THE GUT MICROBIOTA AND THE LIVER: COLLABORATORS IN HOST IMMUNITY AND METABOLISM

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The **gut microbiota** consists of over 10^{14} commensal bacteria required for proper gut immunity development. Commensals also augment the host's ability to extract energy from the diet. Although restricted to the gut lumen by intestinal barrier epithelia, commensals shed microbial associated molecule patterns (MAMPs) into the circulation where they augment aspects of systemic immunity. Commensals also release fermentation byproducts into the portal blood stream. Since the liver receives 80% of its blood via the portal vein and contains a unique repertoire of immune cells particularly enriched in **Kupffer Cells** (KC) and **Natural Killer T cells,** we proposed that gut-derived MAMPs contribute to the development of residential hepatic leukocyte subsets. Because of the contributions of gut bacteria to digestion, we suspected that gut bacteria add an additional level of regulation to host metabolism and would generate a specific hepatic metabolic gene profile.

Results showed that a cocktail of MAMPs translocate into the portal circulation of normal conventional (CL) mice stimulating KC expansion. ICAM1 expression, thought to be constitutive on sinusoidal endothelium, was significantly reduced without gut bacteria and was required for KC accumulation. The finding that constitutive ICAM1 expression by LSEC was dependent on gut bacteria lead us to investigate if the frequency of intra-hepatic lymphocytes known to bind ICAM1 were affected by gut bacteria. Results showed that intra-hepatic T

lymphocyte populations including NKT (TCRβ+NK1.1+) cells and T helper (CD4+TCRβ+) cells were significantly reduced in GF mice and AVMN mice.

In addition to the significant cellular composition changes of the liver related to gut bacteria density, notable changes in murine weight and **metabolic gene profiles** were observed. The average body mass of CL, GF, and AVMN mice was 37.8g, 33.4g, and 34.1g respectively. Our whole-liver gene array analysis included 217 probe sets mapped to 163 differentially expressed genes between groups, of which forty-eight have roles in lipid metabolism.

In conclusion, gut bacteria affect both the hepatic metabolic gene profile and the inflammatory potential of the liver. These finding have implications for many hepatic pathologies including obesity, NAFLD, and autoimmune disease like PBC and AIH mediated by liver leukocytes.

ABBREVIATIONS

APC- Antigen Presenting Cell

AVMN- Ampicillin/Vancomycin/Metronidazole/Neomycin-treated

BEC- Biliary Epithelial Cell

CL- Conventional

DAMP- Damage Associated Molecular Pattern

DAP- Diaminopimelic Acid

DC- Dendritic Cell

FLA- Flagellin

FSP-1- Fibroblast Specific Protein 1

GF- Germ Free

GI- Gastrointestinal

ICAM-1- Intercellular Adhesion Molecule 1

ILF- Isolated Lymphoid Follicle

KC- Kupffer Cell

LPS- Lipopolysaccharide

LRR- Leucine Rich Repeat

LSEC- Liver Sinusoidal Endothelial Cell

MAMP- Microbe Associated Molecular Pattern

MDP- Muramyl Dipeptide

MLN- Mesenteric Lymph Node

MPS- Mononuclear Phagocyte System

NICU- Neonatal Intensive Care Unit

NK- Natural Killer

NPC- Non-parenchymal Cell

NLR- NOD-like Receptors

PAMP- Pathogen Associated Molecular Pattern

PDG- Peptidoglycan

PP- Peyer's Patch

PRR- Pattern Recognition Receptor

RLR- RIG-like Receptors

SAMP- Symbiont Associated Molecular Pattern

SC- Stellate Cell

SCFA- Short Chain Fatty Acid

TCR- T Cell Receptor

TLR- Toll-like Receptor

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PREFACE

‘If anyone has been able to do it before, you can do it too. If no one has ever done it, you can be the first.” –Ralph Marston

Truthfully, I am in disbelief at arrival of this moment when I can pause in time to reflect on my journey and look back at how far I have come. While I carried much of the burden and triumph, I did not get to this place in time on my own.

I would like to thank my mentor, Jake Demetris, for taking a chance on me. We are probably as different as any surgeon is from a pathologist, but we have learned a lot from each other in this venture. Dr. Demetris has given me the freedom to think, design, execute, and fail experiments, which in my opinion, has allowed me to develop into an independent investigator and a better scientist.

My thesis committee has been instrumental in all steps of my graduate training. The suggestions at each committee meeting and individual meetings have been invaluable. The career advice from surgeon-scientists Drs. Billiar and Hackam has only been encouraging. I have no words to express my gratitude for the motivational support and acts of kindness shown to me by Drs. Morelli and Stolz. I can only hope to maintain my relationship with them as I continue my training to become a physician-scientist.

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Lastly I would like to thank my friends in the immunology and pathology graduate programs who have made so many scientific suggestions, provided reagents, and listened to me ramble on about mouse poo. I would not have arrived at this place without them either. Where "this place" is, I am not sure, but I do know that it is a major milestone for me and for my family. No one in my family has made it to this point before, but my family has taken tremendous pride in accompanying me for the ride and for that -I am thankful.

1.0 INTRODUCTION

The contributions of the gut microbiota to host health and disease are being redefined at an alarming pace. The gut microbiota extends its affects to virtually every organ system of the entire body (reviewed in (1)), yet the affects of the gut microbiota on the liver have not been thoroughly explored with the exception of the endotoxin-related studies primarily carried out *in vitro*. In the subsequent sections, the gut microbiota and liver will be characterized in effort to establish the connection(s) between the two regarding liver immunity and metabolism.

1.1 GUT MICROBIOTA

1.1.1 Establishment of the Gut Microbiota

Like all vertebrates, highly complex and genetically diverse communities of microbes inhabit our bodies. The gut microbiota, residential microbes of the gastrointestinal tract, exist in largely stable but dynamically interactive climax communities (2). However, we are not born bearing microbes (3, 4). The complex ecosystem forming the gut microbiota results from the successive establishment of *bacteria* best suited for intestinal niches beginning at birth and extending into early childhood (2) (3, 5-9).

Bacteria make contact with skin and mucosal surfaces such as the urogenital, respiratory, and gastrointestinal tracts (4). Residential gut bacteria (commensals, mutualists, microflora, microbiota) are transferred to offspring primarily from their mothers during delivery. Reservoirs for human gut bacteria include the maternal fecal flora, maternal skin flora, maternal vaginal flora, breast milk, hospital staff, and transmission between infants to name a few (5, 6, 10). Like human infants, rodent offspring acquire gut bacteria from their mothers, littermates, and environment as well. Our environment serves as a reservoir for aerotolerant bacteria (i.e. Enterococci) and bacterial spores (i.e. *Clostridium* spp.) (5).

The influences of the intestinal colonization pattern are multi-factorial. Delivery mode (vaginal delivery versus Cesarean section), feeding mode (breast milk versus formula), diet, family structure (only child versus siblings), course in NICU, hospital hygiene, non-familial care, country of residence, age, gender, and host genome (5) (10-12) (13). Infants delivered by Cesarean section were found to have a lower ratio of Anaerobes to Facultatives at one year of age suggesting Cesarean section contributes to a lag-time in microbiota maturity (5). Breast milk is enriched in many bacteria, specifically Bifidobacteria, which is not typically acquired by formula/bottle-fed infants (5). Children without older siblings also exhibit a lower ratio of Anaerobes to Facultatives at one year of age (5). Differences in colonization patterns were observed in infants of different hospital wards (12). Likewise, differences in colonization patterns were noted in mice from different commercial providers and/or housed in different rooms of a single animal facility (13). High overlap of gut microbiota between family members (most similarity in monozygotic twins) supports host genetics as a contributing factor influencing colonization pattern (10, 14). Diet directly affects gut microbiota development because specific bacteria have preferences for particular metabolic substrates (12). While many factors dictating

colonization pattern of the gut microbiota are fixed (i.e. genetics), many factors can be exploited (i.e. diet) and the capacity to manipulate these factors is evident particularly during the transition to a stable microbiota and perturbations thereafter.

The "Christopher Columbus" colonizers of the gut are heterogeneous and form unstable microbial communities distinct from the adult microbiota (15). The first bacterial species to arrive to the gut are essentially Facultatives and aerobes including *Escherichia coli*, *Enterococci faecalis*, and *Enterococci faecium* (5). Bifidobacterium, Clostridium, and Bacteroides also dominate in infants (5). Neonates are rarely colonized by yeast or Lactobacilli (5). Following the arrival of Facultatives, which are able to survive in aerated environments, obligate anaerobes take foothold concomitant with the transition to weaning (3). As Facultatives expand, they use up oxygen thereby creating an increasingly anaerobic environment promoting the expansion of anaerobes and the decline of Facultatives (5). Some bacteria are suited to survive the transition from milk to solid diet (i.e. *Bacteroides thetaiotamicron*) while others only appear after the introduction of solids (i.e. Peptostreptococci) (5) (10). The introduction of solid food heralds a shift in the immature gut microbiota toward the adult microbiota (6). Maturation and stabilization of the gut microbiota is a continuous process lasting many years (5). However, 2.5 year-old human gut microbiota has nearly the same functional attributes as the human adult microbiota (6).

The climax communities comprising the adult microbiota of vertebrates are reportedly stable and resilient to disruption (2, 6). However, the perturbations imposed by stress, antibiotics, prebiotics, and probiotics are unavoidable and their effects are only recently being fully understood. Human and rodent exposures to stress lead to compositional alterations in the gut microbiota (2). Stress induces neuroendocrine hormones, which increase GI motility and reduces

energy substrate availability (2, 12). Antibiotics target members of the gut microbiota, enabling other members to expand or new members to become established in the microbial community. Changes in gut microbiota composition have been noted with three days of Ciprofloxacin administration in humans (11). Many individuals will return to their pre-antibiotic state demonstrating only a transient response, but others are unable to return to the community structure prior to antibiotic therapy (2, 11). Antibiotic therapy can contribute to permanent long-term effects on microbiota density and diversity especially with repeated perturbation (11, 16). On the other hand, prebiotics (fiber) and probiotics (live non-pathogenic bacteria conferring host benefit) typically only have transient affects on the gut microbiota (10, 16). Incorporation of probiotic bacteria and/or invasion of pathogen strains of bacteria may be predetermined by what phylotypes are already present in an individual (9). At present, the changes in gut bacterial density and diversity resulting from perturbation and/or manipulation of the gut microbiota are not entirely predictable.

1.1.2 Characteristics of the Gut Microbiota

The gut microbiota is primarily composed of bacteria (more than 99%), but also consists of small numbers viral and fungal members that have not been well characterized to date (4, 8, 15, 17). To earn residential status in the gut, autochthonous bacteria must be possess enzymes to harness energy from the host diet, be able to evade bacteriophages, resist their host's immune system, and have the ability to proliferate rapidly avoiding washout from peristalsis and bowel movements (14). Bacteria in the adult gastrointestinal tract are Facultative anaerobes and strict anaerobes. Facultatives can perform aerobic or anaerobic metabolism while strict anaerobes

cannot use oxygen because they lack the enzymes to do so (5). Aerotolerant bacteria (i.e. Enterococci, Lactobacilli, Enterobacteriaceae family) are predominant in the stomach and small intestine and strict anaerobes (i.e. Bacteroides, Bifidobacteria, Clostridium, Fusobacteria, and Peptostreptococci) are predominant in the large intestine (5, 18, 19). Anaerobic species (97% of microbiota density) reportedly outnumber Facultative species (3% of microbiota density) by a factor of one thousand (7, 18). The change in oxygen and availability of substrates allows the gastrointestinal tract to support a complex bacterial community with spatial heterogeneity (8, 18).

In addition to the longitudinal differences in the microbial communities of the gut microbiota, cross sectional differences occur as well. Bacteria with pili and those suited to utilize mucus polysaccharides for fuel are more commonly found adherent to the mucosal epithelium and within the mucus layer (15). Autochthonous gut bacteria belong to two major bacterial divisions, Bacteroidetes and Firmicutes, constituting over 90% of the phylogenetic categories characterized in both human and rodent microbiota (10, 14-16, 20), Despite the fact that only two of fifty-five known bacterial divisions have been identified within the gut, the microbiota exhibit increasing density and diversity in a spatio-temporal manner (Figure 1).

1.1.3 Bacterial Density in the Gut

The microbiota is estimated to contain 100 trillion (10^{14}) bacteria, the highest of any microbial habitat (10, 14, 20). At this density, microbiota members outnumber human cells by a least one order of magnitude (2-4, 10, 18). Bacterial density ranges from less than 10^5/g luminal content in the small intestine to up to 10^{12}/g luminal content in the large intestine (2, 19). The acidity of the

stomach poses as a bottleneck limiting the numbers of upper gastrointestinal bacterial residents (4). The stasis near the ileocecal valve promotes increased bacterial density (5). Lactobacilli, despite their notable immunosuppressive affects, are ubiquitous among individuals but only in low frequency (2, 5). The genus Bacteroides was found to be the most prevalent, but also the most variable among individuals from around the world (15). The most abundant genus in mice was Clostridium and Bacteroides was a close second (2). The mucosal immune system keeps bacterial density within limits, but perturbations (i.e. antibiotics) result in dysbiosis and changes in bacterial abundance.

Stool and intestinal samples have been utilized to analyze bacterial density and diversity. Assessment of bacterial density and diversity was previously quantified and qualified using culture based techniques before the 1970's. The fastidious nutrient requirements and oxygen requirements have made it almost prohibitive to use culture techniques to identify the vast majority of anaerobes colonizing the gut. DNA based techniques of bacterial detection revealed that less than 50% of the microbiota members are cultivatable (5, 18, 19, 21). 16S rRNA gene amplification can be used to enumerate bacterial species, while whole gene or partial gene sequencing of the variable gene region can be used to assess bacterial diversity (3). However, even DNA based techniques are limited by the frequency of bacteria with the microbial community found in the gut (5).

1.1.4 Bacterial Diversity in the Gut

Bacterial diversity in the gastrointestinal tract has received a tremendous deal of attention with the Human Microbiome Project (HMP) and The Human Gut Microbiome Initiative (HGMI).

Both human and rodents harbor massive numbers of microbes (22). Gut bacteria differ between individuals and within individuals spatially descending down the gastrointestinal tract and temporally (5, 15). Within gut microbial communities, most taxa change relative to each other on a day-to-day basis (11). Interpersonal variation is greater among infants than adults (6) and interpersonal variation is less than inter-individual variation (22). Understanding the level of complexity and changes in microbiota diversity is confounded by the complexity of sampling, DNA processing, and variability in sequencing protocols on one side and the host physiology, nutrition, and environment on the other side (15). Still, the human microbiota has an estimated one thousand species (4, 7). Qin et al. found that 18 species were common among 124 individuals, 57 species were common in 90% of the same individuals, and 75 species were common among 50% of the same individuals (20). Manichanh et al. demonstrated that the rat microbiota possessed more species diversity than human samples (in comparison to two sequenced female microbiomes) (16). Mice have more variable diversity, but some species have been documented to possess between 30 and 500 species, which overlap with the human microbiota (2, 9).

The concept of a “core” gut microbiome defined by a conserved consortium of species or bacterial gene pool shared among individuals has been proposed. A core microbiome could ensure conservation of metabolic function performed by gut bacteria (9). However, despite functional redundancy among gut bacteria, a single bacterial phylotype was not detectable among 154 individuals examined in the study performed by Turbaugh et al. (22). Arumugam et al. characterized the microbiota of 22 individuals and categorized individuals with “enterotypes” based on the predominant genera (Bacteroides, Prevotella, Rumminococcus), which functionally differ in carbohydrate consumption (15). In the future, the “core” gut microbiome will have to be

further defined in the face of extensive bacterial diversity influenced by endogenous and exogenous factors. Whether or not a functional or phylogenetic "core" microbiome exists, bacterial diversity or lack thereof may become a prognostic factor many diseases. Blunted microbiota diversity with reduced levels of Bacteroidetes has been identified in both obese mice and humans (10, 14). In addition, both Crohn's Disease and Ulcerative Colitis are associated with reduced microbial diversity in the gut (16, 20).

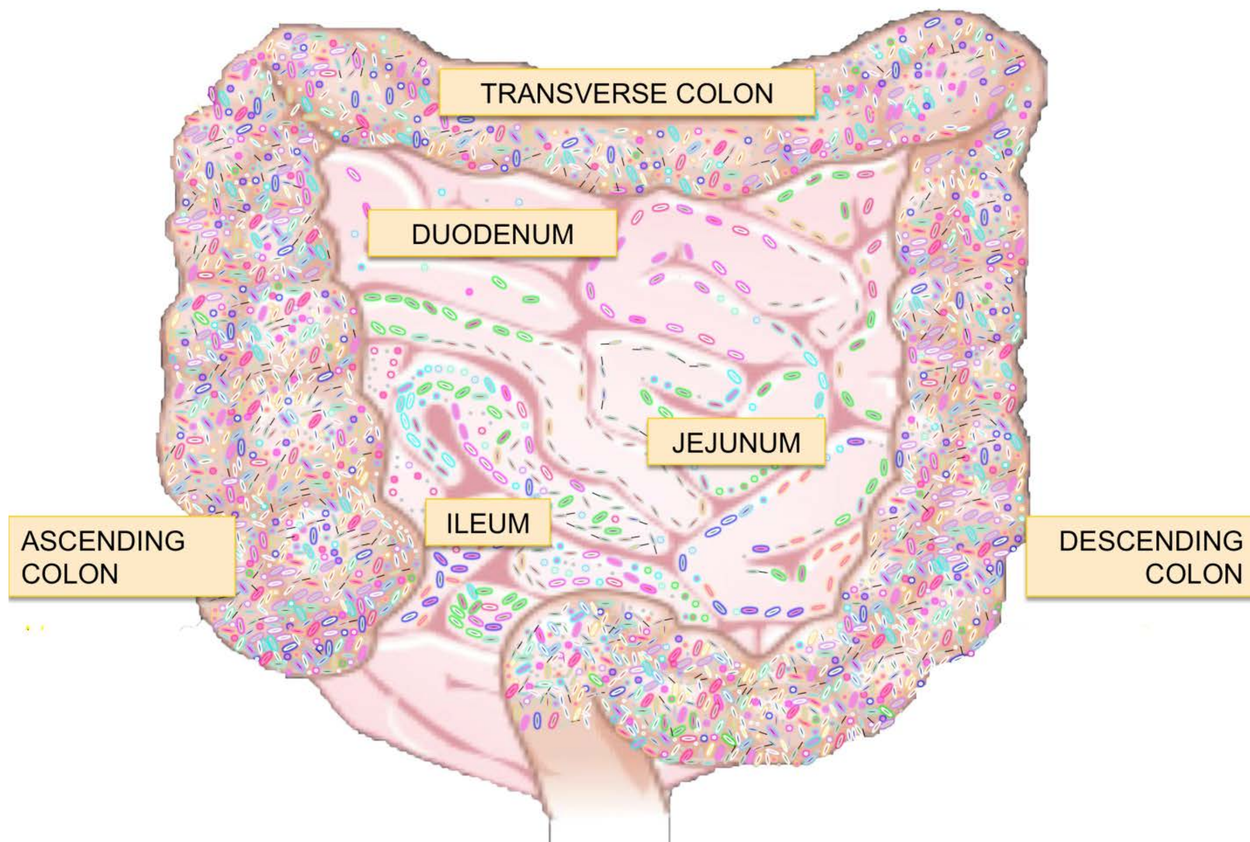


Figure 1. Bacterial Density and Diversity in the Gut.

1.1.5 Functions of Gut Bacteria

Gut bacteria are frequently called commensals inferring that they are akin to non-rent paying tenants, when in actuality they are mutualists conferring many benefits to the host. The co-evolution of bacteria and vertebrates has resulted in a relationship where the normal function of the host gastrointestinal organ system is dependent on gut bacteria for normal function (12). The human microbiota encodes one hundred times as many genes than the human genome in its entirety (20). The extensive gene pool within the gut microbiota enables gut bacteria to participate in metabolism, intestinal function, colonization resistance, and immune system development.

Gut bacteria are major players in host metabolism with unique fermentation capacity. Gut bacteria metabolize complex plant polysaccharides and mucus that would otherwise be indigestible forming short chain fatty acids (SCFA) that are consumed by host cells for energy. The SCFA are acetate, propionate, and butyrate produced in a ratio of 60:25:15 (5, 10). Heart, muscle and brain cells utilize acetate, the liver uses propionate, and enterocytes use butyrate (20). Bacteroidetes are associated with carbohydrate metabolism whereas Firmicutes possess genes encoding transport systems for carbohydrate substrates (6, 10, 12). Moreover, gut bacteria function to metabolize amino acids, xenobiotics, and synthesize essential vitamins (i.e. vitamin K, biotin) (2, 4, 5, 19, 20). Collectively, the metabolic potential of the microbiota is thought to mimic that of the liver (4).

In addition to the basic metabolic functions performed by gut bacteria, hosts receive aid with intestinal development, immune system development, and colonization resistance from their abundant neighbors (10, 11, 18). Regarding intestinal development, gut bacteria increase mucosal thickness, increase crypt depth, and cause broader and shorter villi thereby increasing

the over mass of the small intestines (5). Moreover, gut bacteria promote intestinal epithelial cell turnover, mucus production, and peristalsis (5). The development of the mucosal immune system is only partially driven by intrinsic factors and requires cues from gut bacteria for full development (2, 3, 5). Studies in Germ Free rodents underscore the importance of gut bacteria to host immunity and will be discussed more extensively later. Lastly, just by being present, the gut microbiota function to prevent proliferation and establishment of new bacteria strains by competing for space and resources (4, 5, 7, 9, 12, 19). Loss of colonization resistance is a common side effect of antibiotic therapies that essentially reduce bacterial density and alter community structure (7). In summation, the functions of the gut microbiota are numerous and likely incomplete as further studies continue to examine host-microbial interactions.

1.2 BACTERIAL TRANSLOCATION

In order to maintain the homeostatic and delicate relationship between the intestinal compartment and gut bacteria, the host skillfully implores several physical and immunological components to restrain residential microbial communities. Without these defenses, the host would succumb to infection, illness, and possibly death terminating the mutualistic relationship. At the physical level, the gut is characterized by a single layer of epithelium enforced by tight junctions, intermediary junctions, and desmosomes between cells (4, 23). The tight junctions restrict macromolecules with their small pore size permitting passage of molecules < 600 Daltons (23). Barrier function is reinforced by a lubricating mucus layer, which serves to prevent bacteria from directly adhering to epithelium and invading the single cell layer (4, 19).

Antimicrobial molecules (i.e. α-defensins) produced by paneth cells further strengthen barrier function in the mucosal compartment (4, 19, 24).

The gut barrier works in concert with the mucosal immune system to limit gut bacteria to the intestinal lumen. Both IgG and sIgA are produced in the mucosal compartment in response to colonization. Secretory IgA blocks adherence and penetration of bacteria to and past the intestinal epithelium (4, 25). Bacteria that bypass the gut barrier are rapidly removed by macrophages within the lamina propia immediately underneath the epithelium (19). Dendritic Cells patrolling the mucosal compartment occasionally sample gut bacteria, exit to mesenteric lymph nodes (MLN), and home back to the gut limiting their affects to the mucosal compartment (19). Peyer's Patches (PP) and isolated lymphoid follicles (ILFs) also prevent bacterial translocation from the intestinal compartment. Some bacterial species co-localize with such structures in mice, non-human primates, and humans including *Alcaligenes faecalis* (25). The physical and immunological barriers of the gut effectively keep gut bacteria in their place.

The intestine is not impermeable despite the wall created by the gut barrier. The intestine forms a selective barrier, permitting fluxes of nutrient, ions, water, and even dietary antigens (23). Intestinal permeability is facilitated by para-cellular diffusion and trans-cellular transport (23). Dietary antigens and bacterial products potentially travel between cells or across cells (23). Bacterial translocation to extra-intestinal compartments has rarely been proven in the healthy state (26). However, several factors can predispose to bacterial translocation including host immunodeficiency, stress-induced increases in intestinal permeability, enteric nervous system input regulating tight junction synthesis, manipulation of the gut during abdominal surgery, alteration of microbial communities (2, 4, 23, 26, 27). Compromised barrier function and/or increased intestinal permeability result in bacterial translocation.

Bacterial translocation is the exception, not the rule. Nonetheless, perturbations in the gut microbiota and gut barrier function ultimately lead to bacterial translocation and infection or exacerbation of concomitant diseases. For example, antibiotic therapy reducing competitors of *Clostridium difficile* promotes expansion of this intestinal colonizer and causes diarrheal illness plus mucosal injury (5). Likewise, bacterial translocation often results in bacteremia and septicemia in neonates with incompletely established gut microbiota (5). Characteristically non-pathogenic colonizers (i.e. Enterobacteriaceae) could potentially translocate given the right conditions, but obligate anaerobes rarely translocate from the gut lumen, as the oxygenated environment of extra-intestinal tissues would not be suitable for survival (5).

1.3 MICROBIAL DETECTION

1.3.1 Microbiota Ignorance

In spite of the gut bacteria preventing bacterial translocation, the peripheral body is not ignorant of gut bacteria. Historically, mice were thought to be systemically ignorant of microbiota based on the inability of colonizing gut bacteria to induce a humoral response with specific IgG production (19). However, absence of antigen specific IgG toward for microbiota members does not preclude gut-derived microbe-associated molecules from being able to stimulate systemic immunity. In fact, a broader influence of gut microbiota in the regulation of systemic immunity resulting from translocation of luminal bacterial products but not viable bacteria into systemic circulation is a growing area of interest (28). Further, Macpherson et al. proposed that the baseline setting of the immune system is a result of “contamination” of the body with microbial

products that have penetrated the gut barrier (19). This concept will reappear in the results section in relation to liver immunity development.

1.3.2 Pattern Recognition

In 1989, Charles Janeway proposed the idea of pattern recognition. He suggested that hosts should be able to detect conserved structures specific to microbes but not eukaryotic hosts (29, 30). He postulated that these "pathogen-associated molecular patterns" distinguishable from self, or PAMPs as they are commonly called, would also have to be common to a broad class of microbes so they could be detected by a set number of receptors and that the structures would be required for microbe survival otherwise selective pressures would promote their mutation and evasion from detection (31). Truthfully, the innate immune system relies heavily on the recognition of the evolutionarily conserved microbial structures (PAMPs) through a limited number of germ-line encoded pattern recognition receptors (PRR) just as Janeway proposed over 20 years ago (32). While PAMPs, by definition are supposed to be highly conserved, microbes do show significant diversity in PAMPs that may influence how they are recognized (33). The current understanding of microbe-PRR interaction was catapulted by the discovery of Toll-like Receptors (TLR) in 1997 (32). Since then the concept of PAMPs has been criticized because PAMPs are not restricted to pathogens and are present on all members of the microbiota, most of which are not pathogenic or virulent, and were subsequently renamed "microbe-associated molecular patterns" (MAMPs) (33). As our understanding of pattern recognition grows, PRR have been shown to recognize danger signals or "damage-associated molecular patterns"

(DAMPS), which mediate sterile inflammation (32, 34) as well as ligands that orchestrate host-commensal symbiosis labeled "symbiont associated molecular patterns" (SAMPs) (35).

Pattern recognition and the resulting downstream response is confounded by whether or not the host distinguishes between pathogenic and nonpathogenic microbes, detection of single MAMPs by multiple sensors, detection of multiple MAMPs by single sensors, coincident detection of multiple MAMPs by multiple sensors, and where in the host the MAMP is detected (30-33). The advantage of having multiple PRR recognize different components of a single microbe as well as different classes of microbes prevents immune evasion (32). The location within the body and within individual cells (extra-cellular, endosomal compartment, or cytosol) also provides information as to how a particular MAMP should stimulate the host.

Pattern recognition can affect the host in a multitude of ways. PRR, upon binding to specific ligands, often trigger pro-inflammatory and antimicrobial responses via activation of intracellular signaling pathways. The downstream signaling pathways of individual PRR are similar to each other and consist of recruitment of adaptor molecules, kinases, and activation of transcription factors (32). The two major categories of responses induced by MAMPs are transcriptional (i.e. gene expression) and post-translational (activation of genes expressed) (33). The end-result of PRR signal transduction includes (but is not limited to) the synthesis of cytokines, chemokines, adhesion molecules, immunoreceptors, and recruitment of leukocytes (4, 32). Recognition of MAMPs by PRR on antigen presenting cells (APC) like DC and KC triggers cytokine production, co-stimulatory molecule expression, and activation of T cells (4). Therefore, PRR-MAMP interactions are not only important for innate immunity and infection clearance, but also bridges to adaptive immunity.

How the immune system differentiates between the microbiota and infectious microbes remains an unanswered question (35). One theory is that host cells can distinguish between pathogenic live bacteria and dead or non-threatening bacteria/MAMPs based on the PRR that are activated. Live virulent bacteria are more equipped to penetrate the cell cytoplasm or secrete virulent factors into the cytoplasm (via type 3 secretion systems), where they may be detected by cytosolic PRR (33). Another theory is that infectious replicating bacteria release more MAMPs or shed concentrated MAMPs at the site of infection initiating a different response than that elicited by the translocation of MAMPs from the gut microbiota. Moreover, endocytosed bacteria or MAMPs are degraded into a form recognized by specific endosomal PRR and are typically not seen by cytosolic PRR. Therefore the delivery of MAMPs to specific cellular compartments ultimately directs the response. Subsequent sections describe the most well characterized PRR-ligand interactions.

