HEPATIC CHOLESTEROL METABOLISM DRIVES ENTERIC IMMUNITY AND DEVELOPMENT OF NECROTIZING ENTEROCOLITIS

A Dissertation

by

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ABSTRACT

Necrotizing Enterocolitis (NEC) is a disease of the neonatal gastrointestinal tract related to prematurity, low birth weight and formula feeding that has been linked to imbalances in tolerizing versus inflammatory lymphocytes. Lipid metabolism is crucial to T cell phenotype. We hypothesized that derangements in hepatic lipid processing predispose to inflammatory T cell types, including gamma delta T cells (GDTCs), and diminish tolerizing regulatory T cells (Tregs) in the genetically susceptible host.

First, we conducted an epidemiological study of babies predisposed to NEC. Flow cytometric analysis was conducted on cord blood Tregs from susceptible babies. Next, we analyzed microarray data to determine genetic differences in lipid metabolism of babies who developed NEC. We studied strain-specific differences in T cell activation in murine models in response to manipulations of lipid substrates. Finally, we explored the direct effect of low density lipoproteins (LDL) and high density lipoproteins (HDL) on lymphocyte profiles *in vitro* and *in vivo*.

We found that babies who had intrauterine growth restriction (IUGR) and were born to pre-eclamptic mothers had a nine-fold higher risk of developing NEC compared to controls. Prematurity led to higher numbers of poorly suppressing Tregs while IUGR led to low frequencies of normally-functioning Tregs in cord blood. Microarray data showed significant differences in cholesterol metabolism genes in babies with NEC. *In vitro*, LDL supplementation decreased Treg numbers and increased IL-17-producing cells (a pro-inflammatory cytokine). Liver was found to have higher ratios of GDTCs to Tregs. Finally, a neonatal mouse model of formula feeding combined with epithelial

destruction of the intestine showed that formula-feeding primed the immune system with high GDTCs and low Tregs for response to enteric pathogens, as seen in NEC.

The implications of cholesterol metabolism in the development of NEC are novel. Our data suggest that lipid derangements favoring high LDL and low HDL prime the system towards an IL-17-producing GDTC phenotype as mediated in the liver. These findings may be important for both diagnostic and therapeutic purposes, though further studies are needed.

DEDICATION

This work is dedicated to my family, especially my parents Phalguni and Shantasri Mukhopadhyay.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Newell-Rogers (advisor), Dr. Meininger and Dr. DeMorrow of the Department of Medical Physiology at Texas A&M University, Dr. Beeram of the Department of Pediatrics at Baylor Scott & White McLane Children's Medical Center and Dr. McNeal of the Departments of Pediatrics and Internal Medicine at Baylor Scott & White Hospital.

The data analyzed for Chapter 3 were gathered in part by Dr. Lena Perger of the Department of Pediatric Surgery at Baylor Scott & White Hospital and Laura Weaver of the Texas A&M Health Science Center College of Medicine and published in 2014. The data analyzed for Chapter 2 were provided by Dr. David Krauss of the Department of Pediatrics and Dr. Perger and the analyses were conducted in part by Dr. Luka Komidar of the University of Ljubljana and published in 2016.

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NEC is a disease of newborns that results in epithelial destruction of the gastrointestinal tract leading to a cascade of local and systemic effects. The incidence is 1 in 1000 live births with a mortality of 15 - 30% and it is almost exclusively a disease of premature and low birth weight infants.[1–4] NEC is a complex multifactorial disease, as risk factors from the interplay of host, microbiome, and iatrogenic events such as formula feeding have all been independently found to increase the risk of development of NEC.[5,6]

Randomized trials of therapies that appear promising in animal models of NEC, including supplementation with lactoferrin, arginine, glutamine or probiotics, are incompletely characterized and therefore have not been standardized into clinical guidelines of care.[7–10] Current practices that appear to have marginally improved NEC incidence and mortality over the last decade include aggressive use of breast milk, standardization of enteral formula feeding guidelines, delayed cord clamping, minimization of antibiotic use and nil per os (NPO, nothing by mouth) status.[11] However, understanding of the pathogenesis of the disease still remains limited, thus the quest for a unifying effective therapy remains.

The immune response is a critical factor in the development and progression of the disease. Both innate and adaptive immune mechanisms have been implicated. The current understanding of the disease process is that an initial breach of the intestinal epithelial barrier leads to a pro-inflammatory immune response and the

inability of the premature immune system to then counteract the inflammatory cascade leads to downstream effects that manifest as NEC (Figure 1).

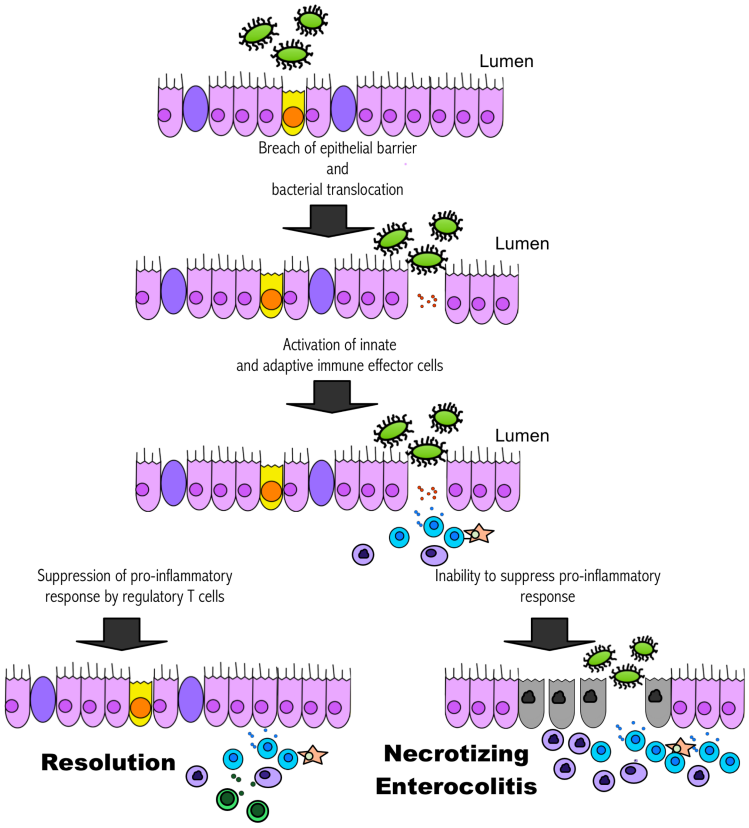

Figure 1. Pathogenesis of NEC

Macrophages from NEC-affected infants appear to be phenotypically different from healthy term neonates, expressing strongly pro-inflammatory behavior.[12–14] Depletion of neutrophils and macrophages from the intestinal lamina propria has been shown to exacerbate colitis in experimental NEC.[15] Intraepithelial lymphocytes (IELs), resident lymphocytes of the gut mucosa that usually express the Yδ T-cell receptor (GDTCR) and unlike classical T cells do not mount an antigen-specific response, are necessary for self-tolerance and habituation to commensal microbiota.[16] Infants with

NEC have been shown to have a reduction in this subset of innate-like lymphocytes, as well.[17]

Additionally, a dysregulation in the ability to mount antigen-specific tolerance to both the microbiome and to self-antigens occurs in the pathogenesis of the disease.[18] Secretory IgA, required for oral and microbiome tolerance, is reduced in preterm infants. In experimental colitis models, decreased exposure to IgA leads to a more pro-inflammatory gut microbiota and intestinal epithelial gene expression associated with inflammatory bowel disease.[18] Tregs, CD4+ T lymphocytes defined by the expression of forkhead box P3 (Foxp3), that promote self-tolerance are also attenuated in the gut epithelium of infants who develop NEC.[19] Pro-inflammatory cytokine expression, especially of IL-6 that disposes T cells to differentiate towards the pro-inflammatory T-cell subsets (including IL-17+ cells), is also proportionately increased during development of NEC.[20]

One of the major foundations of immunology-based NEC research currently centers on the role of toll-like receptors (TLRs), transmembrane molecules that detect pathogen-associated ligands and affect downstream signaling to elicit an immune response.[21] TLR4, the receptor for bacterial endotoxin that is the lipid A domain of lipopolysaccharide (LPS) from gram-negative bacteria, is highly expressed on fetal and neonatal enterocytes. The structure of lipid A has tremendous diversity among various organisms.[22] TLR4 appears to play a crucial role in the development of NEC. Premature infants have higher expression of TLR4 than full term infants due to its physiologic role in gut development.[23] However, TLR4 activation in premature infants can also cause enterocyte death, loss of the epithelial barrier and bacterial

translocation, leading to the local and systemic effects of NEC.[24] Loss or attenuation of TLR4 appears to have a protective effect against experimental NEC.[25]

Metabolic profiles heavily influence T cell differentiation. Quiescent and memory T cells as well as Tregs use different metabolic machinery for energy and appear to be more dependent on oxidative phosphorylation and exogenous fatty acid utilization.[26] Conversely, effector T cells, including Th17/Th1/Th2 cells, are heavily dependent on upregulation of glycolytic pathways through mammalian target of rapamycin- (mTOR)-dependent pathways.[27–29] Dietary supplementation with short chain fatty acids has been shown to induce colonic Tregs and ameliorate experimental colitis.[30–34]

The composition of dietary fat in colitis has generally been shown to have a profound effect on gut inflammation. Dextran sodium sulfate (DSS), a heparin-like polysaccharide, that is used to induce murine inflammatory bowel disease, appears to exert its effects by complexing with medium chain fatty acids.[35] However, in general, medium chain fatty acid supplementation appears to attenuate colitis.[36–38] Meanwhile, dietary supplementation with long-chain fatty acids appear to induce gut inflammation, such as the polyunsaturated omega-3 docosahexanoic acid increasing FoxP3 expression while at the same time attenuating Treg proliferation and inhibitory capacity.[39,40]

This takes on special significance in the neonate, who switches from obtaining nutrients by placental transfer *in utero* to oral ingestion of nutrition after birth. NEC is a disease almost exclusively of formula-fed infants. Breast milk is a complex biofluid of which lipids are the largest source of energy, contributing 40-55% of its composition.[41] The majority of these lipids are in triacylglyceride form (TAG), with the fatty acid

component along the glycerol backbone being highly conserved and in specific positions. Oleic, palmitic, and linoleic acids are commonly found at the sn-1, sn-2, and sn-3 positions.[42] Lipids, including triglycerides (98%), diacylglycerides, monoacylglycerides, free fatty acids, phospholipids, and cholesterol, are packaged into a specialized carrier molecule called the milk fat globule (MFG). The MFG is composed of a TAG core surrounded by a unique triple membrane structure called the MFG membrane.[43,44] Infant formula does not contain MFG and tends to have vegetable source-derived lipids which differ greatly in size and composition.[44,45] Supplementation of formula with MFG appears to protect against *Clostridium dificile*-induced gut inflammation in neonatal rat models and against gut barrier disruption and inflammation in systemic LPS infection.[46,47]

The liver functions as the main effector organ in the processing of dietary lipids throughout life, as well as a primary lymphoid organ prenatally. Fatty acids are either burned for energy or processed and packaged by hepatocytes into very low density lipoproteins (VLDL) for delivery of fatty acids and cholesterol for cellular use. HDLs ("good cholesterol") then return lipids from peripheral tissues to the liver for disposal through bile (Figure 2).

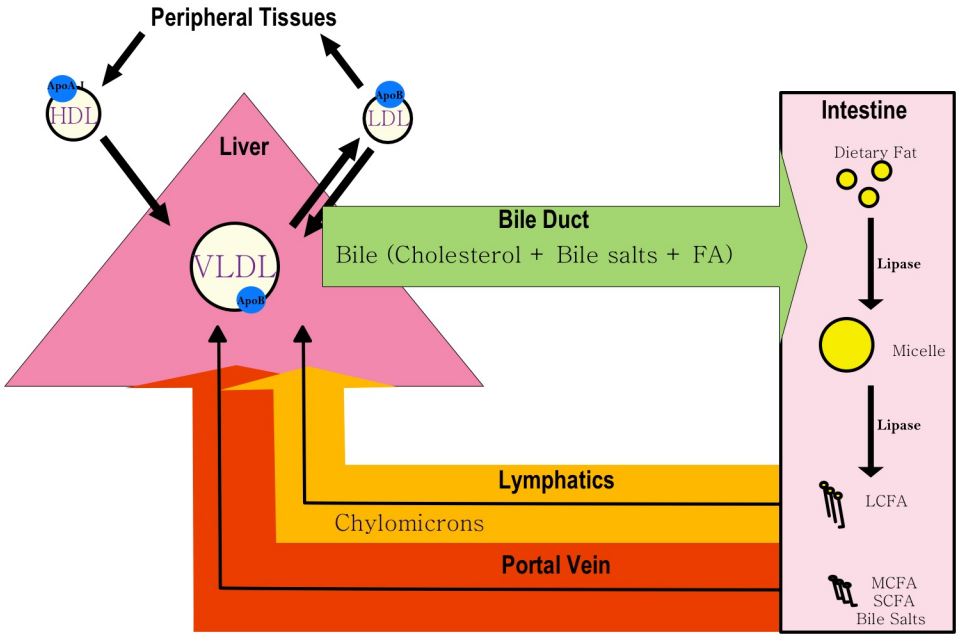

Figure 2. Lipid metabolism in the enterohepatic circulation

Recently, oxidized LDL ("bad cholesterol") has been demonstrated to play a role in IL-17-dependent inflammation in atherosclerosis.[48–52] Conversely, HDL appears to promote a Treg-dependent reduction in inflammation.[51,53] A high-fat diet has been demonstrated to cause Treg apoptosis in the liver without a parallel effect on splenic Tregs.[54] Independently, infection with rhesus rotavirus, a major intestinal pathogen that also induces experimental biliary atresia in mouse models, has been shown to induce IL-17 production and concomitant decrease in anti-inflammatory IL-10 by intrahepatic T cells with no significant similar effect seen in mesenteric lymph nodes or gut mucosa.[55] B-cells, which present either CLIP (class II-associated invariant peptide) or antigen in the groove of MHC II to promote T cell activation by engagement with the T cell receptor, have been shown to have a high density of LDL receptors and the ability to

internalize LDL for processing in the lysosome, where antigen association with MHC II occurs as well (Figure 3).[56]

Together, we therefore hypothesized that the liver plays a significant role in development of intestinal pathology such as NEC via crosstalk of the immune system and lipids and that fat content of dietary intake likely drives immune cell differentiation toward tolerance or inflammation (Figure 3).

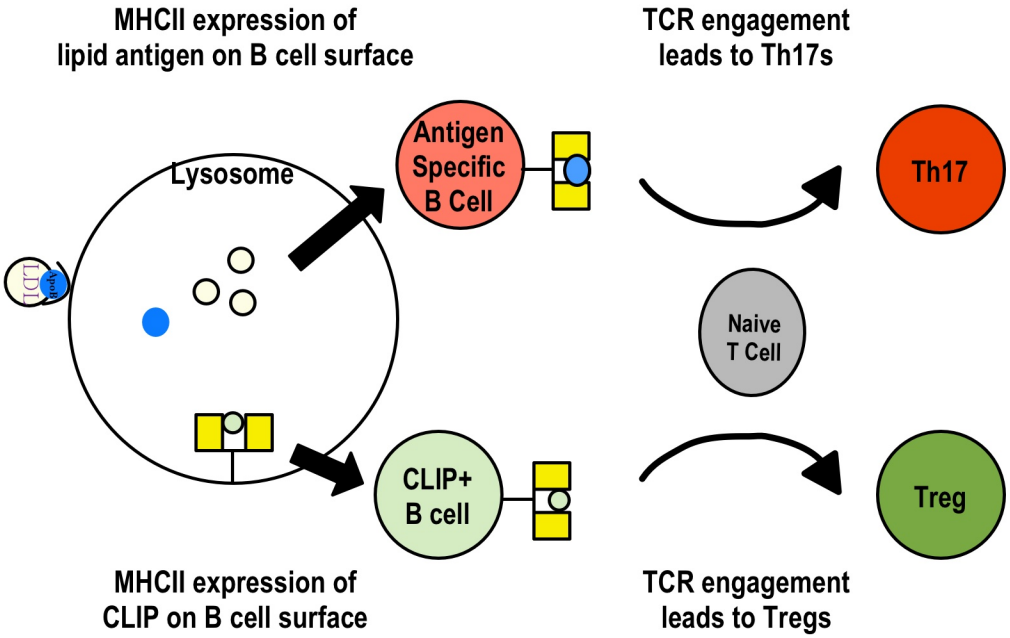

Figure 3. Proposed mechanism

II. INCREASED RISK OF NECROTIZING ENTEROCOLITIS IN GROWTH-RESTRICTED INFANTS BORN TO PRE-ECLAMPTIC MOTHERS*

II.1 Introduction

Maternal health during pregnancy can fate a baby to a lifetime of disease. The development of pre-eclampsia (PE), a syndrome of hypertension and proteinuria that occurs after the 20th week of gestation in 5-8% of pregnancies in the U.S., has, in particular, profound and long-reaching consequences for infant health.[57] Babies born to pre-eclamptic mothers have higher risks of perinatal complications with increased frequency of IUGR, immunosuppression, sepsis, and feeding problems.[58–63] Endocrine, metabolic, nutritional and hematologic diseases continue to plague these children into early adulthood.[64] In an era when the rising expense of medical care is increasingly at the forefront of debate, babies born to pre-eclamptic mothers generally have longer postnatal hospital courses, increased numbers of hospitalizations and higher overall health care costs.[65]

Because PE is the basis for 15% of premature births, diseases of prematurity including retinopathy of prematurity, bronchopulmonary dysplasia, and NEC have all been associated with PE.[59,66–69] However, when compared to age- and weight-matched controls, the presence of PE does not appear to increase the odds of retinopathy of prematurity, bronchopulmonary dysplasia, and NEC or intraventricular hemorrhage and

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* Reprinted with permission from "Maternal pre-eclampsia as a risk factor for necrotizing enterocolitis" by Lena Perger, Dhriti Mukhopadhyay, Luka Komidar, Katie Wiggins-Dohlvik, Mohammad N. Uddin & Madhava Beeram, 2015. The Journal of Maternal-Fetal & Neonatal Medicine, 29:13, 2098-2103, Copyright 2015 by Taylor & Francis. Available online: http://wwww.tandfonline.com/10.3109/14767058.2015.1076386.

may even play a protective role in these disorders due to aggressive protocolized prenatal treatment regimens of the mother.[70–74]

NEC, destruction of the gastrointestinal epithelium with invasive transmural bacterial infection leading to a cascade of local and systemic effects, is a highly morbid disease of newborns that is particularly frequent and severe in premature and growth-restricted infants.[1,4,75,76] Population-based estimates of the incidence of confirmed NEC in the United States are approximately 1 in 1000 live births with a mortality of 1 in 7 for babies hospitalized for NEC.[2] Unlike other diseases of prematurity, an imbalance in the pro-inflammatory response has been shown to be critical to the development of NEC in the newborn.[19] Interestingly, similar derangements in immunity underlie the development of PE in the mother.[77] We hypothesized that maternal PE will be linked to the development of NEC in their newborns through the common pathophysiologic mechanisms at play in both mother and fetus.

The goal for this study was twofold. First, we compared the incidence of NEC in babies born to PE mothers to the incidence of NEC in babies born to non-PE mothers to determine if a pre-eclamptic environment was linked to the disease. Second, we investigated the relative importance of PE and several infant and maternal factors when predicting the development of NEC.

II.2 Patients and Methods

After approval by the Institutional Review Board, the study cohort of all live births between January 2008 and December 2011 was retrospectively constructed using the Scott & White Labor and Delivery database (n=10,078). Inclusion dates were selected

due to availability of an integrated neonatal intensive care unit and hospital electronic medical record system starting in 2008. Exclusion criteria included non-viability of less than 23 weeks estimated gestational age (GA) (n=44) and inaccessible infant medical records (n=41) (Figure 4).

Infant information including method of delivery, gestational age, birth weight, sex, and Apgar scores at 1 and 5 minutes was gathered. Presence of IUGR was identified by classifying babies with birth weights less than the 10th percentile as small-for-gestational age (SGA) in contrast to non-growth-restricted appropriate-for-gestational age babies (AGA).[26] Babies with a diagnosis of all stages of NEC were identified using ICD-9 codes 777.5-777.53. Maternal information, including age, race, and number of pregnancies and deliveries, was also identified and linked to baby information. ICD-9 code 642 for PE was used to define and separate out the study group of pre-eclamptic mothers. In patients with missing individual data points, manual chart review was performed to complete the database.

