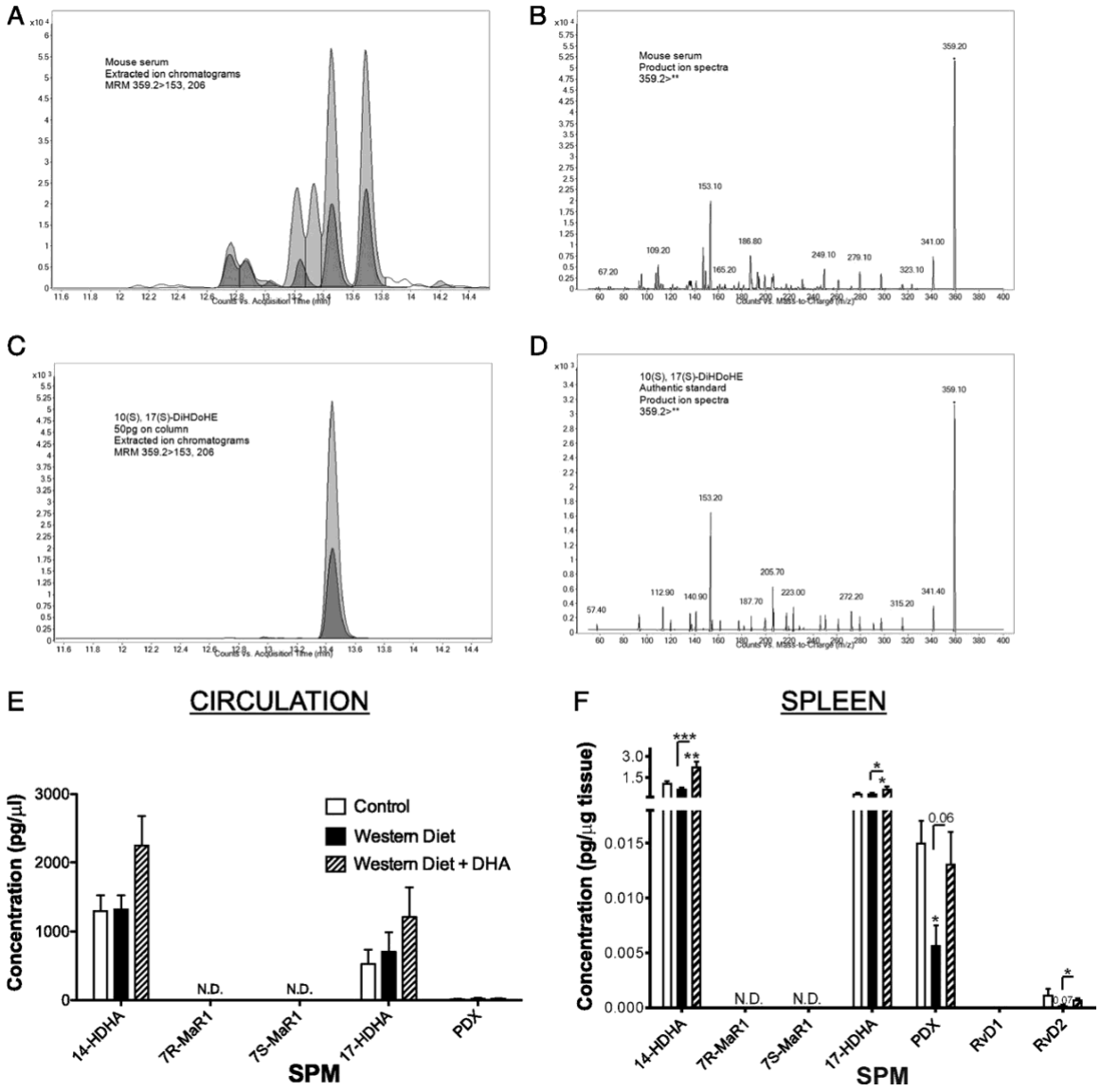
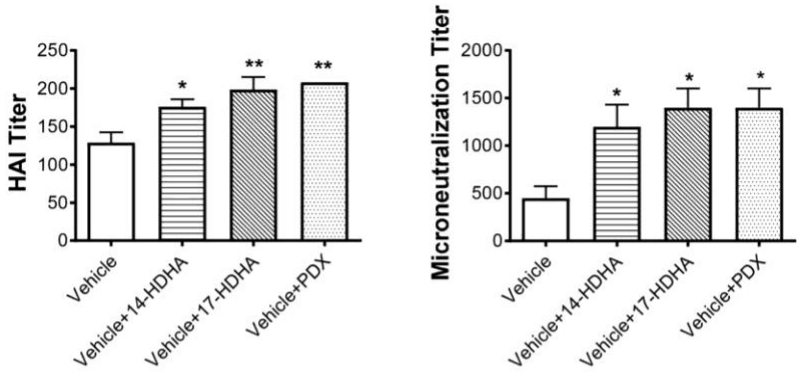


Figure 4.3: Supplementation of the Western diet with DHA enhances the levels of DHA-derived SPM precursors and SPMs in the spleen in an influenza model.

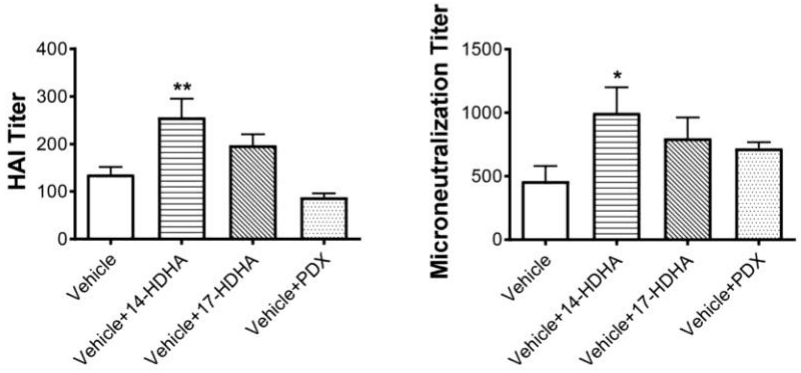
Raw histograms of the extracted ion chromatograms (A and C) and product ion spectra (B and D) for 10(S),17(S)-DiHDoHE (PDX) in mouse serum (A and B) and authentic standard (C and D). DHA-derived SPM precursors and SPM concentrations in murine (E) serum and in the (F) spleen at 7 days post infection upon consumption of a control, Western, or Western + DHA diet for 15 weeks. Samples for serum analysis required pooling of 2 control animals to achieve a signal. N=5-10 mice per diet. *P<0.05, **P<0.01, ***P<0.001 by one-way ANOVA followed by a Bonferroni post-test.

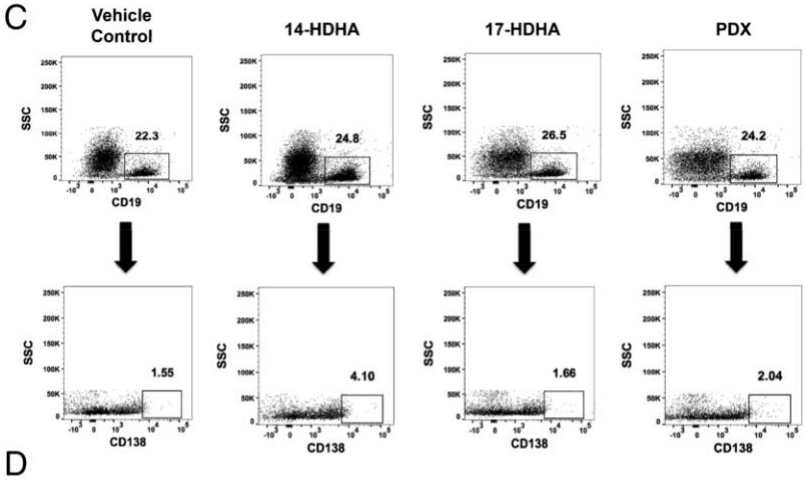
Administration of 14-HDHA in parallel with influenza infection enhances antibody titers and increases the number of $CD138^+$ plasma cells in the bone marrow. As DHA increased the levels of 14-HDHA, 17-HDHA, and PDX in the spleen, we wanted to determine if administration of these metabolites in parallel with infection and after infection could enhance the antibody response. Our results revealed that HAI (Figure 4.4A, left panel) and microneutralization (Figure 4.4A, right panel) titers were elevated by all three metabolites when administered 24 hours post-infection. Interestingly, when administered with the influenza infection, only 14-HDHA elevated HAI (Figure 4.4B, left panel) and microneutralization (Figure 4.4B, right panel) titers. Similarly, flow cytometry analysis (Figure 4.4C) revealed that mice receiving the 14-HDHA injection had a 2-fold or greater elevation in the percentage (Figure 4.4D, left panel) and number (Figure 4.4D, right panel) of $CD138^+$ plasma cells. Mice that received 17-HDHA or PDX injections did not have significant increases in the percentage or number of plasma cells. Collectively, our results demonstrate that SPM precursors and SPMs have different effects on antibody production depending on the time of administration.

A Administration 24 hours post-infection



B Administration with infection





D

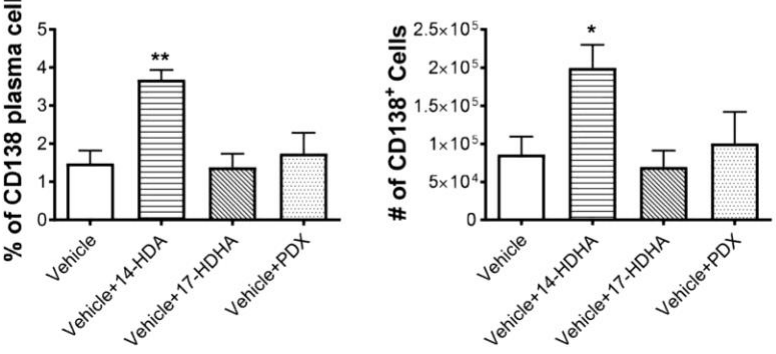


Figure 4.4: 14-HDHA enhances antibody titers and increases the percent and number of CD138$^+$ cells upon influenza infection in mice consuming a Western diet. (A) HAI and microneutralization titers upon administration of the SPM precursors, 14-HDHA and 17-HDHA as well as the SPM, PDX 24 hours post-infection. (B) HAI and microneutralization titers upon administration of the SPM precursors and SPMs in conjunction with the influenza infection. HAI and microneutralization titers were assayed in both A and B 14 days post-infection. (C) Sample flow cytometry gating of CD138$^+$ cells on day 21 post-infection in mice administered 14-HDHA, 17-HDHA, and PDX in parallel with influenza infection. (D) Average percentage and number of CD138$^+$ plasma cells on day 21 post-infection in mice administered 14-HDHA, 17-HDHA, and PDX in parallel with influenza infection. Data are average $\pm$ S.E.M. N= 3-5 mice per treatment. *P<0.05, **P<0.01 by unpaired Student's t test.

DISCUSSION

B cell activity is impaired in obesity and is improved with supplementation of DHA in the diet. Obesity impairs B-cell antibody production likely through several mechanisms. We showed that animals fed a high fat diet had a clear reduction in $CD138^+$ plasma cells, which was a similar trend found in mice consuming the WD. This decrease could be driven by a reduction in BLIMP1, a transcription factor critical in plasma cell differentiation (342). In addition, we previously found that bone marrow B cells from these HF animals are reduced as well as the transcription expression of several key transcription factors that are necessary for B cell development such as IL-7, Pax5, Oct-2, and BLIMP-1 (103,281). It is important to point out that the reduction in these transcription factors could simply be due to the decreased numbers of B cells and B cell subsets in the bone marrow.

