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The Impact of Maternal Obesity on the Neonatal Immune System

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Dedication

I would like to dedicate this work to all of my family.

I love you all. Thank you for helping make me who I am.

To my parents and my brother, thank you for your continuous love and support. Thank you for everything you've ever done for me. Thank you for always being my stalwart support group, my weekend distractions, and for always being my home away from home. I also want to say thank you to my best friend and wife, Kelsi. Without your steady encouragement, positive point of view, and unconditional love I would never have made it. With your support I was able to make it to this milestone in my life, and can move on to the next great thing with no regrets.

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Chapter 1: The impact of maternal obesity during pregnancy on offspring immunity

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Abstract

In the United States, approximately 64% of women of childbearing age are either overweight or obese. Maternal obesity during pregnancy is associated with a greater risk for adverse maternal-fetal outcomes. Adverse health outcomes for the offspring can persist into adulthood, increasing the incidence of several chronic conditions including cardiovascular disease, diabetes, and asthma. Since these diseases have a significant inflammatory component, these observations are indicative of perturbation of the normal development and maturation of the immune system of the offspring in utero. This hypothesis is strongly supported by data from several rodent studies. Although the mechanisms of these perturbations are not fully understood, it is thought that increased placental inflammation due to obesity may directly affect neonatal development through alterations in nutrient transport. In this review we examine the impact of maternal obesity on the neonatal immune system, and potential mechanisms for the changes observed.

Introduction

Obesity is defined as the accumulation of excess body fat and is traditionally reported by the body mass index (BMI), which is a calculation of weight (kg) divided by height (meters) squared. Healthy individuals have BMI values >19 and <25 kg/m^2 whereas persons with a BMI ≥25 kg/m^2 are considered overweight and those with a BMI ≥30 kg/m^2 meet the criteria for obesity. Obesity is a metabolic disease that results in a chronic low-grade inflammation (1-4), characterized by high levels of inflammatory mediators such as C-reactive protein (CRP), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNFα). More importantly, excess weight/adiposity is associated with several co-morbidities including: diabetes, cardiovascular disease, pulmonary complications, and cancer (5) resulting in decreased life expectancy (6). According to the Centers for Disease Control and Prevention (CDC), as of 2012, 69% of the adults aged 20 years and older in the United States have a BMI>25, and 35.1% are obese (7). The American Heart Association estimates that in 2013, 154.7 million people were overweight, and of these, 78.4 million were obese (8). Unfortunately, the rates of obesity are expected to continue to rise (9).

Clinical Impact of maternal obesity on maternal health

It is generally believed that a high BMI entering pregnancy, and excess weight gain during pregnancy can exacerbate the natural inflammatory state associated with pregnancy resulting in detrimental health outcomes for the

mother (2, 10). A retrospective study by Leung et al. that assessed adverse outcomes associated with maternal obesity in a cohort of 29,303 Chinese women between 1995-2005, found that high maternal BMI assessed during the first antenatal visit correlated with increased rates of preeclampsia, gestational diabetes (GDM), caesarean section, and preterm delivery (11). Similarly, a study of 6,959 singleton pregnancies from 2001 to 2005 by Gaillard et al., in the Netherlands found that maternal obesity prior to pregnancy and excessive weight gain during pregnancy were both associated with increased incidence of preeclampsia, GDM, hypertension, and caesarean section despite adjusting for familial and lifestyle factors (12). A third retrospective study of 30,298 subjects from 2004-2011 by Scott-Pillai et al. also reported similar outcomes in the UK, and showed that these risks are further exacerbated as BMI (measured during first pre-natal visit) increases above 35 (13).

Maternal environment and origin of disease

The “origins of fetal and infant disease hypothesis,” was first proposed in 1990 by Dr. Barker to serve as an explanation for the increase in coronary heart disease in western countries (14, 15). In 2001, Drs. Hale and Barker expanded the “origins of fetal and infant disease hypothesis” into the “thrifty phenotype” hypothesis, which was based on the observations that children born to undernourished women during famine were better able to survive than children born to normally nourished women. This adaptation, while beneficial in times of

famine, became a disadvantage when conditions became plentiful and children became predisposed to obesity and impaired glucose tolerance (16, 17). This theory was further expanded by Dr. Barker in 2002 into the “Fetal Origins of Adult Disease” (FOAD) based on the observation that children who were small at birth/infancy, as a result of intrauterine growth restriction in undernourished mothers, subsequently underwent accelerated weight gain in childhood and experienced higher incidence of coronary heart disease, type 2 diabetes, and hypertension as adults compared to normal birth weight children (18, 19). The FOAD proposes that changes in the gestational milieu directly impact fetal growth and development (18, 20), and consequently disease incidence later in life (21). Two cornerstones of the FOAD hypothesis are developmental plasticity and compensatory growth (19). Developmental plasticity refers to changes in neural connections during development as a result of in utero environment. Compensatory growth, or “catch-up growth”, is accelerated growth following a period of slowed development. Therefore, adaptations acquired in utero during limited nutritional intake were later detrimental in the adult offspring or when food became plentiful.

Although the initial Barker hypothesis focused primarily on nutritional deficiencies and low birth weight, there is evidence that over-nutrition also alters in utero programming (22). The “ fetal over-nutrition hypothesis” was developed based on epidemiological evidence that greater maternal adiposity, (self-reported/measured pre-pregnancy/ first antenatal visit weight), increased lifetime

risk of type 2 diabetes (23) and obesity (24) in the offspring. In the latter study, both maternal and paternal BMIs were taken before pregnancy, and the child's BMI was assessed at birth, 5 years, and 14 years. Only maternal BMI had an effect on offspring BMI, which wasn't evident until 14 years of age.

Taken together, there is strong evidence that in utero instruction by maternal nutritional environment can influence growth rate and disease risk during adulthood (18, 22, 24, 25). A fetus can adapt its physiology and metabolism to the supply of nutrients crossing the placenta (25). Excess supply of nutrients can lead to permanent changes in metabolism, behavior and appetite regulation in offspring resulting in adverse health outcomes later in life (26). In summary, this phenomenon is known as "early-life programming" which recognizes that events in utero have long term influences on risks of disease later in life (26).

Clinical Impact of maternal obesity in offspring:

Increased incidence of stillbirths, abnormal growth, cardiac defects, and neural tube defects has been reported in the offspring of obese women after adjustment for various factors including age, familial and lifestyle factors, and ethnicity ((11-13, 27, 28) and Table 1). Furthermore, children born to women who enter pregnancy in an obesogenic state are at higher risk for several adverse long-term health outcomes including increased incidence of obesity (29), cognitive development deficits and ADHD (30), type-2 diabetes (31, 32),

cardiovascular disease (33, 34), cancer (35), and greater all-cause mortality (33) in comparison to children born to lean mothers.

A Finnish study of coronary heart disease found that men born to mothers with a high BMI (assessed at time of labor admission) experienced a higher incidence of coronary related deaths (34). A prospective cohort study in the Netherlands, utilizing data from the Generation R study in which growth and development, behavior, asthma childhood disease and genetics were documented from fetal life until young adulthood, found that high maternal pre-pregnancy BMI was associated with adverse cardiometabolic profiles in the offspring (36). Adverse cardiovascular outcomes were detected despite the exclusion of women with both pre-pregnancy, and pregnancy complications including diabetes/GDM (33, 34, 36). In addition to cardiovascular disease, the periconceptional environment affects gene expression for at least 10 years and may result in lifelong increased risk of cancer in the offspring (35). Finally, increased incidence of childhood asthma and wheezing is emerging as a common adverse health outcome for the offspring of mothers with a pre-pregnancy BMI >30 or excessive weight gain during pregnancy following adjustment for sex of child, maternal age, smoking, maternal history of asthma/allergy, maternal BMI, and maternal weight gain during pregnancy (37-39). The immune system plays a critical role in the pathogenesis of cardiovascular disease (40), asthma (41-43), and diabetes (1). Since maternal

Species	Finding	Time of maternal BMI Assessment	Age of offspring assessed	Covariates adjustment*	References	Association with offspring BMI
Human	Increased incidence of abnormal growth	Pre-gravid	Birth	1- 4, 19	11	Unknown
Human	Increased incidence of stillbirth, abnormal growth	Pre-gravid, and throughout	Birth	1, 2, 5, 9	13	Unknown
Human	Increased incidence of neural tube defects	Pre-gravid	Unknown	1, 4, 6-7, 14, 16, 17	28	Unknown
Human	16x higher likelihood of detectable CRP	Pre-gravid	12 years	9, 10	29	Unknown
Human	Increased coronary artery disease and all-cause mortality	Pre-gravid	34-61 years	1, 2, 8, 10-12, 19	33	Yes
Human	Increased coronary heart disease	Pre-gravid	37-70	1, 2, 8, 10, 11, 12, 19	34	Yes
Human	Increased adverse cardiometabolic risk	Pre-gravid	24 month	4, 8, 14, 15, 17, 19, 20	36	Yes
Human	Increased risk of childhood asthma, and wheezing	Pre-gravid	14 months to 16 years	1-2, 10-11, 14, 16, 18,	37	Unknown
Human	Increased risk of childhood, wheezing	Pre-gravid	6 months. 2, 4, and 6 years	5, 12, 14, 16, 19, 21	39	Unknown
Human	Increased risk of childhood asthma, ectopic eczema, and hay fever	2nd trimester (16 weeks)	6 and 18 months, and 7 years	1, 4, 8, 10-11, 17-19, 21	38	Unknown
Human	May increase risk of ADHD/cognitive deficits	Pre-gravid	From birth to childhood	2, 8, 10, 12, 14, 16	30	Unknown
Murine	Lower IgG and higher IgE responses	HFD at start of study	17-19 weeks		74	Yes
Murine	Lower systemic responses and higher colonic inflammation	HFD at start of study	5-6 weeks		75	Yes
Baboon	PBMC gene expression changes	HFD at start of study	Pre-term: 165 days gestation		76	Yes

Table 1.1: Impact of pre-gravid obesity on infant outcomes. * Covariates: 1) maternal age, 2) parity, 3) social deprivation, 4) smoking, 5) maternal diabetes, 6) drug use, 7) maternal chronic conditions (diabetes, high blood pressure, history of seizures), 8) birth weight, 9) Tanner stages, 10) offspring gender, 11) socio/economic status, 12) current age of offspring, 13) placental weight, 14) maternal education, 15) paternal BMI, 16) Maternal ethnicity, 17) alcohol consumption, 18) mode of delivery, 19) gestational age, 20) childhood height, 21) history of maternal allergy (asthma, atopic eczema, hay fever)

obesity is associated with increased incidence of these diseases; it is highly likely that maternal obesity disrupts normal development and maturation of the offspring's immune system in utero.

Overview of the immune system

The immune system is our first line of defense against microbial infection and cancer. It is composed of leukocytes that secrete various immune molecules such as cytokines, chemokines, immunoglobulins, and complement proteins in order to eliminate pathogens as well as mediate cell-to-cell communication. The immune system can be divided into two main branches: innate and adaptive immunity (44). The innate immune branch is composed of neutrophils, eosinophils, basophils, natural killer (NK) cells, monocytes/macrophages, and dendritic cells (DCs); as well mucosal barriers and antimicrobial peptides (45). These cells can distinguish self from invading pathogens by expressing pattern recognition receptors (PRR) that recognize pathogen associated molecular patterns (PAMPs). Signaling through these PRRs results in signaling cascades that culminate in the production of cytokines, chemokines and complement proteins that can interfere with pathogen replication and recruit additional immune cells to the site of infection. The innate response responds immediately to pathogens but, in contrast to T and B cells, it lacks pathogen specificity.

Activation of the innate immune branch is critical to the initiation of the adaptive immune response; which is required to fully clear pathogens and

establish long-term immunological memory. The adaptive immune branch is comprised of T cells and B cells. Innate immune cells such as macrophages or dendritic cells can activate T cells by presenting pathogen-derived peptides in the context of major histocompatibility molecules (44). T cells can be divided into CD4+ T helper (Th) cells and CD8+ cytotoxic T cells. CD4 T cells can be further divided into Th1, Th2, Th17, regulatory (Treg), and follicular helper (Tfh) cells. Th1 cells produce cytokines such as IL-2 and IFNγ and play a critical role in anti-viral and anti-bacterial pathogens; Th2 cells primarily produce cytokines such as IL-4 and IL-10 and mediate responses to parasites; Th17 cells are responsible for immunity against extracellular bacteria and fungi; Tfh cells regulate development of B cell immunity; finally, Treg cells are negative regulators and play a role in the termination of the immune response and protect against auto-immunity (46). The primary function of B cells is to secrete antibodies that coat pathogens thereby targeting them for destruction through opsonization, activation of the complement system, and neutralizing their ability to infect new target cells (44). Activation of both innate and adaptive immune responses results in acute inflammation, which is critical for pathogen clearance. However, chronic inflammation can cause and exacerbate several diseases such as cardiovascular disease (40, 47-49), diabetes (50-54), and asthma (55, 56).

The development of the immune system begins in conjunction with the vascular and cardiac systems early during embryogenesis (57). In both mice and humans, blood cells in the yolk sac develop into hematopoietic stem cells (HSC)

(57). In humans, the thymus can be detected at 8 weeks of gestation and is colonized by hematopoietic stem cells (57). Early T cell development begins between 14 and 17 week of gestation (58) and mature T cells emigrate to form the peripheral T cell pool 16-20 weeks of gestation (57, 58). By the 20th week, organogenesis of the thymus is complete (57). Secondary lymphoid tissues such as the lymph nodes are detectable by 12 weeks of gestation (59). T cells appear in the lymph nodes at approximately week 14 of gestation, shortly after the beginning of T cell development (57, 60).

In addition to the thymus, the liver is also seeded early on by HSCs from the yolk sac around week 3-4 of gestation (57, 61). By 9 weeks of gestation the liver functions as both an early source of red blood cells and pre-B lymphocytes prior to formation of bone marrow (60). In parallel with pre-B cell development, the spleen is detectable by 8 weeks (59). B cells are present in the primary follicles of lymph nodes by 17 weeks of gestation (57, 60). It has been seen that humans HSCs travel from the liver to the bone marrow around 20 weeks of gestation (62) which takes over as the producer of red blood cells, B cells and phagocytic cells.

Some of the first phagocytic cells seen in the fetus are embryonic macrophages around week 3-4 of gestation (61, 63, 64). These early monocytes are produced by the liver as their appearance precedes the development of bone marrow (61). Blood circulating monocytes and dendritic cells, as seen in adults, develop in the bone marrow starting in the second trimester (61). The population

of monocytes and dendritic cells increase throughout gestation (57, 64, 65), but dendritic cell levels don't reach those of adults until almost 5 years of age (57). In contrast to monocytes/macrophages and dendritic cells, granulocytes, specifically neutrophils, are not present until 31 weeks (66). Neutrophils are derived from granulocyte/monocyte progenitors in the bone marrow (61). By birth, neutrophils become the dominant cell population (62). In utero, the fetal immune system is predominantly Th2 biased, presumed to prevent Th1 type alloimmune responses to maternal tissues (67).

The adaptive immune system of the newborn lacks antigenic experience as evidenced by the exclusive presence of naïve T and B cells (44, 68), and therefore it must rely on its innate immune system (69). Additional protection against infection during the first 6 months of life is provided by maternal antibodies acquired in utero and through breast-feeding that help protect against gastrointestinal and respiratory infection (70). Moreover, newborns display Th-17 (IL-6, IL-23) responses, low production of type-I interferons, and weak Th1 support (TNF, IL-1) following TLR stimulation (71). In the early weeks following birth, there is a large increase in the number of peripheral lymphocytes (57). However, T cell responses remain biased towards Th2 phenotype, with low IL-2 and IFNγ production (72). This bias is believed to be due to functional differences in dendritic cells that may prevent differentiation of naïve CD4 T cells towards Th1 (68). Through infancy and early childhood, the newborn’s immune system undergoes a shift from a predominantly Th-2 to Th-1 responses, which promote

cellular immunity and macrophage activation (67, 69). By two years of age, production of Th1 supporting cytokine IL-12p70 by dendritic cells is detected, which further promote development of Th1 responses (71). With age and antigen experience, T cell responses increase, and Cytotoxic and Helper T cells develop central and effector memory populations (44, 57). Similarly, B cells are largely naïve at birth and generate limited responses (57), most likely due to both extrinsic factors such as reduced dendritic cell co-stimulation and intrinsic factors such as decreased B cell receptor signaling in naïve neonatal B cells (68). Immature B cells undergo maturation in the spleen where switch from high expression of IgM to high expression of IgD (73).

Impact of maternal obesity on immune system development

Recent studies have begun to characterize the effect of maternal obesity on the development of the neonatal immune system. Children born to obese mothers have a 16 fold higher risk of having detectable CRP levels at 12 years of age after adjustment for BMI z-score, Tanner stages (scale of physical development), and gender (29) compared to children born to lean mothers, suggesting that higher CRP levels in the offspring are a result of high maternal BMI during pregnancy. Studies using murine models have shown detrimental changes in the immune system of pups born to dams fed a high fat diet during gestation regardless of postnatal diet (74, 75). In one study, pups born to dams fed a high fat diet (HFD) during gestation and maintained on a HFD after

weaning generated lower ovalbumin (OVA)-specific IgG, but higher OVA-specific IgE, after immunization compared to pups born to control diet fed dams and pups (74). The increase in IgE production could potentially explain the increased incidence of allergy in children born to obese mothers. In addition, *ex vivo* stimulation of splenocytes from pups born to dams fed a HFD during gestation with lipopolysaccharide (LPS), produced higher levels of TNFα regardless of their post-weaning diet (74).

Another rodent study found that pups born to dams fed a western diet (WD) both prior to and during gestation exhibited worse outcomes in response to bacterial infection and experimentally induced autoimmunity, higher colonic inflammatory responses, and muted systemic responses to antigen despite being weaned on a control diet and not being obese or diabetic themselves (75). Specifically, the offspring exhibited greater mortality rates in an *E.coli* sepsis model, and exhibited greater bacterial burden and larger abscesses after infection with Methicillin-resistant *Staphylococcus aureus* (MRSA) (75). These pups were also statistically more likely to develop signs of experimental autoimmune encephalitis (EAE) following injection of myelin oligodendrocyte glycoprotein than mice born to dams fed a control chow diet (75). Frequency of Tregs was reduced in both colonic lamina propria lymphocytes (LPL) and splenocytes collected from pups born to dams fed a WD. In addition, *ex vivo* stimulation of colonic LPLs isolated from pups born to dams fed a WD with LPS resulted in greater levels of IL-6, IL-1β, and IL-17, whereas LPS stimulated

splenocytes from the same group generated decreased levels of TNFα and IL-6 compared to control groups (75).

A nonhuman primate study compared the transcriptome of fetal peripheral blood mononuclear cells (PBMC) from offspring of obese and lean baboons (maternal BMI was assessed prior to pregnancy). Analysis conducted using Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed 29 significantly affected pathways including antigen presentation, complement and coagulation cascades, leukocyte transendothelial migration, B cell receptor signaling pathways, tight junctions, and MAPK and VEGF signaling pathways (76). Taken together, these studies support the concept that high fat intake during pregnancy or a high maternal BMI can interfere with normal development of the offspring's immune system in utero.

Mechanisms of neonatal immune system dysregulation

The mechanisms underlying maternal obesity-induced changes in immune system of the offspring are just beginning to emerge. The placenta is responsible for transport of nutrients to the fetus and is likely to influence fetal growth and development and therefore play a key role in the development of the neonatal immune system (16) (Table 2). Accumulation of lipids has been seen in the placentas of obese women, notably in the villi stroma (77). In a nonhuman primate study, excess lipid build up in the microvilli created a structural impairment that resulted in decreased amino acid transport by *System A* (76).

Increased lipid accumulation can also result in higher expression of inflammatory genes in the placenta. Indeed, increased concentrations of pro-inflammatory mediators TNFα, IL-1, and IL-6 in the placentas of obese compared to lean pregnant women (2, 78, 79) as well as increased production of Th2 cytokines (80) have been reported. Increased inflammation within the placenta may alter fetal cytokine levels thereby affecting immune system development and resulting in differential response to vaccination and allergens later in life (81, 82).