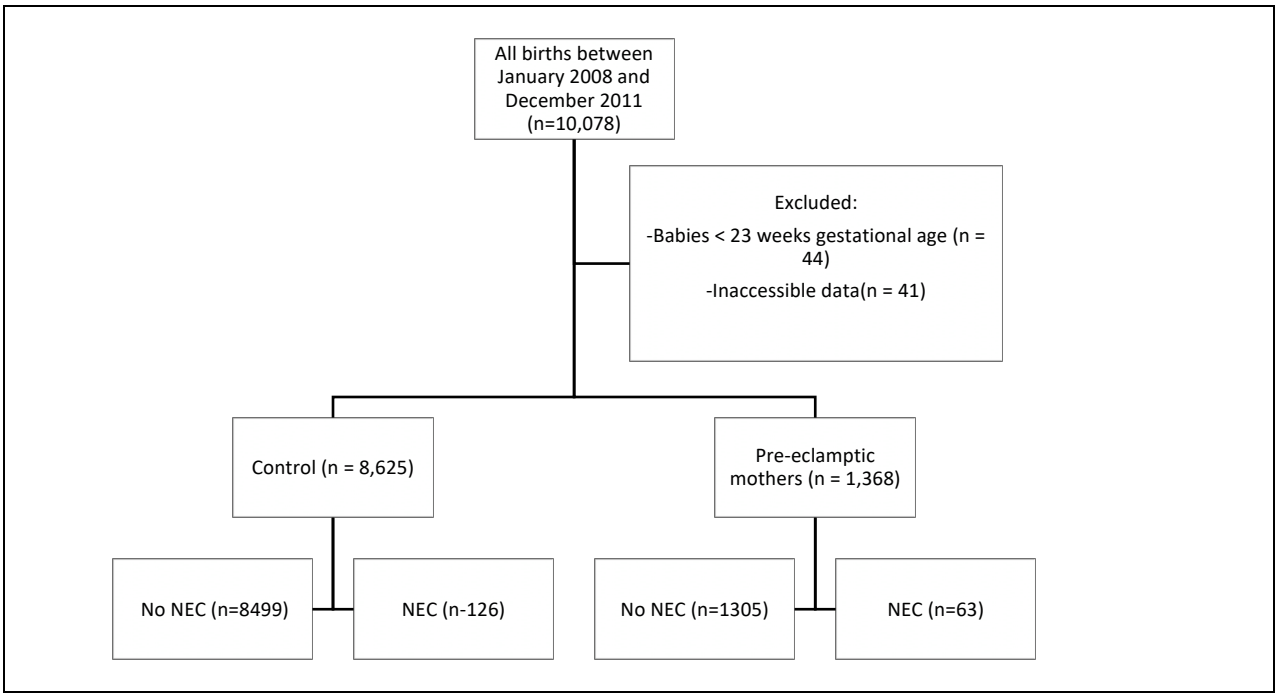


Figure 4. Inclusion criteria of PE mothers and NEC infants

Statistical analysis was done using SPSS 20.0 software. We used non-parametric Mann-Whitney U and Pearson's $\chi 2$ tests to test the differences between the control and the pre-eclamptic group. To estimate effect size, Pearson's correlation coefficient (r) was used for continuous variables and Cramer's correlation coefficient (Cramer's V) for categorical variables. To determine the predictive power of PE, infant, and maternal factors on the incidence of NEC, a hierarchical binary logistic regression was conducted. These three variables were entered into the model in three consecutive blocks. To evaluate the predictive power of PE first as a sole predictor and afterwards in combination with infant and maternal predictors, the presence of PE was the only variable entered into the first block by using the default Enter variable selection method; by using the Enter method, PE stayed in the model regardless of its statistical significance. In the case of infant (block 2) and maternal factors (block 3), the stepwise Forward: Likelihood Ratio method was used to retain only those predictors whose

inclusion resulted in a statistically significant improvement of the model fit. The Wald statistic was used to estimate the relative importance of a certain predictor with a higher Wald statistic translating to higher relative importance.

II.3 Results

First, we characterized the population of infants and mothers analyzed in this study according to significant demographic and clinical factors. Overall infant demographics showed a predominance of babies of Caucasian descent of equal gender distribution born at term with normal birth weight, modeling the semi-rural population served by our institution. Clinical status at birth, reflected by Apgar scores at 1 and 5 minutes, were similar between the two groups. However, detailed analysis showed that the pre-eclamptic group had moderately higher percentages of African-American babies, pre-term births and low-birth weight and growth-restricted infants (Table 1).

Pre-eclamptic mothers were slightly older than control mothers. Both groups had a median of two pregnancies with one birth at the time of gestation of the study population. Multiple gestations and delivery by cesarean section were more prevalent in pre-eclamptic mothers compared to the general population (Table 1).

In the next step, we carried out a hierarchical binary logistic regression to predict NEC by PE, infant, and maternal factors (Table 2). When PE was entered as a sole predictor, it proved to be a statistically significant predictor of NEC; infants born to pre-eclamptic mothers had approximately 3.3 times higher odds of developing NEC. However, when we added the infant factors in the second step, the effect of PE became non-significant, most likely due to the large effects of infant weight and age. It is important to note that the large effects of weight and age also resulted in the exclusion

of gestational size in the model. Adding the third block predictors revealed that maternal age, multiple gestation, and delivery method also significantly contribute to the development of NEC.

Table 1. Infant and maternal demographics

INFANT CHARACTERISTICS	Control (n=8625)	Pre-eclamptic (n=1368)	p-value	Effect size*
Gender (% female)	4229 (49.0)	677 (49.5)	0.75	Cramer's V<0.01
Race			<0.001	Cramer's V=0.08
White	4229 (49.0)	674 (49.3)		
Black	1233 (14.3)	293 (21.4)		
Hispanic	1980 (23.0)	265 (19.4)		
Asian	143 (1.7)	16 (1.2)		
Other	919 (10.7)	103 (7.5)		
Birth weight (g) – median (IQR)	3293 (2920-3617)	2938 (2287-3394)	<0.001	*r*=0.18
Birth weight groups (%)			<0.001	Cramer's V=0.21
Normal (>2500 g)	7693 (89.2)	943 (68.9)		
Low (1500–2500 g)	683 (7.9)	280 (20.5)		
Very Low (1000–1500 g)	111 (1.3)	71 (5.2)		
Extremely Low (<1000)	138 (1.6)	74 (5.4)		
Presence of Intrauterine Growth Restriction (% SGA)	542 (6.3)	140 (10.2)	<0.001	Cramer's V=0.05
Gestational Age (wks) – median (IQR)	39.1 (37.9-39.9)	37.2 (35.0-38.9)	<0.001	*r*=0.25
Preterm births (%)	1248 (14.5)	566 (41.4)	<0.001	Cramer's V=0.24
Apgar at 1 minute – median (IQR)	8.4 (7.6-9.0)	8.0 (6.8-8.8)	<0.001	*r*=0.13
Apgar at 5 minutes – median (IQR)	8.9 (8.4-9.5)	8.8 (8.2-9.4)	<0.001	*r*=0.14
Cases of necrotizing enterocolitis (%)	126 (1.5)	63 (4.6)	<0.001	Cramer's V=0.08
MATERNAL CHARACTERISTICS	Control (n=8471)	Pre-eclamptic (n=1308)	P-value	Effect Size
Mean age (SD)	25.6 (5.6)	26.8 (6.1)	<0.001	*r*=0.07
Gravidity – median (IQR)	2.2 (1.3-3.4)	2.0 (1.2-3.5)	0.14	*r*=0.01
Parity – median (IQR)	0.7 (0.1-1.7)	0.6 (0.0-0.5)	<0.001	*r*=0.04
Number (%) single gestations	8323 (98.3)	1252 (95.7)	<0.001	Cramer's V=0.09
Number (%) delivered vaginally	6396 (74.2)	787 (57.6)	<0.001	Cramer's V=0.13

*Interpretation of effect size: Small (0.10-0.30), Medium (0.30-0.50), Large (>0.50)
IQR – interquartile range
SGA – small for gestational age
SD – standard deviation

Table 2. Predictors of NEC development

Predictor[#]	R^2	Model (df)	Wald	Odds ratio	95% CI for OR
Block 1	.028	38.44 (1)***			
Constant			1775.31	0.01	
Pre-eclampsia			44.90	3.27***	2.31–4.62
Block 2[a]	.448	633.76 (4)***			
Constant			10.14	50.91	
Pre-eclampsia (present)			0.35	1.14	0.75–1.73
Infant age (weeks)			5.81	0.88*	0.79–0.98
Infant weight (100 g)			16.36	0.88***	0.83–0.94
Gestational status			6.23	2.56*	1.23-5.55
Block 3[b]	.463	656.56 (7)***			
Constant			9.49	70.51	
Pre-eclampsia			0.01	1.03	0.66–1.59
Infant age (weeks)			7.55	0.86**	0.77–0.96
Infant weight (100 g)			13.34	0.89***	0.84–0.95
Gestational status			6.73	2.7**	1.28-5.88
Mother's age (years)			4.44	0.97*	0.93–0.99
Multiple gestation			7.02	2.16**	1.22–3.82
Delivery method			13.64	2.17***	1.44–3.27

[#]All dichotomous categorical predictors were coded with 0 and 1; the value of 0 indicates the reference value for interpreting the corresponding odds ratio. The reference values were set as follows: PE (absence of PE), gestational status (term), multiple gestation (single), and delivery method (vaginal).
[a]Excluded predictors: Gestational size, Apgar 1, Apgar 5, Infant gender, Infant race.
[b]Excluded predictors: Gestational size, Apgar 1, Apgar 5, Infant gender, Infant race, Gravidity, Parity.
*p<0.05, **p<0.01, ***p<0.001

In sum, we found three infant and three maternal factors that significantly contribute to development of NEC independently if all other measured variables are controlled. For every one week increase in age and every hundred grams increase in weight, the odds of developing NEC decrease by a factor of 0.86 and 0.89, respectively. Babies who were pre-term had a 2.7 (i.e. 1/0.37) times higher odds of NEC than term

babies, babies who were a product of multiple gestation had a 2.16 higher odd of NEC than singletons, and babies delivered by C-section had a 2.17 times higher odd of developing NEC than vaginally delivered babies.

Although PE did not turn out to be a significant predictor of NEC when controlling for infant and maternal factors, we suspected an effect hidden in interactions with the most important predictors. Therefore, we conducted additional analyses in terms of incidence and relative risks (RR) of developing NEC in the presence of PE compared to non-PE controls (Figure 5A-D). This was done separately for groups defined by gestational size, gestational status, multiple gestation, and delivery method and then RRs for each group calculated as a ratio of the incidence of NEC in the PE group (% of NEC infants with PE mothers) to the non-PE group (% of NEC infants with non-PE mothers). These additional analyses revealed important interactions of PE with gestational size, multiple gestation, and method of delivery when predicting the incidence and risk of developing NEC.

In the case of AGA babies, PE had a rather small effect, for babies born to PE mothers were only 2.5 times ($p<0.01$) more likely to develop NEC (Figure 5A). Within the cohort of SGA babies, the incidence of NEC in non-PE babies was 1.5%, similar to the population incidence. However, this was dramatically different from the PE group, which had an incidence of 13.6% ($p<0.001$). SGA babies born to pre-eclamptic mothers were therefore 9.2 times more likely to develop NEC than SGA babies born to non-PE mothers ($p<0.001$).

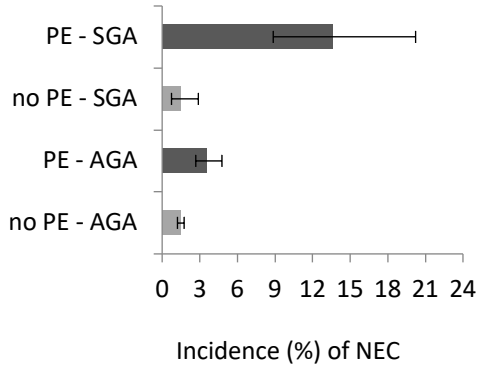
Term babies had an incidence of 0.18% of NEC in non-PE and 0.25% in PE babies. Within the preterm population, 9.0% of control babies and 10.8% of

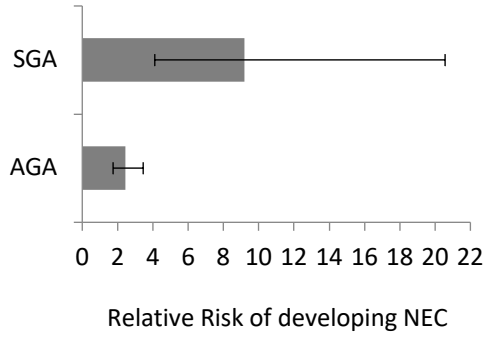
PE babies developed NEC (p=0.25). Pre-term birth clearly represents a risk factor for developing NEC (Figure 5B). However, the RRs revealed that PE did not appear to play an important role in term or pre-term babies, because ratios were quite small and did not significantly differ from 1.00 (P>0.05).

Other significant factors found were that babies who were the product of a multiple gestation were more likely to develop NEC than single born babies (Figure 5C). Interestingly, the presence of PE was associated with an increased risk of NEC in singletons, yet not in multiple gestations; multiple gestation babies born to PE mothers were only 1.1 times (P>0.05) more likely to develop NEC, whereas single born babies born to PE mothers were 3.5 times (P<0.001) more likely to develop NEC than those born to non-PE mothers. Additionally, babies delivered by C-section who were born to PE mothers were 3.1 times (P<0.01) more likely to develop NEC than their non-PE counterparts (Figure 5D). In the case of vaginally delivered babies, however, PE did not significantly increase the risk of developing NEC.

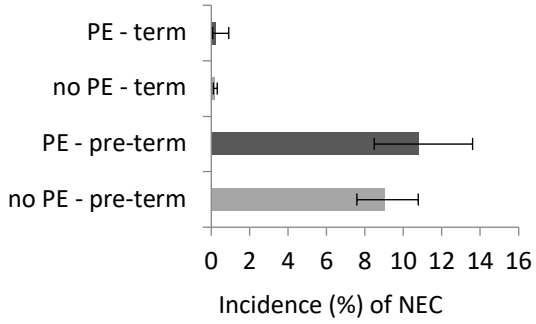
Figure 5. Incidence of NEC. Data is stratified by presence of PE and infant (gestational size and status) and maternal (multiple gestation, method of delivery) factors. Relative risks in the right column were calculated as a ratio between incidence of NEC in the pre-eclampsia group (PE) and incidence of NEC in the non-PE group. All whiskers represent 95% confidence intervals for incidence of NEC (left column graphs) and for relative risks (right column graphs). AGA = appropriate for gestational age birth weight > 10th percentile; SGA = small for gestational age < 10th percentile; C/S = cesarean section; VAG = spontaneous vaginal delivery.

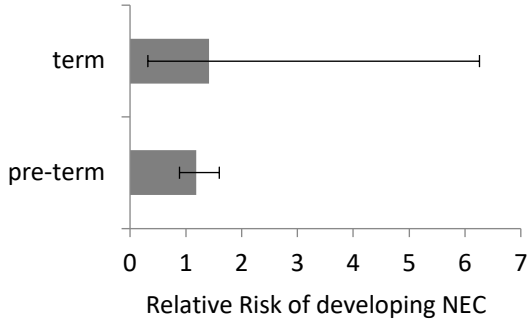
A - Gestational size



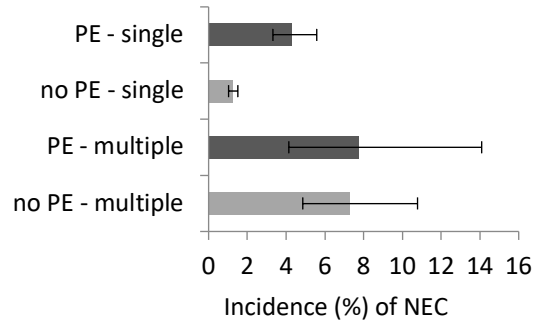


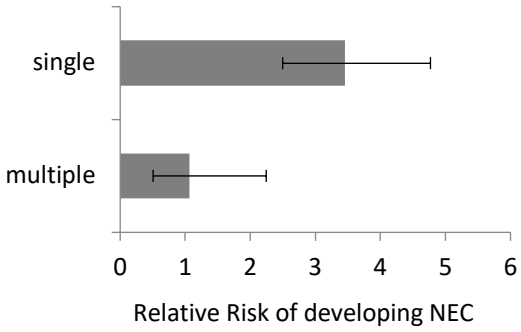
B - Gestational status



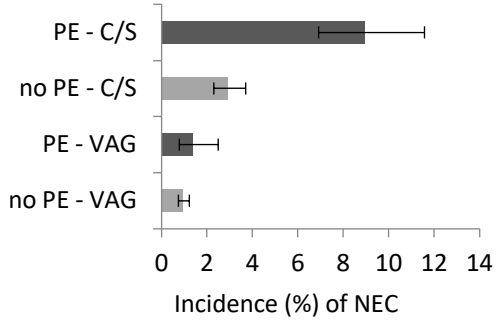


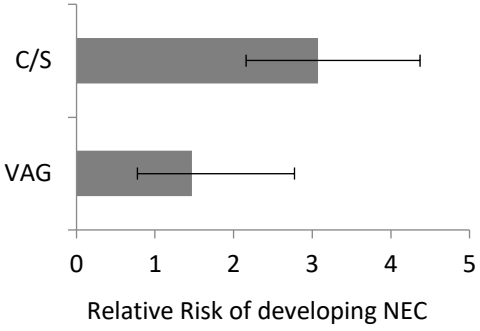
C - Multiple gestation





D - Method of delivery





II.4 Summary

We have shown that, overall, a baby born in a pre-eclamptic environment has a threefold increase in the odds of developing NEC. Though the power of maternal PE in predicting infant NEC is dampened by the much stronger and already well-characterized infant characteristics of low birth weight and prematurity, our subgroup analyses show dramatic associations that have never been described previously. In the subset of babies who are born SGA, a comparative birth weight measure that signifies intrauterine growth restriction, a pre-eclamptic intrauterine environment imparts a nine-fold higher risk ratio of NEC development than similar babies born to non-pre-eclamptic mothers. Interestingly, a similar pattern is not found in premature infants; babies born early secondary to PE have no significant increased risk of NEC compared to those born pre-term for other causes.

Importantly, other maternal characteristics including multiple gestation and mode of delivery have significant associations with the development of NEC as well, which have not been previously described or examined in the literature. After controlling for the infant factors, we found a 2.16 higher odds of NEC in babies who were a product of multiple gestation and 2.17 higher odds of babies delivered by cesarean section. Because this study was not designed to investigate these associations, further research is needed to explore these findings.

As with any retrospective database review, there are two significant weaknesses in this study. First, because data was not collected prospectively, findings suggest associations but not causality. Second, though some of the inherent drawbacks of database review were mitigated by manual correction, data collected by mining for ICD

(International Classification of Diseases) codes is only as strong as the accuracy with which codes were entered in the first place. Additionally, because this is a single center study in a semi-rural setting, there may be a component of selection bias with some of the associations being population-dependent and not translatable to the general population.

Our study had an incidence of Bell's stage II and III NEC (39/9993 ≈ 4/1000 live births) similar to previously reported population-based findings.[1] Other strengths of the study are that our results do accurately model the disease and patient characteristics of our stable semi-rural patient population due to the cohesiveness of our hospital system. Unlike other population-based studies, our study captures all patients who were suspected to have developed NEC, thereby properly capturing babies with NEC responsive to medical treatment without overt radiographic signs (Bell's Stage I). Finally, because of the comprehensiveness of our integrated electronic medical record, we were able to manually correct missing or aberrant data in a manner that most other retrospective database studies cannot.

PE and NEC are complex heterogeneous disease states and their etiologies are incompletely understood. However, our hypothesis stems from and our results can in part be explained by a shared pathophysiology driven by the placenta that leads to a poorly regulated pro-inflammatory state in both processes.

The current paradigm is that the underlying vascular insufficiency of the placenta drives both PE and NEC. In PE, multifactorial aberrations in the normal mechanisms of pregnancy initiation and maintenance lead to its development in two stages. First, early in pregnancy, aberrant spiral artery remodeling by the cytotrophoblast leads to poor

placental blood supply. Poor perfusion precipitates an inflammatory response by the placenta, which, in the susceptible host leads to the second stage, a systemic response that manifests in the classic findings of PE.[62,78] Current research supports that placental hypoxia causes an upregulation of the transcription factor hypoxia-inducible factor 1α (HIF-1α) in maternal circulation.[77,79,80] HIF-1α is critical for tipping the balance of differentiation of naïve CD4+ TH cells toward a pro-inflammatory TH17 phenotype over the anti-inflammatory regulatory T cell (Treg) phenotype.[81] PE has been shown to have a TH17–predominant CD4+ T cell lymphocyte pool compared to the significant Treg expansion found in normal pregnancy.[77,82–84,84–86]

The placenta, which serves as the sole source of oxygen and nutrient delivery to the infant, is a critical organ for fetal growth and development.[87,88] Abnormalities in placental development, necessary for PE, also underlie pathologic IUGR.[26] Just as in the maternal circulation, placental hypoxia causes HIF-1α up-regulation, and similar processes likely occur *in utero* during fetal development. In fact, babies who develop necrotizing enterocolitis have been found to have a relative dearth of intestinal Tregs.[19,89] SGA infants have also been shown to have lower proportions of circulating Tregs immediately at birth prior to encounter of foreign antigen.[90] This suggests that babies who are exposed to intrauterine hypoxia, manifested as growth restriction, are immunologically primed to mount a pro-inflammatory response due to the same transcriptional changes that have been found in the mother.