Our previous results revealed that B cells from male mice consuming a high fat diet were hyperstimulated upon no antigen challenge (103). This hyperstimulation could be a result of increased circulating leptin in obesity (101). To elaborate, B cells from lean individuals treated with leptin *in vitro* had increased phospho-STAT3 levels, which is crucial for decreased TNF-α production (101). Therefore, leptin could be promoting excessive antibody production under basal conditions from the B cells of obese mice.

Another potential mechanism that could be contributing to the impaired antibody response in obese individuals could be decreased levels of DHA and their corresponding SPM productions. Obese children and adults have low levels of circulating essential fatty acids including DHA, which could be limiting an adequate innate and adaptive immune response (146,241–243,343). This decreased could be driven by lower dietary consumption rates in obese subjects or potential modifications to the LOX and COX

enzymes that metabolize DHA to its downstream products. Similarly, our lipidomic data revealed that mice consuming a WD had decreased PDX levels relative to those mice consuming a control diet. RvD2 also exhibited a strong trend to be diminished compared to the control mice. Decreases in PDX and other SPMs could be driving an inadequate antibody response to influenza (344). Several SPM precursors and SPMs are lowered in the AT of obese humans (159,182,282). In addition, PDX, 14-HDHA, and 17-HDHA are lowered in the adipose of obese mice relative to their control counterparts (159,210). As SPMs promote tissue homeostasis in the immune response, their diminished presence contributes to chronic inflammation (159,210). 14-HDHA and 17-HDHA were also decreased in obese diabetic mice upon wound closure (345). Therefore, future studies will need to mechanistically address how the lowering of DHA and its downstream metabolites could impair B-cell responses such as signaling networks that regulate class-switching and antibody production.

DHA exerts its actions through its downstream metabolites, SPM precursors and SPMs.

Ramon et al. established that 17-HDHA enhances antibody production through the increased production of $CD138^+$ plasma cells in the bone marrow (284). Therefore, we wanted to see if the parent fatty acid of 17-HDHA, DHA could enhance plasma cell production. We showed that DHA rescued the decreased plasma cell pool in mice consuming the WD upon influenza infection. Contrary to our initial hypothesis, when given parallel with influenza 14-HDHA enhanced antibody titers and increased the number of $CD138^+$ plasma cells in lean mice. 17-HDHA and PDX, however, did not increase the percentage and number of plasma cells in lean mice. 14-HDHA and DHA did not improve

the body weights of mice after infection and DHA did not affect survival upon infection (data not shown). Therefore, DHA and its metabolites may be enhancing antibody production without conferring protection in obese mice. A recent study showed that obese mice had no defects in the generation of neutralizing and non-neutralizing antibodies upon adjuvanted influenza vaccination. However, these antibodies were not protective upon challenge with influenza virus (37). Furthermore, our results could also imply that one dose of DHA or one injection of 14-HDHA is not enough to improve body weights in an infection.

It is worthwhile to note that DHA probably exerts its effects through other downstream metabolites rather than solely through 14-HDHA. When supplemented in the diet, DHA increased the levels of other SPM precursors and SPMs in mice consuming the WD. Therefore, DHA's effects are likely driven by a combination of several SPMs, which may exert their actions at different time points. Our data show that his could be a real possibility as 14-HDHA, 17-HDHA, and PDX enhance antibody titers differently in lean mice depending on if the SPM was given in parallel or 24 hours after the infection. Future studies will require different combinations of SPM precursors and SPMs tested in various disease models in order to address this concern. Furthermore, future experiments using specific SPM receptor knockouts and knockdowns will need to be generated to establish the mechanistic underpinnings of how DHA is modulating and targeting B-cell antibody production. Lastly, SPM precursors and SPMs can directly target other immune cells such as macrophages and T cells. As T_{FH} cells are directly involved in B cell differentiation to an antibody-producing plasma cells, it is necessary to determine if SPMs are targeting T_{FH} cells.

Conclusions

Our results provide evidence that B cell antibody production is modulated in other tissues outside of the AT in obesity. Modifications to the B-cell immune response could be a major contributing factor towards the poor responses to infection and vaccinations in obese populations. Furthermore, our results reveal that loss of essential fatty acids such as DHA could also be contributing to impaired antibody responses in obesity. This is likely driven by a SPM-mediated mechanism. Our studies warrant further investigation into DHA-derived SPM precursors and SPMs for clinical populations that have a diminished antibody response such as the obese or the aged.

CHAPTER 5: DISCUSSION

Obesity is associated with an impaired B-cell driven humoral immune response. However, mechanisms by which the B cell response is impaired in obesity is lacking in the literature. We hypothesized that the loss of SPM precursors and SPMs in tissues that are necessary for B cell development and function could be driving the diminished B cell response in the obese population. Therefore, the primary goal of this dissertation was to establish whether restoration of diminished SPM precursor and SPM levels in obese mice could enhance the impaired B cells response. Our studies have demonstrated that SPM precursors and SPMs directly target B cell recruitment, alter B cell subset numbers in various tissues, and regulate antibody production in obese mice. Furthermore, we established that SPMs can increase B cell function during the course of an infection. The findings of this dissertation not only highlight the importance of these metabolites in the B cell response, but also identify them as a potential target that can modulate the impaired B cell phenotype in the obese population.

Obesity impairs the B-cell mediated immune response potentially driven by decreased levels of DHA-derived metabolites.

The findings of this dissertation present a complex model as to how DHA-derived metabolites are regulating B cell recruitment and B cell function. We first established that obesity results in an impaired B cell response. In Chapter 2, we found that total B cell numbers and numbers of B cells subsets are decreased in the bone marrow of obese mice. In addition, we found a modest decrease in the percentage of germinal center B

cells in obese mice, which are important for the generation of high affinity, class-switched antibodies as well as memory B cells. We have also previously established that B cell supernatants from obese mice had increased basal IgM and IgG antibody levels upon no antigen challenge suggesting that B cells are hyperstimulated due to the chronic inflammatory environment established in the obese spleen (103,302,303).

In Chapter 2, we identified that levels of SPM precursors, 14-HDHA and 17-HDHA, along with the SPM, PDX are decreased in the spleens of obese mice. 14-HDHA was also decreased in the bone marrow of obese mice. We established that this decrease in DHA-derived metabolites was not due to reduced levels of the parent molecule DHA in the obese mice and was not driven by impaired B cell SPM production in the obese mice. Rather, levels of the n-6 PUFA, linoleic acid (LA) were significantly enhanced in obese mice compared to their control counterparts. As LA binds to the 15-LOX enzyme with higher affinity when present in excess, our findings suggest that DHA-derived metabolites are decreased in obese mice due to LA serving as the substrate more than DHA (141,260,346,347). Future studies need to determine if LA is boosting the production of LA-derived mediators while lowering DHA-derived mediators. These would include measuring the production of the HODEs, which can be generated via the actions of 15-LOX and COX-2 as well as the production of their downstream metabolites, the oxo-ODEs (348).

ALOX5 is not driving decrements in B cell numbers in the bone marrow and spleen.

In Chapter 2, we established that B cells from obese mice had decreased transcripts of 5-LOX and 12/15-LOX. Therefore, we used an Alox5$^{-/-}$ mouse model to

determine if reductions in 5-LOX were driving reduced B cell numbers in the bone marrow. Surprisingly, we found that decrements in B cell numbers and subsets in the bone marrow and spleen were only slightly decreased by the 5-LOX enzyme, which suggested that reductions in B cell numbers and subsets in the bone marrow were not driven by the reduction of this enzyme in obese mice. One limitation to these Alox5$^{-/-}$ studies was that these animals were whole body knockouts and were not B cell specific. In addition, a recent study also confirmed that leukocytes from obese individuals had decreased 5-LOX levels as we reported (214). Interestingly, this study reported that 15-LOX was the most critical factor for the deficient production of SPMs by obese leukocytes (214). It should be noted that B cell subsets and antibody levels were reported to be elevated in 12/15 LOX$^{-/-}$ mice (259). However, based on these new findings by Claria et al., it would be worthwhile to revisit the 12/15-LOX to determine if B cell impairments in obesity could be partially attributed to this enzyme.

Administration of DHA-derived metabolites that are deficient in obese mice increases B cell numbers in the bone marrow.

Several studies have reported that B cell function is impaired in obese mice and humans in response to different antigen challenges (26,37,101,132,222,229). Furthermore, as SPM precursors and SPMs are decreased in several tissues in obese mice, we wanted to explore if decrements in these DHA-derived metabolites could be contributing to the impaired B cell response. As a result, we injected our mice with a cocktail of 14-HDHA/17-HDHA/PDX and investigated whether these DHA-derived metabolites could rescue decrements we see in B cell subsets in obese mice.

Administration of 14-HDHA/17-HDHA/PDX to obese mice increased the percentage and number of total B cells in the bone marrow as well as the number of B cell subsets including pro, pre, immature, and mature B cells (Figure 5.1). We hypothesized that these DHA-derived metabolites were enhancing B cell numbers in the bone marrow by targeting B cell development markers in the bone marrow. However, to our surprise we found that this was not the case as these transcription factors were also decreased in the bone marrow to the same degree as their obese counterparts. It is worth noting that there are limitations to our study. For example, we measured these transcripts in the whole bone marrow rather than in sorted B cells from all three groups. Thus, future studies will need to measure these transcripts in sorted B cells to fully conclude whether SPMs regulate B cell development at the transcriptional level.

As a result, many possibilities could be occurring in obese mice to explain the bone marrow PCR data. First, decrements in transcript levels of the developmental markers in the obese population could be driven by the reduction of B cells in the bone marrow and thus less B cells expressing these transcription factors. In addition, B cells from the obese mice could be shuttled and recruited to other tissues at a faster rate, therefore depleting B cell pools in the bone marrow. DHA-derived metabolites could be preventing this recruitment to other tissues resulting in higher B cell numbers in the bone marrow of these mice receiving the metabolites. This hypothesis would mean that B cell lymphopoiesis is not necessarily decreased as B cells are still being produced in adequate amounts. Therefore, both obese groups may be targeting alternative B cell generation pathways through transcription factors such as EBF, which needs further exploration (312,313). Several studies also suggest that the chemokine CXCL12 and CXCR4 could be

enhancing B cell numbers in the bone marrow of obese mice receiving the DHA-derived metabolites (44,314). 14-HDHA/17-HDHA/PDX could be targeting and upregulating this chemokine and its receptor to increase B cell numbers in the bone marrow, which are both greatly involved in the generation of pre-pro B cells, pro B cells and the homing of plasma cells to the bone marrow (44). Whether the SPM precursors and SPM used in our studies can target this chemokine is not known and could be a potential area of study. Therefore, our results suggest that B cells from obese mice are being shuttled and recruited to other tissues at a faster rate resulting in depletion of bone marrow B cells.

Splenic B cells from obese mice are slightly increased in number and are hyperstimulated.

