Long-term consequences of maternal breast milk antibodies on offspring intestinal immunity

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Abstract

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Breast milk is a key regulator of host-microbiome interactions in early life. In addition to nutrients, breast milk contains cytokines, growth factors, and antibodies, which help shape the composition of the microbiota and regulate infant immunity to gut microbes. Accumulating evidence links breastfeeding with a decreased risk of developing immune-mediated intestinal and metabolic diseases later in life. As such, current WHO guidelines recommend that mothers exclusively breastfeed for the first six months of life. However, this is not always possible for all women and children. While infant formulas are designed to meet the basic nutritional needs of growing infants, they lack certain components of human breast milk. Understanding which components and how these components of breast milk may influence long-term health, can inform the development

of treatments or supplements to breast milk to promote the healthy growth and development of neonates, particularly in infants who are not breastfed.

Using a mouse model, our lab discovered that mice deficient in breast milk antibodies mount increased mucosal T cell- dependent immune responses, including elevated T follicular helper (Tfh) cells and germinal center (GC) B cells in the gut-draining lymphoid tissues at the time of weaning. This process is driven by microbes as germ-free mice lacking breast milk antibodies do not generate aberrant mucosal Tfh and GC B cell responses. Due to the profound impact of the gut microbiota on the development of the immune system coupled with the lifelong persistence of activated adaptive immune cells, a current paradigm is that inappropriate adaptive immune responses to gut bacteria drive adverse immune and health outcomes in the long-term.

To address this, we first investigated whether the elevation in the GC B cell response observed in offspring that did not receive maternal antibodies resulted in differences in the intestinal memory B cells or plasma cell compartment. To supplement these studies, we developed a novel method to isolate and characterize B cell subsets and plasma cells from the intestine. We observed that offspring that did not receive maternal antibodies showed trending increases in memory and plasma B cells in the colon. However, we did not observe differences in serum or fecal antibody quantity of IgA or any other isotype. Additionally, there were no differences in the microbiota-reactivity of IgA or any other isotype generated in offspring that received or did not receive maternal antibodies.

In parallel, we also performed functional readouts of the T cell-dependent adaptive immune response seen in offspring that did not receive maternal antibodies. We asked

whether maternal antibodies affect the offspring's intestinal microbiota composition and function in the long term. We found equivalent composition, diversity, and abundance of microbes across different parts of the small intestine and colon between offspring that received or did not receive maternal antibodies. Additionally, we found similar susceptibility to the small intestinal pathogen *Salmonella* typhimurium or colonic pathogen *Citrobacter rodentium* between offspring that received or did not receive maternal antibodies. Finally, intestinal length and intestinal transit time of offspring that received or did not receive maternal antibodies were equivalent.

Finally, as a first step towards translating this research to humans, we comprehensively mapped the relative contributions of all subclasses of antibodies found in the breast milk of a cohort of healthy, lactating women.

Overall, my thesis work has contributed to our understanding of the long-term consequences of maternal antibodies on offspring intestinal immunity. This work may inform the development of therapeutics or supplements to formula to foster beneficial relationships with the gut microbiota and promote long-term health.

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Bach2: BTB Domain And CNC Homolog 2

Bcl6: B-cell lymphoma 6 protein

Blimp-1: B lymphocyte-induced maturation protein-1

BMI: body mass index

CCL28: Chemokine C-C motif ligand 28

CFU: colony forming units

Cre-ERT2: tamoxifen-inducible Cre

CXCL16: chemokine ligand 16

DTT: dithiothreitol

DPI: days post infection

EGF: epidermal growth factor

ELISA: enzyme-linked immuno assay

ELISpot: enzyme-linked immunosorbent spot

FACS: fluorescence-activated cell sorting

FcRn: neonatal Fc receptor

FoxP3: forkhead box protein 3

GAPs: goblet-cell-associated antigen passages

GC: germinal center

GF: germ free

IgA: immunoglobulin A

IgD: immunoglobulin D

IgE: immunoglobulin E

IgG: immunoglobulin G

IgM: immunoglobulin M

ILC: innate lymphoid cells

IQR: interquartile range

LP: lamina propria

matAb+: maternal antibody sufficient (received maternal antibodies)

matAb-: maternal antibody deficient (did not receive maternal antibodies)

mFLOW: microbiota flow cytometry

mLN: mesenteric lymph nodes

NEC: necrotizing enterocolitis

NKT: natural killer T cells

Pax5: paired box protein 5

PD-L1: programmed death-ligand 2

pIgR: polymeric Ig receptor

PP: Peyer's patches

qPCR: quantitative polymerase chain reaction

S1PR2: sphingosine-1-phosphate receptor 2

SI: small intestine

SIRPa: Signal-regulatory protein alpha

SFP: specific pathogen free

swIg: switched immunoglobin

TACI: transmembrane activator an CAML interactor

TCR: T cell receptor

tdTOM: tdTomato fluorescent protein

Tfh: T follicular helper cells

TGF-beta: transforming growth factor beta

Treg: regulatory T cells

WT: wildtype

YFP: yellow fluorescent protein

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Chapter I

Introduction

In this introduction, I will provide background on the epidemiologic studies linking breastfeeding and health. I will discuss the components of breast milk compared with formula and the efforts to fortify formula with immune-modulatory molecules naturally present in breast milk. I will discuss our background research aimed at understanding how breast milk antibodies regulate adaptive immune responses in the neonatal gut. I will then provide background on the role of T cell-dependent and -independent antibody responses to the commensal microbiota. I will also discuss the concept of maternal "immune imprinting" in offspring. Finally, I will conclude with the goals for this thesis.

1.1 Breastfeeding and health

As an evolutionarily conserved trait among all mammals, breastfeeding provides early nourishment to offspring. Compared to formula feeding, breastfeeding is associated with an overall benefit in a number of health outcomes, including a reduction in the burden of diarrheal diseases, decreased respiratory infections, and decreased long-term risk of developing asthma, diabetes, and inflammatory bowel disease (2-6). As such, several health organizations have issued guidelines around breastfeeding. The World Health Organization recommends that infants be exclusively breastfed for the first six months of life (8). The American Academy of Pediatrics also recommends breastfeeding for at least 12 months (11). Recently, the Academy of Nutrition and Dietetics stated that breastfeeding provides optimal nutrition and health benefits for the first six months of life,

and that breastfeeding with complementary foods from six months until at least 12 months of age is the ideal feeding pattern for infants (12). However, due to complex socioeconomic and biological factors, breastfeeding is not possible for all women and children (13, 14). Worldwide, only 38% of infants are exclusively breastfed. In the United States, only 75% of infants initiate breastfeeding from birth; however, by three months, 67% of them rely on infant formula for some portion of their nutrition. Among new mothers, only 13% exclusively breastfeed for six months (11). In these circumstances, infant formulas serve to meet the basic nutritional needs of growing infants and are undoubtedly beneficial in reducing infant morbidity and mortality. However, considering the accumulating evidence linking breastfeeding with short and long-term health benefits, we need a better understanding of the specific components of human breast milk and the molecular mechanisms by which they function in order to design appropriate and effective infant formulas that maximize health.

1.2 Composition of breast milk

Human breast milk is a complex matrix with 87% water, 3.8% fat, 1.0% protein, and 7% lactose. Fat and lactose, respectively, provide 50% and 40% of the total energy of the milk (15).

Macronutrients	g/dL
Protein	0.9
Fat	3.5
Carbohydrates (mainly lactose)	6.7

Table 1.1. **Macronutrients in human breast milk.** Adapted from (10).

Aside from these macronutrients, breast milk also contains vitamins and minerals that aid in the development of the neonate(16). The composition of human breast milk is dynamic and changes over time, adapting itself to the changing needs of the growing child. For example, during each nursing session, the milk that is initially expressed is thinner with a higher content of lactose, which is thought to satisfies a baby's thirst. In contrast, the milk that is expressed later is creamier with a much higher content of fat, which is thought to meet the baby's nutritional needs (16). Remarkably, it has been observed that a mother's breast milk is almost always adequate in essential nutrients for the infant's growth and development, even when her own nutrition is inadequate (16).

Anti-microbials	Immunoglobulins- Secretory IgA, IgM, IgG Lactoferrin, Lactadherin/MFG E8, Lysozyme, Complement C3, Antiviral mucins- MUC1, MUC4
Growth Factors	Epidermal growth factor (EGF), Nerve growth factor (NGF), Insulin-like growth factor (IGF), Transforming growth factor (TGF), taurine, polyamines, Heparin Binding EGF like growth factor (HB-EGF), Vascular Endothelial growth factor (VEGF), Erythropoietin
Cytokines, Chemokines and Anti-inflammatory factors	Tumor necrosis factor-alpha (TNF-α), Interferon-gamma (IFN-γ), Transforming growth factor-beta (TGF-β), prostaglandins, α_1-antichymotrypsin, α_1-antitrypsin, platelet-activating factor: acetyl hydrolase Interleukins- IL-6, IL-7, IL-8, IL-10 Chemokines- Granulocyte colony stimulating factor (G-CSF), Macrophage Migratory inhibitory factor (MIF)
Hormones	Calcitonin, Somatostatin, Adiponectin, Leptin, Ghrelin
Digestive enzymes	Amylase, Bile acid-stimulating esterase, Bile acid-stimulating lipases, Lipoprotein lipase
Transporters	Lactoferrin, Folate binder, Cobalamin binder, IGF binder, Thyroxine binder, Corticosteroid binder
Oligosaccharides and Glycans	Human Milk Oligosaccharides (HMOs), Gangliosides, Glycosaminoglycans

Table 1.2. **Bioactive molecules found in human breast milk.** Table from (10).

Aside from nutritional components, breast milk also contains bioactive factors that help reinforce proper immunity for the infant, particularly at a time in which the infant's immune system is still immature (17). In addition to maternal cells like macrophages, neutrophils, and lymphocytes, these bioactive components include anti-microbial peptides, cytokines, chemokines, growth factors, and hormones (7, 14, 15). For a complete list, reference Table 1.2

1.3 Composition of infant formula

In the U.S., the development of infant formulas is tightly regulated by the FDA, which specifies the minimum and maximum values of the majority components of formula, including protein, lipids, and carbohydrates. Cow's milk is the basis for most infant formula. However, because raw cow milk has higher levels of fat, minerals, and proteins compared to human breast milk, cow's milk is skimmed and diluted to more closely resemble human breast milk (18, 19). Additionally, cow's milk is fortified with vegetable oils, vitamins, minerals, and iron. Soy-based formulas and hypoallergenic formulas with hydrolyzed proteins also exist for those infants with lactase deficiency or protein allergy (20). Apart from several exceptions, infant formulas largely lack the bioactive molecules found in human breast milk.

<u>Human milk oligosaccharides</u>

Human milk oligosaccharides (HMOs) are a group of molecules produced by the mammary gland and abundant in human milk. These molecules have a lactose backbone with additions of various sugars like glucose and galactose, and range from 3 to 15 added carbohydrate groups (21, 22). The lactose backbone itself can be fucosylated,

glycosylated, or sialylated, forming different structural isomers (21, 22). Around 200 different HMOs have been identified in human milk (21). Interestingly, these molecules are not broken down by the digestive enzymes of the infant and thus can reach the distal small intestine and colon. Studies conducted in the early 2000s showed that HMOs are critical in maintaining intestinal homeostasis. First, HMOs have important roles in protecting neonates from pathogens. HMOs act as decoys for the attachment of pathogenic bacteria like *Streptococcus pneumoniae* and *Campylobacter jejuni* to intestinal epithelial cells (23, 24). They also have been shown to inhibit the growth of certain strains of *Escherichia coli* by binding to its secreted toxins (25). HMOs prevented disease onset in mouse models of necrotizing enterocolitis, a disease that primarily affects pre-term infants (26-29). Concurrent with preventing pathogens, HMOs also promote the expansion of beneficial microbes such as Bifidobacterium and Bacteroides, which use HMOs as a food source (30). These microbes, in turn, produce short-chain fatty acids, which serve to regulate immune function and provide energy to the infant (31-33). Abbott, a large manufacturer of infant formulas worldwide, conducted clinical trials and found that infants fed formula supplemented with HMOs had lower amounts of serum inflammatory cytokines compared to those fed regular formula, at levels that were similar to those in breastfed infants (34). This evidence, amongst other results, resulted in the eventual addition of HMOs to infant formula in 2016. The story of the addition of HMOs to formula serves as an example of the beneficial impact basic sciences research can have and underscores the necessity for continuing to research the mechanisms of action of the other bioactive molecules found in breast milk.

1.4 Maternal breast milk antibodies

One of the components found in breast milk is antibodies. Antibodies have a wide variety of functions, including binding to pathogens and engaging with other immune cells to target the pathogen for elimination. In breast milk, antibodies bind bacteria present in the gut lumen of suckling infants. Maternal antibodies are transferred to breast milk via the mammary glands, which are structures of epithelial origin that contain ducts for transporting milk and alveoli for synthesizing milk. IgA, IgG, and IgM are routinely found in the breastmilk, with IgA present at the highest concentration ((35) and Chapter 5 in this work).

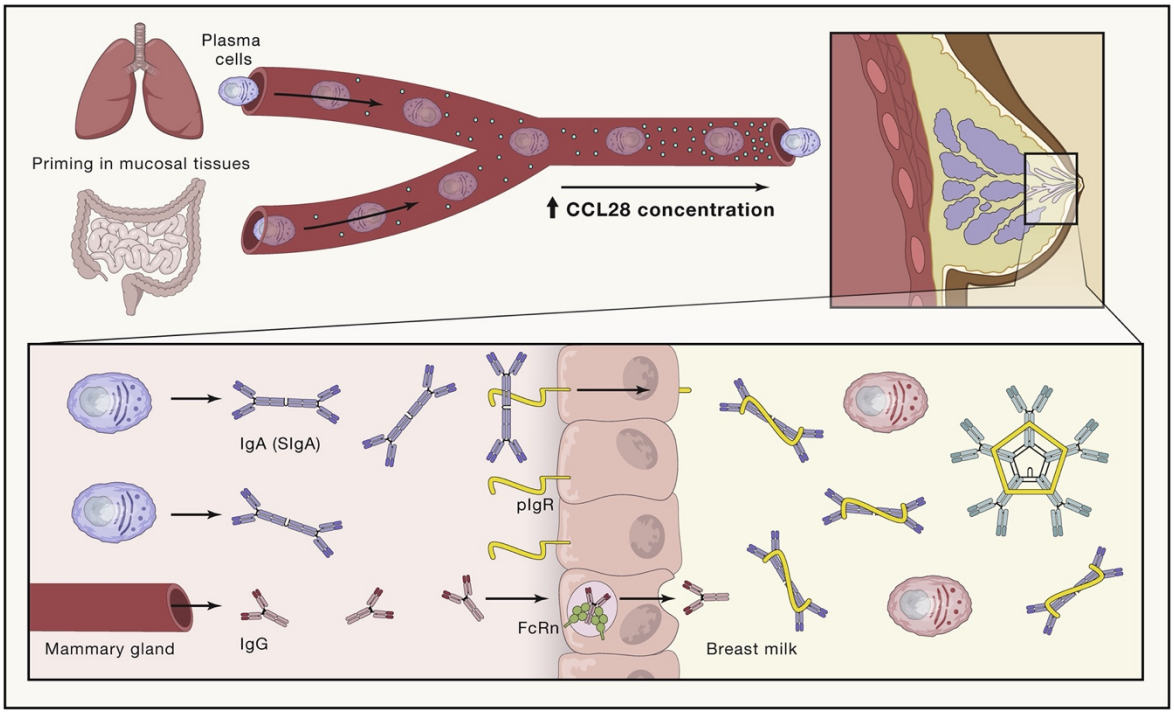


Figure 1.1. **Transfer of antibodies to breast milk.** Top: plasma cells from mucosal tissues home to the mammary gland, following a CCL28 chemokine gradient generated by the evolving mammary tissue. Bottom: these plasma cells produce IgA and IgM, which is passed into breast milk via pIgR on the mammary epithelium. IgG is thought to be transferred into the breast milk via FcRn expressed on the mammary epithelium cells. Figure from (9). The origin of breastmilk IgG is unclear.

