A Role for B-1 B cells and IgM antibodies in Obesity-Induced Glucose Intolerance and Insulin Resistance

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ABSTRACT

Obesity, characterized by an excess of adipose tissue, is a leading cause of metabolic disease. It is now well accepted that obesity-induced adipose tissue inflammation contributes to systemic insulin resistance and glucose intolerance that can lead to type 2 diabetes. B cells and their secreted antibodies have recently emerged as important regulators of adipose tissue inflammation and insulin resistance associated with obesity, but roles for specific B cell subsets are still unclear. The helix-loop-helix factor *Id3* mediates B cell function and obesity development, suggesting it may link B cells and metabolism. Here, we used a mouse containing a B cell-specific deletion of *Id3* ($Id3^{Bcell\ KO}$) to study the role B cells play in diet-induced adipose tissue inflammation and glucose intolerance. In addition, we assessed an obese human cohort for associations between adipose tissue B cells, natural IgM antibodies, and indices of inflammation and insulin resistance. $Id3^{Bcell\ KO}$ mice had increased numbers of visceral adipose tissue B-1b B cells and attenuated high-fat diet (HFD)-induced glucose intolerance compared to littermates. Omental visceral fat from $Id3^{Bcell\ KO}$ mice displayed enhanced local natural IgM secretion. Furthermore, $Id3^{Bcell\ KO}$ mice fed a short-term HFD had less inflammation and improved insulin signaling in omental fat compared to controls. Transfer of B-1b B cells null for *Id3* was sufficient to attenuate diet-induced glucose intolerance in *Rag1*$^{-/-}$ hosts, while B-1b B cells unable to secrete IgM had no effect. In humans, a recently identified $CD20^{+}CD27^{+}CD43^{+}$ B cell with B-1-like characteristics was identified within omental fat, and correlated with serum natural IgM levels. In addition, IgM antibodies were inversely associated with the

inflammatory chemokine MCP-1 and insulin resistance. Results presented here provide the first evidence that IgM antibody-producing B-1b B cells attenuate diet-induced glucose intolerance in mice. In addition, we link anti-inflammatory natural IgM antibodies with reduced inflammation and improved metabolic phenotype in obese humans. Together, these findings suggest role for B-1 B cells and natural IgM antibodies in mediated obesity associated metabolic dysfunction.

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LIST OF ABBREVIATIONS

BAFF-R	B cell activating factor receptor
BCR	B cell receptor
bHLH	Basic helix-loop-helix
B_{REGS}	B regulatory cells (undefined family)
CLS	Crown-like structures
CRP	C-reactive protein
DIO	Diet-induced obesity
dsDNA	Double-stranded DNA
FACS	Fluorescence-activated cell sorting
FALC	Fat-associated lymphoid clusters
FcμR	IgM Fc receptor
FFA	Free fatty acids
FO B cells	Follicular B cells (B-2 family)
FO B cells	Follicular B-2 B cells
GTT	Glucose tolerance test
HDL	High-density lipoprotein
HFD	High-fat diet
HLH	Helix-loop-helix
HOMA-IR	Homeostatic model assessment of insulin resistance
HSC	Hematopoietic stem cell
i.p.	Intraperitoneal
Id	Inhibitor of differentiation
Id3$^{-/-}$ mouse	Mouse globally null for *Id3*
$Id3^{B\ cell\ KO}$	B cell-specific *Id3* knockout mouse
IgM-IC	IgM immune complexes to apoB
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
lNFγ	Interferon gamma

IRS-1	Insulin receptor substrate-1
ITT	Insulin tolerance test
LDL	Low-density lipoprotein
LP-IR	Lipoprotein insulin resistance
LPS	Lipopolysaccharide
MBL	Mannose binding lectin
MCP-1	Monocyte chemoattractant protein-1
MDA-LDL	Malondialdehyde-low density lipoprotein
MHC	Major histocompatibility complex
MIP-2	Macrophage inflammatory protein-2
MS	Milky spots
MZ B cells	Marginal zone B cells (B-2 family)
NMR	Nuclear magnetic resonance
PAI-1	plasminogen activator inhibitor-1
PC	Phosphocholine
SC	Subcutaneous
$sIgM^{-/-}$ mouse	Mouse incapable of secreting IgM; does express surface IgM and secretes IgA, IgE, and IgG
SLE	Systemic Lupus Erythematosus
STAT3	Signal transducer and activator of transcription-3
SVF	Stroma-vascular fraction
T15-IgM; PC-IgM	IgM antibodies that recognize PC
T2D	Type 2 diabetes
TCR	T cell receptor
TGFβ	Transforming growth factor beta
T_H1	Type 1 helper T cells
T_H17	IL-17-producing helper T cells
T_H2	Type 2 helper T cells
TLR	Toll-like recptor
TNFα	Tumor necrosis factor alpha
TOSO	Fas apoptotic inhibitory molecule
T_{REGS}	T regulatory cells
VAT	Visceral adipose tissue

VAT	Visceral adipose tissue
VLDL	Very low-density lipoprotein
μMT mouse	Mouse that lacks mature B cells

CHAPTER 1:

Introduction

Pathogenesis of obesity

Obesity, defined by an excess of body fat, is an epidemic in the Western world, and dramatically increasing rates in developing countries have made it one of the prominent global health concerns of the 21st century. According to the World Health Organization, obesity rates have nearly doubled since 1980. Worldwide, 35% of adults are overweight, with 11% being obese. Obesity is a systemic disease that contributes to a wide range of metabolic, cardiovascular, and neurological disorders[1-3]. However, many of these pathophysiological effects stem from obesity-induced insulin resistance and glucose intolerance, two main factors that lead to the development of diabetes[4]. Thus, understanding how obesity impacts glucose metabolism is a key area of study with the ultimate goal of developing preventative treatments for many downstream consequences brought on by obesity.

Adipose tissue dysfunction during obesity

While obesity induces wide-ranging detrimental effects throughout the entire body, its impact on adipose tissue signaling and function is especially pronounced (Figure 1). Adipose tissue is a highly complex and essential metabolic organ made up of fat cells (adipocytes), connective tissue, vascular cells, and immune cells. In addition to being the predominant site for energy storage, adipose tissue secretes a wide variety of cytokines and hormones that regulate diverse processes including, but not limited to, satiety, blood pressure,

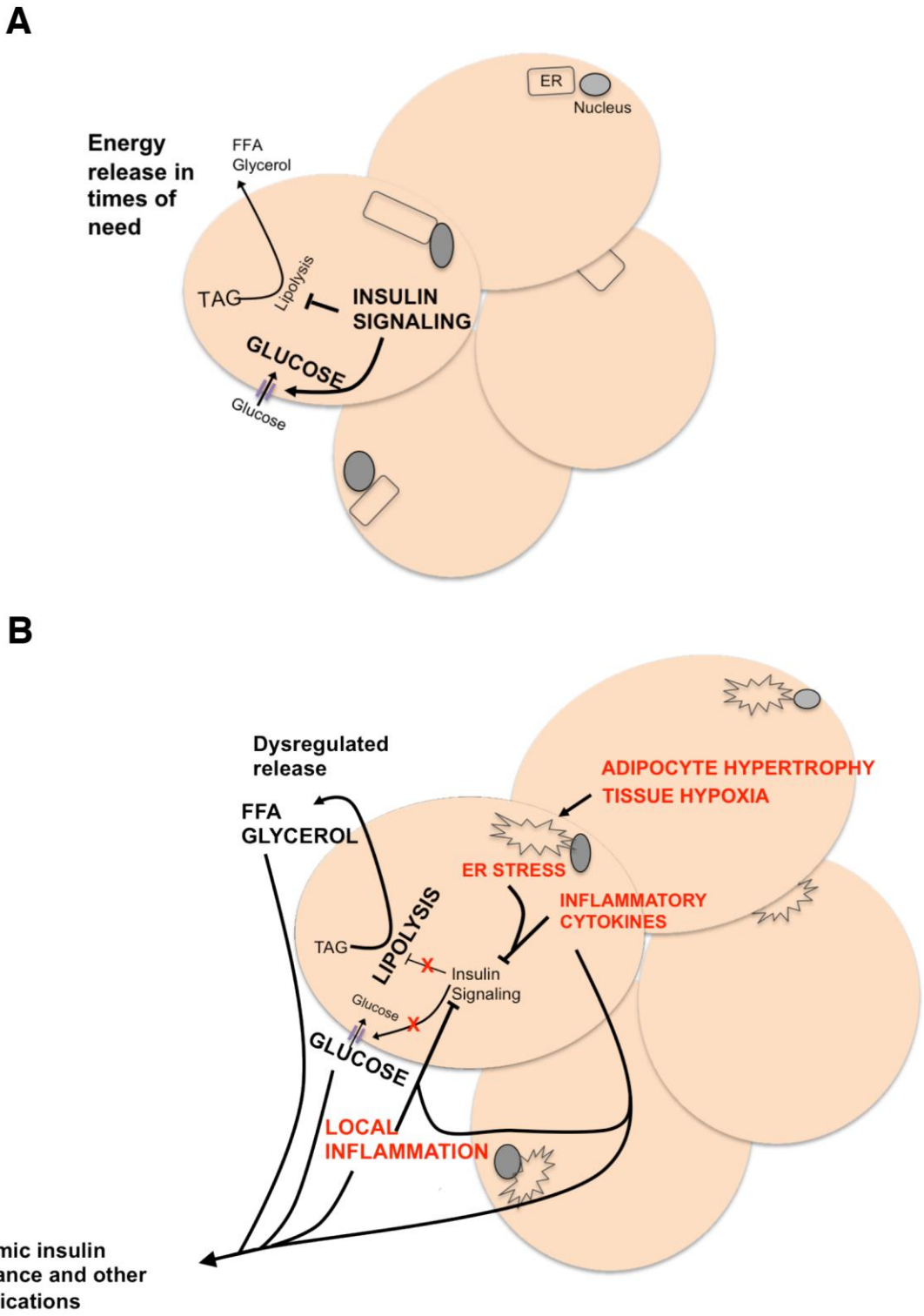

A
Energy release in times of need
FFA
Glycerol
ER
Nucleus
TAG
Lipolysis
INSULIN SIGNALING
GLUCOSE
Glucose
B
Dysregulated release
FFA
GLYCEROL
ADIPOCYTE HYPERTROPHY
TISSUE HYPOXIA
ER STRESS
INFLAMMATORY CYTOKINES
TAG
LIPOLYSIS
Insulin Signaling
Glucose
GLUCOSE
LOCAL INFLAMMATION
Systemic insulin resistance and other complications

Figure 1. Adipose tissue dysfunction during obesity. (A) Under normal conditions, glucose uptake and energy release are tightly regulated by insulin signaling. This promotes energy storage when nutrients are in excess and energy release during times of need. **(B)** In obese adipose tissue, ER stress caused by adipocyte hypertrophy and hypoxia, along with inflammatory cytokines, contribute to impair adipocyte response to insulin. Insulin resistant adipocytes display reduced glucose uptake and increased lipolysis. The uncontrolled release of metabolites and inflammatory factors by adipose tissue can lead to systemic lipo- and gluco-toxicity, insulin resistance, glucose intolerance, and other complications associated with obesity.

inflammation, and pancreatic insulin secretion[5, 6]. During the development of obesity, adipocyte hypertrophy leads to cellular ER stress and causes tissue growth. Expanding adipose tissue that outpaces the available oxygen supply creates a local hypoxic environment, which can further impair cellular function and cause cell death. In parallel, adipocytes, along with immune cells within adipose tissue, increase production of pro-inflammatory factors that serve to directly impact local signaling as well as recruit additional inflammatory cells as part of a feed-forward process. All of these factors contribute to impair adipocyte function and insulin signaling[7-15], which reduces adipocyte glucose uptake and promotes lipolysis. The resulting breakdown of stored triacylglyceride and release of free fatty acids (FFA) and glycerol contributes to systemic gluco- and lipo-toxicity that impairs metabolic function in other tissues. Utilization of tissue-specific knockout mice has provided evidence that enhancing insulin sensitivity in adipocytes improves hepatic and skeletal muscle insulin sensitivity[16-18], whereas impairing adipocyte insulin signaling leads to insulin resistance in these insulin-sensitive tissues[19, 20]. Interestingly, altering insulin sensitivity in either liver or muscle tends to only impact local signaling[21-24]. These findings indicate that obesity-induced adipose tissue insulin resistance has widespread systemic metabolic effects.

Adipose tissue distribution

Nearly 70 years ago, it was first observed that adult humans display two distinct patterns of fat distribution[25]. Subsequent studies demonstrated that

accumulation of upper-body fat, rather than lower-body fat, was associated with various chronic diseases[26]. Since then, many studies comparing anatomical fat distribution (reviewed elsewhere[27-29]) confirmed that intra-abdominal visceral adipose tissue (VAT) accumulation is more closely associated with impaired insulin sensitivity and type 2 diabetes development than increased subcutaneous (SC) fat[30-33]. Adipocytes from VAT are more metabolically active, and VAT is a greater source of obesity-induced pro-inflammatory cytokines than SC fat[8, 10]. In addition, surgical removal of VAT[34, 35], but not SC fat[36, 37], results in improved insulin sensitivity. In light of these findings, it is well accepted that obesity-induced changes in VAT signaling and function contribute strongly to downstream metabolic disease.

Inflammation and insulin resistance

Anti-inflammatory drugs have long been known to lower blood sugar levels in diabetic patients[38, 39], and studies in the 1980s noted systemic insulin resistance in pathological inflammatory states such as sepsis[40, 41], endotoxemia[42], and trauma[43]. However, it was not until 1991 that treatment of adipocytes with the inflammatory factor tumor necrosis factor alpha (TNFα) was shown to impair insulin-stimulated glucose uptake[44]. Several years later, this effect was rescued in obese rats by TNFα neutralization[45]. Additional mechanistic studies indicated that TNFα promotes insulin resistance through insulin receptor substrate-1 (IRS-1) serine phosphorylation, which inhibits insulin-induced IRS-1 tyrosine phosphorylation and prevents downstream insulin signaling[46-49]. The *in*

vivo relevance of these studies was confirmed when two groups independently generated TNFα knockout mice that were protected against high-fat diet (HFD)-induced glucose intolerance and insulin resistance[50, 51]. These findings sparked clinical studies that demonstrated circulating levels of TNFα, as well as other inflammatory factors such as interleukin-6 (IL-6) and C-reactive protein (CRP), were not only positively associated with human obesity, insulin resistance, and diabetes[52-56], but were also predictive of future type 2 diabetes (T2D) diagnosis in healthy patients[57, 58].

While analyzing circulating factors helped identify a novel link between inflammation and obesity-induced metabolic dysfunction, it was still unclear where this immune response was located or which cells were involved. In the same study that identified the insulin sensitizing effects of TNFα neutralization, Spiegelman and colleagues demonstrated that TNFα expression was elevated in adipose tissue of obese rodents[45]. Additional studies showed that TNFα expression in human adipose tissue correlated strongly with obesity and insulin resistance, and local expression decreased following weight loss and improved insulin sensitivity[59-61]. Further analysis of human and murine adipose tissue revealed enriched expression of various inflammatory markers including plasminogen activator inhibitor-1 (PAI-1), IL-6, and transforming growth factor beta (TGFβ)[62-65], together suggesting that adipose tissue may harbor previously unknown immune activity. Indeed, studies shortly thereafter in mice[66] and humans[67] identified macrophages in adipose tissue. Finally, in 2003, two seminal publications[68, 69] demonstrated that bone marrow-derived macrophages

accumulate in obese adipose tissue and produce several of the previously identified pro-inflammatory factors, thus contributing substantially to the local inflammatory environment. These findings strengthened the hypothesis that obesity is a disease of chronic inflammation, and that adipose tissue may be the epicenter of a detrimental immune response.

Macrophages and T cells in adipose tissue

Since these initial reports, much work has gone into characterizing the immune cell phenotype within adipose tissue, and how cell composition changes during obesity (Figure 2). Elegant studies utilizing genetic knockouts of inflammatory factors and transfer or ablation of specific immune cell subsets have helped identify key roles for macrophages and T cells in regulating adipose tissue function. Here, a brief summary of these findings is provided, as they have been reviewed extensively elsewhere[70-75]. In lean adipose tissue, alternatively activated M2 macrophages are found in abundance and secrete the anti-inflammatory cytokine interleukin-10 (IL-10) that blunts inflammatory cell activity and directly promotes adipocyte insulin sensitivity. IL-10 is also secreted by regulatory T cells (T_{REGS}), and type 2 helper (T_H2) T cells produce interleukin-4

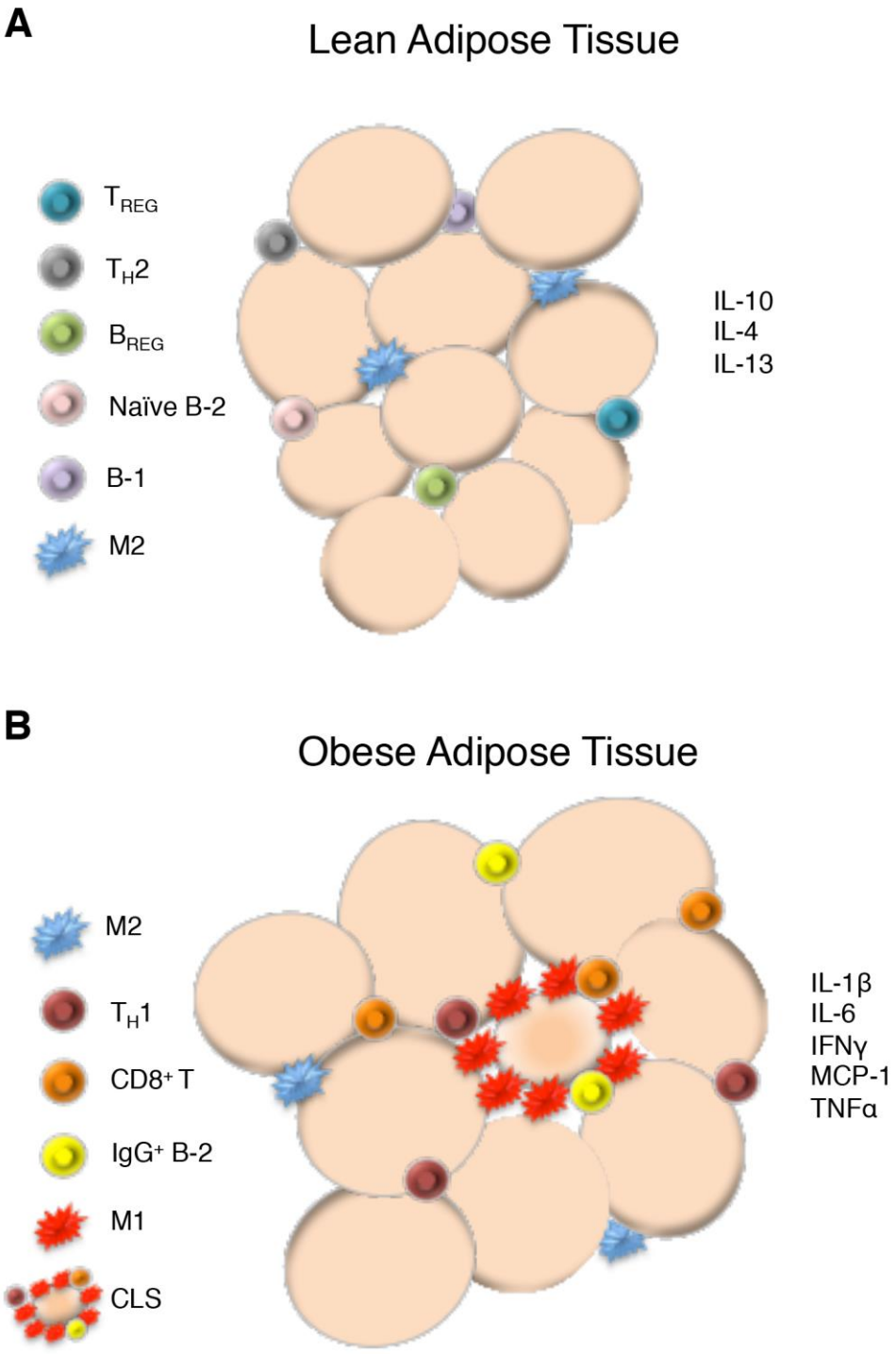

A
Lean Adipose Tissue
T_{REG}
T_H2
B_{REG}
Naïve B-2
B-1
M2
IL-10
IL-4
IL-13
B
Obese Adipose Tissue
M2
T_H1
$CD8^+$ T
IgG^+ B-2
M1
CLS
IL-1β
IL-6
IFNγ
MCP-1
TNFα

Figure 2. Obesity causes adipose tissue inflammation. (A) Resident immune cells in lean adipose tissue primarily possess regulatory functions and secrete anti-inflammatory cytokines. **(B)** During obesity, inflammatory immune cells infiltrate adipose tissue. Obese adipose tissue is characterized by elevated pro-inflammatory cytokines and clusters of M1 macrophages surrounding dead adipocytes called crown-like structures (CLS).

(IL-4) and interleukin-13 (IL-13) that help maintain M2 macrophage signaling. During the onset of obesity, visceral adipose tissue experiences an influx of inflammatory cells. Obese adipose tissue produces elevated levels of monocyte chemoattractant protein-1 (MCP-1) that serves to recruit circulating monocytes that differentiate into classically activated M1 macrophages once they enter the tissue. M1 macrophages form clusters around dead and dying adipocytes called crown-like structures (CLS) where they secrete high levels of TNFα and interleukin-1 beta (IL-1β). These factors impair adipocyte function by inhibiting insulin sensitivity and inducing secretion of inflammatory cytokines and lipids that further activate M1 macrophages. This feed-forward loop is further exacerbated by type 1 helper (T_H1) and $CD8^+$ cytotoxic T cells that enter obese adipose tissue and secrete interferon gamma (INFγ), a cytokine that also serves to activate M1 macrophages and impair insulin sensitivity in adipocytes. Together, these findings have not only identified a clear pathogenic effect of adipose tissue inflammation, but have illustrated that immune function within adipose tissue is a highly complex process involving many more participants than originally thought.