1.3.2.1 Transmembrane Pattern Recognition Receptors

TLR are the most extensively studied receptors under the umbrella of pattern recognition receptors. TLR are homologs of *Drosophila melanogaster's* Toll family of proteins, which also function to detect microbes (36). The Toll protein of *Drosophila* was characterized for its involvement in dorso-ventral patterning, but was later found to be important to the antifungal response in the fruit fly (32, 36). TLR are key components of innate immunity triggering host antimicrobial responses when activated (37). As a subset of PRR, TLR function to recognize a broad spectrum of MAMPs.

TLR resemble each other in structure with common organization. They possess an extra-cellular domain, a transmembrane domain, and an intracellular domain. The extracellular domain

contains leucine rich repeats (LRR domain) (4). The LRR domain functions specifically in MAMP recognition either through direct binding or interaction with adaptor molecules. The LRR domains of TLR are horseshoe shaped and ligands may bind to both the convex and concave faces (29). The transmembrane domain connects the intracellular domain to the extracellular domain of TLR. The intracellular domain is called the TIR (Toll/IL-1R) signaling domain due to its homology to the IL-1 receptor (29, 32).

TLR initiate intracellular signaling upon ligand recognition. Signaling pathways involve primarily adaptor protein recruitment, and activation of mitogen activated protein kinase (MAPK) and transcription factors. The major adaptor protein for all TLR except TLR-3 is Myeloid Differentiation factor-88 (MyD88) (32, 38). MyD88 allows association of IRAK-1 (IL1 Receptor Associated Kinase) with the TIR domain (39). Other adaptor proteins that are recruited to the TIR domain include TIRAP (TIR domain containing Adaptor Protein), MAL (MyD88 Adaptor Like protein), TRIF (TIR domain containing adaptor protein Inducing IFNβ), TICAM (TIR domain Containing Molecule-1), and TRAM (TRIF Related Adaptor Molecule) (38). Alternatively, TLR-3 signaling relies on TIR domain interaction with TRIF and not MyD88 (32). Association of adaptor molecules with the TIR domain of activated TLR allows activation of MAPK, p38, ERK, and JNK (30, 38). Ultimately, inhibitor of NF-κB is released following phosphorylation, which allows NF-κB to translocate to the nucleus where it can transcriptionally regulate the expression of hundreds of genes involved in the inflammatory and antimicrobial responses. In addition to NF-κB activation, TLR signaling activates members of the Interferon Regulatory transcription factor (IRF) family for the production of Type 1 Interferons (32).

Mammals have approximately eleven TLR that are restricted to cell membrane and endosomal compartments and are not involved in the detection of cytosolic MAMPs (32, 37, 39).

TLR-1, -2, -4, -5, -6, and -10 are located on the cell membrane (32). TLR-3, -7, -8, and -9 are found in the endosomal compartment (32). TLR-11 is non-functional in humans, but functions in protozoan detection in mice (32). TLR-10 is expressed in humans, but not in mice (40). TLR-8 is expressed in both humans and mice, but does not signal in mice (40).

TLR expression patterns differ by tissue and cell type and can be influenced by genetic background. TLR expression has been documented in the intestine, liver, heart, kidneys and lungs (41, 42). Further, TLR expression has been described in epithelial cells, endothelial cells, neutrophils, T cells, B cells, NK cells, Dendritic Cells, Monocytes, and Macrophages (37, 39).

Transmembrane PRR are not exclusively TLR. Alongside the surface TLR is also the C-type lectin Dectin-1, which functions to detect the MAMP beta-glucan of fungus (31). See Table 1 for transmembrane PRR and their respective ligands. Collectively, TLR can be classified according to the type of ligand they bind, whether they bind directly to their ligand (i.e. TLR-5) or require adaptor molecules (i.e. TLR-4), whether they bind a single ligand (i.e. TLR-9) or multiple ligands (i.e. TLR-2), and whether they form homo-dimers (i.e. TLR-5) or hetero-dimers (i.e. TLR-1/2). The broad range of TLR recognition allows TLR to alert the host to the presence of a diverse range of microbial products including bacterial, fungal, and viral MAMPs as well as host factors (DAMPs).

The end result of TLR ligation is induced gene expression and post-translational modification of expressed genes, which trigger inflammatory and antimicrobial responses. For example, IL-6 and IL-12 production follow TLR activation and mediate the inflammatory response (38). Conversely, TLR-2 signaling contributes to IL-10 production and elicits an anti-inflammatory response (35). Moreover, the induction of Type 1 Interferons (i.e. IFNβ) by TLR-3 and -4 confers viral protection to the host (32, 39). Besides the transcription of genes that

promote cytokine/chemokine production, TLR ligation promotes the differentiation/activation of immune cells. TLR signaling favors Th1 skewing over Th2 (43). TLR signaling results in upregulation of MHC Class II and co-stimulatory molecule expression on APC (12). Even IgG antibody production by B cells can be TLR dependent in specific circumstances (31). Because hundreds of genes can be expressed in response to various MAMPs, transcribed genes should require multiple levels of regulation within the TLR pathway (44).

Notably, immunodeficiency diseases have been linked with impaired TLR signaling, only further substantiating how critical MAMP detection is to the host. Mutations in NEMO, IκBα, IRAK4, MyD88, and TLR-3 causing immunodeficiency have been documented in humans (32). TLR SNPs have also been identified, although their role in inflammatory responses remains controversial. SNPs Asp229Gly and Thr399Ile in TLR-4 are associated with a blunted TLR response (45), but TLR-5 stop polymorphism is associated with protection from Systemic Lupus Erythematosus (46). Therefore, there are some deficiencies in the current understanding of the benefits and drawbacks of TLR in immune-related diseases.

Table 1. Transmembrane Pattern Recognition Receptors

PRR	Location	Ligand
TLR-1/2	Cell Membrane	Bacteria: Triacylated Lipopeptides
		Synthetic: PAM_3CSK_4
TLR-2	Cell Membrane	Fungus: Zymosan
	Phagosome	Fungus: Phospholipomannan
		Fungus: β-glycan
		Bacteria: Lipoproteins
		Bacteria: Lipotechoic Acid (LTA)
		Bacteria: *Peptidoglycan (PDG)
		Virus: Glycoprotein
		Virus: CMV, HSV
		Self: Heat Shock Proteins (HSP)
TLR-2/6	Cell Membrane	Fungus: Zymosan
		Bacteria: Diacylated Lipopeptides
TLR-3	Endosome	Virus: dsRNA
TLR-3/7	Endosome	Virus: RNA
TLR-4	Cell Membrane	Fungus: mannan
	Early Endosome	Bacteria: Lipopolysaccharide (LPS, Lipid A)
		Virus: Glycoproteins
		Self: HMGB1
		Self: HSP
		Self: ECM products (Fibronectin, etc)
		Synthetic: Eritoran (Antagonist)
		Synthetic: TAK-242 (Antagonist)
		Other: Free Fatty Acids
TLR-5	Cell Membrane	Bacteria: Flagellin
TLR-7	Endosome	Virus: ssRNA
TLR-8	Endosome	Virus: ssRNA
TLR-9	Endosome	Bacteria: Unmethylated CpG motifs (DNA)
TLR-10	Cell Membrane	Unknown
TLR-11	Cell Membrane	Unknown

References: (27, 32) (38, 39) (29, 30, 36, 40, 43, 45) (4, 46)
*Unclear if PDG is recognized by TLR2 because of contradictory reports in the literature.

1.3.2.2 Cytosolic Pattern Recognition Receptors

PRR stationed in the cell cytoplasm complement the TLR localized to the cell membrane and endosomal compartments. Cytosolic PRR consists of NOD-like Receptors (NLR), RIG-like Receptors (RLR), and others that have recently been identified but do not share homology with NLR or RLR. The commonly studied NLR include NOD1 and NOD2 which both contain a nucleotide-binding oligomerization domain (NOD) and function to detect PDG (30, 32, 47). Two other newly identified member of NLR family include Ipaf (NLRC4) and Naip5, which both function to detect cytosolic flagellin (30). NLR have conserved structures like TLR, consisting of a centrally located NOD, a LRR domain on one end for ligand sensing, and a CARD domain on the other end that initiates signal transduction. Like NLR, RLR also have a CARD domain, but they differ by having a central helicase domain instead of the NOD domain found on NLR (32). The RLR family includes RIG-1 and MDA5 (Melanoma Differentiation Associated gene 5), which are both RNA helicases and function to detect exogenous cytosolic RNA (31, 32).

Like TLR, NLR expression differs by tissue and cell type. NOD2 has been found on monocytes, macrophages, T cells, dendritic cells, granulocytes, intestinal epithelial lymphocytes, and hepatocytes just to name a few (48). However, expression of Ipaf is limited to cells of myeloid lineage (30). NLR expression has not been as extensively studied as TLR expression.

The MAMPs detected by cytosolic PRR are nearly as diverse as those detected by TLR. PDG actually binds to two members of the NLR family, both NOD1 and NOD2 (49). However, NOD1 and NOD2 differ in the particular residues of PDG that they recognize. Diaminopimelic acid (DAP) of gram-negative bacteria binds NOD1 and Muramyl Dipeptide (MDP) of gram-negative and gram-positive bacteria binds NOD2 (26, 32, 47, 48). NOD2 mutations are associated with Crohn’s disease and juvenile granulomatous diseases (31, 32).

Two other NLR, NALP1 and NALP3, also recognize the bacterial cell wall component PDG (MDP specifically). In addition, NALP3 detects host uric acid crystals, ATP, viral RNA, and bacterial DNA (32). As for RLR, RIG1 detects short viral dsRNAs and MDA5 detects long viral dsRNAs (31, 32). Cytoplasmic viral and bacterial DNA is recognized by two DNA sensors: AIM2 and DAI (DNA dependent activator of IFN regulatory factors) (32).

Unlike TLR activation upon MAMP detection, cytosolic PRR activation typically leads to inflammasome formation and cleavage of pro-molecules versus the transcription/translation of new antimicrobial and inflammatory molecules (33) although NLR signaling does induce the secretion of pro-inflammatory cytokines IL-6 and IL12 (30). The activation of pro-molecules via NLR signaling is mediated through the activation of caspase 1 (30). Caspase 1 can functionally cleave molecules like pro-IL1β and pro-IL-18 into the active forms of these cytokines. Aberrant IL-1 production associated with NLR mutations results in periodic fever syndromes (32).

Another mechanism of NLR signaling that differs from TLR signaling is pyroptosis. Pyroptosis is programmed cell death that differs from apoptosis and is primarily mediated by caspase 1 functioning to protect "damaged" cells and limiting "infection" spread (30). Similar to TLR signaling, RLR activation leads to Type 1 Interferon production mediating the cellular anti-viral response (32). The characteristic diversity, functional redundancy, and sheer frequency of TLR, NLR, RLR and other MAMP sensors is astonishing.

1.3.3 Microbe-Associated Molecular Patterns of Interest

1.3.3.1 Peptidoglycan

Peptidoglycan (PDG) is a structural component of bacterial cell walls. Although, cell walls are characteristic of gram-negative bacteria, both gram-negative and gram-positive bacteria have a

layer of PDG (26). In gram-negative bacteria, PDG is located under the bacterial outer membrane that contains LPS (4, 32). PDG is formed by polymerization of alternating N-acetylglucosamine and N-acetylmuramic acid residues connected by a short stem peptide made of diaminopimelic acid (DAP) (49). As mentioned previously, monomeric components of PDG include DAP and MDP which are recognized by PRR (NOD1 and NOD2 respectively). Lysozymes, secreted by the host, are bacteriocidal effectively liberating muropeptide fragments including DAP and MDP from the bacterial cell wall (50). Then, PDG recognition proteins (PGRP) pick up soluble PDG residues that are released upon bacterial degradation (51). Alternatively, PDG fragments can be released during bacterial cell wall synthesis occurring during bacterial replication (50).

Clark et al. demonstrated that luminal PDG can efficiently cross the gut barrier and enter systemic circulation and penetrate the bone marrow of hosts (12, 28). The natural structure of PDG following translocation is unknown at this time (26), although PRR can detect polymer PDG as well as monomers DAP and MDP. We also do not know of translocated PDG enters the cell cytoplasm to be picked up by NLR, although PDG-specific transporters PEP1 and PEP2 have been identified in the gut and lungs respectively (50). We do know that PDG is detectable in healthy patient plasma (1.76 μg/mL) (26) and can conclude that PDG is present in circulation under homeostatic conditions.

1.3.3.2 Flagellin

Flagellin (FLA) is a major subunit of the fllament component of bacterial flagella (39). The entire flagellum is comprised of a basal body, hook, motor, and filament (39) and these components together make a whip-like structure on both gram-negative and gram-positive bacteria enabling their motility (30). FLA itself is shaped like a boomerang and consists of four

domains (D0-D3) that resemble the capital Greek letter gamma (Γ) (30). Of the four domains, one is a polymerization domain, one a signaling domain, and the remaining are hypervariable domains (39). An estimated 30,000 subunits of FLA are required to form the filamentous portion of a single flagellum (30).

As previously mentioned, FLA is recognized by two major PRR, one inside the cell (Ipaf) and one on the cell membrane (TLR-5). The D1 domain of FLA binds directly to the concave face of TLR5 despite the isolation of this domain within the flagellar filament (30). Virulent bacteria utilize T3SS and T4SS to secrete FLA into the cell cytoplasm where it can be detected by Ipaf (30). Regardless of the PRR that detects FLA, NF-κB activation will result upon ligation and the subsequent response is largely cell/tissue specific. As little as 1μg of FLA was sufficient to elicit NF-κB activation in murine livers, but maximal NF-κB activation has been achieved with just 10ng in the lung (41). The degree of NF-κB activation induced by FLA was lowest in the lungs and intestines and highest in the liver of the murine organs examined by Rolli et al. (41). Accordingly, the highest level of TLR-5 expression was found in the liver, although TLR-5 expression did not increase following exposure to exogenous FLA (41).

The significance of FLA-PRR interactions on host health was made obvious by TLR-5 knockout studies and in vivo FLA challenges by various groups. TLR-5 knockout mice have altered microbiota composition (12) as well as a five-fold increase in microbiota density (46). Moreover, TLR5 knockout mice display hyperphagia and hallmarks of metabolic syndrome for reasons not completely understood (12). Further, flagellin has been consistently detected in plasma of septic patients at a concentration ranging from 2ng/mL to 20ng/mL (39, 42). The translocation of luminal FLA into circulation under homeostatic conditions has not been documented.

1.3.3.3 Lipopolysaccharide

Lipopolysaccharide (LPS), commonly referred to as endotoxin, is composed of an O-linked polysaccharide attached to a hydrophobic Lipid A moiety by a non-repeating core polysaccharide (4, 32). The Lipid A portion is a phosphorylated disaccharide backbone acylated with fatty acids (32). The degree of phosphorylation and acylation determines the immunogenicity of this lipid moiety (32). Moreover, the Lipid A portion of LPS is one of the most potent MAMPs characterized and is responsible for the inflammatory response observed in endotoxic shock (32). Lipid A is highly diverse among gram-negative bacterial species and the particular isoform of lipid A dictates the binding affinity for its cognate PRR, TLR-4 (32, 33).

Liberated LPS immediately associates with LPS binding protein (LBP), which facilitates the transfer of LPS to MD2 and association of CD14 with the LPS-MD2-TLR-4 complex to mediate TLR-4 signaling. High circulating levels of LPS are characteristic of septic shock and multi-organ dysfunction secondary to systemic inflammation (41). Moreover, plasma LPS concentrations are tightly correlated with patient survival in sepsis and liver deterioration in patients with cirrhosis (26, 52). Even small amounts of LPS (as little as 0.1 μg) can skew the immune response (39). The primary method of LPS detection in blood is the Limulus Amebocyte Lysate assay, which has a detection limit of 5pg/ml (26). However, the translocation of luminal LPS into circulation has not been reliably reported using this assay. Kim et al. reported LPS detection in half of the healthy control patients enrolled in their study (26). Crispe et al. report concentrations of LPS in portal blood up to 1 ng/ml under normal conditions, but no LPS in systemic circulation (53). Gregory et al. reported a LPS concentration ranging from 10-1000pg/ml in portal venous blood (54). Yet, Singh et al. later reported that LPS was undetectable in normal rats and healthy humans (27). Accurate determination of LPS translocation from the

gut is likely complicated by: (1) interfering molecules such as CD14, LBP, and lipoproteins (26), (2) circulating cells expressing TLR-4 that trap LPS (26), and (3) the uptake of luminal LPS into gut-derived chylomicrons, which is often overlooked (55).

1.3.4 Pattern Recognition Receptor Regulation

PRR signaling must be tightly regulated due to the convergence of PRR pathways into the inflammatory response. Inducible negative regulators, production of anti-inflammatory cytokines, and alterations in PRR signaling complexes function as the "off switch" for PRR-associated inflammation (44). PRR left "on" for too long will result in non-specific tissue damage (40). Examples of negative PRR regulators include PI3 kinase (interferes with TLR-5 signaling), ST2, RP105, SARM, MyD88s (competes with MyD88), IRAK-M, Tollip, and SOCS (suppressors of cytokine signaling) (30, 32, 40).

Although different classes of genes may be induced by the same PRR, their regulation does not appear to be at the signal level (44). Foster et al. performed a microarray on macrophages that were stimulated with LPS once, stimulated with LPS twice, or left un-stimulated (44). Resulting gene expression from this experiment was then grouped into two classes: one gene set could only be elicited after a single LPS stimulation and not thereafter and the other gene set could be induced after a single LPS stimulation and was induced at ≥ magnitude with a second LPS stimulation (44). The gene set that was silenced after a single stimulation consisted mostly of inflammatory genes, while the gene set that was inducible over and over was comprised of antimicrobial genes. The induction of particular gene sets and the regulation of their expression following PRR ligation is intricately connected with PRR "tolerance".

1.3.5 Pattern Recognition Receptor "Tolerance"

PRR "tolerance" or hypo-responsiveness is characterized by poor inflammatory mediator production and can be mediated by several TLR. The lack-luster response of TLR-bearing cells following multiple stimulations or prolonged stimulations with a specific MAMP produces a "tolerant" or hypo-responsive state. Previous studies suggested this phenomenon was due to receptor desensitization (44). However, when trying to unravel the differential regulation of gene sets activated following TLR activation, Foster et al. elucidated the mechanism behind TLR tolerance as well (44). At present, it is clear that gene specific regulation following TLR activation occurs at the level of chromatin and includes nucleosome remodeling and covalent histone modification (44). Simply put, negative transcriptional regulators induce chromatin remodeling and loss of inflammatory gene expression with repeated TLR stimulation (44). The chromatin modification model was developed while examining the TLR-4 pathway, but this model can certainly be applied to other TLR that exhibit "tolerance." Key examples of MAMP tolerance occur with TLR-5 ligation with FLA and TLR-4 ligation with LPS. Both receptors elicit reduced inflammatory cytokine production for a short period (hours to days) following repetitive stimulation with their cognate ligands and even reduce responsiveness to unrelated PRR agonists during this period (39, 40). PRR "tolerance" can be exploited therapeutically to manipulate host-microbial interactions.

1.3.6 Pattern Recognition Receptors in the Liver

Some would agree that no obvious inflammation occurs in the healthy liver (40). By definition, inflammation infers that something has gone awry. Yet, the healthy liver in rodents and humans accommodates a multitude of cells that express various PRR and encounters translocated MAMPs in every day life. The next section will cover the various cells of the liver (with emphasis on Kupffer cells), but in order to set the scene-Table 2 describes the dangers of TLR signaling in the liver.

Table 2. Pattern Recognition Receptors and Liver Pathogenesis

TLR	Condition	Reference
NOD2	Con A hepatitis	(48)
MyD88	Liver Regeneration	(51)
	CCL4 fibrosis	(40)
TLR-3	Attenuates Liver regeneration	(51)
TLR-4	Attenuates Acetaminophen-induced Injury	(51)
	Acute Liver Failure	(40)
	Endotoxic Shock	(40)
	NASH progression	(40)
	CCL4 fibrosis	(40)
	Hepatocellular Carcinoma (HCC)	(40)
	Acute Allograft Rejection	(45)
Nonspecific TLR	*Alcoholic Liver Disease	(40, 64)
	Granulomatous Disease	(40)
	Infectious Disease	(40)
	I/R injury	(40, 64)
	Liver Regeneration	(40)
	NASH	(40, 64)
	Autoimmune Hepatitis (AIH)	(40, 64)
	Primary Biliary Cirrhosis	(40, 64)
	Primary Sclerosing Cholangitis (PSC)	(40, 64)
	Fibrosis	(40, 64)
	Hepatocarcinogenesis	(40, 51)
	Chronic HCV	(40)

*Acute alcohol exposure down-regulates TLR signaling and chronic alcohol exposure up-regulates TLR.

1.4 THE LIVER AND NONPARENCHYMAL CELLS

1.4.1 The Liver

1.4.1.1 Blood Supply and Liver Architecture

The liver is the largest solid organ in the body (54, 56-58). On gross examination, the liver appears to be a large red (well-perfused) organ located in the upper right quadrant of the abdomen under the diaphragm. Histologically, the liver appears to be a homogenous section of hepatocytes with occasional sighting of vascular tissue and bile ducts (59). The liver has multiple connections to the body's vasculature, with two major entryways and one exit for circulating blood. Blood supply to the liver depends on the hepatic artery (branch from abdominal aorta), which provides oxygenated blood and supports bile ducts and tissues of the hepatic portal tract (53, 60). However, the majority of the liver's blood supply comes from the portal vein, a confluence of the splenic and superior mesenteric veins. The liver receives 80% of its blood from the portal vein, the draining tributary of the intestines, spleen, and pancreas, and the remainder from the hepatic artery (57, 58, 61, 62). The hepatic artery and portal vein drain into liver capillaries also called hepatic sinusoids. Hepatic sinusoids form terminal hepatic venules that drain into the hepatic vein and then the inferior vena cava, which recirculates blood back to the heart (60, 61). The narrow diameter (5-7μm) of the liver sinusoids favors interaction between the liver's cellular components and blood components (58, 60, 63). Our entire blood volume circulates through the liver about 360 times per day and a third of our blood volume passes through the liver each minute (58, 60, 63). Constant recirculating blood enhances exposure to gut-derived MAMPs (58).

Blood flows from portal tract (periportal region) to hepatic venule (centrilobular region) through the hepatic sinusoid, while bile flows in the opposing direction (64). The portal tract (portal triad) consists of a portal vein, bile duct, hepatic artery, lymphatic vessels, and sympathetic nerves too (59). The architecture of the liver is highly reflective of its function. The functional unit of the liver is the lobule and the cellular unit is the hepatocyte. Liver lobules are plates of hepatocyte in hexagonal arrangement (58, 64). The liver can be also divided by tissue system comprised of elements from the vascular system, hepatocytes, biliary cells, immune cells, and stroma (64).

1.4.1.2 Cellular Components of the Liver

The healthy liver is not considered to be inflamed (40). However, like primary and secondary lymphoid organs, the liver is enriched in various immune cell types capable of executing innate immunity functions (53, 58, 61). Immune cells found in the liver include Kupffer Cells (KC), Dendritic Cells (DC), Natural Killer (NK) cells, Natural Killer T (NKT) cells, and other lymphocytes (53, 56, 57, 63, 64). In addition to traditional immune cells, hepatocytes, biliary epithelial cells (BEC), and stellate cells (SC) also possess immunological function (61). Hepatocytes predominate the liver mass (60-80%) and constitute the parenchymal cell population. All other cells fall into the non-parenchymal cell (NPC) forming 20-40% of the liver's cellular mass (65). The exact pattern of PRR expression and functionality in the liver has not been fully elucidated.

1.4.1.3 Functions of the Liver

The liver as its name indicates is essential for life. During the fetal period, the liver serves as hematopoietic tissue before maturing to function as the central organ for metabolism managing

exocrine, endocrine, and immunity responsibilities (56, 66). Liver functionality can be divided into two major groups: (1) metabolism-related and (2) immune-related. Firstly, the liver is responsible for protein, carbohydrate, and lipid metabolism as well as bile synthesis and drug detoxification (58). Secondly, the liver modulates PRR-MAMP interactions and also contributes to innate immunity via the synthesis of acute phase proteins (i.e. metallothioneins), cytokines, and chemokines (40, 56-58, 62, 65). Antimicrobial proteins like complement help the host combat viruses, bacteria, and tumors (56). The liver's expansive number of immune cells help in this regard as well.

As mentioned before, the liver functions in the metabolism and catabolism of dietary elements (63). Hepatocytes located within different zones of the liver carry out different metabolic functions. The acinar zones are prime location for β-Oxidation. Cholesterol synthesis takes place in the periportal region. Glutathione functions to protect hepatocytes from oxidative stress and is produced primarily in the periportal region (67). Urea and glutamine synthetase strictly occur around the central veins (67). Bile synthesis is highest in central areas too (67).

The secreted immune-related proteins are diverse and possess many functions, all of which will not be covered. However the acute phase proteins and complement proteins are of particular interest. The production of acute phase proteins is best characterized during infection and inflammation, however the mechanism behind constitutive production of dozens of these proteins has not been elucidated (58). Still, the acute phase response is characteristic of innate immunity, rapid and non-specific, acting as a first line of defense before more specialized cells are recruited to resolve either infection, inflammation, or tissue injury (62). By definition, acute phase proteins synthesized by the liver must change their baseline concentration (up or down) by at least 25% in response to perturbations (62). Pro-inflammatory cytokines Interleukin-1,

Interleukin-6, and Tumor Necrosis Factor-α as well as glucocorticoids particularly enhance acute phase protein synthesis by hepatocytes, further enhancing the inflammatory response (62). Complement protein production confers cytotoxic, opsonization, and inflammatory function (57). Secreted soluble PRR made by the liver aid in microbial opsonization as well. The liver also serves as a scavenger to clear circulating antigens in order to prevent systemic inflammation (57, 65). While assuming immunological responsibilities, the liver has to circumvent dispensable immune activation and tissue injury to maintain its metabolic function, which is essential for survival.

1.4.2 Parenchymal and Non-Parenchymal Cells

1.4.2.1 Parenchymal Cells

Parenchymal cells perform the vast majority of metabolic processes carried out by the liver. Hepatocytes, parenchymal cells, are the predominant cell type of the liver representing 70% of hepatic cells and 80% of total liver volume (57-60, 64). Hepatocytes form anastomosing plates of cells of one cell thickness and communicate with each other via gap junctions (67). On one side, hepatocyte face the Space of Disse separated from the hepatic sinusoid by a layer of endothelial cells. On the other side, hepatocytes face the bile canaliculus responsible for bile collection (59, 64).

Hepatocytes are essentially synthesizing factories. They contribute to innate immunity by releasing soluble PRR, complement protein, and acute phase proteins into the blood stream (57, 58, 62). Hepatocytes synthesize C1-9, Cbp, Factor B, mannan binding lectin, CfI, CfH, and C1 inhibitor (57). Intra-hepatic immune cells make minor contributions to complement synthesis as well (57). Although hepatocytes are separated from the cells of the sinusoid by endothelial cells

lining the sinusoid, hepatocytes do express MHC class I, Intercellular Adhesion Molecule 1 (ICAM1), and CD1d (MHC Class 1-like molecule that engages the TCR of NKT cells). Therefore, hepatocytes have the potential to interact with lymphocytes (58, 62). Many have speculated that hepatocytes contribute to liver tolerance by inducing T cell death by neglect when engaging TCR (signal 1) but not providing co-stimulatory signals (signal 2) (53, 62), but the likelihood of hepatocytes engaging T cells *in vivo* under homeostatic conditions seems relatively slim considering liver architecture. Nonetheless, in the course of hepatic inflammation, T cells target hepatocytes for destruction (64).

1.4.2.2 Liver Sinusoidal Endothelial Cells

Liver Sinusoidal Endothelial Cells (LSEC) form half of the non-parenchymal cell (NPC) population in the liver (60, 68). Their lack of basement membrane and clusters of fenestrations (100nm) appearing like "sieve plates" facilitate communication between hepatocytes and sinusoidal content (53, 58, 62, 63, 66-68). LSEC differ from endothelial cells in other organs by their distinct pattern of adhesion molecules. For example, LSEC lack E-selectin and P-selectin, but constitutively express ICAM1, Vascular Adhesion Protein 1 (VAP1), and CD44 (LYVE1) (61, 63, 64, 69). ICAM1, VAP1, and CD44 are critical adhesion molecules involved in leukocyte recruitment to the liver (61). ICAM1 and CD44 facilitate firm adhesion, while VAP1 facilitates brief rolling adhesion of leukocytes (61). ICAM1 binds to Lymphocyte Function-associated Antigen 1 (LFA1 also known as CD11a) found on lymphocytes, macrophages, and neutrophils (62). Further, VAP1-leukocyte interactions upregulate ICAM1 (CD54) and VCAM1 (CD106) expression on LSEC (61). VCAM1 interacts with the integrin CD49d on lymphocytes (63). VCAM1 and MadCAM1 (the gut homing adhesion molecule) are inducible under inflammatory conditions (61, 70). In addition, LSEC express numerous ligands for chemokine receptors found

on circulating leukocytes including CCL3-5, CXCL9-11, and CXCL16 (61, 64). Both constitutive adhesion molecule expression and chemokine expression enable accumulation of liver leukocytes.

LSEC have been characterized as non-immune cells with immune function. Whether LSEC actually serve as APC remains a point of contention (53, 62). LSEC have the most potential of all of the liver cells to interact with circulating leukocytes (63, 65). Moreover, LSEC express MHC class I, MHC class II, and costimulatory molecules CD40, CD80, and CD86 (53, 58, 60, 63, 71). Moreover, LSEC undoubtedly produce both pro-inflammatory cytokines (i.e. IL-1) and anti-inflammatory mediators (i.e. IL-10, PGE2) (53, 62, 63). LSEC also clear macromolecules from circulation (53, 58, 60, 62). At least five different endocytosis receptors have been identified on LSEC, which can function in antigen uptake (57).