A different paradigm may be that the underlying aberration is instead secondary to altered lipid metabolism driven by the fetus rather than vascular insufficiency and hypoxia. PE is known to lead to increased cardiovascular risk later in life, though the

cause for this remains unknown. Multiple studies have shown measurable alterations in serum fatty acid binding proteins, specifically fatty acid binding protein 4, in women with PE, though levels are not predictive of severity of PE.[91–93] However, severe PE, which manifests as the HELLP syndrome (hemolysis, elevated liver enzymes, low platelets), and other pregnancy-related liver disease, including acute fatty liver of pregnancy, appear to be related to a defect in fetal fatty-acid oxidation secondary to a deficiency in long-chain 3-hydroxyacyl-coenzyme A (CoA) dehydrogenase, a portion of the mitochondrial trifunctional protein necessary for fatty acid beta-oxidation.[94–96] Furthermore, placental proteins are encoded by the fetal genome, and genetic polymorphisms in lipoprotein metabolism of the fetus have been shown to influence maternal lipid profiles during pregnancy independent of maternal genetics.[97] Women with PE have been found to have lower HDL levels and more atherogenic LDL particles.[98] Severe early-onset PE (before 20 weeks' gestation) has been linked to hypertriglyceridemic dyslipidemia and significantly lower levels of ApoB-100.[99] Underlying defects in fetal lipid metabolism that drive modification of maternal lipid profiles by the placenta not only explain the disproportionate increase in IUGR secondary to PE, but also the increased risk of maternal cardiovascular disease later in life despite no differences in lipid profiles or hypertension after delivery.

We postulate a two-hit model in which babies who develop IUGR are born immunologically primed to respond with an overwhelmingly pro-inflammatory phenotype due to genetic polymorphisms in lipid metabolism, and require a second hit from the external environment that allows the development of NEC. Further clinical and basic

studies are needed to elucidate the prenatal interventions that may modify these risks and provide targets for future diagnosis and therapy.

III. INTRAUTERINE GROWTH RESTRICTION AND PREMATURITY INFLUENCE REGULATORY T CELL DEVELOPMENT IN NEWBORNS**

III.1 Introduction

The immune system is a complex network of cellular and humoral checks and balances. CD4+ regulatory T cells (Tregs), characterized by the simultaneous expression of an IL-2 receptor α-chain (CD25) and transcription factor forkhead box P3 (FoxP3), are a potent segment of the negative regulatory arm of immune system in their physiologic role in suppressing adaptive immune responses.[100,101] Their importance as effectors of self-tolerance and regulators of overzealous immune activation to foreign antigen is well characterized for primary diseases of immunity such as autoimmunity and allergy.[102,103] Yet, much remains unknown about their diverse role in a variety of physiologic and pathologic situations.

Specifically, Tregs have been implicated in the development of NEC.[104] However, baseline values of circulating Tregs in infants at birth have not been established. Their dynamics in the early post-natal period and in newborn health and disease remain to be characterized. Tregs are currently defined as CD3+/CD4+/FoxP3^{high}/CD25^{high} cells by flow cytometry, a method of analysis that enables precise counting of blood cells using intra- and extracellular markers. The first objective of this study, therefore, was to establish normal values of Treg frequency and activity in cord blood of healthy newborns using contemporary criteria.

Local and systemic injury in NEC is mediated by an overwhelming pro-inflammatory response of the immune system. Lack of suppression of this response may be attributed to decreased numbers or activity of Tregs. Previous studies utilizing flow cytometric analysis of Tregs suggest that numbers of CD25+/CD4+ T cells and expression of FoxP3 are both inversely correlated with gestational age.[104] FoxP3 expression also appears to be attenuated in SGA infants compared to age-matched controls.[105] Because NEC disproportionately affects babies who are born prematurely or with IUGR in frequency and severity, our secondary objective was to determine whether such incomplete or restricted development during the fetal period translated to decreases in Treg populations or activity prior to development of disease.[106]

III.2 Methods/Materials

Selection of Subjects

A prospective study was conducted between June 2012 and January 2013 with IRB approval (protocol 710737). All live births were included in the study with the exception of babies born to mothers with active infection or transfers from outside hospitals due to cord blood unavailability. Historical data was not available to power the study but a projected sample size of ten per study group was determined through feasibility analysis. The study was closed once this sample size was achieved in each group.

Patients were assigned by gestational age and birth weight according to standard AAFP growth curves into the following groups:[107]

1. Term (>37 weeks) and appropriate for gestational age (AGA > 10th percentile on the growth curve),

2. Term and small for gestational age (SGA ≤ 10th percentile),

3. Pre-term (<37 weeks EGA) AGA

a. Late (born between 33-36 weeks),

b. Early (born between 23-32 weeks),

4. Pre-term SGA.

Samples

Cord blood routinely collected in EDTA-coated vacutainers (BD, Franklin Lakes, NJ) following birth was obtained from the institutional blood bank within three days of birth. Two hundred and ten total samples were collected and processed until all study groups were filled. Two to three mL of blood per patient was processed to isolate cord blood mononuclear cells (CBMCs) using cellular density gradient centrifugation according to the manufacturer's instructions (Lymphoprep, Accurate Chemicals, Westbury, NY). Samples processed not according to protocol or with technical issues were excluded from analysis.

Flow Cytometry

Isolated CBMCs were stained for cell-surface markers using fluorescence-conjugated primary mouse anti-human antibodies, anti-CD3 (Pacific Blue), anti-CD4 (PE-Cy7), and anti-CD25 (APC) as well as intracellular anti-FoxP3 (PE) (all antibodies from BD Biosciences, San Jose, CA), with a PE-anti-mouse/rat FoxP3 staining set (e-

Biosciences, San Diego, CA). Cells were analyzed using flow cytometry (BD FACS Canto II Flow Cytometer, Franklin Lakes, NJ).

Statistical Analysis

Samples were analyzed by comparison to isotype controls. Using standard gating for all samples, we determined CD4+ T cell numbers (defined as CD3+, CD4+) and within this population, Tregs were counted (defined as CD25high, FoxP3high). Due to significant variability in number of cells per sample, final analyses were performed by determining cell ratios rather than absolute numbers. Flow cytometry data was used to calculate the percentage of CD4+ T-cells of all CBMCs and the percentage of Tregs of all CD4+ T-cells. Mean fluorescence intensity (MFI) was calculated as geometric mean of FoxP3 in CD4+ T-cell populations per sample (FlowJo 10.0.0 software), which measures the expression of FoxP3 and has been validated in the literature as proportionate to Treg suppressive ability.[101] In order to normalize suppressive ability, we defined an index for the comparison of MFI per Treg (total FoxP3 MFI/absolute number of Treg) as the suppressive index (SI) of each sample to determine suppressive capability of individual Tregs in each patient.

GraphPad Prism 6.0c software was utilized for statistical analysis using Mann Whitney test, Kruskall-Wallis and linear regression in order to compare groups with non-parametric distributions. Significance was defined as $p<0.05$.

III.3 Results

Demographics

Study populations were similar to the control group in terms of gender and ethnic distributions as well as method of birth (Table 3). Median Apgar scores at 1 and 5 minutes were significantly lower with younger gestational age (9/9 in term infants, 8/9 in late pre-term and 7/7 in early pre-term). SGA babies were significantly more often born to nulliparous women than AGA babies, regardless of gestational age (nulliparous: 28% controls, 33% late pre-term, 38% early pre-term vs. 50% term SGA, 80% pre-term SGA).

Table 3. Characteristics of study population

	TERM		**PRE-TERM**		
	Term AGA n=80	*Term SGA n=18*	*Late Pre-term AGA (33-36 weeks) n=33*	*Early Pre-term AGA (23-32 weeks) n=13*	*Pre-term SGA n=10*
Gestational Age	39.2 ± 1.0	38.9 ± 1.2	35.3 ± 1.0	28.9 ± 2.4	32.0 ± 3.7
Birth Weight	3452 ± 401	2495 ± 284	2576 ± 440	1278 ± 512	1388 ± 485
Gender (%male)	41%	44%	61%	62%	40%
Mode of Delivery (%SVD)	46%	56%	45%	85%	60%
Ethnicity					
% Black	15%	28%	21%	23%	10%
% White	50%	39%	52%	62%	60%
% Hispanic	28%	17%	9%	15%	20%
Apgar at 1 minute	8.5 ± 0.7	8.3 ± 1.5	7.7 ± 1.8	6.2 ± 2.0	8.3 ± 0.9
Apgar at 5 minutes	9.1 ± 0.3	9.0 ± 0.5	8.7 ± 1.0	6.7 ± 1.6	8.9 ± 0.4
Maternal gravidity (% primagravida)	21%	50%	33%	31%	80%
Maternal parity (%nulliparous)	28%	50%	33%	38%	80%

Mode of Delivery

Comparisons of babies born by uncomplicated spontaneous vaginal delivery (SVD) and elective Cesarean section (C-section) were conducted in the term AGA group. No significant differences were found in CD4+ T-lymphocyte or Treg percentage, MFI or SI between the two groups. The mode of delivery did not appear to have a significant immediate effect on regulatory T-cell populations or activity in newborn peripheral blood, so babies born by both methods were included as the control group for all subsequent analyses (Table 4).

Table 4. Effect of mode of delivery

	Spontaneous Vaginal Delivery (n=40)	**Cesarean Section (n=29)**	**p-value**
%CD4 T cells of total cells counted	13 ± 11	10 ± 6	0.9542
% Treg of CD4+ T cells	7 ± 4	9 ± 6	0.1166
Treg mean fluorescence intensity	4603 ± 3165	4011 ± 2147	0.2177
Treg suppressive index	177 ± 272	127 ± 272	0.9398

Intrauterine Growth Restriction

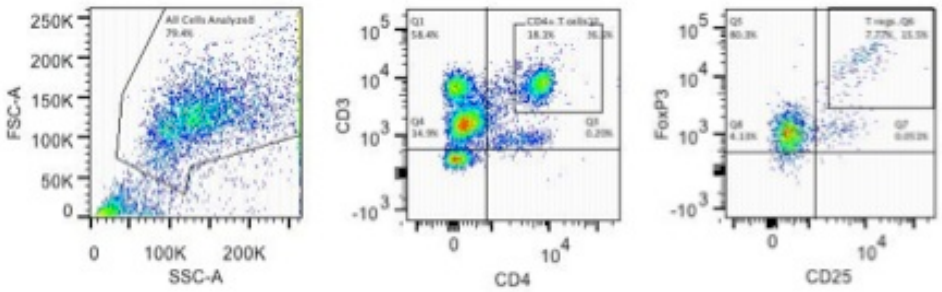
Term appropriate-for-gestational-age babies (AGA, n=80) were compared to term small-for-gestational-age babies (SGA, less than 10th percentile birth weight, n=18) to determine the relationship between IUGR and regulatory T cells. Results are summarized in Figure 6. Median Treg percentage of CD4+ T cells was found to be significantly lower in the SGA group (7% in AGA vs. 6% in SGA, p=0.0121). The SGA group also had FoxP3 MFI that was significantly lower than in AGA babies (3602 in

AGA vs. 2686 in SGA, p=0.0021). However, when the Treg suppressive index of the two groups was compared (67 in AGA vs. 81 in SGA, p=0.6905), no significant difference was found. Together, this suggests a significant decrease in normally functioning Tregs secondary to IUGR. These differences, though seemingly small, are likely clinically relevant due to the small number of Tregs necessary in the circulation for function.

Gestational Age

AGA babies born at 37 weeks estimated gestational age (EGA) or later (term, n=80) were compared to AGA babies born between 33 and 36 weeks EGA (late pre-term, n=33) as well as between 23 and 32 weeks EGA (early pre-term, n=13) to investigate the changes in Tregs through gestation. Results are summarized in Table 5 and Figure 6. A significant decline in median CD4+T cell percentages was observed early in the third trimester, between the early pre-term and late pre-term groups (16% in early vs. 12% in late pre-term, p=0.0218), then remained steady through the completion of gestation (10% in the term group). Treg numbers also declined through gestation, but this occurred later in the third trimester, upon completion of gestation. Early pre-term and late pre-term groups had no significant difference in Treg percentages (10% in early and 11% in late) but there was a significant decline in babies who were born at

A.



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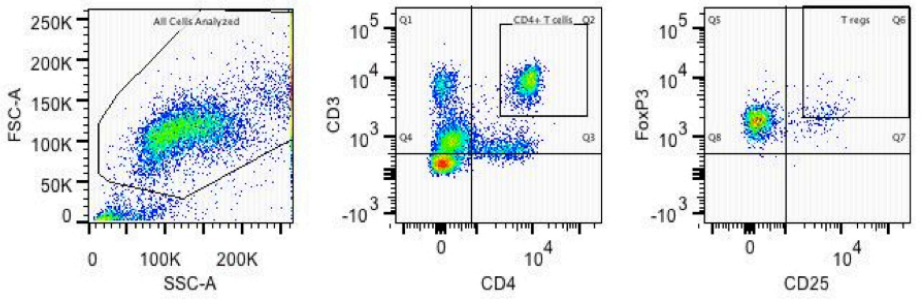

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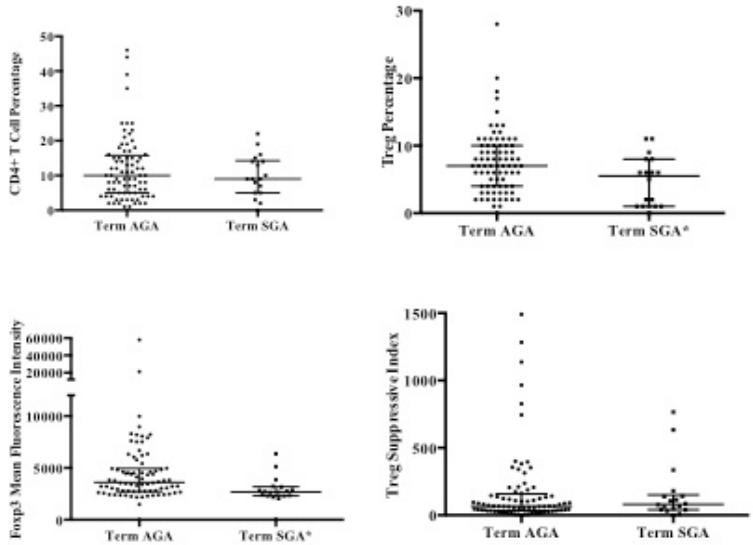


Figure 6. CD4+ and Treg population characteristics according to birth weight in term infants. A. Representative samples of flow cytometric analysis of term appropriate for gestational age infant showing all cells gated in first panel, CD3+/CD4+ cells gated in second panel representing CD4+ T cells and FoxP3high/CD25high cells gated in third panel representing Tregs. B. Flow cytometric analysis for term small for gestational age infant. C. Appropriate for gestational age (AGA) are compared to small for gestational age (SGA) infants with median and interquartile range denoted by long and short horizontal bars, respectively. Significance defined as $p<0.05$ marked with an asterisk*.

Table 5. Changes in CD4+ and Treg populations through gestation

	Early Pre-term (23 – 32 weeks) n = 13	Late Pre-term (33 – 36 weeks) n = 33	Term (37+ weeks) n = 80	p-value
%CD4 T cells of total cells counted	20 ± 11	12 ± 8	12 ± 9	0.0218
% Treg of CD4+ T cells	10 ± 4	11 ± 6	8 ± 5	0.0025
Treg mean fluorescence intensity	4720 ± 1753	4879 ± 2878	5054 ± 6586	0.4332
Treg suppressive index	56 ± 72	76 ± 110	173 ± 285	0.0037

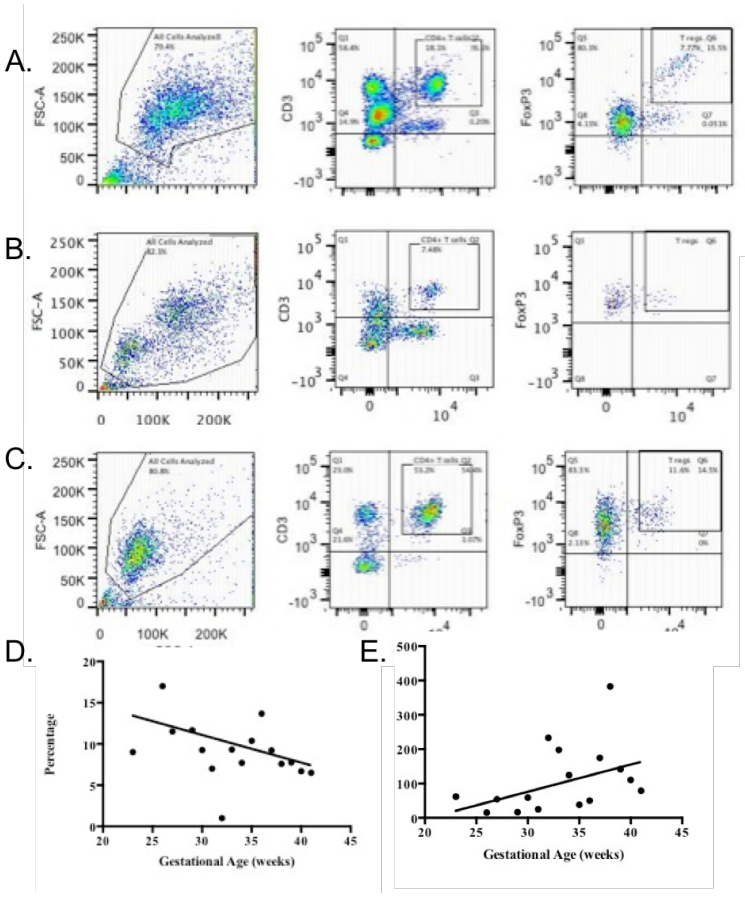
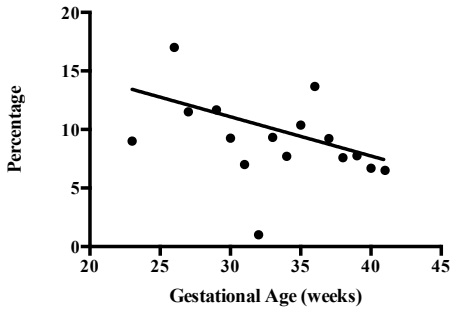


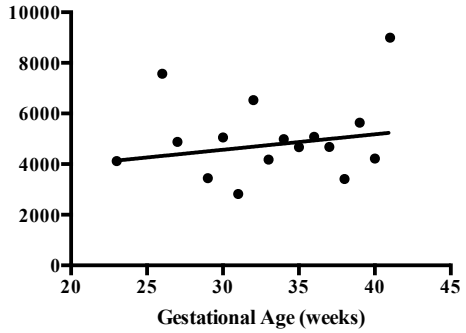
Figure 7. Treg characteristics through gestation. A. Representative samples of flow cytometric analysis of term appropriate for gestational age infant showing all cells gated in first panel, CD3+/CD4+ cells gated in second panel representing CD4+ T cells and FoxP3high/CD25high cells gated in third panel representing Tregs. B. Flow cytometry for late pre-term appropriate for gestational age infant (33 – 36 weeks). C. Flow cytometry for early pre-term appropriate for gestational age (23 – 32 weeks). D. Linear regression shows a gradual decrease in Treg proportions from 23 weeks gestational age until term (r = -0.222). E. Concurrently, there is an increase in the Treg suppressive index (r = 0.119).

Though MFI remained steady throughout gestation (4720±1753 in early pre-term, 4879±2878 in late pre-term, and 5054±6586 in term, p=0.4332), suppressive index rose steadily through gestation, which was statistically significant (56±72 in early pre-term vs. 76±110 in late pre-term vs. 173±285 in term, p=0.0037), as shown in Figures 7 and 8. This data may suggest attenuation of Treg numbers due to clonal selection of more powerfully suppressive Treg subsets through fetal maturation.

a. T_{reg} percentage through gestation



b. T_{reg} mean fluorescence intensity through gestation



c. T_{reg} suppressive index through gestation

Figure 8. Decline in Tregs through maturation with accompanying increase in MFI and SI.

III.4 Summary

The interplay of intrinsic and environmental factors in a developing infant is so complex that no single insult has yet been identified that reproducibly leads to the development of NEC. Infant characteristics such as prematurity and growth restriction, as well as environmental factors like microbial colonization and formula feeding have all been implicated as risk factors. It is clear from the extensive body of literature that a multitude of derangements at any point during uterine life can predispose a baby to this potentially devastating disease.[108–111] The stage for its development is likely set prenatally in a genetically susceptible infant who then sustains a postnatal environmental insult. Bacterial translocation through damaged epithelium combined with a pro-inflammatory immune state leads to intestinal necrosis, systemic sepsis and the short- and long-term manifestations of NEC.[106] An overwhelming systemic and local immune response is an important etiologic factor in its pathogenesis and the role of Tregs as powerful regulators of the immune system needs to be defined in order to target them as a potential prophylactic or therapeutic option in NEC.[112–114]

This study thus establishes how Tregs, a preventive arm of adaptive immunity, are affected by the two most well-characterized risk factors for NEC, growth restriction and prematurity, immediately at birth prior to environmental influences.[107,111,115] Tregs have been shown to be critical for protection against intestinal ischemic and inflammatory injury.[27,116] IUGR is associated with placental insufficiency and fetal hypoxia, which likely lead to hypoxic conditions shown to affect the development of Treg populations.[19,117,118] Given that epithelial ischemia is part of the cascade that ultimately

leads to NEC, a decrease in numbers or function of Tregs not only locally but systemically may be a contributing factor.