We next wanted to determine if loss of B cells in the bone marrow of obese mice could be accounted for in other tissues such as the spleen and adipose tissue and whether DHA-derived metabolites were influencing this B cell recruitment. As displayed in our model (Figure 5.1), we found that obese mice had a trending increase in the total number of B cells compared to both the control and obese mice receiving the DHA-derived metabolites. This slight increase could be due to the fact that obesity is associated with a low-grade chronic inflammatory tone that is stimulating splenic B cells without the need of addition stimulation (302,303). Our data from Chapter 3 also points to this finding as we found that splenic B cells from obese mice had increased IgM and IgG levels without antigen challenge. Whether 14-HDHA/17-HDHA/PDX is mediating this chronic inflammation to prevent hyperstimulation is not known and is an area for future studies.

As B cells were not depleted in the spleen of obese mice, these findings further support our hypothesis that B cells are being recruited from the bone marrow in greater amounts.

DHA-derived metabolites regulate B cell recruitment in the VAT suggesting a role for chemokines and chemokine receptors.

B cells have been implicated as a major contributor to obesity-induced inflammation and insulin resistance in the visceral adipose tissue (88,89,285). In addition, several studies have reported that B cells are found in higher numbers in the VAT of obese mice compared to their control counterparts (89,289,290). Based on these findings, we hypothesized that B cells from the bone marrow could be trafficking to the adipose tissue in obese mice, which could further explain the depletion of bone marrow B cells in the obese. The existence of the bone marrow-adipose axis suggests that B cell recruitment from the bone marrow is possible (304). Furthermore, whether DHA-derived metabolites can target B cells in the adipose tissue is currently not known at least to our knowledge. As depicted in our model, our findings from Chapter 3 revealed that obese mice had increased percentage and number of total B cells and number of B2 cells in the VAT compared to their control counterparts as previously reported (89,349). Interestingly, obese mice administered 14-HDHA/17-HDHA/PDX had a reduction in the percentage and number of total B cells as well as reduced number of B2 cells in the VAT relative to their obese counterparts (Figure 5.1). In addition, we found that obese mice had increased antibody concentrations of IgM and IgG in the VAT. This is likely driven by the release of “self” antigens that are chronically stimulating B cells in the hypoxic environment seen in the VAT (303). Obese mice receiving the DHA-derived metabolites had significant

reductions in IgM and IgG levels in the VAT, which further supports our hypothesis that these metabolites are resolving chronic hyperstimulation of B cells in various tissues.

Chemokines secreted from adipocytes are involved in the recruitment of immune cells to tissues and many inflammatory chemokines such as MCP-1 are upregulated in obesity (291,292,295). As a result, our studies focused on whether SPM precursors and SPMs could be limiting B cell recruitment to the adipose tissue by regulating chemokines and chemokine receptors. As shown in our model (Figure 5.1), we found that transcript levels of inflammatory chemokines that are expressed in B cells were not reduced in obese mice receiving the DHA-derived metabolites. However, transcript levels of their corresponding receptors including CCR2, CCR2, and CXCR3 were significantly decreased in obese mice administered the SPM cocktail. Thus, it appears that SPM precursors and SPMs are limiting recruitment to the adipose tissue through regulation of chemokine receptor interaction with chemokines.

However, these studies present limitations that require further exploration. To elaborate, decreased number of B cells in the adipose tissue expressing these receptors could be driving the decrements in chemokine receptor expression that we are seeing at the transcript level. However, as many of these chemokines that were elevated in the obese mice were also elevated in obese mice receiving 14-HDHA/17-HDHA/PDX, we believe that these metabolites are in fact influencing recruitment and migration of B cells to the adipose tissue. Therefore, future studies addressing chemotaxis of B cells in the presence of adipocytes, chemokines, and these DHA-derived metabolites must be performed. Results from these studies will give us a clear indication whether DHA-derived metabolites are in fact limiting recruitment through modulation of chemokine receptor

interaction with their respective chemokine ligands in B cells from both the bone marrow and spleen.

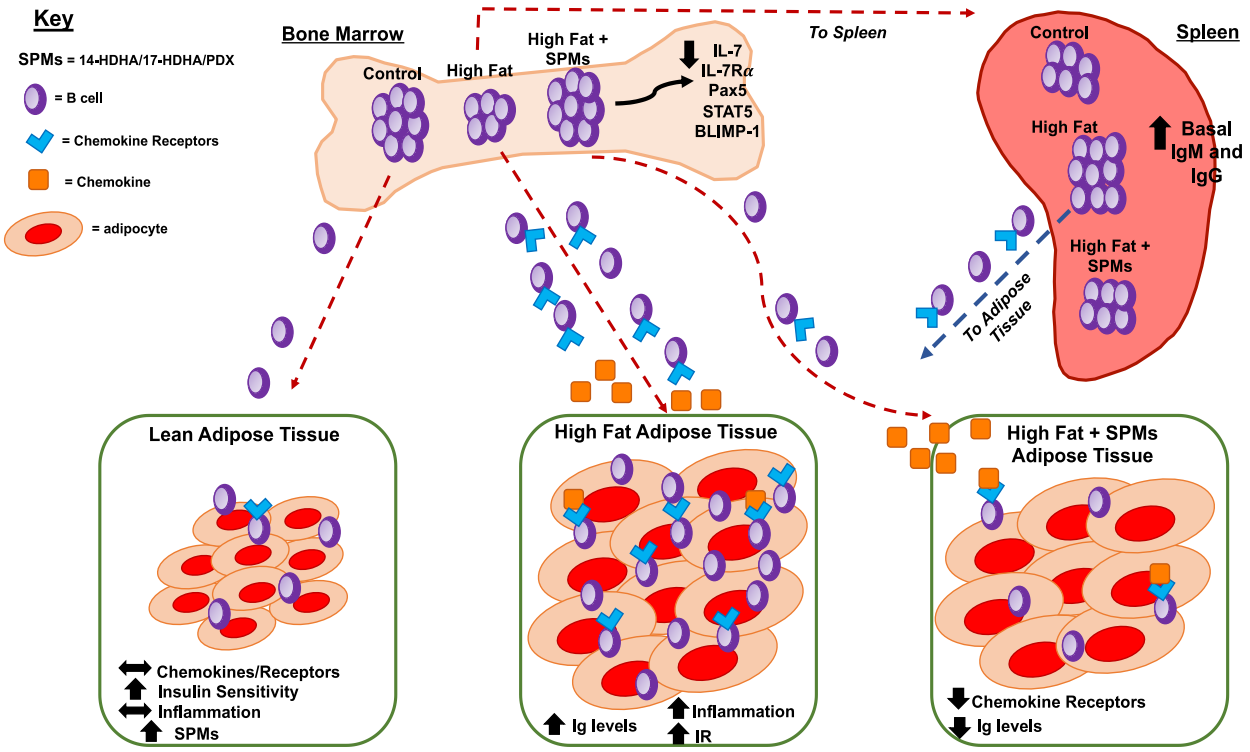


Figure 5.1: DHA-derived metabolites regulate B cell recruitment to various tissues in obesity. Obesity decreases the number of B cells in the bone marrow, which is rescued upon administration of 14-HDHA/17-HDHA/PDX to obese mice. Bone marrow cells isolated from obese mice receiving these DHA-derived metabolites had decreased transcript levels of B cell development markers suggesting that these SPMs may not be targeting these transcription factors to enhance B cell numbers. B cells from obese mice are likely being shuttled to the spleen more often than B cells from control mice and obese mice administered the 14-HDHA/17-HDHA/PDX cocktail as evident by increased IgM and IgG levels in B cells from obese mice that were not challenged with antigen. B cells from obese mice are also recruited to the VAT and this recruitment seems to be regulated by 14-HDHA/17-HDHA/PDX potentially through modification of inflammatory chemokine receptors. In the adipose tissue, obese mice administered DHA-derived metabolites have reduced numbers of total B cell numbers, reduced number of B2 cells, and decreased levels of various Ig levels that were increased in the obese mice. Therefore, DHA-derived metabolites can regulate B cells in the adipose tissue.

DHA and its downstream metabolite, 14-HDHA enhance antibody production in lean mice infected with influenza.

In Chapter 4, we established that incorporation of DHA in a Western diet resulted in increased levels of 14-HDHA, 17-HDHA, and PDX in the spleens of obese mice. We also showed that DHA rescued the percentage and number of plasma B cells in obese mice. As DHA-derived metabolites can increase antibody production that is protective against influenza infection in mice, we hypothesized that DHA was exerting its effects through downstream metabolites (284). Specifically, we showed that administration of single injections of 14-HDHA, 17-HDHA, and PDX into lean mice resulted in differences in antibody response and production in lean mice infected with influenza. To exemplify, we reported in Chapter 4 that when these metabolites were administered as a single injection 24 hours post-infection in lean mice both HAI and microneutralization titers were elevated relative to the lean mice that received vehicle control. Interestingly, when these metabolites were administered in parallel with infection only, 14-HDHA resulted in elevated HAI and microneutralization titers and increased the percentage and number of $CD138^+$ plasma cells in the bone marrow. Thus, these results indicate timing and dosage of DHA-derived metabolites are crucial in attaining their effects. Several studies have revealed that obese mice do not have defects in generating neutralizing and non-neutralizing antibody upon influenza infection. However, these antibodies are not protective upon challenge. As a result, whether increased antibody production by DHA and its downstream metabolites are actually protective in the obese population needs further exploration.

Female mice do not have impaired B cell numbers in the bone marrow or spleen and do not have decreased levels of SPM precursors and SPMs.

Current literature on how obesity influences the B cell response in females is limited. The studies that do address this suggest that females generally exhibit a stronger response to vaccination compared to males during premenopausal years (350,351). Findings from this dissertation attempt to address this gap. In Chapter 2, we reported that obese females do not display large reductions in the B cell populations of the bone marrow and spleen as we reported in obese males. Studies from obese women revealed that the percentage of plasma cells was increased relative to their control counterparts. Females are known to have increased B cell numbers compared to males (352,353). Thus, obese females could have a constant flux of B cells due to an increased pool of B cells, which could explain increased plasma cells in these females. We also established in Chapter 2 that obese females displayed no deficiencies in 14-HDHA, 17-HDHA, PDX, or RvD1. This was likely due to several reasons. First, females synthesize DHA at higher rates than males do (272,273,275). Next, the capacity to generate DHA from the parent n-3 PUFA, ALA is much higher in women suggesting that DHA is probably converted to its downstream mediators more efficiently in obese females than obese males. Therefore, these results highlight lipid metabolism as a potential therapeutic target in obese females and further studies should explore whether differences in androgens between the two sexes could be contributing to the differences in lipid metabolism.

Finally, we established that obese female mice have drastically different metabolic profiles compared to their obese male counterparts, which could also account for the differences in SPM precursors and SPM levels in the females. Obese female mice

weighed less than obese males even though their fat mass was relatively similar. Obese females also did not display drastic adipose tissue inflammation as seen in the obese males and fasting insulin levels were not elevated. In addition, when challenged with CpG + α-IgM, B cells from obese female mice produced more IL-10 compared to their control counterparts. These increased IL-10 levels were not found when B cells from obese male mice were challenged. Thus, IL-10 and the effects of estrogen may exert protective B cell responses in the obese female mice as estrogen has been shown to increase somatic hypermutation and class switching recombination (246,278,279). A recent study found that females may benefit more from anti-leukotriene (LT) therapy more than males due to androgen differences. This was evident as female blood and leukocytes treated with testosterone displayed decreased survival compared to those treated with the 5-LOX-activating protein inhibitor, MK886 (276).