1.4.2.3 Biliary Epithelial Cells

The liver houses specialized cells, Biliary Epithelial Cells (BEC), which function to transport bile. Bile leaves the liver and is stored in the gall bladder. Bile is released into the intestine, in response to dietary fat consumption, where it functions to emulsify fats for breakdown and uptake. BEC only make up a small amount of the total liver mass in comparison to other NPC like LSEC (62). Notably, BEC do express PRR to detect invading microbes in the bile ducts (58). Despite their low frequency, BEC are specifically targeted in liver diseases such as Primary Biliary Cirrhosis (PBC), Primary Sclerosing Cholangitis, and vanishing bile duct disease (62).

1.4.2.4 Dendritic Cells

Intra-hepatic Dendritic Cells (DC) are derived from the bone marrow (60, 62). DC frequency within the liver is sparse, but are can be identified around portal tracts (58, 62). The liver

contains five types of DC: myeloid DC ($CD8\alpha^{-}B220^{-}CD11b^{+}$), lymphoid DC ($CD8\alpha^{+}B220^{-}$ $CD11b^{-}$), plasmacytoid DC ($CD8\alpha^{-}B220^{+}$), a mixture of lymphoid and myeloid DCs ($B220^{-}$ $CD11^{-}$) and natural killer DC ($B220^{-}CD11c^{int}$ $CD69^{+}2B4^{+}DX5^{+}$) (53, 57, 58). Plasmacytoid DC are more abundant in the liver than in the spleen (53). Notably, lymphoid and natural killer DC are specific to rodents livers and have not been identified in humans (53).

Intra-hepatic DC are the only professional APC present among NPC (62, 65). Activated DC migrate to the space of Disse to exit into the lymphatic system where they are then transported to extra-hepatic lymph nodes for initiation of adaptive immunity (62). Liver DC are poor T cell stimulators in comparison to extra-hepatic populations of DC (i.e. spleen and bone marrow derived DC) (57, 58, 62). Their poor immunostimulatory capacity is attributed to an immature phenotype and lack of costimulatory molecule expression (58, 60). The cytokine milieu in the microcirculation of the liver containing high amount of IL-10 appears to render residential DC tolerogenic (60, 62). Intra-hepatic DC are key players in the balance between immunity and liver tolerance given their poor immunostimulatory capacity yet efficient antigen capture and MAMP detection capabilities (58, 62).

1.4.2.5 Hepatic Stellate Cells

Hepatic stellate cells (SC), localized to the Space of Disse, make up 5-10% of NPC in the liver (65, 68). SC, also called Ito cells and fat-storing cells, function to store vitamin A, store fat, and modulate hepatic microcirculation (58, 59). The ability of SC to function as APC is controversial, although they express MHC Class I, MHC Class II, and CD1d (58).

SC are quiescent in the healthy liver (57). SC are activated in various liver pathologies. Upon activation, SC proliferate and acquire a myofibroblastic phenotype (57, 58). Myofibroblast-like SC secrete large amount of extracellular matrix proteins and collagens

thereby promoting liver fibrosis (57, 59, 65, 72, 73). SC can be activated by Kupffer Cells producing TGFβ and Platelet Derived Growth Factor (PDGF) (57, 72, 73).

1.4.2.6 Mast Cells and Granulocytes

In the healthy liver, granulocytes are rarely identified representing less than 1% of the liver NPC (54, 58). Mast cells are also extremely rare in the healthy liver and have not been identified consistently in humans and rodents in absence of liver pathology (58). Liver pathologies may recruit large numbers of granulocytes (neutrophils, basophils, and eosinophils) (58).

1.4.3 Kupffer Cells

1.4.3.1 Origin and Lifespan

Kupffer Cells were identified by pathologist C. von Kupffer in 1876 (73, 74). In the 1970's and 1980's, the mononuclear phagocyte system (MPS) was defined as a family of cells that includes bone marrow progenitors, circulating monocytes, and tissue macrophages (75). Circulating monocytes enter the liver where they differentiate into KC (60, 65, 75, 76). In the traditional MPS, cell division occurs at the level of the monoblast and proliferation of differentiated tissue macrophages rarely occurs (75). Ralph van Furth, the father of the MPS, provided evidence that tissue macrophages lacked self-renewing capabilities through thymidine incorporation assays (74, 75). Likewise, Croton et al. reported that less than 2% of Kupffer Cells proliferate under steady state conditions (76).

The MPS is challenged by the separate embryonic phagocytic lineage (75). During the fetal period, macrophages are found in the yolk sac although they differ from adult macrophages

and do not express F4/80 (75). Although, the bone marrow compartment has not formed during the early gestational period, macrophages still appear in the liver, although they disappear at later developmental stages and then reappear at low frequency before birth (66). Additionally, some KC may be derived from the spleen based on splenectomy studies (77). Presently, there is no consensus on the lifespan of Kupffer Cells. KC may turnover after weeks or survive for up to a year (74, 76).

1.4.3.2 Frequency

Kupffer Cells account for 80-90% of all tissue macrophages in the body, 15% of liver cells, and 20-35% of non-parenchymal cells (54, 58-60, 62, 68, 71, 73, 74, 76, 78). The sheer numbers of KC present in the liver signify their importance to this organ.

1.4.3.3 Location and Distribution

Kupffer Cells are localized to the hepatic sinusoid adherent to LSEC (54, 59-61, 63, 68, 74). KC have heterogeneous distribution within the liver and are consistently absent in the sinusoids within 50 microns of central veins (68). KC predominate in the periportal region (43%) and lesser so in the mid-zonal and centrilobular regions (25% and 29% respectively) (54, 58, 73, 77). Although KC are reportedly fixed macrophages, several groups have refuted the claim that KC are sessile, insisting that KC are in fact mobile within the sinusoid (63, 73, 78).

1.4.3.4 Phenotype

Although KC are localized throughout the liver, differences exist in phenotype based on proximity to the portal vein (74). Periportal KC are larger with higher lysosomal enzyme content and greater phagocytic potential compared to centrilobular KC (67, 74). Larger KC have been

shown to produce more TNFα, PGE2, and IL1 than smaller KC (74). Intermediate and large KC also release IL-6, IL-10, and MCP1 (monocyte chemotactic protein 1) to exert antimicrobial functions (58). Centrilobular KC are smaller and produce more NO (74). Smaller KC can also make TNF-α and may have a more prominent role in immunoregulation (58).

F4/80 can be used as a pan-macrophage marker for tissue macrophages (68, 75, 77). The F4/80 antibody binds to family of seven transmembrane domain receptors (EMR1-4 in humans and EMR1-2 in mice) related to Epidermal Growth Factor found exclusively on macrophages (75). The F4/80 antigens are difficult to detect on lung, lymphoid, and fetal macrophages (75). The function of these receptors is unclear, but they bind to proteoglycans (68, 75). The ED1 and ED2 antigens, which function as hemoglobin scavenger receptors, are used to identify KC in rats (74). KC also exclusively express heme oxygenase 1, which is an inducible KC marker (74). There are few other surface markers that distinguish KC from professional APC like DC (75). When liver macrophages were being characterized in the 1970's, investigators used esterase staining to non-specifically identify KC (76).

Several investigators (including myself) have tried to identify distinct subsets of liver macrophages by identifying phenotypic/functional differences. Two types of macrophages have been characterized outside of the liver termed M1 an M2. M1 macrophages are thought to be activated classically while M2 macrophages are activated by an alternative mechanism (61). M1 macrophages make pro-inflammatory cytokines (i.e. IL-6) and anti-microbial products and M2 macrophages make anti-inflammatory cytokines (i.e. IL-10) to promote tissue repair (61, 65). M1 macrophages interact with Th1 cells (cellular immunity) and M2 macrophages interact with Th2 cells (humoral immunity) (72). Alas, Kupffer Cells do not appear to polarize into M1 and M2 subtypes (77). However, they may be divided into CD11b+ and CD11b- subsets based on

function. CD11b is found on myeloid cells, granulocytes, and NK cells and functions as a complement receptor. CD11b- KC appear to be less phagocytic, more sensitive to gadolinium chloride and liposome entrapped clodronate (macrophage depleting agents), and produce lower amounts of ROS, TNFα, and IL-12 following antigenic stimulation (77). The percentages of CD11b- versus CD11b+ KC have not reported (77).

1.4.3.5 Function

KC are phagocytes first and antigen presenting cells second. As phagocytes, KC clear the sinusoids of circulating debris, senescent erythrocytes, activated neutrophils, bacteria, LPS, immune complexes, chemicals, and injectable colloids (54, 57, 58, 62, 65, 68, 73, 74, 78). KC perform both immune and non-immune mediated phagocytosis to clear the sinusoid of insoluble, toxic, and infectious agents. Immune mediated phagocytosis is carried out using Complement and Fc Receptors (62). Non-immune mediated phagocytosis utilized opsonins and lectin receptors (62). In addition, KC partner with neutrophils to clear bacteria from circulation by adhering bacteria on their cell surface so that neutrophils can eliminate bound but not phagocytosed bacteria (63, 74).

As antigen presenting cells (APC), KC present antigen to activated effector T cells via MHC class II, but not as efficiently as spleen macrophages or bone marrow derived macrophages likely for the same reasons that liver DC are poor APC (63, 65, 73). KC, within the hepatic sinusoid, are in a prime location to interact with lymphocytes as they pass through the liver (61).

KC are important to innate immunity and host defense (52, 73, 74). They secrete many immune mediators to fulfill their immunological responsibilities, which affect the activity of neighboring cells (60). Cytokines reportedly produced by KC (constitutively or upon activation) include the following: TGF-β, TNF-α, IL-1, IL-6, IL-10, IL-12, and IL-18 (54, 60, 62, 63, 65,

71, 73). TNF-α, produced by KC in soluble or membrane-form, is a cytotoxic factor in the liver, which can cause hepatocyte death and promotes the infiltration of granulocytes to the liver (71). It is worth mentioning that both pro-inflammatory and anti-inflammatory cytokines are produced by KC when exposed to MAMPs (63). Moreover, the production of pro-inflammatory cytokines upregulate adhesion molecule expression by LSEC (61), while anti-inflammatory cytokines can downregulate MHC Class II and costimulatory molecule expression on KC and other NPC. KC also release leukotrienes, thromboxane A2 (TXA2), prostaglandin E2 (PGE2) and ROS, which may be immunosuppressive or immunostimulatory depending on context (54, 60, 63, 73).

1.4.3.6 Activation

Upon activation, KC characteristically produce pro-inflammatory cytokines, but also IL-10 which serves a negative feedback mechanism to reduce TNF-α and IL-6 production and promotes liver tolerance (71). KC activators include ischemic injury, complement components C3a and C5a, MAMPs (i.e. LPS), and cytokines (74, 78). Prostaglandins typically suppress KC activation (78). The major signal transducer of activated KC is phospholipase C (PLC), which activates Protein kinase C (PKC) and NADPH oxidase (oxidative burst) as well as prostaglandin synthesis (PGE2 and TXA2) (74). Strong activation signals permit KC to activate T cells, leading to T cell proliferation (58). Activated KC producing IL-12 and IL-18 seem to be particularly important for the maturation of Natural Killer T (NKT) cells and subsequent IFN-γ production (60, 62, 71).

One of the most intriguing characteristics of KC is their ability to become tolerant (hypo-responsive) upon antigenic stimulation. KC depletion experiments have demonstrated that KC are involved in oral tolerance, portal venous tolerance, and liver allograft tolerance (53). KC

promote tolerance partially by inducing apoptosis of intra-hepatic T cells (74). The mechanisms behind KC mediated tolerance are not fully understood.

1.4.3.7 The Good, The Bad, and The Ugly

KC are implicated in many liver pathologies including Alcoholic Liver Disease (ALD), Nonalcoholic Fatty Liver Disease (NAFLD), Liver failure secondary to Acetaminophen (APAP) toxicity, and Ischemia/Reperfusion (I/R) injury (73, 74). In I/R injury, KC produce tissue damaging oxygen radicals and hepatotoxic TNF-α (73). Reperfusion activates KC, presumably via LPS buildup from portal vein occlusion during surgery (74). In hepatic fibrosis, KC produce SC activation factors (PDGF and TGF-β) driving myofibroblast production of extracellular matrix and reducing matrix metalloproteinases activity (72, 73). In ALD, KC are activated by LPS to produce excessive TNF-α and inflammatory mediators (73, 74). There is little evidence suggesting that alcohol actually affects KC, but overwhelming evidence showing increased LPS translocation from the gut secondary to alcohol mediated by increased intestinal permeability (73). Acute alcohol exposure affects KC differently than chronic alcohol exposure associated with ALD. In fact, pro-inflammatory cytokine production by KC is inhibited by acute alcohol exposure (74).

There is a limited set of experimental models of liver inflammation. One of the major models of liver inflammation and fibrosis is mediated by Carbon Tetrachloride (CCl_4). Activated KC cause more tissue damage than inactivated KC or the absence of KC in this model (73). Circulating monocytes differentiate into NO producing KC with chronic CCl_4 exposure (72). In the model of hepatic inflammation mediated by APAP, KC are directly activated by acetaminophen and deplete liver glutathione stored causing tissue damage (74). KC further

exacerbate drug-induced liver injury by producing TNF-α, IL-1, and NO (61). Conversely, KC can be hepato-protective in the model of liver injury mediated by Concanavalin A (65).

Increased Kupffer Cells frequency or activation does not have adverse consequences in all scenarios. For example, increased KC numbers allow for more efficient clearance of bacterial infections and resolution of septicemia in mouse models (73). In addition, KC prevent metastasis of GI primary tumors to the liver via phagocytosis of metastatic tumor cells (73, 74). Finally, the importance of KC in liver regeneration was demonstrated by KC depletion studies (74).

1.4.4 Liver Lymphocytes

The human liver houses 10^{10} lymphocytes (60, 62). The heterogeneous group of intra-hepatic lymphocytes is predominantly localized to the periportal regions of the liver, but lymphocytes are occasionally scattered throughout the parenchyma (62). Circulating lymphocytes travel into the liver to become resident lymphocytes and undergo maturation (62). Leukocyte recruitment to the liver was previously attributed to physical trapping in narrow sinusoids often obstructed by Kupffer Cells (61). However, lymphocytes have been shown to adhere to ICAM1 on LSEC under physiological conditions and ICAM1 knockout mice demonstrate reduced lymphocyte adhesion to hepatic sinusoids (61, 63). Leukocyte migration into the liver is also dependent upon the appropriate chemotactic gradient generated by chemokine produced by parenchymal and non-parenchymal cells (64).

Intra-hepatic lymphocytes (IHL) are comprised of: T lymphocytes, B lymphocytes, Natural Killer (NK) cells, and Natural Killer T (NKT) cells. Conventional T lymphocytes include CD4+ and CD8+ T cells (65). Unconventional T cells are categorized according to NK cells markers (i.e. NKT cells) and those that do not (i.e. γδ T cells) (62). Nonetheless, over 80%

of intra-hepatic T cells express the αβ TCR leaving γδ T cells in the minority (62). In comparison to other organs, NK (30% of IHL) and NKT (20% of IHL) cells are particularly abundant in the liver (57, 62, 65). B lymphocytes only make up 6-10% of the IHL population in humans and rodents (58, 62).

1.4.4.1 Hepatic B Cells

Due to the small number of residential B cells in the liver, not much is known about their function (60). Traditionally, B cells are activated, differentiate, and proliferate in lymphoid organs (60). In the mouse, hepatic B cells are similar to B2 splenic lymphocytes (58). In humans, hepatic B cells are CD5+ and are distinctly different from murine hepatic B cells (58).

1.4.4.2 Double Negative Hepatic T Cells

Conventional hepatic T cells are typically CD4+ or CD8+ T cells. However, cells that express neither CD4 nor CD8 are double negative T cells, which are found in secondary lymphoid organs, the appendix, and the liver (58). This pool of T cells is extremely difficult to detect, hence not much is known about their role in liver immunity (62).

1.4.4.3 Conventional Hepatic T Cells

Conventional hepatic T cells comprise CD4+ and CD8+ T cells, which recognize antigens presented by MHC Class II and MHC class I respectively (60, 62). The liver differs from the periphery in that it possesses a higher proportion of CD8+ T cells relative to CD4+ T cells (58, 60, 62). Hepatic CD8+ T cells that only express alpha chain of CD8 have been reported (62). The vast majority of hepatic T cells produce IFN-γ, IL-2, and TNFα (62). Few hepatic T cells produce IL-4 (62).

1.4.4.4 Hepatic Regulatory T Cells

Regulatory T cells constitute less than 1% of the IHL population (79). In comparison to lymphoid organs like the spleen and draining lymph nodes, the liver has at least one hundred fold fewer FoxP3 expressing CD4+ T cells (58). The suppressive activity of regulatory T cells has been deeply investigated, however their role in liver tolerance and transplantation may be limited due to low numbers of cells (58).

1.4.4.5 Hepatic Gamma Delta T Cells

Gamma delta T cells differ from conventional T cells by expressing an alternative T cell receptor made of γ and δ subunits. Γδ T cells are abundant in the skin, the genitourinary tract, the gut, and the liver (57, 58, 62). In the liver, γδ T cells make up 15-20% of IHL (58, 62). The TCR of γδ T cells is thought to recognize a limited range of antigens although antigen recognition by γδ T cells is not restricted by MHC class I/II presentation, thus exact target of these cells in the liver is unknown (60, 62). This hepatic T cell subset may play a role in combating viral and bacteria infection (57).

1.4.4.6 Hepatic Natural Killer Cells

Hepatic Natural Killer cells, also called Pit cells, are 10% of liver lymphocytes in mice and 30-50% of liver lymphocytes in humans (57). Natural Killer (NK) cells can be identified by the absence of the T cell marker CD3 and expression of CD56 (neural cell adhesion molecule isoform) in humans (58, 62). NK cells are large granular lymphocytes with roles in antimicrobial and antitumor immunity by killing target cells as their name implies (57, 58, 60). Activated NK cells lyse target cells via perforin and granzyme molecules upon recognition of changes in target cell membrane glycoproteins (58, 62). KC activate NK cell cytotoxicity by secreting IL-18. NK

cells can promote T cell immunity by recruiting T cells and producing IFN-γ (58). Also, NK cells can modulate the local immune response in the liver by balancing production of pro-inflammatory and anti-inflammatory cytokines upon activation of their activating and/or inhibitory receptors (i.e. NK2GD) and Inhibition typically dominates over activation (57, 60).

1.4.5 Hepatic Natural Killer T Cells

1.4.5.1 NKT Cell Origin and Development

NKT cells arise in the thymus and mature in peripheral tissues (60, 62, 80). NKT function in recognizing lipid antigens such as the glycolipid components of microbial cell walls (i.e. glycosphingolipid from Sphingomonas spp. and Diacylglycerol from Borrelia spp.) (58, 60, 81). In addition to exogenous lipid antigens, NKT cells can recognize endogenous glycolipids (82). Many cells in the liver can present glycolipid antigens to NKT cells via CD1d including hepatocytes, liver DC, SC, and KC (81, 83, 84).

1.4.5.2 NKT Cell Frequency

Natural Killer T (NKT) cells are highly enriched in mouse and human livers accounting for up to 20% of liver lymphocytes in mice and up to 10% of liver lymphocytes in humans (57, 58, 62, 79, 84, 85). NKT cells are far less abundant in the blood only found at a 0.5% frequency (62). NKT frequency is highly variable between mouse strains and humans (86). NKT cell numbers depend on chemokine and integrin interactions to localize to the liver similar to conventional T cells (86).

1.4.5.3 NKT Cell Phenotype

NKT cells express markers characteristic of both NK cells and conventional T cells (57, 58, 81, 84). Unlike conventional T cells, NKT cells have a restricted TCR repertoire and recognize antigen in the context of the MHC Class I like molecule CD1d (57, 58, 62, 79, 82). The invariable TCR is typically Vα14Vβ8 for mice and Vα24Vβ8 or Vα24Vβ11 for humans (62, 82).

CD1d-dependent NKT cells can by either type 1 invariant (CD4+) or type 2 subtypes (double negative), but type 1 invariant NKT cells greatly outnumber non-invariant type 2 NKT cells (57, 58, 79-81, 83). As expected, CD1d-dependent NKT cell numbers are drastically diminished in CD1d knockout mice (82). Moreover, the number and proportion of CD1d-dependent NKT cells in the liver increases with age (80). Cd1d independent NKT cells are characterized as third non-classical subtype functioning to recognize other ligands outside of lipid antigens and their ontogeny is unclear (57, 82).

1.4.5.4 NKT Cell Function

NKT cells play an important role in innate and adaptive immunity since they can produce large amount of cytokines very rapidly (minutes) upon lipid antigen stimulation (60, 86). CD1d-dependent and CD1d-independent NKT cells differ in their cytokine production (57). Most classical CD1d-dependent NKT cells are activated by IL-12 (from KC or DC) to perform perforin and Fas-mediated cell killing like NK cells (60, 84, 87). Moreover, CD1d-dependent NKT cells in the liver constitutively produce IFNγ (83). Generally, NKT cells are capable of producing pro-inflammatory (i.e. IFNγ, TNF-α) and anti-inflammatory (i.e. IL-4, IL-10, IL-13) cytokines when activated similar to NK cells (58, 62, 82).

1.4.5.5 The Good and the Bad

NKT cells have overlapping function with NK cells in anti-microbial and anti-tumor defense (57, 87). NKT cells have been implicated in a broad spectrum of diseases including cancer, Primary Biliary Cirrhosis, viral hepatitis, NASH, NAFLD, Type 1 Diabetes, allergy, allograft rejection, and GVHD (62, 79, 82, 84, 86, 88). Moreover, NKT cell activation in acute liver injury models (i.e. Concanavalin A, α-galactosylceramine, LPS) has demonstrated the hepatotoxicity potential of NKT cells (57, 62). However, NKT cells can also be hepatoprotective as demonstrated by liver injury models mediated by CCl_4 and bile duct ligation (57). In addition, NKT cell deficiency can abrogate alcohol induced liver injury (57).

1.4.5.6 Murine Model of Auto-immune Hepatitis

The synthetic glycolipid α-galactosylceramide can specifically induce NKT cell activation in murine models in a fast and consistent manner (81, 88). Although α-galactosylceramide is commonly used to induce an Autoimmune Hepatitis phenotype in mice, α-galactosylceramide injection does not cause hepatic inflammation in humans (57). Basically, α-galactosylceramide causes rapid reduction of NKT cells (apoptosis) followed by repopulation within a few days (80, 81). The response is characterized by increased cytokine production (IFNγ and TNF-α) and concurrent liver damaged associated with increased liver enzymes ALT/AST (79, 81, 88, 89). Hepatic inflammation induced by α-galactosylceramide is a good model of AIH given the induction of autoantibody production (88). Mice of different background do exhibit differences in α-galactosylceramide susceptibility. For example, BL6 mice are high susceptible while BALB/c are characteristically resistant (81). The hepatic inflammation induced by α-galactosylceramide is not dependent on KC (81).

2.0 SPECIFIC AIMS

The liver has several characteristics that distinguish it from other organs. Firstly, the liver receives approximately 80% of its afferent blood supply from the portal vein, the major draining tributary of the gastrointestinal organs. Secondly, Kupffer Cells and Liver Sinusoidal Endothelial Cells (LSEC) of hepatic sinusoids function primarily to scavenge components of the incoming portal blood. They are uniquely positioned to interact with circulating lymphocytes like Natural Killer T cells as well as gut-derived microbial products (MAMPs) (90). Consequently, KC (as well as other liver leukocytes) are strongly influenced by specific microbes (91-93). KC play essential roles in host defense *and* tolerance through their interaction with microbes as phagocytic cells (94) *and* interaction with lymphocytes as antigen presenting cells (APC) respectively (95). While PAMP detection by specialized cells in the liver is critical for innate immunity and tolerance induction by liver leukocytes is undoubtedly characteristic of adaptive immunity, the location and distinctive characteristics of KC enable this cell type to functionally bridge these separate arms of the immune system. Surprisingly, few studies have explored the affects of the gut microbiome on liver tolerance although it is a major source of hepatic PAMPs. Therefore, **we hypothesize that the gut microbiome contributes to the tolerogenic properties of the liver by 1) promoting the expansion and/or recruitment of KC that mediate tolerance or 2) modulating the tolerogenic properties of KC.** We will test this hypothesis with the following aims:

Specific Aim 1: To identify Pattern Recognition Receptor (PRR) pathway(s) by which components of the gut microbiome utilize to interact with hepatic cells. PRR ligands (i.e. PDG and LPS) translocate from the gut lumen despite normal gut barrier function. Many NPC express PRR including KC and LSEC, however the culmination of effects resulting from PRR ligation on hepatic cells has been poorly characterized.

<u>Sub-aim 1A:</u> To characterize the PRR pathway(s) influenced by gut-derived microbial products in the liver. PRR pathway activation by MAMPs most commonly leads to production of pro-inflammatory cytokines. However the liver is *not* constantly inflamed in spite of constant bombardment by gut-derived MAMPs (which presumably activate their PRR). We hypothesize that hepatic 'conditioning' by gut-derived microbial products contributes to the tolerant state of the liver. A comprehensive genomic study on hepatic PRR expression in the absence of gut flora has yet to be performed. Therefore, we plan to perform an affymetrix whole genome microarray study using mRNA isolated from the liver's of mice +/- gut bacteria followed by comprehensive PRR pathway analysis.

<u>Sub-aim 1B:</u> To characterize the PRR pathway(s) influenced by gut-derived microbial products in KC. Single PRR ligation on KC can induce expression of hundreds of genes. We hypothesize that the gut microbiome serves as a source of gut derived bacterial products with which KC interact via their PRRs leading to downstream alterations in gene expression that collectively make KC tolerogenic. Whole-liver microarray results will serve as a platform to analyze specific PRR expression patterns of KC isolated from mice +/- gut bacteria. The expression of genes resulting from PRR ligation on KC will be analyzed using RT-PCR, Immunofluorescence (IF), and Western Blot (WB) methodologies.

Specific Aim 2: To elucidate the effects of intestinal colonization on the growth of the KC population. Preliminary studies indicated that intestinal colonization by commensal microbes augments the number of hepatic leukocytes. However the underlying mechanisms are not clear. The following studies will further clarify the contribution of commensal bacteria to KC population development.

<u>Sub-aim 2A:</u> To evaluate the relationship between gut microbiota density & KC population size. KC make up the largest population of tissue macrophage in the body. Our preliminary results indicate KC numbers are greater in adult mice with a stable gut microbiome in comparison to adult germ free (GF) mice (which lack a gut microbiota). We will further characterize the role of gut bacteria in KC population development by quantitatively evaluating KC population size during murine adolescence and in late adulthood using IF and Flow Cytometric (FC) methodologies in concert with bacterial load evaluation.

<u>Sub-aim 2B:</u> To evaluate the relationship between gut microbiota density & KC progenitor frequency. As tissue macrophages, KC are part of the Mononuclear Phagocyte System (MPS) and populate the liver via monocyte recruitment and differentiation thereafter. Monocytes are derived from the bone marrow (BM) and can make up to 10**%** of the blood's white cell count in humans. Clarke et al. has shown that the gut microbiome can influence BM-derived cells (28). To determine if the gut microbiome influences the frequency of KC progenitors, we will isolate PBMC from mice +/- gut bacteria to analyze monocyte frequency using FC.

<u>Sub-aim 2C:</u> To evaluate the role of gut microbiota density in KC recruitment by LSEC. LSEC express many adhesion molecules to facilitate monocyte recruitment and differentiation into KC within the liver. Interestingly, the hepatic expression of Inter-Cellular Adhesion Molecule 1 (ICAM1) is reduced in mice lacking gut bacteria. To determine if LSEC facilitate

monocyte recruitment via up-regulation of ICAM1 in response to gut derived microbial products, we will track monocyte influx following KC depletion in mice +/- gut bacteria +/- ICAM1 blockade. We will also track monocyte influx in ICAM1$^{-/-}$ mice following depletion of gut bacteria.

Specific Aim 3: To elucidate the effects of intestinal colonization on the function and tolerogenic potential of KC. A previous study from our lab has shown that LPS exposure alters the maturity state and IL-6 production of liver DC. We suspect that microbial product exposure alters KC properties as well.

Sub-aim 3A: To evaluate the relationship between gut microbiota density & KC phenotype and phagocytosis. KC are a heterogeneous population of tissue mϕ. Periportal KC have been characterized as larger, more mature, and more phagocytic whereas centrilobular KC are smaller, less mature, and exhibit less phagocytic activity. We hypothesize, that in the absence of gut bacteria (elimination of gut derived microbial products in the hepatic sinusoids), KC heterogeneity and phagocytic function will be reduced. We will measure the expression of surface markers CD80, CD86, and MHC Class II to analyze KC maturation in mice +/- gut bacteria via FC. We will also evaluate KC phagocytic activity in periportal and centrilobular regions of the liver by injecting phagocytosable latex beads (micro-beads) into the tail vein.

Sub-aim 3B: To evaluate the role of gut derived microbial products in KC tolerance. KC and their neighbors (LSEC) have the potential to release inflammatory cytokines (i.e. IL-6) in response to PRR ligation. However, KC also have the potential to release anti-inflammatory cytokines (i.e. IL-10) and/or reduce pro-inflammatory cytokine production in response to PRR ligation (termed KC tolerance). We suspect that ligation of multiple PRR by gut derived microbial products (either in sequence or simultaneously) results in KC tolerance. We plan to

assess the affects of the gut microbiota on KC in situ. To identify components of the gut microbiota that may condition KC to become tolerant, we will: **1)** Isolate KC from mice +/- gut bacteria, **2)** Stimulate KC with PRR ligands in vivo & **3)** Measure cytokine production (IL-10 and IL-6) resulting from PRR ligation using IF and IHC.