Previous studies have demonstrated a decreased ratio of Tregs to effector CD4+ cells locally in the intestinal tissue of premature newborns with NEC.[104] Systemically, it has been suggested that Foxp3 expression decreases with decreasing birth weight and increasing gestational age.[101,119] Our study further qualified previous findings by definition of the Treg suppressive index. Using this measurement, we showed that despite decreased percentages of circulating Tregs in the cord blood, growth-restricted babies actually had intact individual Foxp3 expression. This suggests that an intrauterine insult that results in growth-restriction may also result in an immune phenotype skewed towards a pro-inflammatory state; Tregs therefore remain functional but are fewer in number. In contrast, premature babies showed an increased percentage of Tregs in their CD4+ T-cell population, resulting in the appearance of intact Foxp3 expression. However, suppressive indices were lower in younger infants and actually increased through gestation, suggesting a physiologic clonal deletion of less functional Tregs through gestation, preparing the infant for interaction with the external environment. Through these different mechanisms, Treg numbers or function may play a role in the multifactorial etiology of NEC.

Establishment of normal baseline values of Tregs in human babies and how they are quantitatively and qualitatively affected by prematurity and growth restriction lays the groundwork for future research in diseases of premature and IUGR infants, especially necrotizing enterocolitis. Linking the findings of Treg populations and dynamics in peripheral blood and how they change in the subset of babies who go on to

develop NEC has exciting implications for risk-stratification, diagnosis and even therapy. Regulatory T cells remain an exciting branch of immunology research, but much remains to be discovered with respect to their role in diseases of prematurity and how they are influenced by the combination of baby, mother and environment.

IV. LIPID METABOLISM INFLUENCES TREG PROLIFERATION

IV.1 Introduction

In recent years, the field of immunometabolism has begun to explore the importance of metabolic strategy and metabolic byproducts on immune profiles. Within the CD4+ T cell compartment, quiescent and memory T cells and anti-inflammatory Tregs appear to utilize different substrates for mitochondrial functions compared to rapidly proliferating, pro-inflammatory conventional T cells (Tcons), which include IL-17-producing Th17 cells, Th1 and Th2 cells. This difference rests keenly on the dichotomy between fat and glucose metabolism.[27,120–123] Memory cells and Tregs appear more dependent on oxidative phosphorylation and exogenous lipid utilization, including fatty acids (FA).[26,124,125] Meanwhile, Tcons also utilize oxidative phosphorylation but are primarily dependent on upregulation of glycolytic pathways through mammalian target of rapamycin (mTOR)-dependent pathways.[27–29]

Nutrient availability and composition therefore have a significant impact on immune profile and disease progression as well. Examination of the effect of various lipid components on gut mucosal immunity has provided key insights into this process. Dietary supplementation with short chain FA induces colonic Tregs and ameliorates experimental colitis.[30–34] Medium chain FA supplementation appears to attenuate colitis.[36–38] Meanwhile, dietary supplementation with long-chain FA appear to induce gut inflammation, including treatment with the polyunsaturated omega-3 docosahexanoic acid that increases FoxP3 expression while at the same time attenuating Treg proliferation and inhibitory capacity.[39,40] Ma et al. have shown that a

high-fat diet leads to Treg apoptosis in the liver without a demonstrable effect on splenic Tregs.[54]

Systemically, lipid trafficking largely occurs via complex lipoprotein particles that consist of triglycerides, cholesterols, and surface apolipoproteins. These include chylomicrons that transport from the gut to the liver, VLDL, intermediate-density lipoprotein (IDL), and LDL that delivers triglycerides and cholesterol to extrahepatic tissues, and HDL that traffics cholesterol back to the liver for excretion. The effect of lipoproteins on T cells has mainly been explored in relation to atherosclerosis and cardiovascular disease. LDL has been demonstrated to play a role in Th17-dependent inflammation in atherosclerosis.[48–52] HDL, conversely, appears to promote a Treg-dependent reduction in inflammation.[51,53] Clinically, the acute phase response of inflammation affects cholesterol levels by increasing serum triglycerides and decreasing HDL levels.[126–129] Systemic inflammation has also been shown to decrease serum LDL, although enteric inflammation caused by celiac disease, *H. pylori, Vibrio cholera* and derangements in the microbiome in psoriatic arthritis, appears to cause the opposite response.[130–137]

Together, these observations suggest that the serum lipid balance between available FA and the complex lipoproteins LDL and HDL may play a direct role in the choice between the anti-inflammatory Treg and pro-inflammatory Tcon phenotype. Thereby, the lipid balance is capable of priming the immune system towards exaggerated inflammation or autoimmunity. We hypothesized that genetic dysmorphisms in, or blockade of, mitochondrial FA oxidation would lead to impaired Treg proliferation and conversely inhibition of high rate glycolysis would cause the

reverse. Furthermore, we expected that supplementation of complex lipoproteins in the form of LDL would cause a pro-inflammatory IL-17-predominant response, while supplementation with HDL would affect proliferation of suppressive Tregs.

IV.2 Materials and Methods

Mice

BALB/cByJ (BALB/c) mice, which express a deficiency in short-chain acyl-CoA dehydrogenase (SCAD) needed for FA β-oxidation, and C57BL/6J (B6) mice used as controls, 3-6 months of age, were purchased from The Jackson Laboratory (stock #001026) and maintained at the Baylor Scott & White/Texas A&M animal facility, Temple, TX.[138] All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee.

Cell Isolation and Medium

Splenocytes were isolated from 3-4 pooled spleens using a 40 μm nylon strainer. Red cells were lysed using either Gey's or ACK lysing buffer (ThermoFisher Scientific).[139] Cells were washed twice in phosphate buffered saline and suspended in RPMI 1640 medium (Gibco) supplemented with 5% fetal bovine serum for plating at concentrations of 0.5 – 1.0 x 10^6 live cells/mL.

Metabolic Reagents

Etomoxir (Sigma Aldrich), a malonyl-CoA analog that blocks carnitine palmitoyl transferase in FA oxidation, was used at doses of up to 100 mM.[140] 2-deoxyglucose (2DG) (Sigma Aldrich), an inhibitor of high rate glycolysis, was used at a 4 mM

concentration.[141,142] Purified human plasma LDL and HDL (>95% SDS-PAGE) were purchased from Lee Biosciences and used at concentrations of up to 500 mg/dL to mimic levels measured in serum.

T Cell Activation

To mimic T cell receptor (TCR) recognition of antigen and MHC and co-stimulation, the requirements for T cell activation, cells were activated with 1 μg/mL of purified anti-CD3ε antibody (clone 145.2C11, eBioscience) and 1 μg/mL purified anti-CD28 antibody (eBioscience) at 37° for 0-48h as specified.

Flow Cytometry Analysis for Surface and Intracellular Markers

Eight-color flow cytometry was performed using the BD FacsCanto II instrument and Flow Jo software (TreeStar). The following antibodies were used: rat anti-mouse FITC-CD4, PE-Cy7-CD8a, APC-CD25, PE-Cy7-CD25, PacBlu-CD4 (BioLegend), PE-FoxP3, PerCP Cy5.5-FoxP3, APC-IL-17 (eBioscience), and APC-Cy7-CD3e (BD). Cells were permeabilized using an anti-mouse/rat FoxP3 staining set (eBioscience) for intracellular staining of IL-17 or Foxp3 when indicated. Mitochondrial membrane potential (MMP) was measured using MitoTracker Red (ThermoFisher Scientific).

Statistical Analysis

Parametric tests, including Student's t test for two comparisons and two-way analysis of variance (ANOVA) with Dunnett's for multiple comparisons, were used to calculate differences between means. Chi square was used to calculate differences in

frequencies in pooled data between control and treatment groups using Prism 7.0d (GraphPad Software). Statistical significance was set at $p < 0.05$.

IV.3 Results

Strain-specific Genetic Differences in Metabolism affect Treg Proliferation upon Antigen Encounter

To investigate how genetic differences in lipid metabolism affect the T cell response to antigen, we performed experiments comparing B6 and BALB/c splenocytes, due to previously described genetic differences in β-oxidation in the BALB/c mouse.[138] BALB/c splenocytes had significantly higher CD8+ and CD4+T lymphocyte frequencies prior to TCR activation. TCR stimulation resulted in a four-fold increase in Treg numbers, which persisted through 48 hours (Figure 9). Without stimulation, Tregs were close to undetectable by 48 hours. Concurrent measurement of MMP showed a higher proportion of MMP^{high} cells, supporting a heavier dependence on glycolysis. On the other hand, though B6 splenocytes had lower frequencies of all T cells, a 20-fold increase in Tregs was observed at 24 hours with TCR activation. By 48 hours, however, Treg numbers had decreased to a similar proportion as BALB/c (four-fold increase from baseline).

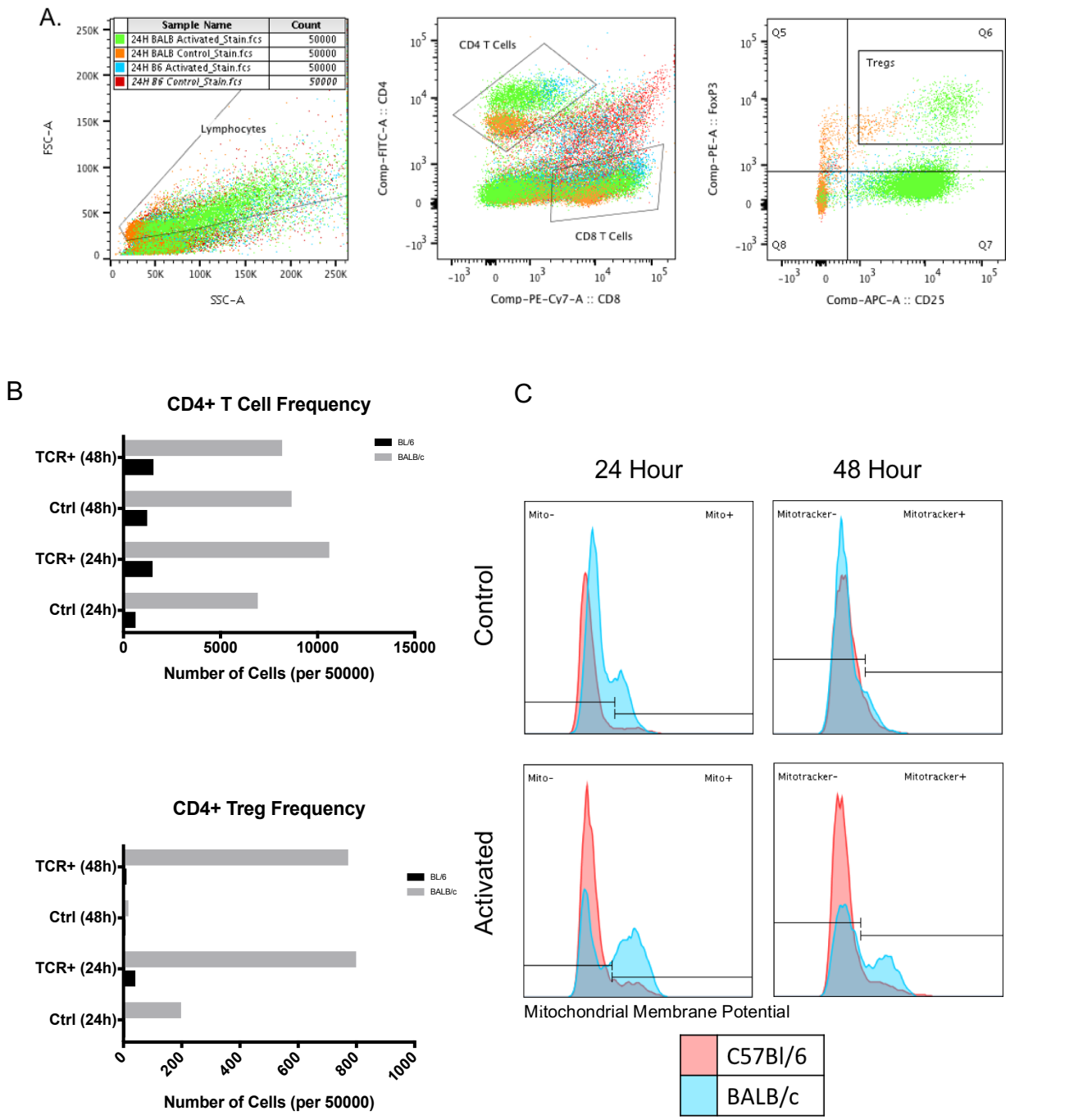


Figure 9. Analysis of CD4+ lymphocytes, CD4+ Tregs and CD8+ T cells after TCR activation. A. Representative flow cytometry for B6 and BALB/c at 24h. B. Frequencies of CD4+ T cells and Tregs at 24 and 48h. C. Mitochondrial membrane potential (MMP) of splenocytes after TCR activation with Mito+ population representing high MMP. All analyses showed statistically significant difference using Chi square at $p<0.05$.

Strain-specific Responses to Metabolic Inhibitors on Tregs

We then explored the separate effects of blocking glycolysis and lipid oxidation on Treg frequency (Figure 10). Treatment with 2-DG to block glycolysis led to decreased activation of Tregs in both strains, with a greater response seen in BALB/c mice. Inhibition of FA oxidation by etomoxir initially led to a decrease in Treg activation in B6 mice but conversely an increase in Treg activation in BALB/c mice. After 48 hours, both groups showed increased Treg activation with a higher response seen in the BALB/c mice. Both groups showed higher populations of MMP^{low} cells with 2-DG treatment, supporting increased lipid utilization. However, though blocking FA oxidation with etomoxir led to the reverse in the C57BL/6, it did not affect MMP in the BALB/c mice. Additionally, T cell activation in glucose-free and lipid-free media with isolated FA supplementation led to complete loss of T cells, suggesting that FA alone cannot be sufficiently metabolized for growth and survival. Both oxidative and glycolytic machinery are necessary in tandem.

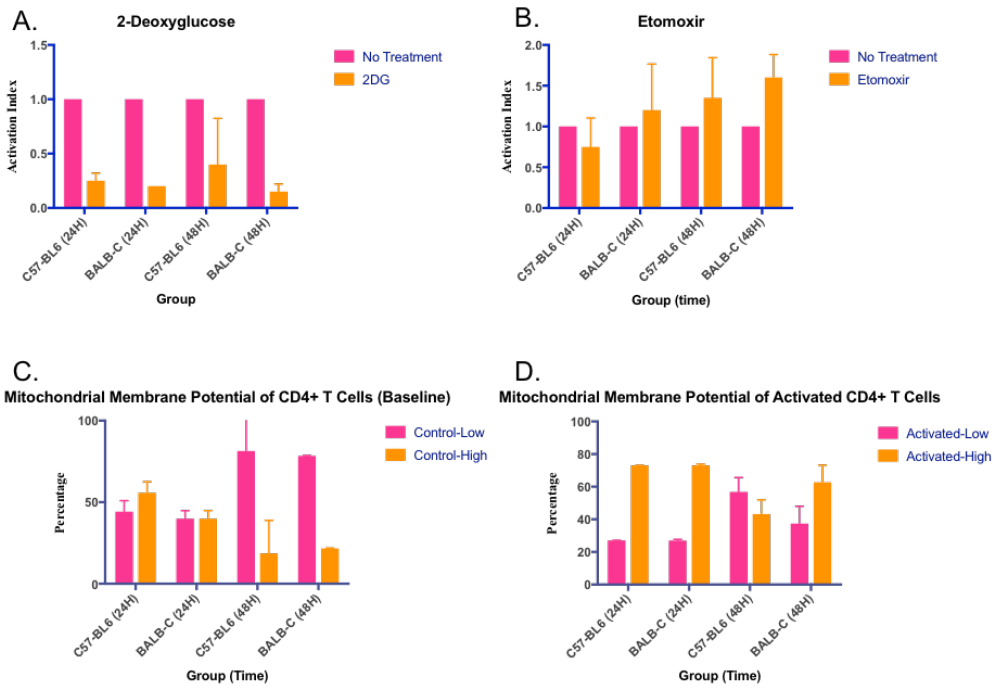


Figure 10. Treatment of B6 and BALB/c splenocytes with 2-DG. A. Activation index was calculated by normalizing Treg numbers to the control group; B. Treatment of B6 and BALB/c splenocytes with etomoxir; C. Baseline MMP of CD4+ T cells; D. MMP of TCR-activated CD4+ T cells. Statistical significance was set at $p<0.05$.

Lipids Complexed into LDL and HDL affect Treg and IL-17+ T Cell Frequency

Since the above experiments showed that genetic differences in lipid metabolism appeared to affect the response to T cell activation, we next aimed to determine whether changes in lipid availability – specifically complex lipoproteins in the form of LDL and HDL – would have a direct effect *in vitro* on lymphocytes without TCR stimulation. Analysis of the effect of increasing doses of LDL and HDL on Treg and IL-17+ cell proliferation on B6 splenocytes showed that there was not a significant dose-dependent effect of LDL on Treg or IL-17+ cell frequency ($p=0.49$ and $p=0.06$, respectively). Tregs did show a significant linear decrease in LDL-supplemented medium as a function of time ($p<0.0001$). There was a significant time-dependent increase in IL-17+ cells in LDL-supplemented and HDL-supplemented media, especially

at 12 hours ($p<0.0001$). Meanwhile, HDL was found to have both a significant time- and dose-dependent effect on Treg frequency ($p=0.03$ and $p=0.01$, respectively), though an unexpected attenuation of Treg numbers was observed (Figure 11).

Differences in T Cell Activation due to Lipoproteins

Finally, we sought to show that, upon antigen encounter, differences would be observed in Treg and IL-17+ cell proliferation, dependent on lipid availability. We found that LDL exposure was associated with a decrease in Treg and an increase in IL-17+ proliferation at 12h and 24h ($p<0.0001$) but, again, a relationship to increasing dose was not observed compared to control. However, HDL again showed a time- and dose-dependent decrease in Tregs ($p<0.0001$ and $p=0.02$, respectively) but also in IL-17+ cells ($p=0.01$ and $p=0.02$, respectively) (Figure 12). The effect on Tregs appeared to diminish once an extremely high dose of HDL (500 mg/dL) was utilized.

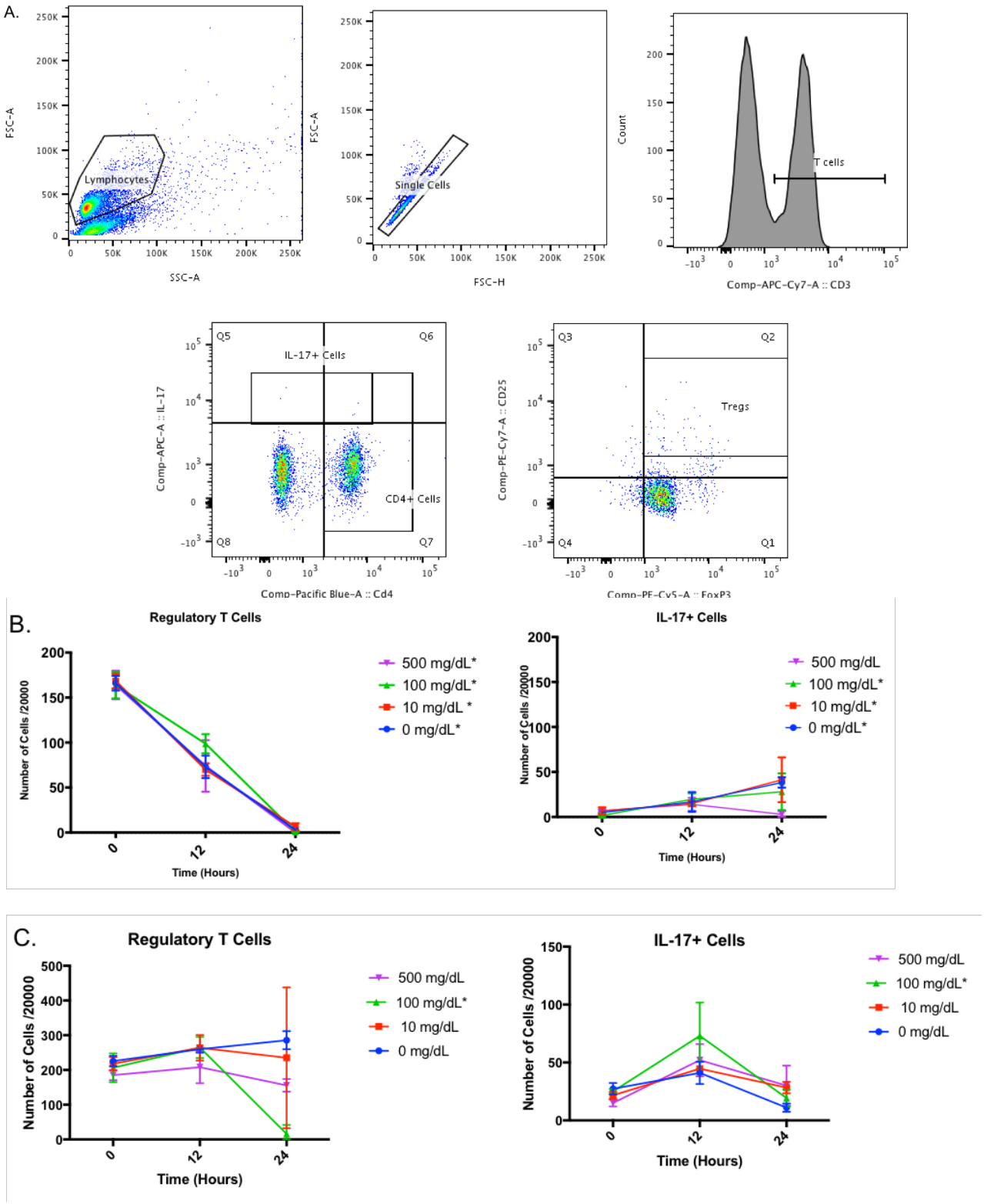


Figure 11. Effect of lipoproteins on Tregs and IL-17+ cells. Pooled B6 splenocytes were cultured at 37° in triplicate in increasing levels of lipid. A. Representative flow cytometric analysis for Tregs and IL-17+ cells. B. Treg and IL-17+ cell proliferation in response to differing concentration of LDL at 12 and 24h. C. Treg and IL-17+ cell proliferation in response to differing concentration of HDL at 12 and 24h. * depicts statistically significant difference on time-dependent change at $p<0.05$.