The origin of IgA antibodies is particularly well-established. One group transferred labeled cells from the mesenteric lymph nodes (mLN) or unlabeled peripheral lymph nodes and found that the mammary glands in mice consisted of labeled cells from the mLN (36). Another group found that the IgA repertoire found in the mammary glands mirrored the IgA repertoire found in the small intestines (37). Additionally, another group used the photoconvertible Kaede-transgenic mice and visually tracked the migration of IgA plasma cells from the gut to the mammary glands in lactating mice (38). The migration of IgA plasma cells depends on surface expression of CCR10 in IgA plasma cells following the chemokine CCL28 found in the lactating mammary gland (39). These studies collectively demonstrate that mammary gland IgA plasma cells are derived from the intestines. Since IgA plasma cells in the intestines are specific to commensals, it would make sense that IgA in breast milk is directed toward commensal microbes as well (40-43).

The origin of IgG and IgM antibodies in breast milk is not well characterized; however, it has been proposed that these antibodies may accumulate in mammary glands via direct transfer from maternal blood (9).

Once in the mammary glands, the polymeric Ig receptor (pIgR) transfers secretory IgA (SIgA) and IgM (SIgM) into the breast milk (44). pIgR binds to polymeric IgA or IgM. The complex undergoes endocytosis and then is transported to the luminal surface (45). pIgR is proteolytically cleaved, releasing SIgA or SIgM still bound to the external domain of the pIgR (46). This domain, called the secretory component, stabilizes the antibodies from cleavage by proteases in the gut (47, 48). The neonatal Fc receptor (FcRn) transfers IgG into breast milk (49-51). However, we have shown low levels of IgG in the breast milk

of FcRn deficient mice (our unpublished data), indicating that FcRn-independent mechanisms may be involved.

Transfer of breast milk antibodies into the systemic circulation

In ungulates and rodents, IgG antibodies are transferred from the breast milk into systemic circulation via the gut for up to 48 hours or 21 days after birth, respectively (52, 53). This process is mediated by the declining gradient of FcRn on intestinal epithelial cells, which binds IgG and transfers it across the intestinal barrier. SIgM and SIgA from breast milk bind to the pIgR. Since pIgR only transfers IgA unidirectionally, SIgM and SIgA are not absorbed by offspring. The intestines of adult mice have 1000x less FcRn compared to the intestines of neonatal mice (54). Though present at considerably lower levels, FcRn in the adult mouse continues to transfer IgG bi-directionally from the serum to the intestinal lumen (54-58) and vice versa and has been shown to protect the host from infection by certain intestinal pathogens (59, 60).

In humans, the transfer of breast milk antibodies into the systemic circulation of the infant is less clear. While several well-cited reviews state that breast milk antibodies do not reach the infant's systemic circulation (9, 61, 62), there are several lines of evidence suggesting that the transfer of breastmilk IgG or IgA to the infant could occur under certain contexts. One study analyzed FcRn expression in the human neonatal intestine, which was done via intestinal biopsies in children and teenagers or using fetal tissue. Interestingly, FcRn was found to be expressed by the intestinal epithelium, and levels were constant over time, though there was a low sample size (63). Additionally, one study done in the 1970s administered colostrum containing anti-poliovirus IgA to strictly formula-fed infants and recovered poliovirus-specific IgA in the serum of infants

fed within 24 hours of birth but not after (64). These data, combined with studies demonstrating the human infant intestinal barrier are permissive to larger sugars and proteins (65), suggest that the low-level passage of antibodies, including IgG and IgA, across the infant intestinal epithelium may occur, at least during the first few days of life. The mechanisms mediating this process remain unclear, though we note that transfer of IgA and IgM is unlikely to involve pIgR, as murine studies have found this receptor to be unidirectional, transferring from breast milk to the intestinal lumen (66). Whether FcRn is involved in this process remains an open question.

1.5 Maternal breast milk antibodies in maintaining intestinal homeostasis

At birth, the infant is exposed to a massive influx of microbes from the mother's vaginal canal, skin-derived nursing microbiome, breast milk, and the environment. These generally harmless microbes colonize the infant’s gut and serve to establish the gut microbiota. These microbes are vital to the health and development of the offspring by enhancing digestion, promoting immune development, and resisting infection (67). To reap these benefits, the host must mount specialized intestinal immune responses that promote local tolerance to resident commensal microbes while limiting pathogenic infection.

In adults, secretion of mucus and anti-microbial factors by specialized intestinal epithelial cells helps deter bacterial translocation, thereby limiting immune activation by beneficial gut bacteria (68, 69). Additionally, the adult lamina propria harbors a complex network of immune cells (including IgA-secreting plasma cells and specialized regulatory

T cells) that work in concert to maintain a balance between tolerance to commensal microbes and resistance to harmful pathogens.

In infants, however, many mechanisms that maintain tolerance to commensal microbes in adults are either absent or not yet developed. For example, mucus secretion and anti-microbial peptide production by IECs are significantly reduced in neonates (70, 71). Similarly, IgA-producing plasma cells and microbiota-specific regulatory T cells do not arise until after weaning (72, 73). These observations indicate that there are unique mechanisms that serve to establish intestinal homeostasis in early life.

Breast milk is ideally poised to regulate nascent host-microbiota interactions. As discussed above, breast milk contains immunoregulatory elements, including cytokines, chemokines, growth factors, and hormones (74), which likely support homeostatic immune responses to newly acquired gut bacteria. In this next section, I will explore our study, and other studies, which demonstrate the importance of breast milk antibodies in maintaining intestinal homeostasis.

Breast milk antibodies dampen mucosal Tfh response in early life

Our lab showed that maternal antibodies, delivered via breast milk (and not acquired in utero), play a key role in establishing intestinal homeostasis in early life (75).

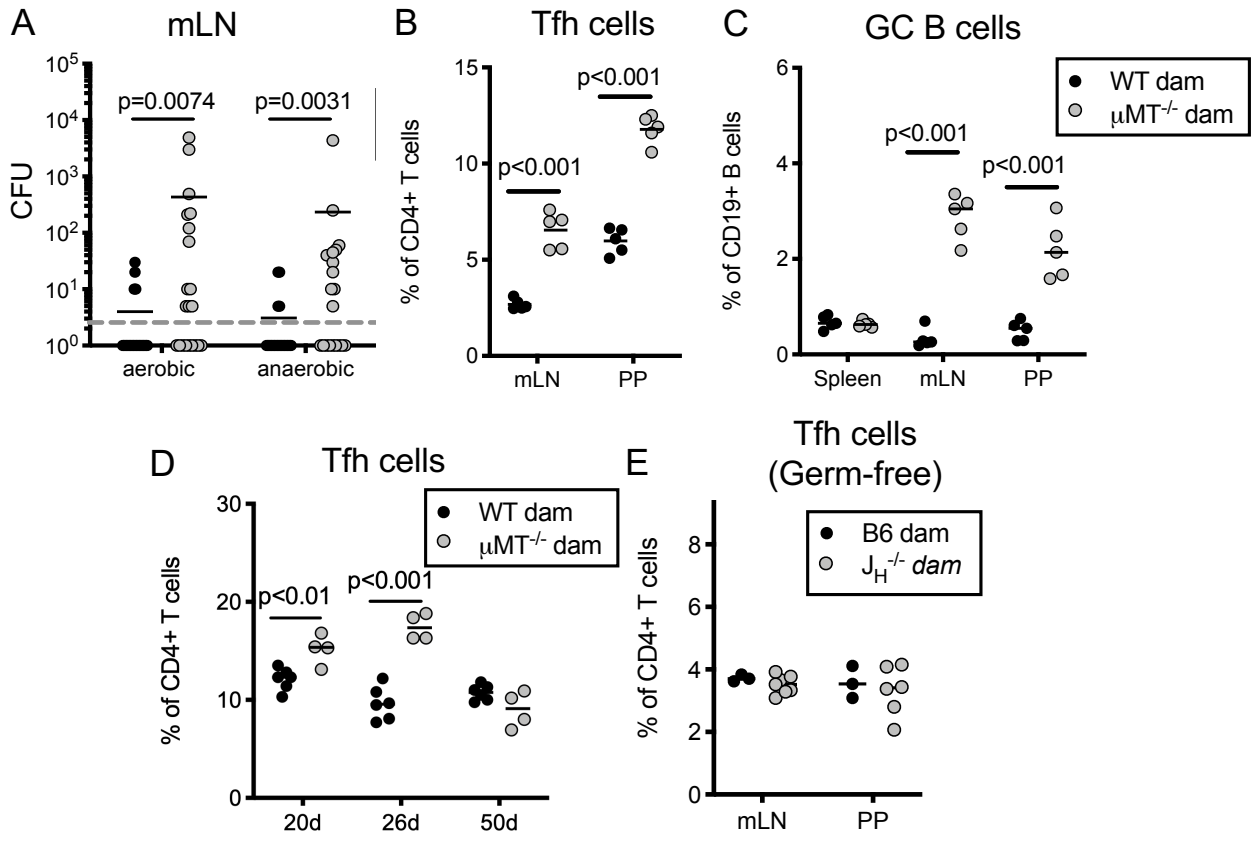


Figure 1.2. **Aberrant mucosal immunity in neonates lacking maternal breast milk antibodies**. A) Level of commensal bacteria in day 21 (d21) pups, B) % of Tfh cells at d25 in the indicated tissues, C) GC B cells at d25 in the indicated tissues, and D) percent of CD4 Tfh cells in the mLN at the indicated ages of offspring born to indicated dams (muMT$^{-/-}$ mice lack B cells). E) % of Tfh cells in d25 germ-free pups born to indicated dams (JH-/- mice lack B cells). Data consists of 4-6 mice per group, statistical significance was determined using two-way ANOVA. Data from (1) and have been repeated by me.

Heterozygous progeny of B cell-deficient (muMT$^{-/-}$) dams crossed with wild-type sires harbored increased numbers of live commensal bacterial in the gut-draining mesenteric lymph nodes (mLN) compared to control pups at the time of weaning (day 21 of age; Figure 1.2A), demonstrating that maternal antibodies prevent the accumulation of intestinal bacteria in the mLN. Correlating with these observations, we detected elevated numbers of T follicular helper cells (Tfh) and germinal center (GC) B cells in gut-

associated lymphoid tissues (mLN, Peyer's patches (PP)) of offspring lacking maternal antibodies (Figure 1.2B, C). These aberrant adaptive immune responses were transient, peaking at day 25 post-birth (Figure 1.2D), and were restricted to gut-associated lymphoid organs but not distal tissues, like the spleen (Figure 1.2C and data not shown). Under germ-free conditions, neonates lacking maternal antibodies did not generate aberrant mucosal Tfh and GC B cell responses when compared with antibody-sufficient progeny (Figure 1.2E and data not shown), establishing the importance of the microbiota in driving these phenotypes. Maternal antibodies were ingested via breastfeeding, where they bound to bacteria colonizing the infant's gut. We observed similar results in follow-up experiments wherein newborn wildtype B6 pups were fostered to lactating antibody-sufficient or deficient dams, demonstrating that these immune phenotypes resulted from the absence of breast milk antibodies and were not dependent on maternal antibodies acquired *in utero.*

It is important to note that this response occurs at homeostasis. That is, neither the dam nor the offspring was infected with pathogenic microbes, and all mice were given access to food and water ad libitum. While pathogen-specific maternal antibodies (transferred both in breast milk or *in utero*) have been well-established in preventing infection from said pathogen in the offspring (76), the mechanisms underlying the function of non-pathogenic (presumably anti-commensal) maternal antibodies and their consequences on long-term health remain a gap in our knowledge.

<u>Accumulating evidence from other studies</u>

Several other studies have highlighted the importance of breast milk antibodies in maintaining intestinal homeostasis. Wildtype mice pups nursed by pIgR-deficient dams,

and thus deficient in breast milk IgA and SIgM, exhibited decreased epithelial barrier function, marked by an increase in the translocation of pathogenic aerobic bacteria into the mLN compared to pups nursed by wildtype dams (70). Mice that did not receive SIgA in breast milk had subtle alterations in the gut microbiota, which persisted through adulthood. These alterations include an increase in Proteobacteria (70), which have also been shown to be elevated in individuals with inflammatory bowel disease (IBD) (77, 78). In humans, formula-fed infants exhibit increased gut inflammation (79). In mice, maternal antibodies transferred via breast milk were found to be protective against neonatal enteric infection with enterotoxigenic *Escherichia coli.* The study found that gut colonization with Pantoea, a commensal species closely related to Enterotoxigenic *Escherichia coli* (ETEC), elicited maternal antibodies that were cross-reactive to ETEC (80).

Breastfeeding, as opposed to formula feeding, has been shown to decrease the incidence and severity of necrotizing enterocolitis, an intestinal illness that disproportionately affects premature infants. One group found that infant samples with NEC were associated with a decrease in IgA-bound bacteria and dominated by unbound Enterobacteriaceae. They developed a mouse model of NEC and found that breast milk IgA coated Enterobacteriaceae in the gut, preventing intestinal damage and mortality (10).

Cumulatively, these data support the hypothesis that maternal antibodies transferred through breast milk are important for both fostering the development of a healthy microbiota and for protecting offspring from pathogenic infections, even if the mother is not infected herself.