3.0 METHODS

3.1 ANIMALS

All mice received humane care according to the guidelines in the "Guide for the Care and Use of Laboratory Animals". Germ Free (GF) and Conventional (CL) Swiss Webster (SW) male mice aged 3-4 weeks or 6-9 weeks were purchased from Taconic (New York) and the Center for Gastrointestinal Biology and Disease Core at the University of North Carolina (Chapel Hill). GF SW mice were maintained inside of a sterile vinyl isolator for 48 hours with sterilized food and water. CL SW mice were maintained under specific pathogen–free conditions according to the National Institutes of Health guidelines for the use and care of live animals. Antibiotics (Ampicillin 1g/L, Vancomycin 0.5g/L, Metronidazole 1g/L, and Neomycin 1g/L) were delivered to CL SW mice (age 4 weeks) in drinking water for two weeks to generate the AVMN experimental group.

3.2 CELL PREPARATIONS

3.2.1 Non-Parenchymal Cells

Livers were perfused with cold Hanks Balanced Salt Solution (HBSS) and digested with 2ml cold collagenase (1mg/ml) (Sigma Aldrich). Perfused livers were then excised following removal of the gallbladder and placed in a 1:1 solution of cold Phosphate Buffered Saline (PBS) (Invitrogen, San Diego, CA) and Complete Media (RPMI 1640, Sigma Aldrich, The Woodlands, TX) supplemented with 5% FBS, nonessential amino acids, sodium pyruvate, HEPES, L-glutamine, 55µM ß-ME, and penicillin/streptomycin (Invitrogen). Excised livers were minced through a cell dissociation sieve (size 40, Sigma Aldrich) using a glass pestle for single cell suspension. The resulting cell suspension (50ml volume) was separated into hepatocytes (pellet) and NPC (supernatant) by centrifugation (500 rpm in off break setting for five minutes). The NPC fraction was passed through a 40µm cell strainer (BD Falcon, Franklin Lakes, NJ) and cell populations were stained for flow cytometry (FC). More than 85% of isolated NPC expressed common leukocyte antigen (CD45 AF700, Biolegend, San Diego, CA) as assessed by FC.

3.2.2 Intra-hepatic Lymphocytes

For intra-hepatic lymphocyte isolation, NPC were first separated from hepatocytes. Lymphocytes were separated from non-lymphocyte NPC, red blood cells, and debris using Lympholite M (Cedarlane, Burlington, NC) according to the manufacturer’s instructions.

3.2.3 Peripheral Blood Mononuclear Cells

Peripheral Blood Mononuclear Cells (PBMCs) were obtained from systemic blood suspended in 2ml HBSS for Ficoll (Invitrogen) gradient separation. The buffy coat was removed, washed, filtered (40µm cell strainer, BD Falcon, Franklin Lakes, NJ) and stained for FC.

3.2.4 Bone Marrow

Bone marrow was flushed using a 1:1 solution of PBS and Complete Media. Bone Marrow derived cells (BMDC) were washed, filtered (40µm cell strainer, BD Falcon), and stained for FC.

3.2.5 Blood

For systemic leukocyte analysis, 500µl systemic blood (heparinized) was obtained by cardiac puncture. Blood samples were analyzed by the Cell Counter (Beckman Coulter, Fullerton, CA). For ELISA assays, 500µl systemic blood was obtained by cardiac puncture and allowed to coagulate for 48 hours. Collected serum was used as directed to measure ALT (ALT ELISA, Novateinbio, Cambridge, MA) according to the manufacturer's instructions.

3.3 FLOW CYTOMETRY

Antigen	Clone	Host	Conjugate	Source
Flow Cytometry				
Gr1 (Ly6G)	RB6-8C5	Rat	PE	eBioscience
IgG2 Isotype control		Rat	PE	eBioscience

Cells were resuspended in Facs Buffer (1% BSA, 0.1% Sodium Azide in PBS) and analyzed on an LSR II with Facs Diva software. FC data were analyzed with FlowJo using antibodies listed in Table 3.

F4/80	BM8	Rat	PE-Cy7	eBioscience
IgG2a k Isotype control		Rat	PE-Cy7	Biolegend
CD11b	M1/70	Rat	Pacific Blue	Biolegend
IgG2b k Isotype control		Rat	Pacific Blue	Biolegend
MHC Class II (I-A/I-E)	M5/114.15.2	Rat	Alexa Fluor 700	eBioscience
IgG2b k Isotype control		Rat	Alexa Fluor 700	eBioscience
CD80 (B7-1)	16-10A1	Armenian Hamster	PE	eBioscience
IgG Isotype Control		Armenian Hamster	PE	eBioscience
CD86 (B7-2)	GL1	Rat	FITC	eBioscience
IgG2a k Isotype control		Rat	FITC	eBioscience
Ly6C	HK1.4	Rat	PE	Biolegend
IgG2c k Isotype control		Rat	PE	Biolegend
CD281 (TLR1)	TR23	Rat	PE	eBioscience
CD282 (TLR2)	6C2	Rat	FITC	eBioscience
CD284 (TLR4/MD2 complex)	MTS510	Rat	PE	eBioscience
CD289 (TLR9)	M9.D6	Rat	FITC	eBioscience
TLR5	85B152.5	Mouse	PE	Imgenex
CD16/CD32 (Fc Block)	93	Rat		eBioscience
Tissue Staining				
F4/80	CI:A3-1	Rat		Serotec
ICAM1	3E2	Hamster		BDPharmingen
CD44 (LYVE1)	ALY7	Rat		eBioscience
Ki67	SP6	Rabbit		GeneTex

Table 3. Cellular Staining Antibodies

3.4 BACTERIAL DETECTION

3.4.1 Stool Isolation

The cecum was opened aseptically to collect stool samples. Total DNA was extracted from 80 mg of cecal stool using a QIAmp DNA stool mini kit (Qiagen, Santa Clarita, CA) for bacterial

density assessment. Alternatively, fresh stool pellets were also collected for analysis of bacterial density variation over time and assessment of colonization dynamics.

3.4.2 Bacterial Density Measurements

Extracted DNA was submitted to real time PCR of the conserved region of the bacterial 16S rRNA gene using universal primers (Forward 5-TCCTACGGGAGGCAGCAGT-3 and Reverse 5-GGACTACCAGGGTATCTAATCCTGTT-3) (96), calibrated using DNA from E. coli (max efficiency DH5α competent cells, Invitrogen). Amplification and detection of DNA by Q-PCR were performed with the 7500 Fast Real-Time PCR Instrument (Applied Biosystems, Foster City, CA). Bacteria enumeration used Invitrogen's Bacteria Counting Kit.

3.5 PRR LIGAND SCREENING

Portal and Systemic Blood were collected from adult CL SW mice, plasma isolated and alkaline phosphatase activity was heat inactivated by 30 minute incubation at 56°C. PRR stimulation was tested by Invivogen (San Diego, CA) by assessing NF-κB activation in HEK293 cells expressing individual mouse PRR. Briefly, Invivogen performed PRR ligand screening by assessing NF-κB activation in HEK293 cells expressing a single PRR. NF-κB activation was monitored by measuring secreted alkaline phosphatase, a reporter gene in HEK293 cells induced by NF-κB. Plasma from CL mice was tested on mouse NOD1, NOD2 and on mouse TLR-2, -3, -4, -5, and -9. Plasma was tested in duplicate using a 1:10 dilution and compared to control ligands. The

following were used as positive controls: Heat-killed *Listeria monocytogenes* (HKLM) (TLR-2), Poly (I:C) (TLR-3), E. coli K12 LPS (TLR-4), *S. typhimurium* flagellin (TLR-5), CpG ODN 1826 (TLR-9), C12-iEDAP (NOD-1), L18-MDP (NOD-2), and TNFα (NF-κB control cells). NF-κB Control cells (no PRR) and unstimulated PRR-expressing HEK293 cells were used as negative controls. The results are provided as mean OD values. For semi-quantification of ligands, plasma was concentrated 2-fold for testing (Amicon Ultra 3K Centrifugal Filters, Millipore, Marlborough, MA). Tenfold dilutions of plasma and positive control ligands were then tested in triplicate for activation of TLR-2, TLR-5, and TLR-9.

3.6 PRR STIMULATION

Flagellin (FLA-ST, Invivogen) (dose of 100ng, 100ul) was injected via tail vein into CL and GF mice. Controls were injected with 100µl PBS. NPC were isolated 24 hours post-challenge.

3.7 TISSUE STAINING

FFPE and OCT embedded tissue was sectioned (4 µm) and stained with antibodies listed (Table 3). H&E staining was performed according to standard protocol. For Immunofluorescence, Qdot® Streptavidin conjugates (Invitrogen) were used following application of biotinylated rat secondary (BD pharmingen) or biotinylated hamster and rabbit secondary (Vector Laboratories, Burlingame, CA) antibodies. Mirax Whole Slide Scanner created whole slide images for analysis (Zeiss, Thornwood, NY) using Axiovision Software. When comparing H&E sections

between groups, histology scoring was performed blindly by two individual pathologists.

3.8 PROTEIN EXPRESSION

Protein was isolated from the left lateral lobe of CL, GF, and AVMN livers by tissue homogenization in RIPA buffer containing complete protease inhibitors (Roche, Indianapolis, IN). Protein quantification was assessed by BCA Protein Assay Kit (Pierce, Chicago, IL). Seventy-five micrograms of protein was run on SDS-PAGE. Following electrotransfer to a nitrocellulose membrane, 5% milk in TBST (Tris buffered saline tween) was used for blocking. Monoclonal β-actin (clone AC-15, Sigma Aldrich), CD1d (clone Y17, Santa Cruz, Santa Cruz, CA), and ICAM1 (clone M19, Santa Cruz, Santa Cruz, CA) were used to probe the membrane. ECL western blotting substrate (Thermo Scientific, Ashville, NC) was used to visualize the protein.

3.9 GENE EXPRESSION

3.9.1 RNA Isolation, RT-PCR, and PCR for murine genes.

Total RNA was extracted from homogenized liver tissue using Trizol (Invitrogen). Reverse transcription was performed using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) according to the manufacturer’s instructions. To determine expression of *csf1* and *csf2* relative to the *hprt* internal control, 25ng cDNA was used in a 10µl reaction using the

Taqman Fast Universal PCR Master Mix (Applied Biosystems) and Taqman array 96 well fast plates for Mouse Immune Response (Applied Biosystems). Analysis was performed on a 7500 Fast Real-Time PCR Instrument (Applied Biosystems).

3.9.2 Microarray

Using the GeneChip Mouse Gene 1.0 ST Arrays from Affymetrix (Santa Clara, CA), we examined hepatic gene expression levels in CL, GF, and AVMN mice. Livers were collected from 18 mice (6 animals per experimental condition) and placed in Trizol (Invitrogen) for RNA extraction. Genome Explorations performed RNA extraction, labeling, and hybridization of cDNA onto gene chips (Memphis, TN) according to standard operating procedure. Gene signal values were calculated by RMA for background correction, normalization, and summarization. Differential Gene Expression was assessed using the following criteria: (1) Differentially expressed Genes (DEGs) had signal values ≥ 8 for all samples in one group, (2) False Discovery Rate (FDR)-corrected ANOVA p values were ≤ 0.05, (3) Absolute fold change was ≥ 1.5 and FDR corrected t-test p-values ≤ 0.05 with FDR correction performed by the Benjamini-Hochberg method (corrects type 1 error associated with multiple comparisons). Log 2 signal values for DEF probe sets were row mean centered and subjected to unsupervised hierarchal clustering by the complete linkage algorithm using Pearson Correlation as the similarity metric. Samples were clustered by Complete Linkage based on Pearson Correlation permitting identification of outliers. Results from the micro-array and pathway analysis were primarily performed by the statistician and CRO of Genome Explorations, Bob Rooney.

3.10 PHAGOCYTOSIS

Mice were injected (tail vein) with Sphero™ Carboxyl Magnetic Particles (1.22 µm) once (200µl, ~5.4×10^8 particles, Spherotech Inc., Libertyville, IL). NPC were isolated 24 hours later and active liver phagocytes were isolated by magnetic separation. Phagocytes were then counted and analyzed by FC.

3.11 KUPFFER CELL REPOPULATION MODEL

Kupffer Cells can be depleted using liposome entrapped clodronate (van Rooijen). CL mice were treated as described: Day 0: Clodronate Liposome injection (300µl, i.p.). Day 3: Blocking antibody injection i.p. using 50µg (100µl) anti-ICAM1 (YN1/1.7.4, Abcam) and/or 20 µg (100 µl) anti-LYVE1 (CD44, IM7.8.1, Cedarlane, Burlington, NC). Rat IgG2b (50 µg, 100µl) (BD Biosciences, San Jose, CA) was used as an isotype control. Day 6: KC Repopulation and Monocyte frequency assessment by FC.

Cl_2MDP (clodronate) was a gift from Roche Diagnostics GmbH, Mannheim, Germany. Other reagents to make liposome entrapped clodronate include: Phosphatidylcholine (LIPOID E PC) from Lipoid GmbH, Ludwigshafen, Germany; Cholesterol (SIGMA Chem.Co. USA); CL_2MDP was synthesized by the laboratory of Dr. N. van Rooijen in Amsterdam, The Netherlands.

3.12 AUTOIMMUNE HEPATITIS MODEL

Synthetic glycolipid antigens induce NKT cell mediated hepatitis modeling Autoimmune Hepatitis (81). CL SW and GF SW mice were treated as follows: α-Galactosylceramide (KRN7000, Funakoshi, Japan) was injected i.v. (tail vein) with a dose of 5μg (50μl). Controls were injected with 50 μl PBS. Blood and tissue were collected at 0, 12, and 24 hours post-injection.

3.13 STATISTICAL ANALYSIS

The Student *t* test was used for statistical analysis of data when sample size was > four per group; the nonparametric Mann-Whitney U test was used when sample size was ≤ four per group. ANOVA was used when comparing more than 2 groups. Data are presented as the arithmetic mean. Error bars represent standard error of the mean.

4.0 RESULTS: KUPPFER CELL POPULATION DEVELOPMENT

4.1 GUT BACTERIA ARE A MAJOR SOURCE OF SYSTEMIC MAMPS

Gut bacteria shape normal gut morphology as evidenced by cecal dilation in GF and AVMN mice, which has been previously attributed to water and mucus accumulation and a thinner cecal muscularis propria (1, 4) (Figure 2).

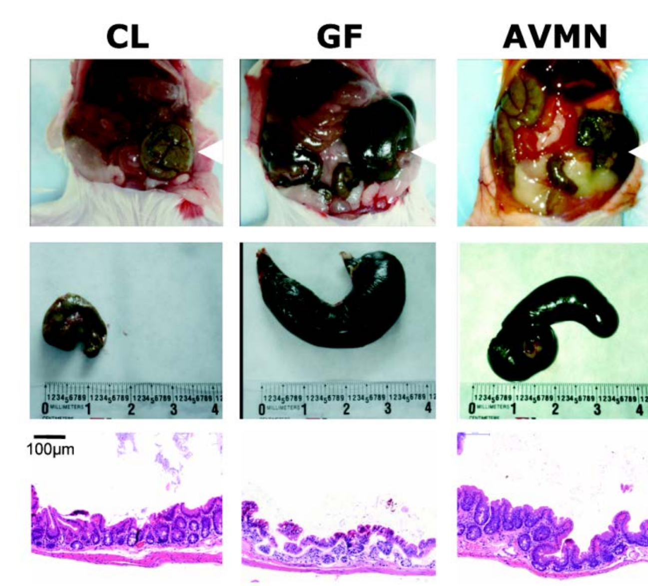


Figure 2. Gut Morphology. Top, representative images of intestines show cecal dilations in GF and AVMN, but not in CL mice (white arrow). Middle, representative images of the isolated cecum highlights dilation in GF and AVMN mice. Bottom, longitudinal H&E section of cecal wall shows thinned muscularis propria in the GF and AVMN mice (100μm scale bar). Conventional (CL), Germ Free (GF), and Antibiotic Treated (AVMN) mice were used.

Gut microbial density increases temporally and spatially with greatest bacterial loads found following weaning during adulthood and in the distal gut (4, 97) (Figure 3A and 3B). Bacterial density, however, varies with environment, mouse background, gender, and age (13, 21). Since > 50% of gut bacteria cannot be cultivated (21, 25), we assessed cecal bacterial density via PCR amplification of the bacterial 16S ribosomal gene and via flow cytometry. Cecal bacterial density measurements showed that adult CL mice average > 12 million bacteria per gram of stool, but CL siblings housed in the same room, cage, and specific pathogen free conditions yielded a wide range of bacterial levels (Figure 3B). This variation is likely attributable to bacterial population dynamics. As expected, cecal bacteria were undetectable in GF mice (Figure 3A and 3B), whereas AVMN mice showed an 8.5-fold reduction in bacterial DNA relative to CL mice (Figure 3B, p=0.005).

Live whole bacterial translocation to the liver rarely occurs under non-pathogenic homeostatic conditions (2, 23) (data not shown), but it is widely accepted that gut-derived MAMPs shed by commensals are able to penetrate the gut barrier. Yet, the amalgam of MAMPs able to translocate into portal and systemic circulation and to cause activation of TLR-expressing cells has not been fully characterized. It is also unclear whether physiologic levels of individual or combinations of MAMPs exert downstream effects on the liver. Therefore, we identified gut-derived PRR ligands by screening portal and systemic blood for MAMPs using a NF-kB activation bioassay (Figure 3C). PRR ligation by MAMPs from portal and systemic blood was observed for TLR-2, TLR-5, and TLR-9, but not for TLR-3, TLR-4, NOD-1, or NOD-2 (Figure 3C). Although TLR-4 and NOD ligands (LPS and PDG respectively) are known to be detectable in the portal circulation, their physiological concentrations may not be detectable in this bioassay or they might be in a form that does not trigger PRR signaling. Alternatively, LPS and PDG

might be responsible for the observed TLR-2 activation since both are known to bind to this receptor.

We next examined NF-kB activation in response to TLR-2 (Figure 3D), TLR-5 (Figure 3E), and TLR-9 (Figure 3F) ligation by portal blood MAMPs in comparison to concentrations of known TLR ligands (HKLM, flagellin, and CpG respectively). Figure 3E and 3F indicate TLR-5 ligand concentration (presumably flagellin) ranges between 1-10ng/ml and TLR-9 ligand concentration (presumably bacterial DNA) ranges between 0.01-0.1 μg/ml in portal circulation. The relative concentration of TLR-2 ligand is indeterminate due to infidelity of TLR-2.

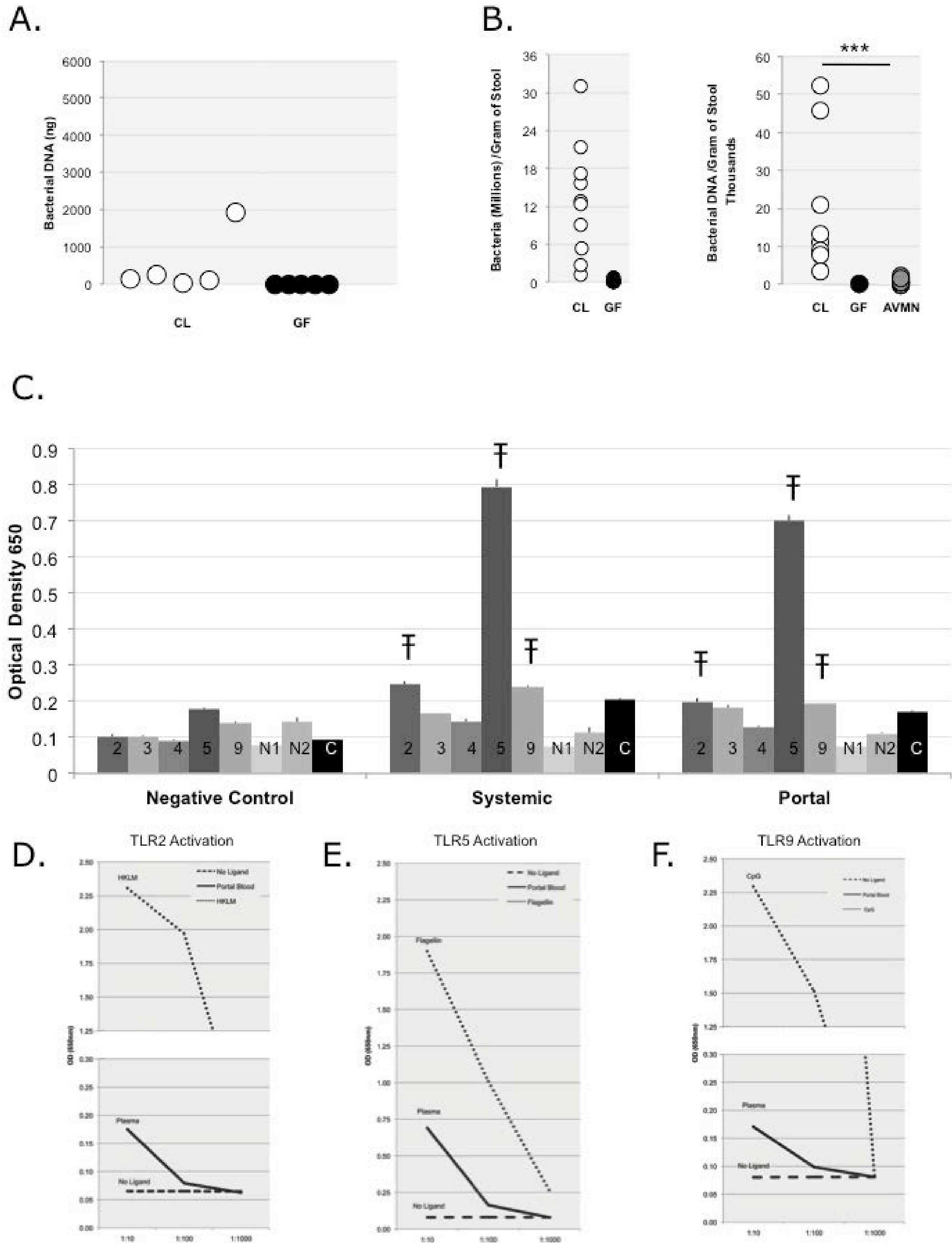


Figure 3. Gut Bacteria and Intestinal Permeability. (A) Bacterial DNA (ng) in 80mg cecal stool assed by real time PCR amplification of 16S rRNA gene in adolescent mice (3-4 weeks old) shows little difference in bacterial

load between GF and CL mice. (**B**) Left, bacterial density of cecal stool assessed by a Flow Cytometry Bacterial Counting Kit confirms the gut bacterial status of adult (6-8 weeks) CL and GF mice and shows the marked variability in CL mice. Right, bacterial DNA (ng) in cecal stool assessed by real time PCR amplification of 16S rRNA gene again shows absence (GF) or significant reduction (AVMN; ***p=0.005) and considerable variability of gut bacteria in CL mice (n=10). (**C**) Pattern Recognition Receptor (PRR) ligand detection in CL portal and systemic blood assessed by NFkB activation in HEK293 cells expressing mTLR2 (2), mTLR3 (3), mTLR4/CD14/MD2 (4), mTLR5 (5), mNOD1 (N1), mNOD2 (N2), or no PRR on NFkB Control Cells (C). Results represent blood pooled from 10 mice, averages are representative of two experiments performed in duplicate (Ŧ = activation levels above negative controls from the pooled sample of 10 mice; repeated twice). (**D-F**) Estimation of plasma TLR ligand concentrations for TLR2, TLR5, and TLR9 relative to known controls: (**D**) TLR 2 Activation on HEK293 cells by no ligand (Dashed line), Portal Blood (Solid line), HKLM (heat-killed Listeria monocytogenes (Dotted line) at 10^8, 10^7, and 10^6. Blood pooled from 10 mice. Samples were run in triplicate. (**E**) TLR5 Activation on HEK293 cells by No ligand (Dashed line), Portal Blood (Solid line), Flagellin (Dotted Line) at 100, 10, and 1 ng/ml. (**F**) TLR9 activation on HEK293 cells by No ligand (Dashed line), Portal Blood (Solid line), CpG ODN 1826 (Dotted Line) at 1, 0.1, and 0.01 µg/ml. Conventional (CL), Germ Free (GF), and Antibiotic-Treated (AVMN) mice were used.

4.2 KUPFFER CELL FREQUENCY AND MHC CLASS II EXPRESSION INCREASES WITH BACTERIAL DENSITY

Ligation of a PRR (NOD1) by a gut-derived MAMP (PDG) results in expansion of specific mucosal immune cells (i.e. regulatory T cells) (69, 98). Since liver NPC, including KC and LSEC, express many PRRs for MAMP detection (40, 99), we determined whether gut bacteria stimulate expansion of liver immune cell populations. Results show nucleated sinusoidal cell number is reduced in GF livers (Figure 4, p=0.009). Similar results were obtained for the

absolute numbers of NPC isolated from CL and GF livers assessed by trypan blue exclusion of dead cells and cell counting (6.34 ± 1.2 million and 4.17 ± 1.2 million respectively, p=0.28).

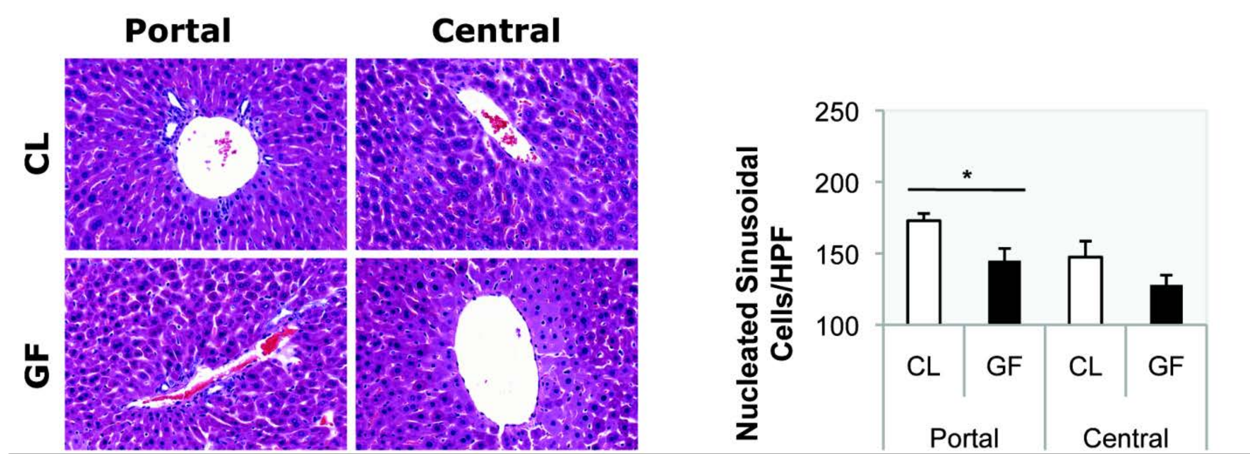


Figure 4. Sinusoidal Cells and Germ Status. Nucleated Hepatic Sinusoidal Cell Quantification; 10 High Power Fields (HPF, 40x) per region per group (n=3) of H&E sections were used to quantify sinusoidal cell count. Germ Free (GF) and Conventional (CL) mice were used. Statistical Significance indicated by: *P<0.05.

Accordingly, we hypothesized that gut bacteria contribute to quantitative and/or qualitative characteristics of KC. First, we evaluated KC frequency during adolescence when the gut microbiome is not fully established. During adolescence, CL mice have 15.2% F4/80+ NPC and GF mice have 17.11% F4/80+ NPC, though both CL and GF KC are relatively immature/inactivated as reflected by the insignificant MHC Class II expression (0.5% and 1.5 % F4/80+MHCII+ NPC respectively) (Figure 5A). However, the KC population size doubles during the transition from adolescence to young adulthood in CL mice whereas KC frequency in age-matched GF counterparts remains unchanged (CL 32% and GF 14% F4/80+ NPC in adults, Figure 5B). Moreover, adult CL mice have a significant population of MHC Class II+ KC

(14.4% NPC) while adult GF mice (7.4% F4/80+MHCII+ NPC) resemble their juvenile counterparts (Figure 5B, Figure 6).

Perplexed by the variability in CL KC compared to GF KC frequency, we hypothesized that the former might be related to the aforementioned differences in gut bacterial loads among CL mice (Figure 3B). Results showed that gut bacterial load is directly related to the number of mature KC (F4/80+MHC II+ NPC) (Figure 5C, p=0.024). If more gut bacteria are associated with higher KC numbers, then less gut bacteria should result in lower KC numbers. As expected, AVMN mice showed a significant reduction in KC number compared to CL mice (AVMN 3.5% and CL 20.4% NPC) and either lose or fail to develop MHC Class II+ KC (AVMN 0.6% and CL13.5% NPC) (**Figure 5D**).