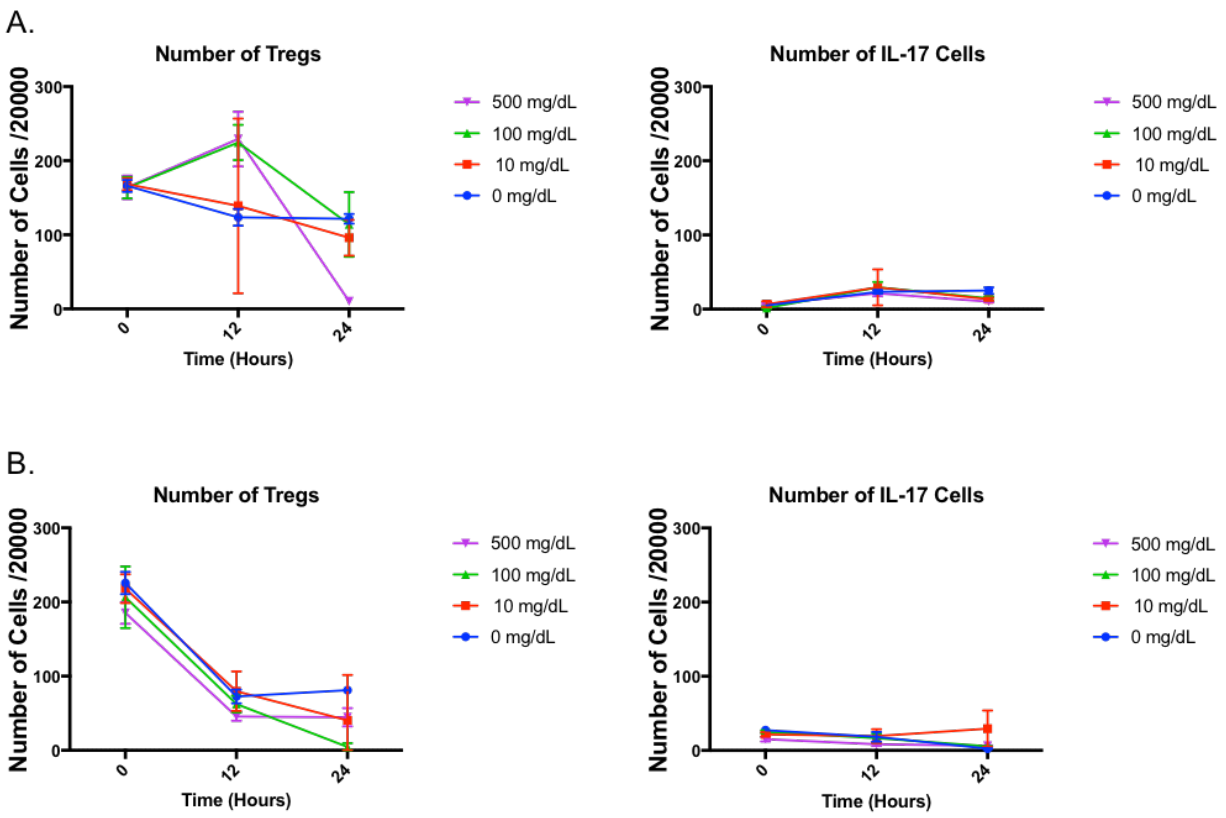


Figure 12. Effect of lipoproteins on TCR-activated Tregs and IL-17+ cells. Pooled B6 splenocytes were cultured at 37°C in triplicate in increasing levels of lipid and activated with anti-CD3e and anti-CD28. A. Treg and IL-17+ cell proliferation in response to differing concentration of LDL at 12 and 24h. B. Treg and IL-17+ cell proliferation in response to differing concentration of HDL at 12 and 24h.

IV.4 Summary

Lipid availability and metabolism have been shown to be profoundly important in enteric, autoimmune, and vascular disease. This implies that underlying derangements in lipid metabolism may be correlated with increased autoimmunity or predisposition to exaggerated inflammatory responses. The present study was designed to explore whether strain-specific differences in lipid oxidation lead to observable differences in the T cell/Treg compartment of adaptive immunity.

We showed that BALB/c mice, demonstrated to have upregulated glycolysis via higher proportions of MMP^{high} cells, had both increases in baseline CD8+ and CD4+ T

cells as well as exaggerated responses of overall CD4+ T cells, including Tregs, when antigenic TCR activation was mimicked. Without stimulation, Treg levels diminished to insignificant levels, despite continued high numbers of overall CD4+ cells. This suggests that TCR activation is necessary in the BALB/c mice for sustaining circulating Tregs due to its decreased ability to utilize short chain fatty acids, leading to an overall inflammatory CD4+ T cell phenotype. Meanwhile, though the B6 mice had significantly lower absolute numbers of CD8+ and CD4+ T cells, Treg proportions were higher and increased relatively more in comparison when antigen exposure occurred. Therefore, with intact lipid oxidation machinery, the B6 mice had a more anti-inflammatory phenotype and an ability to mount a significantly higher suppressive response to turn off the inflammatory cascade earlier than the BALB/c mice.

BALB/c mice had a greater decrease in Tregs in response to inhibition of glycolysis with 2DG that was unaffected by a blockade of carnitine palmitoyl transferase, further supporting the idea that the BALB/c mouse is dependent on glycolysis and mounting of a pro-inflammatory response for Treg proliferation. While much of the susceptibility of the BALB/c mouse to diverse autoimmune diseases, including autoimmune encephalitis, systemic lupus erythematosus, myocarditis and arthritis, has largely been attributed to differences in MHC alleles (I-A^d I-E^d) compared to B6 alleles (I-A^b), our study therefore also implicates this defect in lipid utilization in the susceptibility.[143–146]

Our next step was to address whether, in the absence of metabolic abnormalities, lipoprotein availability can be manipulated to improve Treg frequency or decrease IL-17+ production. This has implications for diseases linked to Treg

derangements, as simple dietary manipulation or targeted initiation of lipid-lowering agents may improve disease outcome. In clinical studies, statins, HMG-CoA reductase inhibitors that decrease LDL and increase HDL, have been shown to attenuate colitis as well as improve autoimmune diseases such as inflammatory bowel disease and multiple sclerosis.[126,147,148] We found that LDL did not appear to have a direct effect on CD4+ or Treg numbers. Additionally, HDL decreased both Tregs and IL-17.

Multiple mechanisms may explain our findings. We showed that both oxidative phosphorylation and glycolysis are required for T cell proliferation, and rapidly dividing cells may have metabolized available excess lipoproteins for energy without direct toxic effects. HDL may have preferentially attenuated proliferation of inflammatory CD4+ subsets, rather than increasing Treg numbers. In parallel to previous observations in clinical acute phase responses, TCR activation may have decreased available HDL levels as well, preventing a positive effect on the infrequent Tregs, or preferentially used pro-inflammatory HDL subsets.[149] Finally, our findings may also mean that instead of a direct effect on T cells, lipoproteins exert their effect through the professional antigen presenting cells (APCs). In fact, B cells have recently been shown to have high levels of LDL receptors and affect T cell responses differentially based on lipid availability and costimulatory molecules.[49,56,150]

Thus, the major limitation of our study is that the use of *in vitro* assays prevents modeling of the complex anatomic, cellular and cytokine milieu of the lymphatic system. Additionally, lipoprotein metabolism is coordinated in the liver, through which the majority of dietary lipids are trafficked for delivery or excretion and likely have high direct interaction with immune cells. This microenvironment cannot be modeled in a Petri

dish. Though our studies implicate the importance of genetic differences in lipid-directed immunity and show a correlation between T cell activation and lipoprotein availability, more studies are needed to further explore these relationships.

V. LIPIDS AFFECT IMMUNE PROFILES THROUGH CLIP+B CELL AND GAMMA DELTA T CELL INTERACTIONS IN THE LIVER

V.1 Introduction

In the classical teachings of immunology, the focus has been on two arms of the immune system: innate immunity, which includes neutrophils and macrophages that are immediate responders to an inflammatory insult, and adaptive acquired immunity, in which antigen-specific B- and T- lymphocytes are the most well-characterized. Professional APCs, including B cells, macrophages, and dendritic cells, function as the intermediaries between these two arms of immunity. APCs engulf and process protein antigens into peptides in the lysosome that are then presented on the cell surface complexed with MHC class II (MHCII) molecules. CD4+ T cells, or helper T cells, in turn recognize these antigenic peptides through their TCR, made of a heterodimer of an alpha and beta chain ($\alpha\beta$ T cells), to effect activation through the production of cytokines that either assist in the humoral B cell response or that assist in activation of the CD8+ T cell, cytotoxic T cell, responses.[151,152]

T cell activation therefore requires recognition of antigen presented in the context of MHC. However, this alone is not sufficient for a response. Co-stimulation, mediated by ligand-receptor interactions between APC and T cell, is necessary as a second signal.[153,154] The most well-studied co-stimulatory molecules are the B7 molecules on APCs (B7-1/CD80 and B7-2/CD86) that interact on T cells with CD28 to activate or CTLA-4 to downregulate the T cell response.[155–162]

However, closer examination of B- and T-cells has shown subsets of lymphocytes that do not follow this paradigm. B cells that carry invariant chain-derived (CD74) class II-associated invariant chain peptides (CLIP), rather than antigen complexed with MHC II, are activated by innate immune signals. CLIP+ B cells serve at the interface of innate and adaptive immunity by promoting inflammatory responses without antigenic stimulation and may play a significant role in the chronic inflammation of autoimmune disease.[163–166] T cells that carry the γδ-TCR, gamma delta T cells (GDTC), are usually CD4-/CD8+, but can also be CD4+ or double negative, do not necessarily require peptide presentation in MHC and can recognize lipid antigens.[167–172] Many of the GDTC produce the inflammatory cytokine IL-17 and develop in thymus-independent viscera, such as the gut, as intraepithelial lymphocytes (IELs).[17,55,84,120,172–178]

The liver functions as the main effector organ in the processing of dietary lipids throughout life, as well as a primary lymphoid organ prenatally. Fats are either burned for energy or processed and packaged by hepatocytes into VLDL for delivery of FA and cholesterol for cellular use in the peripheral tissues. HDLs then return cholesterol from peripheral tissues to the liver for disposal through bile (Figure 13).

Recently, LDL has been demonstrated to play a role in pro-inflammatory IL-17-dependent inflammation in atherosclerosis.[48–52] Conversely, HDL appears to promote an anti-inflammatory Treg-dependent reduction in inflammation.[51,53] Independently, infection with rhesus rotavirus, a major intestinal pathogen that also induces experimental biliary atresia in mouse models, has been shown to induce IL-17 production and concomitant decrease in IL-10 by intrahepatic T cells with no significant

similar effect seen in mesenteric lymph nodes or gut mucosa.[55] B cells have been shown to have a high density of LDL receptors and the ability to internalize LDL for processing in the lysosome, where antigen association with MHC II occurs as well (Figure 13).[56] Cholesterol content has also recently been linked to differential regulation of GDTC activation.[179]

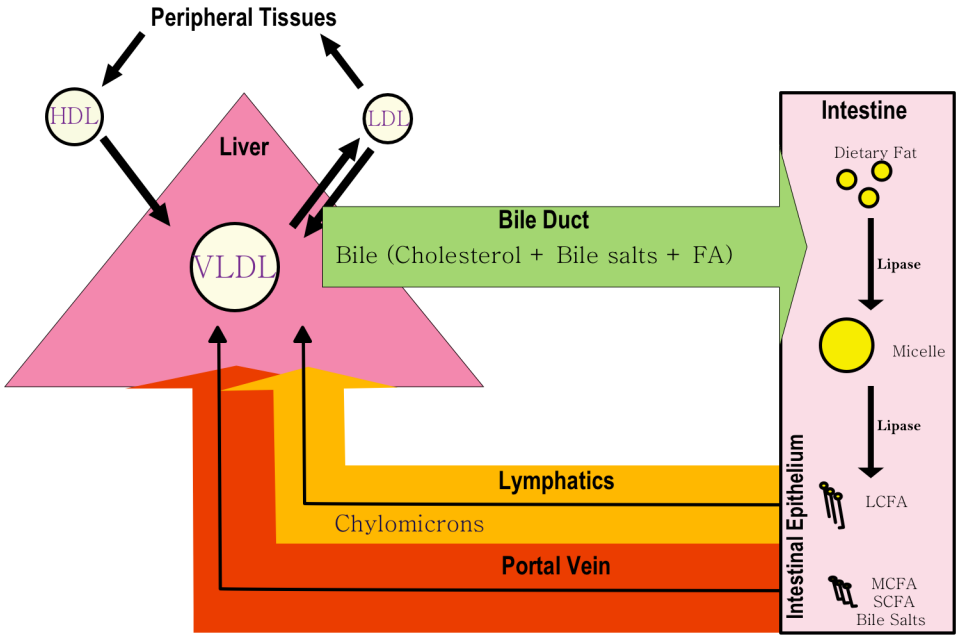


Figure 13. Circulation of lipids as mediated through the liver

The study outlined in our preceding chapter aimed to show a direct effect of LDL and HDL on the Treg/Th17 axis. We found that strain-specific deficiencies in lipid processing indeed affected Treg dynamics. HDL level correlated with both Treg and IL-17+ cell frequency. From this, we hypothesized that CLIP+ B cells and GDTC as the main source of IL-17 are the mediators of the lipid-dependent inflammatory responses

that lead to attenuation in Treg numbers.[51–53,126,127,129,130,133,135,137,148,180,181] In this chapter, we aimed to determine if lipid processing in the liver is the key to directing the body towards enteral tolerance or autoimmunity/inflammation via differential expression of co-stimulatory molecules on CLIP+ B cells and/or activation of GDTCs.

V.2 Materials and Methods

Mice

C57BL/6J (B6) mice, 3-6 months of age, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the Baylor Scott&White/Texas A&M animal facility, Temple, TX.[138] All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee.

Cell Isolation and Purification

Splenocytes and hepatocytes were isolated from B6 mice and single cell suspensions made using a 40 μm nylon strainer. Discontinuous 40%/70% Percoll density gradient centrifugation was used for leukocyte enrichment.

Cell Culture

Spleens were pooled (3-4 per group) for adequate cell numbers of splenocytes and cultured in 96-well plates. RPMI 1640 medium (Gibco) supplemented with 5% fetal bovine serum was used for plating cells in triplicate at concentrations of 1.0×10^6 live cells/mL. Cells were incubated at 37° for up to 30h.

Reagents

Purified human plasma LDL and HDL (>95% SDS-PAGE) were purchased from Lee Biosciences and used at concentrations of up to 200 mg/dL to mimic serum levels in humans. Recombinant human Fas:Fc (Enzo Life Sciences) was used at a concentration of 0.75 μg/mL. The rhFas:Fc binds to Fas ligand (FasL) on T cells, including GDTC, B cells, natural killer (NK) cells and some innate immune cells and inhibits soluble FasL-mediated lysis of cells sensitive to Fas-induced cell death. The rhFas:Fc can also block FasL-dependent cell proliferation.[182] Cells were incubated in lipoproteins for 6h to allow acclimation prior to treatment for 12h (18h group) and 24h (30h group) with reagents.

T Cell Activation

To mimic TCR stimulation and co-stimulation by antigen, cells were activated with 1 μg/mL of purified anti-CD3e antibody (eBioscience) and 1 μg/mL purified anti-CD28 antibody (eBioscience) at 37° for up to 30h as specified.

Flow Cytometry Analysis for Surface and Intracellular Markers

Eight-color flow cytometry was performed using the BD FacsCanto II instrument (BD Biosciences) and FlowJo software (TreeStar). FACS staining was performed according to standard protocols. The following antibodies were used: rat anti-mouse PacBlu-CD4, PE-Cy7-CD25, PacBlu-CD19 (BioLegend), APC-GD TCR, PE-B71, PE-Cy5-B71, APC-B72, PE-Cy5-MHC II (eBioscience), PacBlu-B220, APC-Cy7-CD3e (BD), and FITC-CLIP (Santa Cruz). Per group, 10000 events were collected and

visualized according to forward (cell size) and side scatter (cellular granularity). Isotype controls were used for populations of interest.

Statistical Analysis

Parametric tests, including Student's t test with Holm-Sidak correction for multiple comparisons, were used to calculate differences between means. Linear regression and Pearson r correlation was used to show dose-dependent relationships of absolute cells numbers and percentages of cells. Prism 7.0d was used for all analyses (GraphPad Software). Statistical significance was set at $p < 0.05$.

V.3 Results

Preferential Dose-dependent Increases in T cell Subsets in Response to HDL and LDL Supplementation

In vitro, we observed a dose-dependent increase in Treg frequency and in the percentage of Tregs from total cells in response to HDL, with no similar effect seen with LDL supplementation. This dose-response appeared to occur at concentrations greater than 50 mg/dL, the clinically defined target for serum HDL levels. This suggests that Tregs can utilize HDL but not LDL, a finding we have observed in our previous studies. This may also suggest that availability of HDL allows naïve CD4+ T cells to differentiate preferentially into Tregs over effector conventional T cells (Figure 14C, 14D).

Meanwhile, the opposite effect was seen with GDTCs, with a significant dose-dependent increase in both absolute frequency and proportion seen in response to LDL (Figure 14C). HDL appeared to also cause an increase in absolute GDTC frequency, but when the proportion of overall T cells was analyzed, this effect vanished (Figure

14D). These observations lead us to believe that GDTCs may proliferate in response to LDL availability, either via primary utilization of LDL as an energy source as a consequence of FA oxidation or recogntion of LDL particles associated with MHCII as a lipid antigen or lipidated peptide.

LDL Decreases Number of CLIP+ B Cells

Our next set of experiments was conducted to explore the relationship of lipoprotein levels and B cells. We found that HDL did not affect the total number of CLIP+B cells. However, LDL appeared to cause a decrease in CLIP+B cell numbers (Figure 15B). This is consistent with previous observations that B cells have a high density of LDL receptors.[56] LDL is endocytosed into lysosomes by B cells, the compartment where the peptidic antigens can displace CLIP in the groove of MHCII to enable antigen presentation. It has been described that exogenous lipid availability changes acidity of the lysosome and surface expression of MHCII.[183] We reasoned that components released from LDL digestion, either FA, cholesterol or proteins, likely interacted with, or even replaced, CLIP in the binding groove of MHCII to cause the observed decrease in $CLIP^{high}$ B cells, priming the immune system to target lipidated antigen possibly via recognition by GDTCs.

We then explored whether treatment with rhFas:Fc would reverse the observed loss of CLIP+B cells or improve Treg proliferation by blocking lipoprotein-induced Fas-mediated killing of either population. Use of rhFas:Fc to block FasL-mediated cell death by GDTCs has been described previously.[184] Increased GDTCs in response to LDL was inversely correlated with Treg and CLIP+B cell frequency (Figures 14C, 14D, 15B).