Implications of the Research

Obesity is associated with an impaired humoral immune response, which results in decreased responses to vaccinations and increased susceptibility to infections (84,222,333). B cells, which mediate the humoral immune response are a major cellular target in DIO models as they are necessary for high-affinity antibody production and seem to have a pathological role in tissues such as the adipose (88). Mechanisms detailing how the B-cell response is impaired in obesity are few and far between in the literature. Studies have suggested that impaired leptin signaling and impairments in class-switching recombination could be potential mechanisms driving B cell impairments in the obese population (101,285102,354). However, many questions involving B cell function in the

context of obesity remain unclear. Various studies have shown that specialized pro-resolving lipid mediator (SPM) precursors and SPMs, which promote resolution of the inflammatory response are decreased in various tissues in obesity (159,210,211). As these mediators can target and regulate B cells and select subsets, antibody production, and class switching, we investigated whether deficiencies in DHA-derived metabolites could be driving impaired B cell responses in the obese population.

Overall, findings from this dissertation propose that administration of DHA-derived metabolites that are deficient in obese mice can regulate B cells and enhance antibody production. Throughout this thesis, we identified that B cells are dysregulated in various tissues in obese mice including the bone marrow, spleen, and adipose. Specifically, in the bone marrow we found that obese mice had reduced number of B cells and number of B cell subsets including long-lived plasma cells. Administration of these DHA-derived metabolites to obese mice rescued decrements in the number of total B cells, B cell subsets, and long-lived plasma cells. We also established that DHA can enhance antibody production in obese mice infected with influenza, whereas 14-HDHA enhances antibody production in lean mice infected with influenza. Similarly, another study also found that 17-HDHA can enhance antibody production in mice that were infected with influenza (216). Therefore, results from our work and others demonstrate these DHA-derived metabolites have immune-stimulatory properties in B cells via enhancement of antibody production.

A novel finding from this dissertation was that DHA-derived metabolites not only limit recruitment of B cells to the VAT in obese mice, but also can directly modulate B cell subsets and antibody production in the adipose tissue. The VAT of obese mice is a

chronic inflammatory environment that is classified by excessive production of pro-inflammatory cytokines, chemokines, and increased recruitment of immune cells, including B cells (131,355). B cells from obese mice contribute to obesity-induced insulin resistance and produce pathogenic, IgG2c antibodies (89). Therefore, regulating the pro-inflammatory B cell phenotype in the obese VAT could be incremental in improving the impaired B cell response in obese individuals. Our findings reveal that DHA-derived metabolites directly regulate B cell numbers into the VAT of obese mice potentially through modulation of chemokine receptors. Interestingly, we also found that obese mice receiving 14-HDHA/17-HDHA/PDX had reduced antibody levels of various immunoglobulins that are produced by B cells in the VAT, which is likely driven by decreased numbers of B cells in the VAT. Overall, these findings advance the field by demonstrating that deficiencies in SPM precursors and SPMs could be exacerbating B cell-induced inflammation and impairments in the obese VAT.

Finally, we establish that the impaired B cell immune response seems to be sex-specific. Literature on the humoral immune response in obese females is very limited. However, data thus far suggest that women respond better to vaccinations and are less susceptible to certain diseases and viruses compared to men (351). This is attributed to increased numbers of B cells in females. To address this gap in the literature, we determined whether obese females had an impaired B cell response similar to that of their obese male counterparts. Interestingly, obese female mice had slight reductions in the number of B cells in the bone marrow, decreased adipose tissue inflammation, improved metabolic phenotype, and had increased B -cell production of IL-10. Obese female mice also did not display deficiencies in 14-HDHA, 17-HDHA, or PDX in the bone marrow and

spleen. Therefore, our findings set an important precedent that sex must be considered in the development of future vaccinations and therapeutics as females will more than likely respond differently.

Future Directions

An adequate B cell response is crucial in proper response to vaccinations and defense against foreign pathogens in addition to improving inflammation. Therefore, understanding the mechanisms by which the B-cell response is impaired in obesity is essential to improving vaccinations which protect the obese from various infections. While the findings of this dissertation suggest a potential mechanism by which the B-cell response is impaired, many unknowns still remain. Future studies should first and foremost address the role of DHA-derived metabolites in the VAT of obese mice. How SPMs regulate B cells in the VAT is not clear. As B cells display a pathogenic phenotype in the obese VAT, identifying potential novel therapeutics that could mediate or prevent this phenotype in the VAT would be crucial. As a result, future studies must address how SPMs are limiting B cell recruitment and whether these metabolites can alter B cell function in the adipose tissue. This would need to be answered by examining chemotaxis of the B cells to various tissues including the bone marrow, spleen, and bone marrow. Furthermore, B cell function in the VAT needs to be explored by analyzing B cell subsets such as germinal center B cells, plasma cells, and memory cells in the VAT and whether class switching can occur in the adipose tissue. In conclusion, much remains to be discovered in how the SPM family is regulating B cells in various tissues. Data from this dissertation suggest that these metabolites directly affect the B cell response and could

potentially modulate the pro-inflammatory B cell phenotype in the adipose and boost antibody production in the context of influenza. Therefore, these metabolites could potentially be a therapeutic target to enhance the impaired humoral response in obese individuals in the future.

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APPENDIX A: ANIMAL USE PROTOCOL

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION NOVEMBER, 2013

Project Title:

High fat diets modulate adaptive immune responses

	Principal Investigator	Secondary Contact
Name	S. Raza Shaikh	William Guesdon
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AUP # C059C			
New/Renewal Renewal 2/14/17			
Full Review/Date	DR/Date		
Approval Date 2/15/17			
Study Type obesity			
Pain/Distress Category D			
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC 11/4/14	EHS
OHP Enrollment		influenza PR8,	
Mandatory Training		transgenic	
Amendments Approved			

1. Please list all of the potentially painful or distressful procedures in the protocol:

Influenza infection.

2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):

Date Search was performed:	December 16, 2016
Database(s) searched:	Pubmed, Google Scholar
Time period covered by the search (i.e. 1975-2013):	All
Search strategy (including scientifically relevant terminology):	Obesity, infection, saturated fatty acids, monounsaturated fatty acids, n-3 and n-6 polyunsaturated fatty acids, antigen presentation, T cell proliferation, high fat diets, mechanisms, alternatives, immunological synapse, B-T cell synapse, lipid rafts, lipopolysaccharide (LPS), lymphocyte activation, membrane microviscosity, cell culture, influenza, B cell activation, LPS stimulation, B cell subsets, PR8 methods, pain control, distress and influenza
Other sources consulted:	Google

3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress.

When searching for the effects of high fat diets on the targets and mechanisms of adaptive immunity, the only alternative possibility is to use fatty acids in cell culture (i.e. replacement). However, this method does not depict the in vivo condition of consuming a high fat diet, and results from cell culture can lead to artifacts. In order to avoid these artifacts, feeding mice high fat diets and then isolating specific cell types for experiments is the closest to the in vivo condition. However, to address fundamental mechanisms, we implement the use of cell culture experiments to minimize the use of animals.

We also investigated the possibility of using heat-inactivated influenza in our studies in order to conduct cell culture studies on how influenza modulates B cell mediated immunity (again,

I. Personnel

A. Principal Investigator(s):

Dr. S. Raza Shaikh

B. Department(s):

Biochemistry

C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name/Degree/Certification	Position/Role(s)/ Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
William Guesdon	Postdoc	Yes	Extensive over 5 years with feeding and infecting.
Madison Sullivan	PhD student	Yes	Handling and feeding. Also, doing insulin/glucose tests for the past 3 years.
Ross Pennington	PhD student	Yes	Handling and feeding for the past two years.
Miranda Crouch	PhD student	Yes	Handling and feeding for the past 1.5 years and infecting with virus for the past 6 months.
Rasagna Kosaraju	Technician	Yes	Extensive over 5 years with feeding and infecting.
Kym Gowdy	collaborator	Yes	Extensive training with differing animal models and infectious agents.
S. Raza Shaikh	PI	Yes	Extensive training over the past 10 years in feeding, infecting.
Click here to enter text.	Click here to enter text.	Choose an item.	Click here to enter text.
Click here to enter text.	Click here to enter text.	Choose an item.	Click here to enter text.
Click here to enter text.	Click here to enter text.	Choose an item.	Click here to enter text.

II. Regulatory Compliance

A. Non-Technical Summary

Using language a non-scientist would understand, please provide a clear, concise, and sequential description of animal use. Additionally, explain the overall study objectives and benefits of proposed research or teaching activity to the advancement of knowledge, human or animal health, or good of society. (More detailed procedures are requested later in the AUP.)
Do not cut and paste the grant abstract.

We are studying how the composition of dietary fat influences the immune response of mice. We are particularly interested in understanding how we can improve the diet of obese mice (by changing the type of fat they consume) in order to improve their ability to combat inflammation and infection. Obese individuals are more prone to inflammation and infection; thus, aiding this population has long-term health consequences.

The study objectives are to determine if supplementing the diet of lean and obese mice with omega-3 fatty acids enhances the ability of mice to clear inflammation and infection. This will have an impact on developing sound clinical trials in humans with omega-3 fatty acids.

B. Ethics and Animal Use

B.1. Duplication

Does this study duplicate existing research? No
If yes, why is it necessary? (note: teaching by definition is duplicative)
Click here to enter text.

B.2. Alternatives to the Use of Live Animals

Are there less invasive procedures, other less sentient species, isolated organ preparation, cell or tissue culture, or computer simulation that can be used in place of the live vertebrate species proposed here? No
If yes, please explain why you cannot use these alternatives.
Click here to enter text.

B.3. Consideration of Alternatives to Painful/Distressful Procedures

a. Include a literature search to ensure that alternatives to all procedures that may cause more than momentary or slight pain or distress to the animals have been considered.

1. Please list all of the potentially painful or distressful procedures in the protocol:

Influenza infection.

2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):

Date Search was performed:	December 16, 2016
Database(s) searched:	Pubmed, Google Scholar
Time period covered by the search (i.e. 1975-2013):	All
Search strategy (including scientifically relevant terminology):	Obesity, infection, saturated fatty acids, monounsaturated fatty acids, n-3 and n-6 polyunsaturated fatty acids, antigen presentation, T cell proliferation, high fat diets, mechanisms, alternatives, immunological synapse, B-T cell synapse, lipid rafts, lipopolysaccharide (LPS), lymphocyte activation, membrane microviscosity, cell culture, influenza, B cell activation, LPS stimulation, B cell subsets, PR8 methods, pain control, distress and influenza
Other sources consulted:	Google

3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress.

When searching for the effects of high fat diets on the targets and mechanisms of adaptive immunity, the only alternative possibility is to use fatty acids in cell culture (i.e. replacement). However, this method does not depict the in vivo condition of consuming a high fat diet, and results from cell culture can lead to artifacts. In order to avoid these artifacts, feeding mice high fat diets and then isolating specific cell types for experiments is the closest to the in vivo condition. However, to address fundamental mechanisms, we implement the use of cell culture experiments to minimize the use of animals.

We also investigated the possibility of using heat-inactivated influenza in our studies in order to conduct cell culture studies on how influenza modulates B cell mediated immunity (again,

replacement and reduction strategy). However, the mechanisms by which heat-inactivated influenza activated B cells is not the same as the in vivo condition, where select cytokines from T cells aid in the development of antibody directed against influenza.

We have also investigated how distress and pain management will be addressed with the influenza studies (i.e. refinement). Distress will be minimized by providing a long sipper tube and/or hydrogel for hydration, as needed, in addition to placing food and nesting material on the bottom of the cage. We also employ humane endpoints to minimize pain and distress.