Inflammation within the placenta may also be a result of increased accumulation of macrophages (78). Increased macrophage infiltration in the placenta as a result of maternal obesity was also described in a baboon model (76). It has been hypothesized that activated peripheral monocytes or adipose

Species	Finding	Maternal HFD diet/BMI measurement	References
Human	Increased Macrophages, increased expression TNFa, IL-1, IL-6	Pre-gravid	78
Baboon	Increased Macs, increased expression TNFα, IL-1, IL-6 Lipi accumulation in placenta	Pre-gravid	75
Human	Lipid build up in villi stroma	Pre-gravid	76, 77
Rhesus Macaque	Reduced uterine blood flow, increased TNFα, IL-1β, MCP-1, CCL2	Pre-gravid	79
Human	Increased placental surface thickness; chronic and impaired endothelial activation function; increased production of IL-6	Pre-gravid	80, 86
Human	Activation of placental p38-MAPK and STAT3 signaling	Pre-gravid	87
Sheep	Upregulation of NF-kB and JNK pathways	Mid-Gestation	88

Table 1.2: Impact of maternal obesity on placental health

tissue-derived macrophages are recruited to accumulate in the placenta of obese pregnant women due to increased levels of monocyte-chemoattractant protein-1 (MCP-1), IL-6, and TNFα secreted by adipose tissue (83) and the greater circulating levels of CRP and IL-6 seen in obese pregnant women (78, 84-86). Increasing maternal BMI and fat mass also correlates with increased placental surface and thickness, and chronic endothelial cell activation (87). These changes can lead to reduced blood flow and structural damage in the placenta. Indeed, in a nonhuman primate study using a cohort of 24 Japanese Macaques, a high fat diet during pregnancy resulted in a 38-56% reduction in uterine volume blood flow regardless of the dam's weight (79). This obesity-induced placental ischemia was associated with the up-regulation of 13 pro-inflammatory cytokines including GM-CSF, IL-2, IL-4, IL17, MCP-1, and TNFα (79).

Some studies have implicated p38-MAPK and STAT3 as key pathways involved in placental inflammation, while others have suggested NF-kB and JNK pathways (88, 89). A study by Aye et al. showed that maternal BMI is associated with activation of placental p38-MAPK and STAT3 signaling but not NF-kB, JNK, or caspase 1 pathways (88). Work by Basu et al found that in pregravid obesity, the stromal vascular cells of adipose tissue express higher levels of TNF-α and IL-6 (90). Increased secretion of TNFα and IL-6 provide a potential explanation for the activation of the p38-MAPK and STAT3 pathways. TNFα activates p38-MAPK, whereas TNFα and IL-6 activate STAT3 (91, 92). Finally, Zhu et al. found that, maternal obesity resulted in up-regulation of NF-kB and JNK pathways in

mid-gestation sheep placenta (89). The authors proposed that free fatty acids (due to excess adiposity) activate the Toll-Like Receptor (TLR) 4 pathway, which in turn activates the NF-kB and JNK pathways leading to placental inflammation.

Another potential mechanism by which maternal obesity can modulate development of the offspring's immune system is through changes in gut microbiome. Recent studies have shown that the diversity of microbes in the gut is instrumental for the normal function and development of the immune system (93). In one of the rodent studies discussed in the previous section (75), microbiome analysis of colon section from pups born to dams fed a western diet showed an increased Firmicute/Bacteriodete ratio that corresponded to increased colonic inflammation (75). In a recent human study, maternal BMI was found to be a strong predictor of increased Shannon Diversity Index (SDI, or the measure of overall diversity within a microbial community) in gut microbiota of toddlers between 18 and 27 months (94). The authors proposed that the increase in both the alpha and beta diversities was due to reduced immune surveillance resulting in greater bacterial growth. Additional studies are required to understand the role of bacterial diversity in shaping the developing immune system.

Epigenetic and in utero programming

Changes in the transport of nutrients and levels of inflammatory mediators in the placenta are also likely to influence the development of the neonatal

immune system through epigenetic modifications, which in turn lead to alterations in gene expression (76). Epigenetic modifications include posttranslational modification of proteins and histones (such as ubiquitination, methylation, acetylation, and phosphorylation) and DNA methylation that lead to either suppression or activation of gene expression (95). For instance, the histone marker H3K4me is associated with active transcription whereas the marker H3K9me3 is associated with transcriptional repression (96). DNA methylation is the addition of a methyl group to the 5-position of a cytosine (97) which either inhibits transcription directly, or recruits methyl-CpG-binding domain proteins which repress transcription by chromatin compaction through chromatin remodeling complexes (97). DNA methylation is a highly regulated process that is inherited by daughter cells. Several studies have reported epigenetic changes in response to diet (21), environmental pollutants (98, 99), or chronic conditions such as asthma (100) and cancer (101).

Maternal BMI before and during pregnancy affects DNA methylation in the offspring's peripheral blood cells. A recent study demonstrated that maternal pre-pregnancy BMI inversely correlated with overall methylation levels in cord blood samples, with the greatest methylation changes occurring within genes associated with cancer (WNT16) and diabetes (BTN3AI) (102). In a zebrafish model, WNT16 was found to regulate the hematopoietic stem cell specification during embryonic development; therefore, increased methylation could potentially interfere with Notch ligand signaling required for HSC specification (103).

BTN3A1 is a regulator of molecules that activate CD4+ T cells (104); therefore, increased methylation could theoretically result in lower CD4+ T cell responses. Another recent study that examined DNA methylation patterns in cord blood leukocytes obtained from children born to obese mothers and then again at three years of age showed lower global methylation relative to children born to lean mothers at 3 years (105). Lower global methylation in leukocytes could result in dysregulated immune responses or cellular differentiation. An additional study by Godfrey et al. compared cord blood methylation profiles at 68 CpGs sites from 5 genes to adiposity measurements in the same children at 9 years of age (106). This study showed that methylation at retinoid X receptor-a (RXRA) and endothelial nitric oxide synthase (eNOS) correlated with increased adiposity in children at 9 years (106). RXRA is a receptor that enhances Th2 development following stimulation by RXRAs agonist Vitamin A (107). Endothelial nitric oxide synthase (eNOS), is important in the up-regulation of inos and other inflammatory factors such as NF-kB (108). Finally, pups born to dams fed a western diet during gestation exhibited greater H3K9Me3 histone methylation at TLR4 and LPS binding protein loci in splenocytes compared to pups born to dams fed a control diet (75). These data provide a rationale for the lower cytokine response generated by splenocytes isolated from pups born to obese dams compared to those born to dams fed a control diet following LPS stimulation (75). Moreover, these methylation profiles were identical to those observed in the obese dams indicative of germ line inheritance (75).

Alterations in DNA methylation of circulating PBMCs of children due to maternal obesity (102, 105, 106) can have significant impact on gene expression and consequently function of immune cells. For instance, reduced DNA methylation at genes associated with T cell activation can lead to aberrant cytokine production and inflammation. Indeed, it has been seen that in activated CD4 and CD8 T cells, loci of cytokines have less methylation compared to naïve cells (109). Additional studies are needed to better understand the relationship between maternal body composition during pregnancy and epigenetic changes, differences in gene expression and functional alterations of immune cells in the offspring.

Conclusion:

Prevalence of obesity has increased dramatically over the last 20 years, especially in women of reproductive age. Entering pregnancy in an obese state and/or gaining excessive weight during pregnancy are associated with several complications for the mother including increased rates of preeclampsia, GDM, caesarean section, and preterm delivery. Adverse health outcomes for the neonate include abnormal growth and birth defects. Long-term adverse health outcomes for the offspring include increased incidence of chronic inflammatory diseases such as cardiovascular disease, diabetes and asthma (Table 1). The mechanisms by which maternal obesity induces changes in immune system of the offspring are poorly understood, but it is thought that increased accumulation

of lipids in the placenta result in increased infiltration of macrophages, a reduction placental blood flow which lead to hypoxia and exacerbate placental inflammation, and altered nutrient transport thereby resulting in poor fetal health outcomes (Figure 1 and Table 2).

Changes in nutrient transport and levels of inflammatory mediators can contribute to epigenetic modifications and changes in gene expression. This hypothesis is supported by reported changes in global methylation and transcriptome analysis of PBMC or umbilical cord blood mononuclear cells. Future studies will need to focus on epigenetic changes within individual immune cell populations, as important changes in small populations may not be apparent when analyzing total leukocytes. Specifically, it might be interesting to examine epigenetic changes in monocytes or CD4 T helper cells as we have shown recently a dampened response by monocytes and myeloid dendritic cells stimulated by TL4 or TLR1&2 agonists and a reduction in IL-4 production by CD4 T cells (110). In addition to CpG islands, future epigenetic studies should include variably methylated regions (VMR). VMRs are regions of DNA that have a great propensity for changes in methylation, can sometimes overlap with CpG islands, are involved in embryonic patterning, and vary across individuals including family members (111). VMRs can be used in identifying disease vulnerability traits (112). In addition, further studies examining changes in the transport of lipids, nutrients, and immune molecules across the placenta would better elucidate their role in modulating epigenetic modifications and regulation of gene expression.

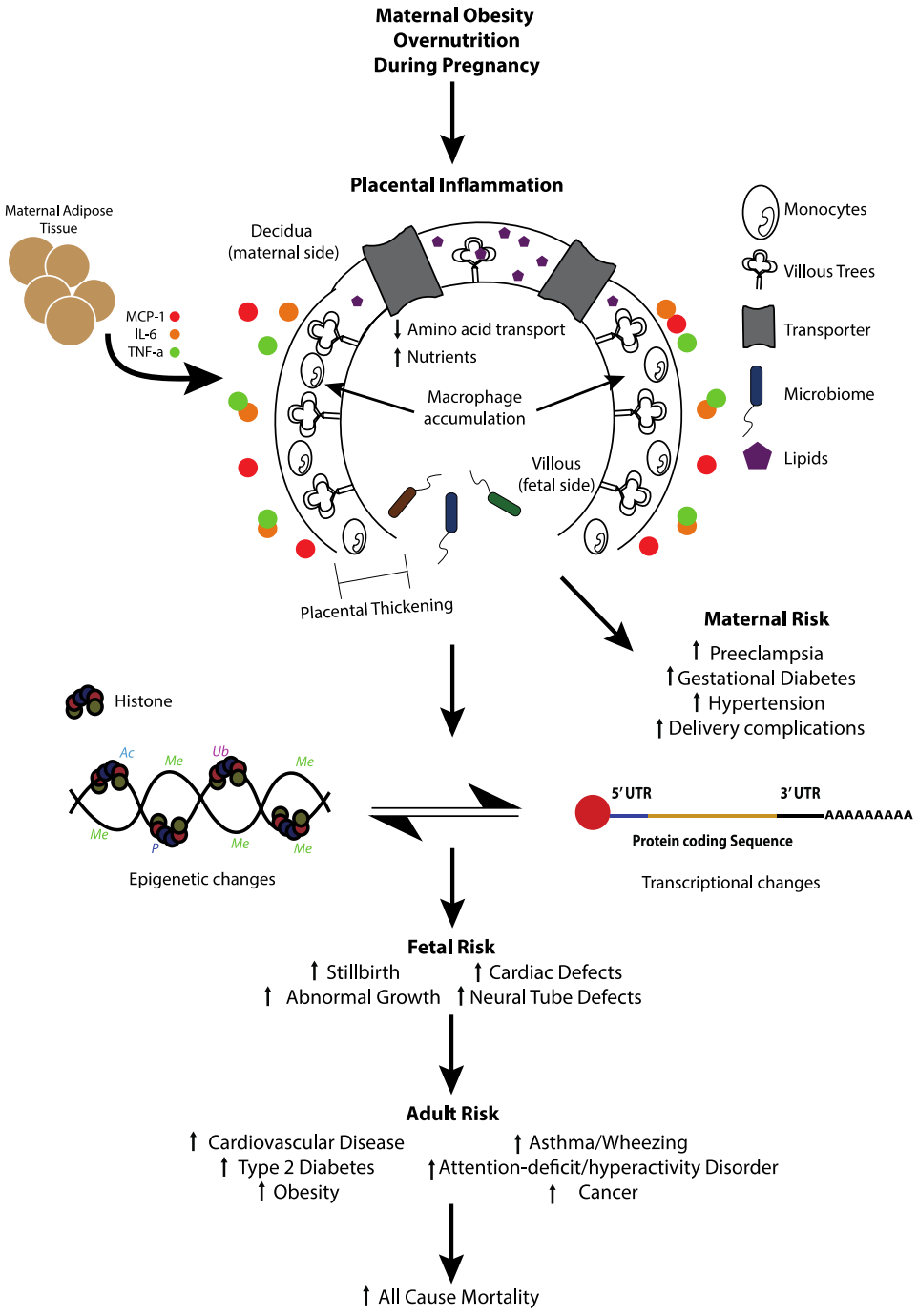

Figure 1.1: Overview of how obesity or over nutrition during pregnancy leads to adverse health outcomes for the offspring

Additional studies into the role of miRNAs in mediating the effects of maternal obesity on placental and fetal health are also needed given their integral role in in regulating gene expression. Future work should also address the effect of maternal obesity in subsequent generations to assess whether these long-term epigenetic changes are compounded over successive generations.

Finally, an important consideration for future studies is the choice of model organism. There are several advantages to working with rodent models: short time of fetal development and pup maturation, and the availability of recombinant strains that facilitate mechanistic studies. However, rodent models also present several challenges when studying neonatal immunity because the peripheral adaptive immune system develops only after birth in mice whereas primates are born with a fully populated peripheral immune system (113). Large animal models such as nonhuman primates provide robust models for studying immunity at the maternal-fetal interface and in the offspring. Their large size facilitates longitudinal studies, imaging of the placenta, and importantly, exhibit greater similarity to humans in their susceptibility to metabolic disease (114).

In summary, maternal obesity is becoming one of the biggest threats to the offspring's health. However, the effects of maternal obesity on the development of the offspring's immune system are poorly understood. A better understanding of the mechanisms by which maternal obesity interferes with normal development as well as maturation of the offspring's immune system will lead to treatment strategies and prevention of diseases such as asthma,

diabetes, and atherosclerosis which are more prevalent in the offspring of obese mothers.

Chapter 2: Maternal Obesity alters immune cell frequencies and responses in umbilical cord blood samples

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Abstract

Maternal obesity is one of several key factors thought to modulate neonatal immune system development. Data from murine studies demonstrate worse outcomes in models of infection, autoimmunity, and allergic sensitization in offspring of obese dams. In humans, children born to obese mothers are at increased risk for asthma. These findings suggest a dysregulation of immune function in the children of obese mothers; however, the underlying mechanisms remain poorly understood. The aim of this study was to examine the relationship between maternal body weight and the human neonatal immune system.

Introduction

Almost 60% of women of childbearing age are overweight (body mass index (BMI) ≥25 kg/m^2) or obese (36%, BMI ≥30 kg/m^2)(7). Maternal obesity is associated with several adverse health outcomes for both mother and infant (12, 25, 33, 115). Overweight and obese women are at increased risk of developing gestational diabetes (25, 115), pre-eclampsia (25, 115), and requiring cesarean delivery (115). Complications for the fetus include restricted growth (25), stillbirth (25, 115), and increased risk of congenital anomalies including neural tube defects (25, 115). Long-term risks for the offspring include type-2 diabetes (25), cardiovascular disease, (33) and significantly higher all-cause mortality (33).

Recent studies in murine models have shown dampened immune response to ovalbumin, increased serum IgE and pro-inflammatory cytokines such as TNFα, and worse outcomes in response to bacterial infection and experimentally induced autoimmunity in pups born to dams fed a high fat diet during gestation regardless of postnatal diet (74, 75). Human children born to obese mothers have elevated levels of circulating pro-inflammatory factor C-reactive protein (CRP) (29), and are at increased risk of developing asthma and wheezing (26, 37). These observations suggest dysregulation of the immune system of children born to obese mothers, and that these defects are acquired in utero. However, the impact of maternal obesity on neonatal immune homeostasis and function has not been rigorously examined. The goal of this study was to determine the impact of maternal BMI on the neonatal immune system by

characterizing the frequency and function of major innate and adaptive immune cell populations and assessing the concentration of circulating cytokines, chemokines, and growth factors in cord blood samples collected from babies born to obese, overweight and lean mothers.

Methods

Subjects

All studies were approved by the Institutional Ethics Review Board of Oregon Health and Science University (OHSU), and the University of California Riverside. All subjects provided signed consent before study. A total of 39 non-smoking mothers without diabetes who had an uncomplicated, singleton gestation at term (>37 0/7 weeks) were enrolled: 11 lean mothers with a mean age of 31.5 ± 4.95 years and pre-pregnancy BMI of 22.27 ± 1.95 kg/m^2; 14 mothers with a mean age of 31.5 ± 6.5 years and pre-pregnancy BMI of 27.3±1.42 kg/m^2; and 14 with mean age of 29.6 ± 5.9 years and pre-pregnancy BMI of 37.5 ± 5.0 kg/m^2. The racial distribution was: 30 white, 3 Asian-American/Pacific Islander, 1 American-Indian/Alaskan native, 2 African-American, and 3 unknown.

Umbilical cord blood mononuclear cell and plasma isolation

Umbilical cord blood (UCB) samples were collected into heparinized vacutainers during delivery and processed within twelve hours. Complete blood

counts were obtained by Hemavet instrument (Hemavet, Dallas, TX). Umbilical cord blood mononuclear cells (UCBMC) and plasma were obtained by standard density gradient centrifugation over Ficoll (BD Bioscience, San Jose, CA). UCBMC were suspended in 10%DMSO/FBS, frozen using Mr. Frosty Freezing Containers (Thermo Scientific Waltham, MA) and stored in liquid nitrogen while plasma was stored at -80 until analysis.

Flow cytometric analysis of UCB immune cells

UCBMC (1-2x10^6 cells) were stained using CD4 (OKT4, eBioscience, San Diego, CA), CD8β (2ST8.5H7, Beckman Coulter, Brea, CA), CD95 (Dx2, Biolegend, San Diego, CA), CD28 (28.2, Biolegend) and CCR7 (G043H7, Biolegend) to delineate CD4 and CD8 T cells populations as follows: naïve ($CD28^+CD95^-$); central (CM; $CD28^+CD95^+CCR7^+$), transitional (TEM; $CD28^+CD95^+CCR7^-$) and effector memory (EM; $CD28^-CD95^+CCR7^-$) ((116), Fig. 1A). UCBMC were also stained using CD20 (2H7, eBioscience), CD27 (O323, TONBO, Temecula, CA), and IgD (C4211, Southern Biotech, Birmingham, AL) to delineate: naïve (CD20+CD27-IgD+), marginal zone-like (MZ-Like; CD20+CD27+IgD+), and memory (CD20+CD27+IgD-) populations ((117), Fig. 1D). After surface staining, UCBMC were fixed, permeabilized and intracellularly stained with Ki67 (B56, BD Bioscience).

A second tube of UCBMC (1-2x10^6 cells) was stained as follows: CD3 (UCHT1, ebioscience), CD20 (B9E9, Beckman Coulter), HLA-DR (LN3,

Biolegend), CD14 (M5E2, Biolegend), CD11c (3.9, Biolegend), CD123 (6H6, Biolegend), CD56 (RPA-T8, BD Biosciences) and CD16 (3G8, Biolegend) to delineate monocytes ($CD3^{-}CD20^{-}HLA\text{-}DR^{+/-}CD14^{+}$) (Fig. 2A), myeloid dendritic cells (mDC; $CD3^{-}CD20^{-}CD14^{-}HLA^{-}DR^{+}CD11c^{+}$); plasmacytoid dendritic cells (pDC; $CD3^{-}CD20^{-}$ $CD14^{-}HLA\text{-}DR^{+}CD123^{+}$), and natural killer cells (NK; $CD3^{-}$ $CD20^{-}56^{bright/dim}$) (Fig. 2F). Monocytes were further divided into classical and non-classical based on CD16 expression, and natural killer cells were subdivided by both CD56 bright/dim expression and CD16 into four distinct subsets (Fig. 2A, F). All flow cytometry samples were acquired with LSRII instrument (Becton Dickinson, Franklin Lanes, NJ) and analyzed using FlowJo (TreeStar, Ashland, OR).

In vitro UCBMC stimulation

For analysis of T cell cytokine production, $1\text{-}2x10^{6}$ UCBMC were stimulated for 24 hours at 37°C in supplemented RPMI (10% FBS) in the presence or absence of anti-CD3/CD28 (OKT3, TONBO; CD28.2, BD Biosciences); Brefeldin A (Sigma, St. Louis, MO) was added after 1-hour incubation. Cells were stained for CD4 and CD8, fixed, permeabilized and stained intracellularly for TNFα (MAb11, ebioscience), IFNγ (4S.B3, eBioscience), IL-4 (Biolegend), IL-2 (MQ1-17H12, Biolegend), and IL-17a (BL168, Biolegend).

To measure cytokine production by monocytes and dendritic cells (DC), a second tube of UCBMC was cultured for 14 hours at 37^0C in RPMI supplemented (10%FBS) alone or in the presence of heat killed Listeria monocytogenes (HKLM, TLR1 agonist) and synthetic triacylated lipoprotein (PAM3CSK4, TLR2 agonist), or lipopolysaccharide (LPS, TLR4 agonist) (Invivogen, San Diego, CA). Brefeldin A (Sigma) was added after 1-hour incubation. Cells were stained with CD3 (ebiosciences), CD20 (Bioloegend), HLA-DR (L243, Biolegend), CD14 (Biolegend), CD11c (Biolegend), and CD123 (Biolegend), fixed, permeabilized and stained intracellularly for IL-6 (MQ2-13A5, eBioscience), and TNFα (ebioscience). All samples were acquired and analyzed as described above.

Cytokine, chemokine, and growth factor analysis

Plasma samples were analyzed using human multiplex panels (Millipore, Temecula, CA) per the manufacturer's instructions in duplicates using MAGPIX multiplexing platform (Luminex, Austin, TX). Values below the limit of detection were designated as not detected.