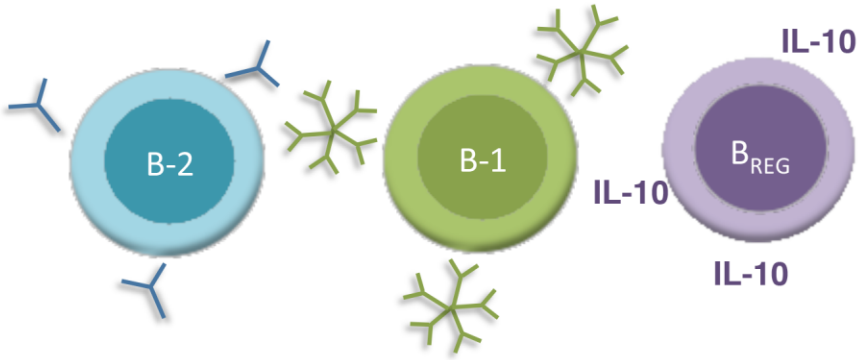
A role for B cells in obesity

B cells are unique immune cells that secrete antibodies, which bind specific antigens. While B cells are also found in adipose tissue[76-82], their role(s) in mediating local inflammation and adipose tissue function have not been fully explored. $CD19^+$ B cells have been reported to infiltrate murine epididymal adipose tissue prior to M1 macrophages during the early stages of diet-induced

obesity (DIO)[78], and B cells were found in sites of inflammation within SC adipose tissue of obese humans[80]. In addition, B cell-deficient (μMT) mice fed a HFD gain less weight, have reduced adipose tissue inflammation, and display improved insulin signaling and glucose tolerance compared to WT controls[77, 81], suggesting that B cells do play a role in regulating the metabolic effects of obesity. While only a few studies have looked in depth at specific roles B cells may play in this context, results highlighted below indicate that, similar to what we know about macrophages and T cells, B cell regulation of adipose tissue inflammation and insulin resistance is likely subset-dependent.

B cell subsets

B cells are divided into two functionally distinct families, B-1 and B-2 (Figure 3). Follicular (FO) B-2 B cells undergo class switching and somatic hypermutation in response to T-dependent antigen. These cells then either differentiate into long-lived plasma cells that spontaneously secrete high amounts of antibody, or become memory B cells that are primed for a rapid antibody response after antigen re-exposure[83]. Antibodies secreted from terminally differentiated FO B-2 cells are highly antigen specific and comprise an essential component of the adaptive immune system[84, 85]. Marginal zone (MZ) B cells are another class of B-2 B cells that reside in the splenic marginal zone[86]. MZ B cells



Major function	T-dependent, long-lived humoral immunity	T-independent, early defense, tissue homeostasis	Inflammation resolution
Major secreted product	High affinity, class-switched antibodies	Low affinity, IgM natural antibodies	IL-10
Role in obesity	-Increased inflammatory cytokine production in obesity -IgG antibodies promote adipose tissue inflammation, insulin resistance, and glucose intolerance	-Present in adipose tissue, but function unknown	-Decreased IL-10 production in obesity -B cell-derived IL-10 attenuates inflammation and insulin resistance

Figure 3. B cell subsets and known roles in obesity. B cells can be divided into two major subsets: B-1 and B-2. It is unclear whether IL-10-secreting B_{REG} B cells belong to either subset or develop independently. B cell subsets differ in their function within the immune system, and our limited understanding suggests their roles in obesity may vary as well. However, much more study is needed to understand how B cells function in adipose tissue and how they may impact obesity-associated metabolic disease.

are components of the innate immune system that respond immediately to circulating antigens filtered through the spleen.

B-1 B cells develop from a distinct lineage earlier in ontogeny than B-2 B cells and reside primarily in the coelomic cavities and spleen[87-89]. Although most characterization of B-1 B cells stems from murine studies, recently a human B cell subset with B-1-like characteristics was identified[90]. Despite being fewer in number than B-2 B cells, B-1 B cells possess the unique ability among B cells to self-renew[91], allowing for a smaller number of cells to maintain a functional population. B-1 B cells are further divided into B-1a and B-1b B cells that have overlapping functions, but exhibit differences in activation and response to infection[92-95]. Both B-1 subsets secrete antibodies that are termed “natural antibodies” because they are produced without previous antigen exposure. As antibodies from B-1 B cells are produced spontaneously or in immediate response to stimulation, are independent of T cell help, and do not require affinity maturation, B-1 humoral responses are quicker than those from B-2 B cells and play key preventative roles during the early stages of infection[96].

Regulatory B cells (B_{REGS}) are a recently discovered group of B cells that suppress inflammation primarily through IL-10 production[97, 98]. As B cells expressing a mix of surface markers generally used to define B-1 and B-2 B cell subsets have been shown to secrete IL-10, it is currently unclear whether these B cells represent unique developmental subsets or if IL-10 production is a shared feature among various B cell families. Given their potential therapeutic use in

treating autoimmune and inflammatory disease, additional studies of these unique B cells are needed.

B cell regulation of adipose tissue inflammation and obesity-induced metabolic dysfunction

Several studies have demonstrated specific roles for B cells in obesity-induced inflammation and downstream metabolic dysfunction (Figure 3). Mice null for B cell activating factor receptor (BAFF-R) have a severely reduced B-2 B cell population[99] and, when challenged with a HFD, display attenuated adipose tissue inflammation as well as improved insulin sensitivity and glucose tolerance compared to WT controls[100]. Another study found FO B cells from obese mice to secrete increased IL-6 and reduced IL-10 compared to cells from lean controls, suggesting an inflammatory phenotype[77]. Interestingly, mice fed a HFD displayed an influx of class-switched B-2 B cells, as well as IgG antibodies, within visceral adipose tissue[81]. Adoptive transfer of WT B-2 B cells, but not B-2 B cells with impaired ability to present antigen, were able to drive insulin resistance in μMT mice. This group went on to show that IgG, but not IgM, from DIO mice activated macrophage TNFα production and was sufficient to induce systemic insulin resistance and glucose intolerance. Together, these findings suggest that B-2 B cells promote diet-induced insulin resistance through an adaptive IgG humoral response.

Very little is known about potential roles of other B cell subsets in obesity and diet-induced metabolic dysfunction. B cells from type 2 diabetic patients

display impaired IL-10 secretion compared to B cells in non-diabetics[101]. A recent study identified IL-10-producing B cells within adipose tissue and showed that B cell-specific deletion of IL-10 attenuated the inflammatory effects of obesity and protected against HFD-induced insulin resistance and glucose intolerance[102]. These findings suggest that different B cell families may have contrasting functions in the context of obesity-induced metabolic disease. Although B-1 B cells are enriched in adipose tissue compared to other compartments[81] and comprise a large fraction of milky spots found in omental fat[82], their role in obesity is unknown.

Natural antibodies

Natural antibodies[103], primarily produced by B-1 B cells in mice, are generally of the IgM isotype and arise independent of antigen exposure in mice and humans. Unlike antibodies derived from B-2 B cells, natural antibodies do not undergo affinity maturation and contain few point mutations or non-templated nucleotide additions[104]. Interestingly, natural antibody repertoires of naïve individuals demonstrate similarity both within[105-108] and across[109, 110] species, providing evidence that these antibodies are not generated randomly. Indeed, natural antibody repertoires demonstrate preferential heavy and light chain pairings that allow for recognition of conserved structures such as carbohydrates, nucleic acids, and phospholipids that are often found on the surface of foreign pathogens[111-114]. In some cases, these same epitopes are expressed on damaged self-cells[109]; thus providing natural antibodies dual roles in preventing

infection and maintaining tissue homeostasis. The best-studied example of this multi-functionality is the T15 family of natural antibodies that recognize a phosphocholine (PC)[115] moiety expressed on pneumococcal cell membranes as well as on the surface of apoptotic cells[116] (Figure 4). IgM specific for PC (T15-IgM or PC-IgM) plays important roles in protecting against lethal *Streptococcus pneumoniae* infection[117, 118] and facilitating apoptotic cell clearance[116, 119, 120]. These studies have helped demonstrate a unique regulatory niche for natural antibodies in a layered immune system.

Natural IgM and inflammation

Multiple factors inherent to natural antibodies contribute to their general anti-inflammatory function[121, 122]. First, IgM is less inflammatory than IgG. The μ constant region of IgM antibodies cannot bind activating Fcγ receptors responsible for triggering inflammatory responses induced by certain IgG isotypes (i.e., IgG2a/c and IgG2b in mice). Second, natural IgM antibodies promote efficient apoptotic cell clearance that prevents the accumulation of dead cells[116, 119, 120, 123-125]. The cellular contents from uncleared dead cells promote inflammation and can introduce the body to normally hidden auto-antigens[126, 127]. Indeed, mice lacking the ability to secrete IgM have defective apoptotic cell

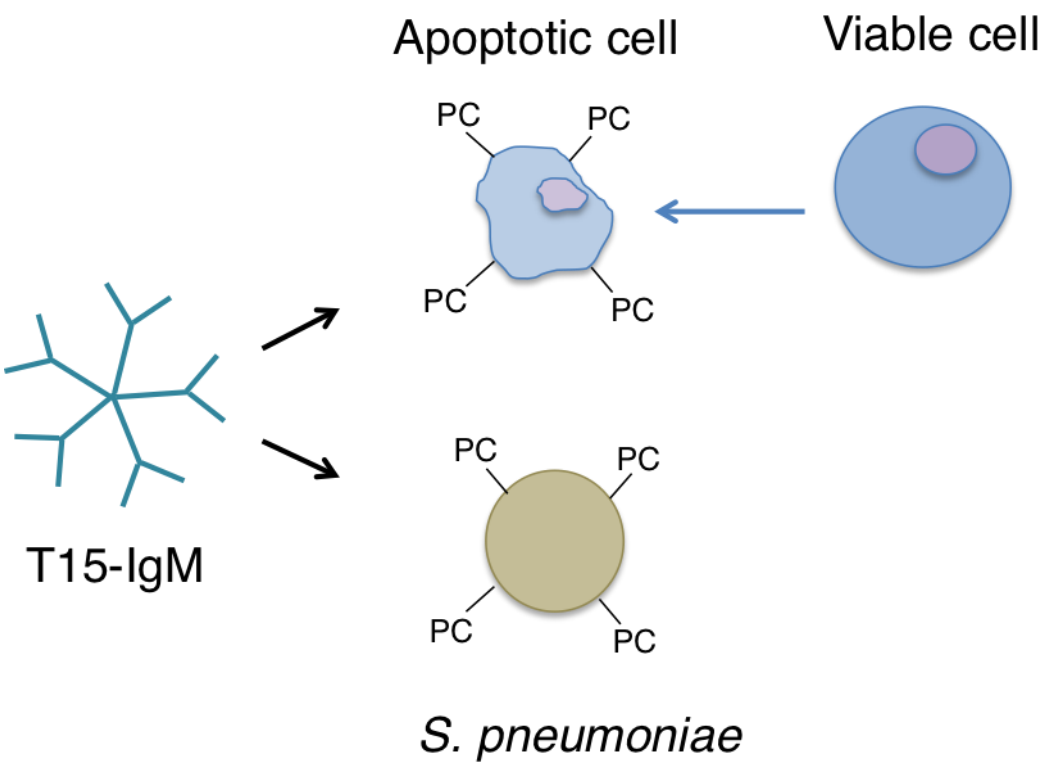

Apoptotic cell
Viable cell
PC
PC
PC
PC
PC
PC
PC
PC
T15-IgM
S. pneumoniae

Figure 4. Multifunctionality of natural IgM antibodies is best summarized by T15. Natural antibodies arise without previous antigen exposure and often recognize evolutionarily conserved moieties. The T15 family of natural antibodies recognizes a phosphocholine (PC) epitope that is expressed on certain gram positive bacteria such as *S. pneumoniae*, as well as on apoptotic cells. PC-recognizing IgM can promote bacteria neutralization and facilitate apoptotic cell clearance, thus playing important roles in initial defense against invading pathogens and in maintaining tissue homeostasis. (Adapted from *Binder, Nat. Med., 2002*[128])

clearance[123] and develop a Systemic Lupus Erythematosus (SLE)-like phenotype characterized by inflammatory IgG auto-antibodies directed at double-stranded DNA (dsDNA)[129]. In addition, IgM antibodies have been shown to reduce apoptotic cell numbers and macrophage content of atherosclerotic plaques[130], suggesting a protective role in a model of diet-induced inflammation. Third, recent evidence suggests IgM natural antibodies can directly attenuate inflammation in myeloid cells. While the mechanism has yet to be elucidated, T15-IgM treatment significantly attenuated lipopolysaccharide (LPS)-stimulated TNFα and IL-6 production by RAW264.7 macrophages and impaired primary dendritic cell activation[119]. Together, B-1 B cell-derived natural IgM antibodies can function through various pathways to attenuate inflammation. However, while IgM antibodies have been reported in adipose tissue[81], their ability to regulate diet-induced adipose tissue inflammation or downstream insulin resistance has not been evaluated.

Omental adipose tissue

The omentum is a unique fatty tissue attached to the greater curvature of the stomach that comprises the largest visceral adipose tissue depot in humans. The omentum has long been known for its role in preventing infection and promoting wound healing and tissue regeneration within the peritoneal cavity (reviewed elsewhere[131-133]). While recent studies in humans have shown omental adipose tissue inflammation associates with insulin resistance[134, 135], most rodent studies on adipose tissue function in obesity focus on perigonadal depots

(epididymal in males, parametrial in females) that are not present in primates or humans. Although these fat deposits make up the largest adipose regions in mice, their systemic drainage allows for secreted metabolites and cytokines to bypass the liver, thus diluting their metabolic impact. In contrast, inflammatory factors produced by portally-draining omental adipose tissue go directly into the liver where they can impact systemic metabolism[136, 137]. Indeed, epididymal adipose tissue transplants led to substantial systemic insulin resistance in recipients when connected portally compared to systemically[138]. While mice do have omental adipose tissue that displays similar structural characteristics as in humans[139], surprisingly few studies have evaluated murine omental fat in the context of obesity.

Omental milky spots

In addition to differences in drainage, the immune cell composition and organization distinguishes murine and human omental adipose tissue from other depots. In SC and epididymal fat, immune cells are primarily found interspersed between adipocytes. However, omental adipose tissue contains organized clusters of macrophages and B cells that reside adjacent to adipose-rich areas. These cell clusters are called milky spots due to their opaque white appearance, and have been described since the mid-nineteenth century[140]. Milky spots (MS) trap intraperitoneal (i.p.)-injected antigens, expand during peritoneal infection, and contribute moderate immunity in mice lacking traditional lymphoid tissues such as lymph nodes and Peyer's patches[82, 133, 141, 142]. While thorough immuno-

phenotyping of MS is lacking in humans, the majority of B cells residing in murine MS are B-1 B cells[82]. This is in contrast to the recently discovered fat-associated lymphoid clusters (FALCs) that populate human and murine mesenteric fat and contain large numbers of “natural helper” lymphocytes, but few B cells[143]. Thus, further study of omental fat may enhance our understanding of how B cells function within adipose tissue, and particularly, how their activity may mediate the metabolic dysfunction caused by obesity.

Inhibitor of differentiation proteins

Inhibitor of differentiation (Id) proteins are helix-loop-helix (HLH) factors that regulate transcription in a dominant-negative manner[144-146]. While Id proteins are known to regulate cell cycle progression, differentiation, and survival, their exact function can vary widely between cell types[147]. Four known Id homologs – named sequentially in order of discovery Id1-Id4 – exist in humans and rodents. In general, Id expression is highest in developing tissues and proliferating cells, while decreasing in healthy adult tissues and terminally differentiated or quiescent cells[147, 148]. Genetic knockout studies have demonstrated that while no single Id gene is essential for viability, various combinations of double Id knockouts confer embryonic lethality[149], suggesting that Id proteins have overlapping but non-redundant functions.

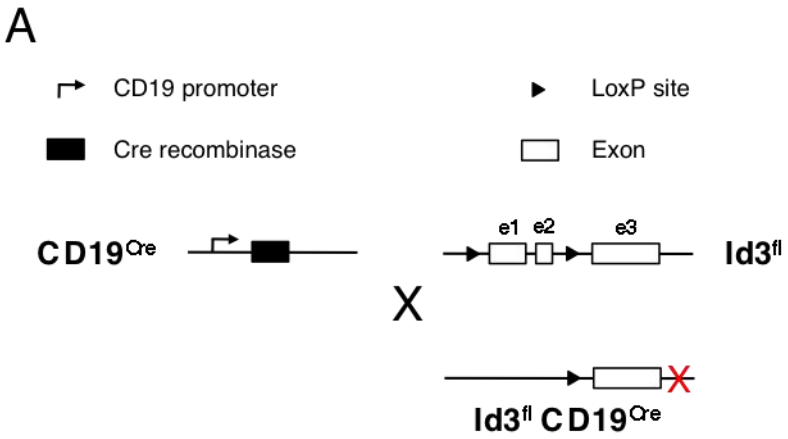
Id3: a link between B cells and obesity?

Id3 has been implicated in B cell development, activation, and antibody response. While *Id3* is similar to other Ids in that it is highly expressed in the embryo and progressively declines during development[150-152], *Id3* is present in both developing and mature lymphocytes[153, 154]. In developing lymphocytes and B cell progenitors, *Id3* expression prevents maturation by inducing apoptosis[155, 156] and downregulation of *Id3* is essential for B cell lineage commitment[157]. In contrast, *Id3* is quickly upregulated following antigen stimulation by B cell receptor (BCR) crosslinking and promotes BCR-mediated proliferation in B-2 B cells[158]. Furthermore, *Id3* has been shown to regulate immunoglobulin class-switching in activated B-2 B cells[158-160], suggesting that *Id3* may be involved in various aspects of antibody response.

Studies using mice globally null for *Id3* (*Id3*$^{-/-}$) suggest that *Id3* may be a key regulator of both B cell function and obesity development. While *Id3*$^{-/-}$ mice have relatively normal numbers of splenic B-2 B cells[158, 161], they have impaired antigen-specific antibody responses[158]. In contrast, several studies have indicated that *Id3*$^{-/-}$ mice have increased titers of IgM antibodies at baseline[162, 163]. Recently, we showed that global loss of *Id3* results in reduced VAT expansion and attenuated DIO[164]. In this study, we identified high *Id3* expression in the stroma-vascular fraction (SVF) of VAT, suggesting that *Id3* may function in a non-adipocyte cell in adipose tissue. Together, these findings suggest that *Id3* likely plays important roles both in regulating B cell function and obesity development.

B cell specific *Id3* knockout mouse

The use of the *Id3*$^{-/-}$ mouse has generated many questions regarding *Id3*'s role in B cell biology and obesity progression. However, *Id3*'s ubiquitous expression during development and known regulation of T cell function[165-169] made it necessary to generate a B cell-specific knockout mouse to address whether *Id3* function in B cells directly impacts DIO. To accomplish this, we crossed floxed *Id3* (Id3$^{fl/fl}$) mice[170] containing LoxP sites flanking the first two exons of *Id3* with CD19cre (CD19$^{Cre/+}$) mice containing the Cre recombinase gene knocked into the first coding exon of the pan B cell marker, CD19 (Figure 5A). The resulting *C57Bl/6 Id3$^{fl/fl}$ CD19$^{Cre/+}$* (Id3$^{B\ cell\ KO}$) mouse lacked *Id3* only in B cells (Figure 5B). As described in chapter 3, this mouse provided us a unique tool to explore potential ways B cells regulate HFD-induced metabolic dysfunction.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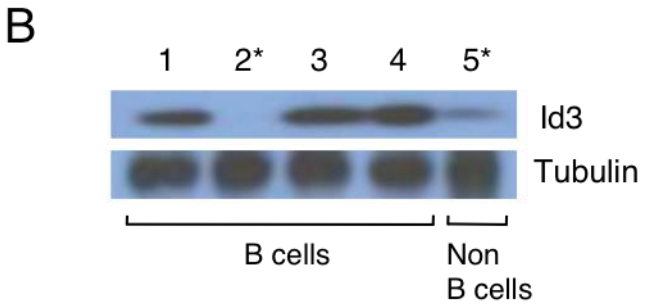

B
1 2* 3 4 5*
Id3
Tubulin
B cells
Non
B cells

Figure 5. Mouse containing a B cell-specific *Id3* deletion. **(A)** Schematic showing CD19cre x Floxed Id3 cross to generate Id3BcellKO mouse. **(B)** Id3 Western blot of purified splenic B cells (lanes 1-4) or B cell-depleted splenocytes (lane 5). Numbers represent cells from the following genotypes: 1=Id3fl/+ CD19cre/+; 2=Id3fl/fl CD19cre/+; 3=Id3fl/fl CD19+/+; 4=Id3+/+ CD19cre/+; 5=Id3fl/fl CD19cre/+. *Cells from $Id3^{Bcell\ KO}$ mice.

CHAPTER 2:

Materials and Methods

Animals:

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. *C57Bl/6J* and μMT mice were purchased from Jackson Laboratory (stock# 000664 & 002288, respectively). *Id3*$^{-/-}$ and *Id3*$^{fl/fl}$ mice were a generous gift of Dr. Yuan Zhang (Duke University), *CD19*$^{cre/+}$ and *Rag1*$^{-/-}$ mice were gifted by Timothy Bender (University of Virginia), and *sIgM*$^{-/-}$ mice were kindly provided by Dr. Peter Lobo (University of Virginia). *Id3*$^{fl/fl}$ mice were crossed to *CD19*$^{cre/+}$ mice to generate *Id3*$^{fl/fl}$*CD19*$^{cre/+}$ mice. These mice were then bred to *Id3*$^{fl/fl}$*CD19*$^{+/+}$ mice to generate *Id3*$^{fl/fl}$*CD19*$^{cre/+}$ (Id3$^{B\ cell\ KO}$) and littermate control *Id3*$^{fl/fl}$*CD19*$^{+/+}$ (WT) mice. All mice were on a pure C57BL/6 background, and confirmation of B cell specific *Id3* deletion was confirmed by PCR and Western blot[171]. Only male mice were used for experiments, and all animals were given standard chow diet and water *ad libitum* until they were genotyped. For high-fat feeding studies, littermates were placed on a 60% kCal fat diet (Research Diets, D12492) for the designated length of time.