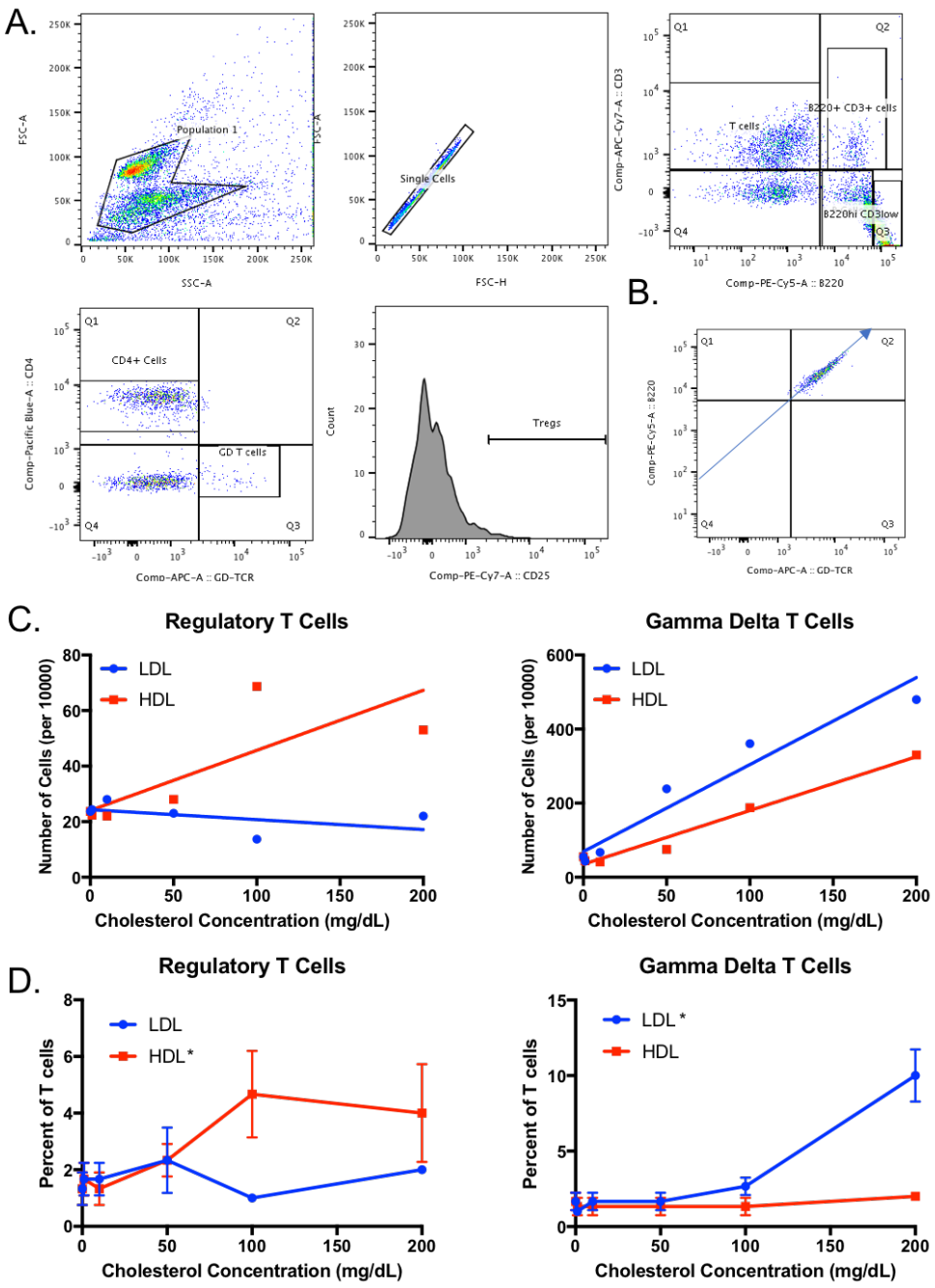


Figure 14. Effect of LDL and HDL on GDTCs and Tregs. A. Representative flow cytometric analysis of $1x10^4$ cells analyzed after culture with increasing concentrations of LDL and HDL for 18h. T cells defined as CD3+B220- cells, GDTCs defined as CD3+CD4-GDTCR+ cells, Tregs defined as CD3+CD4+CD25high cells. B. CD3+B220+ cells were found to be 100% GDTCR positive in all populations, with a direct increase in expression of both markers observed. C. Linear regression of frequency of Tregs and GDTCs for dose-response effect of exposure to increasing concentrations of LDL and HDL for 18 hours, $p<0.05$ for difference between slopes for both datasets. D. Pearson r correlation for dose-response effect on Treg and GDTC percentage of total T cells. * denotes statistically significant correlation set at $p<0.05$.

This may have resulted from the inhibition of Fas-dependent proliferation of CLIP+B cells. In the absence of lipid, there was actually a significant decrease in CLIP+B cells when treated with rhFas:Fc, and any addition of lipid negated this effect (Figure 15C). However, rhFas:Fc treatment improved Treg proliferation at low doses of HDL and decreased Tregs at low doses of LDL, suggesting that lipid-primed GDTCs may reduce Tregs, though this did not reach statistical significance (Figure 15E).

Additionally, we observed that CLIP+ B cells were largely CD80+CD86+^{high} cells, as determined by flow cytometry, which has not previously been described in the literature (Figure 15C). As a final step, this led us to examine the expression of the B7 co-stimulatory molecules to determine whether these molecules may also play a role in differential immune responses to lipid.

Co-stimulatory Molecules CD80 and CD86 on B Cells are Differentially Affected by Lipid

Further *in vitro* studies showed that exposure of B cells to LDL for 30h led to increases in CD80+ (B71) B cells in a dose-responsive manner, but no significant decrease in CD86+ (B72) cells. Meanwhile, HDL led to significantly decreased numbers of B71, B72 cells, double positive, and total B cells at high doses (200mg/dL), though the effect on B71 cells first became significant at a lower dose (100mg/dL) (Figure 16B). B72 cell frequency ran parallel to total B cell frequency, in comparison to other subsets, suggesting that at baseline B72 is constitutively expressed on B cells and an external signal is required for expression of B71, which serves to stimulate a stronger inflammatory response. This further implies that increased expression of the co-stimulatory molecule B71 in response to LDL and decreased expression secondary

to HDL exposure may be a key point at which divergence to an inflammatory or tolerizing state occurs, respectively.

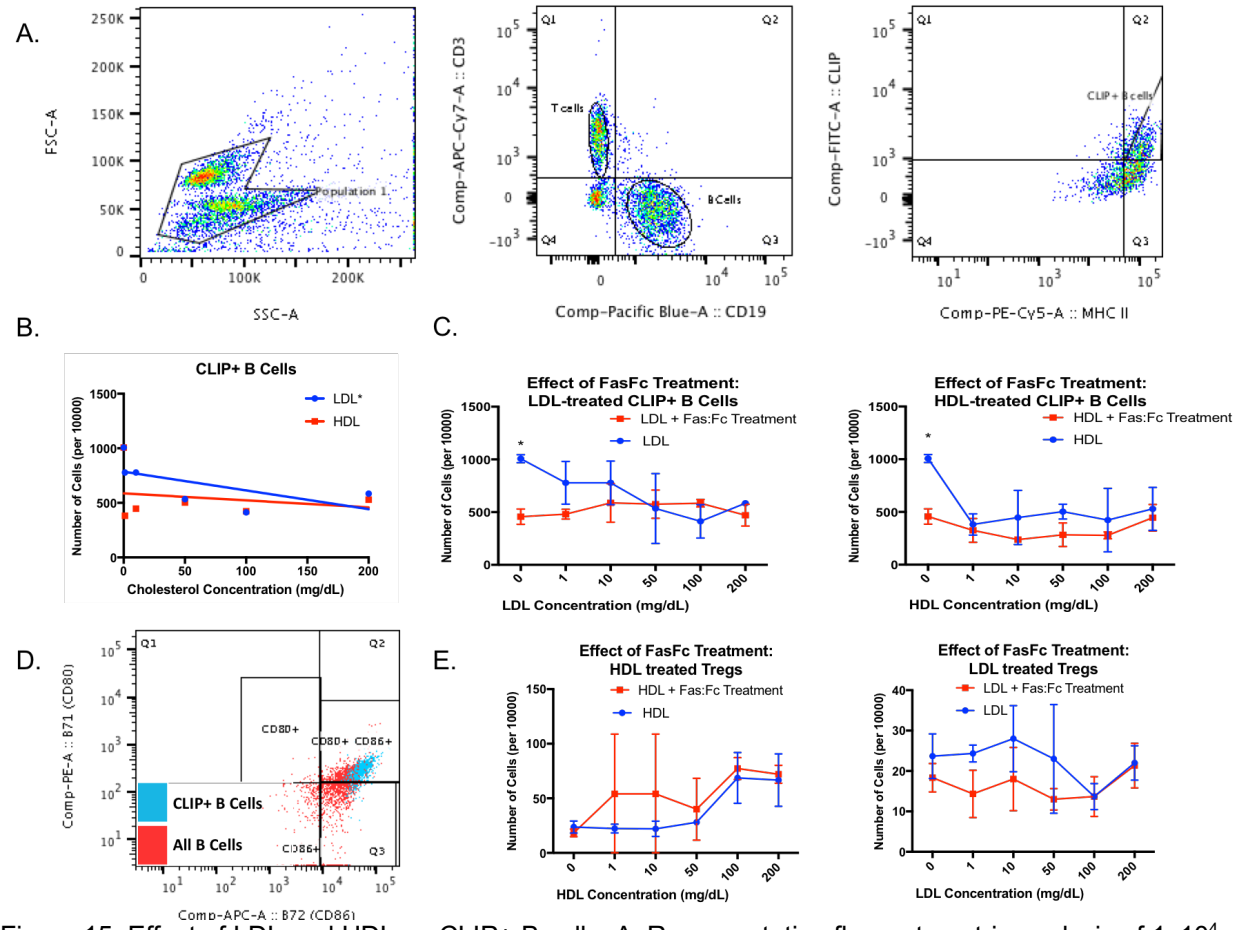


Figure 15. Effect of LDL and HDL on CLIP+ B cells. A. Representative flow cytometric analysis of $1x10^4$ cells analyzed after culture with increasing concentrations of LDL and HDL for 18h. B cells defined as $CD3^-CD19+$ cells, CLIP+B cells defined as $MHCII^{high}$ CLIP+ cells from B cell gate. B. Linear regression of CLIP+B cell frequency in response to increasing doses of LDL and HDL showed a significant decrease with LDL but no relationship to HDL. C. Treatment with Fas:Fc showed significant decrease of CLIP+B cells without lipoproteins, which disappeared with lipoprotein treatment. D. CLIP+B cells were found to be almost exclusively $CD80+^{high}CD86+^{high}$. E. Fas:Fc treatment improved Treg proliferation at low doses of HDL but decreased Treg proliferation at low doses of LDL. Multiple Student's t-test with Holm-Sidak correction for multiple comparisons used to detect differences between means. * denotes statistically significance defined as $p<0.05$.

We also wanted to determine whether the immune changes fostered by lipoprotein availability were mediated by differential MHCII expression. By measuring the MFI of MHCII staining on B cells to indicate MHCII expression, we found no effect in

response to differing doses or types of lipoproteins (Figure 16C). Interestingly, we observed that each subset of B cells (CLIP+, B71, B72 and double positives) appeared to have a unique MHCII expression fingerprint that was unchanged by lipoprotein availability, with CLIP+ cells having the highest and B71 having the lowest MHCII levels per cell. This may suggest that co-stimulatory molecules also direct MHCII expression, a finding that warrants further investigation.

Hepatic B Cell Populations Differ from Spleen Populations in Mice

Because the liver is the central organ for lipid metabolism, we theorized that lymphocyte populations found in the liver might reflect a role in promoting enteral tolerance to dietary fats. Our previous observations supported LDL-induced decreases in CLIP+ B cells and increases in GDTCs. In fact, we found similar proportions in the liver compared to spleen of B6 mice. Significantly lower numbers of B cells and CLIP+ B cells were found in the liver compared to spleen, though the overall proportion of B cells that were CLIP+ was higher in the liver (Figure 17A). Conversely, liver had more T cells, including CD4+ and GDTCs, but fewer Tregs, which trended towards significance (Figure 17B). Hepatic GDTC:Treg ratio (defined as the inflammatory index – Figure 17C) favored GDTCs, while a statistically significant reversal was seen in the spleen. Overall, these observations suggest that the key mediators of enteral tolerance to lipid propagated by the liver are GDTCs, promoted by its lipid-rich microenvironment.

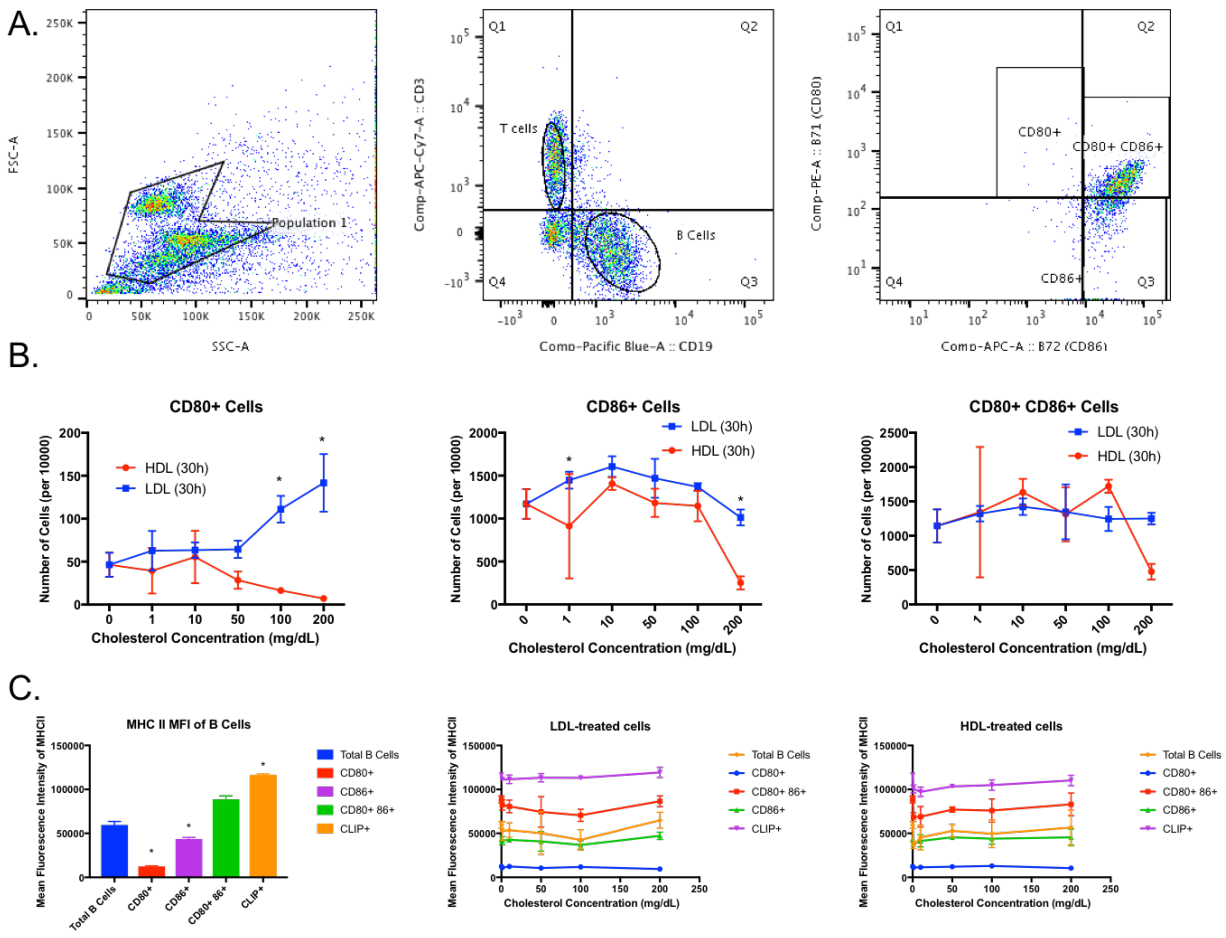


Figure 16. Effect of LDL and HDL on B cell co-stimulatory molecules and MHCII expression. A. Representative flow cytometric analysis of $1x10^4$ cells analyzed after culture in increasing concentrations of LDL and HDL for 30h. B cells defined as CD3-CD19+ cells, B71/CD80+ cells defined from B cell gate as CD80+CD86-, B72/CD86+ cells defined as CD80-CD86+ and double positive population identified as CD80+CD86+. B. Comparison of proliferation of CD80+, CD86+ and double positive cells in response to LDL and HDL. C. Geometric mean fluorescence intensity (MFI) of MHCII expression evaluated by flow cytometry for B cell subsets and in response to LDL and HDL treatment. * denotes statistically significance defined as $p<0.05$.

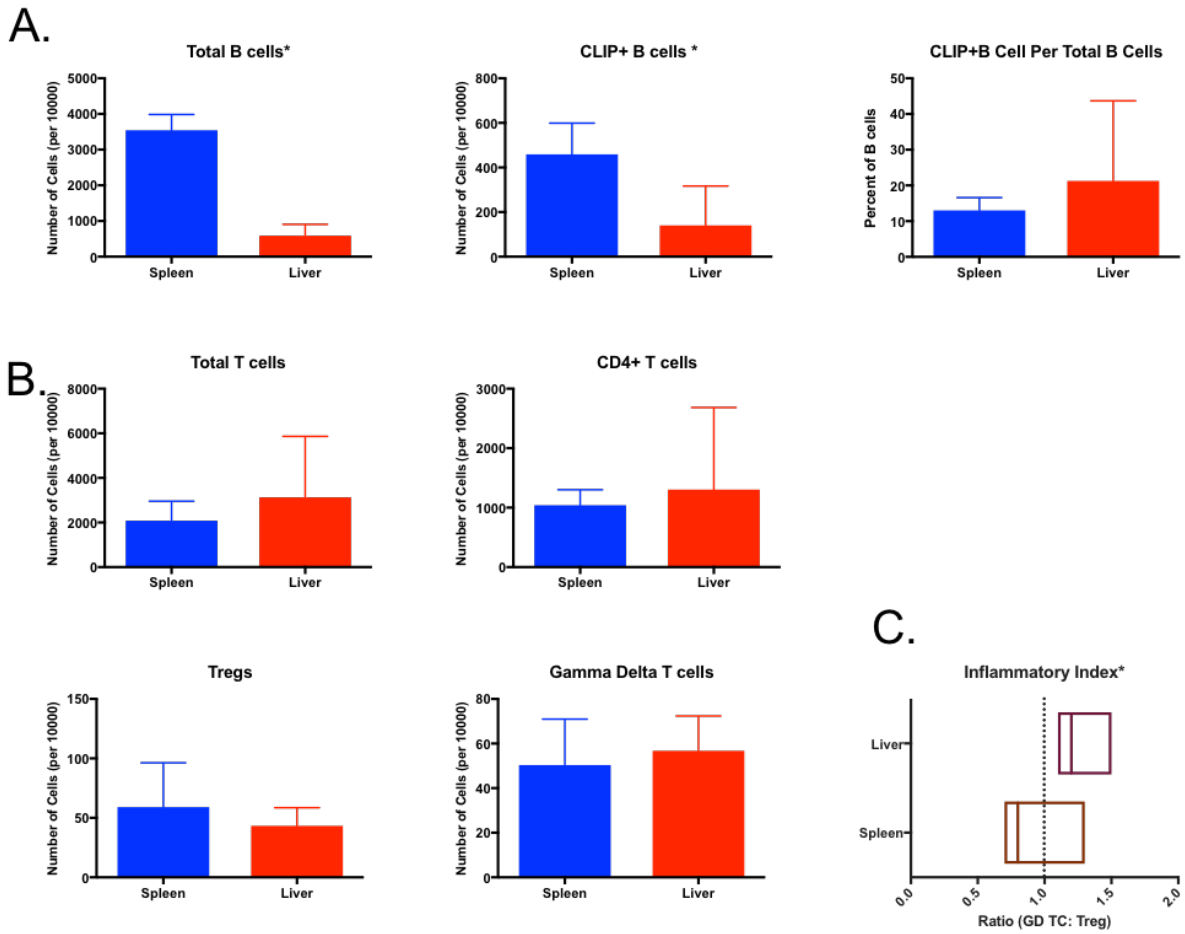


Figure 17. Frequency of lymphocyte populations and subsets in spleen and liver. A. B cells in spleen and liver (n=6-12 per group). B. T cells in spleen and liver. C. Inflammatory index of lymphocytes - defined as ratio of GDTCs to Tregs. Student's t test used to compare differences between means. * denotes statistically significance defined as $p<0.05$.

V.4 Summary

Overall, our data shows the importance of lipoprotein particles on immune profiles. First, we were able to demonstrate that LDL is important in the proliferation of GDTCs. We also found that LDL caused decreases in CLIP expression on B cells. Together, this suggested that CLIP was replaced by lipid particles on B cells for presentation to GDTCs. GDTCs in the gut are largely pro-inflammatory IL-17-producing cells.[173,174] IL-17 GDTCs in the liver in response to pathogen have also been shown to

promote IL-17-induced inflammation or promote PE during pregnancy.[55,185] However, tolerizing roles to acute infection in other anatomic compartments, including a protective effect against LPS-induced lung injury have been described as well.[186] Furthermore, GDTCs in the form of IELs have been shown to be critical for maintenance of gut epithelial integrity.[17,175] This, along with our previous data, suggests that in low LDL environments, GDTCs may promote tolerance to lipid, but in states of excess lipid can promote IL-17-based inflammation.[173,179] Furthermore, improved HDL levels may ameliorate this effect, based on their ability to increase Tregs to suppress an inflammatory response.