Statistical analysis

All data sets were first assessed for normal distribution. Normal data sets were then tested for variance homogeneity. Data sets not displaying homogeneity were subjected to weighted one-way analysis of variance (ANOVA). If group differences were observed, Dunnet posttest correction was applied. If

data sets displayed homogeneity, they were subject to unweighted one-way ANOVA and Dunnet posttest if differences were present. Data sets that were not normally distributed, alternative transformations to normality were explored. When a suitable transformation could be found, the analysis strategy discussed above was followed. Data sets that could not be transformed were subjected to Kruskall-Wallis one-way ANOVA. If group differences were observed, a customized Dunnet posttest was applied.

Results

Maternal obesity and fetal complete blood counts

We first compared complete blood counts (CBCs; lymphocytes, monocytes, eosinophils, neutrophils, and basophils) between the three groups (Table 1). A trend towards reduced white blood cell was observed in UCB from babies born to overweight and obese compared to lean mothers. This difference was due to a significant reduction in eosinophil counts and a slight reduction in basophils as no changes in total lymphocytes, neutrophil, or monocyte counts between the three groups were observed.

	BMI Category		
	Lean	Overweight	Obese
White Blood Cell (cells x 10^3/mL)	16065 ± 1719	12497 ± 1240	13530 ± 996
Neutrophil (cells x 10^3/mL)	3281 ± 1158	3764 ± 1065	4231 ± 668
Lymphocytes (cells x 10^3/mL)	2470 ± 180	2424 ± 317	2506 ± 228
Monocytes (cells x 10^3/mL)	2011 ± 213	2078 ± 311	2124 ± 301
Eosinophils (cells x 10^3/mL)	7336 ± 1141	3852 ± 611*	4472 ± 708*
Basophils (cells x 10^3/mL)	514 ± 75	324 ± 45.1	292 ± 34
*P<0.05 vs Lean.			

Table 2.1: Complete umbilical cord blood counts

Maternal obesity and fetal lymphocyte populations

We next investigated the impact of maternal obesity on $CD4^+$, $CD8^+$, and B cell subset (total, naïve and memory) frequencies in UCBMC samples using flow cytometry (Fig. 1). Analysis showed significantly lower total and naïve CD4 T cell counts in babies born to obese mothers compared to those born to lean mothers (Fig. 1B). Although there were no significant changes in CD4 CM and EM frequencies, a trend of increased CD4 EM cells occurred with increasing maternal BMI was noted (Fig. 1B). We also observed a trend towards lower total and naïve CD8 T cell counts but these changes were also not significant (Fig. 1C). In contrast to T cells, we did not detect any differences in the total or sub-populations of B cells between the three groups (Fig. 1E). Finally, we did not detect differences in the rate of homeostatic proliferation within $CD4^+$, $CD8^+$ or $CD20^+$ subsets (data not shown).

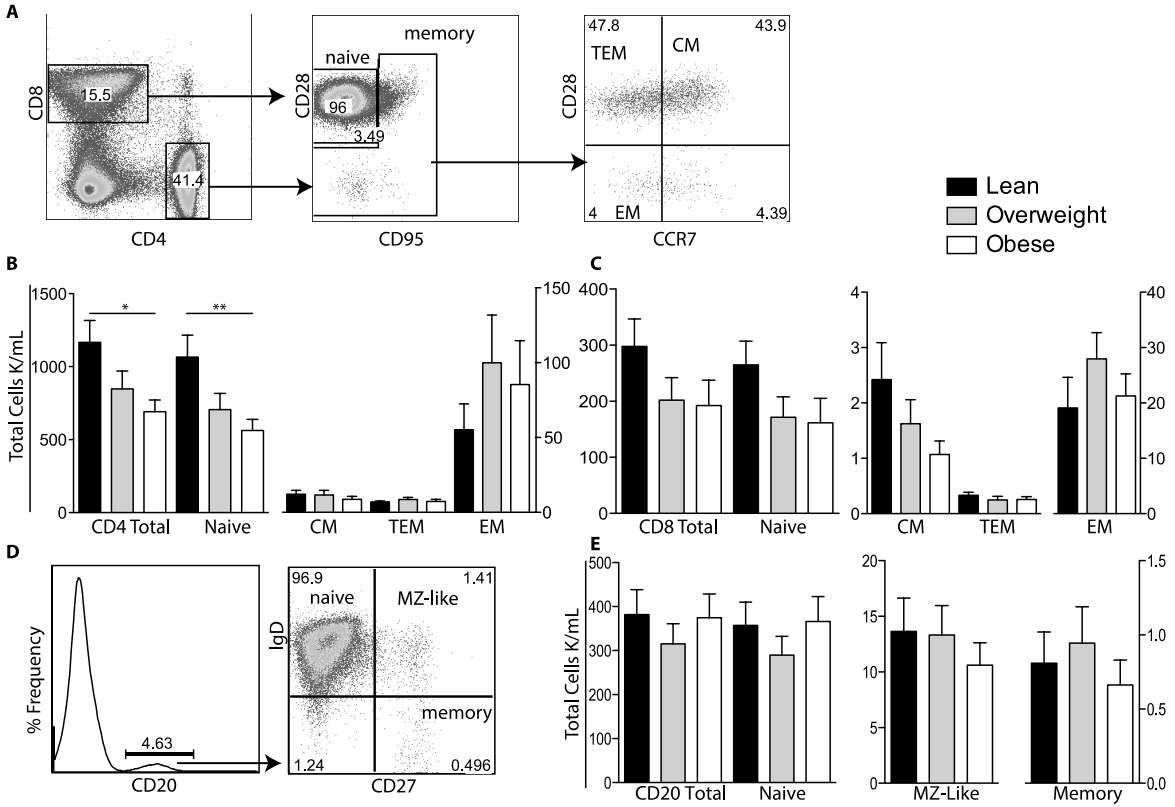

Figure 2.1: Impact of maternal BMI on umbilical cord blood T and B cell populations. (A) Flow cytometry was used to delineate CD4 and CD8 T cells subsets: naïve, central (CM), effector (EM), and transitional effector memory (TEM) based on expression of CD28, CD95 and CCR7. (B) Numbers of total, naïve, CM, TEM and EM CD4 T cells per µl of cord blood were determined by multiplying subset frequency by the number of lymphocytes obtained by the hematology analyzer. (C) Number of total and CD8 T cell subsets were determined as described for CD4 T cells. (D) Flow cytometry was used to delineate total CD20+ B cells as well as naïve, marginal-zone like (MZ-like), and memory subsets. (E) Number of total CD20 B cells and subsets were determined as described for T cells. +/- SEM (n=11-14 UCB samples per group) *p≤0.05 **p≤0.01

To further investigate changes in T cell populations, we compared TNFα, IFNγ, IL-2 (Th1), IL-4 (Th2), and IL-17 (Th17) production following stimulation of UCBMC with anti-CD3 and anti-CD28 (Fig. 2A and B). We detected no differences in $CD4^+$ (Fig. 2C) or CD8+ (data not shown) TNFα, IFNγ, and IL-2 production. In contrast, there was a significant decrease in $CD4^+$ IL-4 production in the overweight group, a trend of decreased $CD4^+$ IL-4 production and increased IL-17 production in the obese group.

Maternal obesity and fetal innate immune cell subsets

We next investigated the impact of maternal BMI on the frequency of total as well as subsets of monocytes, DCs and Natural Killer cells (Fig. 3A, F). No significant differences in total monocytes, DCs and natural killer cells (Fig. 3B-C,G) or their sub-populations (Fig. 3D-E, H) were observed between all three groups, with the exception of pDCs, which were significantly increased in babies born to obese mothers (Fig. 2E).

We then compared the ability of monocytes and DCs in UCBMC to respond to TLR agonists PAM3CSK4/HKLM (TLR1&2) and LPS (TLR4) (Fig. 4A and B). Our analysis revealed significant reduction in IL6 and TNFα production by monocytes in UCBMC of babies born to overweight and obese mothers in response to PAM3CSK4/ HKLM (Fig. 4C) and LPS (Fig. 4D). We also saw decreased production of IL-6 and TNFα by mDCs in the overweight babies in response to PAM3CSK4 & HKLM (Fig. 4E) and decreased TNFα in the

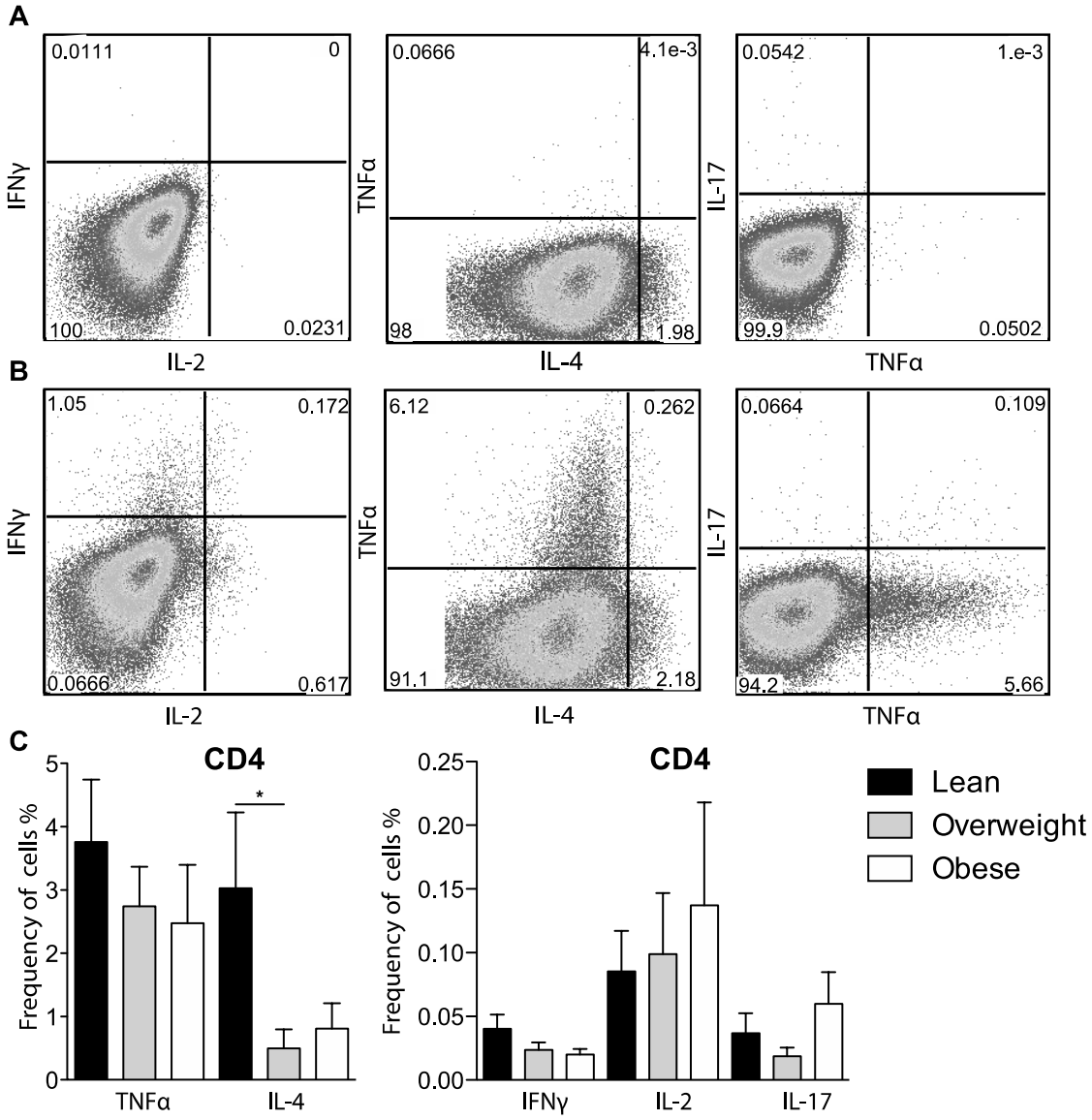

Figure 2.2: T cell stimulation of umbilical cord blood mononuclear cells. (A-B) Representative umbilical cord blood CD4+ T cell response (A) before stimulation and (B) following anti-CD3/CD28 co-stimulation determined by flow cytometry. Cells were first stained with antibodies against CD4 and CD8, permeabilized, and intracellularly stained to detect TNFα, IL-4, IFNγ, IL-2, and IL-17. (C) Average frequency of CD4 T cells producing TNFα, IL-4, IFNγ, IL-2 and IL-17 after adjusting for background using unstimulated controls. +/- SEM (n=11-14 UCB samples per group) *p≤0.05.

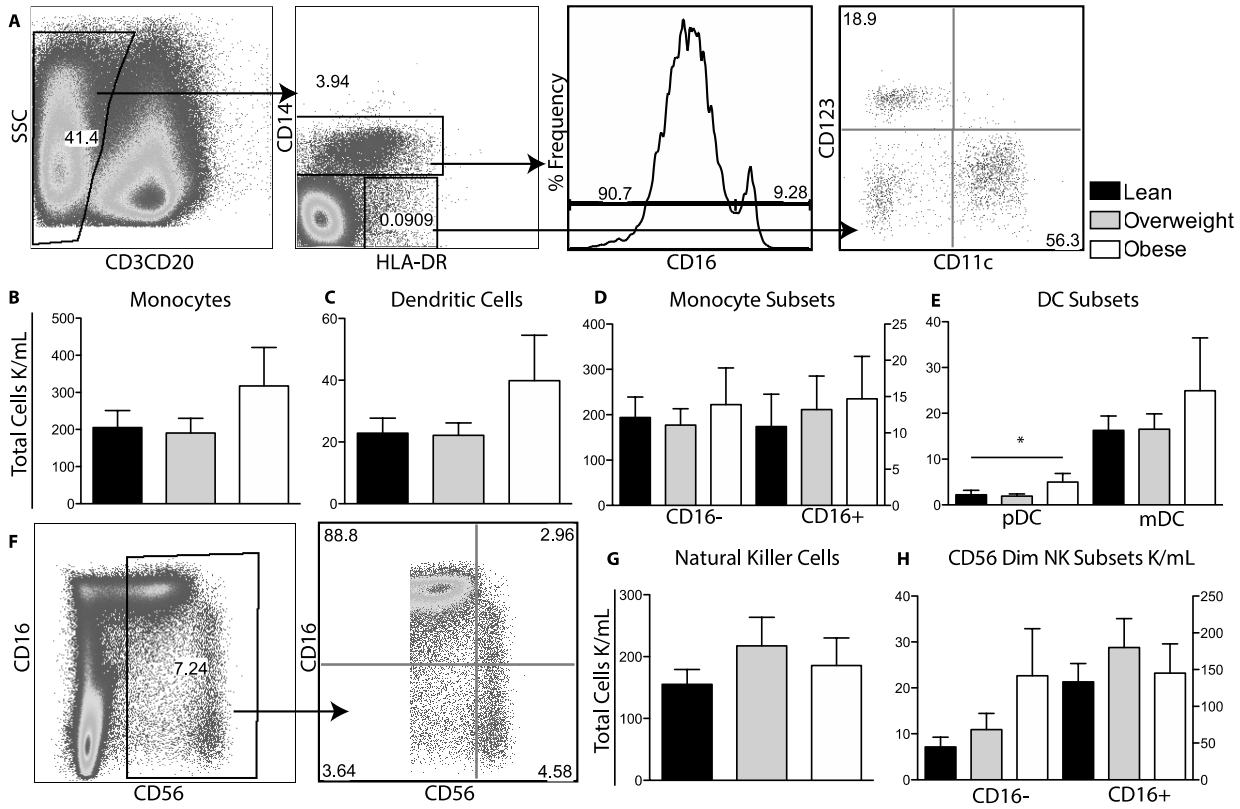

Figure 2.3: Impact of maternal obesity on circulating innate immune cells. (A) Flow cytometry was used to delineate innate cell populations by first gating out CD3 and CD20 lymphocytes and then using CD14 and HLA-DR to define monocytes and dendritic cells. Monocyte subsets were further delineated by CD16 expression and DC subsets by CD11c and CD123 expression. (B-E) Total number of monocytes, dendritic cells and their subsets per µl of cord blood were determined by described above for T cells. (F) Flow cytometry was used to delineate total natural killer cells by gating on CD3 and CD20 negative cells and then based on CD56 and CD16 expression. (G, H) Numbers of total and natural killer subsets were determined as described for monocytes. +/- SEM (n=11-14 UCB samples

obese babies in response to LPS (Fig. 4F) No significant changes in the response of pDCs (Fig. 4G-H) to agonists were detected.

Maternal obesity and fetal blood levels of circulating factors

Finally, we compared circulating levels of cytokines (IFNα2, IFNγ, TNFα, IL-1Ra, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IP-10, IL-12p40, IL12p70, IL-13, IL-15, Flt-3L), chemokines (MCP-1, MIP-1a, MIP-1b, GRO, MCP-3, MDC, eotaxin, and fractalkine), adipokines (insulin, leptin, total PYY, adiponectin, lipocalin-2/NGAL, adipsin, resistin, total PAI-1), and growth factors (VEGF, EGF, FGF-2.2, TGF-a, G-CSF, GM-CSF) in the UCB plasma between the three groups (Fig. 5).

Although only IFNα2 and IL-6 levels were statistically increased in UBC plasma of babies born to obese mothers, there were several notable trends. We saw small increases with maternal BMI in insulin (Fig. 5A), adipsin (Fig. 5B), VEGF (Fig. 5D) and IFNγ (Fig. 5F) as well as a small decrease in resistin (Fig. 5C) in UCB plasma levels. Analytes IL-13, IL-15, IL-17a, IL-1a, IL-2, IL-3, IL-5, TNFβ were below detection and while RANTES and sCD40L were above detection, we did not see differences in the remaining analytes.

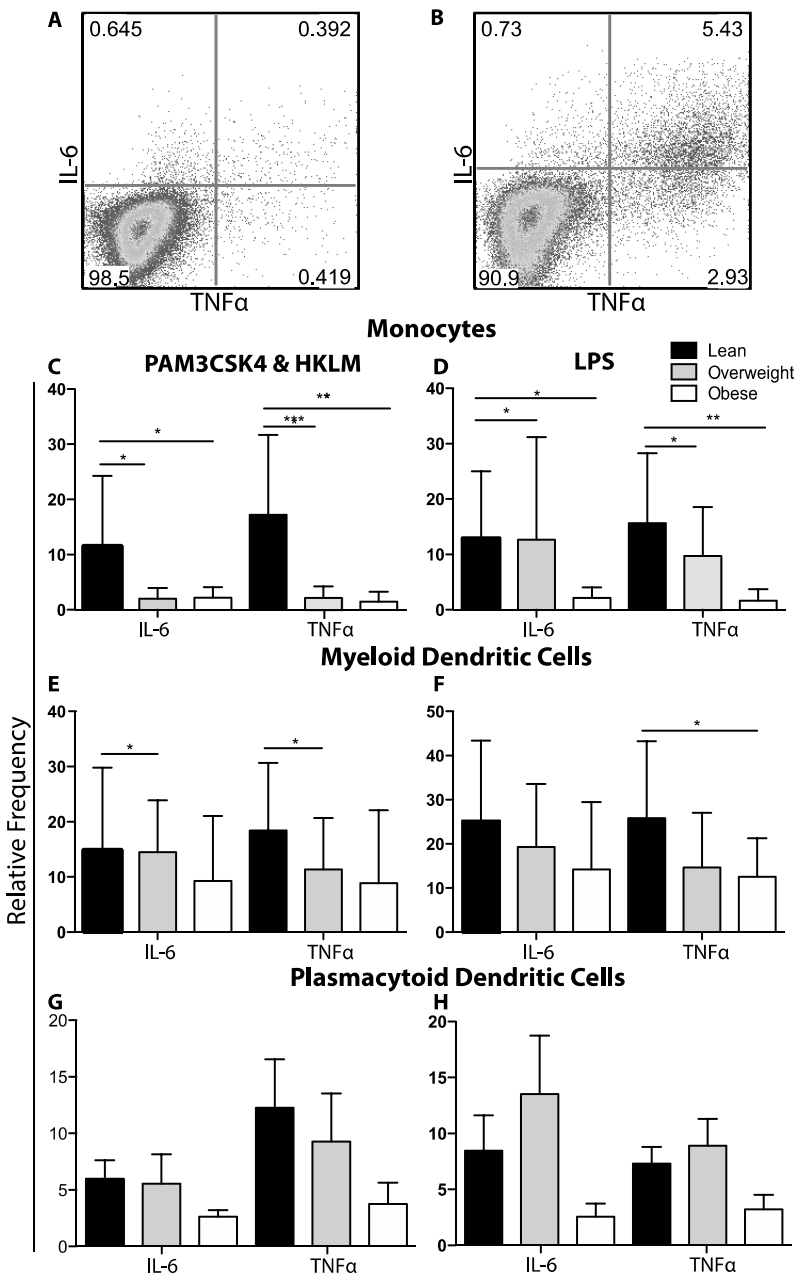

Figure 2.4: Toll-like receptor stimulation of UCBMC (A-B) Representative image of monocytes from UCBMC (A) before stimulation and (B) following LPS stimulation. UCBMC were stimulated with PAM3CSK4&HKLM (A,C,E) or LPS (B,D,E). Cells were then stained to define monocytes (A,B), mDCs (C,D), or pDCs (E,F) followed by intracellular staining for IL-6 and TNFα. The mean frequency of stimulation was adjusted for background using unstimulated controls.+/- SEM (n=11-14 UCB samples per group)* $p \leq 0.05$. **$p \leq 0.01$ ***$p \leq 0.001$