C. Hazardous Agents

1. Protocol related hazards (chemical, biological, or radiological):

Please indicate if any of the following are used in animals and the status of review/approval by the referenced committees:

HAZARDS	Oversight Committee	Status (Approved, Pending, Submitted)/Date	AUP Appendix I Completed?
Radioisotopes	**Radiation**	Click here to enter text.	Choose an item.
Ionizing radiation	**Radiation**	Click here to enter text.	Choose an item.
Infectious agents (bacteria, viruses, rickettsia, prions, etc.)	**IBC**	Approved 8/7/13 for influenza.	Yes
Toxins of biological origins (venoms, plant toxins, etc.)	**IBC**	Click here to enter text.	Choose an item.
Transgenic, Knock In, Knock Out Animals---breeding, cross breeding or any use of live animals or tissues	**IBC**	ALOX5 and ALOX12 knockouts. Approved April 13, 2016.	N/A
Human tissues, cells, body fluids, cell lines	**IBC**	Click here to enter text.	Choose an item.
Viral/Plasmid Vectors/Recombinant DNA or recombinant techniques	**IBC**	Click here to enter text.	Choose an item.
Oncogenic/toxic/mutagenic chemical agents	**EH&S**	Click here to enter text.	Choose an item.
Nanoparticles	**EH&S**	Click here to enter text.	Choose an item.
Cell lines, tissues or other biological products injected or implanted in animals	**DCM**	Click here to enter text.	Choose an item.
Other agents		Click here to enter text.	Choose an item.

2. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

No

III. Animals and Housing

A. Species and strains:

C57BL/6. ALOX5 and ALOX 12/15 are also on a C57 background

B. Weight, sex and/or age:

Generally 20g, males and females, and age starting arounf 4-8 weeks

C. Animal numbers:

1. Please complete the following table:

Total number of animals in treatment and control groups	Additional animals (Breeders, substitute animals)	Total number of animals used for this project
C57BL/6 - 655 males and 655 females ALOX5 – 80 males and 80 females ALOX12/15 30 males and 30 females	0	=1530

2. Justify the species and number (use statistical justification when possible) of animals requested:

The rationale for using mice is a continuation of our ongoing work with the NIH and industry groups. Based on the F-test in the ANOVA, we seek to have an effect size of 20% (for small to moderate changes). Thus, we require 10 mice per diet group to be statistically valid for our ex vivo studies.

3. Justify the number and use of any additional animals needed for this study:

Click here to enter text.

a. For unforeseen outcomes/complications:

None

b. For refining techniques:

None

c. For breeding situations, briefly justify breeding configurations and offspring expected:

We are no longer breeding

d. Indicate if following IACUC tail snip guidelines: Choose an item.
(if no, describe and justify)
N/A

4. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health, behavioral, physical abnormalities, or cause debilitating effects in experimental manipulations? Yes **(if yes, describe)**

No with the ALOX5 knockouts. In fact, we expect that by knocking these mice out, we will improve the response to inflammation and obesity.

With the ALOX12/15 knockout mice, we are aware of their cancer phenotype (myeloproliferatve disease/leukemia, anemia) (http://jem.rupress.org/content/203/11/2529.long); therefore, these mice will be used within 2-3 weeks of receiving them prior to the mice developing any complications. This will be essential given that the mice develop the disease starting at an age of 10 weeks.

5. Are there any deviations from standard husbandry practices?
No **If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, prolonged weaning times, wire-bottom cages, etc.):**
Click here to enter text.

6. The default housing method for social species is pair or group housing (including mice, rats, guinea pigs, rabbits, dogs, pigs, monkeys). Is it necessary for animals to be singly housed at any time during the study?
No **(If yes, describe housing and justify the need to singly house social species):**
Click here to enter text.

7. Are there experimental or scientific reasons why routine environmental enrichment should not be provided? No
(If yes, describe and justify the need to withhold enrichment)
Click here to enter text.

8. If wild animals will be captured or used, provide permissions (collection permit # or other required information):
N/A

9. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.
N/A

IV. Animal Procedures

A. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design. Briefly state surgical plans in this section. Surgical procedures can be described in detail in IV.S.

Overview: There are four major ongoing studies in the lab. Each project is described below:

Study #1: Assess the influence of dietary fatty acids on the ex vivo B cell immune response of male and female C57BL/6 mice in the absence of infection.

The objective of this study to understand if dietary fat improves B cell function. We will administer male and female mice the following diets that were developed by Dr. Barbara Mickelson at Envigo diets:

1. Control diet – mice will be consuming a purified mouse chow that matches in ingredients to the experimental diets. The control diet is essentially standard mouse chow except the ingredients are highly purified.
2. High fat diet – mice will administered a high fat diet (45% or 60% of total kcal from fat depending on the study).
3. High fat diet + omega -3 fatty acids. We will supplement the high fat diet with fish oil that is enriched in omega-3 fatty acids. The dose of fish oil is 4 grams per day, which is biologically relevant for humans.
4. High fat diet + monounsaturated fatty acids. Here we use olive oil that contains oleic acid and/or palmitoleic acid. The dose is 4 grams of oil per day, which is biologically relevant for humans.

For each study, 10 C57BL/6 mice per diet will be put on the diet for 10-20 weeks (depending on current study results). Here is a breakdown of the numbers required:

1. Ex vivo B cell phenotyping study for bone marrow and spleen. 10 male mice per diet x 4 diets = 40 mice x 3 time points (10 weeks, 15 weeks, 20 weeks) = 120 male mice
2. Ex vivo B cell phenotyping study for bone marrow and spleen. 10 female mice per diet x 4 diets = 40 mice x 3 time points (10 weeks, 15 weeks, 20 weeks) = 120 female mice

Study #2: Assess the influence of dietary fatty acids on the ex vivo B cell immune response of male and female C57BL/6 mice upon influenza infection.

The objective of this study is the same as study #1, except we are now assessing B cell function upon influenza infection. We will administer male and female mice the following diets:

1. Control diet – mice will be consuming a purified mouse chow that matches in ingredients to the experimental diets. The control diet is essentially standard mouse chow except the ingredients are highly purified.
2. High fat diet – mice will administered a high fat diet (45% or 60% of total kcal from fat).
3. High fat diet + omega -3 fatty acids. We will supplement the high fat diet with fish oil that is enriched in omega-3 fatty acids. The dose of fish oil is 4 grams per day, which is biologically relevant for humans.
4. High fat diet + monounsaturated fatty acids. Here we use olive oil that contains oleic acid and/oe palmitoleic acid. The dose is 4 grams of oil per day, which ia biologically relevant for humans.

For each study, 10 mice per diet will be put on the diet for 10-20 weeks (depending on the outcome). Here is a breakdown of the numbers required:

1. Ex vivo B cell phenotyping study for bone marrow and spleen. 10 male mice per diet x 4 diets = 40 mice x 3 time points (10 weeks, 15 weeks, 20 weeks) x 3 immunization/infection protocols = 360 mice
2. Ex vivo B cell phenotyping study for bone marrow and spleen. 10 female mice per diet x 4 diets = 40 mice x 3 time points (10 weeks, 15 weeks, 20 weeks) x 3 immunization/infection protocols = 360 mice

Infection protocols – there are three infection protocols (one is immunization, one is infection with influenza PR8, and the last is influenza CA09).

1. Protocol for immunization. After completing the experimental diets, mice will be immunized with recombinant HA protein derived from the human adapted influenza H1N1 A/California/04/2009 (2 µg; BEI Resources, Manassas, VA). Control animals will get a mock immunization injection, which is defined as PBS with 0.4% ethanol by volume without HA protein. HA immunizations will be delivered by i.m. injection in the left flank of the mouse.

2. Protocol for immunization followed by influenza infection with either CA/09 or PR8.After completing the experimental diets and the mice have been immunized for 3 weeks with either HA protein or PBS, the mice will be i.m. injected with 2.5 plaque forming units of the human adapted influenza H1N1 virus (A/California/4/2009). Alternatively, the mice will be infected with 7.5 plaque forming units of influenza PR8. The mouse adaptive influenza PR8 will be administered intranasally. When mice are ready to be infected with PR8, the mice will be anesthetized with isoflurane. Isoflurane will be administered in a bell jar under a hard-ducted biosafety cabinet. A small amount of isoflurane will be placed on a cotton ball, which will not be in contact with the mouse. Mice will be removed from the container under anesthesia in order to infect with the PR8 virus. Upon completion, the cotton ball will be bagged in an appropriate disposal bag and the container will be disinfected inside and outside with Clidox for 10 minutes (for those materials that are not autoclavable) or autoclaved.

The dose of influenza virus A/Puerto Rico/8/34/H1N1; mount Sinai strain (30ul, 7.5 plaque-forming units) that is being used corresponds to 2 hemagglutinating units of A/PR8, wich can lead to 20% weight loss (Smith et al. Journal of Nutrition, 2007, 137: 1236-1243). The LD50 for PR8 is 100 plaque forming units. Peak viral replication occurs in the lungs at 2-4 days post-infection. To be safe, we define infectivity as 96 hours post-infection.

Adverse Effects/Humane endpoints. We anticipate no adverse effects with just immunization. Studies in which we will immunize and then infect mice will require us to monitor body weights. We do not expect any significant change in body weight given that the mice are immunized and will be protected against influenza infection. We will use humane endpoints which entail monitoring of body weight on a daily basis. Loss of 20% body mass and/or failure to groom will result in immediate euthanasia. In addition to weight loss, mice may exhibit lethargy, ruffled hair coat, hunched posture, dehydration, dyspnea, atonic bladder, or immobility and these are also criteria for euthanasia. These additional clinical signs (besides weight loss) are highly unlikely given our preliminary data (soon to be published) with the proposed dose of either CA/09 or PR8 .

Clinical signs and monitoring frequency. The key clinical sign that we will monitor will be body weight and failure to groom. Weight loss or of greater than 20% or lack of grooming will result in termination of the mice. Upon infection, the mice will be monitored 3 times per day up to four days post-infection. After this time frame, mice will monitored once per day until the termination of the study. With the dose that is being used, immobility, major weight loss, and other signs of distress are not anticipated.

Study #3: Determine how omega-3 derived lipid mediators influence the immune response after influenza infection

We have found that omega-3 fatty acids improve the ability of mice to combat influenza infection. Omega-3 fatty acids are converted to omega-3 lipid mediators in the spleens of mice. Thus, to establish mechanisms, the objective of this study is to inject omega-3 derived lipid mediators into lean and obese mice and measure ex vivo B cell responses.

1. Mice will be administered a control or high fat diet for 15 weeks. The control and high fat diets are described above in Study #1.

2. We will conduct select studies in which mice will be i.p. injected with 1 microgram or less of one or mixtures of several omega-3 derived lipid mediators. These mediators are protectin D1, maresin, resolvin D1, resolvin D2, and 17-HDHA. All are commercially available from Cayman Chemicals .

3. Either concominant with the injection of the omega-3-derived mediators or 2 weeks after injection, the mice will be subjected to one of the three infection protocols desdribed in Study #2.

The number of C57BL/6 mice for this study are the following:

5 mice per diet x 2 diets x 5 omega-3 derived mediators x 3 infection protocols (PR8, CA09, or vaccine) x 2 genders = 300 mice (150 male and 150 female). The protocols will rely on one of two timepoints, either concominant or after injection, depending on ongoing studies currently in the lab.