Metabolic analysis

For all metabolic studies, mice were fasted for the designated time in individual wood chip-lined cages. Mice had access to water *ad libitum* throughout all experiments.

Glucose tolerance test (GTT): Mice were fasted overnight. At the beginning of each experiment, a small tail snip was made and baseline blood glucose levels were determined. Mice were then injected i.p. with 1.4-2.0g dextrose (Hospira)

per kg body weight, and blood glucose levels were measured at 10, 20, 30, 60, 90, and 120 minutes post-injection.

Insulin tolerance test (ITT): Mice were fasted for four hours. Baseline blood glucose levels were determined as in GTT. Mice were injected with (0.75U/kg) insulin (Eli Lilly), and blood glucose levels were measured at 15, 30, 60, 90, and 120 minutes post-injection and normalized to baseline readings.

Insulin signaling studies: Following an overnight fast, mice were injected with 10U/kg insulin (Eli Lilly). Mice were euthanized after five minutes and omental adipose tissue was removed and flash-frozen for later analysis (see tissue processing and Western blot protocol below).

Adoptive transfers:

B-2 adoptive transfer: Donor *C57Bl/6* and *Id3*$^{-/-}$ mice, along with host μMT mice were fed a HFD for eight weeks prior to transfer. Splenic B-2 cells (>98% purity) were harvested from donor animals as described previously[162]. Hosts received a single i.p. injection of either 10^7 B-2 B cells in 300μl saline or saline alone (vehicle control), and were continued on a HFD for the remainder of the experiment.

B-1b adoptive transfer: Pooled peritoneal fluid from 8-12 week old WT, Id3$^{\text{B cell KO}}$, or *sIgM*$^{-/-}$ donor mice was stained with fluorophore-labeled antibodies (see below). CD19^{+}B220$^{\text{mid/lo}}$IgM$^{\text{hi}}$CD5^{-} B-1b B cells were sorted and resuspended in 200μl saline. Eight-ten week old *Rag1*$^{-/-}$ hosts were injected with either $8.0\text{x}10^4$ B-

1b B cells or with saline alone (vehicle control). Hosts were allowed one week to recover before being placed on a HFD for the remainder of the experiment.

Tissue processing

Murine flow cytometry: Splenocytes, peritoneal cells, and epididymal stroma-vascular fraction were isolated as previously described[164, 171] Cells from omental adipose tissue were obtained using a "walk-out" method[172].

Human flow cytometry: Omental and subcutaneous adipose tissue was processed using methods adapted from Zimmerlin et al.[173]. In brief, adipose tissue was placed in PBS supplemented with 5.5mM glucose and 50μg/ml gentamicin and processed as soon after collection as possible. Ten (g) adipose tissue was minced with scissors and digested in 30ml PBS containing 1% BSA (Gemini) and 2.5g/L Collagenase II (Worthington) in a shaking 37° incubator for 15 minutes. PBS containing 0.1 % BSA and 1mM EDTA was added to stop the collagenase activity. The SVF was then successively passed through 425μm and 180μm sieves (WS Tyler), and finally through a 40μm filter. The remaining SVF was stained for flow cytometry.

Adipose tissue lysates for ELISA: Adipose tissue was homogenized in 2ml RIPA buffer containing protease inhibitors and lysed on ice for 30 minutes. Protein lysate was collected and used for ELISA analysis.

Adipose tissue lysates for Western blot: Ten (mg) adipose tissue was homogenized in 250μl protein lysis buffer (10% glycerol, 1% NP-40, 137mM NaCl, 25mM HEPES pH 7.4, 1mM EGTA) containing protease inhibitors (Sigma-

Aldrich) and phosphatase inhibitors (Sigma-Aldrich) and lysed on ice for 30 minutes. Protein lysate was collected and used for Western blot analysis.

Flow Cytometry:

Red blood cells were lysed if necessary with RBC lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.4). All cells were strained through 70μm filters and incubated with Fc-block (FCR-4G8, Invitrogen) for 10 minutes on ice prior to staining. Cells were stained on ice and protected from light for 20 minutes. Fc-block and antibodies were diluted in either FACS buffer (PBS containing 1% BSA and 0.05% NaN_3) for flow cytometry or sorting buffer (PBS containing 1% BSA) for cell sorting experiments. Murine flow cytometry antibodies: B220 (RA3-6B2), CD3ε (145-2C11), CD5 (53-7.3), CD11c (N418), CD19 (1D3), CD21 (4E3), CD23 (B3B4), F4/80 (BM8), and IgM (II/41) were purchased from eBioscience; Annexin V (Cat# 556420), BrdU (B44), and CD43 (S8) were purchased from BD Bioscience; CD206 (C068C2) was purchased from BioLegend. Human flow cytometry antibodies: CD43 (84-3C1) was purchased from eBioscience, CD3 (5KY), CD20 (L27), CD27 (M-T271), and CD45 (2D1) were purchased from BD Bioscience. Viability was determined by either LIVE/DEAD® fixable yellow cell staining (Invitrogen) or DAPI (Sigma-Aldrich). Cells were run on a CyAN ADP (Beckman Coulter), or sorted on a Reflection Cell Sorter (iCyt) or an Influx Cell Sorter (Benton-Dickenson). Data were analyzed with FlowJo software (Tree Star Inc) using fluorescence minus-one controls for

gate determination when appropriate. Counting beads (CountBright™ Absolute Counting Beads, Molecular Probes) were used for quantitation.

Gating strategies: Adipose tissue macrophages[174], peritoneal and adipose tissue B cells[175], and splenic B cells[176, 177] were identified as described previously.

ELISA:

Self-coated ELISAs: 96 well microtiter plates (Corning) were incubated at 4°C overnight with capture antibody diluted in coating buffer (0.1M disodium phosphate pH 9.0). Capture antibodies and concentrations for IgM and IgG assays: unlabeled IgM (625ng/ml), IgG1 (625ng/ml), IgG2b (1250ng/ml), IgG2c (2500ng/ml), or IgG3 (625ng/ml) (Southern Biotech). For T15-IgM assays: AB1-2 (303ng/ml) (ATCC, HB-33). For PC-IgM assays: PC-BSA (10μg/ml) (Biosearch Tech). Plates were blocked (PBS containing 0.5% BSA, 0.1% TWEEN-20, and 0.01% NaN_3), incubated with sample, and then treated with IgM or IgG detection antibody conjugated to alkaline phosphatase for two hours at room temperature. Detection antibodies and dilutions used: murine IgM-AP (IgM: 1:8000; T15-IgM: 1:1000), murine IgG-AP (IgG1: 1:8000; IgG2b: 1:4000; IgG2c: 1:4000; IgG3: 1:8000), human IgM-AP (PC-IgM: 1:1000) (all purchased from Southern Biotech). Plates were then developed with pNPP solution (Southern Biotech) for 30-60 minutes and read at 405nm using a SpectraMax 190 (Molecular Devices). IgM and IgG isotype concentration was determined through a standard curve of purified immunoglobulin (Southern Biotech) using a range of 0.78-200ng/ml. T15-IgM and PC-IgM levels were determined by normalization to serial dilutions of

standardized mouse (range: 1:50-1:25,600) or human serum (range: 1:50-1:12,800), respectively. Serum from *sIgM*$^{-/-}$ or *Rag1*$^{-/-}$ mice was used as negative controls, and EO6 (Avanti Polar Lipids Inc) was used as a positive control for T15-IgM assays. All dilutions were determined through careful titration, and only values within the range of standard curves with readings at least 3-fold higher than negative controls were used.

Commercial and previously validated ELISAs and metabolic assays: Mouse TNFα (eBioscience, 88-7324), IFNγ (R&D, MIF00), and FFA (Zen-Bio, SFA-1) levels, along with human MCP-1 (SABiosciences, SEH00192A) levels were determined with commercial kits. IgM-IC, IgG-IC, IgM MDA-LDL, and IgG MDA-LDL were determined as previously described[178].

Confocal microscopy

Epididymal and omental adipose tissue were fixed in 4% PFA overnight at 4°C. Samples were blocked and permeabilized for three hours at room temperature in PBS containing 5% normal rat serum and 0.3% Triton X-100. Adipocytes were stained with BODIPY Green (Invitrogen) for 20 minutes at room temperature and then with B220-AF647 (BioLegend) at 4° overnight. Samples were then stained for 20 minutes at room temperature with CYTOX Orange (Invitrogen) for nuclei detection. After washing, samples were mounted in a 50:50 PBS/glycerol solution on gelatin-coated slides. Whole-mounted specimens were imaged by confocal microscopy (Nikon; model TE200-E2). Z-stack Images (24μm range, 3μm step) were taken using a 10x objective. Images were adjusted using ImageJ software.

Omental ex vivo *culture*

Murine omental adipose tissue was removed, weighed, and placed in sterile 48 well cell culture plates containing 200µl media (RPMI-1640, 10% FBS, L-glutamine, penicillin-streptomycin, 55µM β-mercaptoethanol). Cultures were incubated overnight (TNFα and IFNγ studies) or for four days (T15-IgM studies) and supernatant was collected for ELISA analysis.

B-1b Proliferation and survival study

Mice (8-12 weeks of age) were injected with 40µg LPS (Sigma-Aldrich) or a saline vehicle control at t=0. All mice were injected with 1.5mg BrdU at t=24 and t=36 hours. At t=48 hours, mice were euthanized and omental adipose tissue was processed for flow cytometry and stained for B-1b B cell surface markers. Cells were then split into two groups: group 1 was stained for BrdU incorporation using a FITC BrdU Flow Kit (BD Biosciences), and group 2 was stained for Annexin V surface expression in the presence of Annexin V-binding buffer (10mM HEPES pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$). Cells were fixed and analyzed by flow cytometry as described above.

Western blot

Protein lysates were supplemented with 11% β-mercaptoethanol and 0.5% bromophenol blue. Samples were resolved on 4-20% Tris-glycine gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (BioRad).

Western blotting was carried out using an antibody against Thr308 pAKT (1:1,000, Cell signaling) or AKT (1:1,000, Cell signaling), followed by horseradish peroxidase-linked secondary antibody (Jackson). Immunoreactive bands were visualized by enhanced chemiluminescence after incubation with ECL or ECL plus reagent (Amersham Pharmacia Biotech). Relative AKT phosphorylation was determined by normalizing pAKT to total AKT in each sample.

Human studies

Patients were recruited through the Bariatric Surgery Clinic at the University of Virginia. All patients were ≥18 years of age and obese (BMI ≥30), and provided informed written consent prior to participation in the study. The study was approved by the Human IRB Committee at the University of Virginia, and all procedures were in accord with the declaration of Helsinki. Patients were divided into two groups, and both cohorts of bariatric patients were consented under the same criteria at UVA. Cohort 1 represents patients where we performed flow cytometry on fresh adipose tissue and blood (surgery dates between October, 2012 and October, 2013). Blood, subcutaneous adipose tissue, and omental adipose tissue were collected and processed within two hours and analyzed by flow cytometry. Cohort 2 represents patients from whom frozen tissue samples were analyzed by ELISA (surgery dates between May, 2009 and August, 2010). Metabolic syndrome was defined as any three of the following: large waist circumference (men: >40in; women: >35in), hypertension, low HDL (men: <40mg/dl; women: <50mg/dl), high triglcyerides (>150mg/dl), high blood glucose

(>100mg/dl). In all patients, pre-operative creatinine and blood glucose values were used. Serum was stored for future ELISA analysis and/or NMR lipid analysis (LipoScience Inc).

Statistics

For mouse data, a student's t-test was performed on data with normal distribution and equal variance. If data sets had unequal variance, a t-test with Welch's correction was used. For non-Gaussian distributed data, a Mann-Whitney test was performed to determine statistical significance. For comparisons of three or more independent variables, a one-way ANOVA test was used with a Kruskal-Wallis post-hoc test. Results are displayed containing all replicated experiments, and values shown are mean ± SEM unless otherwise noted. For human clinical data, baseline characteristics are reported as mean ± standard deviation for normally distributed variables, and as median with interquartile range for non-normally distributed variables. Spearman correlation coefficients were used to determine the strength of association between variables. Data were analyzed with Prism 6.0a (GraphPad Software, Inc.) or SAS 9.3 and displayed using Prism 6.0a.

CHAPTER 3:

Loss of *Id3* in B-1b B cells attenuates high-fat diet-induced glucose intolerance

Introduction

Obesity is an epidemic in the Western world, and dramatically increasing rates in developing countries have made it one of the prominent global health concerns of the 21st century. Obesity-induced visceral adipose tissue (VAT) inflammation leads to systemic insulin resistance and glucose intolerance[11]. While macrophages and T cells have been implicated in this process[70, 72], emerging evidence suggests that B cells also modulate obesity-induced adipose tissue inflammation and insulin resistance.

B cells have been identified in murine and human adipose tissue[76, 78, 79, 81, 82, 141, 179], and localize to sites of inflammation[80]. Recently, IgG, but not IgM, from DIO mice was shown to drive adipose tissue inflammation and promote systemic insulin resistance[81]. These pathogenic IgG antibodies were localized in VAT and came from adaptive B-2 B cells – the major B cell subset that differentiate into memory B and plasma cells capable of producing class-switched, high affinity antibodies[180, 181]. In mice, the majority of IgM derives from B-1 B cells, a self-renewing innate B cell population that differ from B-2 cells in response to stimuli and antibody repertoire[96]. B-1 B cells protect against early infection and are further divided into B-1a and B-1b subsets that have overlapping functions, but exhibit differences in activation and response to infection[92-95]. While B-1 B cells have known anti-inflammatory properties and are found in similar proportions within adipose tissue as B-2 B cells[81], their role(s) in mediating obesity-induced adipose tissue inflammation and insulin resistance remains unknown.

A major proportion of B-1-derived IgM are termed “natural antibodies” as they arise without previous antigen exposure and contain few mutations or N-additions[182]. IgM is less immunogenic than IgG[121] and natural antibodies often recognize epitopes expressed on both invading pathogens and damaged self-cells[182, 183], providing dual roles in curbing inflammation and promoting tissue homeostasis. Among the many known natural antibodies, the best studied are members of the T15 family that bind phosphocholine (PC)[115], an epitope present on pneumococcal cell membranes as well as on oxidized phospholipids of oxidized LDL and apoptotic cells[116]. T15 natural IgM antibodies (T15-IgM or PC-IgM) have direct and indirect anti-inflammatory functions[119, 120, 184], and are thought to protect in specific instances of diet-induced chronic inflammation[184, 185]. IgM antibodies localize to areas of adipose tissue inflammation[81], yet it is unclear whether they are produced locally or if they serve any role in mediating the metabolic dysfunction brought on by obesity.

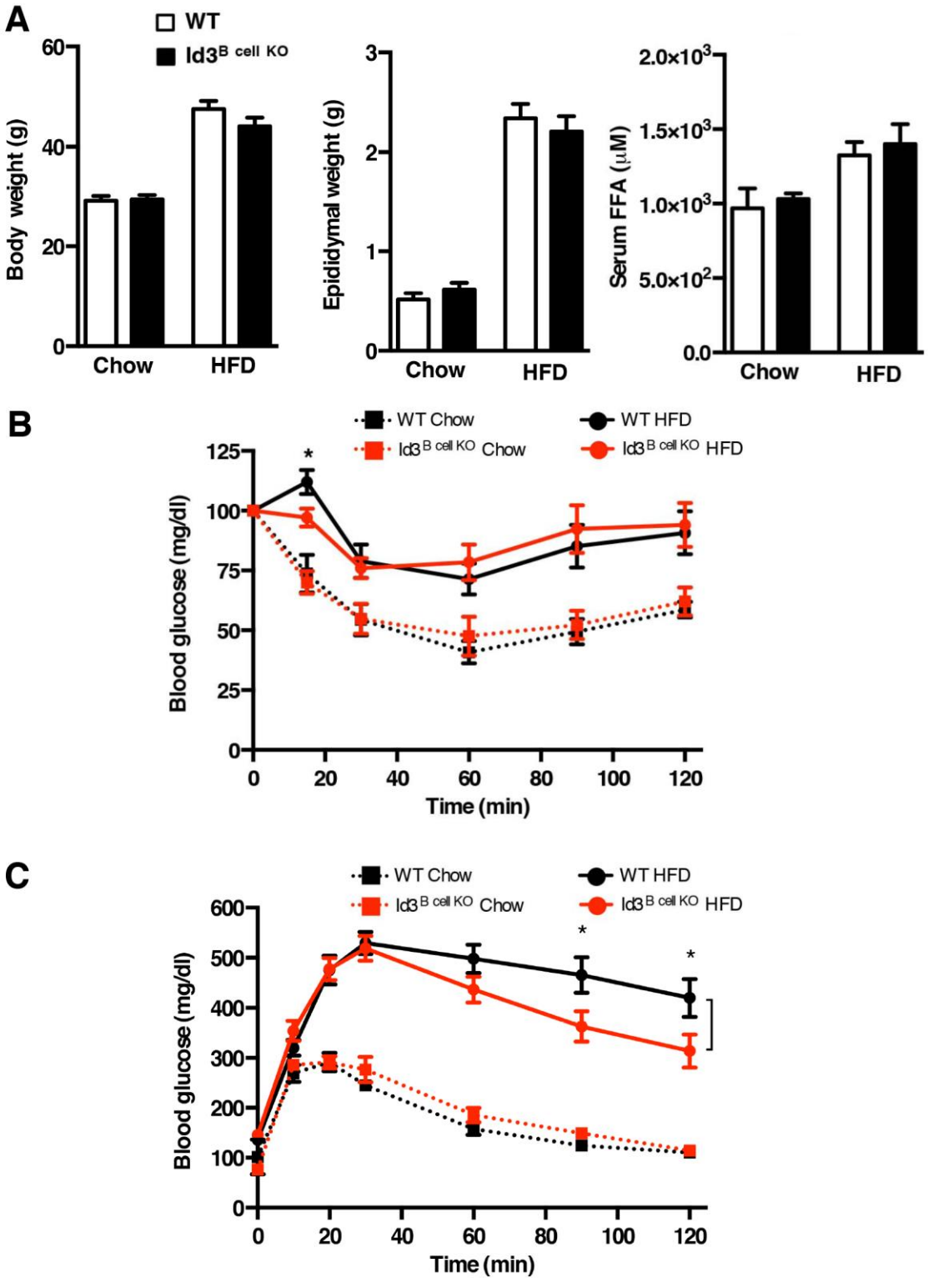
The HLH protein *Id3* is a ubiquitously expressed dominant-negative transcription regulator that, along with its binding partners, mediates various stages of B cell development and function[154, 158]. Mice globally null for *Id3* have impaired antigen-specific antibody responses[158] and increased levels of circulating IgM[162, 163]. Recent work from our group has shown a role for *Id3* in B cell regulation of diet-induced chronic inflammation[162, 186]. Additional studies using a mouse model of obesity showed that mice with global deletion of *Id3* are protected against diet-induced VAT expansion[164]. Together, these findings suggest *Id3* may be a key factor that links B cell function and obesity.

Results

B cell specific deletion of Id3 *leads to improved diet-induced glucose intolerance.*

To evaluate whether *Id3* expression is important for B cell-mediated effects of obesity, mice null for *Id3* specifically in B cells ($Id3^{Bcell\ KO}$) and WT littermates were fed either chow or HFD for 12 weeks. No genotype-dependent differences in epididymal adipose tissue mass, body weight, or serum FFA levels were detected (Figure 6A). Insulin tolerance tests (ITT) revealed that while $Id3^{Bcell\ KO}$ mice fed a HFD displayed a slightly improved initial response to insulin compared to HFD WT controls, this difference did not persist throughout the experimental time course, suggesting little differences in systemic insulin resistance (Figure 6B). Interestingly however, administration of glucose tolerance tests (GTT) showed that $Id3^{Bcell\ KO}$ mice fed HFD did have improved glucose clearance compared to controls (Figure 6C), suggesting a role for *Id3* in B cell regulation of HFD-induced metabolic dysfunction.