1.6 T cell-dependent immune responses, microbes, and imprinting by maternal factors

As mentioned above, we reported that offspring that do not receive maternal antibodies via breast milk exhibit microbiota-dependent immune dysregulation during the weaning period. This immune dysregulation is characterized by a transient increase in Tfh and GC B cells in gut-associated lymphoid tissues during the weaning transition. My thesis work aimed to understand the long-term consequences of this immune dysregulation. There are three motivations for this line of inquiry, which will be expanded upon in the next section. One, T cell-dependent adaptive immune responses can persist up to the lifetime of the host. Second, accumulating evidence links disruptions to host-microbiota interactions in early life with lasting consequences on host immune responses and health. Third, maternal factors can lead to immune imprinting in the offspring, which has lasting consequences on health.

T cell-dependent and independent antibodies

Following antigen-specific activation, T follicular helper (Tfh) cells interact with B cells within specialized immune structures called germinal centers (GCs). The GC response has been studied extensively, and many signaling molecules, like cytokines and CD40/CD40L interaction, orchestrate this process (81). I would like to highlight several molecules involved in the germinal center response as I utilize them in my research strategy. The first molecule is sphingosine-1-phosphate receptor 2 (S1PR2), which is expressed by GC B cells (amongst several other cell types) and confines the B cell into the center of the GC (82). The second is Bcl6, a transcription factor essential for Tfh differentiation and not for the differentiation of other helper cell subsets. Bcl6 is also an

important transcription factor for GC B cells and it allows B cells to be in a proapoptotic state and tolerate DNA damage (83).

GC reactions generate a panel of mutated B cells that are then selected based on affinity and help from Tfh cells to expand and differentiate into antibody-secreting plasma cells and memory B cells. The resulting antibodies, called T cell-dependent (TD) antibodies, have high affinity, and are highly specific to their antigen and are particularly helpful in fighting against viral pathogens, including influenza, HIV, or bacterial pathogens like Citrobacter (84). The serum titers of TD antibodies can persist without measurable declines for years in mice and decades or longer in humans (85-89). Of relevance to our finding, some of the gut bacterial species present at the time of the elevated T-dependent GC cell response persist through adulthood (90) and are, therefore, likely to feed a long-lived GC response.

A discussion about TD immune responses would be incomplete without a discussion about the activation of B cells independent of T cell help (T independent, TI). Unlike TD antigens, which are predominately proteins or peptides, TI antigens are multivalent polysaccharide antigens such as LPS on bacterial cell walls. The specialized B cells that respond to such antigens are marginal zone B cells found in the spleen and lymph nodes or B1 cells found in the peritoneal cavity and at mucosal sites (91). The antibodies generated, called T cell-independent (TI) antibodies, are generally polyreactive and low-affinity and can be produced abundantly and more rapidly than TD antibody responses, providing a first response to initial exposure to an antigen (92). In the gut, one study cloned out many antibodies from IgA+ intestinal plasma cells and found that they target a diverse pool of commensal bacteria via conserved molecular structures

common to a variety of bacterial taxa (93). While originally thought to be short-lived responses, recent studies have shown that TI plasma cells can persist in the bone for upwards of 2 years, approaching the lifespan of the mouse (94).

Specific to the commensal microbiota, TD and TI antibody responses coordinate to maintain host-microbe mutualism (84, 93, 95). Microbiota-reactive TD antibodies are predominantly of the IgA isotype and can be secreted into the intestinal lumen, where they promote tolerance to gut commensals by several mechanisms, including restricting bacterial motility and limiting microbial association with host tissue (96-98). In line with this, TD IgA antibodies preferentially bind “border dweller” bacteria that reside proximal to the intestinal epithelium, such as segmented filamentous bacteria (SFB), Prevotella, and *Helicobacter* species (93, 99-101). Defects in the generation of TD IgA is associated with increased levels of these atypical commensal species in the gut, consistent with the model that TD IgA hinders gut bacteria to maintain intestinal homeostasis. TD antibodies are not always beneficial to intestinal homeostasis. One study found that TD IgG promotes intestinal inflammatory responses by engaging activating Fc receptors on mucosal immune cells (102). In further support of a pathogenic role for microbiota-reactive IgG, genome-wide association studies have linked variants of IgG Fc receptors with reduced affinity for IgG with protection from ulcerative colitis (102).

Microbe and immune interactions in early life

Early life interactions between the microbiota and the developing immune system are carefully orchestrated, and disruptions to this process can have lasting consequences on health (103-105). Studies in mice show that antibiotic treatment before weaning, but not later, can drive allergic disease (106, 107) and that colonization of germ-free mice

before weaning, but not as adults, reduces allergic outcomes (108). Tregs that mediate tolerance to specific bacteria are generated before weaning (109, 110). Additionally, exposure of dendritic cells to the intestinal microbiota before weaning induces a transient increase in PD-L1 (programmed death-ligand 1) required for the generation of Tregs (111). Finally, at the time of weaning, the microbiota induces a strong immune response that results in the expansion of a specialized subset of RORgamma+ Tregs, which serve to protect the host from pathological gut inflammation (90). Germ-free mice, which do not experience colonization of microbes at birth, develop high levels of IgE (112). These mice accumulate invariant natural killer T cells in the lungs and intestine due to the absence of microbiota-induced epigenetic modification of Cxcl16, which, when active, recruits NKT cells to the intestine (108). Thus, appropriate interactions between microbes and the developing immune system are crucial for health.

Similar results have been found in human studies as well. Antibiotics use in children in the first year of life is associated with increased allergic outcomes (113, 114). Antibiotic use also increases the risk of metabolic disease and inflammatory bowel disorders (115-117). These studies demonstrate the importance of microbes in driving healthy development.

Maternal imprinting

There is growing evidence that maternal factors mediate the important immune reactions towards the microbiota in early life, which result in long-term immune imprinting and functional consequences in the offspring.

In mice, low epidermal growth factor (EGF) levels found in breast milk, specifically after day 10 of lactation, were shown to be important for opening colonic goblet-cell-

associated antigen passages (GAPs), allowing for the delivery of microbiota-derived antigens that induce the generation of Tregs. Such Tregs then maintain long-term tolerance against similar bacteria (110). Recently, it was reported that maternal IL-6, induced by a mild maternal infection, caused epigenetic imprinting of fetal intestinal stem cells, resulting in increased numbers of intestinal Th17 in offspring through adulthood. This maternal 'immune imprinting' resulted in overall protective responses following offspring infection with the enteric pathogen *Salmonella typhimurium*, but led to increased morbidity following induction of colitis (118). Finally, in an elegant study, maternal IgA transmitted via breast milk was found to regulate the differentiation of RORgamma+ Tregs, which was essential for controlling intestinal inflammation for multiple generations (38). These studies indicated that maternal factors have long-lasting influences on offspring immunity and health.

Our hypothesis

Given the diverse role of TD antibodies in promoting both beneficial and pathogenic adaptations, the longevity of TD immune responses, the importance of appropriate early life host immune-microbe interactions, and the influence of maternal factors in this process, the overarching hypothesis is that perturbations in adaptive immunity, triggered by the absence of breastmilk antibodies, drive sustained impairments in intestinal immunity. To test this hypothesis, I investigate whether offspring accumulate increased IgA+ plasma and memory cells in the intestines in the absence of maternal antibodies. Additionally, I investigate whether offspring accumulate microbiota-reactive IgA in the absence of maternal antibodies. Finally, I test if offspring deficient in maternal antibodies have alterations in the composition and functional capacity of the intestinal microbiota.

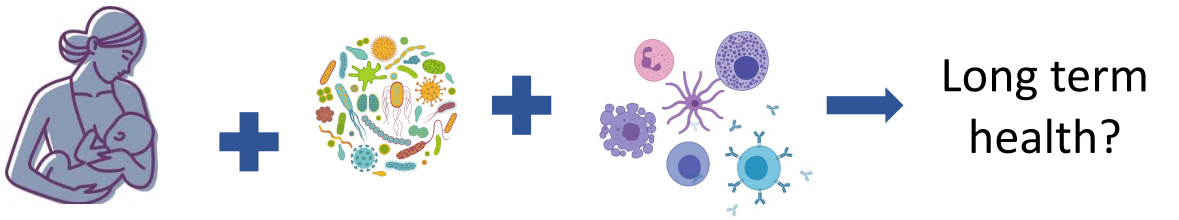


Figure 1.3. **Research question of this thesis**. What are the effects of breast milk antibodies and the microbiota-directed GC B cell response on long-term health?

1.7 Goals for this thesis

The overall goal of this thesis is to investigate the long-term consequences of maternal antibodies delivered via breast milk on offspring intestinal immunity. This information can be informative for the development of treatments or therapeutics aimed at promoting beneficial host-microbiome interactions in early life, particularly in children who are not breastfed.

As described above, offspring fed breast milk deficient in antibodies generate dysregulated B and T cells intestinal immune responses at the time of weaning, which we hypothesize can result in long-lived immune responses to gut bacteria, thus altering host-microbiota mutualism and driving adverse health outcomes in the long term.

In chapter II, I describe a novel method to isolate and characterize intestinal B cell subsets. In chapter III, I use this method to assess whether offspring deficient in maternal antibodies have shifts in intestinal B cell subsets over time. I complement these analyses by evaluating antibody titers and anti-commensal antibody responses in maternal antibody-sufficient and -deficient offspring. In chapter IV, I present results from our research aimed at understanding whether the absence of maternal antibodies results in alterations in microbiota composition, localization, and function. Finally, in chapter V, I

comprehensively analyze the relative contributions of different subclasses of antibodies found in human breast milk.

Chapter II

Identification of intestinal lamina propria plasma cells by surface transmembrane activator an CAML interactor (TACI) expression

This chapter is adapted from the following publication:

Wang, B, Martinez, S, Rojas, O, Koch, MA. (2023) Identification of intestinal lamina propria plasma cells by surface transmembrane activator and CAML interactor (TACI) expression. *Submitted. Journal of Immunology.*

2.1 Introduction

Antibodies are crucial effectors of the humoral immune response and can target pathogens, impair tumor growth, and maintain homeostasis with beneficial gut microbes (119, 120). Following antigen encounter and activation in lymphoid organs, B cells form plasmablasts, which can then migrate to the bone marrow or mucosal tissues and differentiate into long-lived antibody-secreting plasma cells (121-123). The intestinal lamina propria contains the largest number of IgA-producing plasma cells in the body (100, 124). IgA is actively translocated into the lumen to maintain a homeostatic relationship with our commensal microbes (125, 126).

Plasma cells are terminally differentiated B cells that express high levels of the transcription factor Blimp-1, which is essential for plasma cell differentiation (127, 128). Blimp-1 represses the transcription of genes important for B cell identity, including Pax5, Bach2, and Bcl6 (129-131). As such, surface proteins that typically define B cells, including CD19 and B220, are downregulated on plasma cells (132). Within lymphoid tissues, such as the bone marrow, CD138, also known as Syndecan-1, is one of the most frequently used surface proteins to identify plasma cells (132). In contrast to bone marrow plasma cells, intestinal lamina propria plasma cells are embedded in a matrix of collagen and liberating them requires a time-intensive enzymatic digestion process. In our hands, the detection of CD138 on gut immune cells following tissue digestion is inconsistent. The generation of reporter mice for which YFP or GFP expression is controlled by the gene *Prdm1* (encoding the transcription factor Blimp-1) has enabled the identification of Blimp-1 plasma cells without relying on CD138 (133). However, it is not always feasible or practical to use Blimp-1 reporter mice for studies of mucosal B cells. Thus, identifying

surface markers other than CD138 that reliably detect gut plasma cells would enhance studies of mucosal immunology.

Here, we show that detection of CD138 surface expression is impaired following collagenase treatment, making this protein an unreliable marker to identify tissue-derived plasma cells. Transmembrane activator and CAML interactor (TACI) is a survival receptor that is upregulated on activated B cells and plasma cells and has been used to identify plasma cells in several organs (134-136). Using Blimp-YFP reporter mice, we confirm TACI as a robust marker expressed by most gut plasma cells. Additionally, we describe a comprehensive flow cytometry panel that identifies key intestinal B cell subsets, including plasma cells, by expression of surface markers. We demonstrate the utility of this approach using an *in vivo* fate-mapping system to track the differentiation of activated germinal center B cells into mature gut-resident B cell subsets.

2.2 Materials and Methods

Mice

Wildtype C57BL/6J, Blimp1-YFP (B6.Cg-Tg(Prdm1-EYFP)1Mnz/J (133), and Rosa$^{lsl\text{-}tdTOM}$ ^{m} (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG\text{-}tdTomato)Hze}$/J) Specific Pathogen Free (SPF) mice were purchased from The Jackson Laboratory (stock #000664, #008828, and #007914 respectively). IgA knockout (B6.129S7-*Igha*^{tm1Grh}/Mmmh) mice were obtained from the MMRRC (stock #031019-MU) (137). S1PR2^{CreERT2} mice were a gift from Dr. Tomohiro Kurosaki (Riken) (82). All animals were bred and maintained under SPF conditions at the Fred Hutch. Experiments were approved by Fred Hutch IACUC, and the study was conducted in strict compliance with the PHS Policy on Humane Care and Use of Laboratory Animals.

Tamoxifen administration

5mg of tamoxifen (Sigma) was dissolved in 100uL of corn oil for 4 hours at 37C via agitation and administered by oral gavage every day for two consecutive days.

Collagenase treatment of bone marrow cells

Bone marrow lymphocytes were isolated by centrifugation as described (138). 30e6 lymphocytes were digested in collagenase Type II, Type III, Type IV (all from StemCell Technologies), 4, A, D (all from Roche), or collagenase VIII (Sigma) in 5 mL digestion buffer (RPMI, 10% FBS, 15mM HEPES, 0.025 mg/mL DNAse I, 0.5 mg/mL respective collagenase) for 20 min at 37°C. Cells were pelleted and stained for analysis by flow cytometry.