In addition, we will need 50 mice for ethanol only injections as controls.

Study #4: Assess the influence of dietary fatty acids on the immune response using LOX knockout mice

The objective here is to determine if administration of omega-3 fatty acids improves the immune response in LOX knockout mice. LOX knockouts are key to establish mechanism since these enzymes promote inflammation. Two strains will be used to carry out studies described above.

1. B6.129S2-Alox5^{tm1Fun}/J (ALOX5 knockouts). These mice have the 5-lipoxygenase knocked out, which has a central role in generation of prostaglandins and leukotrienes, most notably from macrophages. This enzyme generates arachidonic acid derived mediators for pro-inflammatory responses. Stock number is 004155.

2. B6.129S2-Alox15^{tm1Fun}/J (ALOX 12/15 knockouts). These mice have the 12/15-lipoxygenase knocked out, which has a central role in generation of prostaglandins and leukotrienes in select cell types. This enzyme generates arachidonic acid derived mediators for pro-inflammatory responses. These mice are prone to developing dysregulated splenic and bone marrow architecture driven by changes in myeloid populations. Stock number is 002778.

ALOX5 knockouts

1. We will conduct some in vitro B cell experiments. 20 males and 20 females will be used for isolating B cells from bone marrow and spleen. Total of 40 mice for this *in vitro* study.

2. We will conduct three studies with 10 mice per diet x 3 diets x 2 genders. The diets are lean control, high fat, high fat + omega-3.The studies are the following:

a) Body weight study for tissue collection (mice are fed experimental diets for 15 weeks). Total of 30 male mice for this study and 30 females.

b) Infection study – mice are infected with influenza PR8 (0.1LD50) and euthanized at 14 days post-infection. Total of 30 male mice and 30 females for this study.

Total number of ALOX5 knockouts: 160.

ALOX12/15 knockouts

30 males and 30 females for isolating B cells from bone marrow and spleen. Total of 60 mice for this *in vitro* study.

In sections IV.B-IV.S below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.

Please refer to DCM and IACUC websites for relevant guidelines and SOPs.

B. Anesthesia/Analgesia/Tranquilization/Pain/Distress Management For Procedures Other than Surgery:

Adequate records describing anesthetic monitoring and recovery must be maintained for all species.
If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:
N/A

1. **Describe the pre-procedural preparation of the animals:**
   a. **Food restricted for** N/A **hours**
   b. **Food restriction is not recommended for rodents and rabbits and must be justified:**

Click here to enter text.

c. Water restricted for Click here to enter text. **hours**

d. Water restriction is not recommended in any species for routine pre-op prep and must be justified:

Click here to enter text.

2. Anesthesia/Analgesia *for Procedures Other than Surgery*

	Agent	Concentration	Dose (mg/kg)	Max Volume	Route	Frequency	Number of days administered
Pre-procedure analgesic	Isoflurane	3-5% induction, 1-3% maintenance	N/A	3%	Nasal inhalation	Once	Just one day
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Reason for administering agent(s):

In order to infect with influenza

4. For which procedure(s):

Infection with influenza virus

5. Methods for monitoring anesthetic depth:

Toe pinch, respiratory rate

6. Methods of physiologic support during anesthesia and recovery:

Remove isoflurane and allow exposure to air

7. Duration of recovery:

Mice are monitored after the isoflurane is removed for 10-15 minutes. Mice are also monitored daily for the influenza infection.

8. Frequency of recovering monitoring:
Continuously, see point 7 above
9. Specifically what will be monitored?
Recovery of movement.
10. When will animals be returned to their home environment?
Immediately after infection
11. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:
None

C. Use of Paralytics

1. Will paralyzing drugs be used? No
2. For what purpose:
Click here to enter text.
3. Please provide scientific justification for paralytic use:
Click here to enter text.
4. Paralytic drug:
Click here to enter text.
5. Dose:
Click here to enter text.
6. Method of ensuring appropriate analgesia during paralysis:
Click here to enter text.

D. Blood or Body Fluid Collection

1. Please fill out appropriate sections of the chart below:

	Location on animal	Needle/catheter size	Volume collected	Frequency of procedure	Time interval between collections
Blood Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Body Fluid Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

E. Injections, Gavage, & Other Substance Administration

1. Please fill out appropriate sections of the chart below:

	Compound	Location & Route of admin	Needle/catheter/gavage size	Max volume admin	Freq of admin (ie two times per day)	Number of days admin (ie for 5 days)	Max dosages (mg/kg)
Injection/ Infusion	Influenza PR8	Intranasal	N/A	30ul	once	once	7.5 plaque formin g units (0.1 LD50)
Injection/ Infusion	Inactive influenza vaccinate	IM Left flank	30g	50 microlit ers (2micro grams)	once	once	2 microg rams
Injection/ Infusion	PBS/Ethanol (sterilized with bottle top filters and made fresh the day of an experiment)	IM Left flank	30g	50 microlit ers	once	once	
Injection/ Infusion	Omega-3 derived lipid mediators	i.p.	30g	50 microlit ers (2micro grams)	once	once	1 microg ram
Injection/ infusion	Ethanol	i.p.	30g	50 microlit ers (vehicle control)	once	once	N/A.

3. Pharmaceutical grade drugs, biologics, reagents, and compounds are defined as agents approved by the Food and Drug Administration (FDA) or

for which a chemical purity standard has been written/established by any recognized pharmacopeia such as USP, NF, BP, etc. These standards are used by manufacturers to help ensure that the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy. For all injections and infusions for CLINICAL USE, PHARMACEUTICAL GRADE compounds must be used whenever possible. Pharmaceutical grade injections and infusions for research test articles are preferred when available. If pharmaceutical grade compounds are not available and non-pharmaceutical grade agents must be used, then the following information is necessary:

a. **Please provide a scientific justification for the use of ALL non-pharmaceutical grade compounds. This may include pharmaceutical-grade compound(s) that are not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.** Although researchers at conferences tout omega-3 derived lipid mediator compounds as pharmaceutical, they are indeed only research grade. After speaking with Cayman chemicals (the only company that makes these), they do not provide these as pharmaceutical grade compounds.

b. **Indicate the method of preparation, addressing items such as purity, sterility, pH, osmolality, pyrogenicity, adverse reactions, etc. (please refer to ECU IACUC guidelines for non-pharmaceutical grade compound use), labeling (i.e. preparation and use-by dates), administration and storage of each formulation that maintains stability and quality/sterility of the compound(s).**

Omega-3 derived lipid mediators - These are prepared through organic synthesis and their purity is verified through LC-MS. All of the omega-3 derived mediators are a minimum of 95% purity. Sterility is not an issue since they are in 95% Ethanol. The compounds are labeled with the name of the molecules and generally use-by dates are for one year. If the date is shorter than one year (depending on when it was manufactured), the company will notify us during the order. The stability is ensured by storage at -80C.

F. Prolonged restraint with mechanical devices

Prolonged restraint in this context means *beyond routine care and use procedures* for rodent and rabbit restrainers, and large animal stocks.

Prolonged restraint also includes *any* use of slings, tethers, metabolic crates, inhalation chambers, primate chairs and radiation exposure restraint devices.

1. **For what procedure(s):**
Click here to enter text.
2. **Explain why non-restraint alternatives cannot be utilized:**
Click here to enter text.
3. **Restraint device(s):**
Click here to enter text.
4. **Duration of restraint:**
Click here to enter text.
5. **Frequency of observations during restraint/person responsible:**
Click here to enter text.
6. **Frequency and total number of restraints:**
Click here to enter text.
7. **Conditioning procedures:**
Click here to enter text.
8. **Steps to assure comfort and well-being:**
Click here to enter text.
9. **Describe potential adverse effects of prolonged restraint and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
Click here to enter text.

G. Tumor Studies, Disease Models, Toxicity Testing, Vaccine Studies, Trauma Studies, Pain Studies, Organ or System Failure Studies, Shock Models, etc.

1. **Describe methodology:**
See section IV.A for G.1.-5.
2. **Expected model and/or clinical/pathological manifestations:**
Click here to enter text.
3. **Signs of pain/discomfort:**
Click here to enter text.
4. **Frequency of observations:**
Click here to enter text.
5. **Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

H. Treadmills/Swimming/Forced Exercise

1. Describe aversive stimulus (if used):
Click here to enter text.
2. Conditioning:
Click here to enter text.
3. Safeguards to protect animal:
Click here to enter text.
4. Duration:
Click here to enter text.
5. Frequency:
Click here to enter text.
6. Total number of sessions:
Click here to enter text.
7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

I. Projects Involving Food and Water Regulation or Dietary Manipulation

(Routine pre-surgical fasting not relevant for this section)

1. Food Regulation

a. Amount regulated and rationale:
Click here to enter text.
b. Frequency and duration of regulation (hours for short term/weeks or months for long term):
Click here to enter text.
c. Frequency of observation/parameters documented (i.e. recording body weight, body condition, etc.):
Click here to enter text.
d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

2. Fluid Regulation

a. Amount regulated and rationale:
Click here to enter text.

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):
Click here to enter text.

c. Frequency of observation/parameters documented (body weight, hydration status, etc.):
Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

3. Dietary Manipulations

a. **Compound supplemented/deleted and amount:**
N-3 polyunsaturated fatty acids at 2% of energy (human equivalent of approximately 2.4 grams per day)

b. **Frequency and duration (hours for short term/week or month for long term):**
See protocol above. This ranges in time based on the study but generally 10-15 weeks.

c. **Frequency of observation/parameters documented:**
Body weights are monitored every other day and documented weekly.

d. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
There are no adverse effects of administering n-3 polyunsaturated fatty acids. In fact, we expect to see improvements in immunological outcomes.

J. Endoscopy, Fluoroscopy, X-Ray, Ultrasound, MRI, CT, PET, Other Imaging

1. Describe animal methodology:
Click here to enter text.

2. Duration of procedure:
Click here to enter text.

3. Frequency of observations during procedure:
Click here to enter text.

4. Frequency/total number of procedures:
Click here to enter text.

5. Method of transport to/from procedure area:
Click here to enter text.

6. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

7. Please provide or attach appropriate permissions/procedures for animal use on human equipment:
Click here to enter text.

K. Polyclonal Antibody Production

1. **Antigen/adjuvant used and justification for adjuvant choice:**
   Click here to enter text.
2. **Needle size:**
   Click here to enter text.
3. **Route of injection:**
   Click here to enter text.
4. **Site of injection:**
   Click here to enter text.
5. **Volume of injection:**
   Click here to enter text.
6. **Total number of injection sites:**
   Click here to enter text.
7. **Frequency and total number of boosts:**
   Click here to enter text.
8. **What will be done to minimize pain/distress:**
   Click here to enter text.
9. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
   Click here to enter text.

L. Monoclonal Antibody Production

1. Describe methodology:

Click here to enter text.

2. Is pristane used: Choose an item.

Volume of pristane:

Click here to enter text.

3. Will ascites be generated: Choose an item.

i. **Criteria/signs that will dictate ascites harvest:**
Click here to enter text.

ii. **Size of needle for taps:**
Click here to enter text.

iii. **Total number of taps:**
Click here to enter text.

iv. **How will animals be monitored/cared for following taps:**
Click here to enter text.

4. What will be done to minimize pain/distress:

Click here to enter text.

5. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

M. Temperature/Light/Environmental Manipulations

1. Describe manipulation(s):

Click here to enter text.

2. Duration:

Click here to enter text.

3. Intensity:

Click here to enter text.