To test whether these findings stem from loss of *Id3* function in a B-2 cell, we performed adoptive transfers of splenic B-2 B cells into B cell-deficient μMT hosts. μMT mice contain a deletion in the mu heavy chain that is required for surface BCR expression and is essential for both B-1 and B-2 development[187]. As a previous study demonstrated that B-2 B cells only impact systemic metabolism when derived from a DIO mouse and injected into DIO hosts[81], ten million splenic B-2 cells isolated from HFD-primed WT or $Id3^{-/-}$ donors were


A
WT
Id3B cell KO
Body weight (g)
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Epididymal weight (g)
Serum FFA (μM)
B
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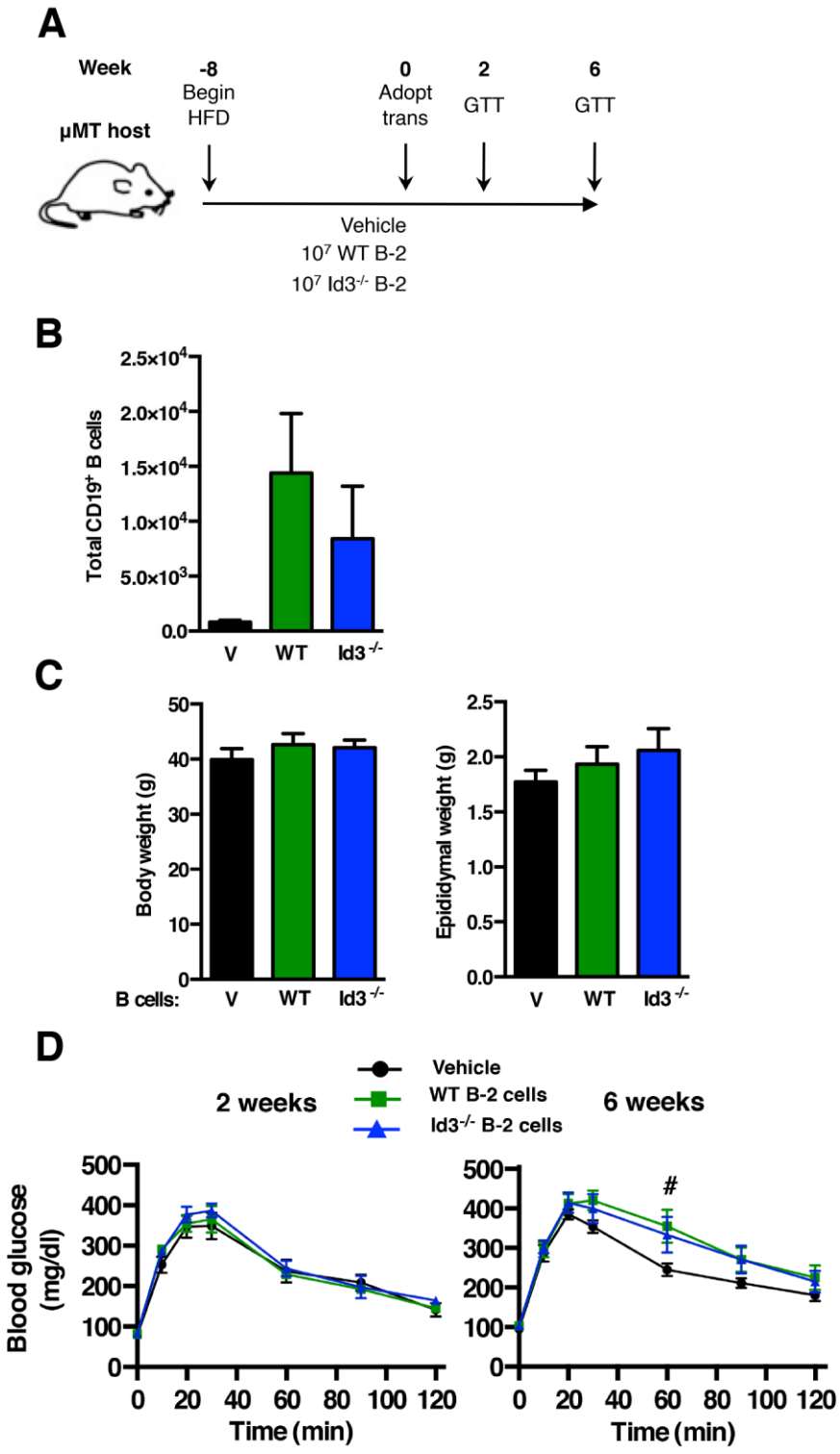
WT HFD
Id3B cell KO HFD
Blood glucose (mg/dl)
Time (min)
C
WT Chow
Id3B cell KO Chow
WT HFD
Id3B cell KO HFD
Blood glucose (mg/dl)
Time (min)

Figure 6. Loss of Id3 in B cells attenuates HFD-induced glucose intolerance. $Id3^{Bcell\ KO}$ and WT littermates were fed standard chow (WT n=6-7; $Id3^{Bcell\ KO}$ n=8-9) or a HFD for 12 weeks (WT n=9-10; $Id3^{Bcell\ KO}$ n=9-14). **(A)** Body and epididymal adipose tissue weights, and serum FFA levels. **(B)** Insulin tolerance test. (**C**) Glucose tolerance test. Error bars represent ± SEM. *$p<0.05$.

injected i.p. into μMT mice that had been fed a HFD for eight weeks. Recipient mice, including a control that received a vehicle saline injection, were continued on a HFD and tested for glucose tolerance at two and six weeks post-transfer (Figure 7A). At the end of the experiment, viable B cells were found in adoptive transfer hosts, and there were no *Id3*-dependent variations in recovery number (Figure 7B). While no differences in body mass or epdidymal adipose tissue were observed (Figure 7C), and all three groups demonstrated similar glucose tolerance at week two (Figure 7D), WT hosts had significantly impaired glucose clearance compared to vehicle controls six weeks post-transfer (Figure 7D). This corroborates previous findings[81] that B-2 cells impair glucose homeostasis. However, hosts receiving WT and *Id3*$^{-/-}$ B-2 cells had nearly identical glucose clearance patterns (Figure 7D), providing evidence that improved glucose tolerance in Id3$^{\text{Bcell KO}}$ mice is not due to loss of *Id3* function in a B-2 B cell, and suggesting other B cell subsets may modulate obesity associated metabolic dysfunction.

Id3$^{\text{Bcell KO}}$ mice fed a HFD have increased B-1 B cells, total IgM, and T15-IgM natural antibodies in adipose tissue.

Immune cells within adipose tissue can impact glucose homeostasis in a subset-dependent manner[70, 72] Flow cytometry studies in epididymal fat from DIO Id3$^{\text{Bcell KO}}$ mice revealed no differences in F4/80^{+}CD206^{-}CD11c^{+} M1 or F4/80^{+}CD206^{+}CD11c^{-} M2 macrophages[174] or total CD3ε^{+} T cells (Figure 8A).


A
Week
-8
Begin HFD
0
Adopt trans
2
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6
GTT
μMT host
Vehicle
10^7 WT B-2
10^7 Id3-/- B-2
B
Total CD19+ B cells
2.5x10^4
2.0x10^4
1.5x10^4
1.0x10^4
5.0x10^3
0.0
V
WT
Id3 -/-
C
Body weight (g)
Epididymal weight (g)
B cells:
V
WT
Id3 -/-
D
2 weeks
6 weeks
Vehicle
WT B-2 cells
Id3-/- B-2 cells
Blood glucose (mg/dl)
Time (min)
#

Figure 7. Id3 not is required for B-2 B cell driven glucose intolerance in DIO µMT mice. (A) DIO µMT mice received either an i.p. vehicle (V, n=6) saline injection or adoptive transfer of 10^7 B-2 cells from DIO WT (n=7) or *Id3*$^{-/-}$ (n=7) donors and were continued on HFD for six additional weeks. **(B)** B cells recovered in epididymal adipose tissue after week 6. **(C)** Body and epididymal adipose tissue weights. **(D)** GTT at two (left panel, representative of two independent experiments) and six (right panel, composite of two independent experiments) weeks post-transfer. Error bars represent ± SEM. #*p<0.05* WT vs. V.

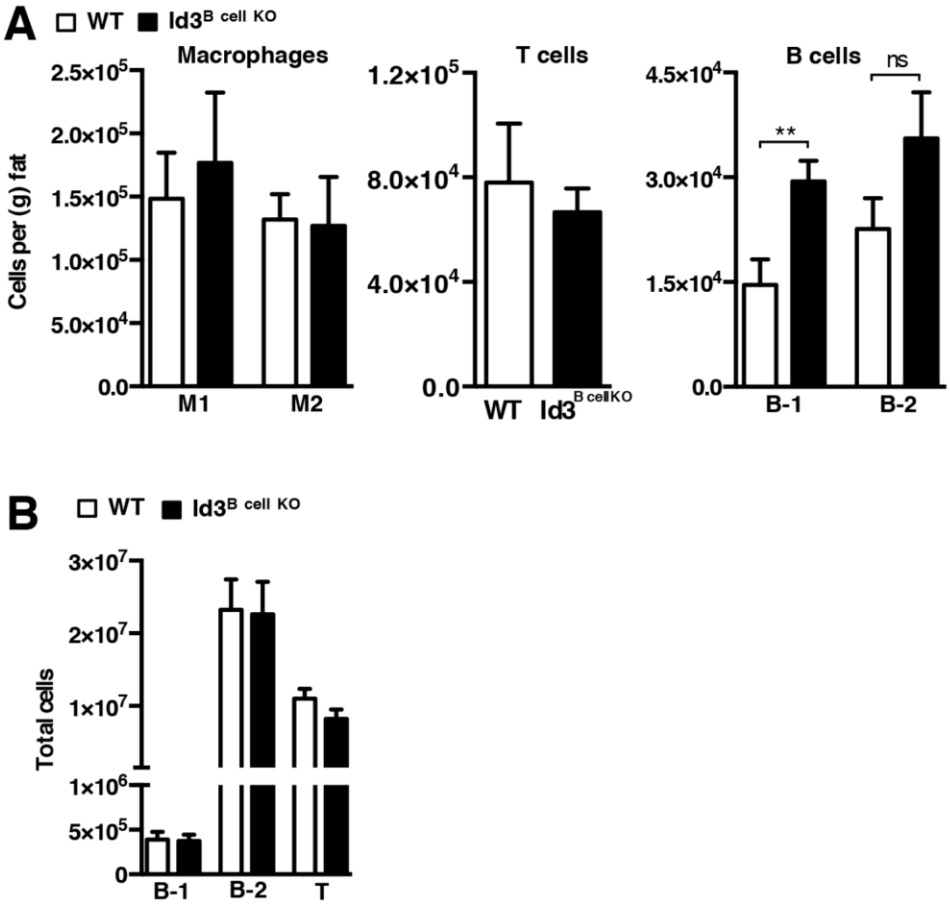

A
WT
Id3B cell KO
Macrophages
T cells
B cells
ns
**
Cells per (g) fat
2.5×10^5
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
M1
M2
1.2×10^5
8.0×10^4
4.0×10^4
0.0
WT
Id3B cellKO
4.5×10^4
3.0×10^4
1.5×10^4
0.0
B-1
B-2
B
WT
Id3B cell KO
Total cells
3×10^7
2×10^7
1×10^7
1×10^6
5×10^5
0
B-1
B-2
T

Figure 8. Adipose tissue-specific increases of B-1 B cells in Id3$^{\text{Bcell KO}}$ mice fed a HFD. Id3$^{\text{Bcell KO}}$ and WT littermates were fed a HFD for 12 weeks. Flow cytometry analysis of **(A)** epididymal adipose tissue F4/80^{+}CD206^{-}CD11c^{+} M1 and F4/80^{+}CD206^{+}CD11c^{-} M2 macrophages (left panel, WT n=6; Id3$^{\text{Bcell KO}}$ n=6), CD3ε^{+} T cells (middle panel, WT n=8; Id3$^{\text{Bcell KO}}$ n=11), and B220$^{\text{mid/lo}}$CD19$^{\text{hi}}$ B-1 and B220$^{\text{hi}}$CD19$^{\text{mid/lo}}$ B-2 B cells (right panel, WT n=6; Id3$^{\text{Bcell KO}}$ n=8). **(B)** Splenic B-1 and B-2 B cells (WT n=6; Id3$^{\text{Bcell KO}}$ n=8) and T cells (WT n=8; Id3$^{\text{Bcell KO}}$ n=11). Error bars represent ± SEM. ***p<0.01*.

There was a trend toward an increase in B-2 cells, although this change did not reach statistical significance. In contrast, Id3$^{Bcell\ KO}$ mice had significantly elevated numbers of B-1 B cells within epididymal fat compared to WT littermates (Figure 8A). No differences were observed in splenic B-1 B cells, B-2 B cells, or T cells (Figure 8B), suggesting that *Id3*-dependent regulation of B-1 cells may be tissue-specific.

B-1 B cells are a major source of natural IgM antibodies, including PC-recognizing T15-IgM, that promote tissue homeostasis and attenuate inflammation[115, 116, 119-121, 182-185]. Epididymal adipose tissue IgM (Figure 9A) and T15-IgM (Figure 9B) levels were elevated in DIO Id3$^{Bcell\ KO}$ mice compared to WT. Neither varied significantly in the circulation, suggesting that differences in adipose tissue were not just a reflection of elevated systemic production. Furthermore, we observed no variations in circulating or adipose tissue IgG antibodies (Table 1). Together, findings demonstrate that loss of *Id3* in B cells leads to increased adipose tissue B-1 B cell numbers and local natural IgM antibodies.

Loss of* Id3 *in B cells leads to elevation of peritoneal and adipose tissue B-1b B cells and increased omental T15-IgM production.

The majority of B-1 B cells are found in the peritoneal cavity and the spleen and can be divided into CD5^{+} B-1a and CD5^{-} B-1b. Since both are capable of producing natural antibodies[96] many studies do not distinguish

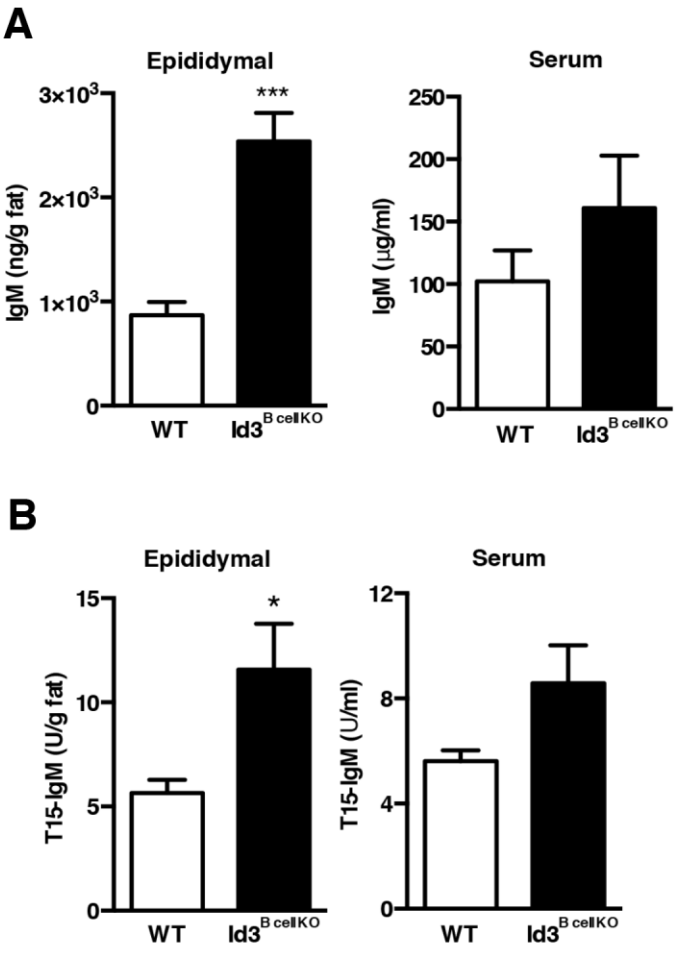

A
Epididymal
Serum
3×10^3
2×10^3
1×10^3
0
***
IgM (ng/g fat)
250
200
150
100
50
0
IgM (μg/ml)
WT
Id3^B cell KO
WT
Id3^B cell KO
B
Epididymal
Serum
15
10
5
0
*
T15-IgM (U/g fat)
12
8
4
0
T15-IgM (U/ml)
WT
Id3^B cell KO
WT
Id3^B cell KO

Figure 9. Adipose tissue-specific increases in total IgM and T15 natural IgM antibodies in Id3$^{\text{Bcell KO}}$ mice fed a HFD. Id3$^{\text{Bcell KO}}$ (n=6) and WT (n=6) littermates were fed a HFD for 12 weeks. **(A)** Total IgM by ELISA in epididymal adipose tissue (left panel) and serum (right panel). **(B)** T15-specific IgM by ELISA in epididymal adipose tissue (left panel) and serum (right panel). Error bars represent ± SEM. *$p<0.05$, ***$p<0.001$.

Table 1. IgG antibody levels in serum and epididymal fat of mice fed a HFD for 12 weeks.

	WT (n=6)	Id3$^{B\ cell\ KO}$ (n=6)	p-value
Serum (µg/ml)			
IgG1	51.7 ± 8.2	33.2 ± 4.4	0.0742
IgG2b	137.2 ± 8.3	121.5 ± 16.9	0.4246
IgG2c	92.3 ± 12.3	67.1 ± 9.0	0.1277
IgG3	45.4 ± 5.4	77.6 ± 23.6	0.2363
Epididymal fat (ng/g fat)			
IgG1	1914 ± 203	1433 ± 423	0.3295
IgG2b	4054 ± 495	4053 ± 759	0.9988
IgG2c	1729 ± 157	1852 ± 322	0.7376
IgG3	2345 ± 706	4514 ± 1572	0.2365

Values presented as mean ± SEM

between the two. However, B-1a and B-1b B cells exhibit differences in activation and response to infection[92-95, 182], indicating they likely have differing functions. Peritoneal fluid from Id3$^{Bcell\ KO}$ mice was analyzed to distinguish whether elevated B-1 numbers in the absence of *Id3* was due to an overall increase in both subsets, or a specific increase in one. Interestingly, Id3$^{Bcell\ KO}$ mice displayed a three-fold increase in peritoneal B-1b B cells, but no difference in B-1a B cells (Figure 10A). Similar to our initial findings (Figure 8), no differences in B-1a, B-1b, FO, or MZ B cell subsets were identified in the spleen (Figure 10B, data not shown). The Id3$^{Bcell\ KO}$ mouse contains only one functional CD19 allele. As alterations of CD19 expression specifically impact B-1b B cell number[94], analysis of peritoneal fluid from *Id3*$^{+/+}$*CD19*$^{Cre/+}$ mice confirmed that our findings were due to deletion of *Id3* in B cells and not CD19 haploinsufficiency (Figure 11).

Similar to findings in the peritoneal fluid, loss of *Id3* in B cells also led to a specific increase in B-1b B cell numbers within epididymal (Figure 12A) and omental (Figure 12B) adipose tissue. Consistent with previous reports[82], substantially more B-1 B cells were found in omental fat than epididymal fat relative to tissue mass (Figure 12A & B), and only omental adipose tissue contained MS clusters that stained heavily for the B cell marker B220 (Figure 12C). In addition, omental fat cultured *ex vivo* from Id3$^{Bcell\ KO}$ mice produced three-fold more T15-IgM than controls (Figure 12D), providing evidence that natural IgM is produced in omental adipose tissue in proportion to the number of B-1b cells. Together, our findings indicate that *Id3* is an important regulator of

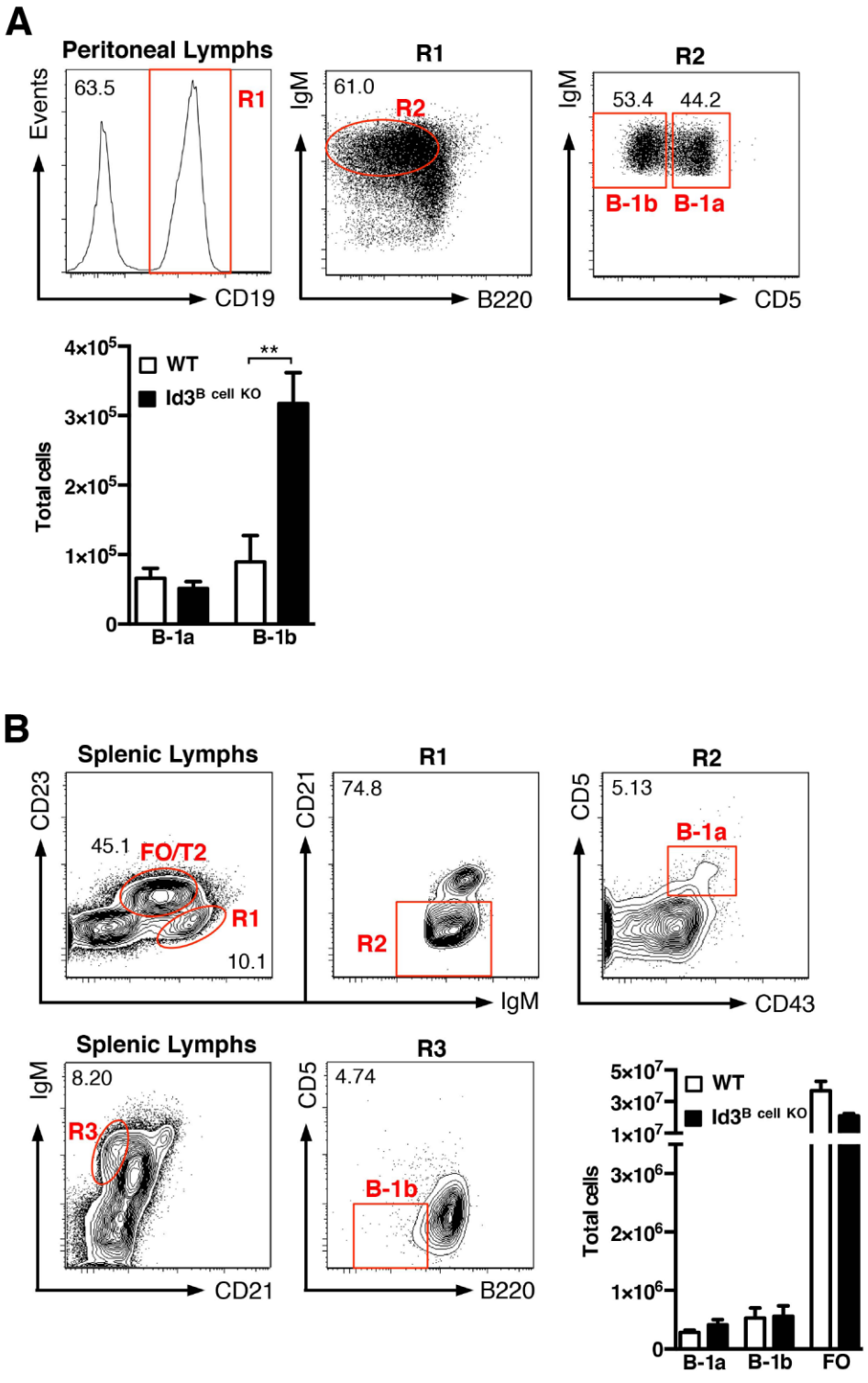

A
Peritoneal Lymphs
Events
63.5
R1
CD19
R1
IgM
61.0
R2
B220
R2
IgM
53.4
44.2
B-1b
B-1a
CD5
4x10^5
3x10^5
2x10^5
1x10^5
0
Total cells
WT
Id3B cell KO
**
B-1a
B-1b
B
Splenic Lymphs
CD23
45.1
FO/T2
R1
10.1
CD21
R1
74.8
R2
IgM
CD5
R2
5.13
B-1a
CD43
Splenic Lymphs
IgM
8.20
R3
CD21
CD5
R3
4.74
B-1b
B220
5x10^7
3x10^7
1x10^7
3x10^6
2x10^6
1x10^6
0
Total cells
WT
Id3B cell KO
B-1a
B-1b
FO

Figure 10. Peritoneal B-1b B cells are elevated in chow-fed Id3$^{\text{Bcell KO}}$ mice. **(A)** B-1a (CD19^{+}B220$^{\text{mid/lo}}$IgM$^{\text{hi}}$CD5^{+}) and B-1b (CD19^{+}B220$^{\text{mid/lo}}$IgM$^{\text{hi}}$CD5^{-}) gating strategy (top) and cell numbers (bottom) in peritoneal fluid of WT (n=5) and Id3$^{\text{Bcell KO}}$ (n=8) mice. **(B)** B-1a (CD23^{-}IgM$^{\text{hi}}$CD21^{-}CD43^{+}CD5^{+}), B-1b (IgM$^{\text{hi}}$CD21^{-}B220$^{\text{mid/lo}}$CD5^{-}), and FO/T2 (CD23^{+}IgM$^{\text{mid/lo}}$) gating strategy and cell numbers in spleens of WT (n=5) and Id3$^{\text{Bcell KO}}$ (n=8) mice. Error bars represent ± SEM. ***p<0.01*.