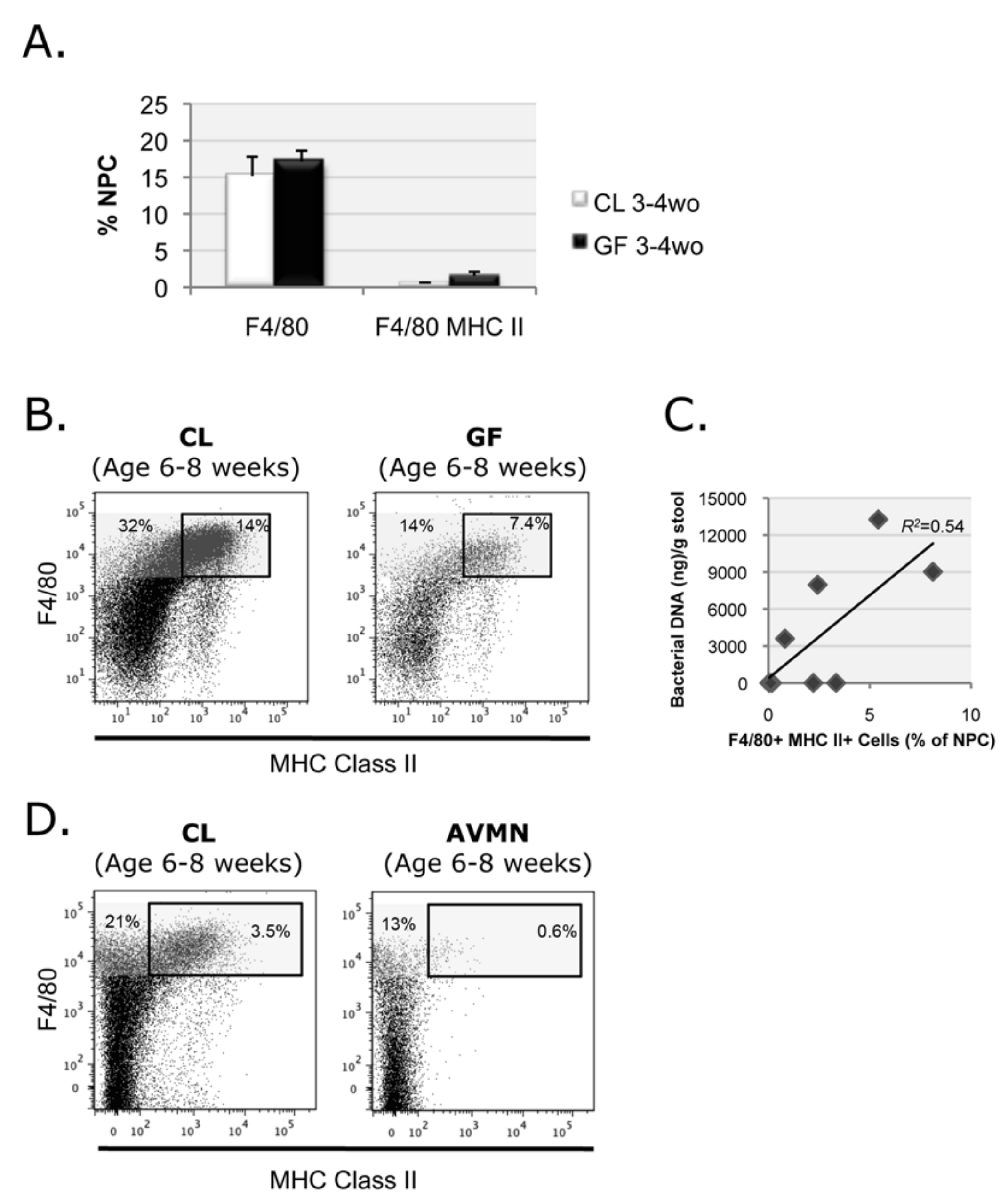
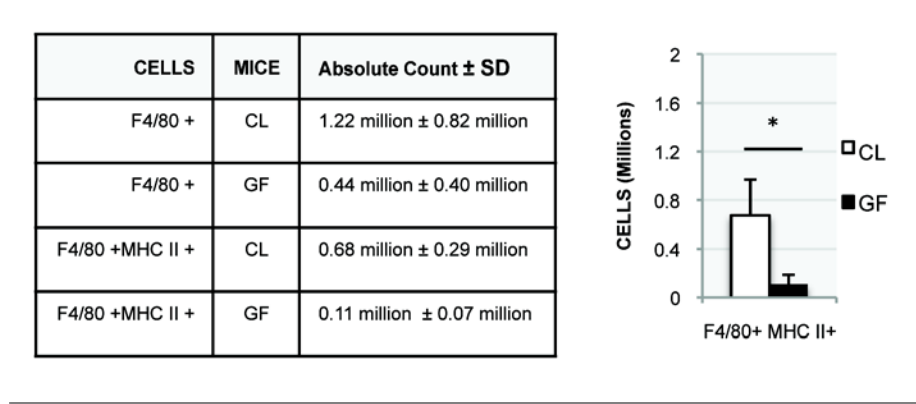


Figure 5. The Gut Microbiota Increase Kupffer Cell Frequency. (**A**) Percentage of NPC isolated from livers of adolescent CL and GF mice expressing F4/80 (macrophage) or F4/80 and MHC Class II (mature macrophage) were similar. Representative of 3 independent experiments (CL n=6, GF n=6) (F4/80+ p=0.40 and F4/80+MHC II+; p=0.06). (**B**) Percentage of F4/80+ NPC (gray filled gate) or F4/80+MHC II+ NPC (black lined gate) isolated from livers of adult CL mice were significantly greater than GF mice (CL n=5, GF n=5; ***p<0.01). Dot Plots representative of 4 independent experiments. (**C)** Correlative analysis showed mature KC frequency (F4/80+MHC II+ NPC) was directly proportional to bacterial load (ng of bacterial DNA/g cecal stool); (Pearson's correlation

coefficient=0.73; p=0.024). (**D**) Percentage of F4/80+ NPC (gray filled gate) or F4/80+MHC II+ NPC (black lined gate) isolated from livers of adult CL mice were significantly greater than AVMN mice (CL n=5, GF n=5; ***p<0.01). Representative of 2 independent experiments. Adult Conventional (CL), Germ Free (GF), and Antibiotic-Treated (AVMN) mice were used.



CELLS	MICE	Absolute Count ± SD
F4/80 +	CL	1.22 million ± 0.82 million
F4/80 +	GF	0.44 million ± 0.40 million
F4/80 +MHC II +	CL	0.68 million ± 0.29 million
F4/80 +MHC II +	GF	0.11 million ± 0.07 million

Figure 6. Kupffer Cell Numbers. Absolute KC Frequency in adult mice (Product of Absolute NPC count and average KC Percentage) (n=6), Representative of 3 independent experiments. Statistical Significance indicated by: *p<0.05. Conventional (CL) and Germ Free (GF) mice were used.

Twice as many KC are located in periportal compared to the centrilobular sinusoids (94). We therefore determined if the KC deficiency observed in GF and AVMN mice was global or regional. CL mice exhibited twice as many KC in the periportal regions (40 KC/Hpf) compared to the centrilobular regions (18 KC/Hpf), but GF and AVMN mice exhibited both reduced periportal (22 KC/Hpf and 26 KC/Hpf) and centrilobular (13 KC/Hpf and 14 KC/Hpf) KC numbers respectively (Figure 7). The change in KC frequency could not be attributed to differences in liver size (Figure 8), lobular size (data not shown), or macrophage proliferation alone as assessed by Ki67 expression (Figure 9).

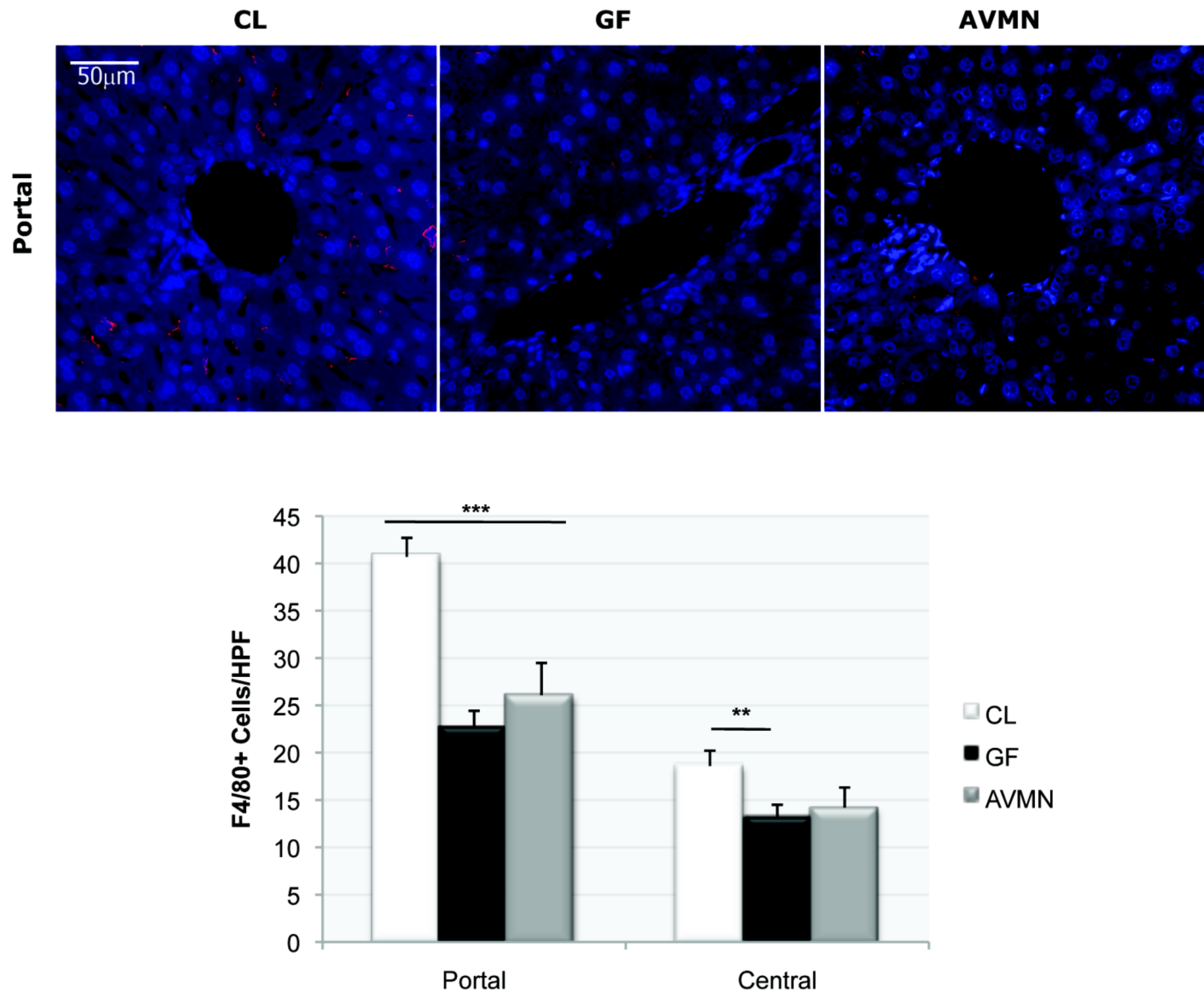


Figure 7. The Gut Microbiota Direct KC Distribution. KC distribution in livers of CL, GF, and AVMN mice assessed by immunofluorescence staining of liver sections with F4/80 (red) and DAPI (blue) nuclear counterstain. CL mice showed significantly more F4/80+ KC/HPF in both periportal and centrilobular sinusoids (**P<0.025; ***p<0.01). Representative images were selected from 3 liver sections per group consisting of 13-20 portal and central areas with vessel diameter between 50-200μm; Scale bar: 50μm. Adult Conventional (CL), Germ Free (GF), and Antibiotic-Treated (AVMN) mice were used.

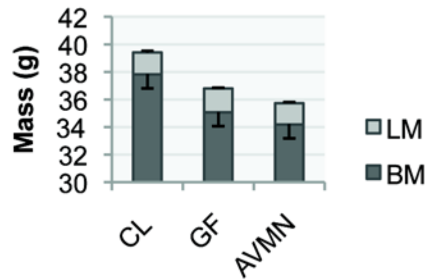


Figure 8. Liver and Body Size. Mean Liver (LM) and Body Mass (BM) shown. Adult Germ Free (GF, n=12), Conventional (CL, n=12), and Antibiotic Treated (AVMN, n=8) mice were used; BM (ANOVA p=0.029), LM (ANOVA p=0.31), LM/BM ratio (ANOVA p=0.14).

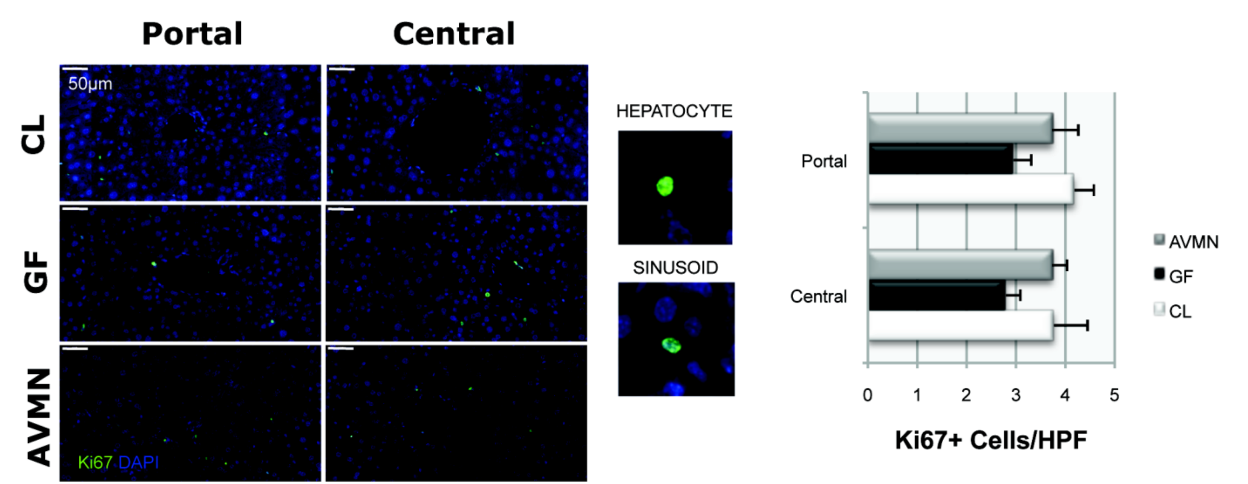


Figure 9. Sinusoidal Cell Proliferation. Ki67 (green) indicates cell that has entered cell cycle. Representative 40X images shown from Conventional (CL), Germ Free (GF), and Antibiotic Treated (AVMN) mice; 15-20 images per region per group (n=3) used for quantification. Although GF showed slightly less sinusoidal cell labeling, the differences were not statistically significant (ANOVA: portal p= 0.14 and central p=0.18).

4.3 GUT BACTERIA AFFECT KC PHENOTYPE AND FUNCTION

KC can be divided into sub-populations based on inflammatory potential (inflammatory FSP1+F480+ cells and quiescent FSP1-F480+ cells) (77, 100). Therefore, we quantified the number of FSP1-positive and FSP1-negative KC in CL and GF mice to further characterize KC in the context of constitutive MAMP exposure. In absence of any supra-physiological stimulation, only a very small subset of F480+ cells also expressed FSP1 (1-2cells/hpf) and no differences in the number of FSP1+F480+ cells were observed between CL and GF mice (Figure 10). Physiological levels of gut-derived MAMPs, therefore, were insufficient to elicit an inflammatory KC phenotype. This does not, however, exclude the possibility that FSP1 expression by KC may change in response to acute or chronic supra-physiological stimulation.

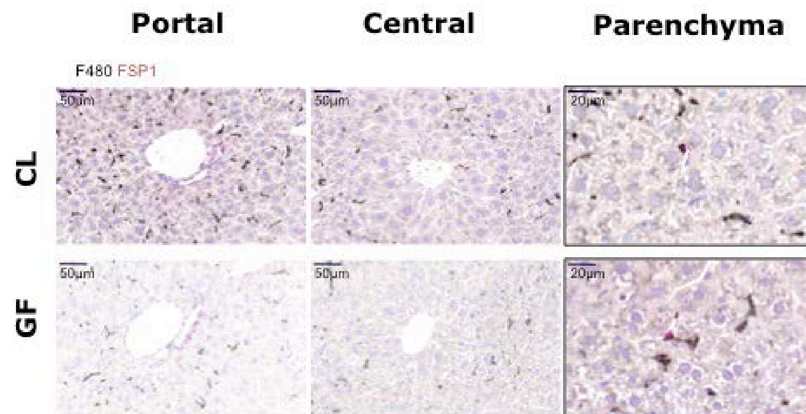


Fig 10. Kupffer Cell Inflammatory Potential. Inflammatory potential of Kupffer Cells (black) assessed by FSP1 (dark pink) staining. Representative 40x images were selected from 2 liver sections per group consisting of 11-20 portal and central areas with vessel diameter between 50-200 µm. Single FSP1+ Kupffer Cell is shown on far right at 100x. Left and Middle Scale bar: 50µm; Right Scale Bar: 20µm. Adult Conventional (CL) and Germ Free (GF) mice were used.

KC link innate and adaptive immunity by interacting with circulating lymphocytes in the hepatic sinusoid via MHC Class II presentation of antigenic epitopes following endocytosis of circulating antigens (74, 101). Loading of MHC molecules with antigenic peptide is often followed by up-regulation of co-stimulatory molecules CD80 and CD86. Because MHC Class II expression increased with higher bacterial loads (Figure 5B and 5D), we next assessed expression of co-stimulatory molecules CD80 and CD86 on KC and phagocytic function (part of exogenous pathway of antigen presentation). Results showed no significant differences in CD80/CD86 expression when comparing KC from CL, GF, and AVMN mice, although KC in GF and AVMN mice consistently expressed modestly lower levels (Figure 11).

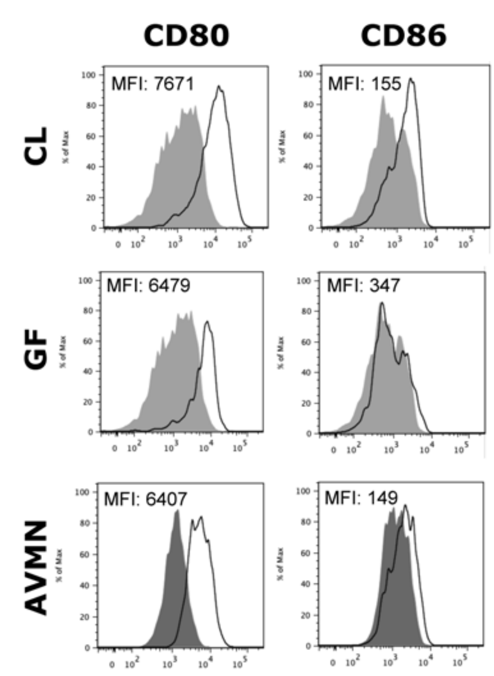


Figure 11. KC Express Low Levels of Co-stimulatory Molecules. Co-stimulatory molecule expression (CD80 and CD86) by KC from CL, GF, and AVMN mice showed no significant difference in percentage of F4/80+ NPC expressing CD80 (>90% for each group, p=0.50) or in percentage of F4/80+ NPC expressing CD86 (>20% for each group, p=0.17). Isotype controls are shown in gray. Histograms are representative of 2 independent experiments (n=5-6 per group). Adult Conventional (CL), Germ Free (GF), and Antibiotic-Treated (AVMN) mice were used.

Since KC characteristically exhibit low levels of co-stimulatory molecules in comparison to other professional antigen presentation cells (90, 99), we assessed co-stimulatory molecule expression twenty-four hours after flagellin stimulation, which is the most highly detectable MAMP found in both portal and systemic blood (Figure 3C, 3E). Flagellin stimulation did not significantly increase or decrease co-stimulatory molecule expression by CL or GF KC (Figure 12A).

We next evaluated *in vivo* phagocytic activity by determining the number of active non-endothelial phagocytes following magnetic isolation of cells containing micro-magnets (1.22 μm). The percentage of active phagocytes was 5.4% (54,716 phagocytes/million NPC) in CL livers versus 9.7% (97,000 phagocytes/million NPC) in GF livers (Figure 12B). The increased phagocytic activity in GF mice suggests that MAMP exposure reduces environmental sampling although overall collective environmental sampling might be maintained in CL mice due to increased KC numbers.

Because LPS has been shown to stimulate KC expansion (78), we determined if flagellin (the most highly detectable MAMP in portal circulation) generated a "CL phenotype" in GF livers (Figure 12C). Neither total KC (F4/80+ NPC) nor mature KC frequency (F4/80+MHC II+ NPC) were altered by flagellin stimulation at physiological concentrations within the limitations of a 24-hour time course to ensure no influence from environmental germ exposures in the GF

mice. However, KC progenitor (monocyte) frequency in GF livers nearly doubled that of CL livers post-challenge (Figure 12C).

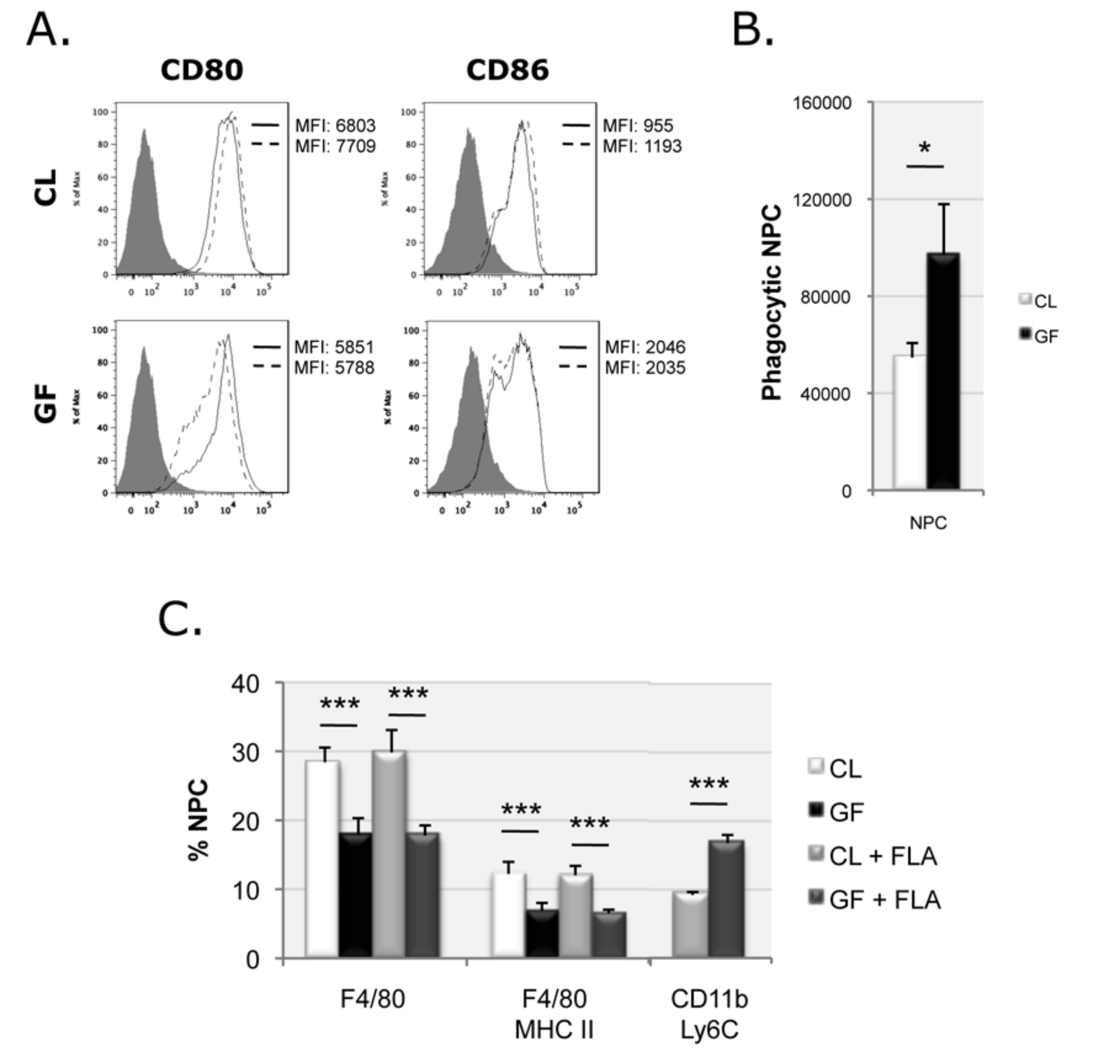


Figure 12. ***In vivo*** **response to Antigenic Stimulation and Phagocytic Function.** (**A**) Co-stimulatory Molecule Expression on KC from CL and GF mice pre- (black solid line) and 24 hours post-treatment (black dashed line) with 100ng of Flagellin (FLA) showed no significant difference; Isotype controls are shown in gray. Histograms are representative of 2 independent experiments (n=3-4 per group). (**B**) Phagocytosis (1.2 micron magnet uptake) assay shows that liver NPC from GF mice have significantly more phagocytic activity than CL mice (*p=0.031)**.** Representative of 2 independent experiments (n=3 per group). (**C**) KC and Monocyte frequency in CL and GF mice pre- (0 hours) and post-treatment (24 hours later) with 100ng FLA shows FLA treatment does not cause significant

differences in KC frequency. However, the difference between CL and GF is obvious (***$p<0.01$). Monocyte Frequency (CD11b+Ly6C+ NPC) 24 hours post FLA challenge results in greater liver monocyte influx in GF mice compared to CL mice (***$p<0.01$). Representative of 2 independent experiments (n=3-4 per group). Adult Conventional (CL), Germ Free (GF), and Antibiotic-Treated (AVMN) mice were used.

4.4 CONSTITUTIVE MAMP EXPOSURE DOES NOT CHANGE PRR EXPRESSION BY KUPFFER CELLS

PRR are conserved germ-line encoded receptors (36, 99), but expression levels of some PRR (i.e. TLR-4) can increase in response to stimulation (40, 102). KC express many PRR constitutively (TLR-2, TLR-4, TLR-5, TLR-9 and NOD-2) (40, 48, 99), but we hypothesized that KC PRR expression might be dependent on constitutive MAMP exposure. Thus, TLR-2, TLR-4, TLR-5, and TLR-9 expression was evaluated before and after flagellin stimulation (Figure 13). Results show that CL KC and GF KC express surface TLR-2, TLR-4, TLR-5, and TLR-9 (Figure 13). TLR-5 expression was modestly increased in response to flagellin, but overall KC surface TLR expression did not appear to be dependent on gut bacteria (Figure 13).

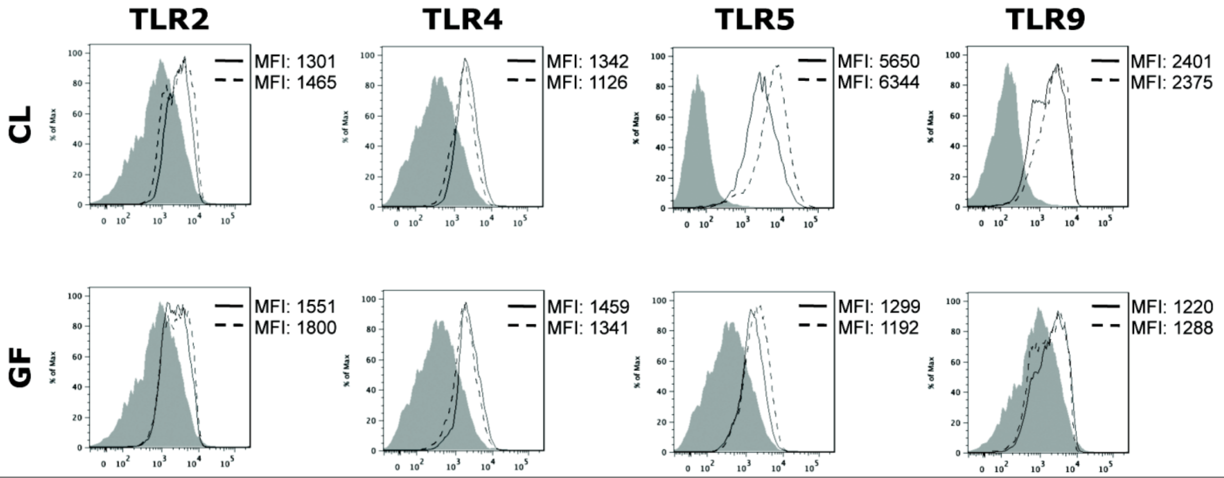


Figure 13. Germ Status does Not Change Pattern Recognition Receptor Expression. TLR2, 4, 5, and 9 expressions on KC (F4/80+ NPC) from CL and GF mice pre- (solid black line) or 24 hours post-treatment (dashed black line) with 100ng Flagellin showed no change in TLR expression. Isotype Controls are shown in gray. Histograms are representative of 2 independent experiments (n=3-4 per group). Adult Conventional (CL) and Germ Free (GF) mice were used.

4.5 CLASSICAL KUPFFER CELL PROGENITOR FREQUENCY IS INDEPENDENT OF GUT BACTERIA

KC arise from bone marrow-derived monocytes under the influence of colony-stimulating factors as defined by the mononuclear phagocyte system (MPS) (75, 94). Therefore, the KC deficiency associated with low gut bacteria could be due to: 1) deficiency of KC progenitors; and/or 2) failure of KC progenitors to differentiate. First, macrophage-colony stimulating factor (*csf1*) and granulocyte macrophage-colony stimulating factor (*csf2*), which drive monocyte differentiation and promote KC survival, were not differentially expressed (cDNA assessed by RT-PCR) between CL (average $2^{-\Delta Ct}$: csf1 0.16, csf2 0.009), AVMN (average $2^{-\Delta Ct}$: csf1 0.02,

csf2 0.01) and GF (average $2^{-\Delta Ct}$: csf1 0.62, csf2 0.0006) livers relative to the *hprt1* internal control (Figure 14). Second, monocyte frequency (%CD11b+Ly6C+ or %CD11b+GR1+ cells) in the liver (<3% of NPC in CL, GF, and AVMN mice) (Figure 15A), circulation (CL 2.1 % PBMC, GF 3.4 % PBMC) (Figure 15B), and bone marrow (CL 51.3% BMDC, GF 53.4% BMDC) (Figure 15C) were unaffected by gut bacteria.