4. Frequency:

Click here to enter text.

5. Frequency of observations/parameters documented:

Click here to enter text.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

N. Behavioral Studies

1. Describe methodology/test(s) used:
Click here to enter text.

2. Will conditioning occur? If so, describe:
Click here to enter text.

3. If aversive stimulus used, frequency, intensity and duration:
Click here to enter text.

4. Length of time in test apparatus/test situation: ***(i.e., each test is ~10 mins)***
Click here to enter text.

5. Frequency of testing and duration of study: ***(i.e., 5 tests/week for 6 months)***
Click here to enter text.

6. Frequency of observation/monitoring during test:
Click here to enter text.

7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

O. Capture with Mechanical Devices/Traps/Nets

1. Description of capture device/method:
Click here to enter text.

2. Maximum time animal will be in capture device:
Click here to enter text.

3. Frequency of checking capture device:
Click here to enter text.

4. Methods to ensure well-being of animals in capture device:
Click here to enter text.

5. Methods to avoid non-target species capture:
Click here to enter text.

6. Method of transport to laboratory/field station/processing site and duration of transport:
Click here to enter text.

7. Methods to ensure animal well-being during transport:
Click here to enter text.

8. Expected mortality rates:
Click here to enter text.

9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

P. Manipulation of Wild-Caught Animals in the Field or Laboratory

1. Parameters to be measured/collected:
Click here to enter text.
2. Approximate time required for data collection per animal:
Click here to enter text.
3. Method of restraint for data collection:
Click here to enter text.
4. Methods to ensure animal well-being during processing:
Click here to enter text.
5. Disposition of animals post-processing:
Click here to enter text.
6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

Q. Wildlife Telemetry/Other Marking Methods

1. Describe methodology (including description of device):
Click here to enter text.
2. Will telemetry device/tags/etc. be removed? Choose an item. **If so, describe:**
Click here to enter text.
3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

R. Other Animal Manipulations

1. Describe methodology:
Click here to enter text.
2. Describe methods to ensure animal comfort and well-being:
Click here to enter text.
3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

S. Surgical Procedures

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species.

1. Location of Surgery (Building & Room #):
Click here to enter text.

2. Type of Surgery (check all that are appropriate):
Click here to enter text.

☐ **Non-survival surgery (animals euthanized without regaining consciousness)**
☐ **Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function)**
☐ **Minor survival surgery**
☐ **Multiple survival surgery**
If yes, provide scientific justification for multiple survival surgical procedures:
Click here to enter text.

3. Describe the pre-op preparation of the animals:

a. **Food restricted for** Click here to enter text. **hours**
b. **Food restricted is not recommended for rodents and rabbits and must be justified:**
Click here to enter text.
c. **Water restricted for** Click here to enter text. **hours**
d. **Water restriction is not recommended in any species for routine pre-op prep and be justified:**
Click here to enter text.

4. **Minimal sterile techniques will include (check all that apply):**

Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species and type of surgery (survival vs. non-survival).

☐ **Sterile instruments**

How will instruments be sterilized?

Click here to enter text.

If serial surgeries are done, how will instruments be sterilized between surgeries:

Click here to enter text.

☐ **Sterile gloves**
☐ **Mask**
☐ **Cap**
☐ **Sterile gown**
☐ **Sanitized operating area**
☐ **Clipping or plucking of hair or feathers**
☐ **Skin preparation with a sterilant such as betadine**
☐ **Practices to maintain sterility of instruments during surgery**
☐ **Non-survival (clean gloves, clean instruments, etc.)**

5. **Describe all surgical procedures:**

a. Skin incision size and site on the animal:

Click here to enter text.

b. Describe surgery in detail (include size of implant if applicable):

Click here to enter text.

c. Method of wound closure:

Click here to enter text.

i. Number of layers

Click here to enter text.

ii. Type of wound closure and suture pattern:

Click here to enter text.

iii. Suture type/size/wound clips/tissue glue:

Click here to enter text.

iv. Plan for removing of skin sutures/wound clip/etc:

Click here to enter text.

6. Anesthetic Protocol:

a. If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:
Click here to enter text.

b. Anesthesia/Analgesia For Surgical Procedures

	Agent	Dose (mg/kg or %)	Volume	Route	Frequency	Number of days administered
Pre-operative analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post-operative Analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

c. Methods that will be used to monitor anesthetic depth (include extra measures employed when paralyzing agents are used):
Click here to enter text.

d. Methods of physiologic support during anesthesia and immediate post-op period (fluids, warming, etc.):
Click here to enter text.

e. List what parameters are monitored during immediate post-op period. Provide the frequency and duration:
Click here to enter text.

f. Describe any other manipulations that will be used to alleviate pain, distress, and/or discomfort during the immediate post-op period (soft bedding, long sipper tubes, food on floor, dough diet, etc.):
Click here to enter text.

g. List criteria used to determine when animals are adequately recovered from anesthesia and when the animals can be returned to their home environment:
Click here to enter text.

7. Recovery from Surgical Manipulations (after animal regains consciousness and is returned to its home environment)

Click here to enter text.

a. What parameters (behavior, appetite, mobility, wound healing, etc.) will be monitored:

Click here to enter text.

b. How frequently (times per day) will animals be monitored:

Click here to enter text.

c. How long post-operatively (days) will animals be monitored:

Click here to enter text.

8. Surgical Manipulations Affecting Animals

a. Describe any signs of pain/discomfort/functional deficits resulting from the surgical procedure:

Click here to enter text.

b. What will be done to manage any signs of pain or discomfort (include pharmacologic and non-pharmacologic interventions):

Click here to enter text.

c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

V. Euthanasia

Please refer to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition and DCM Guidelines to determine appropriate euthanasia methods.

A. Euthanasia Procedure. ***All investigators, even those conducting non-terminal studies, must complete this section in case euthanasia is required for humane reasons.***

1. Physical Method- If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a scientific justification must be provided:

Overdose with CO_2, followed by cervical dislocation. In the case of infected mice in the biocontainment area, mice will be overdosed with isoflurane followed by cervical dislocation.

2. Inhalant Method- Choose an item.

(if other, describe the agent and delivery method)

Click here to enter text.

3. Non-Inhalant Pharmaceutical Method (injectables, MS-222, etc.)- Please provide the following:

a. Agent:

Click here to enter text.

b. Dose or concentration:

Click here to enter text.

c. Route:

Click here to enter text.

B. Method of ensuring death (can be physical method, such as pneumothorax or decapitation for small species and assessment method such as auscultation for large animals):

Absence of heartbeat

C. Describe disposition of carcass following euthanasia:

Biohazardous and non-biohazardous waste are disposed in appropriately labeled coolers in the animal facility.

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature: e-mail **Date:** 2/14/17

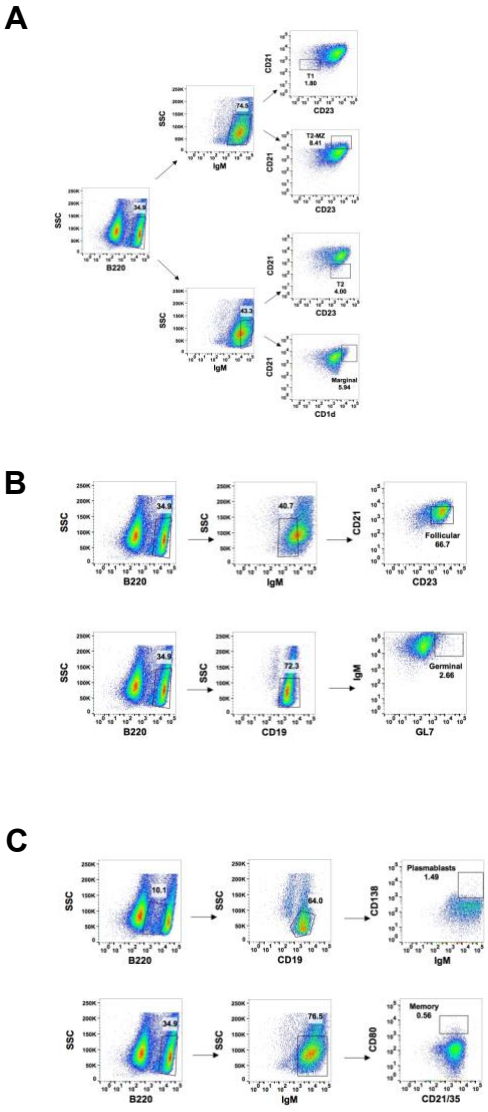
Veterinarian: Karen A. Tippelt **Date:** 2/14/17

IACUC Chair: [signature] **Date:** 2/15/17

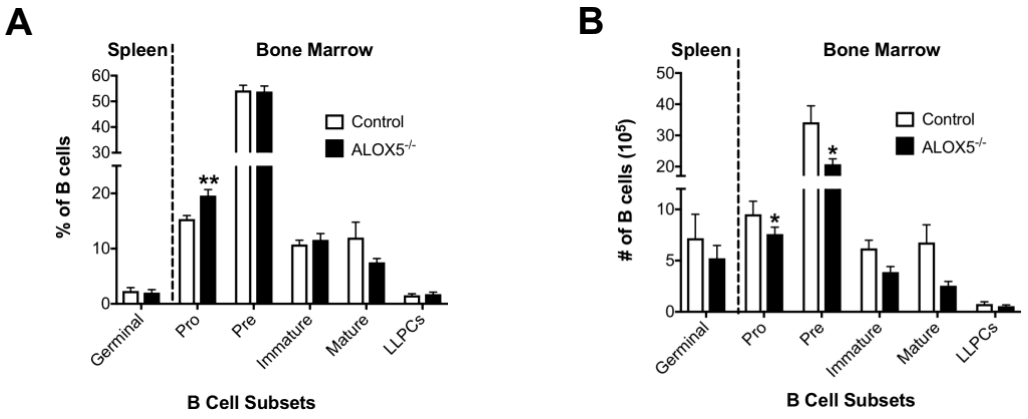
→ Pending IBC approval
KAD 2/14/17
2/15/17

APPENDIX 1-HAZARDOUS AGENTS			
Principal Investigator: S. Raza Shaikh	Campus Phone: 744-2585		Home Phone: (317)409-9565
IACUC Protocol Number: AUP CO59a	Department: Biochemistry & Molecular Biology		E-Mail: shaikhsa@ecu.edu
Secondary Contact: Jarrett Whelan Department: Biochemistry	Campus Phone: 744-2119	Home Phone (252) 414-8700	E-Mail: WHELANJ@ecu.edu
Chemical Agents used:N/A		Radioisotopes used:N/A	
Biohazardous Agents used: Influenza PR8, LPS	Animal Biosafety Level: 2		Infectious to humans? No
PERSONAL PROTECTIVE EQUIPMENT REQUIRED:			
Route of Excretion: Feces, urine, exhalation			
Precautions for Handling Live or Dead Animals: For influenza: With live animals, the major concern is getting bit; thus, we will double glove (even though the mice will be anesthetized). With dead animals, mice will be placed in a biohazard bag and labeled appropriately to indicate infection. For LPS: there are no precautions required.			
Animal Disposal: For influenza: Mice will be disposed in the standard freezers. Infectivity is not an issue since the virus clears in about 48 hours and will not exist in the dead animals. To be safe, we will define infectivity as 96 hours post infection. For LPS: mice will be disposed in the standard freezers.			
Bedding/Waste Disposal: For influenza: During the 48 hours in which the mice can spread infection, proper clothing and precautions with bedding will be required. Bedding should be autoclaved if changed during the 48 hours after infection. Disinfectant should also be used during handling. For LPS: there is no need for special bedding or waste disposal.			
Cage Decontamination: For influenza: During the 48 hours in which the mice can spread infection, the cages should be autoclaved. In addition, disinfectant should also be used during handling. For LPS: no need for special cage decontamination.			
Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment: For influenza: Mice will be in an isolated area that are infected, personnel will be double gloved, and appropriate work will be done in a biosafety cabinet. For LPS: there are no special precautions.			
Initial Approval Safety/Subject Matter Expert Signature & Date Approved 3/25/14 3/25/14			