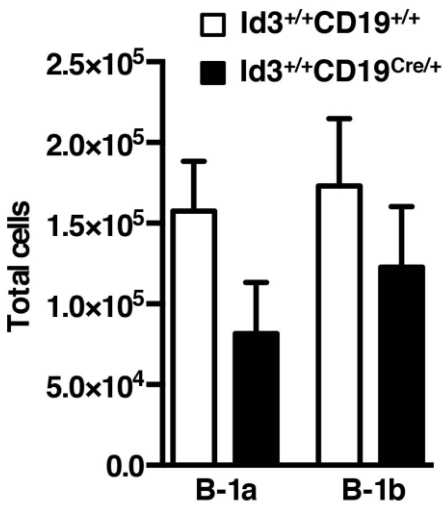

Id3+/+CD19+/+
Id3+/+CD19Cre/+
2.5×10^5
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
Total cells
B-1a
B-1b

Figure 11. No difference in Id3$^{+/+}$CD19$^{Cre/+}$ peritoneal B-1a or B-1b B cells. Flow cytometry analysis of peritoneal fluid B-1a (CD19^{+}B220$^{mid/lo}$IgM^{hi}CD5^{+}) and B-1b (CD19^{+}B220$^{mid/lo}$IgM^{hi}CD5^{-}) B cells in 8-10 week old Id3$^{+/+}$CD19$^{+/+}$ (n=3) and Id3$^{+/+}$CD19$^{Cre/+}$ (n=3) littermates.

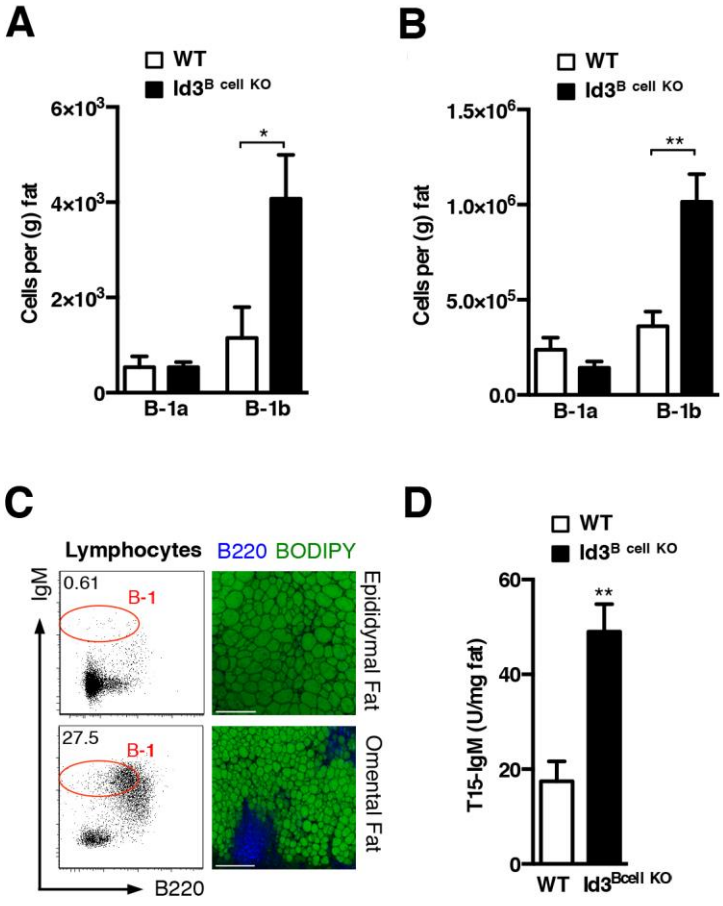

A
WT
Id3B cell KO
6x10^3
4x10^3
2x10^3
0
Cells per (g) fat
*
B-1a
B-1b
B
WT
Id3B cell KO
1.5x10^6
1.0x10^6
5.0x10^5
0.0
Cells per (g) fat
**
B-1a
B-1b
C
Lymphocytes
B220 BODIPY
IgM
0.61
B-1
27.5
B-1
B220
Epididymal Fat
Omental Fat
D
WT
Id3B cell KO
60
40
20
0
T15-IgM (U/mg fat)
**
WT
Id3Bcell KO

Figure 12. Loss of *Id3* in B cells leads to increased adipose tissue B-1b B cells and IgM secretion in chow-fed mice. (A) Epididymal B-1a and B-1b B cells in 8-10 week old $Id3^{Bcell\ KO}$ (n=8) and WT (n=5) littermates. **(B)** Omental B-1a and B-1b B cells in 8-10 week old $Id3^{Bcell\ KO}$ (n=7) and WT (n=5) littermates **(C)** Representative flow cytometry and 10x confocal microscopy images of murine epididymal (top panels) and omental (bottom panels) adipose tissue. Scale bar=200μm. **(D)** T15-specific IgM in supernatant of $Id3^{Bcell\ KO}$ (n=3) and WT (n=4) omental adipose tissue cultures. Error bars represent ± SEM. *$p<0.05$, **$p<0.01$.

adipose tissue B-1b B cell population size, and that loss of *Id3* leads to significantly more B-1b B cells and subsequently elevated local natural IgM production.

Loss of Id3 *promotes omental B-1b B cell survival*

B-1 B cells survive better than B-2 B cells and have the ability to self-renew, allowing for self-governing population maintenance. Our findings in the $Id3^{Bcell\ KO}$ mouse that loss of *Id3* leads to elevated B-1b B cell number in adipose tissue suggest dysregulated mature B-1b B cell population maintenance. However, it is also possible that *Id3* deficiency leads to accelerated B-1b development. To test whether *Id3* regulates population maintenance in mature B-1b B cells, we adoptively transferred equivalent numbers ($8.0x10^4$) of fluorescence-activated cell sorted (FACS) mature B-1b B cells from WT or $Id3^{Bcell\ KO}$ donors into B and T cell-deficient $Rag1^{-/-}$ hosts. $Rag1^{-/-}$ mice were used instead of μMT mice because B-1 B cells do not survive after i.p. transfer into μMT mice[188]. Three weeks after transfer, we confirmed a B-1b B cell population within omental fat (Figure 13A). Moreover, there were a significantly greater number of omental fat B-1b B cells if the donor cells were null for *Id3*. This indicates that the enhanced B-1b B cell numbers in $Id3^{Bcell\ KO}$ mice is intrinsic to loss of *Id3* in mature B-1b B cells.

Population maintenance depends on a balance between cell proliferation and cell death, and increased cell numbers can result from defective regulation of

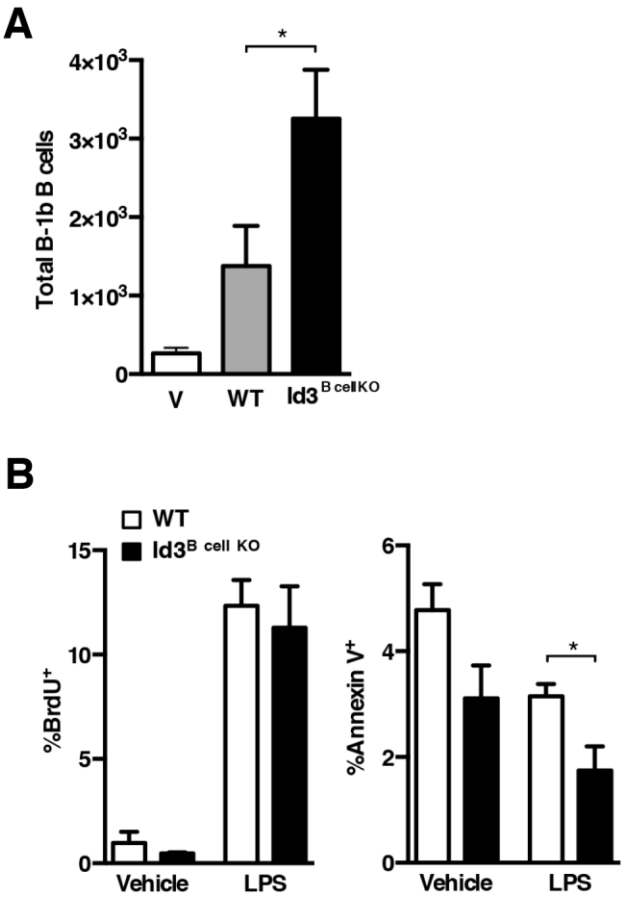

A
Total B-1b B cells
4x10^3
3x10^3
2x10^3
1x10^3
0
*
V
WT
Id3 B cell KO
B
WT
Id3 B cell KO
%BrdU+
15
10
5
0
Vehicle
LPS
%Annexin V+
6
4
2
0
*
Vehicle
LPS

Figure 13. *Id3* regulates survival in mature B-1b B cells. (A) B-1b B cells recovered in omental fat three weeks after adoptive transfer of 80k WT or $Id3^{Bcell\ KO}$ B-1b B cells into $Rag1^{-/-}$ hosts (V (vehicle), n=6 ;WT, n=6 ; $Id3^{Bcell\ KO}$, n=5). **(B)** BrdU incorporation (left) and Annexin V staining (right) of B-1b B cells from WT and $Id3^{Bcell\ KO}$ mice treated with vehicle (n=3-4) or 40μg LPS (n=5-7). Error bars represent ± SEM. *$p<0.05$.

either process. To test whether *Id3* mediates proliferation or survival in B-1b B cells, WT and Id3$^{Bcell\ KO}$ mice were injected with LPS – a rapid B-1b activator[189]. Mice were then injected with BrdU to label proliferating cells, and B-1b B cells in omental fat were analyzed for proliferation and survival. LPS-activated B-1b B cells in omental adipose tissue displayed no *Id3*-dependent differences in BrdU incorporation (Figure 13B). However, loss of *Id3* in B cells led to lower Annexin V staining in omental B-1b B cells in LPS-treated mice (Figure 13B). This indicates reduced apoptosis, and suggests that *Id3* is an important mediator of B-1b B cell survival.

Attenuated HFD-induced inflammation and insulin resistance in omental adipose tissue of Id3$^{Bcell\ KO}$ mice.

Omental fat of Id3$^{Bcell\ KO}$ mice contains higher numbers of B-1b B cells and produces more anti-inflammatory T15-IgM antibodies, suggesting that loss of *Id3* in B cells may attenuate HFD-induced adipose tissue inflammation. To test this hypothesis, WT and Id3$^{Bcell\ KO}$ mice were fed a HFD for two weeks, and inflammatory cytokines secretion in omental adipose tissue was evaluated. Indeed, omental fat from Id3$^{Bcell\ KO}$ mice displayed attenuated diet-induced inflammation as there was significantly less TNFα and IFNγ produced compared to diet-matched WT controls (Figure 14A). There were no genotype-dependent differences in chow-fed animals, suggesting that the local anti-inflammatory function may only occur in response to elevated inflammatory activity, such as

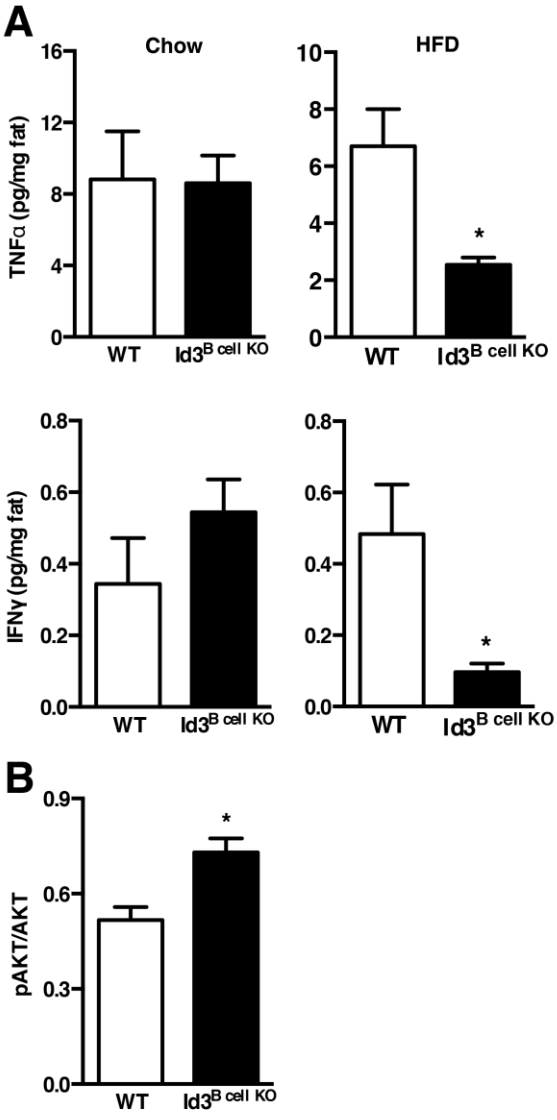

A
Chow
HFD
TNFα (pg/mg fat)
16
12
8
4
0
10
8
6
4
2
0
*
WT
Id3B cell KO
IFNγ (pg/mg fat)
0.8
0.6
0.4
0.2
0.0
*
B
pAKT/AKT
0.9
0.6
0.3
0.0
*

Figure 14. Loss of Id3 in B cells leads to reduced inflammation and improved insulin sensitivity in omental fat of mice fed a short-term HFD. (A) WT and Id3$^{Bcell\ KO}$ littermates were fed chow (n=4-5) or a HFD (n=5-7) for two weeks. TNFα (top) and IFNγ (bottom) ELISA analysis of supernatant of omental adipose tissue cultures from chow-fed (left) and HFD-fed (right) mice. **(B)** AKT phosphorylation normalized to total AKT in omental fat five minutes after insulin injection in WT (n=3) and Id3$^{Bcell\ KO}$ (n=3) littermates fed a HFD for two weeks. *$p<0.05$. Error bars represent ± SEM. *$p<0.05$.

what is known to occur in adipose tissue of HFD mice. In addition, tissue-specific insulin signaling studies revealed better insulin sensitivity in omental adipose tissue of Id3$^{Bcell\ KO}$ mice fed a HFD (Figure 14B). These results demonstrate reduced inflammation and improved insulin sensitivity in adipose tissue in a mouse model with locally elevated B-1b B cells and natural IgM production. Together, this raises the hypothesis that increasing B-1b B cell number may attenuate the metabolic consequences of obesity.

B-1b B cells lacking* Id3 *attenuates diet-induced glucose intolerance, while B-1b B cells unable to secrete IgM have no effect.

Together, findings in the Id3$^{Bcell\ KO}$ mouse suggest that improved B-1b B cell survival leads to increased local IgM production that may protect against downstream metabolic dysfunction. To test this, FACS-sorted B-1b B cells from WT, Id3$^{Bcell\ KO}$, or *sIgM*$^{-/-}$ donors were adoptively transferred into *Rag1*$^{-/-}$ hosts, and the mice were fed a HFD for 12 weeks (Figure 15A). While B cells from *sIgM*$^{-/-}$ mice express surface IgM and secrete IgG, they cannot secrete IgM[175]. Interestingly, while no body weight differences were observed after 12 weeks of HFD (Figure 15B), hosts that received Id3$^{Bcell\ KO}$ B-1b B cells had improved glucose tolerance compared to vehicle controls, and hosts treated with WT cells showed a trend toward improved glucose clearance (Figure 15C). No differences were seen between *sIgM*$^{-/-}$ hosts and vehicle controls (Figure 15C). These results suggest a role for B-1b B cells in attenuating the metabolic consequences of obesity that is

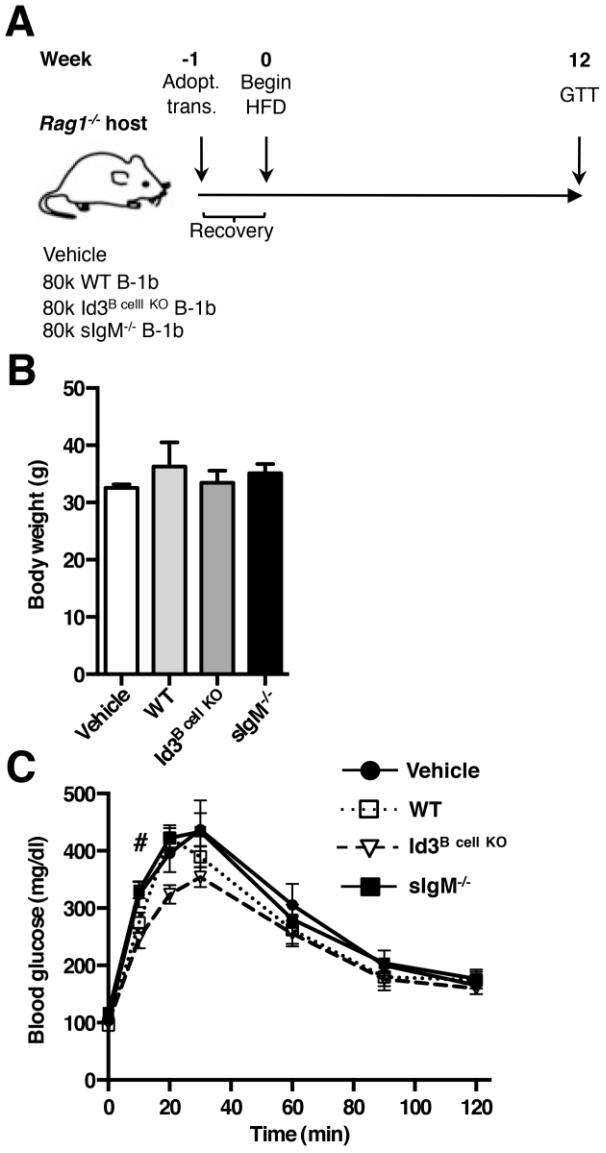

A
Week
-1
Adopt.
trans.
0
Begin
HFD
12
GTT
Rag1-/- host
Recovery
Vehicle
80k WT B-1b
80k Id3B cell KO B-1b
80k sIgM-/- B-1b
B
Body weight (g)
50
40
30
20
10
0
Vehicle
WT
Id3B cell KO
sIgM-/-
C
Vehicle
WT
Id3B cell KO
sIgM-/-
Blood glucose (mg/dl)
500
400
300
200
100
0
#
0
20
40
60
80
100
120
Time (min)

Figure 15. B-1b B cells lacking *Id3* attenuate diet-induced glucose intolerance, while B-1b B cells unable to secrete IgM have no effect. (A) *Rag1*$^{-/-}$ mice received either an i.p. vehicle (V, n=5) saline injection or adoptive transfer of 8.0x10^{4} B-1b B cells from WT (n=4), Id3$^{\text{B cell KO}}$ (n=5), or *sIgM*$^{-/-}$ (n=3) donors. After a recovery week, hosts were placed on a HFD for 12 weeks. **(B)** Body weight of Rag1$^{-/-}$ hosts after 12 week of HFD. **(C)** GTT on Rag1$^{-/-}$ hosts. *#p<0.05* Id3$^{\text{B cell KO}}$ vs V.

enhanced in the absence of *Id3* and is dependent on the ability to secrete IgM antibodies.

Discussion

Recent studies implicate B cells in the metabolic perturbations associated with DIO[76, 78, 80, 81]. As the helix-loop-helix factor *Id3* has emerged as an important factor in regulating obesity development[164] and B cell function[158, 162, 163], we used the Id3$^{Bcell\ KO}$ mouse to test the role of *Id3* and B cells in HFD-induced obesity. B cell-specific deletion of *Id3* led to improved glucose handling and a specific increase in B-1b B cells and natural IgM production within omental adipose tissue. Interestingly, loss of *Id3* in B cells also blunted HFD-induced inflammation and insulin resistance in omental fat, suggesting a protective role for B1-b B cells in DIO. Indeed, adoptive transfer of B-1b B cells from Id3$^{Bcell\ KO}$ mice led to improved glucose clearance in DIO *Rag1*$^{-/-}$ mice, while transfer of *sIgM*$^{-/-}$ B-1b B cells did not. Together, our results provide evidence for a novel IgM-dependent role of B-1b B cells in attenuating HFD-induced metabolic dysfunction.