Isolation of intestinal lamina propria cells

Intestinal lamina propria lymphocytes were prepared (Figure 2.1) by isolating the small or large intestine, dissecting away the Peyer's patches and fat, cutting the intestines open longitudinally and swirling intestines gently in ice-cold PBS to remove feces. Intestines were minced finely with dissection scissors such that individual pieces were no larger than 5mm. Intestines were cleaned further by vortexing pieces in 50mL conical tubes filed with ice-cold wash buffer (HBSS, 2% FBS, 15mM HEPES) for 30 sec. Intestines were strained using a 100 μm strainer (Corning) placed on top a 50 mL conical tube and the washing process was repeated 2 more times. Cleaned intestine pieces were placed in 20mL of room-temperature EDTA buffer (HBSS, 10% FBS, 15mM HEPES, 5mM EDTA), vortexed for 30 sec and placed in an orbital shaker for 20 min at 300rpm to remove epithelial cells. Tissues were strained using a 100 μm strainer and the EDTA wash was repeated. Intestines were strained again using a 100 μm strainer and rinsed with 10mL of ice-cold wash buffer to remove residual ETDA before being incubated with 20 mL of pre-warmed digestion buffer (RPMI, 10% FBS, 15mM HEPES, 0.025 mg/mL DNase I (Roche 10104159001) and 0.75 mg/mL collagenase 4 (Sigma C5138) at 37C for 50 min. To adjust for lot-to-lot variability, we titrated each lot of collagenase to find the optimal concentration for the best viability. During the incubation, tubes were briefly vortexed every 15 min to ensure even digestion. Following this procedure, the supernatant was cloudy and most of the tissue was digested away except for residual fat. Digested cells were filtered over a 70 μm strainer (Corning) placed on top of a 50mL conical. Tubes were rinsed with an additional 20mL of ice-cold RP-5 (RPMI, 5% FBS, 15mM HEPES, 1x

Penicillin-Streptomycin-Glutamine) to collect all residual cells and filtered through the same 70 μm strainer. Cells were pelleted at 475rcf for 5 min at 4C. The supernatant was decanted and cell pellet resuspended in 6mL of 44% Percoll (Cytiva) diluted in plain RPMI. Cells were then transferred to a 15 mL conical precoated with FBS and underlayed with 4 mL of 67% Percoll diluted in PBS. Cells were pelleted at 2800 rpm for 20 min at room temperature without brake. Cells at the gradient interface were transferred into a new conical and topped off with 20 mL of ice-cold RP-5. Cells were pelleted and stained for analysis by flow cytometry.

Flow cytometry analysis

1-3 x 10^6 cells were pelleted and stained in a volume of 50 uL. Cells were incubated with LIVE/DEAD Fixable Aqua viability dye (Thermo Fisher Scientific) and blocked for non-specific binding with anti-CD16/32 (Thermo Fisher Scientific) at 1:200 dilution in PBS for 20 min at 4°C. Cells were then stained for surface protein expression for 20 min at 4°C in FACS buffer (PBS, 2% FCS, 1mM EDTA). For a complete list of antibodies and dilutions used, reference Table 2.1. Cells were washed and stained with Streptavidin-BUV737 (Thermo Fisher Scientific) at 1:300 in FACS buffer for 15 min at 4°C. Cells were sorted immediately using the FACSymphony S6 (BD) or acquired on the FACSymphony (BD) for analysis with FlowJo (TreeStar).

For intracellular staining of immunoglobulins, cells were stained for surface protein expression, fixed and permeabilized with Cytofix/Cytoperm (BD) for 20 min, and stained with anti-IgA (BD) conjugated to AF647 (Thermo Scientific, conjugated per manufacturer's instructions) or anti-IgA FITC (BD) at 1:200 in permeabilization wash

buffer (BD). Cells were washed, resuspended in FACS buffer and subsequently analyzed via flow cytometry.

ELISpot analysis

Multiscreen plates (Millipore) were coated with goat-specific isotype antibodies to murine IgA (BD), IgG, and IgM (Jackson ImmunoResearch Laboratories) at 5ug/mL in PBS and blocked with 5% goat serum diluted in PBS for one hour at 37°C. Plates were washed with PBS and sorted intestinal lamina propria cells were serially diluted in RPMI 1640 (Gibco) supplemented with 10% FBS (HyClone), 1x Penicillin-Streptomycin-Glutamine (Gibco) and 50 μM 2-mercaptoethanol (Gibco) and incubated at 37°C overnight. Cell were serially diluted 1:3 starting with 1200 cells/well. Following washing with PBS, secondary peroxidase-conjugated antibodies specific to IgA (BD), IgG, and IgM (Jackson ImmunoResearch Laboratories) were added at 0.35 to 0.4ug/mL in PBS and incubated for 1 hour at 37°C to detect antibody-secreting cells. Plates were developed with AEC developing reagent (Vector Laboratories) according to the manufacturer's instructions. Plates were read on an ImmunoSpot CTL analyzer and quantitated using ImmunoSpot Pro. For list of all antibodies used, reference Table 2.2.

2.3 Results

Collagenases impair detection of surface CD138 expression by immune cells

Upon terminal differentiation from B cells, plasma cells downregulate many surface proteins routinely used to identify B cells, such as B220 and CD19. CD138, also known as Syndecan-1, is thought to promote cell survival by binding to pro-survival cytokines (139) and is upregulated during plasma cell differentiation. As such, expression of CD138 is commonly used to identify plasma cells in several murine tissues (136, 140). In the bone marrow and spleen, populations of B220+CD138^{mid} short-lived plasmablasts and B220^{-}CD138^{hi} terminally differentiated plasma cells have been reported in numerous studies (136, 140, 141).

Unlike the bone marrow or spleen, liberation of lymphocytes from the intestine requires extensive tissue processing and enzymatic digestion with collagenases. To assess if CD138 expression could be used to identify intestinal plasma cells, we tested whether collagenase treatment impacted the ability to detect CD138 on bone marrow cells. We analyzed expression of CD138 on IgD^{-} Lineage (Lin)$^{-}$ (CD4-, CD8-, Ly6G-, SiglecF-, XCR1-) cells. As expected, we identified a population of B220^{+}CD138^{mid} plasmablasts and B220^{-}CD138^{-} plasma cells (Figure 2.2A) in samples not treated with collagenase (136, 140). Treatment with an array of collagenases commonly used for intestinal lymphocyte isolation (121, 142-147) resulted in a striking loss of these CD138^{+} populations (Figure 2.2B). We obtained similar results of low or undetectable CD138 expression by intestinal B cell subsets following digestion with a subset of these collagenases. Thus, collagenase treatment strongly impairs the ability to detect CD138 on lymphocytes via flow cytometry.

Intestinal plasma cells express TACI

We sought to identify an alternative surface marker of plasma cells that is less sensitive to collagenases. Transmembrane activator CAML interactor (TACI) is a pro-survival receptor that is upregulated on activated B cells and plasma cells (134, 135) and has been used to identify plasma cells in the bone marrow and other organs, such as the spleen (136).

To test whether TACI could be used to identify gut plasma cells, we made use of the Blimp-YFP reporter mice (133). Blimp-1 is a transcription factor that is essential for plasma cell differentiation (129-131). In Blimp-1 reporter mice, enhanced yellow fluorescent protein (YFP) is inserted into the *Prdm1* locus, allowing for detection of $B220^{-}$ plasma cells by high YFP expression in heterozygous animals. After determining the optimal concentration of collagenase 4 to liberate gut immune cells with high viability (Figure 2.3) we sorted Blimp-expressing (YFP+) immune subsets from reporter mice for ELISpot analysis and confirmed that $B220^{-}$Blimp-YFP^{hi} intestinal cells were enriched for IgA antibody-secreting cells (Figure 2.4 (143)). We also developed a 'lineage' (Lin) cocktail containing antibodies to CD4, CD8, Ly6G, XCR1, and SiglecF to precisely isolate gut plasma cells away from other resident immune populations including T cells, neutrophils, eosinophils and other myeloid subsets. Using Blimp-YFP reporter mice, we confirmed that Blimp-YFP^{hi} plasma cells did not express the markers included in our Lin channel (Figure 2.5). Blimp-1 is also expressed by other immune subsets including T cells (148). Consistent with these observations, we observed a population of YFP^{mid} expressing cells within the Lin+ gate, though these cells did not secrete IgA (Figure 2.4).

Using Blimp-YFP reporter mice, we found that TACI enriches for plasma cells, with 66% of the $CD45^+Lin^-IgD^-B220^-TACI^+$ population expressing high levels of Blimp-YFP (Figure 2.6A). Correspondingly, few cells (<5%) of $B220^-TACI^-$ and $B220^+TACI^{mid}$ expressed high levels of Blimp-YFP. Complementary analysis by first gating on Blimp-YFP^{hi} cells revealed that approximately 80% of this subset expresses TACI (Figure 2.6B). Cumulatively, these data demonstrate that TACI can be used to identify the vast majority of gut plasma cells. Of note, though dithiothreitol (DTT) is often used in gut preps to remove mucus, we found that detection of TACI on gut B cells was reduced following DTT treatment (Figure 2.7). Thus, we omitted this reagent for our studies.

Gating strategy to identify B cell subsets in the intestines

We next established a flow cytometry panel to identify key intestinal B cell subsets. We designed a 13-fluorophore surface staining panel that enables the identification of naïve B cells, germinal center B cells, memory B cells, and plasma cells in the murine gut (Figure 2.8).

Among Lin^-CD45^+ cells, germinal center, naïve, and memory B cell subsets all co-express CD19 and B220. Within this group, we identified a small population of $CD38^-GL7^+$ gut germinal center B cells. Though intestinal tissue is not a conventional site of germinal center formation, several reports have identified cryptopatches or isolated lymphoid follicles as sites of germinal center activation (149, 150), consistent with our observations. The $CD38^+GL7^-$ population contains naïve and memory B cells, which can be further classified based on additional markers. Naïve B cells co-express IgM and IgD and accordingly, we observed a population of IgM^+IgD^+ naïve B cells in the intestines.

Gating on IgD^- cells, which we classify as memory B cells, allows for detection of other isotypes, including IgA, IgM, other IgD- switched immunoglobins (swIg). Memory B cells exhibit heterogenous expression of surface markers associated with T cell interactions, including CD73 (151, 152). Accordingly, we observed that gut $CD38^+IgD^-$ cells were heterogenous for CD73 expression.

Among the $Lin^-CD45^+CD19^{mid\text{-}to\text{-}low}B220^-$ population, TACI expression identifies plasma cells. Via surface immunoglobulin expression, we found that the majority of gut plasma cells in our mouse colony were IgA^+, and a small population was either swIg or expressed IgA to levels that could not be detected by our staining method. We did not detect surface IgM expression on gut plasma cells.

Intracellular staining requires fixation and permeabilization of cells, which is often not compatible with downstream applications, including sorting and culturing or usage with fluorescent reporter systems. We confirmed that in the intestine, surface staining of IgA yielded similar staining intensity compared to surface and intracellular staining (Figure 2.9).

Fate tracking B cells in the lamina propria

Having established a flow cytometry staining panel that relies exclusively on cell-surface markers to identify mucosal B cell subsets, we performed a proof-of-concept experiment using an *in vivo* fate tracking approach, which is not compatible with cell permeabilization. We wanted to track the fate of germinal center B cell subsets. Among B cell subsets, germinal center cells preferentially express high levels of sphingosine-1-phosphate receptor 2 (S1PR2) (82). We utilized mice where a Cre recombinase-estrogen

receptor 2 fusion protein (Cre-ERT2) was inserted into exon 2 of S1PR2. Additionally, these mice harbor a transgene encoding the red fluorescent protein tdTomato (tdTOM) downstream of a *lox*P-flanked stop cassette controlled by the constitutively expressed Rosa26 promoter (herein called known as S1PR2-Cre-tdTOM mice). Administration of tamoxifen transiently activates Cre-mediated excision of the floxed stop cassette enabling heritable expression of tdTom fluorescent protein by all cells expressing S1PR2 at the time of treatment. This cutting-edge system has been used by several groups to look at the dynamics and fate of germinal center B cells in the spleen, lymph nodes, and Peyer's patches (153, 154). We administered tamoxifen to S1PR2-Cre-tdTOM mice at 21 days of age, resulting in robust tdTom expression by germinal center B cells (153) and Chapter 3 in this work). Thirteen weeks later, we used our flow cytometry panel to detect a small population of tdTom+ cells in the colonic lamina propria. We found that $tdTom^+$ cells were IgD^-, suggesting that they were previously activated (Figure 2.10A) and were comprised of both plasma and memory B cell subsets (Figure 2.10B). Thus, we have verified our tissue digestion and flow cytometry panel can be combined with other tools, such as fate tracking systems, to assess gut B cell responses.

2.4 Discussion

Intestinal plasma cells and the antibodies they secrete are vital to pathogen defense and maintaining a mutualistic relationship with commensal microbes. One of the major hurdles in investigating intestinal plasma cells is the lack of a marker that will reliably identify these cells, particularly in the context of complex digestion processes with collagenases. We have identified TACI as a cell surface protein that can be detected following collagenase treatment and show that it is enriched on gut plasma cells. Additionally, we described an efficient protocol and staining panel to isolate and identify key intestinal B cell subsets in the gut. This protocol can be accomplished in approximately 3 hours and can be easily scaled up to study many mice at a time. Characterization of antibody isotype expression by plasma cells has been accomplished both by intracellular and extracellular staining methods (136). We showed that extracellular staining of IgA plasma cells in the gut yields the same results as when combined with intracellular staining. In alignment with other studies (93, 101, 143), plasma cells isolated from unmanipulated animals in our mouse colony predominantly secrete IgA, as indicated by ELISpot analysis. However, our gating strategy with TACI may be useful for identifying intestinal plasma cells in the context of perturbations, including dysbiosis or infection, which may skew antibody production in the gut to other isotypes, such as IgG (155).

There are several limitations to our study. While we were able to detect TACI on the majority of Blimp-YFP+ plasma cells, there is a small population that remains $TACI^{lo/dim}$, indicating that some bona fide plasma cells may be 'missed' when using this approach. It is possible that methods to increase the detection of surface TACI (e.g., using

biotin and streptavidin conjugates) may resolve this issue. We note that currently, the anti-TACI reagent used in these studies is not commercially available in a biotinylated format. Additionally, while we tested the impact of eight commonly used collagenases on our ability to detect surface CD138 on bone marrow B cells, we did not exhaustively test all possible parameters, including different enzyme concentrations and incubation times on gut samples. Finally, we focused on detection of surface marker expression to develop a protocol for identifying gut B cell subsets that is compatible with downstream analysis, such as fate-tracking systems. It is possible that intracellular staining for CD138 expression would be useful for identifying gut plasma cells, as collagenase treatment should only impact detection of surface proteins on live cells.

In summary, we revealed TACI expression is enriched on gut plasma cells and described a comprehensive flow cytometry panel to identify B cell subsets in the intestinal lamina propria via surface staining. Our protocol is compatible with several important downstream applications such as sorting and culturing, transcriptomic analysis, and fate-tracking approaches and can aid in the study of intestinal B cells and the mechanisms regulating their activation, differentiation, and maintenance.

2.5 Acknowledgments

We thank Diane Rico and Helena Wynd for their support and technical assistance. We thank Mary Fontana for her feedback on the manuscript.