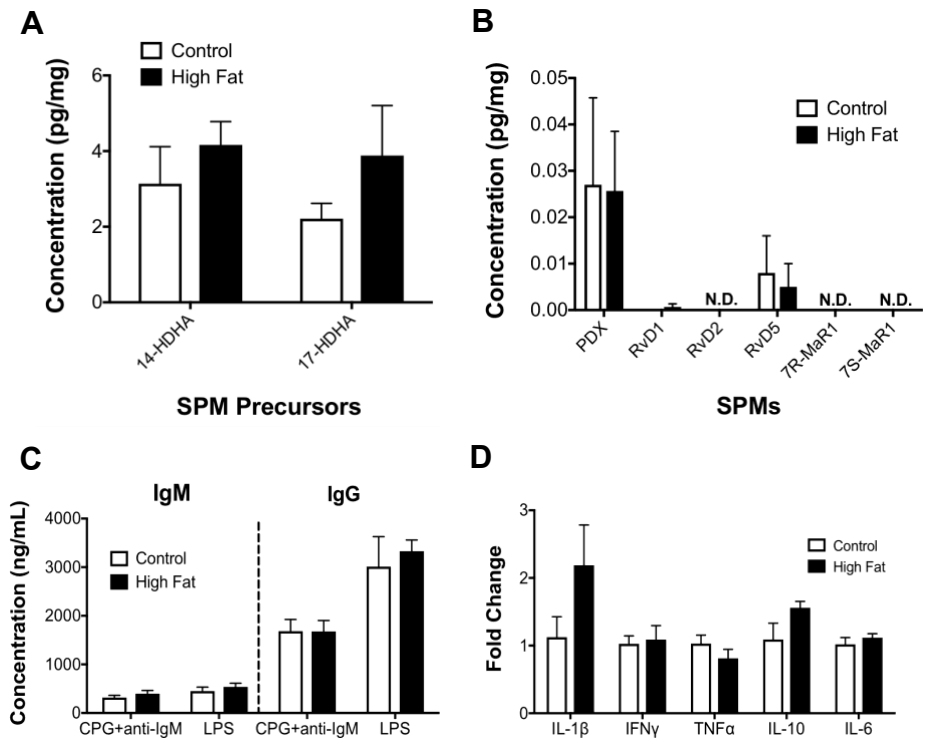
APPENDIX B: SUPPLEMENTAL MATERIAL



Supplemental Figure 2.1: Sample gating strategy for B cell subsets in the spleen. Gating strategy for (A) T1 ($B220^{+}IgM^{+}CD21^{low}CD23$), T2 ($B220^{+}IgM^{+}CD21^{-}CD23+$), T2-MZ ($B220^{+}IgM^{+}CD21^{high}CD23^{+}$), IgM^{+} memory ($B220^{+}IgM^{+}CD35^{+}CD80^{+}$), marginal ($B220^{+}IgM^{high}CD21^{+}CD1d^{high}$), (B) follicular ($B220^{+}IgM^{low}CD21^{int}CD23^{+}$), germinal B cells ($B220^{+}CD19^{+}IgM^{+}GL7^{high}$), and (C) plasmablasts ($B220^{low}IgM^{+}CD138^{+}$).



Supplemental Figure 2.2: 5-LOX is not driving the defects in the percentages and numbers of B cell subsets in the bone marrow and spleen. Flow cytometry analysis of the (A) percentage and (B) number of B cell subsets in the spleen and bone marrow of control and ALOX5$^{-/-}$ mice. Mice were received at 5 weeks of age and aged for 15 weeks to match the age of mice used in obesity studies. N=8 mice per diet for (A, B). Data are average ± S.E.M. *P<0.05 by an unpaired Student's t-test.



Supplemental Figure 2.3: Obese female mice have no defects in the levels of DHA-derived SPM precursor/SPMs and B cell functional responses. Lipidomic analysis of (A) DHA-derived SPM precursors and (B) SPMs in the whole spleen of control and obese female mice. (C) Splenic B cell IgM and IgG levels from control and obese female mice upon stimulation with CpG-ODN+anti-IgM or LPS stimulation for 3 days. (D) qRT-PCR analysis of IL-1β, IFN-γ, TNFα, IL-10, and IL-6 in the splenic B cells of control and obese female mice. Female mice were fed experimental diets for 15 weeks. N=3 mice per diet for (A, B), N=4 for (C), and N=7 for (D). Data are average $\pm$ S.E.M.

Supplemental Table 2.1: Primer sets used for qRT-PCR.

Gene	Forward Primer	Reverse Primer
GAPDH	GGTGTGAACGGATTTGGCCGTATT	GTCGTTGATGGCAACAATCTCCAC
β-actin	GCAGCCACTGTCGAGTC	GCAGCGATATCGTCATCCAT
Alox5	CTGCTGGACAAGGCATTCTA	TCCATCCCTCAGGACAATCT
12/15 LOX	CTCTCAAGGCCTGTTCAGGA	GTCCATTGTCCCCAGAACCT
BLT1	GGCACTAAGACAGATTCAAGGA	AGGATGCTCCACACTACAAAG
ERV1/ChemR23	CGACTTCCTGTTCAACATCT	AAGACGATGGTAAGGTAGCA
ALX/FPR2	CCTTGGCTTTCTTCAACAGC	GCACAGTGGAACTCAAAGCA
PPARγ	GCCTAAGTTTGAGTTTGCTGTG	AGCAGGTTGTCTTGGATGTC
IFNγ	CTCTTCCTCATGGCTGTTTCT	TTCTTCCACATCTATGCCACTT
IL-1β	CTTCCAGGATGAGGACATGAG	GCCTGTAGTGCAGTTGTCTAA
TNFα	GCCTCCCTCTCATCAGTTCTAT	CACTTGGTGGTTTGCTACGA
IL-10	AGAGCCACATGCTCCTAGA	CCTGCATTAAGGAGTCGGTTAG
IL-6	TCTCTGGGAAATCGTGGAAATG	ATCCAGTTTGGTAGCATCCATC
DRV2/GPR18	CTCTCTCTGGGACTGGGCAG	GGTGGCCATCTTACAGCAGG

Supplemental Table 2.2: Subject characteristics for non-obese and obese female subjects. Characteristics of non-obese and obese females used for specific studies on B cell subsets and functional outcomes. Each group of subjects had N=10. The table depicts the average age and BMI for each group. Participants were not type-2 diabetic and were non-smokers. **p<0.01.

Parameters	Non-obese (n=10)	Obese (n=10)
Age (mean and range)	36.2 (33-43)	39.1 (31-52)
BMI (mean and range)	20.5 (18.2-23.3)	46.8 (31.0-47.4) **
Race *n (%)*		
Caucasian	8 (80.0%)	9 (90.0%)
African American	1 (10.0%)	0 (0%)
Asian	1 (10.0%)	1 (10.0%)
Medications *n (%)*		
Proton Pump Inhibitor		1 (10.0%)
Antidepressant		1 (10.0%)
Benzodiazepine		1 (10.0%)
GABA Analog		1 (10.0%)
Vitamin	1 (10.0%)	
Antihistamine	1 (10.0%)	1 (10.0%)
Diuretic		1 (10.0%)
Birth Control	1 (10.0%)	2 (20.0%)

Supplemental Table 2.3. Concentration of 14-HDHA, 17-HDHA, and PDX after 4 days of administering the cocktail in obese mice. An injection of 900ng of 14-HDHA/17-HDHA/PDX was delivered i.p. per mouse for 4 consecutive days. Splenic concentration was measured with mass spectrometry. Mice were fed experimental diets for 15 weeks. N=3 mice. Data are average ± S.E.M.

SPM precursor or SPM	Concentration (ρg/mg of splenic tissue)
14-HDHA	3.11 ± 0.74
17-HDHA	6.73 ± 2.39
PDX	1.06 ± 0.41
RvD5	0.035 ± 0.009

Supplemental Table 3.1: Primer sets used for chemokine qRT-PCR studies.

Primer	Forward	Reverse
CCL2 (MCP-1)	GCTCAGCCAGATGCAGTT	TTGTAGCTCTCCAGCCTACT
CCL8 (MCP-2)	AGTGCTTCTTTGCCTGCT	ATGAAAGCAGCAGGTGACT
CXCL10	TCCCTCTCGCAAGGAC	TTGGCTAAACGCTTTCAT
CCL5	TGCCCTCACCATCATCCTCACT	GGCGGTTCCTTCGAGTGACA
CCR2	ATTCTCCACACCCTGTTTCG	GATTCCTGGAAGGTGGTCAA
CCR3	TTCACCAGAGACAAGTAGAATGG	TGGTGCCCACTCATATTCATAG
CXCR3	GCCATGTACCTTGAGGTTAGT	CAGAGAAGTCGCTCTCGTTT
CCR5	GCCATGTACCTTGAGGTTAGT	CAGAGAAGTCGCTCTCGTTT
β-actin	GCAGCCACTGTCGAGTC	GCAGCGATATCGTCATCCAT

Supplemental Table 4.1: Diet composition for murine obesity models.

Murine High Fat Diet Model			
	Control	High Fat	
Ingredients	g/kg	g/kg	
Lard	18.95	316.6	
Soybean Oil	23.69	32.31	
Corn Starch	298.44	0.0	
Maltodextrin	33.16	161.53	
Sucrose	331.59	88.91	
Cellulose (fiber)	47.37	64.61	
Casein	189.48	258.45	
L-Cystine	2.84	3.88	
Mineral Mix S10026	9.47	12.92	
DiCalcium Phosphate	12.32	16.80	
Calcium Carbonate	5.21	7.11	
Potassium Citrate, 1 H2O	15.63	21.32	
Vitamin Mix V10001	9.47	12.92	
Choline Bitartrate	1.89	2.58	
FD&C Yellow Dye #5/ FD&C Blue Dye #1	0.05	0.06	
Murine Western Diet Model			
Ingredients	Control	Western Diet (WD)	WD + DHA
Soybean Oil	50.0	15.0	15.0
Anhydrous milk fat	0.0	223.0	204.6
DHA ethyl ester	0.0	0.0	18.4
Corn Starch	369.98	98.94	98.94
Maltodextrin	140.0	140.0	140.0
Sucrose	150.0	225.0	225.0
Cellulose (fiber)	50.0	24.0	24.0
Casein	185.0	205.0	205.0
L-Cystine	2.5	3.0	3.0
Mineral mix, AIN-93M-MX	35.0	44.0	44.0
Vitamin mix, AIN-93-VX	15.0	19.0	19.0
Choline bitartrate	2.5	3.0	3.0
TBHQ antioxidant	0.02	0.06	0.06

APPENDIX C: COPYRIGHT TRANSFER AGREEMENTS

May 1, 2019

Miranda Crouch
East Carolina University
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