There are several ways by which IgM antibodies may regulate processes affected by obesity. Natural IgM antibodies produced by B-1 cells bind apoptotic cells[183], facilitating their clearance and promoting tissue homeostasis[121]. Indeed, infusion of T15-IgM rescued impaired apoptotic cell clearance in *sIgM*$^{-/-}$[123] and μMT[119] mice. Uncleared dead cell accumulation in obese adipose tissue contributes to tissue inflammation[190, 191], and B-1-derived IgM reduces inflammation *in vivo*[119, 130]. IgM can also regulate metabolism independent of antigen recognition. Adipocytes treated with polyclonal IgM demonstrated significantly elevated lipogenesis, glucose oxidation, and glucose uptake[192, 193]. These effects were similar to those observed after insulin stimulation and were

Fc-dependent. Thus, identification of this novel mechanism leading to increased local IgM production in adipose tissue may serve to limit inflammation and the metabolic consequences of obesity. Indeed, we found reduced inflammatory cytokines and improved insulin signaling in the omental fat of Id3$^{Bcell\ KO}$ mice.

Results presented here provide evidence that loss of *Id3* in B cells increased B-1b B cell number and enhanced their regulatory role in HFD-induced obesity. Reduced Annexin V staining on B-1b B cells in Id3$^{Bcell\ KO}$ mice suggests that *Id3* is a key regulator of B-1b B cell survival. While *Id3* is known to promote apoptosis in developing bone marrow B cell progenitors through caspase-2[156], further studies are needed to identify whether *Id3* regulates similar pathways in B-1b B cells. Interestingly, *Id3*-dependent regulation of B-1b B cells cell number appears to impact B-1b-mediation of HFD-induced glucose intolerance. Despite transfer of an equal numbers of cells, there were significantly more B-1b B cells in omental fat and a greater improvement in glucose tolerance if the transferred cells lacked *Id3*. These findings are consistent with previous immunization studies[184] showing that enhanced B-1 B cell antibody response attenuated the detrimental effects of diet-induced inflammation. Additional studies are needed to evaluate whether further boosting of B-1b B cell numbers *in vivo* can enhance their regulatory function in obesity-associated metabolic disease.

Results of the present study also highlight the importance of studying omental fat. The omentum is a unique fatty tissue that connects the spleen, pancreas, stomach, and colon, and plays an important role in peritoneal immunity[194]. In humans, omental fat comprises a major portion of VAT that has

high metabolic activity and where inflammation correlates with insulin resistance[135, 195]. In contrast to epididymal fat, inflammatory lipids and cytokines from omental adipose tissue drain portally into the liver where they can impair hepatic insulin sensitivity[138, 196]. Within omental fat, organized clusters of mostly B-1 B cells and macrophages[82, 141] called milky spots reside in close proximity to adipocytes. Mice lacking milky spots have severely reduced T15-IgM titers[82, 179], suggesting that omental fat supports local antibody production. Our findings are the first to demonstrate active production of natural IgM antibodies in murine omental adipose tissue. Furthermore, we demonstrated that mice with increased omental B-1b B cell numbers and elevated IgM production displayed attenuated HFD-induced inflammation and better insulin signaling within omental fat than WT controls, suggesting that this depot may be key to understanding how B cells may regulate metabolic function. Moreover, due to the known structural similarities of omental fat across species[139], results obtained from studies of murine omental fat may have important relevance to human disease.

CHAPTER 4:

Adipose tissue B cells and IgM antibodies in an obese human cohort

Introduction

The use of murine models has been instrumental in developing treatments for many human diseases. However, despite possessing structurally similar immune systems, mouse and human B cells display differences in surface markers, response to cytokines, and intracellular signaling[197-199]. These factors, along with relative inaccessibility of most lymphoid tissues, have made identifying a human B-1 B cell equivalent difficult. However, the well-documented presence of human natural IgM antibodies[107-110] suggests that a B cell with functional capabilities similar to murine B-1 B cells exists in humans.

Recently, Rothstein and colleagues identified a subset of circulating B cells in humans shown to share several unique properties of murine B-1 B cells[90]. This B cell population possessed a $CD20^{+}CD27^{+}CD43^{+}$ surface phenotype and, in contrast to $CD20^{+}CD27^{-}CD43^{-}$ or $CD20^{+}CD27^{+}CD43^{-}$ B cells, spontaneously secreted IgM antibodies. Additional studies on sorted $CD20^{+}CD27^{+}CD43^{+}$ B cells revealed other B-1-like properties including antigen-independent T cell stimulation and tonic intracellular signaling. Furthermore, a fraction of this population was able to bind PC, suggesting the ability to produce antibodies that recognize the same epitope as the T15 family. Importantly, $CD20^{+}CD27^{+}CD43^{+}$ B cells were enriched in umbilical cord blood, suggesting functional capabilities well before the development of humoral memory. Despite these findings, it remains unknown whether, like murine B-1 B cells, this population is enriched in human omental adipose tissue or if natural antibody levels are associated with the number of these B cells.

Similar to mice, natural IgM antibodies in humans are associated with reduced indices of cardiovascular and inflammatory disease[200-204]. In addition to IgM antibodies specific for PC (PC-IgM), IgM to malondialdehyde-low density lipoprotein (MDA)-LDL and IgM-apoB immune-complexes (IgM-IC) are associated with reduced inflammation and decreased risk of death, myocardial infarction, and stroke in prospectively followed subjects from the general community[178, 205]. Thus, although PC-IgM has been the most extensively studied, it represents only one of several IgM antibodies known to be inversely associated with human disease[206-208]. Despite this, to our knowledge, our study is the first to evaluate whether natural IgM antibodies associate with inflammation or insulin resistance in an obese human population.

Several methods are generally used to assess metabolic function and insulin sensitivity in humans. The homeostatic model assessment of insulin resistance (HOMA-IR) uses equations derived from physiological studies to estimate glucose regulation and beta cell function based on fasting insulin and glucose levels[209]. However, this measurement relies on a data taken from a snapshot in time, and acute changes to insulin or glucose levels may skew the results. In addition, this model does not take into account factors such as circulating lipoproteins and triglycerides that are known to be associated with insulin resistance. Recently, a method that evaluates circulating lipoproteins through nuclear magnetic resonance (NMR) was shown to be highly correlated with insulin resistance and was predictive of diabetes development. Because NMR has the capacity to measure both size and particle concentration of low-

density lipoprotein (LDL), very low-density lipoprotein (VLDL), and high-density lipoprotein (HDL), lipoprotein insulin resistance (LP-IR) analysis accounts for parameters missed by normal lipid panels that only measure particle concentration. Indeed, this method is highly predictive of progressive insulin resistance after multivariate analysis[210-213] Furthermore, as LP-IR measurements are reflective of long-term metabolic function, they better reflect overall metabolic health as they are not altered by the acute environmental factors that regulate blood glucose and insulin levels.

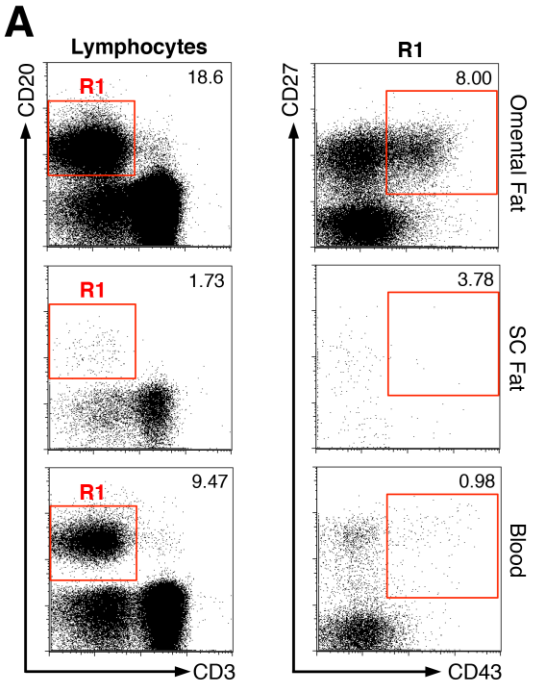
In this study, we analyzed blood and adipose tissue from patients undergoing bariatric surgery at the University of Virginia for B cell subsets and natural IgM antibodies. While all patients were enrolled under the same criteria, individuals were recruited into our studies at two distinct time frames and different endpoints were measured. As such, they are divided into cohort 1 and cohort 2 and analyzed separately (see materials and methods). Blood, SC adipose tissue, and omental adipose tissue samples from cohort 1 were analyzed for $CD20^{+}CD27^{+}CD43^{+}$ B cells via flow cytometry. Samples from cohort 2 were used for protein quantitation and LP-IR analysis. Results show for the first time that $CD20^{+}CD27^{+}CD43^{+}$ B cell reside within human omental adipose tissue and correlate with circulating PC-IgM antibodies. Additional findings provide evidence that PC-IgM antibody levels within omental adipose tissue correlate strongly with circulating PC-IgM. Moreover, analysis of circulating natural IgM antibodies revealed inverse associations with inflammation and insulin resistance. Together, these findings identify additional similarities between

murine B-1 B cells and human $CD20^{+}CD27^{+}CD43^{+}$ B cells, and suggest that elevated levels of natural IgM antibodies may predict an improved metabolic phenotype in obese humans.

Results

CD20$^+$CD27$^+$CD43$^+$ B cells are present in human omental adipose tissue and correlate with circulating PC-IgM levels.

Recently, Griffin et al. identified a subset of circulating B cells (CD20$^+$CD27$^+$CD43$^+$) in humans that shared several unique properties of murine B-1 B cells[90]. Murine B-1 B cells are found in marked abundance within omental adipose tissue relative to other adipose depots. To determine whether the same may be true for the putative human equivalent of murine B-1 B cells, we performed flow cytometry on omental fat in an obese cohort of patients undergoing bariatric surgery (cohort 1). We observed a marked enrichment of CD20$^+$CD27$^+$CD43$^+$ B cells in the omental adipose depot compared to subcutaneous fat or blood in four of 16 patients analyzed. Figure 16A is a representative flow cytometry plot of the patients whose samples displayed marked enrichment. Interestingly, patients with a higher fraction of omental adipose tissue B cells that were CD20$^+$CD27$^+$CD43$^+$ also had elevated serum PC-binding IgM (Figure 16B). While some debate remains over the exact nature of CD20$^+$CD27$^+$CD43$^+$ B cells[214-216], our novel results provide additional evidence supporting CD20$^+$CD27$^+$CD43$^+$ B cells as the human equivalent of murine B-1 B cells. Moreover, consistent with our murine findings, the number of CD20$^+$CD27$^+$CD43$^+$ B cells correlates with increased circulating PC-IgM antibodies.


A
Lymphocytes
R1
CD20
CD3
18.6
1.73
9.47
R1
CD27
CD43
8.00
3.78
0.98
Omental Fat
SC Fat
Blood

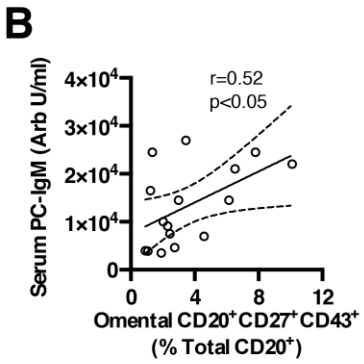

B
Serum PC-IgM (Arb U/ml)
4x10^4
3x10^4
2x10^4
1x10^4
0
r=0.52
p<0.05
0
4
8
12
Omental CD20+CD27+CD43+
(% Total CD20+)

Figure 16. $CD20^+CD27^+CD43^+$ B cells in human omental adipose tissue correlate with serum PC-IgM levels. (A) Flow cytometry on omental fat, subcutaneous (SC) fat, and blood from a single patient. **(B)** Fraction of omental $CD20^+CD27^+CD43^+$ B cells plotted against serum PC-IgM levels in patients from cohort 1 (n=16). Solid line represents Spearman correlation and dashed lines represent 95% confidence intervals.

PC-IgM antibodies in omental adipose tissue correlate with circulating PC-IgM.

In chapter 3, it was shown that PC-recognizing IgM antibodies can be produced locally within omental fat in mice. In a separate cohort of bariatric surgery patients (cohort 2, Table 2), PC-IgM in omental adipose tissue lysates was measured. Results demonstrated a strong positive correlation between PC-IgM in omental adipose tissue and circulating PC-IgM (Figure 17). These findings are consistent with the identification of a B-1-like B cell in omental fat, and suggest the hypothesis that omental adipose tissue may also be a site of natural IgM production in humans. Furthermore, as circulating biomarkers do not always reflect levels within tissue, our findings give credence to the potential use of circulating PC-IgM as a predictor of omental adipose tissue PC-IgM levels.

PC-IgM antibodies in circulation and within omental adipose tissue negatively correlate with MCP-1 levels and age.

PC-recognizing IgM antibodies have anti-inflammatory characteristics, and chapter 3 described attenuated HFD-induced inflammation in mice with elevated T15-IgM production in omental fat. Furthermore, B-1 cells decrease in older mice[217] and humans[90], suggesting that their protective effects may decline with age. We examined serum for the inflammatory cytokines IFNγ, TNFα, and MCP-1 in cohort 2. IFNγ and TNFα levels were below the level of detection in our assay (data not shown). However, MCP-1 – a macrophage chemoattractant

Table 2. Cohort 2 baseline characteristics

Patients	122
Male	29 (24%)
Female	93 (76%)
Metabolic Health[A,B]	
Diabetic	50 (43%)
Non-diabetic, with metabolic syndrome	47 (41%)
Non-diabetic	19 (16%)
Age	44 ± 10
Creatinine (mg/dl)	0.9 (0.8, 1.0)
HDL (mg/dl)	38 (33, 45)
Triglycerides (mg/dl)	125 (100, 174)
LPIR[C]	
Large VLDL-P (nmol/L)	5.8 (3.1, 11.3)
Small LDL-P (nmol/L)	786 ± 281
Large HDL-P (μmol/L)	3.1 (2.1, 4.6)
VLDL size (nm)	55 ± 8.4
LDL size (nm)	20.3 ± 0.5
HDL size (nm)	8.9 (8.7, 9.2)

Values presented as mean ± standard deviation or as median (interquartile range) depending on data distribution

[A]Metabolic syndrome defined as any three of the following: large waist circumference (men: >40in; women: >35in), hypertension, low HDL (men: <40mg/dl; women: <50mg/dl), high triglcyerides (>150mg/dl), high blood glucose (>100mg/dl)

[B]Metabolic health information not available for six patients

[C]LPIR = Lipoprotein measurement of insulin resistance

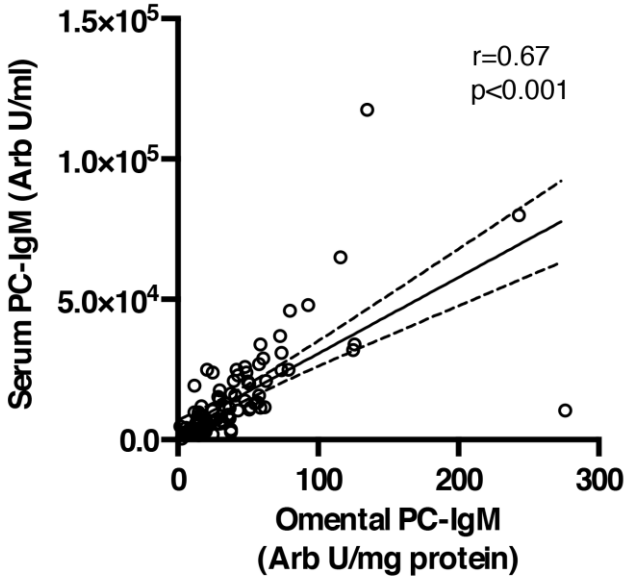

1.5×10^5
1.0×10^5
5.0×10^4
0.0
Serum PC-IgM (Arb U/ml)
r=0.67
p<0.001
0
100
200
300
Omental PC-IgM
(Arb U/mg protein)

Figure 17. Human omental adipose tissue PC-IgM correlates with serum PC-IgM levels. Omental PC-IgM levels plotted against serum PC-IgM levels in patients from cohort 2 (see table 2). Solid line represents Spearman correlation and dashed lines represent 95% confidence intervals.

protein known to be highly predictive of insulin resistance[218, 219] – could be clearly measured and consistent with an anti-inflammatory role for PC-IgM, we found that circulating levels of MCP-1 had inverse correlations with PC-IgM in both the circulation and within omental adipose tissue (Table 3). Interestingly, both circulating and omental PC-IgM were also inversely associated with age (Table 3). Consistent with murine results, our studies provide evidence that PC-IgM antibodies are present in human omental adipose tissue, associate with reduced inflammation, and decline with age.

IgM antibodies against PC and MDA-LDL, along with IgM-apoB immune complexes, are associated with lower indices of inflammatory disease. To determine if these human IgM antibodies and immune complexes were associated with insulin resistance, each patient in cohort 2 was given a score from 0 (most insulin sensitive) to 100 (most insulin resistant) based on NMR-lipid analysis. While we found no correlation with LP-IR scores and circulating PC-IgM (data not shown), LP-IR was negatively associated with IgM-IC and displayed a trending inverse correlation with IgM antibodies to MDA-LDL (Table 4). Both IgM-IC and IgM MDA-LDL were positively associated with HDL. No correlations were observed between either insulin sensitivity or HDL and IgG-IC or IgG MDA-LDL (Table 4). Together, our results suggest that multiple natural IgM antibodies are associated with protective phenotypes in an obese human population.

Table 3. PC-IgM negatively correlates with serum MCP-1 and age.

Measurement	MCP-1 (serum)		Age	
	Spearman r	p-value	Spearman r	p-value
PC-IgM				
Serum	-0.19	**0.05**	-0.25	**0.007**
Omental fat	-0.21	**0.02**	-0.23	**0.01**

Table 4. IgM autoantibodies and apoB-immune complexes correlate with HDL levels and improved LP-IR scores.

Measurement	LP-IR		HDL	
	Spearman coefficient	p-value	Spearman coefficient	p-value
IgM-IC	-0.24	***0.01***	0.32	***0.0005***
IgG-IC	-0.002	0.98	0.08	0.35
IgM MDA-LDL	-0.15	0.12	0.19	***0.04***
IgG MDA-LDL	-0.08	0.38	0.02	0.85

Discussion

Much of the knowledge about immune regulation of disease has come from murine studies, and in many cases, the relevance to human disease is unclear. While immunohistochemical analysis has demonstrated B cells within human omental fat[76, 141], the lack of a known human B-1 cell equivalent had made subset analysis difficult. However, the well-known existence of natural antibodies in humans suggests the presence of a B-1 cell. Recently, a circulating human $CD20^+CD27^+CD43^+$ B cell with several characteristics similar to murine B-1 B cells, such as the ability to spontaneously secrete IgM and bind PC antigen, was identified[90]. Here, we show for the first time that, like murine B-1 B cells, $CD20^+CD27^+CD43^+$ B cells are enriched in human omental adipose tissue compared to subcutaneous adipose tissue and blood. While not all omental adipose tissue samples displayed high numbers of these cells, the variability in omental $CD20^+CD27^+CD43^+$ B cells may be due to uneven clustering and distribution of milky spots within individual depots, variable sampling at the time of surgery, or a reflection of differences in individual patients. Despite the variability, we showed the presence of omental $CD20^+CD27^+CD43^+$ B cells correlated with circulating PC-IgM levels. Our findings add to the known similarities between murine B-1 B cells and human $CD20^+CD27^+CD43^+$ B cells, and indicate that further analysis of omental adipose tissue may improve our understanding of the role B-1 B cells play in metabolic regulation.

Additional studies revealed that IgM autoantibodies and immune complexes were associated with decreased MCP-1 levels, increased HDL, and

improved LP-IR score in a cohort of obese individuals. Natural IgM antibodies have long been associated with reduced risk of cardiovascular disease[178, 200], and our findings suggest they may also be important biomarkers of metabolic function. Interestingly, neither of the IgG antibodies we analyzed had any association with inflammation or LP-IR, supporting the hypothesis that protection is specific to IgM. Our mixed associations with a variety of IgM antibodies suggest that protection may stem from overall increased natural IgM production, and additional studies are needed to identify whether additional IgM antibodies associate with insulin sensitivity. Further understanding of B-1 B cells and natural IgM antibodies in murine and human adipose tissue may lead to novel biomarkers and new strategies to limit inflammation and the metabolic consequences of obesity.

CHAPTER 5:

Summary, General Discussion, and Future Directions

Summary

In chapter 3, a mouse containing a B cell-specific deletion of the HLH factor *Id3* was used as a tool to examine a potential role for B cells in the context of HFD-induced obesity. When challenged with a HFD for 12 weeks, these mice displayed a modest but significant improvement in glucose tolerance compared to HFD-fed littermate controls. While *Id3* has reported roles as a regulator of B-2 B cell function, adoptive transfer studies suggested that loss of *Id3* function in a B-2 B cell could not account for the metabolic phenotype observed in the $Id3^{B\ cell\ KO}$ mouse. Further analysis provide the first evidence that loss of *Id3* in B cells leads to a specific increase in B-1b B cell number and natural IgM production in adipose tissue. Follow-on studies demonstrated that *Id3* likely functions to negatively regulate B-1b B cell survival. Interestingly, increased B-1b B cell number and IgM production correlated with blunted HFD-induced inflammation and improved insulin signaling in omental adipose tissue. Moreover, adoptive transfer of B-1b B cells lacking *Id3* was able to attenuate HFD-induced glucose intolerance in $Rag1^{-/-}$ mice. As transfer of $sIgM^{-/-}$ B-1b B cells had no effect, results suggest that B-1b B cells may play a protective role in mediating metabolic dysfunction associated with obesity through production of IgM antibodies.