The preponderance of CLIP+B cells and GDTCs in the liver compared to spleen suggests that this critical balance may occur in the liver as an anatomic compartment. We hypothesized that lipid processing in the liver is key to directing the body towards enteral tolerance or autoimmunity/inflammation via differential expression of co-stimulatory molecules on CLIP+ B cells and/or activation of GDTCs. In fact, if excess LDL particles begin to displace CLIP in MHCII in B cells and are then presented as antigen to GDTCs, this may be the crucial step in effecting a pathologic response both in the gut through the enterohepatic circulation or systemically via the vena cava to the cardiac circulation. The direct effect seen in CD80 expression suggests that this response may occur in tandem, with CD80 expression increasing only after a pro-inflammatory state has become predominant. If these changes can be demonstrated in the peripheral blood as well, CD80 detection may even be used as a diagnostic target in disease states where the response to enteral lipid is found to be of importance, such as inflammatory bowel diseases, celiac disease, or even necrotizing enterocolitis.[17,187]

Because the liver functions as the anatomic juncture between the enteric circulation and the systemic circulation, these findings may have important implications, not just for enteric tolerance, but even systemic autoimmune disease and transplant rejection.

VI. FORMULA FEEDING PREDISPOSES INFANTS WITH ABERRANT LIPID METABOLISM TO NECROTIZING ENTEROCOLITIS

VI.1 Introduction

NEC is a disease of newborns that results in epithelial destruction of the gastrointestinal tract leading to a cascade of local and systemic effects. The incidence is 1 in 1000 live births with a mortality of 15 - 30% and it is almost exclusively a disease of premature and low birth weight infants.[1–4] Formula feeding has been shown in multiple multi-system clinical studies to significantly increase the incidence of NEC compared to breast-feeding, especially in these high-risk populations.[188–191] However, an incomplete understanding of the underlying pathogenesis of the disease has led to minimal improvements in mortality over the last few decades.[11]

The current paradigm is that an initial breach of the intestinal epithelial barrier leads to a pro-inflammatory immune response and the inability of the premature immune system to then counteract the inflammatory cascade leads to downstream effects which manifest as NEC (Figure 1). A dysregulation in the ability to mount antigen-specific tolerance to the microbiome and to enteric antigens has been shown to be key. Secretory IgA, required for oral and microbiome tolerance, is reduced in preterm infants. In experimental colitis models, decreased exposure to IgA leads to a more pro-inflammatory gut microbiota and intestinal epithelial gene expression associated with inflammatory bowel disease.[18]

T lymphocyte subsets are crucially important in NEC, as well. Tregs, CD4+ T lymphocytes that promote self-tolerance, are attenuated in the gut epithelium of infants who develop NEC.[19] Pro-inflammatory cytokine expression, especially of IL-6 that disposes T cells to differentiate towards the pro-inflammatory T-cell subsets (including CD4+ Th17 cells), is also proportionately increased during development of NEC.[20] IELs, resident lymphocytes of the gut mucosa that usually express the Υδ T-cell receptor (GDTCs) and unlike classical T cells do not mount a peptide antigen-specific response, are necessary during development for self-tolerance and habituation to commensal microbiota.[16] Infants with NEC have been shown to have a reduction in this subset of innate-like lymphocytes, as well.[17]

In previous chapters, we have shown the importance of lipid environment on imbalances in the Treg/IL-17+ and GDTC axis. This takes on special significance in the neonate, who switches from obtaining nutrients by placental transfer *in utero* to oral ingestion of nutrition after birth. Breast milk is a complex biofluid of which lipids are the largest source of energy, contributing 40-55% of its composition.[41] Lipids are packaged into a specialized carrier molecule called the MFG. The MFG is composed of a TAG core surrounded by a unique triple membrane structure called the MFG membrane.[43,44] Infant formula does not contain MFG and tends to have vegetable source-derived lipids which differ greatly in size and composition.[44,45] Supplementation of formula with MFG appears to protect against *Clostridium dificile*-induced gut inflammation in neonatal rat models and against gut barrier disruption and inflammation in systemic LPS infection.[46,47]

We have also previously shown that strain-specific genetic differences in lipid metabolism machinery in mice lead to distinctive T cell profiles. Lipid metabolism is a complex process that initiates in the gut and is regulated in a developmental and tissue-specific manner (Figure 18). The liver plays a key role in mediating this process. Dietary lipids, mostly TAGs, are processed and packaged by the intestinal epithelium into chylomicron particles conjugated to apolipoprotein B48 (apoB48) for delivery to the liver. Hepatocytes produce VLDLs conjugated to apolipoprotein B100 (apoB100) for delivery to the periphery. LDLs of increasing density all contain one molecule of apoB100, which is the major ligand recognized by LDL receptors in extrahepatic tissues for cholesterol uptake.[192] ApoB48 is the truncated form of apoB100, encoded by a single gene on human chromosome 2 and generated as a result of apoB mRNA editing enzyme (encoded by APOBEC), which is developmentally regulated in the small intestine during fetal development.[193–195] ApoB deficiency has been shown to predispose to severe invasive *Staphylococcus aureus* infection.[196] Meanwhile, deletion of microsomal triglyceride transfer protein (MTTP), which assists in assembly of lipoproteins in both liver and intestine, improved mortality and intestinal injury during exposure to pathogen.[197]

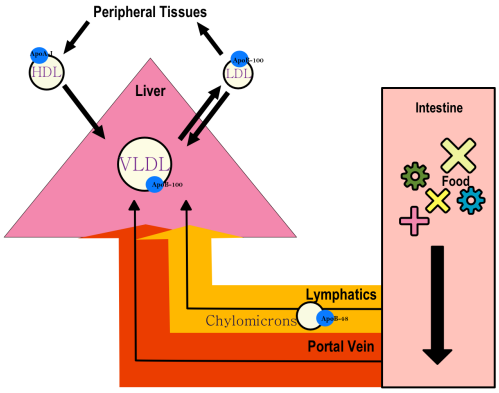


Figure 18. Lipid metabolism mediated by the liver

We propose a two-hit model of NEC, in which formula feeding serves as the first insult in infants with aberrations in lipid metabolism genes, priming the immune system towards creating a repository of pro-inflammatory GDTCs via presentation of lipid antigens by CLIP+ B cells. Exposure to an enteric pathogen causing a breach of the intestinal epithelium serves as the second insult, leading to an exuberant inflammatory response ultimately manifesting as NEC.

VI.2 Materials and Methods

NCBI GEO Database

Differential whole genome microarray analysis had been previously performed on intestinal tissues collected for non-inflammatory intestinal conditions (n=4) compared to those of infants with NEC (n=5) using a human Gene 1.0 ST array system (Affymetrix Inc.) and was publicly available for accession through the NCBI GEO datasets, GEO accession number GSE46619.[198] Four datasets from the GEO series were retrieved in addition to the top 250 differentially expressed genes and data preprocessing carried out using R. A t-test was used to decide which genes were differentially expressed for

each dataset. The four datasets included HLA genes that encode MHC proteins needed for antigen presentation, and genes that regulate polyunsaturated fatty acid metabolism (PUFA), including CYP450 genes that encode metabolic enzymes, FADS genes that encode fatty acid desaturases and genes encoding apolipoproteins needed for lipid metabolism. The GEO2R bioinformatics tool provided by NCBI was used for graphical representation.

Mice

C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the Baylor Scott & White/Texas A&M animal facility, Temple, TX. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee. 5-day-old mice were separated from dams and gavage-fed 75 – 100 μL of Esbilac formula or Esbilac formula + 1.5% DSS every 3 hours for 5 days and sacrificed at day of life (DOL) 10. Thymus, liver and spleen were isolated for further analysis.

Reagents

DSS is a heparin-like polysaccharide that is used to cause destruction of the gastrointestinal epithelium in bowel disease models.[35] A 1:1 combination of DSS of molecular weights 5 kD and 40 kD was used to induce pan-intestinal epithelial injury.[199,200]

Cell Isolation

Spleens and livers were removed and single cell suspensions made using a 40 μm nylon strainer. Discontinuous 40%/70% Percoll density gradient centrifugation was used for leukocyte enrichment for flow cytometry. 2-3 spleens and livers were pooled for 10-day-old mice for adequate cell yield.

Flow Cytometry Analysis for Surface and Intracellular Markers

Eight-color flow cytometric analysis was performed on fresh thymus and spleen cells using the BD FacsCanto II instrument (BD Biosciences) and FlowJo software (TreeStar). FACS staining was performed according to standard protocols. The following fluorochrome-conjugated antibodies were used: rat anti-mouse PacBlu-CD4, PE-Cy7-CD25, PacBlu-CD19, PE-CCR9, hamster anti-mouse FITC-Helios (BioLegend), rat anti-mouse PE-CD3e, PE-Cy5-MHC II, PerCP Cy5.5-FoxP3, hamster anti-mouse APC-GD TCR (eBioscience), rat anti-mouse PacBlu-B220, APC-Cy7-CD3e, hamster anti-mouse FITC-GD TCR, (BD Pharmingen), and rat anti-mouse FITC-CLIP (Santa Cruz). Isotype controls were used to define negative gating for populations of interest.

Immunofluorescence

Livers from dam-fed, formula-fed and DSS-fed neonatal mice were isolated and fixed in 4% paraformaldehyde followed by ethanol. Antigen-retrieval was conducted in a citrate buffer, followed by sucrose protection prior to freezing. Slides were made of 7-μm cryosections stained with DAPI nuclear counterstain (Southern BioTech) and incubated with fluorochrome-conjugated antibodies FITC-GDTCR and PE-CD3, as

detailed above. An Olympus BX51 fluorescent microscope was used for detection of fluorescent staining using 10X magnification and DAPI (excitation: 320-390 nm, emission: 430 -490 nm), FITC (excitation: 455 – 500 nm, emission: 570-560 nm) and TRITC (excitation: 505-560 nm, emission: 575-655 nm) filters.

Statistical Analysis

Parametric tests including Student's t test and ANOVA were used to calculate differences between means. Prism 7.0d was used for all analyses (GraphPad Software). Statistical significance was set at $p < 0.05$.

VI.3 Results

Downregulation of Lipid Metabolism Genes in NEC-affected Intestine

We first evaluated differential gene expression by microarray analysis of intestine collected for non-inflammatory bowel conditions (controls) and NEC.[201] We had expected to find both immunogenetic differences and metabolic differences, but no differences in the expression of HLA genes or in CYP450 and FADS genes were found between the two groups. However, significantly decreased expression of genes critical for cholesterol metabolism and lipoprotein assembly were found in the NEC babies, including various apolipoproteins such as APOA4, which encodes apolipoprotein A-IV ($p = 0.00002$), APOB that encodes apolipoprotein B ($p=0.00003$), and APOC3 that encodes apolipoprotein C-III ($p=0.003$) (Figure 19A). Differences were also found in genes encoding factors and enzymes required for lipoprotein assembly and transport (Figure 19B). *APOBEC*, which encodes the apolipoprotein B mRNA editing enzyme

(p=0.012) needed for post-translational modification of ApoB100 to ApoB48, as well as APOBEC1 complementation factor were both decreased in NEC infants. We also found significantly decreased expression of *Mttp*, necessary for the secretion of apoB-containing lipoproteins.[202]

Infant Liver Contains Higher Treg and GDTC Populations than Adult Liver

Because the liver is the central organ for lipid processing and packaging, we then turned our attention to describing the differences in hepatic lymphocyte populations between adult and exclusively dam-fed neonatal mice, to determine age-dependent differences. We used comparison to spleen as a control within age-groups. B cell populations were significantly higher in both liver and spleen of neonates compared to adults as well as higher in neonatal liver compared to spleen. Meanwhile, frequency of CLIP+ B cells was identical in both organs in neonates and when compared to adults. However, given the total B cell numbers observed, this additionally suggested a much lower actual proportion of CLIP+ B cells in the neonate than in the adult (Figure 20A).

Analysis of T lymphocyte populations, on the other hand, showed no significant differences in total T cells. The key differences were found in the exploration of the various subsets of interest. We observed that neonatal liver and spleen both had half the number of CD4+ T cells of the adult, though this did not reach statistical significance in the liver. Neonatal liver also had significantly higher numbers of Tregs and natural Tregs (nTregs), a subpopulation of Tregs defined by expression of the Ikaros transcription factor, Helios, that develop intrathymically and not in response to foreign antigen, when compared to neonatal spleen as well as to adult liver.[203] Because of the

role of the liver as a primary lymphoid organ in fetal life, this finding likely reflects the need for tolerance to both self and maternal peptide during embryological development.

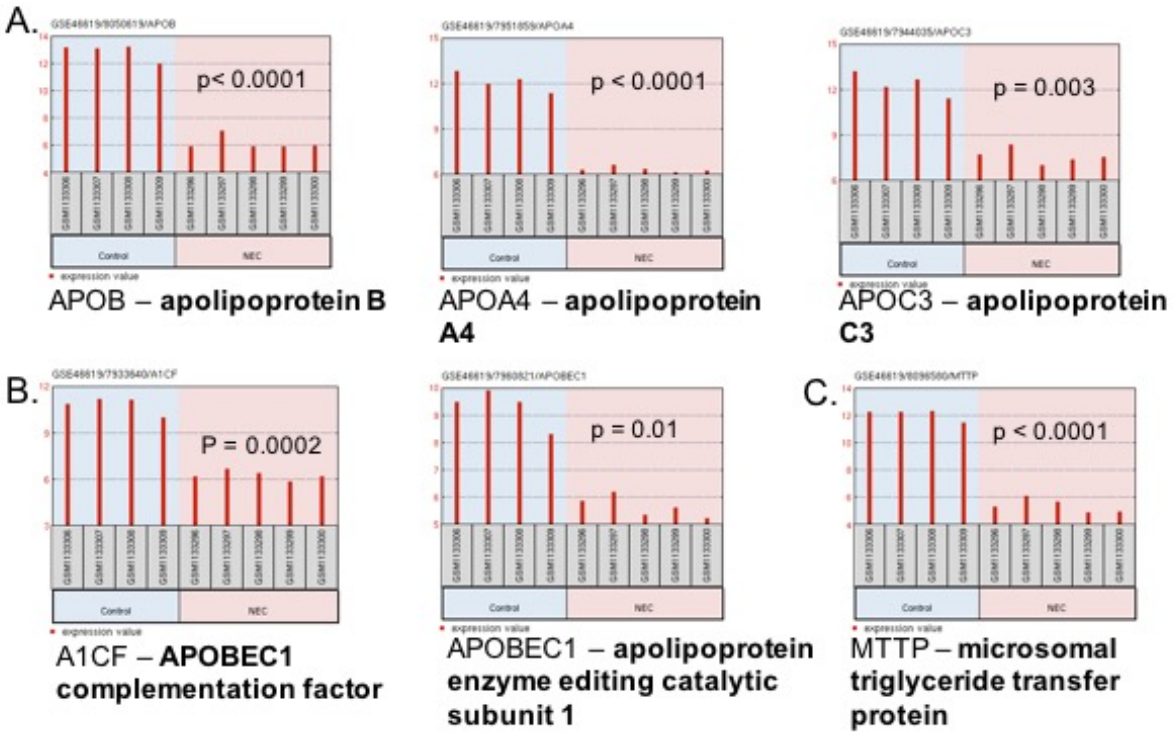


Figure 19. Differential gene expression of genes governing lipid metabolism. Blue column shows control (n=4) and red column shows NEC (n=5) specimens. Statistical significance set at p<0.05

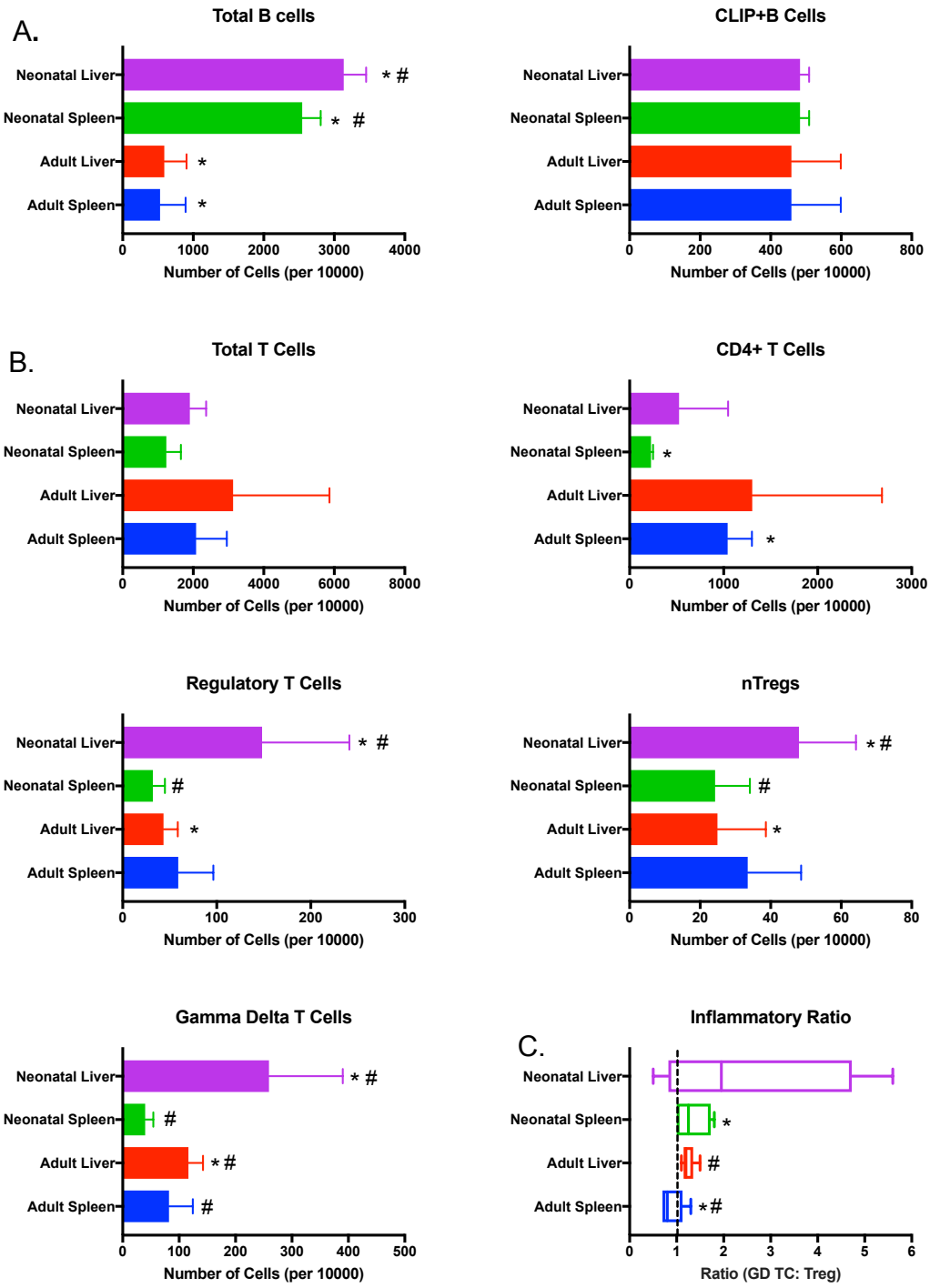


Figure 20. Lymphocyte frequency and subsets in adult and neonatal liver and spleen. A. A significantly higher B cell frequency was found in neonatal liver and spleen compared to adults with no significant difference found in CLIP+B cell frequency, suggesting much lower proportion in neonatal liver and spleen. B. Similar T cell numbers found in all tissues in adult and neonate. Neonatal liver was found to have higher levels of Tregs, nTregs and GDTCs than neonatal spleen and adult liver. C. GDTC:Treg ratio in favor of GDTCs in all tissues except adult spleen. * denotes statistical significance between same tissue in adult v. neonate. # denotes statistical significance between spleen and liver in same group. Statistical significance set at $p<0.05$ for comparisons of means using Student's t test.

Finally, we also found a notable increase in GDTC frequency in neonatal liver compared to adult with a significant decrease in neonatal compared to adult spleen. Because the cytokine-expression profile of these GDTCs was not evaluated, we do not know if they are the inflammatory IL-17-producting GDTC or a tolerizing subpopulation. The ratio of GDTC to Treg in all organs and age groups favored a GDTC-predominant profile except in adult spleen, though these differences appeared more marked in the neonate. Given the accompanying proportions of lower CD4+ and higher Tregs, we anticipate that GDTCs found in the neonatal liver of dam-fed babies are likely a tolerizing subset that proliferate prior to encountering food or enteric pathogens. Because animals were not caged in a germ-free facility, this may also reflect a response to the microbiome inherited from the mother.