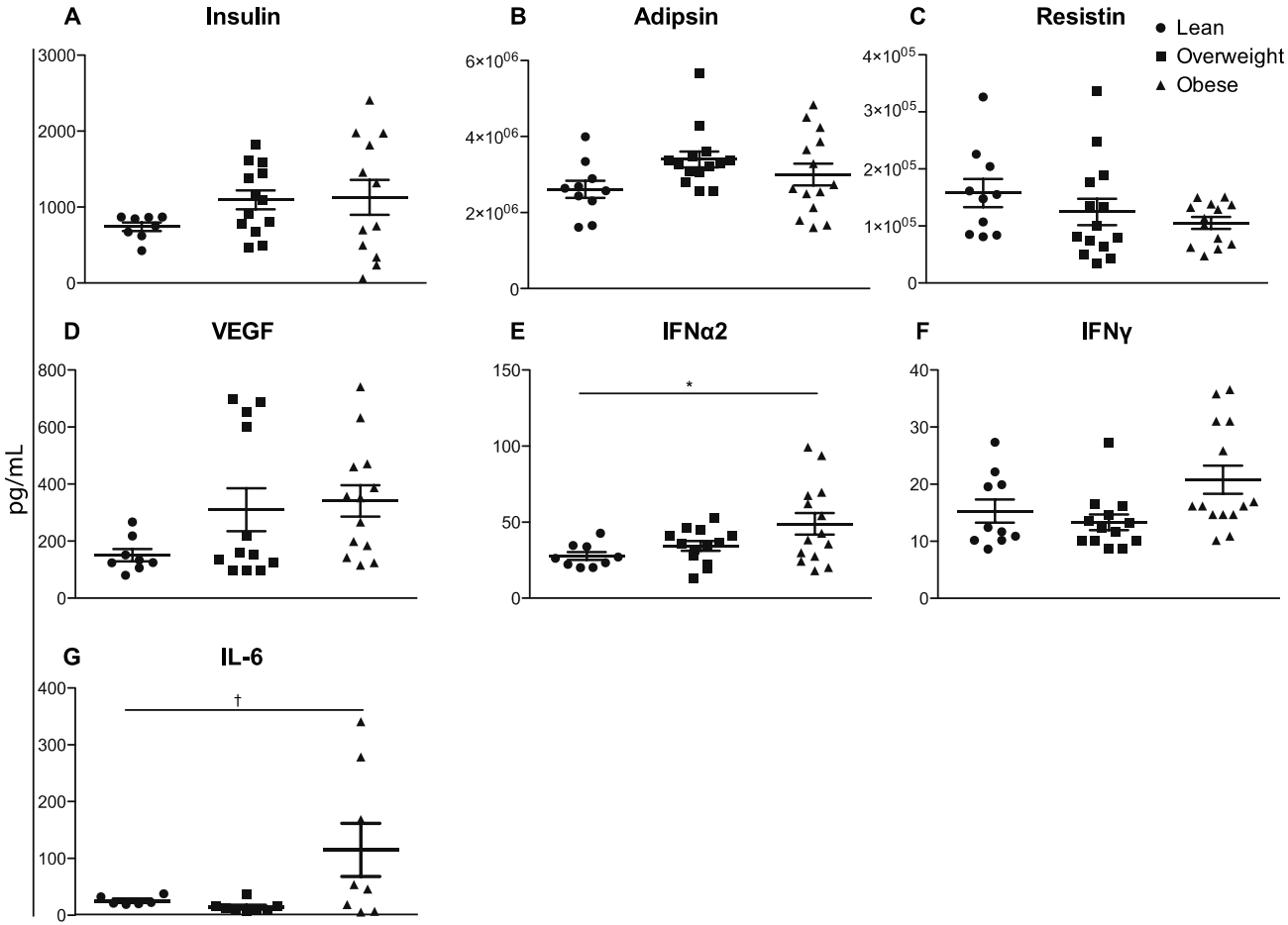

Figure 2.5: Circulating levels of hormones, chemokines, and cytokines UCB plasma. Plasma protein levels were determined by cytometric bead assay using human multiplex. (n=11-14 UCB samples per group) *p≤0.05. †p≤0.10

Discussion

In this study, we investigated differences in the frequency and function of key innate and adaptive immune cells in UCB samples collected from babies born to lean, overweight, and obese mothers to understand the impact of maternal obesity on the development and maturation of the human immune system. Numbers of eosinophils were significantly reduced in UCB of babies born to obese mothers. This observation was unexpected given the role that these cells play in allergic response and asthma pathogenesis and the increased incidence of these diseases in children born to obese mothers (26, 37). One potential explanation is that eosinophils and basophils might already be recruited to peripheral tissues such as the lungs where they have been implicated in airway remodeling and airway hyper-responsiveness (118, 119).

We also report a significant reduction in total and naïve CD4 T cells numbers in UCB collected from babies born to obese mothers. These observations differ from those made in obese adults where total $CD4^+$ counts were increased due to increased homeostatic T cell proliferation (120). The decreased numbers of circulating CD4 T cells could be indicative of dysregulated thymic output or increased peripheral CD4 T cell death and future studies will address this question. Given the important role of CD4 helper T cells, this loss might compromise responses to infection and vaccination in children born to obese mothers. Future studies will aim at evaluating the incidence of childhood diseases and response to vaccination in infants born to obese mothers. Numbers

of total EM CD4 T cells were slightly increased in babies born to obese mothers, suggesting accelerated differentiation.

Changes in CD4 T subset frequency were accompanied by a significant decrease in IL-4 production in babies born to overweight mothers. Decreases in IL-4 production could be linked to the increased plasma levels of IFNα2 and IFNγ observed in the UCB plasma collected from babies born to obese mothers since IFNα2 and IFNγ are known to repress IL-4 expression (121, 122) We also detected significantly higher numbers of pDCs, which could also be linked to the increased plasma levels of IFNα2. In addition to their well-understood role in anti-viral responses, pDCs are thought to play a role in allergic asthma (123). However, these observations need to be validated in a larger cohort study.

Another major observation of our study is decreased TNFα and IL-6 production by monocytes and mDCs in response to TLR1&2 and TLR4 agonists. These observations are consistent with previous studies in adults that showed reduced responses to antigen stimulation with increasing BMI (124). Given the critical role of innate immune responses in the activation of adaptive immunity, these observations are predictive of poor responses to vaccination and infection in infants born to obese mothers. Future studies will investigate the mechanisms for reduced responses to TLR stimulation, including changes in expression of TLR and/or signaling components, and epigenetic modification of cytokine loci.

We saw small increases in UCB plasma insulin and adipsin. Higher insulin resistance in children is correlated with increased childhood diabetes, and

allergic asthma (125, 126). There was also a small decrease in resistin which is thought to play a protective role in asthma (127). Finally, we also saw slight increases in VEGF which has been implicated in promoting airway remodeling and airway hyper-responsiveness (41).

In summary, our data suggest a dysregulation of immune homeostasis and function at birth in babies born to obese mothers. This is in agreement with the Barker hypothesis that adverse conditions during critical development periods in utero lead to lifelong changes in body composition and physiology during adulthood. Our data also provide a potential explanation for the increased incidence of asthma observed later in life in children born to obese mothers, and predict compromised immune responses to vaccination and/or infection. Further studies in a larger cohort are needed to extend these observations and uncover the molecular mechanisms underlying these changes and the implication for responses to vaccination and infection.

Chapter 3: Impact of maternal obesity on gene expression within the offspring's CD4 T cells and monocytes

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Abstract

Maternal obesity during pregnancy is associated with several adverse health outcomes for the offspring that can persist into adulthood. These include a higher lifetime risk of developing obesity, asthma, diabetes, cardiovascular disease, and cancer leading to increased rate of mortality when compared to offspring of lean mothers. These diseases have a significant inflammatory component, which suggest that maternal obesity impacts development and maturation of the neonatal immune system. Indeed, murine studies demonstrated worse outcomes in models of autoimmunity and allergic sensitization in pups born to obese dams or those fed a high fat diet during pregnancy. We have recently shown reduced numbers of CD4 T cells and diminished responses of monocytes and myeloid dendritic cells following stimulation by TLR4 and TLR1/2 agonists in human cord blood samples collected from babies born to obese mothers compared to those collected from babies born to lean mothers. In order to uncover the molecular mechanisms underlying these changes, we compared the transcriptome and DNA methylation patterns using RNA and Methyl Seq analysis of CD4 T helper cells and CD14 monocytes purified from UCBMC collected from babies born to obese or lean mothers.

Introduction

In the United States, greater than one-third of women of childbearing age are obese (7). Obesity during pregnancy is associated with a greater risk for long-term adverse outcomes for the offspring including but not limited to obesity (29), cognitive development deficits and ADHD (30), type-2 diabetes (19), cardiovascular disease (13, 33), and cancer (35). This increase in disease risk has been linked to fetal adaptions to maternal nutritional status (18, 22, 24, 25). This phenomenon is known as early life programing (26). However, since dysregulated inflammatory responses contribute significantly to the development/exacerbation of these diseases, it is also likely that the development/maturation of offspring's immune system is also altered. Indeed, there is growing evidence that the development of the fetal immune system is modulated by maternal obesity during pregnancy. One recent murine study illustrated that pups born to dams fed a high fat diet (HFD) during gestation and weaned on to a HFD had lower ovalbumin (OVA)-specific IgG, but higher OVA-specific IgE, after immunization compared to pups born to control diet fed dams and pups fed a control diet (74). Another rodent demonstrated that pups born to dams fed a western diet both prior to and during gestation exhibited higher mortality rates in an *E.coli* sepsis model (74). Furthermore, children born to obese mothers have a 16 fold higher risk of having detectable CRP levels at 12 years compared to children born to lean mothers (29). These observations

suggest a dysregulation of the immune system of children born to obese mothers, and that these defects are acquired in utero.

One of the mechanisms by which maternal obesity influences development of the offspring's immune system is through introduction of epigenetic changes (29, 105). Changes in DNA methylation patterns cord blood leukocytes have been described in response to maternal diet (21) and correlated with increased incidence of obesity (106), and dysregulated leukocytes responses (105). Moreover, maternal pre-pregnancy BMI was associated with changes in methylation of WNT16 and NTM3AI, genes associated with cancer and diabetes respectively. .

Previously, our lab has shown fewer CD4+ T cells, increased IL-4 production by CD4 T cells, increased frequency of plasmacytoid dendritic cells, increased circulating IL-6 and IFN**α**2 cytokines, and reduced responses to multiple Toll-Like receptor agonists by monocytes in cord blood samples of babies born to obese mothers (110). These observations support the hypothesis that maternal obesity disrupts development and maturation of offspring's immune system in utero. In this study, we investigated the impact of maternal obesity on the transcriptome and the methylome of cord blood CD4+ T cells and monocytes.

Methods:

Subjects

All studies were approved by the Institutional Ethics Review Board of Oregon Health and Science University (OHSU), and the University of California Riverside. All subjects provided signed consent before study. A total of 25 non-smoking mothers without diabetes who had an uncomplicated, singleton gestation at term (>37 0/7 weeks) were enrolled: 11 lean mothers with a mean age of 31.5 ± 4.95 years and pre-pregnancy BMI of 22.27 ± 1.95 kg/m^2; and 14 with mean age of 29.6 ± 5.9 years and pre-pregnancy BMI of 37.5 ± 5.0 kg/m^2. The racial distribution was as follows: 20 white, two Asian American/Pacific Islander, one American-Indian/Alaskan native, one African American, and two unknown.

Magnetic bead based positive cell selection

CD4+ T cells and monocytes were individually purified from umbilical cord blood mononuclear cells (UCBMC) (Figure 1). UCBMC (6-8 x10^6 cells) were stained with anti-CD4+ antibody (Miltenyi Biotec, San Diego, CA) conjugated to a magnetic bead. Cell suspension was loaded into a MS MACS separation column (Miltenyi Biotec); mounted onto OCTOMAX magnetic rack (Miltenyi Biotec), and bound CD4+ T cells were flushed from the column. The CD4 negative fraction was stained with anti- CD14+ antibodies conjugated to magnetic beads (Miltenyi) and was separated as described for the CD4+ T cell above. Cell viability was

assessed by Trypan Blue (Bio-Rad) staining; cell counts were determined using Bio-Rad TC20 (Bio-Rad, Hercules, CA); and population purity was verified by flow cytometry (Figure 2A, 2B). Positive fractions exceeding 70% purity were stored for RNA and DNA extraction. A total of n=2 samples per cell type per group were isolated and used for transcriptome and DNA methylome analysis. All flow cytometry samples were acquired with LSRII instrument (Becton Dickinson, Franklin Lanes, NJ) and analyzed using FlowJo (TreeStar, Ashland, OR).

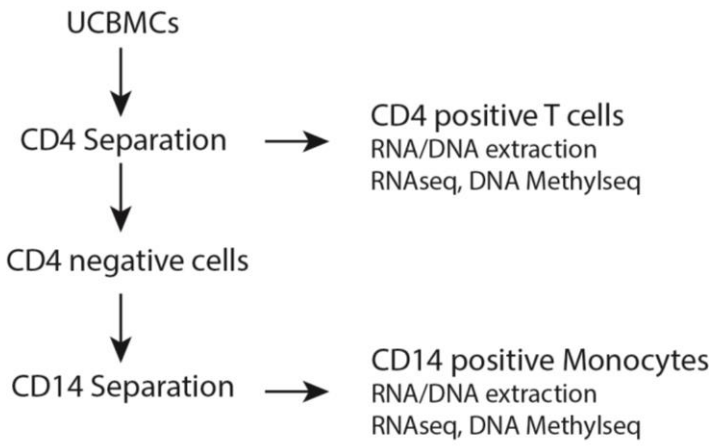

Figure 3.1: Schematic of cell isolation procedure

DNA and RNA extraction

Total RNA and DNA were isolated from CD4+ T helper or CD14+ Monocyte cell fractions collected during positive cell selection process using Illustra triplePrep kit (GE Life Sciences, Little Chalfont, Buckinghamshire, UK) according to manufacturer's instructions. RNA and DNA concentrations were measured using the Qubit RNA High Sensitivity and dsDNA High Sensitivity kits

respectively (Life Technologies, Carlsbad, CA) and the Qubit 3.0 Fluorometer (Life Technologies).

RNA library preparation

A minimum of 1ug of total RNA extracted from either CD4+ T helper cells or CD14+ Monocytes was subjected ribosomal RNA (rRNA) depletion using the Clontech Ribo-Gone rRNA Removal kit (Clonetech, Mountain View, CA). cDNA libraries were constructed from rRNA depleted samples using the Clontech SMARTer Stranded RNA-Seq kit (Clonetech). rRNA-depleted samples were fragmented, converted to double stranded cDNA, ligated with adaptors, and amplified by PCR. Finally, adaptor ligated fragments were ligated with a unique indexing primer that allowed for multiplexed sequencing on the Illumina HiSeq2500 platform (Illumina, San Diego, CA). Overall quality of constructed libraries was determined using DNA HS Chips (Agilent) for the Agilent 2100 Bioanalyzer (Agilent). Samples were multiplexed and underwent single-end 100 base pair sequencing on the HiSeq 2500 at the University of California, Riverside Genomics Core.

Bioinformatics and statistical analysis of RNA-seq

Data analysis was performed using *systemPiperR* on Bioconductor. Reads were demultiplexed, quality trimmed and filtered, and quality reports were generated using the *seeFastq* function. RNA-Seq reads were mapped with the

alignment suite Bowtie2/Tophat2 against the hg19 human reference genome. Raw expression values in the form of gene-level reads were generated by summarizeOverlaps function, counting only reads overlapping exonic regions of genes, and discarding reads mapping to ambiguous regions. Normalization and analysis of differentially expressed genes (DEGs) was performed with the GLM method from the *edgeR* package. DEGs were defined as having a fold change of ≥2 and a false discovery rate (FDR)-P value of ≤0.05.

DNA methylation libraries

A minimum of 1ug genomic DNA isolated from either CD4+ T helper cells or CD14+ monocytes was used to construct DNA methylation libraries. Unmethylated Bacteriophage Lambda Vector was also processed similar to sample genomic DNA and used as a positive control to assess bisulfite conversion rate. Libraries were constructed using the Agilent SureSelect XT Methyl-Seq target enrichment system for the Illumina HiSeq 2500 platform. Genomic DNA was sheared to 100-200 base pairs using Covaris Ultra Sonicator (Covaris, Woburn, MS) at the UC Riverside Genomics Core and verified by Agilent 2100 Bioanalyzer. Ends of sheared DNA were repaired, 3' ends were adenylated and methylated adaptors were ligated. DNA fragments were then hybridized with 120 nucleotide biotinylated RNA library fragments that recognize methylated DNA regions. Methylated DNA fragments were isolated using streptavidin beads. To each sample λ DNA was spiked in to measure conversion

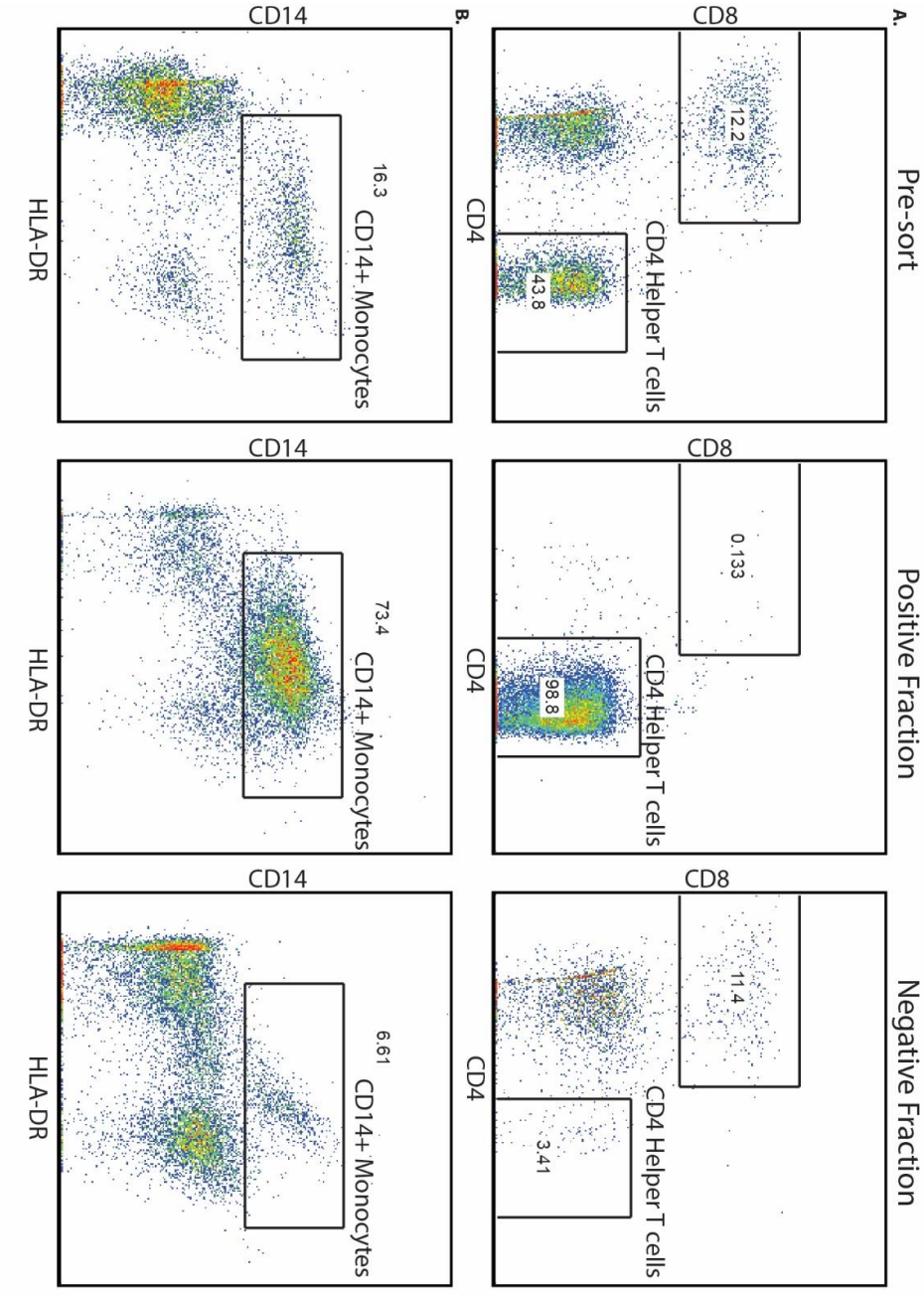

Figure 3.2: Representative results from isolation of fetal CD4+ and CD14+ cells. Representative Isolated CD4+ T cells (A) and CD14+ monocytes were stained with antibodies and determined by flow cytometry.

rate of bisulfite conversion. Samples were then treated using ZYMO EZ DNA Methylation-GOLD Kit (Zymo, Irvine, CA), which converts unmethylated cytosines to uracils. Samples were then PCR amplified to create required amount of DNA library. Next, the amplified samples were ligated with unique indexes, which allowed for multiplexing of multiple samples. Overall quality of constructed libraries was determined using DNA HS Chips on the 2100 Bioanalyzer. Samples were multiplexed and underwent paired-end 50 base pair sequencing on the HiSeq 2500 at the University of California, Riverside Genomics Core.