Notably, however, a population of circulating CD11b-Ly6C+ cells was reduced in GF mice (Figure 15B). CD11b-Ly6C+ cells are not classically characterized as monocytes (myeloid lineage), but there is some plasticity within the MPS (75) and thus this cell population cannot be entirely excluded as precursors to KC or other cell types. Regardless, these findings indicate the frequency of intra-hepatic monocytes (classical KC progenitors) is independent of gut bacteria and the mobilization of monocytes from the bone marrow to circulation is not defective. Therefore, another mechanism is responsible for the KC deficiency associated with gut bacteria deficits.

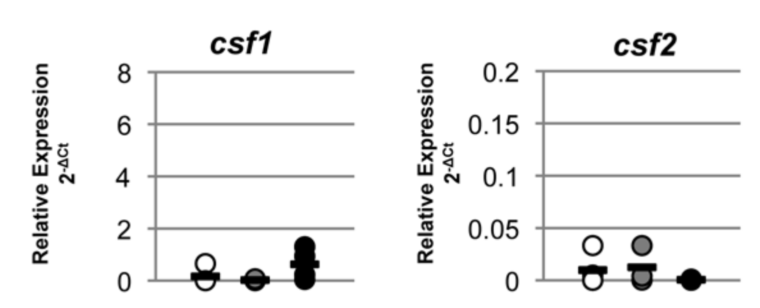


Figure 14. KC Differentiation Factor Expression in the Liver. Relative expression of M-CSF (*csf1*) (ANOVA p=0.50) and GM-CSF (*csf2*) (ANOVA p=0.84) showed no significant differences among the mouse groups. Black line represents mean expression, *hprt* used as internal control; CL (white circle) n=4, AVMN (gray circle) n=4, GF (black circle) n=4. Conventional (CL), Germ Free (GF), and Antibiotic Treated (AVMN) mice were used.

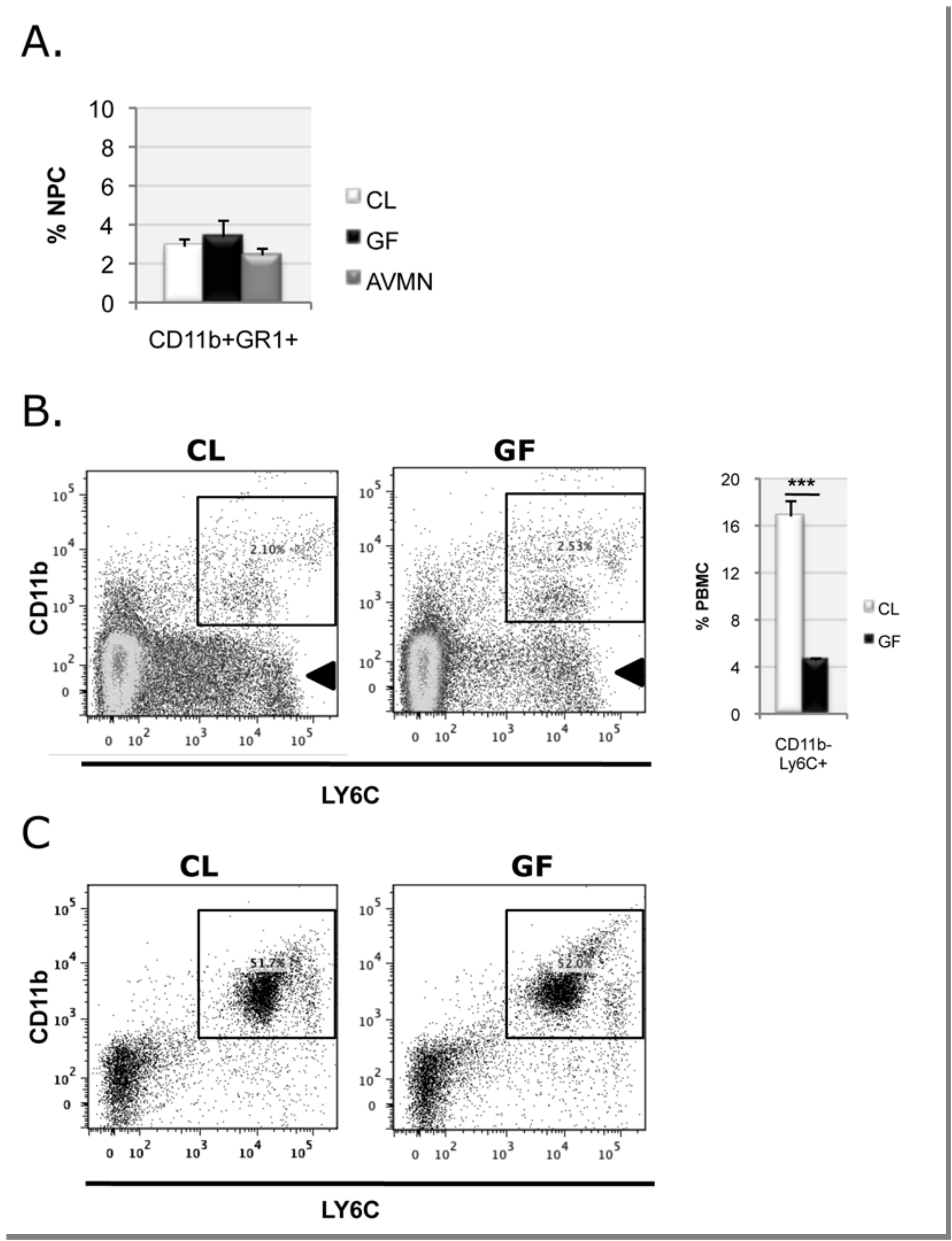


Figure 15. KC Progenitor Development is Independent of Gut Bacteria. (**A**) KC progenitors in the livers of CL, GF, and AVMN mice showed no differences among groups (ANOVA p=0.663). Representative of 3 independent experiments (CL n=3-6, GF n=3-5, AVMN n=3-4). (**B**) KC progenitors in systemic circulation (% Peripheral Blood Mononuclear Cells (PBMC)) in CL and GF mice were not significantly different (p=0.27); CD11b+Ly6C+ PBMC (black lined gate). Circulating CD11b-Ly6C+ PBMC (black arrow; see text), however, were significantly lower in

GF mice; CL n=6, GF n=6 (***p<0.01). (**C**) KC progenitors in Bone Marrow (% Bone Marrow Derived Cells (BMDC)) in CL (n=6) and GF (n=6 mice) were not significantly different (p=0.37); CD11b+Ly6C+ BMDC (black lined gate). Conventional (CL), Germ Free (GF), and Antibiotic-Treated (AVMN) mice were used.

4.6 MAMP EXPOSURE INDUCES ICAM1 AND LYVE1 EXPRESSION BY LSEC INCREASING KC RETENTION

Monocyte recruitment to the liver in the infectious setting is stimulated by upregulation of adhesion molecules on LSEC (48, 103) although some adhesion molecules are thought to be constitutively expressed in the liver, including ICAM1 (70). Thus, we determined if basal adhesion molecule expression is modulated by MAMPs and if a change in adhesion molecule expression alters KC number. We first examined ICAM1 expression on LSEC (LYVE1+ cells), but LYVE1, a liver sinusoidal endothelial cell marker and adhesion molecule (103-105) was not consistently expressed between CL and GF mice (Figure 16A, Figure 17). Compared to expression in CL mice, quantitative morphometric examination of LYVE1 expression in GF livers was reduced (Figure 16A). ICAM1 expression was similarly reduced in GF livers, although particularly so in the centrilobular regions (Figure 16A, Figure 17). We confirmed by Western blotting that whole liver ICAM1 expression was significantly reduced in GF and AVMN mice (Figure 16B, p<0.001).

A re-population model was next utilized to determine if LYVE1 and ICAM1 participate in shaping the KC profile under non-infectious conditions using a liposome-entrapped clodronate (CL_2MDP) KC depletion protocol (Figure 15C). Monocyte influx and KC repopulation were tracked following ICAM1 or LYVE1 blockade. Neither ICAM1 nor LYVE1 blockade

completely significantly limited liver monocyte trafficking following KC depletion (Figure 16D). However ICAM1, but not LYVE1, blockade did impede mature F4/80+MHC II+ KC repopulation (Figure 16E). ICAM1 blockade reduced monocyte influx by (53%, p=0.32) and KC population was stunted by (68%, p=0.04). LYVE1 blockade reduced monocyte influx by (80%, p=0.27) and KC population was stunted by (35%, p=0.41). Results indicate that monocyte recruitment is not dependent on ICAM1 alone, but full restoration of the mature KC population following depletion appears to be ICAM1 dependent (Figure 16D and 16E).

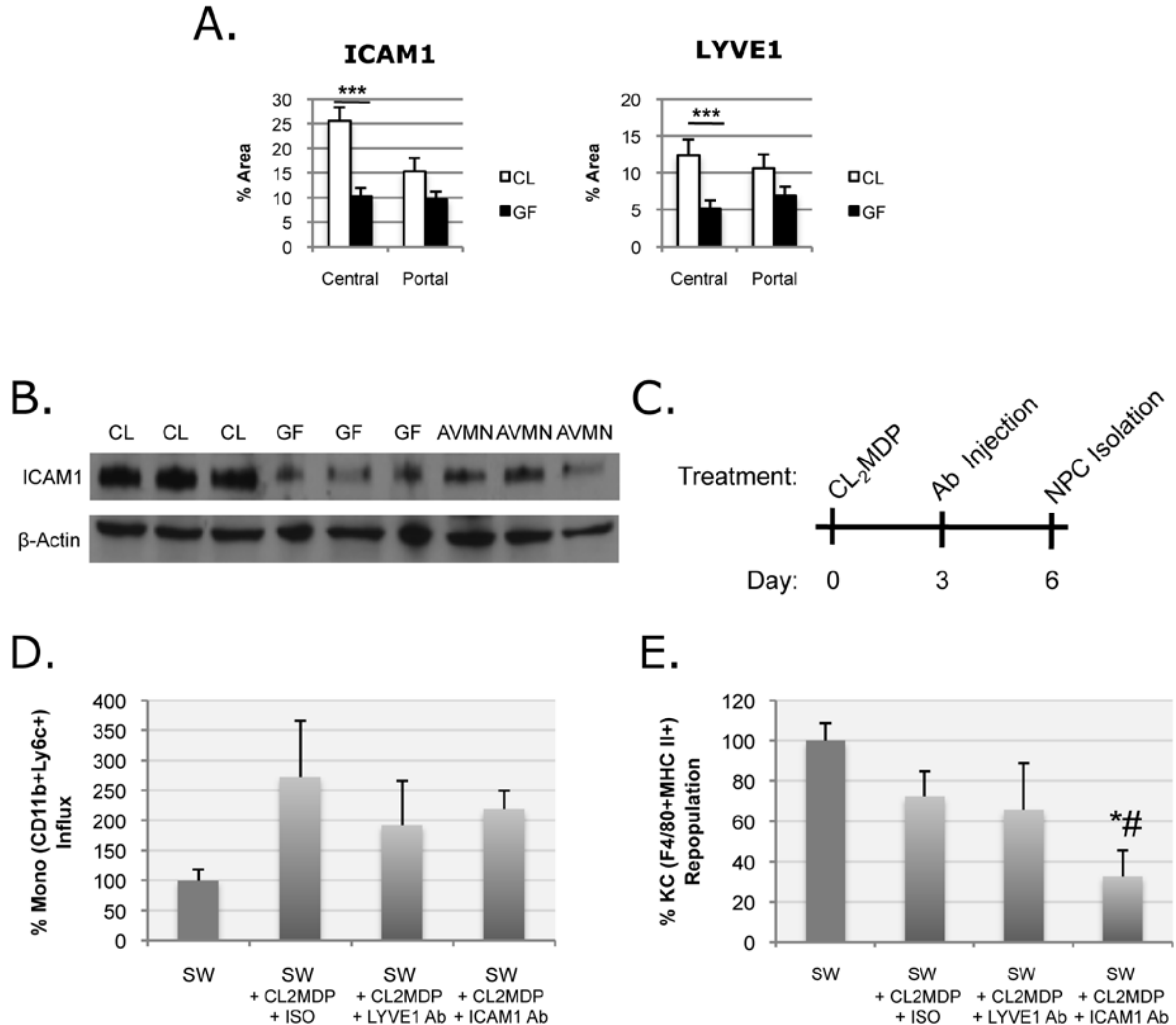


Figure 16. Constitutive ICAM1 Expression by LSEC is induced by the gut microbiota and Permits KC Repopulation. (**A**) ICAM-1 and LYVE1 protein expression in Liver; graphs illustrate quantification of immunofluorescence area per HPF that showed significantly higher ICAM1 and LYVE1 expression in CL mice (CL n=3, GF n=3; ***p<0.01). Five fields/region/mouse total 15 images per group used for analysis. (**B**) Western Blot showing higher hepatic ICAM-1 expression in CL mice compared to GF and AVMN mice (n=3) (ANOVA p<0.001). (**C**) Injection Scheme for Repopulation Model; Liposome entrapped Clodronate (CL_2MDP) was used to deplete KC on Day 0; Either ICAM1 or LYVE1 blockade was performed on Day 3 following KC depletion; KC and monocyte frequency was assessed by Flow Cytometry on Day 6 post KC depletion. (**D-E**) Monocyte influx (CD11b+Ly6C+ NPC) in **D**; Untreated CL SW mice were used as negative controls (100% Influx). KC repopulation (F4/80+MHC Class II+ NPC) in **E**; Untreated CL SW mice were used as negative controls (100% Repopulation). Representative of 2 independent experiments (n=3-4 per group). Statistical Significance indicated by: *p<0.05 experimental groups versus negative control, #p<0.05 experimental groups versus positive control. Conventional (CL), Antibiotic Treated mice (AVMN), and Germ Free (GF) mice were used.

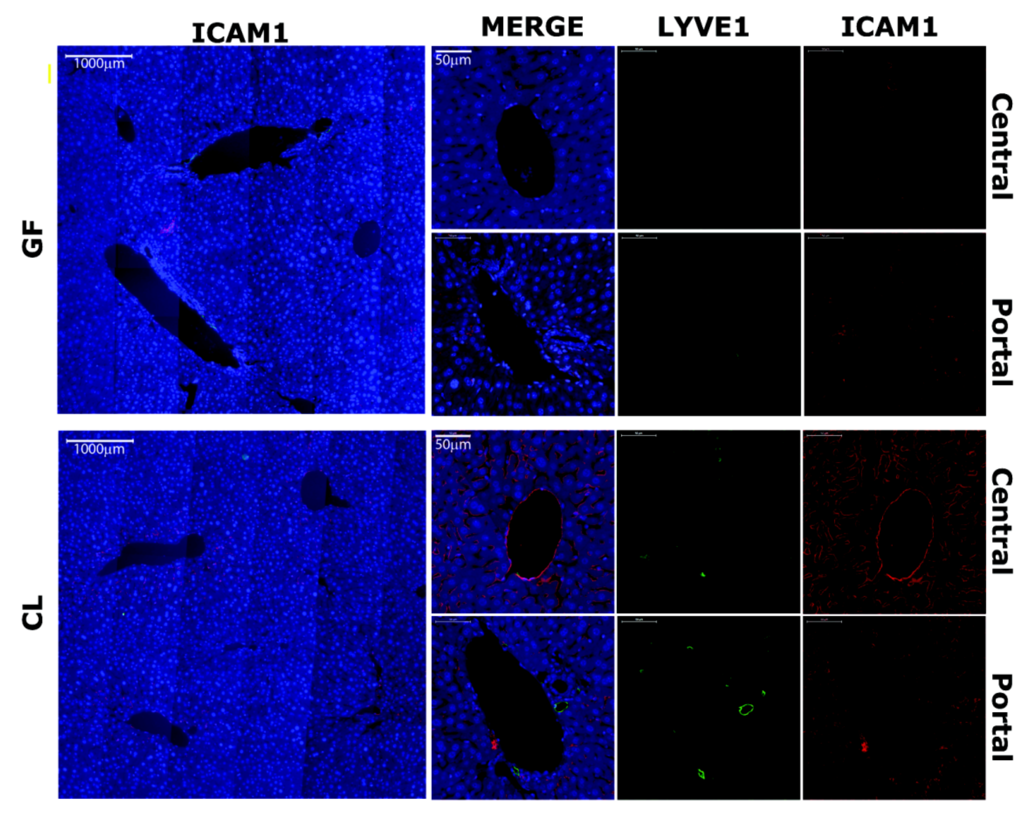


Figure 17. Tissue Expression of Adhesion Molecules in the Liver. ICAM-1 (red) and LYVE1 (green) expression on LSEC; DAPI (blue nuclear stain). Left most column, 10x representative overview image of liver section stained for ICAM-1; Second (MERGE), third (LYVE1) and fourth columns (ICAM-1) are 30x representative images of portal and central regions. Conventional (CL) and Germ Free (GF) mice were used.

5.0 RESULTS: INTRA-HEPATIC LYMPHOCYTE POPULATION DEVELOPMENT

5.1 GUT BACTERIA STIMULATE EXPANSION OF INTRA-HEPATIC T LYMPHOCYTES

T cells can be classified as conventional (CD4, CD8) T cells bearing T cell receptors that recognize MHC class I and MHC Class II restricted antigens as well as nonconventional T cells (NKT, γδ T) bearing nonconventional T cell receptors. Conventional CD4 T cells can be further classified as Th1, Th2, Th17, and regulatory T (Treg) based on cytokine production and function (106, 107). Despite the broad diversity and, at times, opposing functions of classes of T cells, gut bacteria module T cell numbers throughout the body (reviewed in (106)). Gut bacteria increase CD4 T cell numbers in the spleen (108) and peripheral lymph nodes (109), stimulate accumulation of CD4 Th17 cells in the colonic lamina propia (107), expand colonic Treg numbers (98), and boost distinct peritoneal γδ T cell numbers (106). Interestingly, the liver is particularly enriched in lymphocytes including both conventional and nonconventional T cells (58). Because the effects of the gut microbiota on T cell number appear to extend beyond the gut affecting immune organs (spleen), we hypothesized that liver lymphocytes would be secondarily affected by the gut microbiota since the liver is constantly filtering MAMP-laden blood traveling from the gut. Like previous studies in other organs, our results show a two-fold reduction in the percentage of intra-hepatic T cells (CD3+TCRβ+) found in GF livers (Figure 18A) compared to

age-matched CL mice (4.24% NPC and 8.77%NPC respectively, p=0.005). In addition, the percentage of NKT cells (NK1.1+TCRβ+) was nearly depleted in GF livers (Figure 18A, p=0.007). As previously reported, the effect of the gut microbiota appears to predominantly affect T cells (108) and no differences were observed in percentages of intra-hepatic B Cells (5.18% NPC and 2.85% NPC, p=0.07) or NK Cells (1.67% NPC and 1.72% NPC, p=0.87), although intra-hepatic B cells appear to be mildly reduced in GF mice (Figure 18A).

Inside the unique pool of conventional intra-hepatic T cells, the ratio of CD4 to CD8 T cells in the mammalian liver has been classically characterized as CD8 predominant (62, 110). However, the ratio of conventional intra-hepatic T cells differs among mouse species and is under the control of multiple intrinsic (genetic) and extrinsic (microbial) factors (111). Therefore, we further examined intra-hepatic T cells to determine the percentages of CD4 and CD8 T cells in CL and GF Swiss Webster mice. Results show that CD4 T cell predominate the intra-hepatic conventional T cell pool in both CL and GF mice, with a 2-fold reduction in percentage of CD4 T cells in GF mice and similar percentages of CD8 T cells between CL and GF mice (2.04% and 1.34% respectively, p=0.17) (Figure 18B).

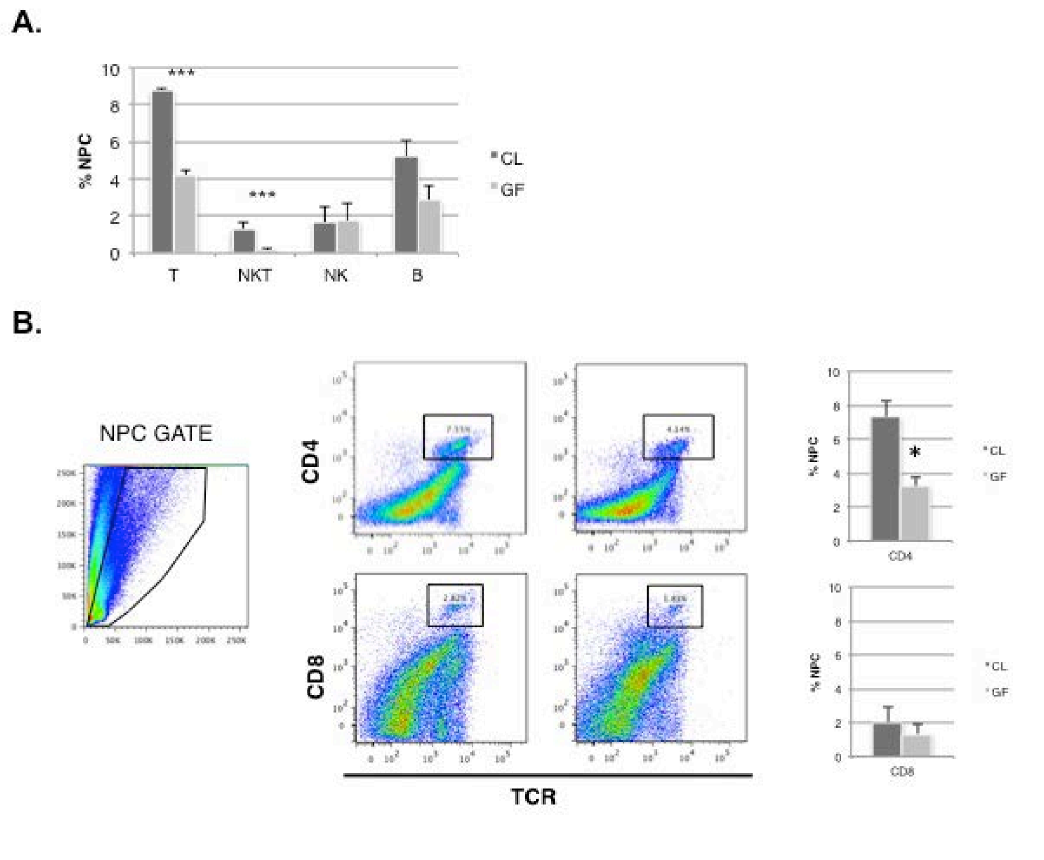


Fig 18. Gut Bacteria modulate liver Non-Parenchymal Cell composition. (**A-B**) Non-parenchymal cells (NPC) were isolated from Conventional (CL) and Germ Free (GF) livers (CL n=6, GF n=6), data representative of one experiment. (**A**) Percentage of NPC that are T cells (CD3+TCRβ+) (p=0.005), NKT cells (NK1.1+TCRβ+) (p=0.007), NK cells (NK1.1+TCRβ-) (p=0.87), and B cells (Cd19+IgD+) (p=0.07). (**B**) Percentage of NPC that are CD4 T (CD4+TCRβ+) (p=0.005) cells and CD8 T (CD8+TCRβ+) (p=0.17) cells. Statistical Significance indicated by: ***P<0.01, **P<0.025, and *P<0.05.

The murine NPC population is comprised not only of lymphocytes, but also of endothelial cells (40-50% NPC), stellate cells (10% NPC), and Kupffer Cells (20-30% NPC) (68, 112, 113). The non-lymphocyte NPC greatly outnumber lymphocytes, therefore, to examine changes in intra-hepatic lymphocyte frequency induced by gut bacteria, we next examined absolute lymphocyte frequency. Results show greater lymphocyte numbers in CL adult murine

livers (415,000 ± 57,780) compared to GF adult murine livers (228,472 ± 35,329) (Figure 19A, $p=0.009$).

Previous reports indicate conventional T and B lymphocytes comprise 43% of intra-hepatic lymphocytes, and up to 65% of intra-hepatic lymphocytes are NK cells and unconventional T cells, which are found at much lower frequencies in circulation (114). However, T lymphocyte numbers vary in mouse strains and humans (86, 111). Results show that the proportion of CD4 T cells among intra-hepatic lymphocytes is significantly reduced in GF mice compared to CL mice (32.06% and 43.77% respectively) (Figure 19B). Similar proportions of CD8 T cells (13.35% and 13.67%), γδ T cells (10.09% and 9.95%), and NKT cells (6.22% and 5.46%) within the intra-hepatic lymphocyte population were observed in GF and CL mice (Figure 19B). However, considering the overall lymphocyte frequency is reduced, the absolute numbers of all T cell subsets were found to be reduced in GF mice (Figure 19C). Results show that GF mice have similar proportions of T cell subsets in the absence of gut bacteria compared to CL mice (Figure 19D), but that the overall intra-hepatic T cell population in GF mice is much smaller in comparison to CL mice and that the CD4 T cell population is particularly affected by the absence of germs compared to other T lymphocyte subsets examined in this study (Figure 19C).

In mice, T cells can be found in the hepatic sinusoids around the portal tracts and also distributed throughout the parenchyma (114). Reportedly, one third of intra-hepatic T cells express NK1.1 (115). Of those, 80% are CD4 NKT cells (iNKT) and the remaining 20% are reportedly double negative non-invariant NKT (79). Results show that NKT (NK1.1+CD3+), T (CD3+NK1.1-) and NK (CD3-NK1.1+) have similar distribution patterns in CL and GF livers (Figure 19E). No difference in NK cell number was observed between CL and GF livers in

accordance with previous data.

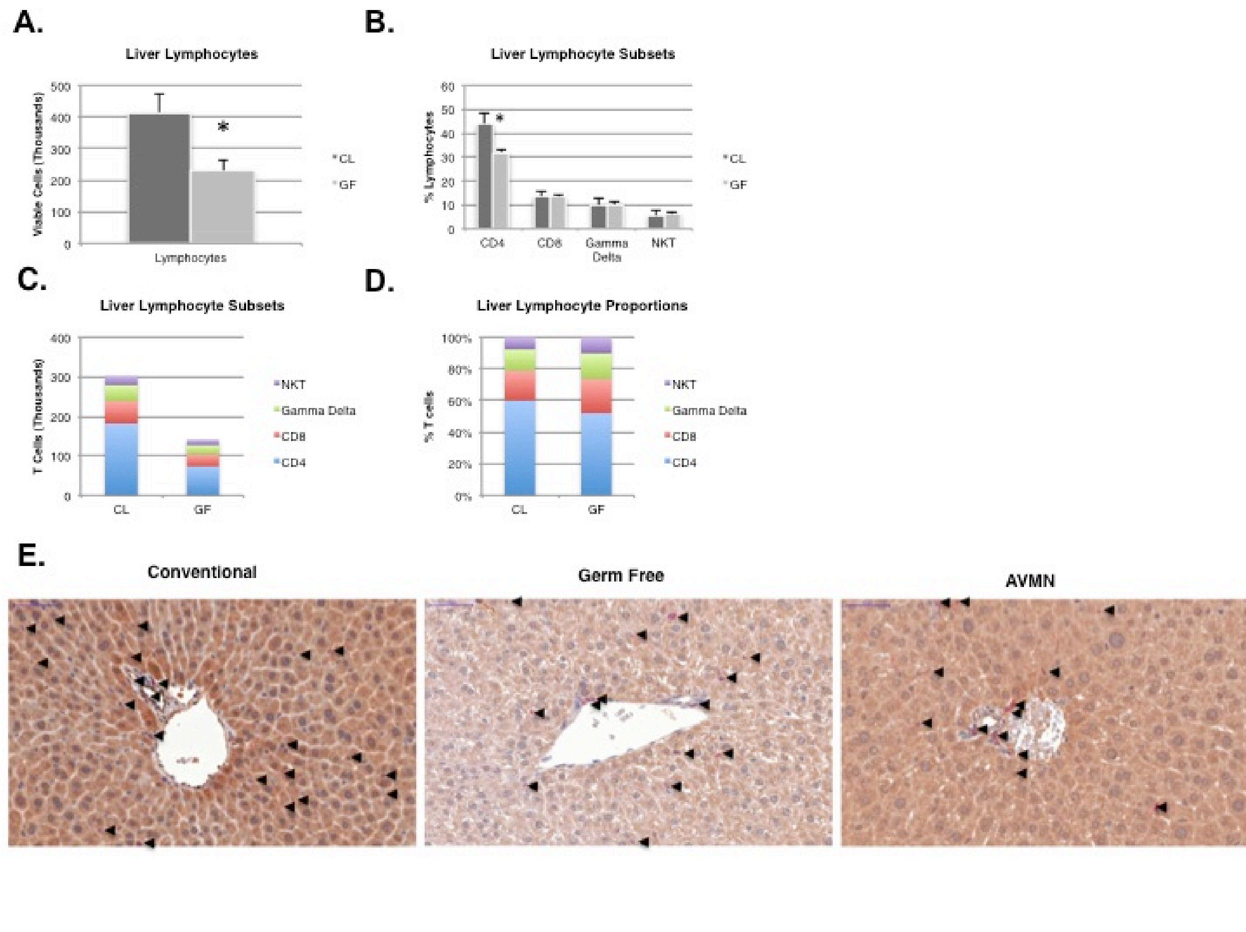


Fig 19. Gut Bacteria modulate intra-hepatic T lymphocyte composition. (**A-D**) Lymphocytes were isolated from adult livers of adult Conventional (CL) and Germ Free (GF) mice. (CL n=6, GF n=6). Data representative of one experiment. **A**) Absolute count of liver lymphocytes in CL and GF mice. (**B**) Percentage of Lymphocytes expressing CD4 and TCRβ, CD8 and TCRβ, TCRγδ (NK1.1-TCRβ-), and NK1.1 and TCRβ. (**C**) Absolute counts of intra-hepatic lymphocyte subsets: NKT (NK1.1+TCRβ+), Gamma Delta T (TCRγδ+NK1.1-TCRβ-), CD8 T (CD8+ TCRβ+), and CD4 T (CD4+ TCRβ+). (**D**) Relative proportions of intra-hepatic T lymphocytes (NKT, Gamma Delta T, CD8 T, and CD4 T). (**E**) Representative images of Immunohistochemical staining of CD3+NK1.1+ cells (black arrow) in the CL, GF, and AVMN (Antibiotic-treated) livers (3 sections per group). Statistical Significance indicated by: *$P<0.05$.