2.6 Figures

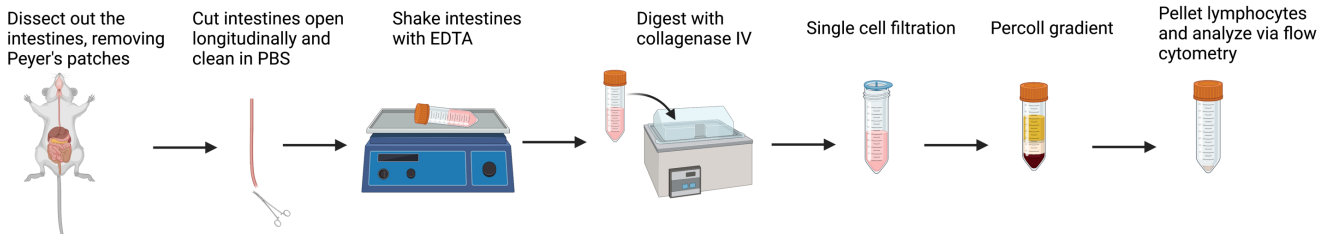


Figure 2.1 **Workflow to isolate plasma cells from the intestinal lamina propria.** Small or large intestines were removed, and the cecum was discarded. Peyer's patches were removed from the small intestine. Intestines were cut open longitudinally and washed in PBS to remove feces. Intestines were then shaken with EDTA to remove epithelial cells, digested to liberate lymphocytes, and passed through a strainer to create a single cell suspension. Lymphocytes were then enriched via a density gradient. Cells at the interface were pelleted and analyzed via flow cytometry.

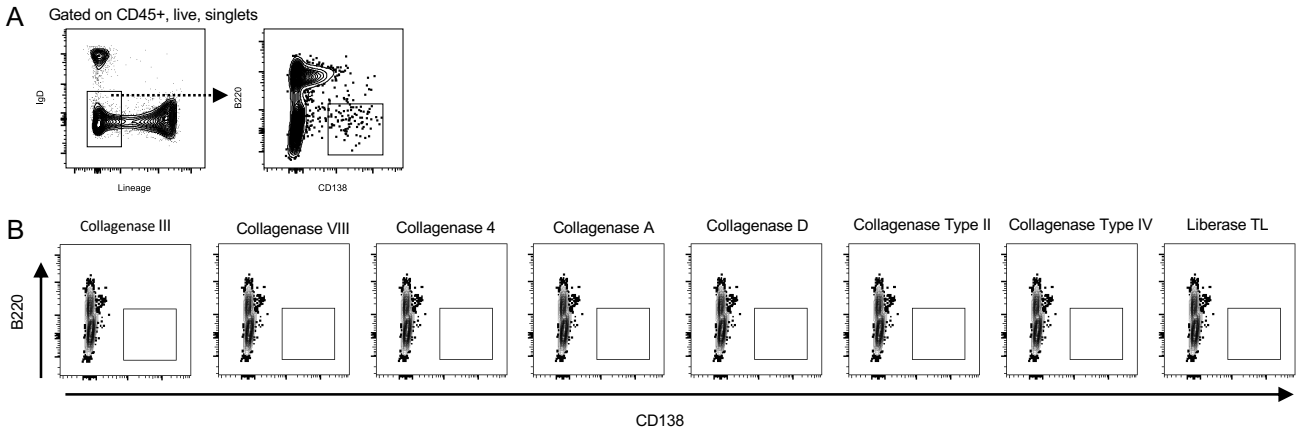


Figure 2.2 **Collagenase significantly diminishes CD138 expression**. A) Bone marrow cells were isolated and stained for analysis by flow cytometry prior to treatment with collagenases. Cells were pre-gated based on size (FSC-A by SSC-A) and viability (not shown). Gating on $CD45^{+}$, IgD^{-}, Lin^{-} (CD4, CD8, Ly6G, SiglecF, and XCR1) enriches for plasma cells, which make up the $CD138^{hi}B220^{-}$ population. B) Expression of B220 vs. CD138 in bone marrow cells after treatment with the indicated collagenases for 20 minutes. Cells are pre-gated as in A). Flow cytometric plots are representative of two independent experiments, each comprised of $n \geq 2$ mice. Mice were analyzed at 10-24 weeks of age.

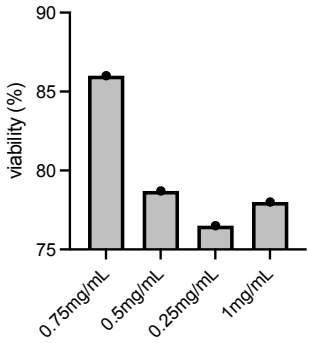


Figure 2.3: **Titration of collagenases for optimal viability**. Small intestines were isolated, incubated with EDTA to remove epithelial cells, and split into fractions, each of which was digested with the indicated concentration of collagenase 4. Cells were then processed as normal via Percoll gradients. Viability was determined by flow cytometry via exclusion of Fixable Aqua viability dye. Data are representative of two independent experiments. 10-week-old sex-matched, littermate mice were used for analysis.

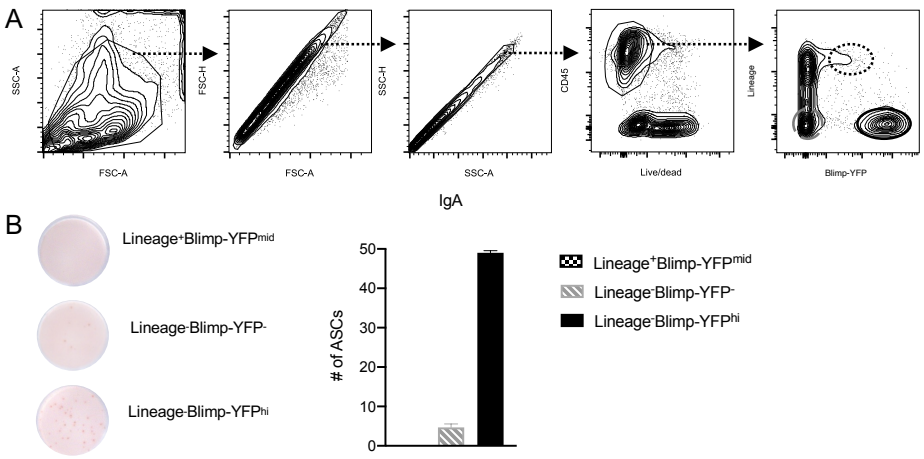


Figure 2.4 **Antibody secreting cells in the intestinal lamina propria express high levels of Blimp-YFP**. A) Small intestinal lamina propria cells were isolated from Blimp-YFP reporter mice and sorted based on expression of Blimp-YFP and Lin markers (CD4, CD8, Ly6G, SiglecF, and XCR1) as indicated for IgA ELISpot analysis. B) Representative image of IgA antibody secreting cells from sorted $Lin^{+}Blimp\text{-}YFP^{mid}$, $Lin^{-}Blimp\text{-}YFP^{-}$, $Lin^{-}BlimpYFP^{mid}$, $Lin^{-}Blimp\text{-}YFP^{hi}$ cells were analyzed for the frequencies of IgA secreting cells by ELISpot. Images (left) show dilution of 44 cells/well using IgA-specific detection reagents and graph (right) indicates number of antibody secreting cells (ASCs) +/- SD from the indicated groups. N=2 mice, analyzed at 10 weeks of age.

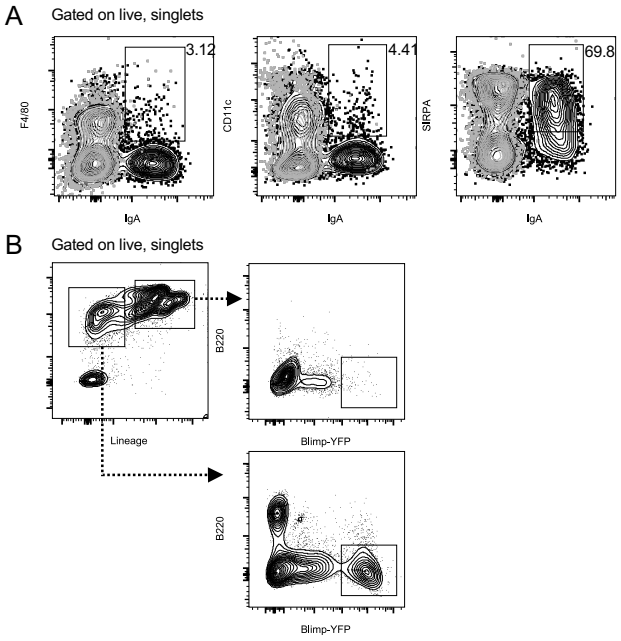


Figure 2.5 **Lineage gate excludes IgA+ cells in the intestine**. A) Small intestinal lamina propria cells were isolated from wild-type B6 mice and stained for analysis for flow cytometry. Cells were gated on live, singlets. Expression of IgA vs. Ly6G, XCR1, F4/80, CD11c, and SIRPA. Numbers indicate frequency of expression of indicated marker within IgA^+ cells. B) Blimp-YFP expression in $CD45^+Lin^-$ and $CD45^+Lin^+$ cells. Lin markers include CD4, CD8, Ly6G, SiglecF, and XCR1, Flow cytometric plots show are representative of two independent experiments, each comprised of $n \geq 2$ mice. Mice were analyzed at 10 weeks of age.

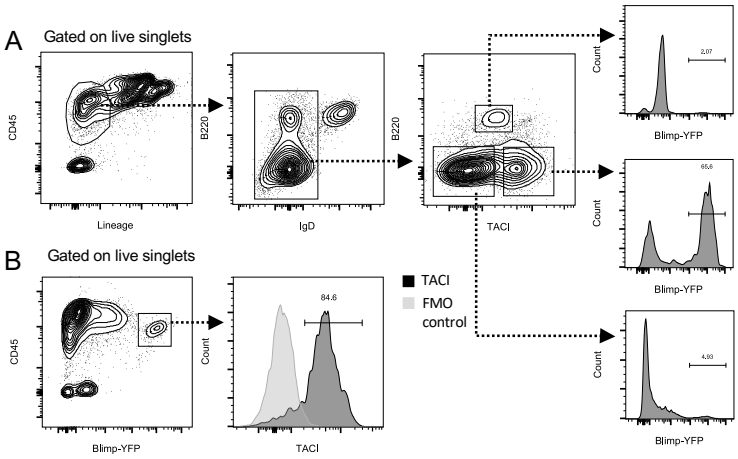


Figure 2.6 **TACI can be used to identify intestinal plasma cells**. A) Small intestinal lamina propria cells were isolated from Blimp-YFP reporter mice and stained for analysis by flow cytometry. Expression of Blimp-YFP in indicated B220 and TACI expressing subsets pre-gated as $CD45^{+}$, Lin^{-} (CD4, CD8, Ly6G, SiglecF, and XCR1), IgD^{-}. B) TACI expression in $CD45^{+}$, Lin^{-}, Blimp-YFP^{hi} cells in the small intestine. Gray histogram indicates fluorescence minus one (FMO) control in samples where TACI antibody was omitted. Flow cytometric plots are representative of four independent experiments, each comprised of n=2 mice Mice were analyzed at 7-10 weeks of age.

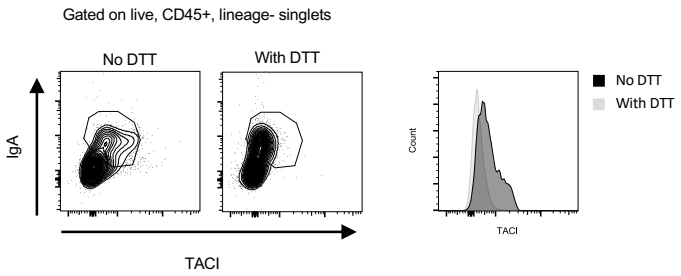


Figure 2.7 **DTT decreases the intensity of TACI staining.** Small intestinal lamina propria cells were isolated from wild-type B6 mice and stained for analysis for flow cytometry. Analysis of IgA vs. TACI expression in tissues treated without DTT or with DTT, as indicated. Histogram shows intensity of TACI staining in samples treated without DTT (black overlay) or with DTT (gray overlay). Cells were gated in live, $CD45^+$, Lin^- (CD4, CD8, Ly6G, SiglecF, and XCR1) singlets. Flow cytometric plots shown are representative of two independent experiments, each comprised of n>3 mice/group. Mice were analyzed at 10 weeks of age.

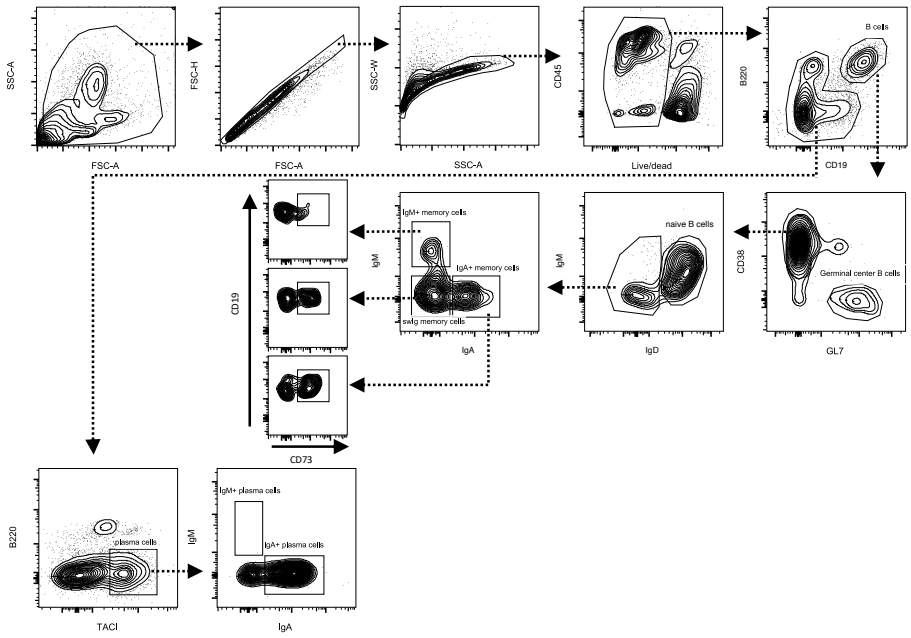


Figure 2.8 **Gating strategy to identify intestinal B cell subsets**. Small intestinal lamina propria cells were isolated from WT B6 mice and stained for analysis for flow cytometry. Gating strategy to identify B cells ($CD19^+B220^+$) subsets including germinal center B cells ($CD38^-GI7^+$), naïve B cells (IgD^+IgM^+), memory B cells (IgD^-CD73^+) and plasma cells ($B220^-IgD^-TACI^+$) is shown. Flow cytometric plots shown are representative of 5 independent experiments, each comprising of n>2 mice. Mice were analyzed at 7-10 weeks of age.