Bioinformatics and statistical analysis of DNA Methyl-seq

Raw reads were first assessed by FASTQC for quality checks. Reads were then trimmed using TrimGalore. Reads were then mapped to the human genome hg19 using Bismark. In addition, the bacteriophage lambda genome control was mapped using Bismark to the lambda genome, to calculate bisulfite conversion rate. Samtools was then used on the aligned samples to remove any PCR duplicates, and convert bam files to sam files. Sam files and R package methylKit were used to calculate methylation percentage at each cytosine. PCR duplicates were removed and filtered so that any reads with <10x coverage were removed. Samples were then normalized for coverage, and logistic regression was performed to identify Differentially Methylated Cytosines (DMC) defined as having a methylation difference ≥25% and an FDR corrected P value ≤0.05. Each DMC was then designated as hyper or hypo methylated, assigned to an

ENSEMBL gene ID as well as within exons (including 1st exon), introns, promoters, and CpG Islands. A weighted score for each gene was derived based on total number of DMCs divided by coverage per DMC, to determine whether it was hyper- or hypo- methylated.

Enrichment Analysis

Functional enrichment analysis of differentially expressed or methylated genes was performed to identify significant biological pathways including gene ontology (GO) terms and disease biomarkers using MetaCore™ software (GeneGo, Philadelphia, PA).

Results

The impact of maternal obesity on CD4 T cell gene expression

Analysis of CD4+ T transcriptome revealed 112 differentially expressed genes (DEG) with 38 up-regulated and 72 down-regulated genes in offspring of obese of mothers compared to those of lean mothers (Figure 3A). Of these DEGs, 22 up-regulated and 16 down-regulated genes were known to encode proteins (Fig. 3 B). Of the 22 up-regulated genes, 3 had a fold change greater than 15: Chemokine (C-C motif) ligand 2 (*CCL2*), Plakophilin 3 (*PKP3*), and LY6/PLAUR domain containing 6 (*LYPD6*). Of the 16 down-regulated, 7 genes were identified to be associated with immune response: Major histocompatibility complex class II (MHC-II) DQ beta 1 (*HLA-DQB1*), MHC-II DR beta 1 (*HLA-*

DRB1), MHC-II DQ alpha 1 (*HLA-DQA1*), MHC-II DR beta 5 (*HLA-DRB5*), complement factor D (*Adipsin*), interleukin 1 receptor type 2, and hexokinase 3 (*HK3*).

The impact of maternal obesity on monocyte gene expression

Analysis of monocytes obtained from cord blood of babies born to obese and lean mothers yielded 78 DEGs; with 72 up-regulated and 6 down-regulated genes (Figure 3A). Interestingly, while most DEGs in CD4 T helper cells were down-regulated, DEGs in monocytes were mostly up-regulated in cells obtained from babies born to obese mothers. Of these DEGs, 31 up-regulated and 3 down-regulated genes encode known proteins (Fig. 3B). Of the 31 up-regulated DEGs, 6 genes mapped to “positive regulation of response to external stimulus”: Wingless-type MMTV integration site family, 5A (*WNT5A*), Interleukin 6 (*IL-6*), Thrombospondin 1 (*TSP-1*), Fatty acid binding protein 5 (*FABP5*), cAMP responsive element modulator (*CREM*), chemokine ligand 3 (*CCL3*). Amongst the 3 down-regulated genes, 2 played a role in immunity: major histocompatibility class II complex DQ β1 and Tumor-associated calcium signal transducer 2 (Table 1).

Impact of maternal obesity result on DNA methylation in CD4 T cells

We identified 38,495 DMCs that mapped to 4,905 genes, 3,140 of which were hyper methylated and 1,765 were hypomethylated in CD4 T cells isolated

from cord blood samples of babies born to obese mothers compared to those born from lean mothers. A schematic of DMG analysis is described in figure 5, and a summary of all genes with known functions can be found in Table 2 and 3. Most of the differentially methylated genes (DMGs) contained 1 to 6 DMCs (Figure 4A). We focused our analysis on DMGs with a weighted score >25 in either direction (Fig. 4C). Of the 4,006 DMGs with a weighted score > 25, 588 had DMCs in the first exon (390 hypermethylated, and 198 hypomethylated). Of the 390 hypermethylated DMGs, 6 had greater than10 DMCs in the 1st exon: *SLC45A4* (DMC=32), *APBB2* (DMC=13), *RP11-977B10.2* (DMC=12), *BAIAP2-AS1* (DMC=11), *HLA-DMB* (DMC=11), and *CERK* (DMC=10). Of the 198 hypomethylated DMGs, 1 gene had greater than 10 DMC in the 1st exonic region ZFP36 Ring Finger Protein-Like 1 (*ZFP36L1*; DMC=26). There were 363 DMGs with DMCs within CpG islands, of which 261 were hypermethylated and 102 were hypomethylated. Of the 261 hypermethylated genes, 11 had greater than 10 DMCs present in CpG islands: *LHX6* (Diff=64), *MPO* (DMC=31), *RP11-867G2.8* (DMC=28), *TSNARE1* (DMC=26), *SLC45A4* (DMC=22), *ZNF516* (DMC=17), *ZNF423* (DMC=16), *CHRNE* (DMC=14), *SLC12A7* (DMC=12), *A1BG* (DMC=10), and *CTD-2619J13.8* (DMC=10). Of the 102 hypomethylated genes, 6 had greater than 10 DMCs in CpG islands: *FP36L1* (DMC=25), *MYT1L* (DMC=25), *SERPINB9* (DMC=19), *PM20D1* (DMC=16), *CCDC57* (DMC=15), and *IFT140* (DMC=10). Finally, 448 DMGs had DMCs within the promoter

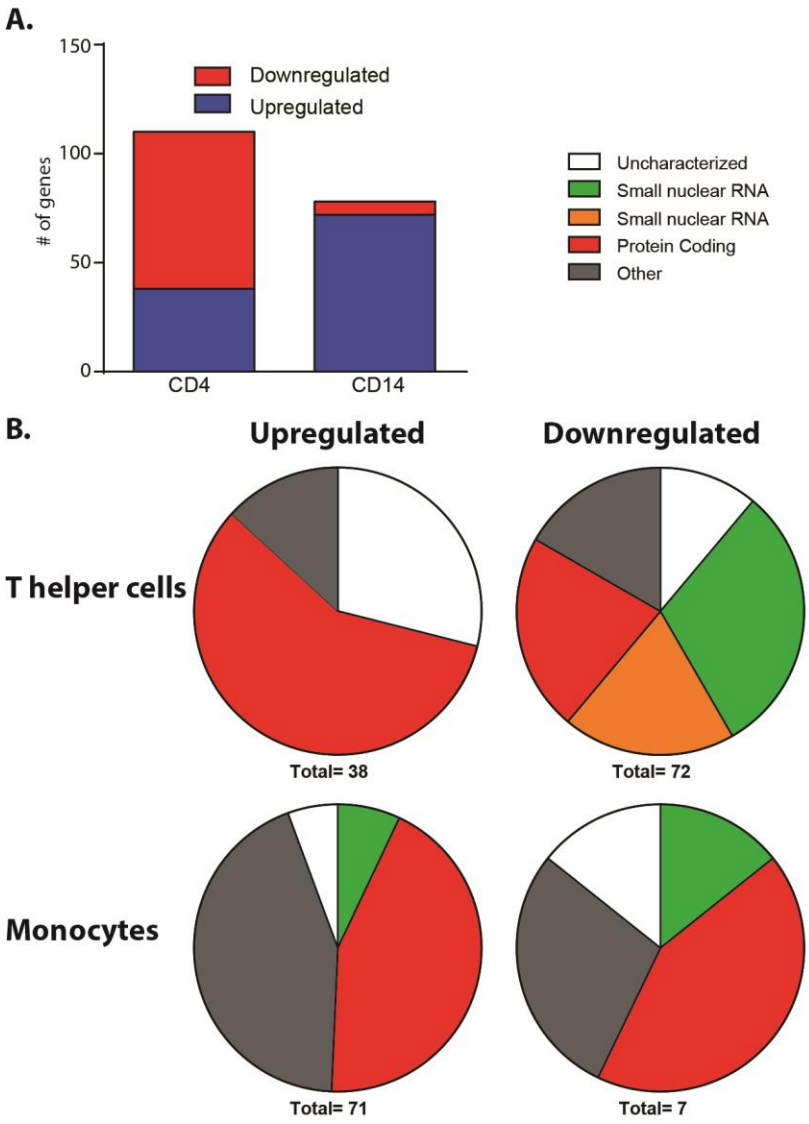

Figure 3.3: Differentially expressed genes in CD4+ and CD14+ cells. Differentially expressed genes were sorted initially by fold change to establish up-regulated and down-regulated genes. (A) Genes were then further divided into protein coding or other various RNA categories. (B)

CD4 T helper Cells						
Up-regulated			**Normalized Counts**			
Gene Name	**Symbol**	**Fold Change**	**Lean 1**	**Lean 2**	**Obese 1**	**Obese 2**
Chemokine (C-C motif) ligand 2	*CCL2/MCP-1*	28.09	0.045	0.046	2.592	2.474
Plakophilin	*PKP3*	15.68	0.012	0.024	0.514	0.302
LY6/PLAUR	*LYPD6*	16.56	0.027	0.082	0.455	0.147
Down-regulated						
Major histocompatibility complex, class II, DQ beta 1	*HLA-DQB1*	11.22	1.040	4.608	0.257	0.439
Major histocompatibility complex, class II, DR beta 1	*HLA-DRB1*	8.41	30.424	147.720	19.914	11.202
Major histocompatibility complex, class II, DQ alpha 1	*HLA-DQA1*	9.55	1.635	3.109	0.165	0.525
Major histocompatibility complex, class II, DR beta 5	*HLA-DRB5*	10.76	7.415	83.009	5.992	6.037
Complement factor D	*Adipsin*	9.55	4.543	34.795	2.902	3.250
Interleukin 1 receptor type 2	*IL-1R type II*	8.7	0.969	3.692	0.484	0.307
Hexokinase 3	*HK3*	7.67	2.318	15.279	1.599	1.719
CD14 monocytes						
Up-regulated	**Symbol**	**Fold Change**	**Lean 1**	**Lean 2**	**Obese 1**	**Obese 2**
Wingless-type MMTV integration site family, member 5A	*WNT5A*	87.8	0	0	0.945	0.319
Interleukin 6	*IL-6*	16.73	1.310	0	12.466	6.671
Thrombospondin 1	*TSP1*	2.4	560.633	458.805	1120.344	1245.558
Fatty acid binding protein 5	*FABP5*	11.97	4.762	1.177	35.647	20.254
cAMP responsive element modulator	*CREM*	2.66	11.886	8.252	33.986	18.665
Chemokine (C-X-C motif) ligand 3	*CXCL3*	3.24	16.973	13.649	56.814	32.378
Down-regulated						
Major histocompatibility complex, class II, DQ beta 1	*HLA-DQB1*	12.32	10.194	9.667	0.751	0.672
Tumor-associated calcium signal transducer 2	*TACSTD2*	95.26	4.495	1.560	0	0

Table 3.1: Differential Gene Expression of CD4+ and CD14+ cells

regions with 312 hypermethylated and 136 hypomethylated. Of the 312 hypermethylated genes, 6 had DMCs greater than 10: *LHX6* (DMC=17), *RNH1* (DMC=15), *IFFO1* (DMC=14), *FGR* (DMC=14), *FANCA* (DMC=10), *PSRC1* (DMC=10). Of the 136 hypomethylated genes, 6 had greater than 10 DMCs: *SERPINB9*, (DMC=22), *LEF1* (DMC=15), *ZCCHC14* (DMC=12), *APBB1* (DMC=11), *RP11-512M8.5* (DMC=11), and *DIABLO* (DMC=11).

The impact of maternal obesity on DNA methylation levels in monocytes

We identified 18,139 DMCs that mapped to 3,008 genes, 1,005 of which were hyper methylated and 2,003 of which were hypomethylated in monocytes isolated from cord blood samples collected from babies born to obese mothers compared to those born to lean mothers. This is in contrast to CD4 T cells where maternal obesity resulted in increased methylation. As described for CD4 T cells most of the DMGs had 1 to 6 DMCs per gene (Fig. 4B). Our analysis was focused on DMGs with a weighted score of >25 in either direction (Fig. 4D). Of the 1,203 DMGs with a weighted score > 25, 284 had DMC present in the first exon, with 78 hypermethylated, and 206 hypomethylated. Of the 78 hypermethylated DMGs, 1 gene had greater than 10 DMCs in the 1st exon region, ZFP36 Ring Finger Protein-Like 1 (*ZFP36L1*, (DMC=10). Of the 206 hypomethylated DMGs, 3 had greater than 10 DMC in the 1st exon: *SLC45A4* (DMC=27), *APBB2* (DMC=12), and *HLA-DMB* (DMC=12).

Of the 148 DMGs with DMCs within CpG islands, 41 were hypermethylated and 107 were hypomethylated. Only one of the 41 hypermethylated genes, *LHX6* (DMC=14), had greater than 10 DMCs within CpG islands *LHX6* (DMC=14). Of the 107 hypomethylated, 5 genes had greater than 10 DMCs within CpG islands: *ERICH 1* (DMC=23), *RP11-867G2.8* (DMC=20), *SLC45A4* (DMC=18), *MPO* (DMC=18), and *ZNF516* (DMC=15). Finally, 87 DMGs had DMCs within promoter regions, of which 25 were hypermethylated and 72 were hypomethylated. None of the 25 hypermethylated genes had greater than 10 DMCs within the promoter region. One of the 72 hypomethylated genes had greater than 10 DMC within the promoter region, *RNH1* (DMC=11).

Concordance of Transcriptome and Methylome analysis

Because DNA methylation can regulate gene transcription, we next compared our list of DEGs to that of DMGs. We identified 9 common genes in CD4+ T helper cells that were differentially expressed and methylated. Of these 9 genes, 3 genes were down-regulated and hypermethylated: Thymidine phosphorylase (*TYMP*, FC=7.40), *HK3* (FC=7.67), and small nucleolar RNA H/ACA box 64 (*SNORA64*, FC=5.02); while 4 genes were up-regulated and hypomethylated: peroxidasin homolog (*PXDN*, FC=6.45), ST6 beta-galactoside alpha-2,6 sialyltransferase 2 (*ST6GAL1*, FC=8.29), transmembrane protein 26 (*TMEM* 26, FC=9.06), and sphinosine-1-phosphate receptor 2 (*S1PR2*,FC=8.47). 2 of the 9 common genes, *HLA-DRB1* (FC=8.41) and *HLA-DQB1* (FC=8.41)

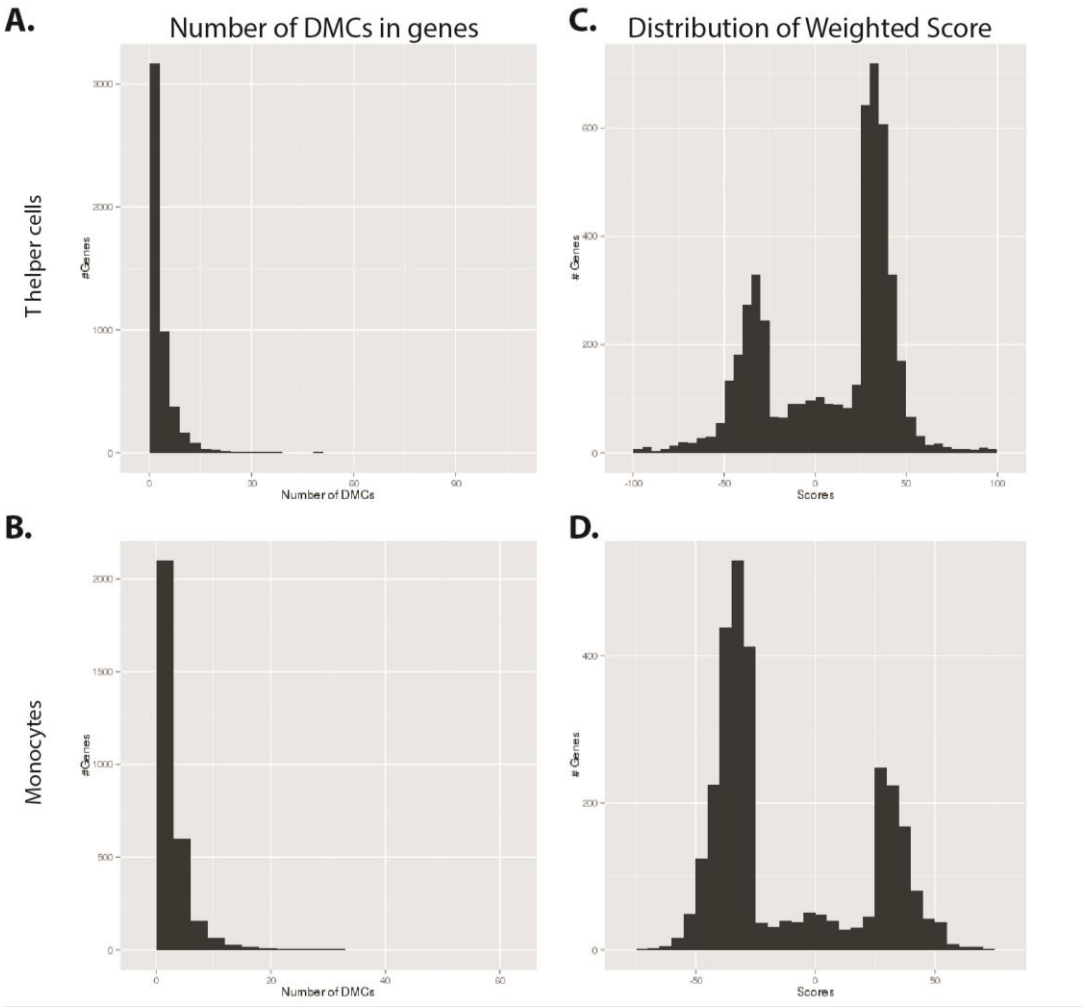

Figure 3.4: Total DMC distribution and weighted average scores. Distribution of total DMCs per gene and average distribution of DMCs as determined based on average weighted score of CD4+ T helper cells (A,C) and Monocytes (B,D).

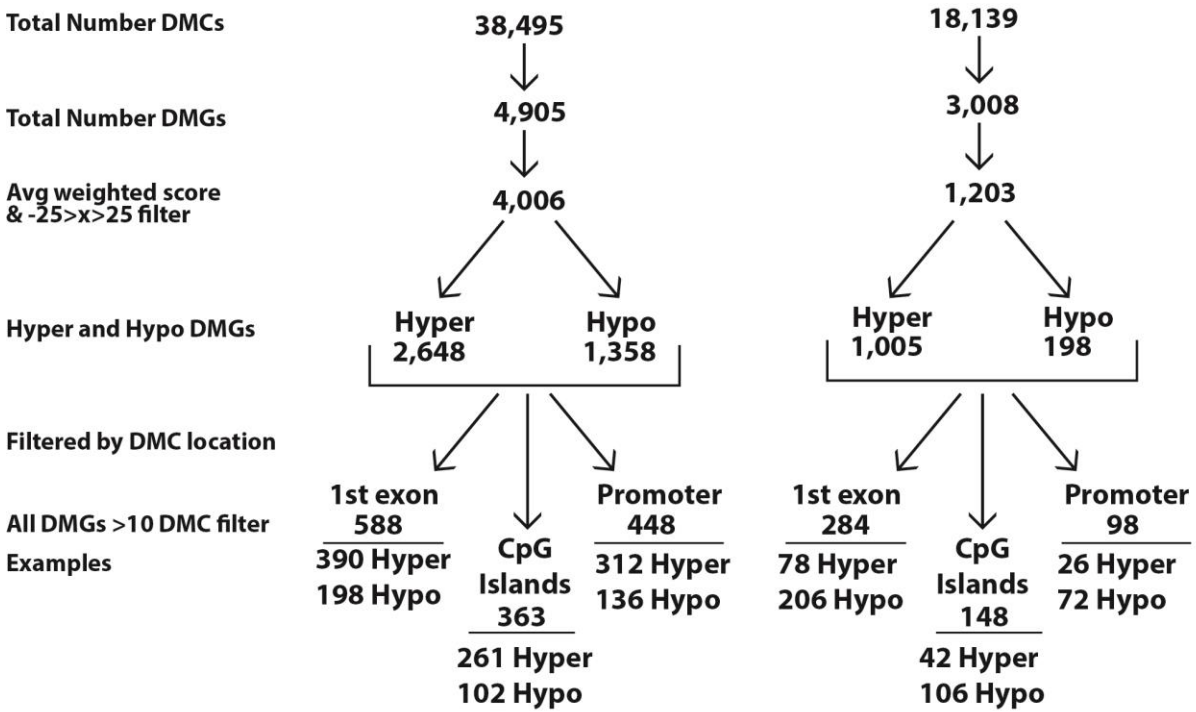

Figure 3.5: Location of DMCs within DMGs. All genes with DMCs present in 1st exon, CpG islands, and promoter regions were compiled. Gene enrichment analysis was completed using Metacore software suite and top immune related GO terms were selected

were down-regulated, but were also hypomethylated. Within the 9 common genes, *S1PR2* contained DMCs within the 1st exon, and *TYMP* and *HK3* contained DMCs within CpG islands. No overlap was identified between DEGs and DMGs within the CD14+ cells.