5.2 BACTERIAL DEPLETION RESULTS IN INTRA-HEPATIC T LYMPHOCYTE REDUCTION

The variability in human and murine intra-hepatic T cell numbers remains unexplained (115). We hypothesized that gut bacteria contribute to the accumulation of intra-hepatic T lymphocytes. We reasoned that if GF mice exhibit reduced intra-hepatic T lymphocyte numbers, then depletion of gut bacteria would yield a similar result. Results show that the intra-hepatic population of CD4 T cells in CL mice is approximately 40% while the intra-hepatic population of CD4 T cells in AVMN (antibiotic-treated) is reduced to 25% of lymphocytes (Figure 20A, $p=0.041$). The percentage of CD4 T cells (CD4+TCRβ+) that are also NKT (NK1.1+) cells is 60.8% and 43.2% in CL and AVMN mice respectively (Figure 20B). CD8 T cells were not significantly reduced by antibiotic treatment (27.9% CL and 15.1% AVMN) (Figure 20C). However, percentages of intra-hepatic NKT cells were significantly reduced by antibiotic-treatment (CL 32.4% lymphocytes and AVMN 15.5% lymphocytes) (Figure 20D, $p=0.043$). Unlike GF mice, the absolute intra-hepatic lymphocyte counts were not significantly different between CL and AVMN mice (385,000 ± 105,573 and 342,500 ± 84,125) ($p=0.37$) (Figure 20E).

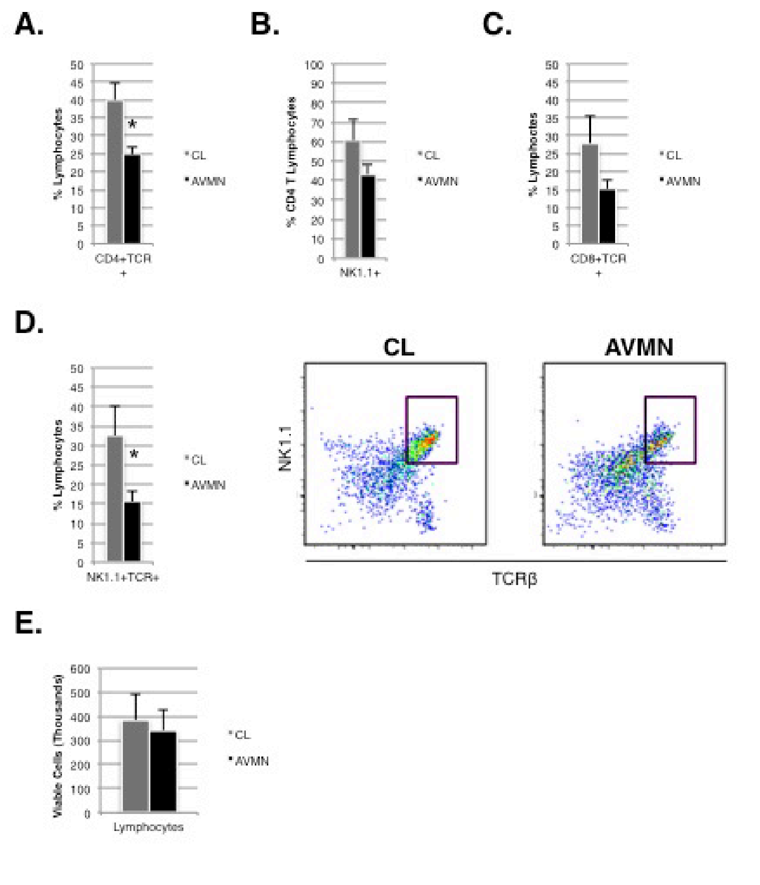


Figure 20. Bacterial Depletion Decreases T lymphocyte Numbers. (**A-E**) Lymphocytes were isolated from adult livers of adult Conventional (CL) and AVMN (antibiotic-treated) mice, data representative of two independent experiments (CL n=3-6, AVMN n=3-5). (**A**) Percentage of Lymphocytes expressing CD4 and TCRβ. (**B**) Percentage of CD4+TCRβ+ Lymphocytes that also express NK1.1. (**C**) Percentage of Lymphocytes expressing CD8 and TCRβ. (**D**) Percentage of NK1.1+TCRβ+ Lymphocytes. (**E**) Absolute Lymphocyte Count for CL (n=5) and AVMN (n=5) mice. Statistical Significance indicated by: *P<0.05.

5.3 NKT CELL MEDIATED LIVER INJURY INDUCED BY A-GALACTOSYLCERAMIDE IS ABROGATED IN GERM FREE MICE

Conventional T cells (CD4 and CD8) recognize antigens via TCR and MHC (Class II and Class I) interactions respectively. NKT cells, bearing TCR with an invariant TCRα chain and restricted TCRβ chain, recognize exogenous and endogenous glycolipid antigens presented by the MHC class I like molecule CD1d (80). CD1d-restricted NKT cells can be activated directly by bacterial glycolipids derived from *Sphingomonas spp.*, *Borrelia burgdorferi*, *Leishmania donovani*, and *Entamoeba histolytica* (116, 117). Moreover, the synthetic glycolipid α-galactosylceramide can activate NKT cells in a fast and selective manner, resulting in non-Kupffer Cell dependent liver injury that resembles acute autoimmune hepatitis (81, 88). Accordingly, the effect of gut bacteria on intra-hepatic NKT cell development would likely be discerned employing the α-galactosylceramide model of liver injury (Figure 21A). Therefore, we hypothesized that germ free mice, containing fewer intra-hepatic NKT cells, would exhibit either mild or absent hepatitis in response to α-galactosylceramide stimulation. Histology results illustrate the degree of liver injury mediated by α-galactosylceramide in CL and GF mice in just 24 hours compared to untreated controls (Figure 21B). Blinded histological scoring of liver tissue isolated from α-galactosylceramide-treated CL and GF mice revealed inflammation was greatest in periportal regions of CL livers and that α-galactosylceramide treatment had a more significant effect on CL livers (Figure 21C, periportal p=0.008, centrilobular p=0.007). Moreover, the frequency of hepatocyte apoptosis (CL, 2.46 apoptotic foci/hpf and GF, 1.13 apoptotic foci/hpf, p=0.01) was greater in CL livers following α-galactosylceramide treatment (Figure 21D). The degree of liver injury was also assessed by measuring serum ALT (Figure 21E). Concordant with liver histology, the serum ALT level was significantly greater in CL α-

galactosylceramide treated mice in comparison to GF α-galactosylceramide treated mice 12 hours after treatment (Figure 21E).

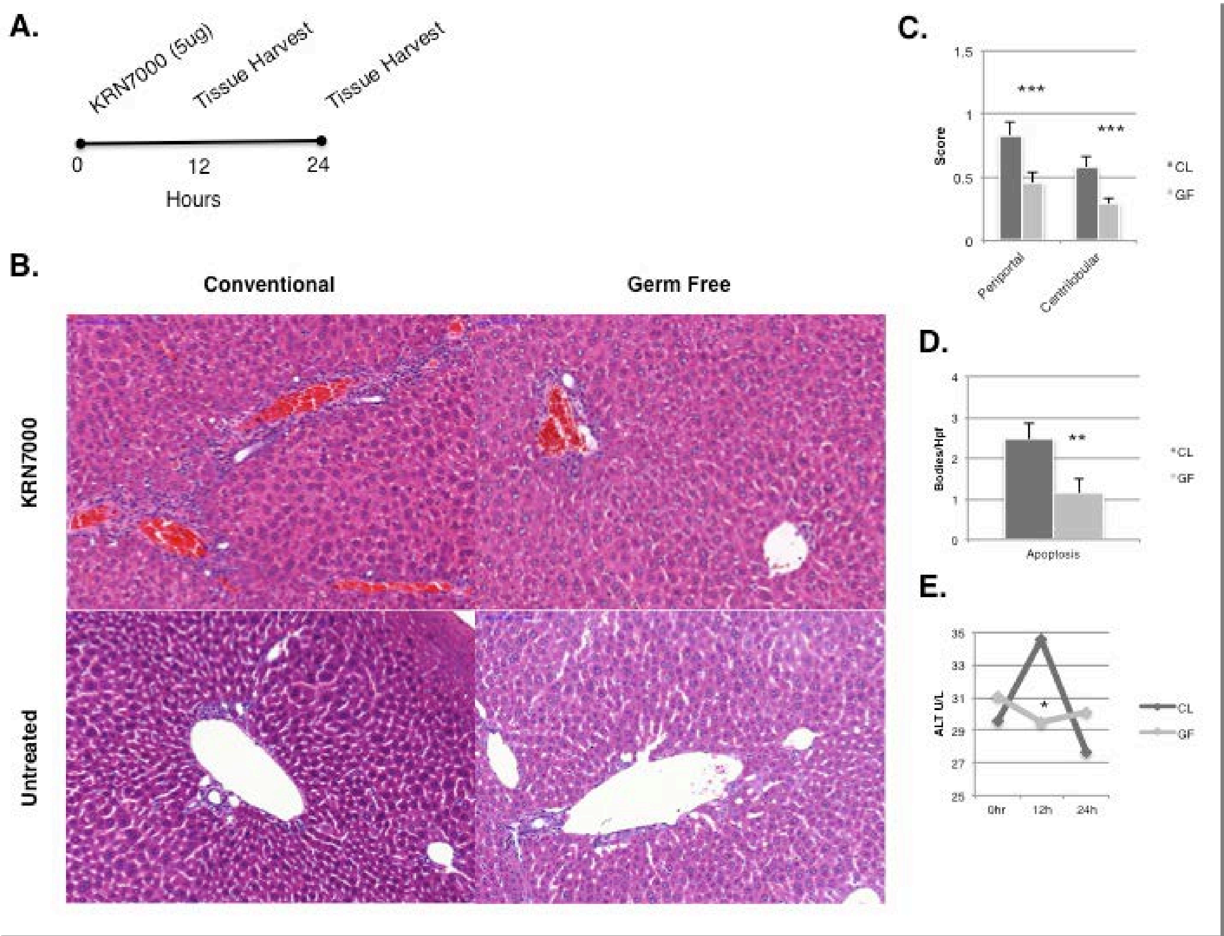


Figure 21. αGalactosylceramide induces NKT cell mediated inflammation. **(A-E)** Conventional (CL) and Germ Free (GF) mice were used in a NKT cell mediated-hepatitis model, data representative of one experiment. **A**) Injection Scheme for NKT cell-mediated hepatitis model; KRN7000 (5µg, 50µl) or PBS was injected (tail vein) into CL and GF mice on Hour 0. Blood and tissue was collected at 0, 12, and 24 hours post-injection. (**B**) Representative H&E images from CL and GF livers (n=6 per group) at 24 hours post-injection of KRN7000, 20x Magnification. (**C**) Blinded Inflammatory scores of H&E images from periportal (p=0.008) and centrilobular (p=0.007) regions of CL and GF livers (6 sections per group) at 24 hours post-injection of KRN7000 (**D**) Apoptosis (apoptotic bodies/hpf, p=0.01) at 24 hours post-injection of KRN7000 (n=6 per group). (**E**) Serum ALT measured by ELISA at 0, 12, and 24 hours post-injection of KRN7000. Conventional (CL) and Antibiotic-Treated (AVMN) mice were

used. GF ALT is significantly lower (P=0.04) at 12 hours post-treatment. Statistical Significance indicated by: ***P<0.01,**P<0.025, *P<0.05.

5.4 GUT BACTERIA DO NOT AFFECT CIRCULATING LYMPHOCYTES, BUT INFLUENCE LYMPHOCYTE ADHESION AND ACCUMULATION IN THE LIVER

Lymphocyte recruitment to the liver differs from other organs, with Intercellular adhesion molecule-1 (ICAM1), vascular cell adhesion molecule-1 (VAP1), and CD44 expressed by LSEC facilitating lymphocyte adhesion and accumulation in the liver instead of leukocyte rolling mediated by selectins as seen in peripheral organs (63, 64). In fact, the number of times that blood circulates through the liver (360 times/day) and the slow rate at which blood flows through the narrow sinusoidal vasculature (25-250 μm/min) promotes contact between circulating lymphocytes and the LSEC (118). Moreover, the number of active binding sites on LSEC is critical for lymphocyte accumulation in the liver (63, 118). We investigated whether gut bacteria affect 1) circulating lymphocyte number or 2) expression of adhesion molecules by LSEC. Results show that neither circulating white blood cell counts (Figure 22A, p=0.09) nor circulating lymphocyte numbers (Figure 22B, p=0.12) were affected by gut bacteria.

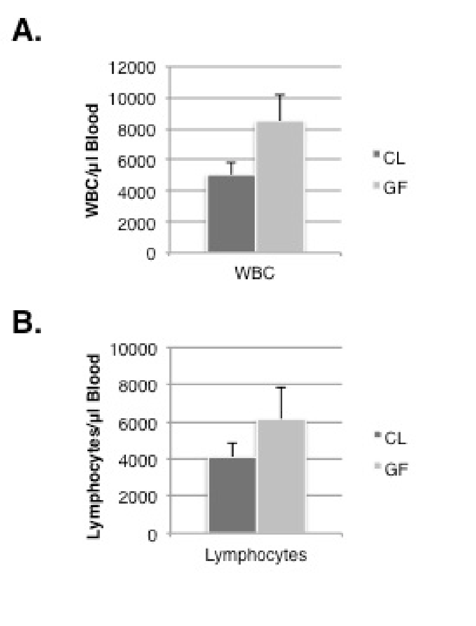


Figure 22 Gut Bacteria do not affect circulating lymphocyte numbers. (A-B) CBC with Differential was performed on systemic blood from Conventional (CL) and Germ Free (GF) mice, data representative of two independent experiments.(A) White Blood Cell (WBC) counts from CL (n=6-8) and GF (n=5-6) mice. (B) Circulating Lymphocyte counts from CL (n=6) and GF (n=5) mice. Statistical Significance indicated by: *P<0.05.

ICAM1 knockout mice characteristically have increased numbers of circulating leukocytes (119). Also, gut bacteria regulate ICAM1 expression in many organs (stomach, spleen, liver, intestines, kidney, skin, and skeletal muscle) (70). As shown previously, we saw reduced intra-hepatic T cell frequency in GF mice concomitant with reduced ICAM1 and CD44 expression by LSEC, molecules required for T cell accumulation in the liver. Results show that both CD44 and ICAM1 expression in GF livers is significantly reduced in comparison to CL counterparts (Figure 23A).

Finally, while circulating lymphocyte-LSEC adhesion contributes to the overall accumulation of intra-hepatic T cells, the development of intra-hepatic NKT cells has an additional level of complexity. NKT precursor cells form in the thymus at the neonatal stage and

migrate predominantly to the liver where they mature with less migration to sites such as the bone marrow and spleen (80, 88, 120). Both type I and II NKT (invariant TCR and non-invariant TCR) require CD1d for development in the mouse as demonstrated by CD1d knockout studies where these cells are absent (27, 57, 86). CD1d expression is particularly omnipresent in the liver as its expression is found on antigen presenting cells like macrophages, Dendritic Cells, and B cells, but also hepatocytes (121, 122). Moreover, CD1d expression levels increase during bacterial infection (116) and MHC class II expression by Kupffer Cells is decreased in absence of gut bacteria (manuscript in submission). Thus, we hypothesized that CD1d expression would be reduced in the livers of GF mice given lower microbial stimulation. Results show reduced hepatic CD1d expression in the livers of GF and AVMN mice in comparison to CL mice (Figure 23B).

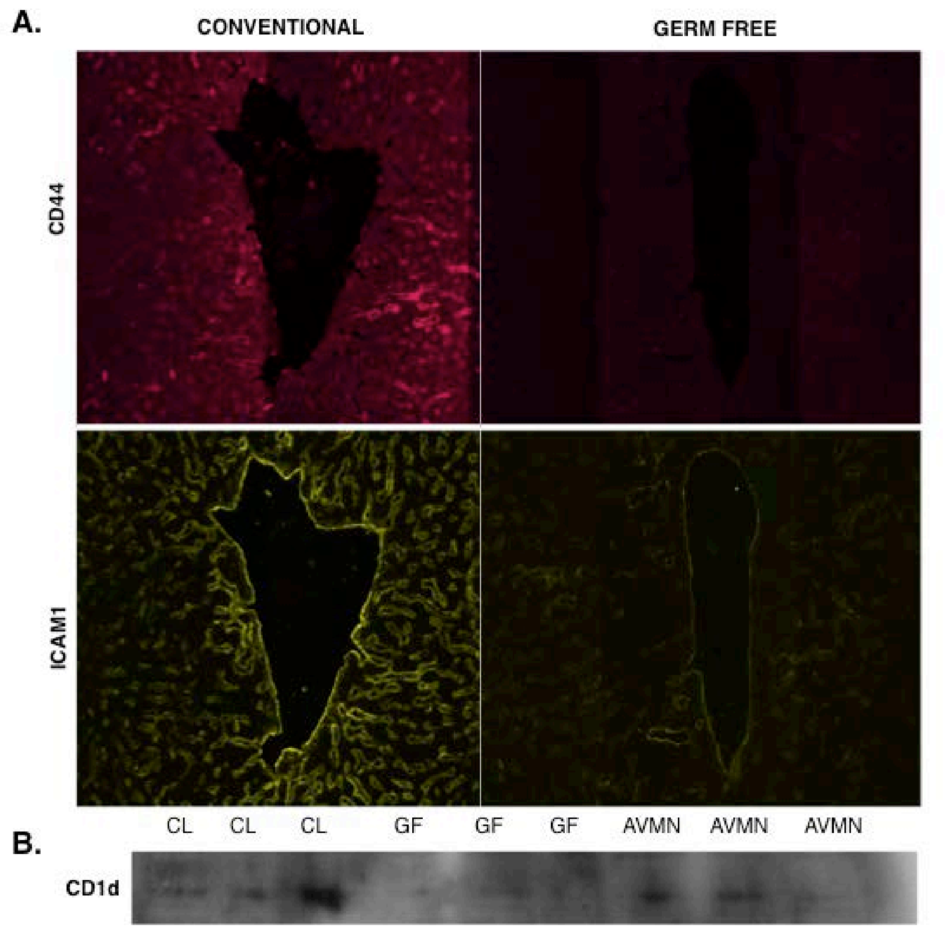


Fig 23. Gut Bacteria induce hepatic CD1d expression. (A) ICAM (yellow) expression and CD44 (red) expression by LSEC (**B**) Western Blot showing hepatic CD1d (37kDa) in CL, GF, and AVMN mice (n=3 per group). Conventional (CL), Germ Free (GF) and Antibiotic-Treated (AVMN) mice were used.

6.0 RESULTS: THE GUT MICROBIOTA REGULATES THE HEPATIC GENE EXPRESSION PROFILE

The liver is the metabolic center, functioning as a sensor and effector in the metabolism of fat, sugars, proteins, and xenobiotics (123, 124). In carbohydrate metabolism, absorbed glucose is taken up by the liver and consumed by the intestine, pancreas and spleen (123). Insulin dependent organs include skeletal muscle, heart and adipose tissue while the insulin independent organs include CNS and blood erythrocytes, which all require glucose. In the fasting state or during exercise, the liver meets the body's glucose demands via glycogenolysis (breakdown of glycogen) or gluconeogenesis (synthesis of glucose from stored substrates). In fat metabolism, absorbed dietary fat is stored in adipose tissue and liberated fatty acids are converted to triglycerides by the liver for release into VLDL. In nitrogen metabolism, ammonia is produced and the urea cycle exclusively occurs in the liver. In addition, the liver functions in conversion of xenobiotics and formation of bile acids.

Because hepatic cellular composition as well as non-parenchymal cell function was affected by the gut microbiota, the gut microbiota aid in host metabolism by metabolizing otherwise indigestible dietary components, the gut microbiota have been shown to alter the systemic metabolic profile, and the significant weight difference observed between GF and CL mice (Figure 8), we hypothesized that the hepatic metabolic profile would differ between GF, AVMN, and CL mice.

Total hepatic gene expression is dependent on gut bacteria as evident by the grouping of individual CL, GF, and AVMN livers together with livers from the same experimental conditions (Figure 25 and 26). The discrimination between CL and GF livers is smaller than the discrimination between CL and AVMN or GF and AVMN livers (Figure 26), which indicates that antibiotics may also affect gene expression in addition to germ status.

The microarray revealed 163 differentially expressed genes grouped into five clusters based on gene relatedness. The genes within these clusters fell into the following categories: Retinol metabolism (13), Metabolic pathways (53), Biosynthesis of unsaturated fatty acids (8), Circadian rhythm (5), Linoleic acid metabolism (7), Steroid biosynthesis (5), PPAR signaling pathway (9), Arachadonic acid metabolism (9), Amino sugar and nucleotide sugar metabolism (7), Metabolism of xenobiotics by cytochrome P450 (7), Drug metabolism by enzymes (6), Primary bile acid biosynthesis (4), Complement and Coagulation cascades (7), Alanine metabolism (4x), Fatty acid metabolism (5), Androgen and estrogen metabolism (4), Prion diseases (4), Terpenoid backbone synthesis (2), Sulfur metabolism (2), Regulation of autophagy (3), Butanoate metabolism (3), Fructose and mannose metabolism (3), and limonene and pinene degradation (2) (Figure 26). These data suggest that genes involved in metabolism are the most differentially regulated.

Of the differentially expressed genes (DEGs) identified, our findings support the influence of commensals in the synthesis of unsaturated fatty acids, linoleic acid metabolism, steroid metabolism, metabolism of arachadonic acid, metabolism of xenobiotics by cytochrome P450, and bile acid synthesis (124, 125). The influence of gut bacteria on the following members of the cytochrome P450 family has been previously reported: Cyp1a2, Cyp2a4, Cyp2b9, Cyp2b13, Cyp2c37, Cyp3a11, Cyp3a13, Cyp3a16, Cyp3a25, Cyp3a41, and Cyp3a44 (125). We

found that the expression of Cyp2a22, Cyp2b10, Cyp2b9, Cyp2c37, Cyp2c50, Cyp2c54, Cyp2c55, Cyp4a10, Cyp4a14, Cyp4a31, Cyp4a32, and Cyp26a1 was higher in GF mice compared to CL counterparts, while expression of Cyp2c39, Cyp3j9, Cyp3a11, Cyp3a41a, Cyp3a44, and Cyp7b1 was lower in GF mice compared to CL counterparts.

The role of gut bacteria in the entero-hepatic cycle of bile is well characterized. Gut bacteria enable recycling of bile acids via transformation of primary bile acids (cholic acid and chenodeoxycholic acid) into secondary bile salts (lithocholic acid and deoxycholic acid) (124), therefore the implicated role of gut bacteria in bile synthesis in our study was unsurprising.

Regarding fat metabolism, Fiaf (angiopoietin-like protein 4) is a prominent mediator of fat storage and energy expenditure via β-oxidation (124). Accordingly, we found that Fiaf was down regulated in GF and AVMN mice compared to CL counterparts, likely due to reduced fat uptake from the gut. Other notable differentially regulated genes involved in fat metabolism include fatty acid synthase and lanosterol synthase (both down-regulated in GF and AVMN mice) involved in synthesis of fatty acids and synthesis of cholesterol respectively.

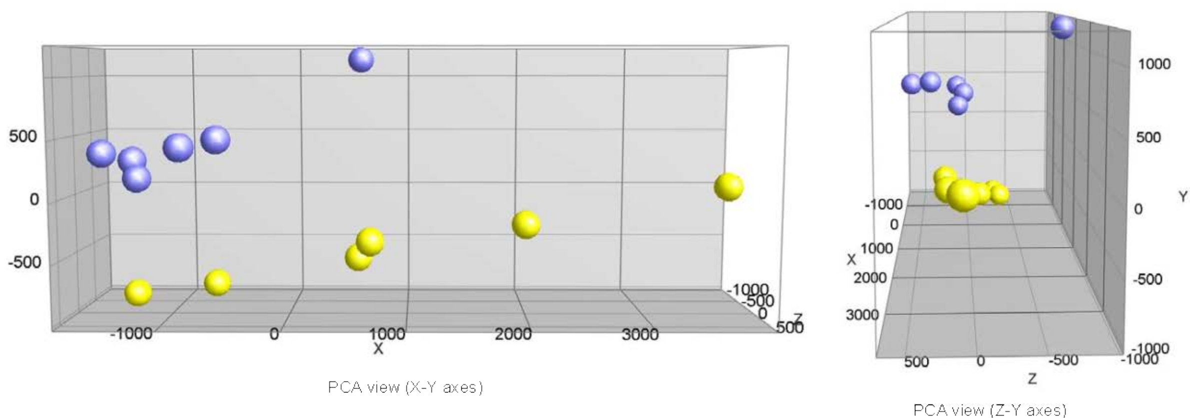


Figure 24. Principle Components Analysis. PCA shows obvious discrimination between GF (yellow) and CL (blue) livers.

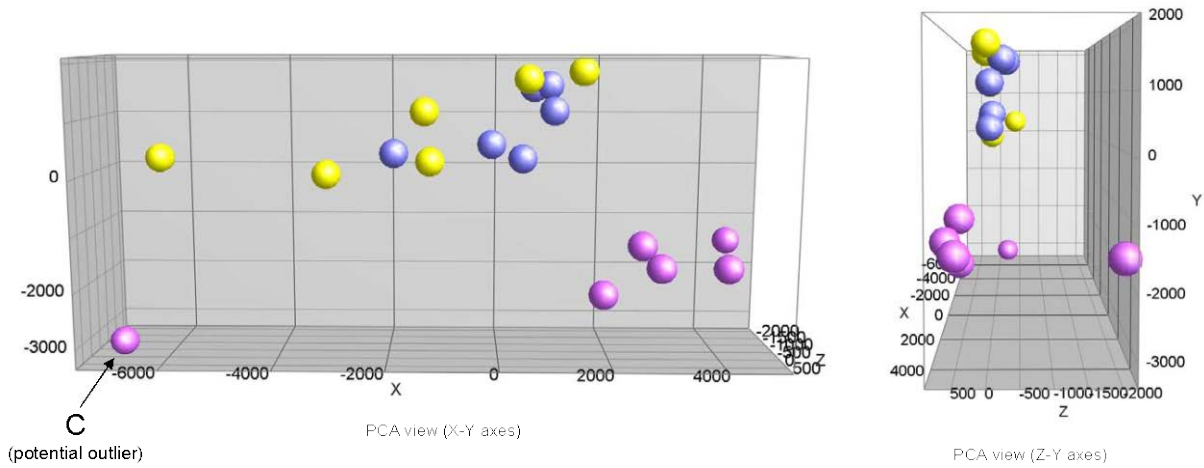


Figure 25. Principle Components Analysis. PCA shows greater discrimination of AVMN (purple) livers as a group relative to GF (yellow) and CL (blue) livers. Sample C from the AVMN group appears as a potential outlier.

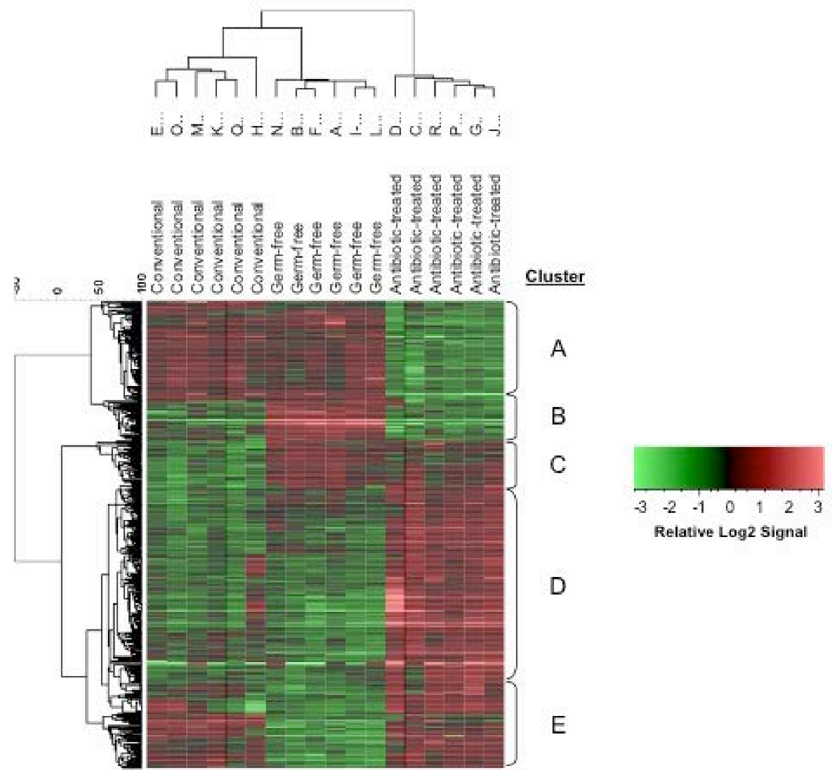


Figure 26. Heat Map of Differentially Expressed Genes. Clusters A-E correspond to DEG similarity.