In chapter 4, studies using human tissue were performed to test the hypothesis that B-1 B cells and natural antibodies are present in human omental adipose tissue. Similar to findings in mice, we identified a human B cell known to have B-1-like qualities in omental adipose tissue of patients undergoing bariatric

surgery. The frequency of these B cells in omental fat correlated with circulating levels of PC-IgM antibodies, providing further support that these cells are the human equivalent of murine B-1 B cells. Additional studies showed that various natural IgM antibodies and immune complexes negatively correlated with systemic inflammation and insulin resistance, suggesting that elevated production of IgM antibody may be associated with improved metabolic function.

Together, studies performed in mice and humans provide the first evidence that B-1 B cell-derived natural IgM antibodies may play important roles in regulating inflammatory and metabolic dysfunction associated with obesity. Future studies are needed to further evaluate B-1 B cell function in adipose tissue, to determine the potential protective mechanism(s) of IgM in the context of obesity, and to test whether enhancing B-1 B cell production of natural IgM antibodies may have therapeutic potential for attenuating obesity-induced metabolic disease.

General discussion and future directions

B cells likely regulate obesity and metabolism in a subset-dependent manner.

Over the last decade, it has become evident that crosstalk exists between metabolic tissues and members of the innate and adaptive immune systems. Recently, B cells have been identified as important players in the emerging field of immuno-metabolism. B cells can be divided into two major subsets with differing roles in the immune system. B-2 B cells respond to T-dependent antigens, undergo class switching and somatic hypermutation, and produce high-affinity antibodies that are crucial components of the adaptive immune system[83]. On the other hand, B-1 B cells produce evolutionarily-conserved natural antibodies, either spontaneously or rapidly after activation, that contribute to initial defense against infection and contribute to tissue homeostasis[96]. Insight into how B cells function in the context of other inflammatory diseases such as atherosclerosis have provided strong evidence that B-1 and B-2 B cells may have differing – and sometimes opposing – roles in disease[220]. Recent studies identifying a pathogenic role for B-2 B cells in obesity-related insulin resistance, combined with results from our studies that suggest B-1b B cells may attenuate metabolic dysfunction in obesity, indicate that differing B cell subsets likely have opposing impacts on adipose tissue function and metabolic health as well.

While B cells had previously been identified in VAT[76-78, 82, 179], a high-impact study by Winer and colleagues in 2011 was the first to identify B-2 B cells as promoters of HFD-induced insulin resistance[81]. In this report, the authors

found that DIO led to a systemic adaptive antibody response highlighted by increased circulating IgG antibodies, along with elevated spontaneous IgG secretion and reduced IgM production by splenocytes. Preliminary studies in our laboratory support the notion that HFD promotes T-dependent antibody responses, as a trend for more germinal center B cells are found in the spleens of DIO mice (Figure 18). Further analysis by Winer and colleagues revealed an accumulation of class-switched IgG^+ B-2 B cells and an abundance of pro-inflammatory IgG2c antibodies within VAT, indicating that DIO may also induce a local adaptive immune response within fat. Adoptive transfer studies showed that B-2 B cells were able to drive glucose intolerance and insulin resistance in B cell-deficient μMT mice. Our studies corroborate these findings, as we obtained similar impairment of glucose tolerance after injecting WT B-2 B cells into μMT mice. Interestingly, Winer et al. found this effect was blunted when donor B-2 B cells lacked functional major histocompatibility complex (MHC) I or II, suggesting that antigen presentation to T cells is required for B-2 B cells to negatively impact metabolic function. Additional studies showed that transfer of IgG purified from obese donors into μMT hosts was sufficient to impair glucose homeostasis. As IgG from chow-fed donors and IgM from DIO mice had no impact, these findings provide evidence that DIO induces a pathogenic T-dependent B-2 IgG antibody response that impairs systemic metabolic function.

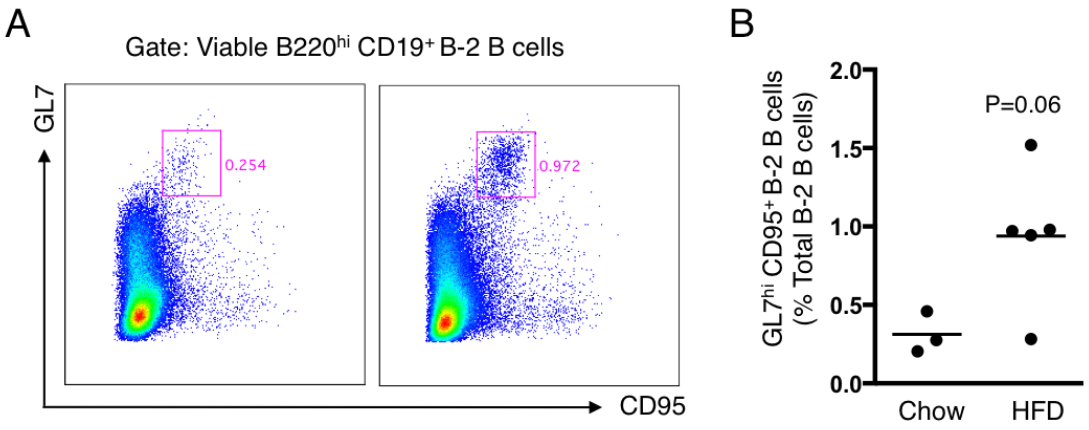

A
Gate: Viable B220hi CD19+ B-2 B cells
GL7
0.254
0.972
CD95
B
GL7hi CD95+ B-2 B cells
(% Total B-2 B cells)
2.0
1.5
1.0
0.5
0.0
P=0.06
Chow
HFD

Figure 18. HFD may lead to splenic germinal center formation. C57Bl/6J mice were fed chow (n=3) or a HFD (n=5) for 12 weeks. **(A)** Flow cytometry staining for splenic $B220^{Hi}CD19^{+}GL7^{Hi}CD95^{+}$ germinal center B cells. **(B)** Quantitation of germinal center B cells. Each dot represents a single mouse.

Identification of class-switched B-2 B cells and IgG2c antibodies in VAT, along with previous reports of VAT-resident T cells containing a restricted T cell receptor (TCR) repertoire[81, 221] suggest a local immune response. Recently, adipose tissue macrophages were shown to induce T cell proliferation and T_H1 polarization through MHC II antigen presentation[222]. A significant fraction of macrophages expressing MHC II were found near proliferating T cells in FALCs, suggesting that these regions may be important for antigen-driven immune responses in fat. Interestingly, obesity progression led to a redistribution of macrophage-T cell interactions toward CLS, which are regions of high inflammatory output surrounding uncleared dead adipocytes[190, 191]. It is possible that apoptotic adipocytes are a source of autoantigen that helps drive the inflammatory IgG response seen in obesity. In support of this, IgG autoantibodies against phosphogluconate dehydrogenase – a highly expressed protein in adipocytes – were found in roughly 40% of overweight insulin-resistant patients[81]. More studies are needed to address the antigen specificity of B-2 B cells in adipose tissue. Identifying whether antibodies against certain antigens are driving a pathogenic response during obesity may allow for targeted immunotherapy aimed at improving metabolic function.

In chapter 3, we show that a mouse model with specifically increased B-1b B cells and elevated IgM production in fat has an improved inflammatory and metabolic phenotype after a HFD compared to WT controls. Adoptive transfer studies showed that B-1b B cells could attenuate the development of diet-induced glucose intolerance only when able to secrete IgM. Most insight into B-

1b B cell function stems from infection studies[223], and our findings are the first to identify B-1b B cells as regulators of obesity-induced metabolic dysfunction and provide evidence that they play a contrasting role to B-2 B cells in this context. As described here in following sections, further analysis regarding B-1b B cell function in obesity and the specific role(s) IgM may play in adipose tissue biology is needed.

In addition to antibody production, cytokines derived directly from B cells may contribute to metabolic regulation. B-2 B cells have the capacity to secrete a multitude of T_H1 and T_H2 polarizing cytokines. Sorted FO B-2 B cells from obese mice secreted more IL-6 and macrophage inflammatory protein-2 (MIP-2), and less IL-10 than lean controls, suggesting a pro-inflammatory phenotype[77]. Similar results were found in T2D patients, where circulating B cells secreted more IL-8 (an ortholog of murine MIP-2) and less IL-10 in response to toll-like receptor (TLR) stimulation than B cells from healthy controls[101]. Very recently, a subset of IL-10-producting B cells, possessing a different surface phenotype than on previously characterized B_{REG} B cells, was identified in adipose tissue[102]. Interestingly, these cells were able to spontaneously secrete IL-10 *ex vivo* – a feature absent in previously described B_{REGS} that require several hours of stimulation for IL-10 production. Adipose tissue IL-10-secreting B cells decreased with progressing obesity, and B cell-specific deletion of IL-10 led to increased adipose tissue inflammation and insulin resistance. As IL-10 has known functions in suppressing activated M1 macrophages[224] and can directly protect adipocytes from TNFα-induced insulin resistance[225], B cell IL-10 production may be an

important mechanism by which B cells can protect against adipose tissue dysfunction. Further analysis of this unique B cell subset is needed to fully understand their function and potential use as a therapeutic agent. Additional study is also needed to identify whether adipose tissue B cells produce cytokines that may have other immuno-regulatory or metabolic functions.

B-1b B cells are poorly understood.

Results described in chapter 3 indicate more study is needed to understand how B-1b B cells function in adipose tissue and what role they play during the progression of obesity. In order to do this, more insight into B-1b B cell biology is required. Owing mostly to the shared surface phenotype of B-1a and B-1b B cells – the only known distinction is their expression of CD5 – many studies do not distinguish between the two, thus making our understanding of B-1b B cells incomplete. However, despite sharing several properties such as self-renewal and natural IgM production, evidence suggests that B-1a and B-1b B cells are distinct B cell subsets with overlapping, but non-redundant functions[96]. The existence of B-1a B cells prior to birth and B-2 development is well documented[87], but B-1b development is less understood. Studies have identified precursor cells that produce only B-1a B cells as early as E8.5 during development in the splanchnopleura region[88]. However, both B-1a and B-1b precursors are found in the fetal liver, well before B-2 B cells develop[89]. Additional studies in adult mice have shown that transfer of lymph node cells can reconstitute B-1b, but not B-1a, populations in *Rag1*$^{-/-}$ hosts[226]. Moreover,

transfer of bone marrow preferentially generates B-1b B cells over B-1a B cells, and this skewed ratio becomes more pronounced with increasing donor age[217]. Elegant work from Ghosn and colleagues showed that transfer of a single $Lin^{-}CD34^{-}cKit^{+}Sca1^{+}C150^{+}$ hematopoietic stem cell (HSC) from an adult Kusabira Orange transgenic mouse was able to replenish splenic and peritoneal B-2 populations of lethally irradiated hosts. Interestingly, a population of B-1b B cells within the peritoneal cavity was partially restored, but no B-1a B cells were generated[227]. To further complicate things, a progenitor population found in fetal liver, and to a lesser degree in bone marrow, was able to reconstitute B-1b and limited B-1a populations, but not B-2 B cells.[228] These findings suggest that B-1b B cells share developmental timing with both B-1a and B-2 B cells, and at least have the potential to differentiate from overlapping progenitor populations. Presently, it is unclear if B-1b B cells generated during development differ functionally from those derived from adult precursors, and further studies are needed to delineate the developmental differences between B-1b B cells and other B cell subsets.

In addition to possessing distinct developmental patterns, B-1a and B-1b B cells appear to have different effector functions. At the onset of infection, both B-1a and B-1b B cells migrate from the peritoneal cavity to the spleen or mucosal tissues where they differentiate into antibody-secreting cells[177, 229]. However, while only B-1a B cells localize in the respiratory tract draining lymph nodes and produce IgM after influenza virus infection[93], B-1b B cells are sufficient to protect against *B. hermsii* bacteria and appear to mount a memory response after

immunization[92, 230]. Furthermore, while B-1a B cells protected against sublethal infection of *S. pneumoniae* in naïve mice, only B-1b B cells conferred immunization-dependent survival in mice treated with a lethal dose of the same bacteria[94]. These findings are supported by studies showing high junctional VH diversity in B-1b compared to B-1a B cells, providing evidence that B-1b B cells have the ability to generate a memory-like response[231-233]. In addition to being critical mediators of immunity, a recent study identified a protective role intrinsic to B-1b B cells in autoimmunity[95]. Here, the authors found that B-1b B cells were required to produce an IgM antibody that attenuated IL-17-producing T_H cells (T_H17) activity and reduced disease progression in SLE-prone mice. Together, B-1b B cells appear to be a unique bridge between the innate and adaptive immune systems and play critical roles in mediating infection and autoimmunity that are not replicated by other known B cell subsets.

B-1b B cells may be an important mediator of adipose tissue and metabolic function.

The results presented in chapter 3 indicate that B-1b B cells may play a protective role in obesity-associated metabolic disease, and additional studies are needed to identify how these cells function in adipose tissue (Figure 19). Our findings are the first to show that cells within omental fat are a source of T15-IgM natural antibodies. As increased production was observed in $Id3^{B\ cell\ KO}$ mice that have specifically elevated B-1b B cell numbers, we hypothesize B-1b B cells are a major source of natural IgM antibodies in adipose tissue. To test whether this is

the case in WT animals, similar T15-IgM ELISAs described in chapter 2 could be performed on culture supernatants from B-1a, B-1b, and B-2 B cells sorted from omental fat of C57Bl/6 mice. The main challenge to these experiments is limited cell yield – several attempts at sorting omental cells in our hands produced no more than 5,000 of any one B cell subset from a single mouse (data not shown) – suggesting that pooling mice or further optimizing cell extraction techniques will be necessary. In parallel, more sensitive ELISPOT assays that quantify antibody-secreting cells could be utilized to determine the fraction of T15-IgM-producing cells from each B cell subset in omental fat. Similar studies looking at antibody production from B cell subsets in obese adipose tissue are also needed to determine whether obesity progression results in altered local IgM production.

As our findings in humans described in chapter 4 suggest that other IgM antibody clones besides those that recognize PC may be associated with insulin sensitivity, full analysis of immunoglobulin repertoires of omental B-1b B cells is needed. High-throughput sequencing can identify heavy and light chain variable regions (V_H and V_L, respectively) that make up the antigen-binding portion of the BCR. Since this technology is generally used to determine the frequency of

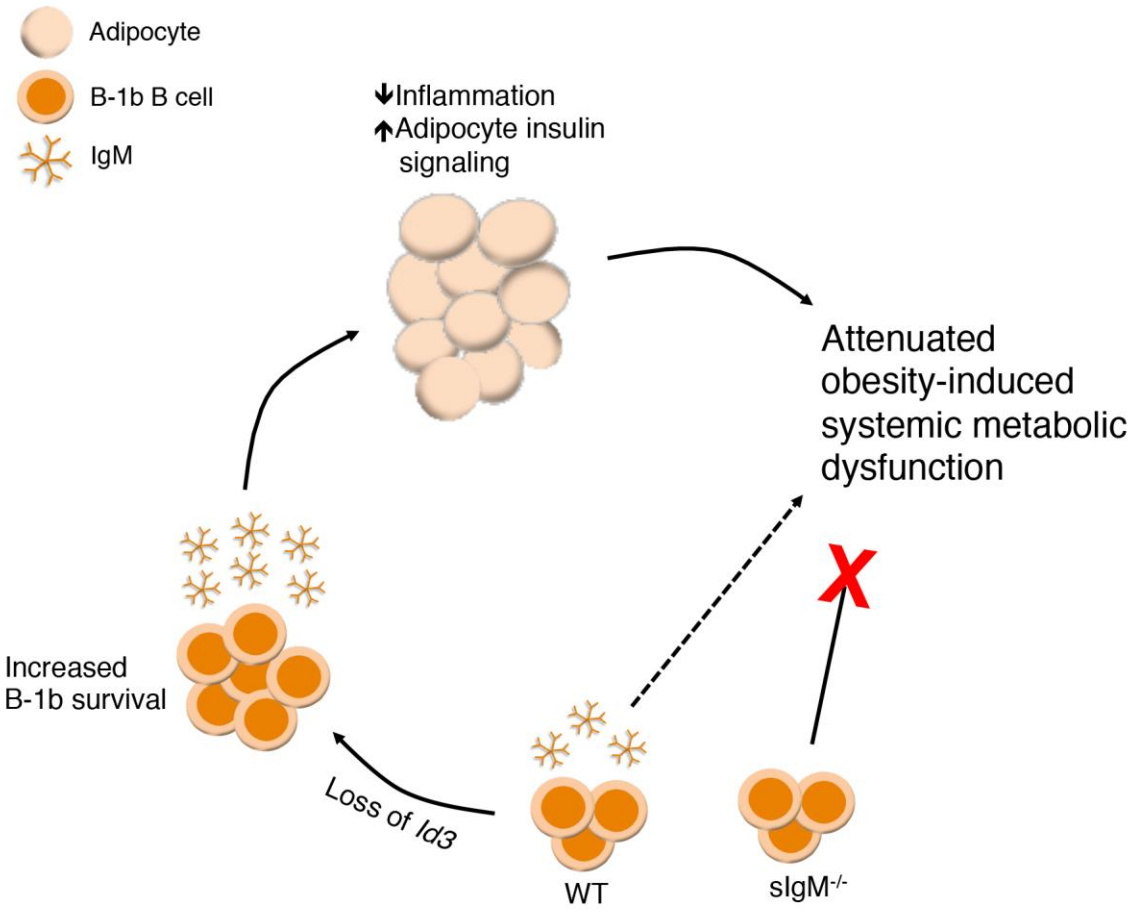

Adipocyte
B-1b B cell
IgM
↓Inflammation
↑Adipocyte insulin signaling
Attenuated obesity-induced systemic metabolic dysfunction
Increased B-1b survival
Loss of Id3
WT
sIgM-/-

Figure 19. Model for how B-1b B cells may play a protective role in mediating obesity-induced metabolic dysfunction. In the absence of *Id3*, B-1b B cell numbers are elevated in adipose tissue. This correlates with reduced inflammation and improved insulin sensitivity in adipose tissue of mice fed a high-fat diet, suggesting that boosting B-1b B cell number may improve adipose tissue function. Furthermore, B-1b B cells null for *Id3* were able to attenuate obesity-induced systemic metabolic dysfunction in *Rag1*$^{-/-}$ hosts, while WT B-1b B cells had a moderate effect. B-1b B cells from *sIgM*$^{-/-}$ mice had no effect, suggesting that secreted IgM may a key regulator of metabolism. Future studies into how B-1b B cells and IgM antibodies function in adipose tissue and whether increasing B-1b B cell number and antibody response via vaccination improves metabolic health are needed.

specific V_H and V_L regions in pooled B cell populations, antigen binding information that can be gleaned from specific V_H and V_L combinations is often lost. However, recent advances in next-generation sequencing allow for the V_H and V_L combination of large quantities of single cells to be determined[234]. This approach to characterizing omental B-1b B cell antibody repertoires could provide an unbiased method for determining specific antigens that antibodies from this B cell population may recognize. Studies that compare V_H and V_L combinations between B-1b B cells in lean and obese omental fat may help determine specific protective antibody clones that decrease with progressing obesity, and conversely, could potentially identify changes in B-1b B cell antibody repertoires that contribute to disease progression. Results from these studies could then be used to determine the therapeutic potential of targeted adipose tissue antibody delivery or neutralization on obesity-induced metabolic dysfunction.

Our results showing that adoptive transfer of B-1b B cells lacking *Id3* attenuated obesity-associated glucose intolerance in *Rag1*$^{-/-}$ mice suggests that immunotherapy using these B cells could improve metabolic function. However, since recipients of WT B-1b B cells displayed a trend that approached significance for improved systemic glucose tolerance, more study is needed to determine whether this effect can also be induced by WT B-1b B cells. Despite the transfer of equivalent numbers of cells, *Rag1*$^{-/-}$ hosts that received Id3$^{\text{B cell KO}}$ B-1b B cells had roughly two-fold more omental B-1b B cells three weeks post-transfer than those that received WT cells, suggesting that increased B-1b B cell

number may lead to an improved metabolic phenotype. As our adoptive transfers consisted of a single injection of $8.0x10^4$ B-1b B cells, it is possible that simply increasing the number of cells transferred, or including additional booster injections, may confer a higher level of protection. Furthermore, since our studies were performed in mice lacking all B cells and T cells, studies are also needed to evaluate B-1b transfer into hosts with a more complete immune repertoire. While no complete B-1b-specific knockout model currently exists, *TLR9*$^{-/-}$ mice have a specific reduction in peritoneal B-1b B cells[95]. Obesity studies comparing these mice and those receiving WT B-1b adoptive transfers would help elucidate whether B-1b B cells have therapeutic potential in the presence of other immune cells known to play a role in mediating the effects of obesity. Due to their ability to self-maintain and self-expand, B-1b B cells have intriguing potential as an immunotherapy agent and additional studies evaluating them in this context may reveal exciting therapeutic roles for B-1b B cells in obesity and other inflammatory diseases.

Immunization is another potential method for enhancing any protective features B-1b B cells may have. B-1b B cells can produce antigen-specific antibodies to various T-independent antigens[223, 226]; a response that aids in the clearance of *B. hermsii* and *S. pneumoniae*[92, 94]. Indeed, immunization of mice with heat-killed *S. pneumoniae* resulted in an IgM antibody response, and attenuated the development of atherosclerosis[184]. As this response is driven by B-1b B cells[94], these findings highlight the potential use of immunization to induce B-1b antibody responses that may protect against diet-induced

inflammatory disease. Interestingly, although milky spots in omental adipose tissue lack follicular dendritic cells required for normal germinal center reactions[82, 235], they are able to trap bacteria and particulates suspended in the peritoneal fluid[236-238] and can support a limited immune response in SLP mice lacking conventional secondary lymphoid organs[82]. These findings suggest that these unique clusters of B-1 B cells and macrophages may be important sites of adipose tissue antibody production. Studies are needed to analyze if immunization with *S. pneumoniae* leads to local B-1b antibody response in adipose tissue, and whether this can protect against glucose intolerance and insulin resistance in obesity.