Gene ID	Avg Score	Location	Methylation Difference	Known Function
SLC45A4	25.847	1st Exon	32	S. cerevisiae homolog is involved in sucrose transport
APBB2	38.017	1st Exon	13	Polymorphisms are associated with Alzheimer's disease
RP11-977B10.2	35.355	1st Exon	12	Antisense RNA
BAIAP2-AS1	37.322	1st Exon	11	
HLA-DMB	40.287	1st Exon	11	Present in CD4+ Tregs
CERK	33.216	1st Exon	10	Converts waxy lipids into Ceramide 1-phosphate bolstering cell survival and cell proliferation
ZFP36L1	-41.963	1st Exon	26	
LHX6	36.840	CpG Island/Total Promoter	64	Transcription factor important in determining neural and lymphoid cell fate
RP11-867G2.8	37.569	CpG Island	28	Antisense RNA
TSNARE1	34.622	CpG Island	26	Predicted role in intracellular protein transport and may be a Schizophrenia/Bipolar susceptibility locus
SLC45A4	25.847	CpG Island	22	
ZNF516	33.207	CpG Island	17	May be involved in transcriptional regulation
ZNF423	31.647	CpG Island	16	May have multiple roles in signal transduction during development

CHRNE	31.880	CpG Island	14	Acetylcholine receptor subunit changes at birth from gamma to epsilon
SLC12A7	29.613	CpG Island	12	
A1BG	30.929	CpG Island	10	
CTD-2619J13.8	30.929	CpG Island	10	
ZFP36L1	-41.963	CpG Island	25	Putative nuclear transcription factor likely functions in regulating the response to growth factors
MYT1L	-37.667	CpG Island	25	May function as a panneural transcription factor associated with neuronal differentiation.
SERPINB9	-36.745	CpG Island/Total Promoter	19	Member of serine protease inhibitor family which inhibits the activity granzyme B
PM20D1	-54.854	CpG Island	16	
CCDC57	-36.423	CpG Island	15	
IFT140	-39.615	CpG Island	10	Component of the retrograde ciliary transport complex
RNH1	34.132	Total Promoter	15	Ribonuclease inhibitor proposed to function in Angiogenin inhibition
IFFO1	35.342	Total Promoter	14	
FGR	33.723	Total Promoter	14	Src family protein tyrosine kinase, may activate NF-kB activation
FANCA	32.599	Total Promoter	10	Hypothesized to operate in DNA repair or as cell-cycle checkpoint
PSRC1	37.666	Total Promoter	10	
LEF1	-36.821	Total Promoter	15	T-cell protein binds to important site in the T-cell receptor-alpha enhancer, conferring maximal enhancer activity
ZCCHC14	-27.676	Total Promoter	12	

APBB1	-39.498	Total Promoter	11	Observed to block cell cycle progression by down-regulating Thymidylate Synthase expression
RP11-512M8.5	-27.632	Total Promoter	11	
DIABLO	-37.193	Total Promoter	11	An inhibitor of apoptosis protein (IAP)-binding protein

Table 3.2: Differential methylated genes of CD4+ T helper cells

Gene ID	Avg Score	Location	Methylation Difference	Known Function
ZFP36L1	34.478	10	1st Exon	
SLC45A4	-37.530	27	1st Exon/CpG Island	The S. cerevisiae homolog is involved in sucrose transport
APBB2	-34.565	12	1st Exon	Polymorphisms associated with Alzheimer's disease
HLA-DMB	-34.119	12	1st Exon	Helps release the CLIP molecule from the MHC class II peptide
LHX6	30.918	14	CpG Island	Transcription factor important in determining neural and lymphoid cell fate
ERICH1	-39.557	23	CpG Island	
RP11-867G2.8	-36.625	20	CpG Island	Antisense RNA
MPO	-41.505	15	CpG Island	Converts peroxides into superoxides
ZNF516	-33.660	15	CpG Island	May be involved in transcriptional regulation
RNH1	-33.977	11	Total Promoter	Ribonuclease inhibitor proposed to function in Angiogenin inhibition

Table 3.3: Differential methylated genes of CD14+ monocyte

Discussion

Recent global studies examining both whole umbilical cord blood and purified cord blood leukocytes have found that maternal pre-pregnancy BMI correlated with epigenetic changes (105, 106). In one study, the methylation levels of retinoid X receptor-α and endothelial nitric oxide synthase were directly correlated with adiposity in children at 9 years in two independent cohorts. A second study demonstrated that decreased global methylation in cord blood leukocytes correlated with increased adiposity at 3 years of age. In this study, we investigated the impact of maternal obesity on gene expression within purified CD4+ T helper cells and CD14+ monocytes isolated from UCBMC collected from babies born to obese and lean mothers. The decision to focus on these cells was based on our earlier findings that maternal obesity leads to altered ratios of IL-4 and IL-17 production by CD4 T cells and diminished cytokine responses by monocytes (110). To further understand the impact of maternal obesity on gene expression in neonatal T helper cells and monocytes, we performed RNA-sequencing to identify differential gene expression and DNA-methylation as methylation of DNA transcripts can affect gene expression. In this study, we explored differences in the transcriptome and the methylome of CD4+ and CD14+ cells isolated from babies born to obese mothers.

Interestingly we saw up-regulation of *CCL2/MCP-1* in CD4 T cells of cord blood of babies born to obese mothers compared to lean. It is possible that CD4+ T cells are up-regulating *CCL2/MCP-1* in response to placental inflammation.

Increases in *CCL2* could induce amylin expression leading to increased insulin resistance (128), which can lead to increased risk of type 2 diabetes and asthma seen in offspring of obese mothers. In murine models, it has been shown that up-regulation of *CCL2/MCP-1* induces migration of monocytes and umbilical cord blood cells during ischemic injury (129). *PKP3* is an armadillo protein that is present in both epithelial and non-epithelial desmosomes (130). *PKP3* is potentially associated with RNA binding proteins and transportation of mRNAs to stress granules (131). It is possible, that up-regulation of *PKP3* could result in translocation of mRNA to stress granules. We hypothesize that translocation of mRNA to stress granules could indicate cellular stress as a result of peripheral inflammation. Lastly, we saw up-regulation of *LYPD6* that has been shown to enhance WNT/β-catenin signaling. WNT/β-catenin signaling promotes differentiation of CD4+ Th2 cells, and increased expression could result in stronger Th2 responses (132).

We saw reduced expression of *HLA-DQB1*, *HLA-DRB1*, *HLA-DQA1*, and *HLA-DRB5* in CD4 T cells from cord blood of babies born to obese mothers. Activated T cells are capable of expressing MHC class II both in vitro and in vivo (133, 134). In addition, a subset of regulatory T cells (Tregs) that can suppress T cell responses can also express MHC-II (133). Therefore, reduced MHC-II expression could indicate fewer Tregs. This would have to be verified in follow up studies using flow cytometry. We also saw down-regulation of complement factor D, which during interaction with APCs leads to increased differentiation and

proliferation of effector memory cells (135). In addition, expression of IL-1R type II, which is important in modulation of inflammatory responses by blocking IL-1β (136), was decreased (137). Decreased expression of IL-1R type II could result in increased autoimmune disease in offspring. Lastly, we saw down-regulation of Hexokinase 3, which converts glucose-1 phosphate to glucose-6 phosphate for use in the glycolysis cycle. Active T cells utilize glycolysis for rapid energy and potentially for macromolecular synthesis while inactive T cells utilize oxidative phosphorylation for energy (138). Down-regulation could result in slower activation of CD4+ T cells of babies born to obese mothers, which could result in poor responses to infection.

In monocytes from cord blood of babies born to obese mothers, we saw up-regulation of *WNT5a*, *IL-6*, *TSP-1*, *FABP5*, *CREM*, and *CXCL3*. Up-regulation of *WNT5a* is known to activate canonical Wnt signaling (139, 140) and activate monocytes through JNK and NF-κB pathway (141) resulting in increased pro-inflammatory cytokines. We also saw increased expression of *IL-6*, which is important in acute phase immune response (142). *IL-6* can activate the JAK/STAT pathways, which are vital for immediate response to pathogens such as gram-negative bacteria (142). *IL-6* is also known to play a role in pathogenesis of atherosclerosis (143), and increased expression of IL-6 could account for increased risk of cardiovascular insult in offspring later in life. However, our previous data showed decreased IL-6 responses in monocytes following stimulation. We hypothesize that monocytes in UBCMC of babies born

to obese mothers may have higher levels of basal IL-6 production, but yet are impaired in their ability to respond to stimulation. *TSP-1* is released during acute inflammation to control and limit immune response by blocking adhesion and activation of leukocytes (144). It has been observed that *TSP-1* can be elevated due to wound healing and ischemia (145). Obesity during pregnancy results in increased incidence of ischemic events in the placenta thereby increasing risk of pre-eclampsia (146). Increased *TSP-1* expression in neonatal UBMC could be a result of fetal stress. *FABP5* is involved in lipid raft composition, inflammation, and a regulator of metabolism in activated monocytes (147). However, *FABP5* may also play a role in insulin resistance (147), which is known to be increased in offspring born to obese mother, which subsequently increases risk of diabetes and allergic asthma (125, 126). Up-regulation of *CREM* could prevent *CREB* factors from acting on their targets (148), which could result in decreased production of IL-6 and TNFα following activation (149), which agrees with our previous data that monocytes had muted IL-6 and TNFα responses (110). Lastly, we saw up-regulation of *CXCL3*, which can control migration, and adhesion of monocytes through CXCR2 (150). *CXCL3* is a chemokine generally expressed during angiogenesis and wound healing (151), and up-regulation of *CXCL3* could be a response to the heightened inflammatory state associated with maternal obesity.

Of the 6 down-regulated genes, 2 were protein coding genes. We saw decreased expression of major histocompatibility complex class II DQ beta 1

which is involved in antigen loading and may signify reduced ability of monocytes to display antigen to T cells. We also saw a decrease in *TACSTD2* a receptor that transduces calcium signals and has been associated with various cancers (152). We hypothesize that since calcium channel activation is important in monocyte/macrophage activation, downregulation of *TACSTD2* could result in worse activation of monocytes from babies born to obese mothers (153).

We also compared DNA methylation within CD4 T cells isolated from babies born to obese and lean mothers at the single nucleotide resolution. We identified 1,399 DMGs with DMCs in the 1st exon, CpG islands, or promoters. Of all the genes that had greater than 10 DMCs, 7 genes had DMCs in the 1st exon, 17 had DMCs present in CpG islands, and 12 had DMCs present in promoters. Of these 36 genes, 10 genes have known or predicted functions. Of these 10, 9 were hyper methylated and 1 was hypomethylated. Of the 9 genes, *RNH1* is a ribonuclease inhibitor with a proposed function in angiogenin inhibition (154). *IFFO1* is a gene for intermediate filament 1. The greater family of intermediate filaments forms components of the cell cytoskeleton and nuclear envelope (155). Hyper methylation of *IFFO1* could result is reduced formation of cell structures such as cytoskeleton and nuclear envelope. This could indicate that the cell is not actively proliferating. *ZNF516* and *ZNF423* are general zinc finger proteins that alter binding specificity of DNA binding proteins, and hypermethylation could indicate changes in general DNA transcription. *FANCA* is a protein that belongs to the Fanconi Anemia Complementation Group and is hypothesized to operate

in DNA repair or as cell-cycle checkpoint (156). Hypermethylation could result in decreased or worsened DNA repair and/or missing vital cell-cycle checkpoints. *FGR* is a member of the Src family of protein tyrosine kinases similar to Lck and Fyn which are present downstream of the T cell receptor during activation. Hypermethylation of *FGR* could suppress NF-kB activation which could result in decreased cell activation, proliferation, and cytokine production (157). Moreover, we saw hypermethylation of *CERK*, which would prevent activation of the phosphatidylinositol 3-kinase/Akt pathway and further downstream signaling and T cell activation (158). Additionally, hypermethylation of *HLA-DMB* would mean reduced levels of beta chain in the class II histocompatibility complex. HLA class II proteins are expressed on CD25+ Tregs and hypermethylation of HLA-DMB could signal lower frequency of Tregs. Decreased Tregs could result in exaggerated immune responses or relate to why children of obese mothers have increased risk of acquiring immune related diseases such as asthma. Finally, *CHRNE*, which is an acetylcholine receptor subunit (epsilon) and is important in neuronal muscular signaling. The function of this gene in T helper cells is not known. In our results *LEF1* was the only hypomethylated gene with greater than 10 DMCs in the 1st exon, CpG islands, or promoters. *LEF1* is present in Treg subsets and hypomethylation would suggest that CD4+ T cells are being polarized and differentiated to a Treg phenotype of babies born to obese mothers.

In monocytes, we identified 530 DMGs with DMCs in the 1st exon, CpG islands, or promoters. Of all genes that had greater than 10 DMCs, 4 genes had DMCs within the 1st exon, 6 genes had DMCs present in CpG islands, and 1 gene had DMCs present in the promoter region. Of the 9 genes, *LHX6* was hypermethylated and may be involved in neural and lymphoid cell differentiation (159). *LHX6* is a transcriptional activator that has the potential to determine cell fate in neural and lymphoid tissue (160, 161). Myeloperoxidase (*MPO*) which was hypomethylated resides in the lysosomes of monocytes and converts hydrogen peroxide and chloride into super oxides such as hypochlorous acid, which is a potent killer of pathogens (162). *MPO* expression is largely seen in neutrophils, but is present in monocytes. Products of *MPO* are also known to play a role in destroying red cells, platelets and lipoproteins. Hypomethylation of *MPO* could result in increased super oxide formation, which could result in increased oxidized low density lipoproteins (LDL) (131). These LDLs have been implicated in atherogenesis, and could result in increased risk of atherosclerosis later in life. We also saw hypomethylated *HLA-DMB* that codes for the beta chain of the HLA class II complex. Hypomethylation could result in increased transcription of this gene which could result in increased antigen loading onto the MHC II complex. *RNH1* is a ribonuclease inhibitor with a proposed function in angiogenin inhibition (154). Hypomethylation of this inhibitor in monocytes, could be in response to the ischemic environment of obese mothers. We saw hyper methylation of *ZFP36L1* and hypomethylation of *ZNF516*, which are zinc finger proteins important in

altering DNA binding specificity of DNA binding proteins. *SLC45A4* a solute carrier family member with no current know function hypomethylated, *ERICH*, also hypomethylated, is a protein-coding gene also with no known function, and *RP11-867G2.8* codes for an antisense transcript was also hypomethylated.

One of the key objectives of this study was to identity genes which were both differentially expressed and differentially methylated. From our CD4+ methyl-seq and RNA-seq data, we identified 9 common genes. We saw decreased expression of Thymidine phosphorylase (*TYMP*), an enzyme that catalyzes the first step in degradation of thymidine to deoxythymidine (163). Deoxythymidine is necessary for synthesis of DNA precursors involved in cell division (164). Decreased *TYMP* could indicate that CD4+ T cells from cord blood of babies born to obese mothers are proliferating less than babies born to lean mothers. *HK3* an enzyme up-regulated in active T cells was down-regulated. Taken together, we hypothesize that hypermethylation and down-regulation of *TYMP* and *HK3*, indicate that CD4+ T cells from cord blood of babies born to obese mothers are less active and proliferating than CD4+ T cells from babies born to lean mothers. We also saw reduced expression of *SNORA64*. The SNORNAs family are important in RNA accumulation (165) and to guide site specific rRNA modification (166). Of the 4 hypermethylated genes with increased gene expression *ST6GAL1* is involved in generating cell-surface carbohydrate determinants. This gene is normally expected in B cells and is related to expression of CD76 (167), but increased expression could be present in T cells

to alter cell-cell interactions. There is little know information regarding *TMEM26* except that is a transmembrane protein that could increase risk of COPD and decrease lung function (168). Increased expression of *TMEM26* could be relevant in increased risk of airway diseases in children. We also saw up-regulation of *S1PR2*, which is a G-coupled receptor related to cell proliferation and survival (169). Increased expression of this receptor would suggest increased proliferation of T cells, however in our previous unpublished work we saw no changes in proliferation by Ki67 staining. Therefore, up-regulation of *S1PR2* could be occurring through a feedback mechanism to compensate for low activation. Lastly, we saw increased expression of *PXDN* a peroxidasin homolog that encodes a heme-containing peroxidase that is likely involved cell proliferation and differentiation (170), but has no known function in CD4+ T cells.

In conclusion, we have shown evidence, that gene expression and DNA methylation patterns are altered in CD4+ T helper cells and CD14+ monocytes from cord blood of babies born to obese mothers. We detected inverse gene and DNA-methylation expression between T helper cells and monocytes. Transcriptome and methylome analysis revealed that genes associated with cell activation and proliferation are dysregulated in CD4+ T cells from cord blood of babies born to obese mothers. CD14+ monocytes had altered gene expression and DNA methylation associated with activation and cytokine production. In our previous data, we showed a reduction in the total counts of CD4+ T cells, which is in agreement with the down-regulation of genes involved in proliferation and

differentiation in this study. Future studies, will be needed to extend this analysis as only 2 subjects per group, per cell type were used. Furthermore, one of the limitations of this study is that our current transcriptome and methylome analysis was not carried out using stimulated cells, which could explain the low frequency of DEGs/DMGs seen in monocytes. Therefore, future studies should utilize a large cohort and include an emphasis on stimulated and non-stimulated samples. Finally, it could be of interest to identify the function of the lincRNA that were present in the data sets.

Chapter 4: Obesity during pregnancy and maternal factors that influence offspring immunity

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Abstract

Obesity during pregnancy is associated with several complications, notably increased incidence of gestational diabetes and preeclampsia, increased susceptibility to viral infection, need for cesarean section, and still birth. Pregnancy during obesity is also believed to result in a heightened inflammatory state, which may exacerbate these complications. Changes in the maternal immune system can also influence placental growth and therefore fetal development and future health of the offspring. Indeed, off offspring of obese mothers are more likely to become obese and develop metabolic disease (diabetes and cardiovascular disease), asthma, cancer, and attention deficit hyperactive disorder. However, obesity-induced changes in maternal immune system are poorly understood. In this study we compared peripheral immune cell frequency, phenotype and function as well s circulating plasma factors between women who began pregnancy in a lean or obese state.

Introduction

As of 2010, 32% of women of childbearing age in the United States are obese (7). Women who enter pregnancy in an obesogenic state are more likely to develop gestational diabetes and preeclampsia, have increased risk of viral infection, labor and delivery complications, need for C-section, and spontaneous abortion (171). Risks to the fetus include in utero growth restriction (IUGR) , stillbirth, and neural tube defects (12, 13, 27) Children born to obese mothers also have increased incidence of obesity (29), ADHD (30), type-2 diabetes (31, 32), cardiovascular disease (33, 34), cancer (35), and greater all-cause mortality (33).

During normal pregnancy the maternal immune system transitions from a pro-inflammatory 1st trimester to an anti-inflammatory 2nd trimester, and then again to a pro-inflammatory third trimester (81), is due in part to modulation by the placenta. For example, from 8 to 12 weeks of gestation, placental circulation is established the maternal peripheral blood is in close contact with villous trophoblasts, and it has been shown that plasma from pregnant women can activate monocytes (172). In contrast, dendritic and natural killer cells are reduced and have more limited responses (172).

Pregnancy is also associated with immune suppression, which leads to increased susceptibility to viral pathogens such as influenza, hepatitis and herpesvirus infections (173), which are detrimental to both the mother and child. In contrast, severity of several autoimmune diseases such as rheumatoid arthritis

and Lupus erythematosus is partially alleviated in pregnant women (174). Since pregnancy is associated with significant immune modulation, entering pregnancy in an obesogenic state may disrupt this delicate balance.

The goal of this study was to determine the impact of obesity on the maternal peripheral immune system by characterizing the frequency and function of major innate and adaptive immune cell populations and assessing the concentration of circulating cytokines, chemokines, and growth factors in peripheral blood samples from obese and lean pregnant women.