In light of our previous findings that leukocyte frequency (KC and T lymphocyte) was drastically diminished in absence of gut bacteria, we expected to observe corresponding changes in the hepatic gene profile of GF and AVMN mice. Reikvam et al. performed a similar study comparing gene expression in the intestines of CL, GF, and antibiotic-treated mice revealing 517 DEGs belonging to the following categories: cell cycle, lipid biosynthesis, inflammatory response, apoptosis, DNA damage response, and oxidative stress regulation (126). Five genes encoding antimicrobial factors were identified in their study (Reikvam). Likewise, we identified 28 DEGs involved in different aspects of immunity (Table 4 and 5).

As previously mentioned, PRR signaling pathways coalesce at the level of transcription factor activation and transcription of genes or post-translational modification, ultimately leading to the production of inflammatory cytokines and antimicrobial molecules. Cytosolic RLRs induce *raet1e* upon activation (Table 4). Both cytoplasmic and transmembrane PRR activate IRFs including IRF2 (Table 4). Viral MAMP recognition induces the production of Type 1 interferons like IFNα5, whose expression is reduced in CL and AVMN livers (Table 4). Moreover, all TLR utilize kinases to transduce signal activation (i.e. MAP2K) (Table 4). The pro-inflammatory cytokine production in response to PRR activation often includes IL-1 (i.e. NOD1/2) and IL-6 production (i.e. TLR4) whose affects are exerted by binding to respective receptors IL-1R and IL-6R (Table 4), both of which are expressed at a lower level in GF livers in comparison to CL counterparts. In addition, IL-22 production can be inflammatory or anti-inflammatory depending on the cytokine microenvironment and its receptor is found to expressed at a lower level in GF livers as well (Table 4). Lastly, TLR-4 signaling is dependent on TLR-4-MD2-CD14-LPS complex formation unlike the other TLR, which simply bind their

cognate ligands irrespective of adaptor molecules. While TLR 4 is a transmembrane PRR, the MD2 adaptor protein is secreted by the liver and loosely associates with the extra-cellular domain of TLR4 (74). MD2 (Ly96) expression is also lower in GF livers reflective of the lack of LPS stimulation.

Several genes related to immune cell phenotype, differentiation, apoptosis, and proliferation were differentially regulated (Table 4 and Table 5). However, the implications of such changes in gene expression are unclear. The increases in lymphocyte 6 Antigen Complex genes are likely compensatory in response to lack of immunostimulation from gut bacteria. However, the decreased expression of genes involved in both proliferation (LIFR) and apoptosis (TRAIL) in GF livers is paradoxical (Table 5).

Interestingly, three genes involved in antigen presentation and processing had reduced expression in GF livers. We previously showed a lack of MHC Class II expression on KC in GF mice as well as AVMN mice (Figure 5). Although, KC are not the only MHC class II expressing NPC in the liver, professional antigen presenting cells characteristically possess all machinery for antigen presentation in this pathway including proteolytic enzymes for the endocytosis and degradation of antigens to be loaded onto the MHC Class II molecules. Cathepsins are involved in antigen processing and loading (127), two of which have reduced expression in GF livers (Table 5). Moreover, the expression of the invariant component of the actual MHC Class II molecule was reduced in GF mice as well (Table 5) (38).

The liver synthesized most of the plasma components of blood (i.e. albumin). An important component of systemic innate immunity includes circulating complement proteins and acute phase reactants, which are also produced by the liver (62). The complement system consists of over 30 proteins (57), both stimulatory and inhibitory in nature. Results show that

Hemolytic Complement, Complement component 2, Complement component 6, and Complement component 9 all had reduced expression in GF livers. Additionally, regulatory components of the complement cascade (Decay Accelerating Factor and Complement component Factor I) exhibited reduced expression as well.

Unfortunately, the microarray data did not yield any inkling about cell deficiency resulting from bacteria deficiency. The raw data of the microarray was extensively checked for even subtle differences in the expression of leukocyte specific chemokines, cytokines, and CD (cluster of differentiation) markers. Moreover, because the microarray did not yield a discernable difference in ICAM1 expression (although two methods of protein expression confirmed a difference in ICAM1 protein expression) between CL and AVMN or CL and GF mice, we reasoned that differential expression of genes of interest might be confirmed using classical PCR. No differences in mRNA expression of leukocyte specific CD molecules (i.e. CD19 for B cells) have been identified at this time (data not shown).

Table 4. Immunity-related DEGs I

DEG	AVMN/CL	GF/CL	AVMN/GF	Pathway
	Log2 Signal Difference			
Raet1e Retinoic Acid Early Transcript delta	0.13	-1.35	1.47	PRR Signaling
IRF2 Interferon Regulatory Factor 2	0.36	0.71	-0.36	PRR Signaling
IFNα5 Interferon α5	-0.65	-0.5	-0.60	PRR Signaling
MAP2k Mitogen activated protein kinase	0.69	0.25	0.44	PRR signaling
Ly96 Lymphocyte Antigen 96 also called MD2	0.62	-0.29	0.90	PRR Signaling
IL22R Interleukin 22 Receptor	0.03	-0.65	0.68	PRR signaling
IL1R Interleukin 1 Receptor	0.03	-0.16	1.99	PRR signaling
IL6R Interleukin 6 Receptor	0.21	-0.62	0.83	PRR Signaling
MIF Macrophage Migration Inhibitory Factor	0.42	-0.21	0.63	PRR signaling
Ly6C2 Lymphocyte Antigen 6 Complex, Locus C2	0.17	0.72	-0.56	Immune Cell
Ly6A Lymphocyte Antigen 6 Complex, Locus A	0.51	2.44	-1.92	Immune Cell
Ly6C1 Lymphocyte Antigen 6 Complex, Locus C1	0.28	1.24	-0.86	Immune Cell
LY6F Lymphocyte Antigen 6 Complex, Locus F	-0.09	0.64	-0.76	Immune Cell

Table 5. Immunity-related DEGs II

DEG	AVMN/CL	GF/CL	AVMN/GF	Pathway
		Log2 Signal Difference		
IL11R Interleukin 11 Receptor	-0.19	0.44	-0.63	Natural Killer Cell Differentiation
LIFR Leukemia Inhibitory Factor Receptor	0.37	-0.35	0.72	Immune Response Proliferation
TNFsf10 TRAIL	1.40	-0.99	2.39	Immune Response Apoptosis
Factor XI Coagulation Factor 11	0.63	0.10	0.53	Blood coagulation
Metallothionein 1	-1.03	-2.90	1.88	Acute Phase Response
Metallothionein 2	-1.02	-1.86	0.84	Acute Phase Response
Hc Hemolytic Complement	-0.18	-0.81	0.62	Acute Phase Response Immune Response
CfI Complement Component Factor I)	0.11	-0.75	0.86	Acute Phase Response Immune Response
DAF Decay Accelerating Factor	0.23	-0.45	0.67	Acute Phase Response Immune Response
C2 Complement Component 2	0.43	-0.33	0.76	Acute Phase Response Immune Response
C6 Complement Component 6 (MAC)	0.11	-1.21	1.32	Acute Phase Response Immune Response
C9 Complement Component 9 (MAC)	-0.04	-0.61	0.57	Acute Phase Response Immune Response
Cathepsin E	-0.22	-2.46	2.24	Antigen Processing
Cathepsin Z	0.27	-0.47	0.73	Antigen Processing

CD74 Invariant Polypeptide of MHC Class II	0.51	-0.53	1.04	Antigen Presentation

7.0 DISCUSSION

7.1 GUT BACTERIA AND KUPFFER CELL DEVELOPMENT

Uniqueness of the liver as an immunologic organ resides, in large part, in the enrichment of specialized leukocyte subsets that are not found at the same concentrations in other organs (60) and the highest concentration of tissue macrophages (KC). Our results show that constitutive exposure to MAMPs via portal blood shapes the sentinel KC population for sinusoidal content monitoring, which in turn, can trigger various innate and adaptive immune responses. These include clearance of bacteria, phagocytosis of malignant cells, platelet aggregates, activated complement components, and antigen presentation to T cells/activation and T cell tolerance (74, 128).

Leukocyte populations in the gastrointestinal tract are largely shaped by luminal gut bacteria (129). Permanent residence and close proximity of bacteria to tissue evolved into a symbiotic and carefully balanced relationship referred to by some as “physiologic inflammation” (129), whereby local immunity is “primed” for reactivity and protection from pathogens, but without commensal bacteria-triggered auto-aggressive tissue damage.

In the liver, we observed germ line KC PRR expression, regardless of gut bacteria, but MAMP exposure triggers hepatic KC recruitment maturation/activation characterized by increased MHC Class II+ KC and decreased phagocytic activity. Co-stimulatory molecule

expression, however, remains low in both CL and GF KC. MAMPs, therefore, help shape the relatively ‘tolerogenic’ KC phenotypic profile that contributes to inefficient antigenic presentation to T cell along with KC secretion of IL-10 and TGFβ (60, 90, 130).

Previous studies show that mono-association of GF mice with *Lactobacillus delbrueckii* (131), inoculation of GF rodents with *Saccharomyces cerevisiae* (93), and mono-association of GF rats with *Escherichia coli* (78) all lead to increased KC numbers. Komatsu et al. previously showed dynamic regulation of liver endothelial ICAM1 expression by gut bacteria (70). Increased LSEC ICAM1 expression has been associated with monocyte/macrophage recruitment during bacterial infection (103) and KC expansion in old age (132). We have confirmed, linked, and furthered these previous observations by showing that under physiological conditions gut bacteria: 1) release specific cocktail of MAMPs into circulation, which upregulate LSEC ICAM1 expression, and are largely responsible for “constitutive expression”; 2) density is directly proportional to KC numbers, phagocytic function, and MHC Class II expression; and 3) density fluctuations occur naturally or can be induced via broad spectrum antibiotic treatment.

KC comprise more than a third of total liver NPC (73) and participate in many critical processes, sometimes playing a protective role and other times a deleterious one depending on context. They have been implicated in: liver fibrosis as activators of hepatic stellate cells; mediators of inflammation and ischemia/reperfusion injury; adverse drug reactions; metastatic tumor cell clearance; liver tolerance induction; liver regeneration; and bacteria/virus clearance (73, 74, 90, 101, 130). By extension, therefore, gut bacteria can directly and indirectly influence many aspects of liver pathobiology.

We observed that development of KC and liver immunity is directly influenced by gut bacteria on a sliding scale: more gut bacteria, more circulating MAMPs, more LSEC

activation/ICAM1 expression, more monocyte adhesion, and finally more KC. Our results are consistent with established clinical observation that selective bowel decontamination before liver transplantation significantly *increases* the risk of infections, whereas as pre- and probiotic treatment shows an opposite trend (133).

It is not unreasonable to speculate that defects in liver immunity or bacterial/MAMP dysbiosis might also contribute to susceptibility, progression, or skewing of other liver diseases as has been shown for the intestine and for allergic reactions (134). This study focused on gut bacterial density, but many other aspects of this intricate system, such as changes in bacterial species and relative proportions, are likely to be fruitful areas for further investigation.

7.2 GUT BACTERIA AND INTRA-HEPATIC LYMPHOCYTES

From person to person, the relative frequencies of intra-hepatic T cell subsets can vary considerably. The variation among individuals is likely reflective of antigenic exposure as much as genetic background (114). However, given the emerging role of gut bacteria in the development of both mucosal and systemic immunity and their role in Kupffer Cell development, it would not be surprising for gut bacteria to shape the intra-hepatic T cell repertoire. Yet, the impact of the gut bacteria on T cell development has primarily been characterized outside of the liver.

Katz et al. demonstrated that the liver is enriched in nonconventional T cells (NKT) and that the conventional T cells (CD4 T) differ functionally from conventional T cells in other organs (110). However, the difference in T cell function was attributed to the interactions between conventional T cells and tolerogenic APC within the context of the liver. In this study,

we confirm that the liver is enriched with nonconventional T cells and expand their findings by demonstrating that gut bacteria stimulate the development of both conventional and nonconventional intra-hepatic T cell subsets. Our results are consistent with the findings of Mazmanian et al. demonstrating that the effect of gut bacteria appears to be limited to CD4+ T lymphocytes although CD8+ T and B lymphocytes were not entirely unaffected (108).

The majority of intra-hepatic nonconventional T cells are NKT cells with NK-like cytotoxic activities (58, 89). The role of gut bacteria on NKT cell development is not entirely clear (106). Park et al. examined NKT cell development in C57BL/6 mice and found that NKT cells in the spleen, liver, and bone barrow develop in the presence or absence of a microbial environment (82). Based on the findings of their small sample size (n=2-4), they concluded that endogenous glycolipids were sufficient for the development of NKT cells in the aforementioned organs (82). The exact identities of endogenous or exogenous glycolipids that promote NKT cell development have been difficult to elucidate. Nonetheless, the unifying structural feature of microbial CD1d restricted NKT cell antigens seems to be a hexose sugar in an α-anomeric linkage with a glycosphingolipid or diacylglycerol and whereas endogenous self lipids include phospholipids, lysophospholipids, and sphingomyelin (116).

Our findings support the concept that gut bacteria as a source of microbial glycolipids are not the single most important factor for intra-hepatic NKT cell development. However, our results clearly demonstrate that gut bacteria contribute to NKT cell expansion in the liver and are in agreement with the NKT cell study performed by Wei et al., which showed decreased iNKT cell numbers in the livers of both Swiss Webster and C57BL/6 germ free mice (135). Moreover, we show that hepatic expression of adhesion molecules CD44 and ICAM1 (required for lymphocyte recruitment to the liver) is regulated to a large degree by exposure to gut-derived

MAMPs. Collectively, our data and previous reports indicate that intra-hepatic T cell development results from combination of factor including genetic traits, antigenic exposure/memory, endogenous self-antigen stimulation, and exogenous stimulation provided by residential microbes.

Identification of the factors that influence intra-hepatic NKT cell developments is critical given that NKT cells coordinate effector and regulatory immune responses, combat microbial infection, and incite chronic inflammatory diseases (99, 135, 136). NKT cells induce hepatic injury following production of copious amounts of immune-regulatory cytokines diseases such as alcoholic hepatitis, primary biliary cirrhosis, and autoimmune hepatitis (85, 89). We have examined NKT cell mediated damage in the mouse model of autoimmune hepatitis following NKT cell activation by α-galactosylceramide. We found that the damage incited by NKT cells is severely reduced in GF mice treated with α-galactosylceramide and can be attributed to lower NKT cell frequency. Regulation of conventional and nonconventional T cell responses is vital for protection from infectious diseases, but also for prevention of autoimmune and immunopathologic disorders (106).

7.3 GUT BACTERIA AND METABOLIC PROFILE

The gut microbiota aid the host in vitamin uptake and energy extraction with the immense number of metabolizing genes they possess and their host's lack. While, their nutrient extraction capabilities are primarily for their own benefit, they increase the available energy available from the mammalian diet. Despite their fastidious nature in culture, they can extract energy from high fat, high fiber, and low calorie diets in the gut. Because of their resourceful nature, gut

microbiota represent an important parameter modulating the amount of energy absorbed from the diet, fat deposition, and susceptibility to metabolic disease in addition to genetics, diet, and exercise (6).

Our data has shown that the gut bacteria influence overall body mass and fat pad size in adult mice. In addition, the whole-liver microarray highlights that nearly all hepatic metabolic pathways are influenced by the presence of gut bacteria. The influence of gut bacteria is both direct and indirect. Gut bacteria directly modulate the liver gene expression profile via MAMP-PRR interactions. By fermenting indigestible carbohydrates and producing acetate, propionate, and butyrate, which can be directly used by various host cells, gut bacteria also indirectly alter the metabolic gene expression profile of the liver. It is possible that gut bacteria influence metabolic processes in adipose tissue and skeletal muscle including fat mobilization, lipolysis, and protein turnover, but this has yet to be investigated.

Microbiota dysbiosis (compositional shift) is a major contributing factor for the development of obesity (22). In fact, a lean person can be distinguished from an obese individual solely on the fermentation profile of their gut microbiota (10). Members of the Firmicutes division are better at salvaging energy than those of Bacteroidetes and overrepresentation by members of Firmicutes in the microbiota undoubtedly leads to weight gain (12). Therefore, obesity studies in rodents should address gut microbial diversity as a contributing factor. Furthermore, fecal transplants have been (sparingly) used to treat *C. difficile* pseudo-membranous colitis in individuals refractory to antibiotic therapy (137). Likewise, fecal transplant might be a viable option to “reset” the microbial community in morbidly obese patients in concert with diet and exercise. The role of gut bacteria in development of metabolic syndrome and obesity has been an area of recent interest.

Obesity and accumulation of fat in the liver as seen in NAFLD and NASH are associated with an inflammatory phenotype. In that regard, the microarray provided some insight into the immune-related gene changes that could have resulted from changes in the metabolic profile attributed to the presence of absence of the gut microbiota to ferment dietary substances or direct affects of gut-derived MAMPs on liver cell subsets directly. Either way, the microbiota stimulate liver cells, presumably hepatocytes, to express genes associated with innate immunity including complement and acute phase proteins.

We are not the first group to stumble upon the finding that the systemic innate immune response is diminished in absence of the gut microbiota. Rouzic et al. previously demonstrated that complement proteins are synthesized at a much lower level in prenatal mice (lacking microbiota), which is consistent with our results (56). Moreover, the ability of CL but not GF KC to augment hepatocyte protein synthesis *in vitro* corroborates our findings as well (52). These data provide more insight into why neonates are more vulnerable to infection.

8.0 FUTURE DIRECTIONS

We primarily focused on bacterial density in liver immune cell population development. However both bacterial density and diversity influence host health and disease. Further, the inconsistencies in bacterial density between mice can be easily rectified by standardizing experimental conditions using a cut-off value of bacterial density to categorize mice as "fully colonized" thereby optimizing homogeneity within an experimental group. However, the composition of the microbiota of mice may never be fully comprehended. It is important to investigate the minimum and maximum numbers of bacterial species required to accomplish metabolic function and appropriate immune-stimulation. Differences between mice colonized by ASF and possessing many more species have been reported suggesting that eight species are enough for mice to reach a specific stage of development but not to reach their full potential. I would propose to classify the murine microbiota species similar to the way that humans have been grouped into enterotypes to standardize mouse experiments, especially those planned to answer metabolic and immunological questions. Fore example, the BL6 enterotype(s) may or may not differ from Swiss Webster enterotype(s) considering that human identical twins have the most closely related gut microbiota but still possess diversity variation. Fundamentally, answering the questions of bacterial diversity between mice and how closely murine microbiota resemble human microbiota would make results from such experiments much more translatable.

The potential to perform experiments characterizing bacterial diversity in the murine gut exists, but the holdup is primarily financial. If I had all the money in the world and unlimited resources, I would perform 454-pyrosequencing on stool from dozens of mice from different animal facilities, academic institutions, and commercial vendors using mice from different backgrounds given the affect of genetics on bacterial diversity. While preliminary less expensive studies like DGGE provide some insight about bacterial diversity between murine individuals, our knowledge is incomplete and identifying the species present or absent will give us the scientific tools necessary to determine which bacteria are really important. Studies performed using individual bacteria overstate the importance of that individual strain. For example, what is so spectacular about *B. thetaiotamicron* other than its ubiquitous nature? It is conceivably possible that there is nothing spectacular about this individual bacterium other than the fact that it serves as a reservoir for MAMPs. Alternatively, this well-studied gut colonizer could possess a unique combination of MAMPs that other colonizers lack enabling it to specifically influence different aspects of the immune system in a combinatorial manner. Without full characterization of murine microbiota diversity, scientists will continue to make gross overstatements of the influence of individual strains of bacteria on host health and development.

Hundreds to thousands of species colonize the gut. It would be inconceivable to perform mono-association studies for each one in order to determine their contribution to liver and systemic immunity. Moreover, it would be grossly inappropriate to do so because such experimentally contrived mono-association situations are nonexistent in nature and therefore the results of such experiments would be essentially useless when trying to discern the significance of the individual results when combinatorial studies would be more translational. The problem with conventional (combinations of gut bacteria) versus germfree (no gut bacteria) models is that

conventional mice have too much going on to figure out which species or classes of species are important, dispensable, or redundant. Identifying the bacterial MAMPs, which translocate into circulation, in my opinion, was a great advance in this respect. In accordance, the immense gut bacterial diversity can be simplified by group classification based on the individual and groups of MAMPs they produce. More and more evidence supports that the host immune system sees bacteria for their parts and not as whole organisms based on the specialization of PRR and detection equipment within particular cellular compartments. In effort not to oversimplify, even members of the same bacterial species secrete different forms MAMPs based on selection pressures and horizontal gene transfer increasing survival fitness. Even in the aforementioned scenarios, FLA is FLA, LPS is LPS, and PDG is PDG because, by definition, MAMPs are conserved microbial structures. Now to say all FLA monomers are created equal would be unfair, but it is unclear how baseline FLA stimulates the immune system or if different isoforms are 'interpreted' differently by the host. To date, no specific soluble PRR have been identified to bind to FLA unlike LBP for LPS and PGBP for PDG, which holds much significance.

Rolli et al. demonstrated that minute amounts of FLA could alter cardiomyocyte contractility (42). The liver makes most of the proteins found in the blood, but the heart pumps the blood around perfusing all organs in the body. The baseline influences of FLA on heart contractility and ejection fraction are of great interest. Further, even the kidneys express TLR-5 and could potentially be influenced by FLA at baseline. The kidneys would be exposed to circulating FLA particularly because of their function in blood filtration and fluid homeostasis, therefore it would be interesting to determine if FLA influences baseline GFR. Unlike mono-association experiments with flagellated bacteria (i.e. *Salmonella* spp.), the proposed experiments could be easily performed using axenic animals on elemental diet (no FLA

exposure), conventional animals, and axenic animals injected with physiological concentrations of FLA systemically. Identifying different stimulatory capacities of various isoforms of FLA could be accomplished with this setup as well.

Microarrays provide a wealth of information that can be a double-edged sword. The whole liver is quite heterogeneous although hepatocytes dominate cell number and tissue volume. The advantage of performing whole liver gene array is that it provides a picture of the forest so to speak, but the drawback is the view of the individual trees is pretty blurry. In other words, the gene content of hepatocytes significantly drowned out the gene content of cells found at lower frequency. While physiologically, having a 5-10% change in lymphocyte composition may mean the difference between mild and severe hepatitis, the gene array was unable to discern these differences among germ free and conventional mice based on gene content alone. It would be very informative to be able to make predictions of cellular compositional changes in the liver based on established high throughput genomic studies. However given the assay limitations, it is not possible at this time to make such predictions. To counter the assay limitations, *deconvolution* of the whole liver gene array could be accomplished by performing multiple gene arrays on isolated hepatic cellular components and then determining their contribution to the total gene pool of the liver. The downside of performing arrays on cell types from heterogeneous tissue is not being able to successfully isolate each individual cell type with no contamination by other cell types. Nonetheless, deconvolution is worth performing if the resulting information would yield further insight into what cell types are collectively influenced by gut-derived MAMPs in the liver. It would also provide functional information as to how the expression of genes in cell subsets changes in response to constitutive exposure to MAMPs. Although, we found many gene changes in response to bacterial colonization including the differential

regulation of CD44, some major physiological differences were not elucidated by the gene array (i.e. differentially regulated ICAM). Therefore deconvolution will only augment our knowledge.

We have shown that many leukocyte subsets are indeed recruited to the liver in a MAMP dependent manner, however the signaling between non-parenchymal and parenchymal cells and their contribution to the recruitment of each cell type adds another layer of complexity. If I were to go back (or go forward) investigating Kupffer Cell development, I would utilize the 'MacGreen' mouse model. These mice have a GFP reporter associated with the *csf-1* receptor specific to monocytes and macrophages thereby making monocytes/macrophages green and easily to identify as well as sort.

Whether or not NKT cell recruitment to the liver is influenced by KC should be more thoroughly investigated. One group reported that KC are not required for NKT cell development in the liver based on the finding that NKT cell frequency was not altered following KC depletion (88). However, I disagree with this interpretation primarily because the establishment of hepatic NKT cells may require KC help while maintenance of hepatic NKT cells does not. Moreover, because KC express CD1d (in addition to parenchymal and nonparenchymal cells), significant KC deficiency as we observed in GF and AVMN mice might alter hepatic NKT cell development. The expression of CD1d on hepatic KC should be assessed individually from total liver CD1d. In addition, the exact ligands of CD1d recognized by NKT cells have not been well described. Methods to detect microbial glycolipid translocation into systemic circulation will need to be developed.

APPENDIX A

CURRICULUM VITAE

BIOGRAPHICAL

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EDUCATION and TRAINING

UNDERGRADUATE:

2002-2005	Howard University of Washington, D.C.	BS: 2005	Biology

GRADUATE:

2005-2007	University of Pennsylvania, PA	N/A	N/A
2007-Pres	University of Pittsburgh, PA	MD/PhD: 2013	Medicine

MEMBERSHIPS in PROFESSIONAL and SCIENTIFIC SOCIETIES

Student National Medical Association, Member	2007-Present
Surgery Interest Group	2007-Present

Oncology Patients and Loving Students, Member	2007-2008
Women in Science and Medicine Association, President	2008-2011
Medical Scientist Training Program Committees, Secretary	2008-2010
Faculty and Students Together (FAST), Mentor	2009-2010
Medical Scientist Training Program Mentorship Committee, Chair	2010-2011
Medical Scientist Training Program Ethics Committee, Chair	2010-2011
Medical Scientist Training Program Mentorship Committee, Mentor	2010-2011
Medical Scientist Training Program Committees, Chair	2011-2012

HONORS

College of Arts and Sciences Honors Association, Howard University	2002-2005
Cum Laude, Howard University	2005
Beta Kappa Chi, Howard University	2005
Golden Key International Honor Society, USA	2005-Present

PUBLICATIONS

Refereed articles:

1. **Natasha Corbitt**, Kumiko Isse, Susan Specht, Lisa Chedwick, Brian R. Rosborough, Noriko Murase, John G. Lunz, Anthony Jake Demetris. 2011. Gut Bacteria Drive Kupffer Cell Expansion in Normal Mice via MAMP-mediated ICAM1 Induction on Sinusoidal Endothelium. Hepatology (In Submission).

2. Isse K, Grama K, Abbott IM, Lesniak A, Lunz JG, Lee WM, Specht S, **Corbitt N**, Mizuguchi Y, Roysam B, Demetris AJ. 2010. Adding value to liver (and allograft) biopsy evaluation using a combination of multiplex quantum dot immunostaining, high-resolution whole-slide digital imaging, and automated image analysis. Clin Liver Dis. 14(4): 669-85.

3. Giorgio Raimondi, Tina L Sumpter, Benjamin Matta, **Natasha Corbitt**, Daisuke Tokita, Zhiliang Wang, and Angus W Thomson. 2009. mTOR Inhibition and Alloantigen-Specific Regulatory T Cells Synergize to Promote Long-Term Graft Survival in Immunocompetent Recipients. J Immunol 184(2):624-36.

4. Yan J, Hokey DA, Morrow MP, **Corbitt N**, Harris K, Harris D, Weiner DB. 2009. Novel SIVmac DNA vaccines encoding Env, Pol, and Gag consensus proteins elicit a strong cellular immune response in cynomolgus macaques. Vaccine 27 (25-26):3260-6.

5. Yan J, Reichenbach DK, **Corbitt N**, Hokey DA, Ramanathan MP, McKinney KA, Weiner DB, Sewell D. 2009. Induction of antitumor immunity in vivo following delivery of a novel HPC-16 DNA vaccine encoding an E6/E7 fusion antigen. Vaccine 27(3):431-40.

6. Laddy DJ, Yan J, **Corbitt N**, Kobasa D, Kobinger GP, Weiner DB. 2007. Immunogenicity of novel consensus-based DNA vaccines against avian influenza. Vaccine, 25(16):2984-9.

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PROFESSIONAL ACTIVITIES

RESEARCH:

Current Grant Support:

NIH/NIDDK support, F31DK089902-01

Prior Grant Support:

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PREP NIH support, R25GM071745

Conferences & Meetings:

1. Medical Scientist Training Program Retreat-Oral Presentation:*, Germs: Behind the Scenes of Liver Immunity, 2011*
2. Cellular and Molecular Pathology Graduate Program Retreat-Oral Presentation:*, Gut Bacteria and Liver Innate Immunity, 2011*
3. 1st International Conference on Immune Tolerance-Delegate, 2009
4. Immunology Graduate Group Retreat - Oral Presentation: *A Novel Engineered HIV-1 Clade C Consensus-Based Envelope DNA Vaccine, 2007*
5. Keystone Symposium - Poster Presentation: *Development of a Novel Engineered HIV-1 Clade C Envelope DNA Vaccine that Enhances Diversity and Breadth of the Elicited Cellular Immune Response, 2006*
6. Leadership Alliance Symposium - Poster Presentation: *A novel Approach to Cervical Cancer that Targets Human Papillomavirus-16-E7, 2004*
7. Annual Biomedical Research Conference for Minority Students - Poster Presentation: *The Correlation Between a Stream's Point Source Pollution and Antibiotic Resistant Bacteria., 2003*

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