Lymphocytes in Liver Show Marked Increase of Gut-homing Chemokine Receptor CCR9

Next, we explored the possibility that the T cells found in the liver expressed markers that directed their interaction with enteric antigens. T-cell homing is regulated by specific chemokine receptor expression and the chemokine receptor CCR9 (CD199) has been shown to be a marker for homing to the small intestine.[204,205] CCR9 has also been shown to be important specifically in GDTC migration and/or development in the intestine, with CCR9 KO animals showing lower frequencies of GDTCs in the small intestine.[206,207]

We examined the expression of CCR9 by fluorescence staining of adult splenic and hepatic T lymphocytes to determine first if there were tissue-specific differences in

intraabdominal viscera. All T cells subsets from the liver had observably increased levels of CCR9 expression, while CCR9 levels in splenocytes were essentially undetectable (Figure 21A&C). This suggests that larger proportions of T cells in the liver are intended for interaction with enteric antigen. Interestingly, CD8+ and CD4+ cells expressed CCR9 more frequently in the adult compared to GDTCs, in contrast to our expected result. We interpreted this to highlight the difference in effector T cells that had encountered enteric pathogenic peptides (CD8+ and CD4+) versus nutritional lipids (GDTC), with the expectation that adults had encountered both higher loads of pathogenic flora as well as a more nutritionally complex diet with advancing age.

We then compared neonatal liver to the adult in order to further examine this theory (Figure 21A-C). Neonatal liver and spleen both had observably higher populations of CCR9+ GDTCs than adult liver, though this was not statistically significant in the liver. Again, this suggested to us a separate subpopulation of GDTCs necessary for neonatal development found in dam-fed infants, rather than inflammatory IL-17+GDTCs promoted by high lipid concentrations.[173,179]

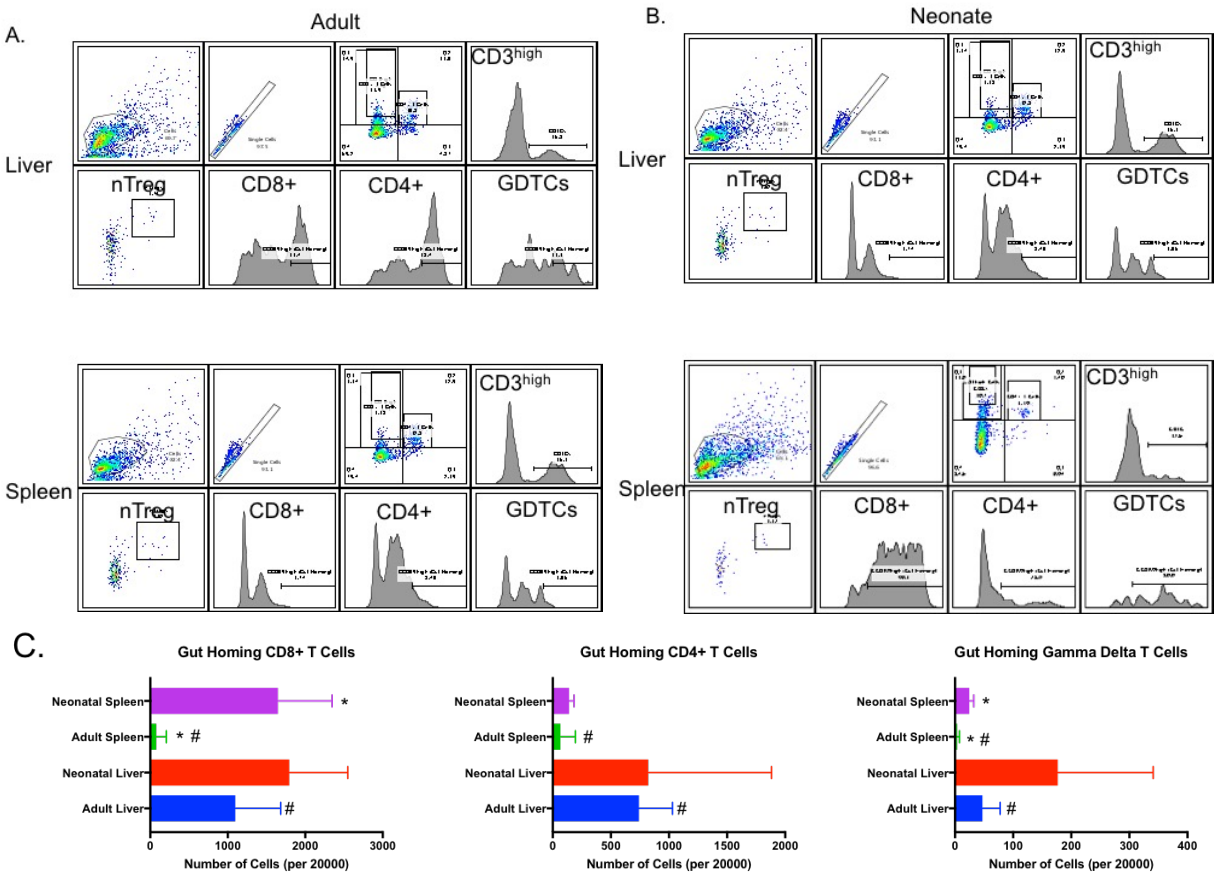


Figure 21. Flow cytometric analysis of T cell populations in adult and neonatal liver and spleen. A. Representative gating analysis of adult is shown in liver and spleen. Upper panel: Cells were first characterization by forward (cell size) and side scatter (nuclear granularity). Doublets were excluded. Staining for CD3 and CD4 was then visualized to isolate CD8+ T cells (CD3+CD4-), CD4+ T cells (CD3+CD4+) and GDTCs ($CD3^{high}$CD4-). Lower panel: nTregs were gated from the CD4+ cells by identifying $FoxP3^{high}Helios^{high}$ cells. CCR9 staining was then identified by comparison to isotype controls in CD8+, CD4+ and GDTCs, respectively. Numbers shown were frequencies of parent gate. B. Similar flow cytometry analysis was conducted in pooled neonatal liver and spleen (3 organs per group). C. CCR9 expression on T cell subsets in adult and neonatal liver and spleen. * denotes statistical significance between same tissue in adult v. neonate. # denotes statistical significance between spleen and liver in same group. Statistical significance set at $p<0.05$ for comparisons of means using Student's t test.

Formula Feeding Increases CLIP+B Cells and GDTCs in Spleen

Finally, we wanted to show the *in vivo* effect on lymphocytes in response to formula-feeding, as the anatomic microenvironment was felt to be crucial to this process. We examined the spleens of 10-day-old neonatal mice which had been either

dam-fed (n=4) or formula-fed (n=8) (Figure 22A and B, respectively). Dam-fed mice showed low numbers of both B and T cells in comparison to formula-fed mice. T-cells populations were comprised of much lower percentages of GDTCs and B cell population of lower percentages of CLIP+ cells. Formula-fed mice had six times more T cells, of which there was a three-fold increase in GDTCs. Formula feeding also led to a doubling of B cell frequency with a 10-fold increase in CLIP+B cells. Comparison of these groups also showed a separate population of B220+CD3+ cells, which has not previously been described in the literature but were predominantly GDTCR+. When we examined the livers of these two groups with immunofluorescence staining, there appeared to be more CD3+ T cells in the formula-fed livers, but equal numbers of GDTCs (Figure 22D top and middle panels).

Our last step was to determine how an insult to the intestinal epithelium, as is assumed to occur in NEC, affected these lymphocyte profiles. We found a doubling of GDTCs detectable in spleen compared to formula-fed controls and a 25-fold increase of in the B220+ GDT cell population. In contrast, CD3+ T cells and GDTCs became undetectable in the liver. Additionally, the population of splenic CLIP+B cells was reduced by half when compared to formula-fed infants.

Together, we interpreted these findings to mean that formula-feeding led to amplified lymphocyte populations, especially GDTCs and CLIP+B cells, which proliferate in the spleen. Higher numbers of hepatic GDTCs were not observed in response to formula-feeding. This leads us to believe that a subset of naïve GDTC proliferate and migrate from the spleen to the liver (including B220+GDTCs), which functions as the repository of gut-homing GDTCs that react in response to

environmental cues from nutritional or infectious factors. Upon breach of the intestinal epithelium, CLIP+B cells from the spleen and gut-homing GDTCs from the liver then migrate to intestine to effect an inflammatory immune response.

VI.4 Summary

Our exploration of differential gene expression of the tissues of actual babies affected by NEC first substantiated the need for further research into this area. Decreased apoB expression and editing may cause an altered ability of both the liver and intestine to utilize and transport lipids in complex lipoproteins, causing an overabundance of lipid in the microenvironment of the cell populations critical to this disease. Aberrant fat digestion through the enterohepatic circulation has been shown to have a direct toxic impact on the small intestine.[208] Previous groups have demonstrated an increase in CD4+ and CD8+ T cells in LDL receptor knockout mice in response to high-fat diets.[48] We had previously been able to demonstrate that lipoprotein availability and character directly affect immune cell populations and that high LDL levels correlate with a pro-inflammatory immune phenotype via IL-17 and/or GD T cells. This study then shows that lipid metabolism is crucial to the development of NEC, a finding that has not been described previously.

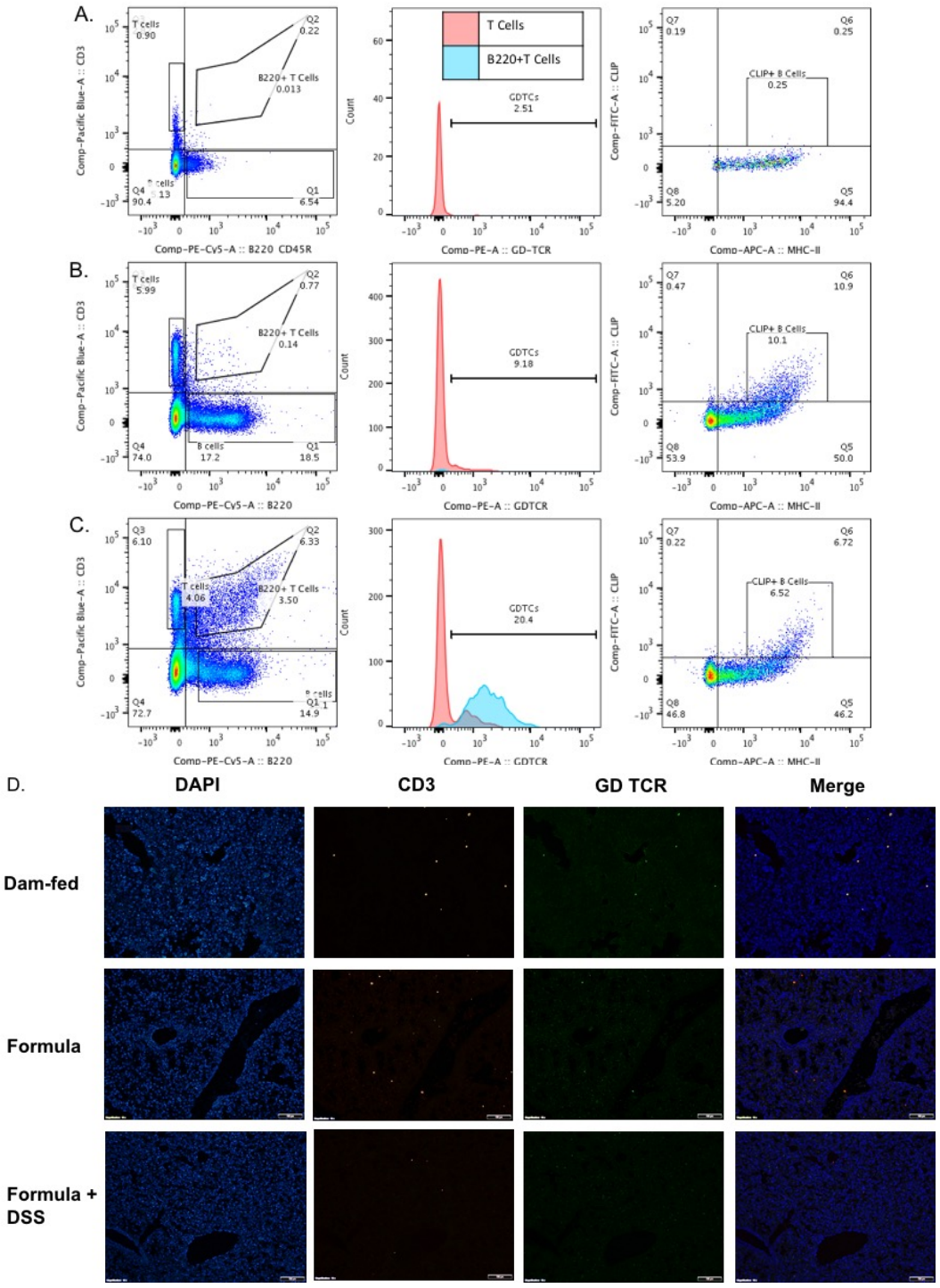


Figure 22. Flow cytometry of pooled spleens of neonatal mice. A. Dam-fed x 10 days (n=4). T cells identified as CD3+B220- cells and B cells as CD3-B220+. T cell gate then analyzed for GDTCR staining compared to isotype control; B cells identified as CD3-B220+ and gated for CLIP and MHCII to identify CLIP+B cells. B. Formula-fed from DOL 5-10 (n=8) or C. Formula-fed and treated with 1.5%DSS from DOL 5-10 (n=8). Numbers shown are percent of parent gate. Statistical analysis was not conducted due to pooling of tissue for adequate cell yield. D. Immunofluorescence staining of liver sections for nuclear counterstain with DAPI (first column), PE-CD3 to identify T-cells (2nd column) and FITC-GDTCR to identify GDTCs (3rd column).

Our previous studies demonstrated that LDL leads to increased IL-17+ cells and GDTCs and decreased CLIP+B cells, while HDL promotes Treg proliferation. We were able to further characterize and implicate this inverse relationship of GDTCs and CLIP+B cells *in vivo* in response to formula-feeding, suggesting this leads to priming of the immune system towards inflammation/autoreactivity and poor enteral tolerance.

We also observed much greater B cell and Treg/GDTC populations in neonatal liver compared to the adult. This is consistent with the very recent finding in humans that fetal liver is critical to lymphopoiesis of lipid-derived tolerizing B cells, which may persist through adult life.[209] The function of the observed GDTCs in our study is not known, but we expect the B220+GDTCs that we have first described here to parallel the ontogeny and function of the tolerizing B cells, in the absence of lipid aberrations or other factors that push them towards autoreactivity and inflammation.

The implications of lipid metabolism in the development of NEC are novel but, we believe, also significant. Further studies are needed to delineate the influence of lipid metabolism on its pathology. Aberrant lipid metabolism or lipoprotein assembly and delivery may even correlate with many of the long-term phenomena observed in the babies who suffer NEC, including the disproportionate incidence of IUGR, intestinal failure-associated liver disease, and even neurodevelopmental disability.[210–213] If our data are further substantiated, these observations will signal the need for a paradigm shift in the treatment of neonatal NEC.

In the future, cholesterol metabolism genes may be utilized for diagnostic purposes to identify babies at highest risk for development of this disease. Because lipid-lowering therapies via statins, changing of lipid formulations in baby formula or

entirely depending on banked breast milk for nutrition are relatively easy therapies, earlier intervention with these agents in identified babies may finally improve the morbidity and mortality of this life-altering disease.

VII. SUMMARY AND CONCLUSIONS

VII.1 Summary

In vitro, we showed that complex lipoproteins LDL and HDL had direct effects on directing the phenotype of T lymphocytes. HDL had an overall anti-inflammatory effect in our studies, as increasing HDL appeared to decrease frequency of IL-17-producing T cells but expand the Treg proportion of CD4+ T cells. Conversely, LDL was shown to increase the GDTC subset of the T cell compartment without having a significant effect on Treg numbers. We then explored whether this effect was due to the B cell acting as professional antigen presenting cell intermediary. In the case of the HDL-Treg dynamic, no such relationship was observed. However, LDL supplementation caused an increase in expression of co-stimulatory molecule CD80 (B71) while at the same time a decrease in CLIP expression. This suggested that LDL likely displaced CLIP in MHCII causing co-expression of CD80, an interaction which led to the observed proliferation of GDTC.

Because the liver is the central organ for processing of dietary lipids into lipoproteins, we then aimed to characterize the differences in hepatic lymphocyte populations. We hypothesized that the liver, well-known to be tolerogenic in hepatic transplant patients, would have a much higher proportion of Tregs than spleen. Interestingly, we found the converse, with a high proportion of CLIP+ B cells and GDTC in adult murine liver. Furthermore, neonatal liver had an even greater subset of hepatic GDTC. Additionally, GDTC of neonatal liver showed a high expression of the gut-homing molecule CCR9. Together, we believe this signifies a differing role for liver-

specific GDTC that is tolerogenic rather than the classic inflammatory IL-17-producing GDTC found in the gut.

Next, our experiments using different strains of mice and mouse models endeavored to show how these effects of lipids and complex lipoproteins on immune cells were significant at an organismal level. Lymphocytes from BALB/c mice with defects in beta oxidation were found to have a preferential decrease in both baseline Treg number and Treg proliferation in response to antigen. This suggested that dysfunctional lipid metabolism would attenuate tolerance to antigen. To explore this *in vivo*, we isolated liver and spleen in our neonatal mouse model of formula-feeding and intestinal epithelial damage using DSS. Here, we found that formula-feeding led to increased CLIP+B cells and GDTC in spleen compared to breast milk feeding. Breach of intestinal epithelium led to an exaggerated proliferation of GDTC, especially B220+ GDTC, but a decrease in splenic CLIP+B cells. Meanwhile, formula feeding did not change hepatic GDTC numbers, though DSS supplementation led to a complete absence of detectable GDTC in the liver.

Our clinical data from human babies recapitulates the importance of these findings and points us to the next stage in these studies. We showed that babies with IUGR born to mothers with PE had a nine-fold higher risk of NEC than IUGR babies born to non-PE mothers. Examination of cord blood in babies with IUGR showed decreased frequencies of normally functioning Tregs, while non-IUGR pre-term babies were found to have high frequencies of less functional Tregs compared to term normal-sized controls. Additionally, dyslipidemia in PE has previously been linked to fetal beta oxidation defects, though this has never been linked to NEC. Using public microarray

data, we found that babies who suffered from surgical NEC had decreased expression of genes necessary at multiple stages of lipoprotein assembly and delivery, including APOB (transcription of apoB-B), APOBEC1 (post-translational modification of apo-B100 to apo-B48), and MTTP (secretion of apoB-B containing lipoproteins). Together, this hints that intact lipid metabolism is crucial for tolerance, and derangements may thus lead to amplified severity of inflammatory conditions such as NEC, especially in the context of an underdeveloped or primed immune system as seen in premature formula-fed infants.

VII.2 Conclusions

Despite being one of the most highly investigated disease processes in pediatric research, the morbidity and mortality of NEC has remained largely unchanged over the last several decades. The fact that it almost exclusively affects premature and IUGR babies who are fed formula rather than breast milk is well-known by even the most junior trainee in both pediatrics and surgery. Yet, this widespread awareness and vast body of research has as yet resulted in no satisfactory methods to either pre-emptively pinpoint those babies who will develop NEC or prevent the progression of low-grade medically manageable NEC to severe surgical disease characterized by transmural necrosis or even perforation. Approximately 20% of NICU costs, totaling about $5 billion annually, continue to be due to NEC, with babies who require surgery spending upwards of three months in the NICU after birth.[214] Of the 70% who survive surgical NEC, long-term problems including neurodevelopment delays, short-gut syndrome, lifetime total parenteral nutrition, hepatic failure or even need for bowel transplant

prevent achieving a normal childhood and acceptable quality of life. Thus, the next steps in this investigation should be to translate our findings into either preventive, diagnostic or therapeutic tools.

Two pathways emerge from our studies that may be used for diagnostic purposes. The first and more obvious one is to screen neonates for lipid metabolism abnormalities, especially those that we have previously described as significant in surgical NEC. Further studies on a population level are needed to determine which derangements are associated with NEC and whether they have predictive value of disease severity. The other option is to quantify either CD80 or GDTC in peripheral blood of formula-fed infants as a predictor of intolerance. This may be less fruitful, however, due to the nonspecific nature of inflammatory markers in general, but may have promise as a screening tool to identify babies at risk.

If population-based studies indeed show a significant causative relationship of aberrant lipid metabolism in the development of NEC, this also points to a role for early-initiation of lipid-lowering therapies in at-risk populations. While breast milk remains the ideal option, perhaps in those babies whose only nutritional option is formula, cholesterol binding agents such as ezetimibe may decrease incidence or severity of NEC. Additionally, this suggests a re-formulation of the lipid component of commercially available baby formula may be needed, potentially through restructuring of lipids to more closely resemble MFG.

Finally, our studies found a novel population of GDTC that proliferated in response to intestinal mucosal damage. Further exploration is needed as to the nature of these lymphocytes as GDTCs remain an enigmatic population. However, if, as our

studies suggest, GDTC are the culprits in the inflammatory manifestations of NEC, this opens up multiple avenues for therapeutic strategies. Previous studies in mouse models suggest a role for adoptive transfer of Tregs in ameliorating intestinal inflammation, which our findings loosely support. However, other strategies such as GDTC depletion therapies or displacement of lipid antigen from MHCII to prevent antigen recognition by GDTCs may prove even more useful to turn off the specific immune response that leads to necrosis and perforation. Though there still remain many more questions than answers in this seemingly incurable disease, we believe our data lay the groundwork for a novel interpretation of this disease and finally point to the promise for a cure.

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