Methods

Subjects

The Institutional Ethics Review Board of Oregon Health and Science University (OHSU), and the University of California Riverside approved all studies. All subjects provided signed consent before entering the study. A total of 26 non-smoking mothers without diabetes who had an uncomplicated, singleton gestation at term (>37 wk) were enrolled: 11 lean mothers with a mean age of 31.5 ± 4.95 yr and pre-pregnancy BMI of 22.27 ± 1.95 kg/m^2 and 15 obese pregnancy women with a mean age of 29.6 +/- 5.9 yr and pre-pregnancy BMI of 37.5 +/- 5.0 kg/m^2. The racial distribution was as follows: 20 White/Caucasian, 2 Asian American/Pacific Islander, 1 American-Indian/Alaskan native, 1 African American, and 2 unknown.

Peripheral blood mononuclear cell and plasma isolation

Peripheral blood samples were collected into heparinized vacutainers during a routine antenatal and enrollment visit and processed within twelve hours. Complete blood counts were obtained by Hemavet instrument (Hemavet, Dallas, TX, USA). Peripheral blood mononuclear cells (PBMC) and plasma were obtained by standard density gradient centrifugation over Ficoll (BD Bioscience, San Jose, CA, USA). PBMC were frozen in 10%DMSO/FBS, using Mr. Frosty Freezing Containers (Thermo Scientific, Waltham, MA, USA), and stored in liquid nitrogen while plasma was stored at -80 until analysis.

Flow cytometric analysis of PBMC

PBMC (1-2 X 10^6 cells) were stained using CD4 (OKT4, eBioscience, San Diego, CA, USA), CD8b (2ST8.5H7, Beckman Coulter, Brea, CA), CD95 (Dx2, Biolegend, San Diego, CA, USA), CD28 (28.2, Biolegend), and CCR7 (G043H7, Biolegend) to delineate CD4 and CD8 T-cells populations as follows: naïve (CD28+CD95-); central (CM; CD28+CD95+CCR7+), transitional (TEM; CD28+CD95+CCR7-), and effector memory (EM; CD28-CD95+CCR7-) (Figure 1A)(116). PBMC were also stained using CD20 (2H7, eBioscience), CD27 (O323, TONBO, Temecula, CA, USA), and IgD (C4211, Southern Biotech, Birmingham, AL, USA) to delineate: naïve (CD20+CD27-IgD+), marginal zone-like (MZ-Like; CD20 +CD27+IgD+), and memory (CD20+CD27+IgD-) populations (Figure 1E) (117). After surface staining, PBMC were fixed, permeabilized, and

intracellularly stained with Ki67 (B56, BD Bioscience). A second tube of PBMC (1-2 X 10^6 cells) was stained as follows: CD3 (UCHT1, ebioscience), CD20 (B9E9, Beckman Coulter), HLA-DR (LN3, Biolegend), CD14 (M5E2, Biolegend), CD11c (3.9, Biolegend), CD123 (6H6, Biolegend), CD56 (RPA-T8, BD Biosciences), and CD16 (3G8, Biolegend) to delineate monocytes (CD3-CD20-HLA-DR+/-CD14+), myeloid dendritic cells (mDC; CD3-CD20-CD14-HLA-DR+CD11c+); plasmacytoid dendritic cells (pDC; CD3-CD20-CD14-HLA-DR+CD123+), and natural killer cells (NK; CD3-CD20-56bright/dim). Natural killer cells were subdivided by both CD56 bright/dim expression and CD16 into four distinct subsets. All flow cytometry samples were acquired with LSRII instrument (Becton Dickinson, Franklin Lanes, NJ, USA) and analyzed using FlowJo (TreeStar, Ashland, OR, USA).

In vitro PBMC stimulation

For analysis of T-cell cytokine production, 1-2 X 10^6 PBMC were stimulated for 24 h at 37°C in supplemented RPMI (10% FBS) in the presence or absence of anti-CD3/CD28 (OKT3, TONBO; CD28.2, BD Biosciences); Brefeldin A (Sigma, St. Louis, MO, USA) was added after 1-h incubation. Cells were stained for CD4 and CD8, fixed, permeabilized, and stained intracellularly for TNFα (MAb11, ebioscience), IFNγ (4S.B3,eBioscience), IL-4 (Biolegend), IL-2 (MQ1-17H12, Biolegend), and IL-17a (BL168, Biolegend). To measure cytokine production by monocytes and dendritic cells (DC), a second tube of PBMC was

cultured for 14 h at 37°C in RPMI supplemented (10% FBS) alone or in the presence of heat-killed Listeria monocytogenes (HKLM, TLR1 agonist) and synthetic triacylated lipoprotein (PAM3CSK4, TLR2 agonist), or lipopolysaccharide (LPS, TLR4 agonist) (Invivogen, San Diego, CA, USA). Brefeldin A (Sigma) was added after 1-h incubation. Cells were stained with CD3 (ebiosciences), CD20 (Biolegend), HLA-DR (L243, Biolegend), CD14 (Biolegend), CD11c (Biolegend), and CD123 (Biolegend), fixed, permeabilized, and stained intracellularly for IL-6 (MQ2-13A5, eBioscience) and TNF-α (ebioscience). All samples were acquired and analyzed as described above.

Cytokine, chemokine, and growth factor analysis

Plasma samples were analyzed using human multiplex panels (Millipore, Temecula, CA, USA) per the manufacturer's instructions in duplicates using MAGPIX multiplexing platform (Luminex, Austin, TX, USA). Plasma samples were also analyzed using CRP and IL-6 ELISA coated well strips (Lifetechnologies, Carlsbad, CA, USA) per manufacturers instructions on the InfiniteF50 microplate reader (Tecan, Maennedorf, Switzerland). Values below the limit of detection were designated as not detected.

Statistical analysis

All data sets were first assessed for normal distribution. Normal data sets were then tested for variance homogeneity. If data sets displayed homogeneity, they were subjected to unweighted student T-test.

Results

Obesity during pregnancy and complete blood counts

We first compared complete blood counts (CBCs; lymphocytes, monocytes, eosinophils, neutrophils, and basophils) between pregnant lean and obese women (Table 1). We observed that there were no differences in lymphocytes, monocytes, eosinophils, neutrophils, or basophils between the two groups.

	BMI Category	
	Lean	Obese
White Blood Cell (cells x 10^3/mL)	7906 ± 565.8	8310 ± 431.1
Neutrophil (cells x 10^3/mL)	4034 ± 819.8	4674 ± 441.3
Lymphocytes (cells x 10^3/mL)	1357 ± 156.5	1466 ± 136.9
Monocytes (cells x 10^3/mL)	486 ± 47.73	492.9 ± 36.71
Eosinophils (cells x 10^3/mL)	1924 ± 470.4	1540 ± 329.9
Basophils (cells x 10^3/mL)	104 ± 18.99	133.6 ± 12.65

Table 4.1 Maternal complete blood counts.

Impact of obesity during pregnancy on lymphocyte populations

We next investigated changes in CD4+ and CD8+ T cell (naïve, and memory) and B-cell subset (naive, Marginal Zone-like, memory, and other)

frequencies in PBMC using flow cytometry (Figure 1). We detected no changes in frequency of CD4+ T helper cells, CD8+ cytotoxic T cells, or CD20+ B cells (Fig.1B) or their naïve/memory subsets between lean and obese pregnant women (Fig. 1C-D, F).

We next determined whether obesity during pregnancy impacts cytokine production by CD4 or CD8 T cells by comparing production of TNFα, IFNγ, IL-2 (Th1), IL-4 (Th2), and IL-17 (Th17) following stimulation of PBMC with anti-CD3 and anti-CD28 (Fig. 2 A, B). No changes in cytokine production by CD8 T cells or CD4+ T helper cells production of TNFα, IFNγ, or IL-2 were detected. On the other hand, a reduction in IL-17 as well as a trend of increased production of IL-4 was observed (Fig. 2C).

Impact of obesity on innate immune cell subsets

We also saw no significant changes in total monocytes, dendritic cells, or natural killer cells (Fig. 3B, C, F). This included the subpopulations of mDCs, pDCs, and natural killer cell subsets (Fig. 3E, G). We then stimulated PBMC with PAM3CSK4&HKLM (TLR 1&2) or LPS (TLR 4) and measured expression of TNFα and IFNγ production by flow cytometry (Fig, 4 A, B). A trend of increased TNFα production by monocytes following stimulation by TLR 1&2 agonists was detected in the obese group (Fig. 4C). No other differences between the lean and obese groups were noted.

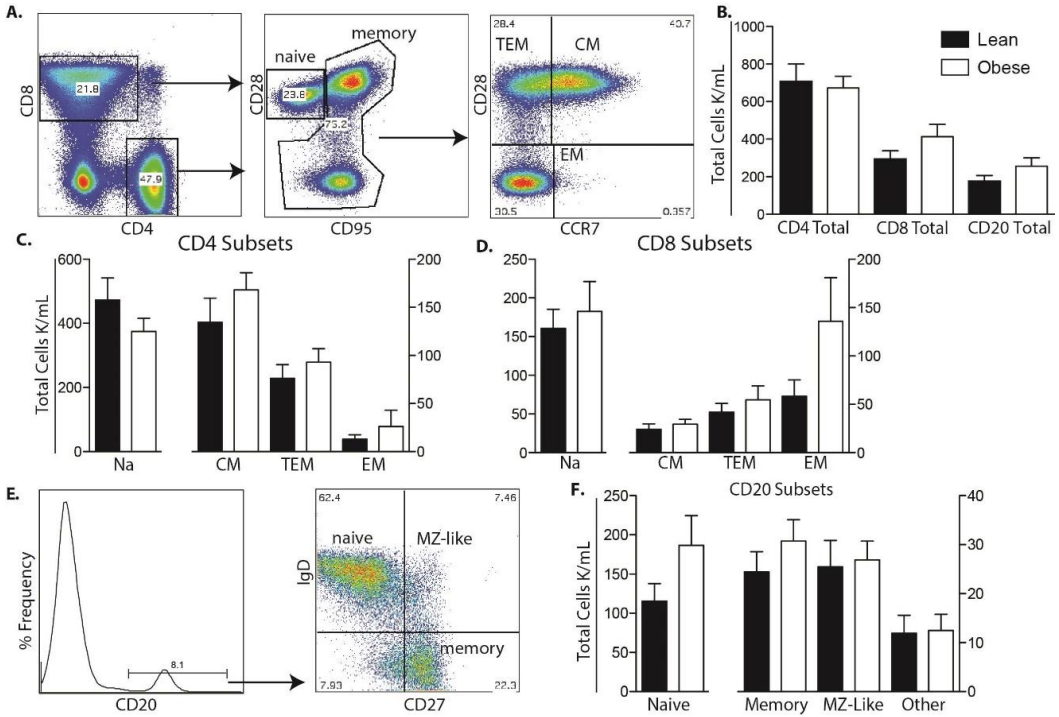

Figure 4.1: Impact of obesity during pregnancy on maternal adaptive immune cell populations. (A) Flow cytometry was used to delineate CD4 and CD8 T cells subsets: naïve, central (CM), effector (EM), and transitional effector memory (TEM) based on expression of CD28, CD95 and CCR7. (B) Numbers of total, CD4 T helper cells, CD8 cytotoxic T cells, and CD2+ B cells were determined by multiplying subset frequency by the number of lymphocytes obtained by the hematology analyzer. (C-D) Number of total naïve, CM, TEM and EM of CD4 and CD8 T cells per µl of cord blood were determined as described for total. (E) Flow cytometry was used to delineate total CD20+ B cells as well as naïve, marginal-zone like (MZ-like), and memory subsets. (E) Number of total naïve, MZ-like, and memory were determined as described for T cells. +/- SEM (n=11-14 per group)

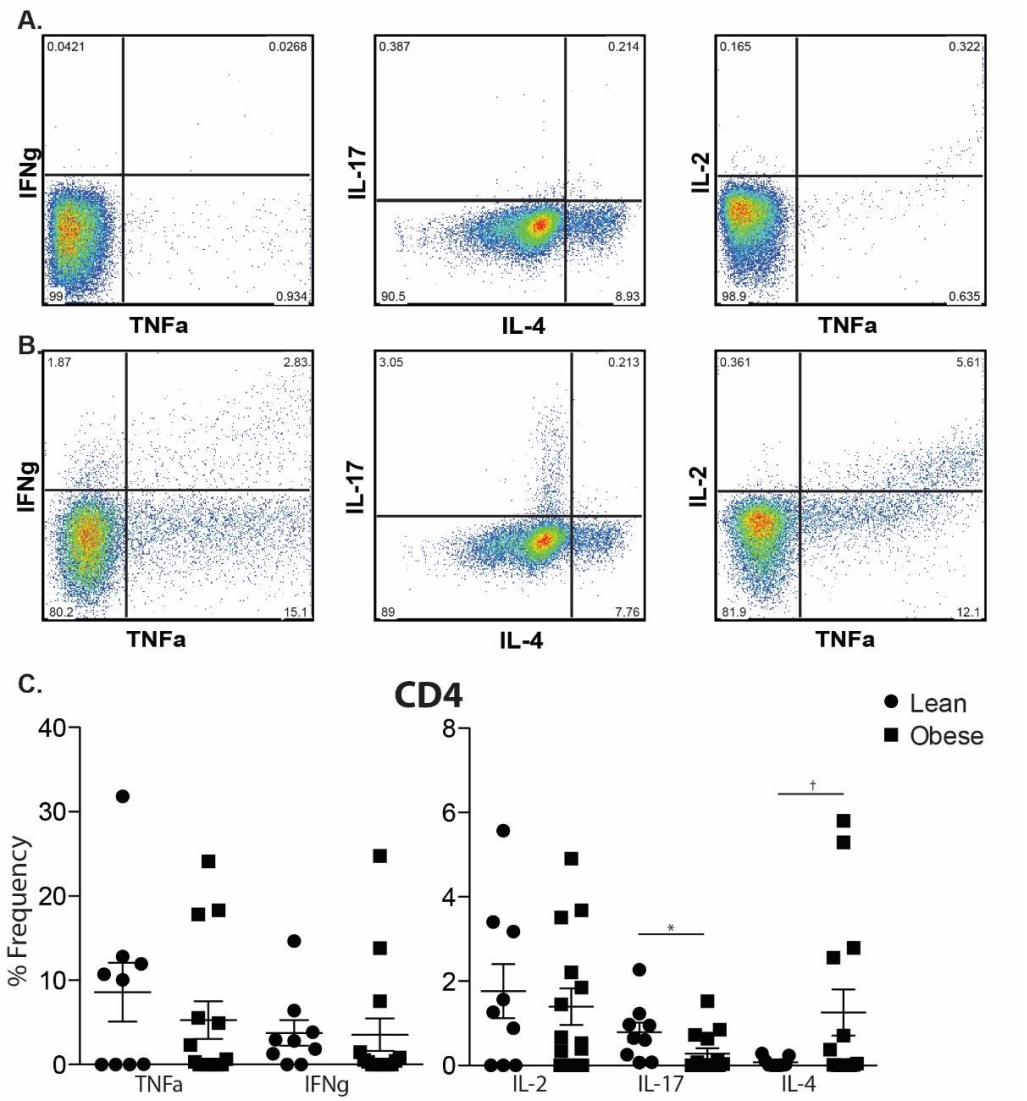

Figure 4.2: Impact of maternal obesity on maternal T cell responses following stimulation. (A-B) Representative CD4+ T cell response (A) before stimulation and (B) following anti-CD3/CD28 co-stimulation determined by flow cytometry. Cells were first stained with antibodies against CD4 and CD8, permeabilized, and intracellularly stained to detect TNFα, IL-4, IFNγ, IL-2, and IL-17. (C) Average frequency of CD4 T cells producing TNFα, IL-4, IFNγ, IL-2 and IL-17 after adjusting for background using unstimulated controls. +/- SEM (n=11-14 samples per group) *p≤0.05, †p<0.10.

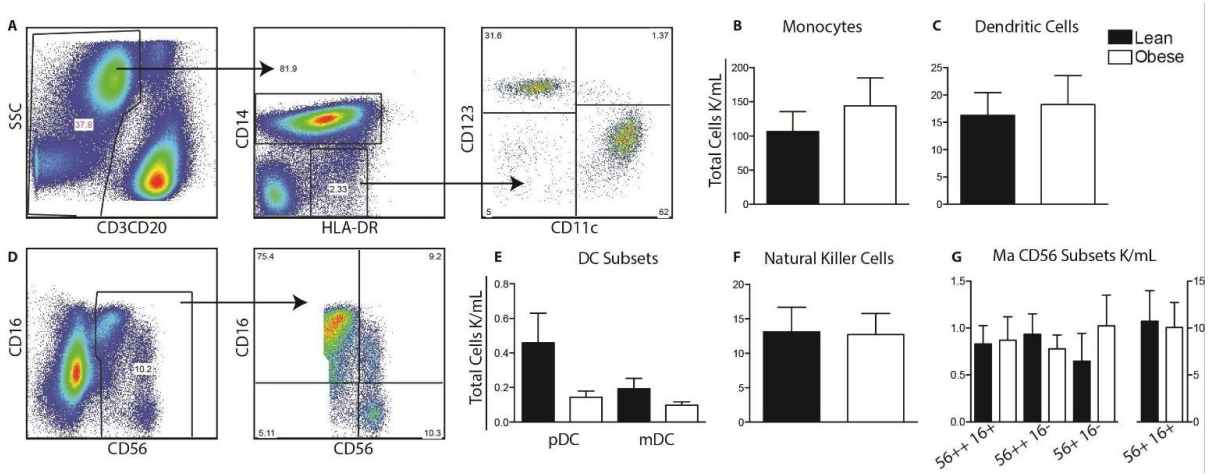

Figure 4.3: The impact of obesity on maternal innate cell counts. (A) Flow cytometry was used to delineate innate cell populations by first gating out CD3 and CD20 lymphocytes and then using CD14 and HLA-DR to define monocytes and dendritic cells, and DC subsets were determined by CD11c and CD123 expression. (B-C; E) Total numbers of monocytes, dendritic cells, and dendritic cell subsets per mL of cord blood were determined as described above for T cells. (D) Flow cytometry was used to delineate total natural killer cells by gating on CD3 and CD20 negative cells and then based on CD56 and CD16 expression. (F, G) Numbers of total and natural killer subsets were determined as described for monocytes. +/- SEM (n=11-14 samples per group)

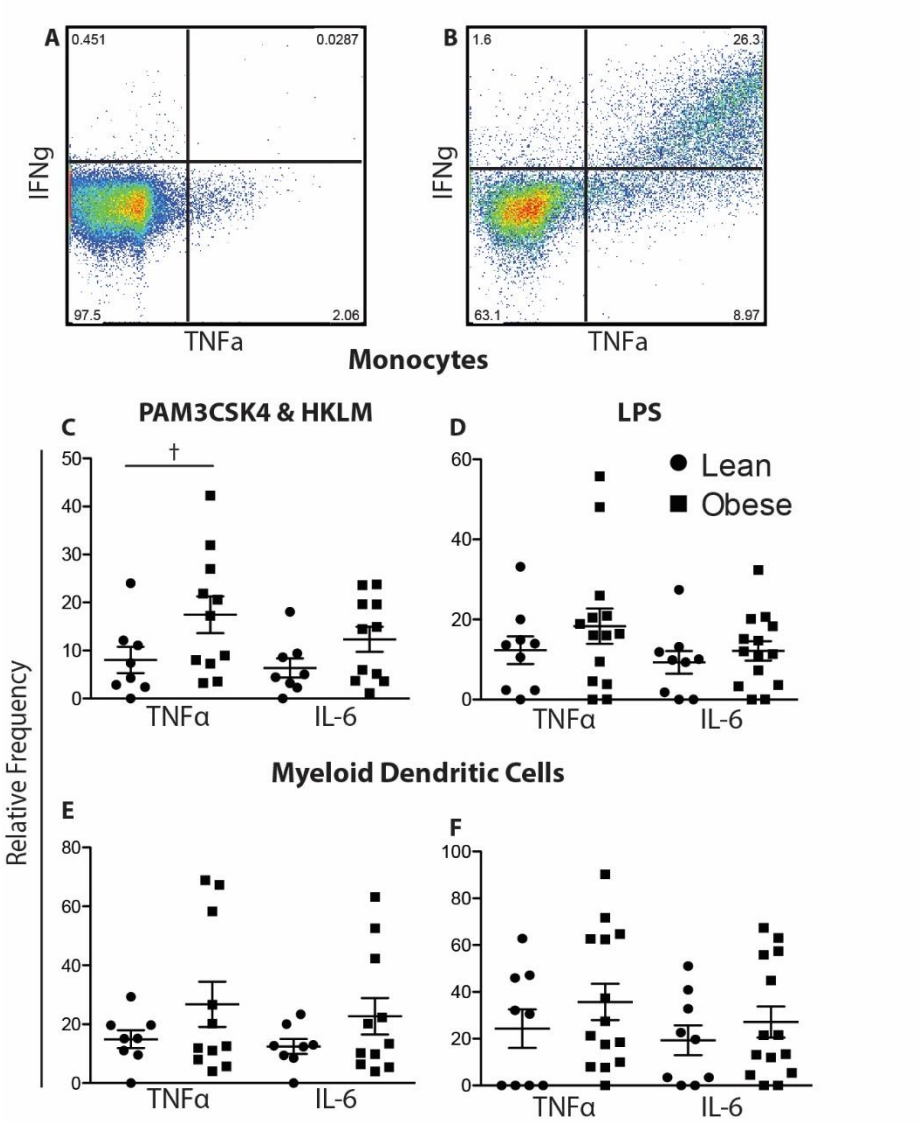

Figure 4.4: Toll-like receptor stimulation of maternal PBMC. (A-B) Representative image of monocytes (A) before stimulation and (B) following LPS stimulation. PBMC were stimulated with PAM3CSK4&HKLM (C,E) or LPS (D,E). Cells were then stained to define monocytes (C,D) or mDCs (E,F) followed by intracellular staining for IL-6 and TNFα. The mean frequency of stimulation was adjusted for background using unstimulated controls.+/- SEM (n=11-14 samples per group) †p<0.10

Impact of obesity during pregnancy and blood levels of circulating factors

Finally, we compared circulating levels of cytokines (IFNα2, IFNγ, TNFα, IL-1Ra, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IP-10, IL-12p40, IL12p70, IL-13, IL-15, Flt-3L), chemokines (MCP-1, MIP-1a, MIP-1b, GRO, MCP-3, MDC, eotaxin, and fractalkine), adipokines (insulin, leptin, total PYY, adiponectin, lipocalin-2/NGAL, adipsin, resistin, total PAI-1), growth factors (VEGF, EGF, FGF-2.2, TGF-a, G-CSF, GM-CSF) and the inflammatory protein marker C-reactive protein (CRP) in maternal plasma (Figure 5). We saw significant increases in leptin, MIP-1b, epidermal growth factor (EGF), IL-6, IL-8, and C-Reactive Protein (CRP) plasma levels and a trend towards increased insulin, Lipocalin-2/NGAL, and macrophage-derived chemokine (MDC) plasma levels in obese pregnant women.