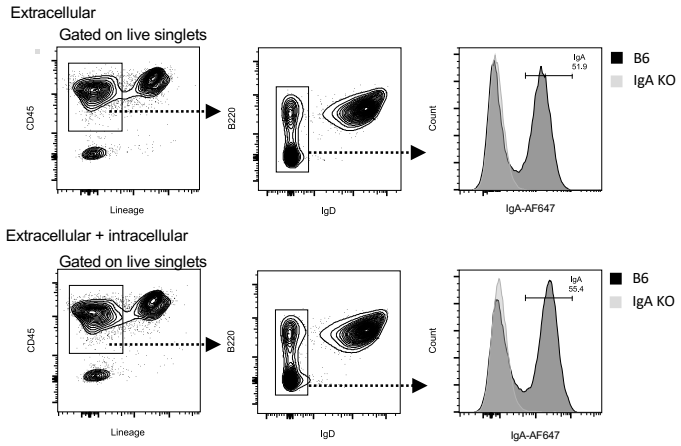


Figure 2.9 **Surface staining for IgA is comparable to surface combined with intracellular staining for IgA .** Small intestinal lamina propria cells were isolated from wild-type B6 or IgA knockout (KO) mice and stained for analysis for flow cytometry. Cells were stained for expression of surface markers only in the extracellular panel (top) or stained for expression of surface markers and then fixed/permeabilized and stained for intracellular IgA in the extracellular and intracellular panel (bottom). Histograms show intensity of IgA staining in $IgD^{-}B220^{-}$ cells in B6 mice (black overlay) and IgA KO mice (gray overlay). N=2-3 mice/group. Mice were analyzed at 21-50 weeks of age.

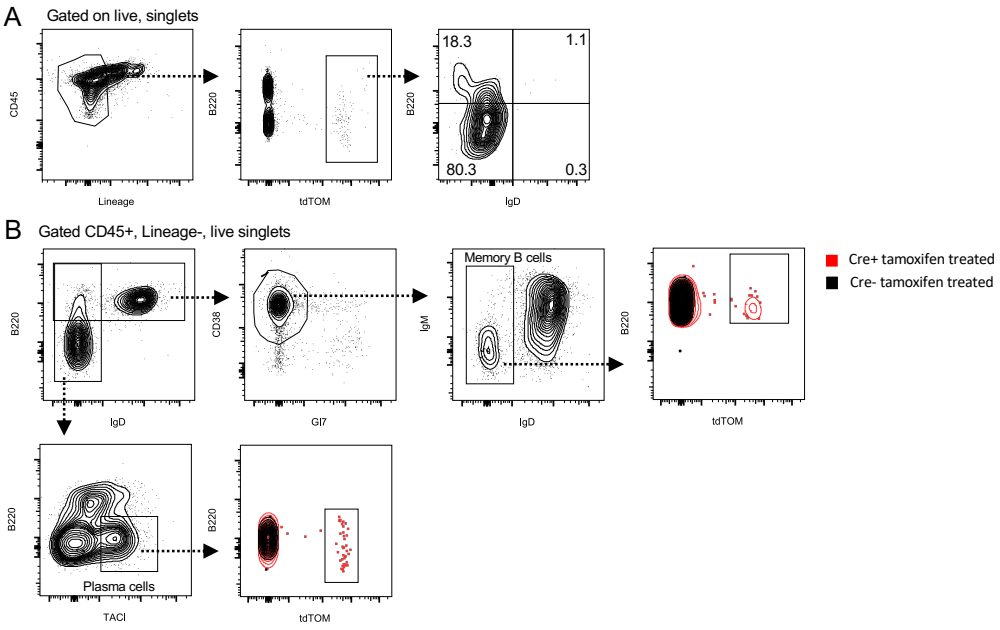


Figure 2.10 **Tracking fate of intestinal B cells in the intestinal lamina propria.** A) Colonic lamina propria cells were harvested and stained for analysis for flow cytometry. Expression of tdTOM in $CD45^{+}Lin^{-}$ cells and IgD expression in $tdTOM^{+}$ cells shown indicated. B) tdTOM expression in $B220^{-}TACI^{+}$ plasma cells and $B220^{+}CD38^{+}IgD^{-}$ memory cells are as indicated. S1PR2-Cre^{+}-tdTOM mice are shown in red, S1PR2-Cre^{-}-tdTOM mice are shown in black. Flow cytometric plots shown are representative of n>2 mice, analyzed at 16 weeks of age.

Gate	Marker	Clone	Fluor	Secondary	Dilution	Vendor	Catalog #
Viability	Live/dead fixable Aqua Stain		BV510		1:500	Thermo Fisher Scientific	L34957
Lineage	CD4	GK1.5	Biotin	SA-BUV737	1:200	Biolegend	100404
	CD8	53-6.7	Biotin	SA-BUV737	1:200	Biolegend	100704
	Ly6G	1A8	Biotin	SA-BUV737	1:200	Biolegend	127604
	SiglecF	S17007L	Biotin	SA-BUV737	1:200	Biolegend	155512
	XCR1	ZET	Biotin	SA-BUV737	1:200	Biolegend	148212
Hemapoetic cells	CD45	30-F11	PerCP-Cy5.5		1:300	Invitrogen	45-0451-82
B cells	B220	RA3-6B2	BV605		1:200	Biolegend	103243
	CD19	1D3	PE		1:200	Invitrogen	12019382
Germinal center B cells	CD38	90/CD38	BUV395		1:200	BD	740245
	GL-7	GL7	PE-Cy7		1:200	Biolegend	144620
Memory B cells	CD73	TY/11.8	APC-Cy7		1:200	Biolegend	127222
Plasma cells	TACI	8F10-3	BV421		1:75	BD	742840
Isotypes	IgA	C10-3	DyLight 650*		1:50	BD	556969
	IgG1	A85-1	FITC		1:200	BD	55443
	IgD	11-26c.2a	BV711		1:200	Biolegend	405731
	IgM	II/41	BV785		1:200	BD	743328

*purifed protein from BD labeled with Thermo Scientific's DyLight 650 Antibody Labeling Kit (cat# 84535)

Table 2.1 **Antibodies for B cell panel.**

Target	Purpose	Concentration	Vendor	Cat
IgA	primary	5ug/mL	BD	556969
IgA	secondary	.35ug/mL	Southern Biotech	1040-05
IgG	primary	5ug/mL	Jackson ImmunoResearch	115-005-146
IgG	secondary	.4ug/mL	Jackson ImmunoResearch	115-035-166
IgM	primary	5ug/mL	Jackson ImmunoResearch	115-005-075
IgM	secondary	.4ug/mL	Jackson ImmunoResearch	115-035-075

Table 2.2 **Antibodies for ELISpots.**

Chapter III

Investigating how maternal antibodies impact humoral immunity in offspring

3.1 Introduction

As elaborated in Chapter 1, the infant gut is colonized by a dense and diverse group of microbiota at birth (156). The composition is dynamic throughout the breastfeeding period, but at weaning, begins to stabilize into a composition that closely resembles that found in adults (157). Starting at birth and through weaning, essential changes in the immune system occur, some of which are triggered by the microbiota. For example, populations of fetus-derived T cells, innate lymphoid cells (ILCs), and myeloid cells are progressively replaced by cells generated in the bone marrow and thymus after birth (158, 159). Additionally, the microbiota induces the generation and expansion of effector and regulatory T cells (Tregs) and ILCs, as well as the formation of isolated lymphoid follicles that produce anti-microbial IgA (160-162).

The balance and interactions between the developing microbiota and the immune system are carefully orchestrated, and numerous studies detailed in Chapter 1 have shown that disruptions to interactions between the immune system and gut commensals result in the deregulation of immune responses later in life (103-105, 108, 112-114).

As detailed in Chapter 1, our lab (1), and others (43, 80), showed that maternal breast milk antibodies (maternal antibodies, matAb) bind to bacteria colonizing the infant's gut and play a key role in establishing intestinal homeostasis in early life. matAb deficient offspring show a transient increased in gut immunity around the time of weaning, which was dependent on colonization by the commensal gut microbiota.

Given that the absence of maternal antibodies results in a disruption of microbiota-immune homeostasis and previous studies have shown such disruptions can result in long-term impacts at the immune level, we hypothesize that the absence of maternal

antibodies results in a pathological immune imprinting in offspring. Here we addressed several lines of inquiry to test this hypothesis: 1. Does the absence of maternal antibodies lead to the alterations in the accumulation of differentiated B cell subsets in the gut? 2. Does the absence of maternal antibodies result in altered systemic antibody titers and/or differential targeting of microbes? 3. Does the absence of maternal antibodies impact the fate of GC-derived B cells activated during the weaning period?

We found trends of increased early-life, GC-derived plasma and memory B cells in the colonic lamina propria of mice that did not receive maternal antibodies. Additionally, these plasma cells show trends of increased IgA expression. We observed equivalent titers of serum and fecal antibodies in mice that received or did not receive maternal antibodies, indicating that maternal antibodies do not substantially alter offspring antibody accumulation. Finally, we found equivalent binding of antibodies to the microbiota in offspring that received or did not receive maternal antibodies suggesting that maternal antibodies do not affect offspring microbiota-reactive antibody pools.

3.2 Materials and Methods

Animals

C57Bl/6 (B6), Rosa$^{lsl\text{-}tdTOM\ m}$ (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG\text{-}tdTomato)Hze}$/J), and muMT$^{-/-}$ mice were purchased from The Jackson Laboratory. S1PR2^{CreERT2} mice were provided as a gift by Dr. Kurosaki Tomohiro (82). Mice strains were maintained under SPF conditions at the Fred Hutch to normalize the microbiota as much as possible. 7-week-old offspring that received maternal antibodies (offspring of muMT$^{-/-}$ dam x B6 sire) or did not receive maternal antibodies (offspring of muMT$^{+/-}$ dam x B6 sire) were used unless otherwise specified. Mice strains were maintained under SPF conditions at the Fred Hutch to normalize the microbiota as much as possible. For experiments comparing two or more groups, mice from each experimental category were co-housed unless otherwise specified. For the breeding of littermate or cohoused dams, females were separated into individual cages and bred for 2–3 nights. Dams were then "re-cohoused" for approximately 18 days. Pregnant females were separated and housed individually until parturition. All mice were maintained on non-acidified water unless specified. All animal experiments were approved by Fred Hutch IACUC, and the study was conducted in strict compliance with the PHS Policy on Humane Care and Use of Laboratory Animals.

Tamoxifen administration

5-7.5mg of tamoxifen (Sigma) was dissolved in 100uL of corn oil via agitation and administered by oral gavage twice at the interval indicated.

Tissue processing

Intestinal lamina propria lymphocytes were prepared by isolating the small or large intestine, dissecting away the Peyer's patches and fat, cutting the intestines open longitudinally, and swirling intestines gently in ice-cold PBS to remove feces. Intestines were minced finely with dissection scissors such that individual pieces were no larger than 5mm. Intestines were cleaned further by vortexing pieces in a 50mL conical tube filed with ice-cold wash buffer (HBSS, 2% FBS, 15mM HEPES) for 30 sec. Intestines were strained using a 100 μm strainer (Corning) placed on top of a 50mL conical tube, and the washing process was repeated two more times. Cleaned intestines pieces were placed in 20mL of room-temperature EDTA buffer (HBSS, 10% FBS, 15mM HEPES, 5mM EDTA), vortexed for 30 sec, and placed in an orbital shaker for 20 minutes at 300rpm to remove epithelial cells. Tissues were strained using a 100 μm strainer, and the EDTA wash was repeated. Intestines were strained again using a 100 μm strainer, and rinsed with 10mL of ice-cold wash buffer to remove residual ETDA before being incubated with 20 mL of pre-warmed digestion buffer (RPMI, 10% FBS, 15mM HEPES, 0.025 mg/mL DNase I (Roche 10104159001), 0.5 mg/mL collagenase 4 (Sigma C5138; we titrated each lot of collagenase to find the optimal concentration for the best viability)) at 37C for 50 minutes. During the incubation, tubes were briefly vortexed every 15 min to ensure an even distribution of digestion. The supernatant is cloudy at this point, and most of the tissue is digested away except for residual fat. Digested cells were filtered over a 70 μm strainer (Corning) placed on top of a 50mL conical. Tubes were rinsed with an additional 20mL of ice-cold RP-5 (RPMI, 5% FBS, 15mM HEPES, 1x Penicillin-Streptomycin-Glutamine) to collect all residual cells and filtered through the same 70 μm

strainer. Cells were pelleted at 475rcf for 5 min at 4C. The supernatant was decanted, and cell pellet was resuspended in 6mL of 44% Percoll (Cytiva) made up in plain RPMI. Cells were then transferred to a 15 mL conical precoated with FBS and underlayed with 4 mL of 67% Percoll made up in PBS. Cells were pelleted at 2800 rpm for 20min at room temperature without brake. Cells at the gradient interface were transferred into a new conical, topped off with 20 mL of ice-cold RP-5. Cells were pelleted and stained for analysis by flow cytometry.

Lymph nodes and organs, including the mLN, PP, and spleen, were harvested, minced with scissors, and digested for 20 min via incubation in RPMI containing collagenase 3 (1mg/mL, StemCell Technologies) supplemented with DNase (5ug/mL, Sigma) for T cell panel or undigested for B cell panel. Following incubation, the collagenase reaction was quenched with RPMI-5 (RPMI, 5% FBS, 15mM HEPES, 1x Penicillin-Streptomycin-Glutamine). Cells were mashed and passed through a 70um filter to prepare a single-cell suspension. Splenic cells were incubated in ACK for 3mins to lyse red blood cells.

Bone marrow lymphocytes were prepared by dissection off skin and muscle from the tibia and femur, disassociating the caps of the femur or tibia, and centrifuging the cells out at 12,000rpm for 1min into RPMI-5. Cells were passed through a 70uM filter to prepare a single-cell suspension. Cells were incubated in ACK for 3min to lyse red blood cells.

For peripheral blood samples, mice were bled retro-orbitally using a heparinized capillary tube and then transferred to a 1.6mL Eppendorf tube containing heparin (Fisher). Cells were incubated in ACK for 3mins to lyse red blood cells and washed twice with PBS.

Flow cytometry

1-3e6 cells were pelleted and stained in a volume of 50uL. Cells were incubated in fixable viability dye (Invitrogen) and blocked for non-specific Fc binding in PBS for 30 minutes at 4C. Cells were then stained for surface proteins in FACS buffer (PBS containing 2% fetal calf serum) for 30 minutes at 4C. Cells were washed and stained with Streptavidin-BUV737 (Thermo Fisher Scientific) at 1:300 in FACS buffer for 15 min at 4°C. Cells were acquired with the FACSymphony (BD Biosciences), and data analyzed with FlowJo software version 10.6.1 (Treestar). For a list of all antibodies used, reference Chapter 2, Table 2.1. To estimate total cell counts, 2e4 AccuCheck Counting Beads (Thermo Fisher Scientific) were added to each sample prior to acquisition on the cytometer.