Id3 *is a regulator of B-1b B cells*

Id3 is a member of the HLH family of transcription factors that also includes basic-HLH (bHLH) proteins containing a basic DNA-binding domain. After bHLH members homo- or hetero-dimerize, they can bind DNA at E-box (CANNTG) motifs and directly activate or repress transcription[144]. *Id3* can also dimerize with bHLH proteins, but due to a lack of a DNA-binding domain, *Id3* acts in a dominant-negative manner to prevent bHLH function[146]. Prior to our studies, no role for *Id3* in B-1b B cells had been identified, and understanding of *Id3's* function in B cells is limited to studies in B-2 B cells. In these cells, much of *Id3's* activity is thought to be through regulation of bHLH proteins E47 and E12 (two splice variants of the *E2A* gene) that play prominent roles in B-2 cell development and function[153, 161, 239, 240]. *Id3* has been shown to regulate various

aspects of B-2 B cell function such as proliferation after BCR crosslinking[158] and class-switch recombination[159, 160]. As both Ig class switching and clonal expansion are critical components of an antibody response, these findings may help explain the impaired T-dependent and T-independent IgG antibody responses in *Id3*$^{-/-}$ mice[158], although the contribution of loss of *Id3* in other cells must also be accounted for. In addition to a potential role in antibody response, *Id3* over-expression induced apoptosis and growth arrest in B-2 progenitors[155, 156], and down-regulation of *Id3* was required for E47-mediated B cell lineage specification[157]. Despite these findings, *Id3*$^{-/-}$ mice have normal mature B-2 B cell numbers[158, 168], suggesting that loss of *Id3* has a limited impact on B-2 B cell development.

In contrast to B-2 B cells, results described in chapter 3 indicate that loss of *Id3* in B cells leads to a specific increase in peritoneal and adipose tissue B-1b B cells. Adoptive transfer studies indicated that this effect is, at least in part, due to loss of *Id3* in mature B-1b B cells. As LPS-injection studies in Id3$^{\text{B cell KO}}$ mice revealed reduced omental B-1b Annexin V staining but no differences in BrdU incorporation, we hypothesize that *Id3* regulates mature B-1b B cell number by limiting survival. Furthermore, the trend for reduced B-1b Annexin V expression in untreated mice could explain the disparity of B-1b B cell numbers at baseline, as consistent small variations in B-1b apoptosis could lead to substantial differences in cell number over time. However, these findings do not rule out the possibility that *Id3* could also regulate B-1b B cell development. While understanding of B-1b B cell development is incomplete, flow cytometry analysis

of fetal liver or bone marrow of Id3$^{B\ cell\ KO}$ mice could be performed to identify differences in progenitors known to be capable of producing B-1b B cells[227, 228]. If *Id3* plays a role in regulating B-1b B cell development, we might expect to see an increase in one of these populations. Two emerging technologies, including one that allows for quantitation of mRNA by flow cytometry, and mass cytometry[241] that can evaluate up to 40 surface or intracellular markers at once, have the potential be instrumental in identifying gene expression and signaling patterns in minute progenitor populations. Utilization of these novel methods will allow for further evaluation of potential checkpoints that *Id3* and other factors may regulate during B-1b development.

While B-1 B cells are able to self-maintain their population in the absence of specific stimulatory signals, the mechanisms that regulate B-1 B cell survival are unclear. Our findings suggest that *Id3* may be a key regulator of B-1b B cell survival, and additional study into how *Id3* functions in B-1b B cells may reveal important differences between these cells and other B subsets. One characteristic that distinguishes B-1 from B-2 B cells is the constitutive expression of various transcription factors in their activated form that are only found in B-2 B cells after stimulation. Two of these factors expressed in their activated, phosphorylated form in B-1 B cells, signal transducer and activator of transcription-3 (STAT3)[242] and extracellular signal-regulated kinase 1/2 (ERK1/2)[243, 244], are known inducers of survival[245, 246]. As *Id3* is activated by the Ras-ERK MAPK cascade in thymocytes[247], it is possible that ERK1/2 signaling activates *Id3* to serve as a regulator against uncontrolled survival in B-1b B cells.

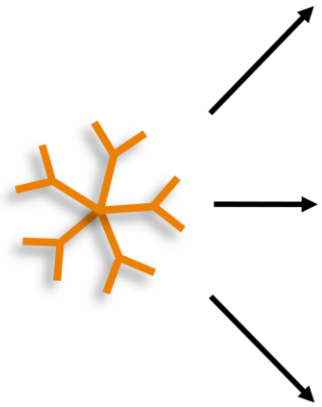
In addition, E2A-mediated STAT6 activation of IgE class-switch recombination in *Id2*$^{-/-}$ B cells was inhibited by *Id2* overexpression[248], suggesting a synergistic role for E2A in and STAT proteins. Future work is needed to determine whether E12 or E47 work with STAT3 to promote survival in B-1b B cells, and whether *Id3* may serve as a negative regulator of this process. Another study demonstrated that TGFβ was able to induce apoptosis in B lymphocyte progenitor cells in a mechanism dependent on *Id3* expression[155]. Follow-up analysis showed that this effect was dependent on caspase 2 signaling[156], and further studies are needed to test whether *Id3* regulates B-1b B cell survival in a similar manner.

In addition to targeted studies derived from known *Id3* function in other cell types, comparative microarray analysis of WT and *Id3*$^{-/-}$ B-1b B cells should also be performed. As *Id3* is known to function very differently depending on the cell type and available binding partners[149, 154], this could reveal important factors that are alternatively expressed in the absence of *Id3* in an unbiased manner. Furthermore, comparison array studies between WT and *Id3*$^{-/-}$ B-1a and B-1b B cells could be performed to identify factors differentially expressed only in *Id3*$^{-/-}$ B-1b B cells. This could identify factors crucial for distinguishing the two similar B cell subsets. Together, these studies have the potential to uncover novel pathways that are not only regulated by *Id3*, but that may be critical for understanding the differences between B-1a and B-1b B cells.

Possible mechanisms for IgM regulation of adipose tissue biology

The studies presented here show that when challenged with a HFD, a mouse model with elevated IgM antibodies displays reduced adipose tissue inflammation, improved adipose tissue insulin signaling, and attenuated systemic glucose intolerance. Adoptive transfer studies suggest that some of these effects may be dependent on IgM secretion by B-1b B cells, and additional studies in humans suggest that reduced levels of IgM may contribute to improved insulin sensitivity. While these findings are consistent with the previously defined role for IgM as a protector against inflammation and chronic disease[121], more work is needed to uncover the mechanism(s) by which IgM functions in adipose tissue (Figure 20).

One possibility is that IgM assists with dead cell clearance and maintaining local tissue homeostasis. In adult humans, billions of dead cells must be cleared every day – it is estimated that throughout the body, roughly one million cells undergo apoptosis per second[249]. Despite this remarkable amount of cell turnover, removal of dead cells occurs so quickly that uncleared apoptotic cells are rarely observed in healthy tissue[249]. However, defective removal of apoptotic cells results in elevated inflammation, and can lead to autoimmune



Possible roles for IgM in adipose tissue	**Remaining questions**
Clear apoptotic cells	-How are dead adipocytes removed? -Can IgM reduce dead cell accumulation in adipose tissue?
Inhibit macrophage activation	-Can IgM inhibit adipose tissue macrophage-derived inflammatory cytokine production? -Does IgM directly bind M1 macrophages? To what receptor? -If binding occurs, does it depend on antigen specificity?
Enhance adipocyte function	-What receptor does IgM bind? -Can IgM rescue glucose uptake and lipogenesis in insulin-resistant adipocytes?

Figure 20. Possible functions for IgM antibodies in adipose tissue. IgM is known to clear apoptotic cells, inhibit macrophage activation, and enhance adipocyte function. Studies are needed to further evaluate how IgM acts in adipose tissue *in vivo*.

diseases including SLE[126, 127], which is associated with insulin resistance in mice[250, 251] and humans[252]. In the same study described above by Winer and colleagues[81], analysis of IgG autoantibodies in obese insulin-resistant and obese insulin-sensitive patients revealed that most autoantibodies enriched in the insulin-resistant cohort recognized intracellular proteins, suggestive of impaired dead cell clearance. Together, these findings suggest that in addition to being primary sites of inflammation, uncleared dead cells in obese adipose tissue might contribute to the pathogenic IgG response observed in obesity.

Secreted IgM is an important mediator of dead cell clearance and tissue homeostasis, and sIgM$^{-/-}$ mice also develop an SLE-like phenotype[129, 253]. Cell surface expression patterns change during apoptosis, and IgM antibodies specific for moieties present on apoptotic, but not healthy, cells promote apoptotic cell clearance[254]. An example of such antibodies are members of the T15-IgM family that recognize the PC phospholipid that is expressed during the early stages of apoptosis. T15-IgM binds apoptotic cells and facilitates the binding of C1q and mannose-binding lectin (MBL)[119, 120, 255] – two proteins that assist phagocyte-mediated apoptotic cell clearance[256-259]. T15-IgM has been shown to promote apoptotic cell phagocytosis both *in vitro*[120] and increase defective phagocytosis *in vivo* in uMT[119] and sIgM$^{-/-}$[123] mice, suggesting that IgM-mediating apoptotic cell clearance is an important physiological process. In addition to PC-IgM antibodies, IgM specific for MDA has been shown to facilitate apoptotic cell clearance in *Rag1*$^{-/-}$ mice[109]. Furthermore, IgM antibodies that preferentially bind apoptotic cells have been identified[260-262], and there are known

IgM clones specific for apoptotic cell surface markers such as cardiolipin, phosphatidylserine, and Annexin V[263-266], suggesting that multiple natural antibodies may participate in the removal of dead cells and thereby prevent downstream inflammation and tissue destruction.

Likely due to a combination of the inflammatory and hypoxic microenvironment of obese adipose tissue, along with the physical demands of excess lipid storage, the progression of obesity strongly correlates with increased adipocyte death in mice and humans[190, 191, 267]. Interestingly, reduction in serum concentrations of MBL strongly correlates with indices of inflammation and reduced insulin sensitivity in mice and humans[268], and genetic MBL polymorphisms associate with gestational diabetes[269], suggesting that reduced concentration or function of apoptotic clearance machinery can impact glucose metabolism. CLS consisting of M1 macrophages surrounding uncleared dead adipocytes are a hallmark of obese adipose tissue, and contribute a major fraction of adipose-derived pro-inflammatory cytokines[270]. Several studies have shown that mice containing global[267] and adipocyte-specific[271] deletions of pro-apoptotic genes have reduced adipose tissue inflammation and display improved glucose homeostasis compared to controls, supporting the hypothesis that adipocyte death contributes to metabolic disease.

It is currently unknown whether IgM participates in removing apoptotic cells in adipose tissue. IgM antibodies have been reported to localize to CLS[81], but these findings must be confirmed using *sIgM*$^{-/-}$ negative controls to avoid the possibility that this staining was detecting IgM^{hi} B-1 B cells within CLS. Additional

studies that quantify CLS or measure apoptotic cells through TUNEL staining in adipose tissue of HFD *sIgM*$^{-/-}$ mice are also needed to determine if loss of secreted IgM leads to a noticeable reduction in apoptotic cell accumulation. If this were the case, follow-up experiments that transfer sorted B-1a or B-1b B cells into *sIgM*$^{-/-}$ hosts would test whether IgM derived from a B-1 subset is involved in preventing apoptotic cell accumulation in adipose tissue. While infusion of IgM antibodies known to assist in dead cell clearance (such as T15-IgM or IgM against MDA-LDL) into *sIgM*$^{-/-}$ recipients would allow for further delineation into whether these antibodies localize to sites of dead adipocytes and promote their clearance, such experiments are costly and require large quantities of antibody.

While CLS identification and TUNEL staining can detect uncleared dead adipocytes, relatively little is known regarding the apoptotic process in adipocytes or the physical factors regulating the removal of dead cells from adipose tissue. Recent studies suggest that dying adipocytes share much of the known apoptotic machinery of other cells[272], but gene overexpression and deletion studies are needed to confirm this. Furthermore, thorough microarray analysis is needed to identify any unique factors to adipocyte apoptosis that may have been missed by studies focusing on well-studied pathways. Understanding how programmed cell death works in adipocytes may allow for cell-specific antagonists of adipocyte apoptosis aimed at improving insulin sensitivity during periods of over-nutrition. In addition, the average adipocyte has a diameter of about 100μm, and can double or triple in size during obesity. As most apoptosis studies focus on clearing 6μm apoptotic thymocytes, obvious physical hurdles must be overcome to remove

these giant, lipid-laden cells. Co-culture studies could be performed to test if IgM promotes adipocyte clearance. In this experiment, labeled apoptotic adipocytes would be incubated with macrophages and treated with either T15-IgM or vehicle control. All studies would be performed in the presence of serum from *sIgM*$^{-/-}$, *Rag1*$^{-/-}$, *or* μMT mice to provide the complement factors, but not IgM antibodies, required for IgM-mediated phagocytosis[119, 120]. After several hours, adipocyte phagocytosis could be quantified by flow cytometry or immunofluorescent microscopy. Results of these studies, along with others that test specific roles of M1 and M2 macrophages in adipocyte clearance, will enhance our understanding of how to improve adipose tissue homeostasis when local cell death increases during obesity.

In addition to promoting apoptotic cell clearance, IgM antibodies have reported direct anti-inflammatory properties. In a murine model of atherosclerosis – another disease characterized by chronic inflammation – B-1a B cells adoptively transferred into splenectomized hosts reduced aortic plaque size and macrophage content in a mechanism dependent on their ability to secrete IgM[130]. Interestingly, IgM antibodies have been identified in atherosclerotic lesions[109] and EO6 – a specific IgM clone of the T15 family[116] – was able to inhibit macrophage uptake of oxidized LDL and prevent foam cell formation[185, 273]. In a separate study, T15/E06 IgM infusion attenuated the development of joint inflammation and cartilage damage in a mouse model of arthritis[119]. Interestingly, EO6 was also able to prevent OxPAPC-induced macrophage activation[274] and TLR-mediated dendritic cell and macrophage activation[119], suggesting that IgM

antibodies may directly reduce inflammatory cell activity. Consistent with these findings, preliminary studies in our laboratory suggest that treatment of omental adipose tissue with IgM or EO6 may reduce TNFα production by M1 macrophages (Figure 21). While no mechanism for this anti-inflammatory activity has been proposed, the previously designated Fas apoptotic inhibitory molecule 3 (TOSO)[275] was recently shown to act as an IgM Fc receptor (FcμR)[276, 277] and to be involved in pro-inflammatory TLR4 signaling[278]. It is possible that IgM can blunt inflammatory macrophage signaling through TOSO binding. Additional studies are needed to test if IgM antibodies can regulate adipose tissue macrophage inflammation, and whether any effect on activity is dependent on IgM binding to this novel FcμR.

A third mechanism by which IgM may regulate adipose tissue function is through direct interaction with adipocytes. Studies in the 1980s showed that polyclonal human IgM was able induce glucose uptake and stimulate lipogenesis in rat adipocytes better than insulin[192, 279]. Interestingly, competition assays showed that IgM did not compete with insulin binding, suggesting that it acts through a different receptor[279]. Additional work by the same group revealed that IgG antibodies have a similar metabolic effect on adipocytes[280], suggesting that

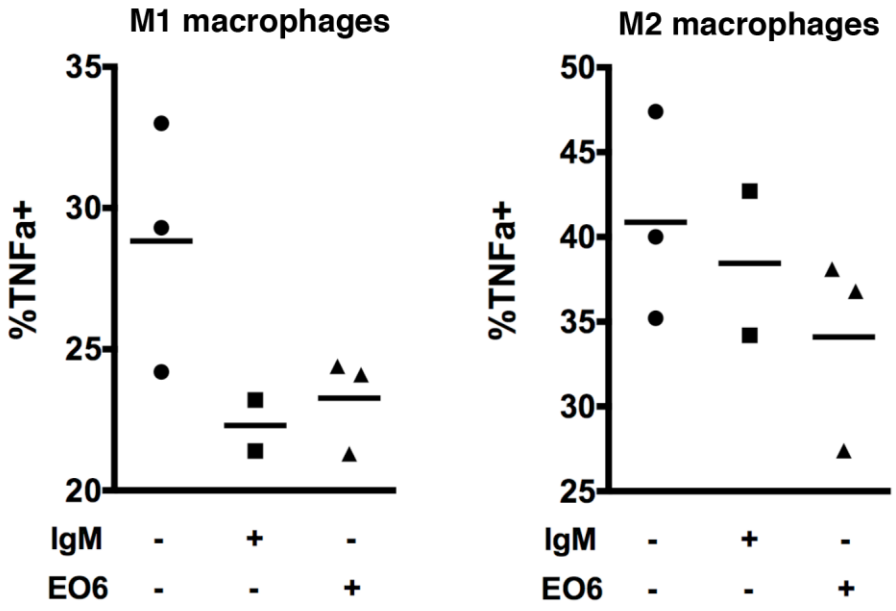

M1 macrophages
%TNFa+
35
30
25
20
IgM - + -
EO6 - - +
M2 macrophages
%TNFa+
50
45
40
35
30
25
IgM - + -
EO6 - - +

Figure 21. IgM antibodies may attenuate omental adipose tissue M1 macrophage TNFα secretion. Whole omental adipose tissue explants from C57Bl/6 mice fed a HFD for three weeks treated overnight with either IgM isotype or EO6 (T15-IgM). Explants were then activated with PMA/Ionomycin in the presence of Brefeldin A for five hours. F4/80^{+}CD11b^{+}CD11c^{+} M1 (left) and F4/80^{+}CD11b^{+}CD11c^{-} M2 (right) TNFα expression was quantified by flow cytometry. Each dot represents one mouse.

regulation may not be limited to IgM antibodies. A more recent study identified IgG Fc receptors on the cell membranes of human adipocytes and showed that Fc fragments of IgG antibodies were sufficient to stimulate lipogenesis and reduce IL-1β and IL-6 expression in adipocytes[76]. Together, these findings suggest that antibodies may have important regulatory effects on adipocytes, and follow-up studies are needed to determine whether adipocytes express TOSO or other receptors that specifically bind IgM. In addition, determining whether IgM treatment can rescue insulin-mediated metabolic function of insulin resistant adipocytes will be of great interest and has the potential to uncover novel signaling pathways that could be exploited for future therapeutic treatment.

Additional characterization of human CD20$^+$CD27$^+$CD43$^+$ B cells is needed.

While the existence of a human B-1 B cell equivalent has long been hypothesized, several factors have made its identification elusive. Unlike in mice, no single cell surface marker is known to identify human B-1 B cells. Murine B-1 B cells were first discovered as a unique CD5$^+$ B cell that spontaneously secreted IgM[281]. Using CD5 expression as a starting point, subsequent studies were able to identify characteristics and surface expression patterns specific for murine B-1 B cells. These studies led to the identification of a CD5$^-$ B-1 B cell[282] and the distinction between CD5$^+$ B-1a and CD5$^-$ B-1b B cells in mice. In humans however, while CD5 is found on IgM-producing B cells[283, 284], it is also expressed by other B cell subsets[285, 286], and no other B-1 distinguishing marker has been proposed. In addition to complications arising from differences in murine and

human B cell surface expression, much of our understanding of B-1 B cells in mice stems from unlimited access to bone marrow, peritoneal fluid, and lymphoid tissue. For obvious ethical and practical reasons, the majority of immune analysis in humans comes from studies of circulating cells. As few circulating B-1 B cells are found in mice, it is not surprising that our knowledge of B-1 B cells in humans has lagged behind.

While Griffin and colleagues' discovery of a novel human $CD20^{+}CD27^{+}CD43^{+}$ B cell population[90] is exciting, follow-up confirmatory studies by other groups will be important. Results from our studies described in chapter 4 indicate additional similarities between this B cell population and murine B-1 B cells, most notably their enrichment in omental adipose tissue and correlation with circulating natural IgM antibodies. Future studies to examine whether these B cells are a source of natural antibodies, if they have the capacity to self-renew, and where else they may be located will be instrumental in determining their true nature. Importantly, now that this cell has been identified, multi-color flow cytometry, microarray analysis, and the recently introduced mass cytometry technology[241] can be used to further characterize its gene expression pattern, antibody repertoire, and surface marker phenotype.

The identification of $CD20^{+}CD27^{+}CD43^{+}$ B cells in human omental adipose tissue highlights the need for additional analysis of these cells in the context of obesity and insulin resistance. Due to the relatively low numbers of patients that we have been able to analyze by flow cytometry, our study is incapable of delineating any correlations between $CD20^{+}CD27^{+}CD43^{+}$ B cells

and clinical outcomes. However, based on our murine findings, we would hypothesize that increased numbers and/or IgM production from these B cells would correlate with reduced adipose tissue inflammation and an improved metabolic phenotype. As recent evidence suggests that obesity and T2D alters B cell activity, further study is needed to evaluate how $CD20^{+}CD27^{+}CD43^{+}$ B cells in omental adipose are altered in this context. B cells from T2D patients secrete higher levels of IL-8, attenuated amounts of IL-10, and are more equipped to induce IL-17 expression in T cells[77, 101], indicating a pro-inflammatory phenotype. Obese individuals also have significantly reduced circulating IgM compared to age-matched controls, suggesting a reduction in either number or function of IgM-producing B cells[287]. Since a significant fraction of our patient cohort were obese and either diabetic or non diabetic with metabolic syndrome, additional studies are needed to determine whether $CD20^{+}CD27^{+}CD43^{+}$ B cell number or function is different in omental fat of healthy individuals. Results from these studies may uncover novel relationships between these B cells and metabolic disease, and allow for further insight into whether this newly discovered B cell population could be a potential immunotherapy agent.

CHAPTER 6:

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