Discussion

In this study we investigated differences in frequency and function of key innate and adaptive immune cell populations in PBMC collected from lean and obese pregnant women. We detected no significant differences in numbers of circulating white blood cells. This is interesting, because normal pregnancy, WBCs, lymphocytes and granulocytes are increased at all stages of pregnancy (175). In addition, during obesity WBCs, lymphocytes, and granulocytes are known to increase in obese women compared to lean women (176). We believe the lack of difference could be due to our smaller population size.

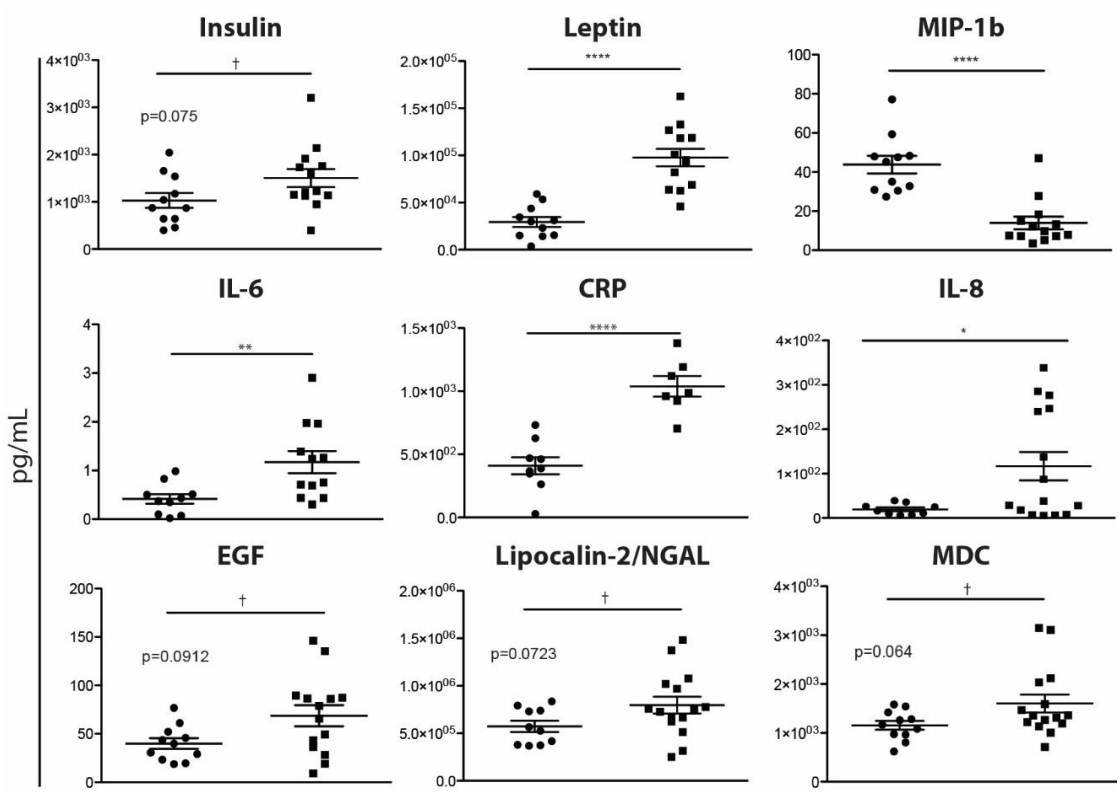

Figure 4.5: Circulating levels of hormones, chemokines, and cytokines in maternal plasma. Plasma protein levels were determined by cytometric bead assay using human multiplex. (n=11-14 samples per group) *p≤0.05. **p≤0.01 ***p≤0.001 ****p≤0.0001 †p≤0.10

In our study we also saw no significant changes in the frequency of CD4+ T helper cells, CD8+ cytotoxic T cells, CD20+ B cells, or their subsets with increasing BMI. In contrast it has been observed that obese women in their 2nd trimester have increased total CD4+ T helper cells (177, 178) compared to their lean counterparts. Additionally, when obese women in their 2nd trimester were compared to lean women, they had lower CD8+ T cells, and increased CD20+ B cell counts (179).

We also examined whether there were any alterations to functional capacity of CD4+ and CD8+ T cells. Results from our stimulation assays illustrated that the obese group had increased production of IL-4 and decreased production of IL-17. Increased secretion of IL-4 by T helper cells in the obese group could be a response to excess lipids in circulation (180). Finally, IL-17 is a mediator that activates production of pro-inflammatory cytokines and chemokines in response to parasites, bacteria, and fungi (181). Decreased IL-17 production could result in decreased bacterial responses as IL-17 acts synergistically with TNFα and IL-1 during pathogen infection. There were no significant changes in production of TNFα and IL-6 by monocytes or myeloid dendritic cells following stimulation by TLR 1&2 or TLR4. One exception was a trend of increased TNFα production in monocytes to TLR 1&2 stimulation in the obese group. While increased TNFα production by monocytes following response to gram-positive bacteria, the resulting inflammation could be harmful to the developing fetus.

Finally, we saw increases in plasma levels of insulin, leptin, IL6, IL8, MIP-1b, EGF, CRP and increasing trends of Lipocalin-2/NGAL, and MDC. Previous studies have shown that increased maternal leptin and insulin impact offspring size and adiposity (182, 183). Higher maternal leptin during pregnancies corresponds with small for gestational age babies (183). Higher maternal insulin also corresponds with higher insulin resistance in offspring (184). Higher insulin resistance has been observed in children born to obese mothers (125) and correlates with increased likelihood of developing childhood diabetes, and allergic asthma; all of which have been observed in children born to obese mothers (125, 126). As previously described (185), we observed a trend of increased Lipocalin-2/NGAL levels, which contributes to insulin resistance. We also detected increased levels of IL-6. Murine studies have linked maternal IL-6 levels with dysregulated formation of neural circuits that control body weight (186). This observation is in line with increased incidence of obesity in children born to obese mothers. Elevated IL-8 in maternal plasma during pregnancy is correlated to increased risk of schizophrenia in adult offspring (187). This is a critical observation given that offspring of obese mothers may have a 3-fold higher increased risk of schizophrenia (188). We also saw decreased MIP-1β levels, which are generally increased with adiposity and inflammation (189).

Furthermore, we saw a trending increase in MDC (CCL22), a chemokine secreted by macrophages and dendritic cells and interacts with CCR4, which is expressed in the villous of the placenta during the first trimester of pregnancy

(190). It has been seen that during inflammation CCR4 is up-regulated on macrophages (191, 192). Increased MDC may be a potential reason for the increased monocytes/macrophages infiltration seen in the placenta of obese women (78). Increased levels of EGF, which promotes placental growth (193), could explain the accelerated placental growth see in obese pregnant women (194). Finally, we saw increases in maternal levels of CRP, a well-known marker of inflammation, which has been linked to a 43% increased risk of autism in the offspring (195). Maternal obesity has also been associated with an increased risk of autism in the offspring (196).

In conclusion, women entering pregnancy in an obesogenic state show some aspects of immune dysregulation which provides insight into increased risk of adulthood diseases such as obesity, diabetes, schizophrenia, and autism in offspring of children born to obese mothers. Further studies in a larger cohort with longer follow up are needed to extend these observations, and uncover the molecular mechanisms of maternal-fetal immune interaction.

Chapter 5: Summary, conclusions, and future directions

Summary and discussion of results

Obesity during pregnancy alters immune cell frequency and response in umbilical cord blood

Chapter 2 described the effect of maternal obesity on the composition and functional responses of adaptive and innate immune cells in umbilical cord blood. Babies born to obese mothers had significantly fewer total and naïve CD4+ T helper cells. This reduction was accompanied by decreased IL-4 production. Analysis of the plasma components from umbilical cord blood found elevated levels of IFNα2, which is known to repress IL-4 production. Elevated IFNα2 may be linked to the elevated pDCs, major producers of Type I interferons, seen in UCBMC of babies born to obese mothers. TNFα and IL-6 production in response to TLR 1&2 and TLR4 agonists by monocytes and mDCs from babies born to overweight and obese mothers were muted.

Surprisingly, babies born to obese mothers had significantly reduced eosinophil counts. Eosinophils are known to contribute to asthma severity (30), and children born to obese mothers are more likely to develop asthma or wheezing (118, 197). Decreased number of circulating eosinophils might indicate early recruitment to the lungs to mediate airway remodeling, but future work will need to address this.

Finally, we saw small increases in insulin and VEGF, as well as a small decrease in resistin in UCB plasma. In contrast to reduced IL-6 production by monocytes and mDCs after TLR stimulation, plasma IL-6 levels were increased. Circulating IL-6 may be produced by endothelial cells (198). Increased IL-6 levels could potentially explain the increased production of IL-17 by CD4+ T cells (110) given their role in Th17 polarization (199).

Genes associated with cell activation and proliferation are down-regulated in CD4+ T cells of Babies of obese mothers and genes associated with activation and cytokine production are up-regulated in monocytes of Babies of obese mothers

In order to uncover the molecular basis for reduced cytokine response by monocytes and altered IL-4/IL-17 cytokine production by CD4+ T cells, in chapter 3, CD4+ T cells and CD14+ monocytes were isolated and subjected to RNA-seq and DNA methyl-seq analysis. Transcriptome and methylome analysis revealed that expression of genes associated with activation and proliferation are dysregulated in CD4+ T cells isolated from babies born to obese mothers, and that expression of genes associated with activation and cytokine production was altered in in CD14+ monocytes. CD4+ T cells from cord blood of babies born to obese mothers had 112 differentially expressed genes (DEGs) compared to those born to lean mothers that were primarily down-regulated. Complement factor D, IL-1R type 2, HK3, and 4 genes associated with the MHC-II complex

were down-regulated in CD4 T cells isolated from babies born to obese mothers. Reduced MHC class II expression on CD4 T cells could suggest decreased Tregs (133). IL-1R type II blocks IL-1β (136) a cytokine important in mediating inflammatory response. Down-regulation of these genes could result in increased autoimmune disease. Hexokinase 3 is responsible for the first step in initiating glycolysis in active T cells, which is the primary method for generating ATP. Down-regulation could result in slower activation of CD4+ T cells of babies born to obese mothers. Conversely, CCL2, PKP3, and LYPD6 were up-regulated. It is possible that CD4+ T cells are up-regulating CCL2/MCP-1 in response to placental inflammation. Increased CCL2 induces amylin expression which is known to be involved in increased insulin resistance (128), which can lead to increased risk of type 2 diabetes and asthma seen in offspring of obese mothers. Cytoplasmic PKP3 is associated with RNA binding proteins which are may play a role in translocation of mRNA to stress granules and influence degradation (131). PKP3 could therefore be up-regulated in response to ischemic injury as has been seen in placentae of obese mothers. LYPD6 is a membrane anchored protein that enhances WNT/β-catenin signaling promoting differentiation of CD4+ Th2 cells (200).

CD14+ monocytes of babies born to obese mothers had 78 DEGs compared to babies born to lean mothers that were primarily up-regulated. WNT5a, IL-6, TSP-1, FABP5, CREM and CXCL3 were up-regulated in babies born to obese mothers. Up-regulation of WNT5a and IL-6 could indicate

activation of monocytes in response to increased lipids (149). Up-regulation of CREM could prevent capacitance of CREB factors (148), which could result in decreased production of IL-6 and TNFα following activation (149), which agrees with our previous data that monocytes had muted IL-6 and TNFα responses (110). Increased TSP-1 could be a response by monocytes to limit activation of leukocytes (144). CXCL3 is associated with angiogenesis and wound healing. Increased expression of CXCL3 could be a marker related inflammation associated with placental ischemia. We also saw decreased expression of HLA-DQB1 and TACSTD2, which could results in reduced antigen loading and presentation to T cells and reduced activation of monocytes from babies born to obese mothers, respectively.

In addition to transcriptome analysis, we also report changes in DNA-methylation in cord blood CD4+ T helper cells and CD14+ monocytes due to maternal obesity. Most of the 4006 differentially methylated genes were hypermethylated in CD4 T cells of babies born to obese compared to lean mother, which correlated with decreased gene expression. FGR, CERK, HLA-DMB were hypermethylated with >10DMCs in the 1st exon, CpG islands, and promoter region. Reduced expression of these genes could lead to lower T cell activation and reduced Treg activity. LEF1, expressed by Treg, was hypomethylated with >10DMC within the 1st exon region, which could lead to increased CD4+ T differentiation to a Treg phenotype. In contrast to CD4+ T cells, most DMGs were hypomethylated in CD14+ monocytes isolated rom

babies born to obese mothers, which correlated with increased gene expression. Notable genes here are MPO, HLA-DMB, and RNH1, which were hypomethylated with >10DMCs present in 1st exon, CpG islands, and promoter region. MPO is important for conversion of peroxide into superoxides, which kill pathogens, and degrade lipoproteins. Hypomethylation of MPO could result in increased super oxide formation, which could result in increased oxidized low density lipoproteins (LDL). These LDLs have been implicated in atherogenesis, and could result in an increased risk of atherosclerosis later in life. This could be due to excess lipoproteins present in cord blood as a result of increased nutrients from obese mothers. Hypomethylation of HLA-DMB could be beneficial in antigen loading, and increased antigen presentation to T helper cells. Interestingly and despite hypomethylation of HLA-DMB, our transcriptome analysis showed a reduced, rather than an increased, expression of HLA-DMB. This could in part be explained by other regulatory mechanisms such as miRNA regulated degradation of HLA-DMB transcripts. Lastly, RNH1, a gene involved in blood vessel formation was also hypomethylated. Increased transcription of RNH1 could decrease blood vessel formation, which is in line with increased risk of ischemic injury seen in placentae of maternal obesity.

Lastly, because the RNA and DNA analyzed originated from the same cells, we looked for concordance between the transcriptome and methylome analysis. In CD4+ T cells, we identified 3 genes that were down-regulated and hypermethylated. TYMP and HK3. Both TYMP and HK3 play roles in T cell

metabolism in active T cells. Down-regulation and Hypermethylation of these genes would indicate that T cells of babies born to obese mothers are less active or have a dysregulated metabolic state. S1PR2 was both up-regulated and hypomethylated. S1PR2 is a G-coupled receptor related to cell proliferation and survival, and may be increased in response to ischemic environment of the placenta. TMEM26, another gene that was up-regulated and hypomethylated, may play a role in the increased incidence of airway diseases in children born to obese mothers. No overlap was identified between differentially expressed genes and differentially methylated genes of CD14+ cells.

Soluble factors in maternal plasma may influence offspring future risk of disease

Chapter 4 establishes additional links between maternal obesity and increased risk of disease in children. CD4+ T helper cells of obese women produced less IL-17 and more IL-4 compared to lean women following T cell stimulation. Decreased IL-17 could result in muted maternal immune responses to bacterial pathogens, while increased IL-4 could result in increased activated B cells and IgE production. IgEs are important in pathogenesis of allergic asthma, and furthermore, IgEs have been shown to cross the placenta barrier, potentially predisposing offspring to asthma. A trend of increased TNFα production in monocytes following TLR1&2 stimulation was detected. Increased TNFα

production could contribute to 1st-trimester loss, later development of gestation diabetes and preeclampsia (201).

Increased plasma levels of insulin, leptin, IL6, IL8, MIP-1b, EGF, CRP and increasing trends of Lipocalin-2/NGAL and MDC, were detected in obese pregnant women. Elevated maternal insulin, Leptin, IL-6, and Lipocalin-2/NGAL are known to influence metabolism in the neonate, correlate with increased childhood adiposity and development of obesity related conditions such as cardiovascular disease and type 2 diabetes. Increases in maternal MIP-1b and MDC as seen in the obese group, could result in increased inflammation, which disrupts placental morphology and impacts neonatal development (202). Likewise, increases in EGF can influence neonatal growth and development as EGF regulates placental growth. Changes in placental growth can influence neonatal development and health outcomes later in life (19, 203). Lastly, increased IL-8 and CRP have been seen to respectively correlate with increased schizophrenia and autism in children (196). Overall, this study illustrates that maternal obesity may directly influence offspring incidence of disease and serves to primarily add to the growing literature on maternal obesity and increased risk of adverse neonatal outcomes.

Future Directions

In our analysis, we found fewer CD4+ T cells in babies born to obese mothers. We saw reduced CD4+ T helper cell IL-4 production that we

hypothesize is due to increased plasma IFN**α**2, and small increases in IL-17 that could be due to increased IL-6 in the plasma. Stimulation studies on CD4+ T cells should be repeated with the use of autologous plasma to confirm these findings. Additionally, future sequencing studies should utilize stimulated CD4+ T cells, because our current analysis is not representative of the functional responses seen in our previous data.

Monocyte and mDC responses to TLR 1&2 and 4 agonists were decreased in stimulation assays. However, gene expression analysis from our study did not reflect the cytokine production we detected in our stimulation assay. This could be because the isolated monocytes in our transcriptome and DNA-methylome analysis were not stimulated. Repeating our analysis using monocytes stimulated with TLR 1&2 or TLR 4 agonist could better illustrate the impact of maternal obesity. Additionally, we identified decreased TNFα and IL-6 cytokine production in monocytes and mDCs, but dysregulation in response to other pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) needs to be further elucidated. Monocytes and mDCs need to be stimulated with other TLR ligands, C-type lectin receptor (CLR) agonists, and Nod-like receptor (NLR) agonists. Lastly, evidence also suggests that stimulating immune cells in autologous plasma from neonates differs from cell culture media and even adult plasma, which results in differential immune responses as seen in monocytes (204).

This study identified that eosinophils were lower in cord blood from babies born to obese mothers. We hypothesize that this is because eosinophils may have already begun migrating to the lungs and/or airways. To verify eosinophil migration was increased to the lungs, we can leverage non-human primate models, which closely mimic human pregnancy to determine whether chemokines such as eotaxin and total eosinophil count were increased in lung tissue (205).

Finally, we have shown that maternal obesity dysregulates aspects of the adaptive and innate immune system of umbilical cord blood mononuclear cells. If children from these or future studies were followed throughout infancy and early years of life, we could evaluate the long-term impact of maternal obesity on response to vaccination and incidence of infections.

Conclusion

In this thesis, we have investigated the impact of maternal obesity on the neonatal immune system. The results from this study provide evidence that children born to obese mothers have a dysregulated innate and adaptive immune system that may contribute to poor responses to infection and increased risk of asthma. We have also shown that monocytes and mDC responses following stimulation were muted. In addition, we found that there are fewer eosinophils in cord blood of babies of obese mothers, we hypothesize that they may be migrating to the lungs, and that these may be key player in early asthma

pathogenesis. This is supported by increased cytokines in cord blood plasma, and increased gene expression in CD4+ T cells of genes associated with asthma. Soluble factors in maternal plasma may influence increased risk of adverse fetal outcomes. In conclusion, maternal obesity dysregulates the neonatal immune system, potentially leading to increased risk of disease in offspring later in life.

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