ELISA

Nunc Hi Affinity ELISA plates (Thermo Fisher Scientific) were coated with isotype-specific antibodies to IgGtotal, IgG1, IgG2b, IgG2c, IgG3, IgM (Jackson Immunoresearch), IgA (BD clone C10-3) or IgE (BD clone R35-72) at 500ng/mL and blocked with PBS with 1% BSA (w/v) and 2% goat serum (Gibco; v/v). Secondary biotinylated antibodies to IgGtotal, IgG1, IgG2b, IgG2c, IgG3 or IgM (Jackson Immunoresearch), IgE (BD clone R35) were used at 1:5000 in PBS. SA-HRP (BD) was added at 1:8000. Detection of IgA was done via a direct IgA-HRP antibody used at 1:5000 in PBS (Southern Biotech). Purified IgG1, IgG2b, IgG3, and IgM standards were from eBioscience, IgGtotal and IgG2c standards from Southern Biotech, and IgA and IgE standards from BD. Plates were developed with TMB (Thermo Fisher Scientific) with 2M H2SO4 stop. Absorbance at 450nm was measured on an Agilent BioTek.

For measurements of immunoglobulins in the lumen, intestinal contents were resuspended at 100mg/mL in PBS, homogenized by vortexing, and centrifuged at 13000xg for 10min. Supernatant was taken and used neat and run in duplicate. Serum samples were diluted to in-range and also ran in duplicate.

Microbiota flow cytometry (mFLOW)

Feces from the terminal ileum or proximal colon were collected, vortexed in sterile PBS, and washed in sterile-filtered PBS with 1% bovine serum albumin (BSA; Fisher) and 0.05% Azide (Thermo Fisher Scientific). Bacteria were resuspended at approximately 10e6 bacteria/mL using an OD600 1= 50e8 bugs/mL. Mouse serum was diluted 1:25 in PBS/BSA/Azide buffer, and 25uL of this solution was mixed with 25uL diluted microbes in a v-bottom plate (Thermo Fisher Scientific). Staining was performed with fluorochrome-conjugated or biotinylated antibodies followed by streptavidin-PECy7 (eBioscience). Cells were washed and resuspended in PBS with SYBR Green (Invitrogen) and analyzed by FACS. For a list of all antibodies and dilutions used, reference Table 3.2. Feces and serum samples for mFLOW were taken from the same mouse or microbes from an 18-day-old muMT neonate$^{-/-}$ was used, as stated. We included both SYBR^{hi} and SYBR^{mid} events in our analyses (referred to as SYBR^{+}).

Statistical analysis

Statistical analyses were performed with Prism software version 9.1 (GraphPad Software). Parametric data were assessed with unpaired t-tests. Nonparametric data

were assessed with Mann Whitney tests. Significance was defined by a p value less than 0.05.

3.3 Results

Experimental set up

To study the long-term effects of maternal antibodies, we bred wildtype, B6 males with B cell-deficient muMT$^{-/-}$ females. Their offspring (muMT$^{+/-}$, also matAb-) can make their own antibodies but do not receive maternal antibodies via breast milk. Because our previous studies show that maternal antibodies transferred in utero had no effect in mediating the Tfh and GC B cell response, this strategy eliminates the need for fostering, which is both time and resource intensive.

Due to cage-to-cage differences in the microbiota that occurs independent of biological variables, studies of microbiota composition and function require careful experimental controls. We used littermate muMT$^{+/-}$ and muMT$^{-/-}$ dams to set up the breeders as previous work from our lab and others have determined equivalent microbiota composition within litters (163-165). Additionally, unless noted, we co-housed offspring at weaning such that each cage had a mixture of matAb+ and matAb- pups to normalize the microbiota as much as possible. We reasoned that by using this strategy, any differences observed in microbiota composition or function, despite our continuous efforts to normalize it, would result from durable changes in intestinal immunity in offspring. The co-housing strategy has been successfully used to identify such changes (166). Finally, unless noted, all mice were placed on sterile non-acidified water.

Equivalent accumulation of intestinal B cell subsets in offspring that received or did not receive maternal antibodies

In the absence of maternal antibodies, offspring mount an increased GC B cell response that is elevated in both frequency and number (1). Additionally, this elevation in GC B cells is only seen at gut-draining lymph nodes like the mLN and PP but not the spleen and only occurs in the presence of colonization by the microbiota.

GC-derived B cells have been shown to develop into long-lived plasma cells and memory B cells that can persist up to the life of the host (121, 122, 167). Furthermore, GC B cells activated in the mLN and PP specific for mucosal-derived antigens are imprinted with homing properties that mediate their migration to the intestinal lamina propria. Second, as the GC B cell response is dependent on microbial colonization, it is likely that these B cells are induced by microbial antigens. Some of the gut bacterial species present at the time of the elevated T-dependent GC cell response can persist through adulthood (90) and can therefore feed a long-lived GC response.

Using flow cytometry (Figure 3.1), we analyzed matAb+ and matAb- offspring at seven weeks of age for the accumulation of B220+TACI+ plasmablasts, B220-TACI+ plasma cells, B220+GI7+ germinal center B cells, B220+CD38+IgD-CD73+ memory B cells, and B220+CD38+GI7- activated precursor B cells in the mLN (Figure 3.2A), PP (Figure 3.2B), colonic lamina propria (Figure 3.2C) and SI lamina propria (Figure 3.2D). While there were changes within individual experiments, overall, we found no stable differences between matAb+ vs. matAb- offspring across the B cell subsets we looked at over the five experimental repeats (Table 3.1). Thus, maternal antibodies do not seem to affect the overall distribution of B cell subsets in the gut and gut-associated lymphoid tissues.

Equivalent distributions of IgA+ and other class-switched activated B cell subsets in offspring that received or did not receive maternal antibodies

The mLN and PP are key sites of IgA induction due, in part, to the high expression of IgA-promoting factors such as TGF-beta (168, 169). Thus, while we did not observe significant differences in the accumulation of B cell subsets, we assessed whether there were differences in the isotype distribution of class-switched, activated B cells between matAb+ vs. matAb- mice.

We analyzed the accumulation of IgA+, IgG1+, IgM+, and other switched-Ig (swIg)+ memory B cells (B220+CD38+IgD-CD73+; Figure 3.3A), GC B cells (B220+GL7+; Figure 3.3B), and plasma cells (B220-TACI+; Figure 3.3C) isolated from the mLN, PP, colonic LP, and SI LP of 7-week old offspring. While we found differences within individual experiments, we found no stable differences between matAb+ vs. matAb- offspring across the five experimental repeats (Table 3.1). These data indicate that maternal antibodies do not substantially alter the frequency of activated B cells expressing different antibody isotypes.

Fate-mapping B cells activated at weaning

The B cell compartment of a seven-week-old offspring comprises of both B cells activated in early life and those activated later. Thus, any potential differences in those B cells activated at the time of weaning (i.e., GC B cells) may be masked by the accumulation of B cells throughout the next four weeks. To specifically study the B cells activated at the time of weaning in matAb+ and matAb- mice, we used a powerful mouse

model system in which GC-derived B cells and their clonal progeny can be heritably labeled.

Among B cell subsets, GC B cells preferentially express high levels of sphingosine-1-phosphate receptor 2 (S1PR2) (82). We bred transgenic animals expressing a tamoxifen-inducible Cre recombinase under the control of the *S1pr2* promotor (82) with reporter mice in which the tdTomato fluorescent protein (tdTOM) has been inserted behind a floxed stop cassette (|s|) in the ubiquitously expressed *Rosa26* locus ($Rosa^{lsl\text{-}tdTOM}$ mice, available from Jackson Labs). Administration of tamoxifen transiently activates Cre-mediated excision of the floxed stop cassette enabling heritable expression of tdTOM by all S1PR2-expressing cells.

Neonates lacking maternal antibodies generate aberrant GC B cell responses between d20-26 of age (1). To assess the feasibility of our system, I administered tamoxifen to S1PR2.Cre^{ERT2} x $Rosa^{lsl\text{-}tdTOM}$ (herein called S1PR2-Cre-tdTOM) mice twice, at day 21 and day 23 of age. Analysis two days later, at day 25 ,revealed that >90% of GC B cells in the mLN and PP were tdTOM+ (Figure 3.4A). Thus, this protocol resulted in highly efficient labelling of GC B cells activated during the weaning transition. We note that up to 40% of GC B cells expressed tdTOM in one control Cre+ mice treated with corn oil , indicating that the Cre expression was leaky and not tightly regulated by tamoxifen (Figure 3.4A). However, we only observed this result with one mouse in one experiment, indicating that this is a possible mistake (e.g., that the mouse was mistakenly gavaged with tamoxifen instead of corn oil).

To further assess this, we looked at B cells in the peripheral blood of older S1PR2-Cre-tdTOM mice for tdTOM expression with the reasoning that GC B cells with a leaky

Cre turned on early in life could exit and circulate in the peripheral blood as tdTOM+ B cells. We were reassured to find no detection of tdTOM+ B cells in the peripheral blood as it indicated that, at the very minimum, those tdTOM+ B cells did not accumulate in the peripheral blood (Figure 3.4B).

Finally, we confirmed that the elevated mucosal GC B cell responses we consistently observed in d25 MatAb- mice was intact in tamoxifen treated S1PR2-Cre-tdTOM mice. To do this, we fostered S1PR2-Cre-tdTOM pups within one day of birth onto lactating wildtype or muMT$^{-/-}$ dams, treated the pups with tamoxifen, and analyzed the frequency of GC B cells at day 25 of life. We observed a statistically significant elevation in GC B cells in matAb- S1PR2-Cre-tdTOM pups treated with tamoxifen compared to matAb+ S1PR2-Cre-tdTOM pups (Figure 3.4C). We also confirmed an increase in tdTOM+ frequency of B220 cells in matAb- pups at day 25, though this was trending towards statistical significance (Figure 3.4D). Count data are not shown but were trending towards statistical significance in the PP. Accordingly, this response is seen at mucosal sites such as the PP and not tissue that drains the systemic circulation like the spleen.

In summary, the S1PR2-Cre-tdTOM system in conjunction with tamoxifen retains the phenotype observed in matAb- mice, making this system useful to probe the fate of the GC B cells activated at weaning.

Increased accumulation of early life GC-dependent colonic IgA memory and plasma cells in mice lacking maternal antibodies.

Utilizing our fate tracking system, we asked if maternal antibodies affected the fate of the B cells activated during the weaning transition. Like before, we fostered S1PR2-

Cre-tdTOM pups within one day of birth onto lactating wildtype or muMT$^{-/-}$ dams, treated the pups with tamoxifen, and harvested tissues at week 16 of life to look at the fate of tdTOM+ GC B cells. As the GC B cells activated in the mLN and PP are imprinted with homing properties that mediate their migration to the intestinal lamina propria, we looked to see if we can find these cells in the intestinal LP. We found that a small subset of memory B cells (Figure 3.5A) and plasma cells (Figure 3.5C) in the colonic LP were tdTOM+, meaning that it had been activated at the weaning transition and had migrated to the colonic LP by 16 weeks of age. Memory B cells and plasma cells can also accumulate in the bone marrow (170), and when we looked at this site, we also found that a small percentage were tdTOM+ (Figure 3.5A, 3.5C). We also looked at the mLN and PP and found similar results (Figure 3.5A, 3.5C).

We also looked at the germinal center cells in the mLN and PP and saw 5-15% of GC B cells were tdTOM+ (Figure 3.5B). It is unlikely that tamoxifen is still activating new cells 13 weeks later, as the half-life of tamoxifen is around a week (171). However, in accordance with a recently published paper, it is likely that these cells represent “winner” B cell clones despite a rapid turnover of cells in the GC (153).

matAb- pups have increased frequency and count of GC B cells at day 25 of life. When we compared whether matAb- pups (Figure 3.5A-C, open red circles) had different accumulations of memory and plasma B cells compared to the matAb+ wildtype controls 13 weeks later, we found trends of increased tdTOM cells in the memory B cells pool (Figure 3.5A) and the plasma B cell pool (Figure 3.5C) from the colonic lamina propria, but no other sites. We did not find increased recovery of the GC B cell pool (Figure 3.5B) across the mLN, PP, bone marrow, or colonic lamina propria. We could have obtained

increased recovery of these cells had we looked at other locations like the SI lamina propria. Alternatively, perhaps these early life GC B cells did not receive the necessary survival signals to persist into 16 weeks of age. Further investigation is warranted. Additionally, the overall number of tdTOM+ CD45+ cells did not differ between the two groups (data not shown). Counts for memory B cells, GC B cells, and plasma cells have not been analyzed from this specific experiment, though based on the extensive testing shown in Table 3.1, we do not expect counts to differ in matAb+ vs. matAb- offspring.

Finally, we were interested to see whether there were differences in the isotype distribution in matAb+ vs. matAb- mice. We specifically looked at IgA in the plasma cells as the mLN and PP are key sites of IgA induction due to high levels of TGF-beta (168, 169). We found a trend for increased IgA+tdTOM+ plasma cells in matAb- mice (Figure 3.5D), suggesting that GC B cells activated during the weaning transition in matAb- mice had the propensity to class-switch to IgA.

Assessing the phenotype of the tdTOM+ cells to look at B cell subsets was challenging due to the low frequency of these cells in the overall sample. Further experiments in which Lin+ cells are depleted from the sample prior to analysis would enrich for the tdTOM+ B cell populations and allow for more robust analysis. Additionally, further analysis should look at the accumulation of tdTOM+ cells in the SI lamina propria.

Overall, we found trends of increased memory and plasma B cells in the colonic lamina propria in the matAb- offspring at 16 weeks of age. Further investigations are needed to confirm these results.

Equivalent serum and fecal antibody titers in offspring that received or did not receive maternal antibodies

Given the propensity of cells activated in the mLN to become IgA-secreting cells, we looked to see if matAb- had higher titers of IgA in the serum. And while there were no differences in the frequency and number of IgA+ plasma cells between matAb+ vs. matAb- mice, we thought that this didn't necessarily preclude the possibility of different quality of plasma cells in matAb+ vs. matAb- mice.

We first looked in the serum to see if maternal antibodies change the quantity of IgA globally. We saw no difference in serum IgA abundance between pups that received maternal antibodies and those that did not (Figure 3.6A, left panel). IgA can be transported to the gut lumen, so we next looked to see if there were differences in the amount of IgA in the feces. We found no difference in fecal IgA between matAb+ vs. matAb- offspring (Figure 3.6A, right panel). We also performed a comprehensive survey of serum IgG1, IgG2b, IgG2c, IgG3, IgM, and IgE, and found that there was no difference (Figure 3.6A, right panel). Overall, maternal antibodies do not alter the quantity of antibodies found in the serum or feces.

Equivalent microbiota-reactive antibody pool in offspring that received or did not receive maternal antibodies

Overall, while the quantity of antibodies can be informative, it does not reveal the quality or function of the antibodies produced. For example, there could be differences in antibody specificity for microbial antigens or alterations in antibody affinity. As a first step toward understanding whether the absence of maternal antibodies resulted in long-term

changes in the microbiota-reactive antibody pool we used a method called microbiota flow cytometry (mFLOW). For mFLOW, we incubated homogenized intestinal contents isolated from the colon or SI of maternal antibody sufficient and deficient mice, which serves as a source of microbes, with host matched serum or fecal supernatant, which serves as a source of antibodies. We then used fluorescent antibody isotype-specific detection antibodies to assess, via flow cytometry, the percentage of microbes bound by host antibodies, and the 'character' (i.e., isotype distribution) of the microbiota-reactive antibody response.

We analyzed matAb+ vs. matAb- offspring at week 6 and week 10 to understand how maternal antibodies influence the kinetics of the microbiota-reactive antibody response in progeny. At week 6, we observed very little binding of serum IgA to fecal microbes (Figure 3.6B, left panel). Additionally, a very small portion of the microbiota was pre-bound by IgA, without incubation with serum of fecal supernatants, indicating that at this timepoint, mice to not harbor high levels of IgA reactive to their microbiota. The quantities of IgA at week 7, just one week after these mFLOW experiments, showed that IgA levels were detectable (Figure 3.6A). This suggests that while IgA is present, they are largely not reactive to the microbiota at week 6.

We also surveyed the microbiota reactivity of other isotypes, including IgG1, IgG2b, IgG2c, IgG3, and IgM. We found that at week 6, IgG2b bound the highest proportion of microbes followed by IgM. At week 10, we observed a higher proportion of fecal microbes bound by serum IgA compared to week 6 (Figure 3.6B, right panel). The fraction of microbiota bound by all antibody isotypes was higher at week 10 than week 6. Interestingly, the proportion of IgM-bound microbiota was higher than IgA at both week 6

and week 10. Despite these kinetic changes, we observed equivalent microbiota-reactive binding profiles in matched samples from matAb+ or matAb- offspring.

Maternal antibodies altered the offspring microbiota-reactive antibody pool to early life microbes

Antibody responses to oral antigens can be detected in the serum at approximately 14-21 post antigen exposure (172). Thus, we reasoned that the majority of the microbiota reactive antibodies present at week 6 may be elicited by gut microbes present during the weaning transition. As discussed previously, the microbiome undergoes changes in abundance and composition during and after the weaning transition. We hypothesized that offspring lacking maternal antibodies, which mount exaggerated microbiota dependent Tfh and GC B cell responses in mucosal tissues, generate heightened or altered antibody responses to gut microbes present during the weaning transition. To test this hypothesis, we incubated fecal microbes isolated from a 18-day-old muMT$^{-/-}$ neonate from our mouse colony, representing a common source of microbes from the weaning transition, with serum from 6-week old matAb+ and matAb- offspring. We chose a muMT$^{-/-}$ mouse as a source of microbes because these mice do not produce antibodies and therefore do not have antibodies pre-bound to their intestinal microbes. We observed an increased proportion of IgA+ bacteria when using day 18 microbes from a muMT-/- donor (Figure 3.7A and B) compared to week 6 host-matched microbes (Figure 3.6B, left panel). Additionally, the fraction of neonatal day 18 microbes bound by all isotypes tested including IgG1, IgG2b, IgG2c, and IgG3, was increased compared to matched microbes isolated from week 6 mice (Figure 3.7A). These data indicate that the majority of anti-

commensal antibodies present at week 6 of life are reactive to microbes present during the weaning transition. Interestingly, in one cohort of mice, we observed a statistically significant decreases in the fraction of neonatal microbes bound by IgA, IgG2b, and IgG2c from 6-week-old matAb- offspring. This result suggests that the overall titer of microbiota-reactive antibodies is reduced in offspring that do not receive maternal antibodies. This was surprising because it was opposite what we had hypothesized. Further investigations are needed with additional cohorts to determine if this response is consistent.

Changes in microbiota composition can drive IgA responses in offspring independent of maternal antibodies

For most of these experiments, we co-housed matAb+ and matAb- offspring at the time of weaning to facilitate homogenization of the microbiota between experimental groups to the extent possible. We performed one experiment wherein we did not use littermate dams nor did we co-house matAb+ vs. matAb- offspring at the time of weaning. Interestingly, we observed decreased IgA binding to commensal microbes in matAb- offspring (Figure 3.7A). In a separate experiment in which we used littermate dams and cohoused offspring at weaning, there was no significant difference in the fraction of IgA+ microbes between matAb+ and matAb- offspring (Figure 3.7B). We speculate that these experimental differences result from changes in the microbiome composition between group and that equilibrating the microbiota via co-housing could mask potential differences.

3.4 Discussion

Our results indicate that the absence of breastmilk antibodies in early life does not substantially alter the accumulation of gut intestinal B cell subsets nor the character of the microbiota-reactive antibody responses. We found trends of increased weaning period-derived plasma and memory B cells in the colonic lamina propria of mice that did not receive maternal antibodies. Additionally, these plasma cells show trends of increased IgA expression.. Additionally, we observed no consistent difference in the titers of specific immunoglobulin isotypes, or the microbiota-reactive antibody binding profiles as assessed by mFLOW between maternal antibody sufficient and deficient offspring.

The lack of long-lived immune responses in the parameters we measured was surprising, especially given recent evidence that maternal factors, including maternal antibodies, can impact offspring immune development throughout generations (38). One possible explanation is that differences in these immune parameters are not evident at the time points analyzed, which was predominately between 6-10 weeks of age. We hypothesize that the Tfh and GC B cell response rose due to microbes found at the weaning transition, specifically the microbes found in the mLN prior to the increase in Tfh and GC B cells. We know that microbes at the time of weaning differ from microbes found in adults. Therefore, the antigenic stimulus that triggers the persistence of these antibodies could have disappeared.

Independent of maternal antibodies, we observed an interesting temporal difference in the antibody response to commensal microbes. Serum and fecal antibodies from 6-week-old mice of bound a higher fraction of neonatal microbes derived from d18 muMT$^{-/-}$ fecal pellet than their own microbes. Microbiota-reactive antibody responses to

endogenous microbes increased by ten weeks of age. Antibody responses to antigens take about 14-21 days to develop (172). Additionally, the gut microbiota changes dramatically over the weaning transition (157), which is one possible explanation as to why serum antibodies from 6-week-old mice do not bind a high fraction of their own intestinal microbes at the same time point. While we do not have data on the reactivity of serum from 10-week-old offspring to neonatal microbes, we speculate that these antibodies bind a smaller fraction of day 18 microbes as the serum responses have since been populated with antibodies against a more "mature" microbiota. Considering the dearth of microbiota-reactive antibodies to endogenous microbes in 6 weeks of age, it would be interesting to test whether perturbations to the intestinal microbiota, by way of infection, would result in different health outcomes compared to infection at 10 weeks of age, when a higher percentage of the microbiota are bound by host IgA, and/or capable of being bound by other systemic isotypes.

Finally, we observed more serum IgM, IgG2b, and IgG3 binding to own microbes compared to serum IgA, independent of maternal antibody status. While secreted IgA has been widely studied and is critical for impacting gut physiology, perhaps serum IgA has a lesser role in microbial defense compared to serum IgM, IgG2b, and IgG3. IgM and IgG are potent activators of complement and engage in different effector functions than IgA (173, 174). Thus, perhaps the high binding of IgG2b and IgG3 has evolved specifically for systemic defense.

3.5 Figures

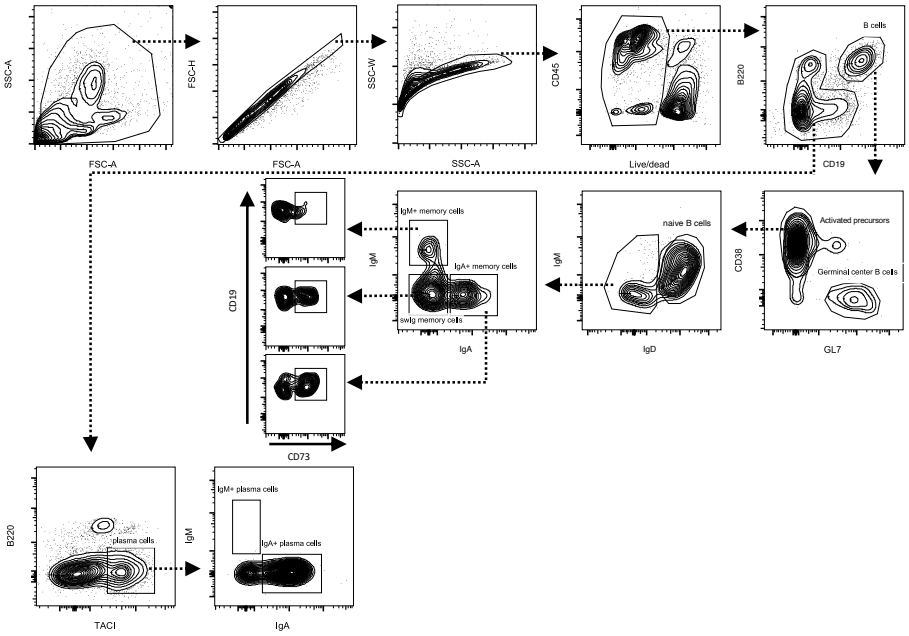


Figure 3.1 **Gating scheme to identify B cell subsets in the small intestinal lamina propria.** Applicable to other organs including the colon, mLN, and PP.

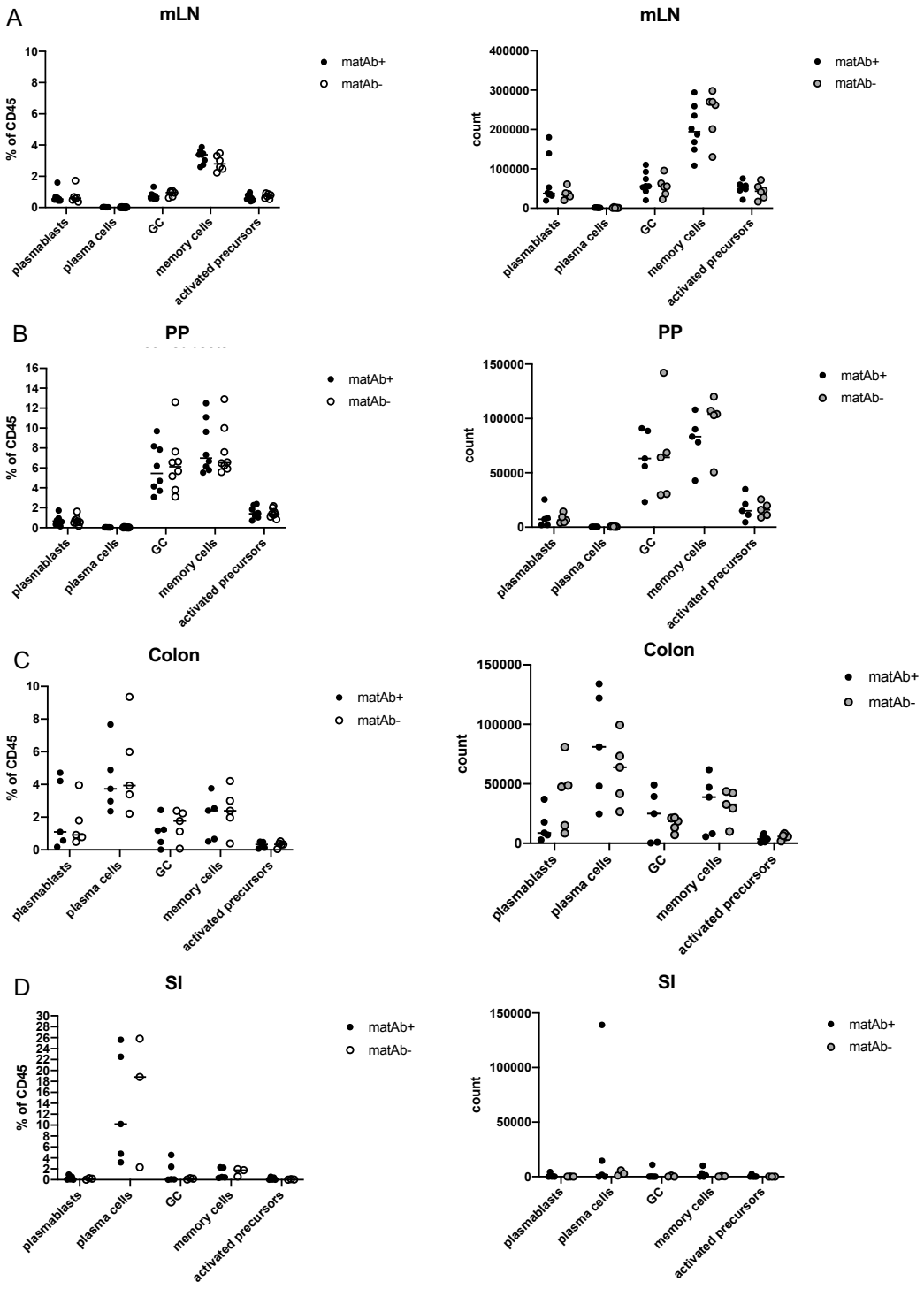

A
mLN
% of CD45
matAb+
matAb-
plasmablasts
plasma cells
GC
memory cells
activated precursors
mLN
count
B
PP
PP
C
Colon
Colon
D
SI
SI

Figure 3.2 **Accumulation of B cell subsets.** Offspring that either received or did not receive maternal antibodies were analyzed at 7 weeks of age for the accumulation of B220+TACI+ plasmablasts, B220-TACI+ plasma cells, B220+GI7+ germinal center B cells, B220+CD38+IgD-CD73+ memory B cells, and B220+CD38+GI7- activated precursor B cells. Frequency out of CD45+, live singlets (left) or as of counts (right) are shown in matAb+ offspring (filled circles) and matAb- offspring (open/gray circles) in the mLN (A), PP (B), colonic lamina propria (C) and SI lamina propria (D). Data representative of 3 independent experiments with n>=5/group. Bars indicate median. Significance was determined by unpaired t-tests.

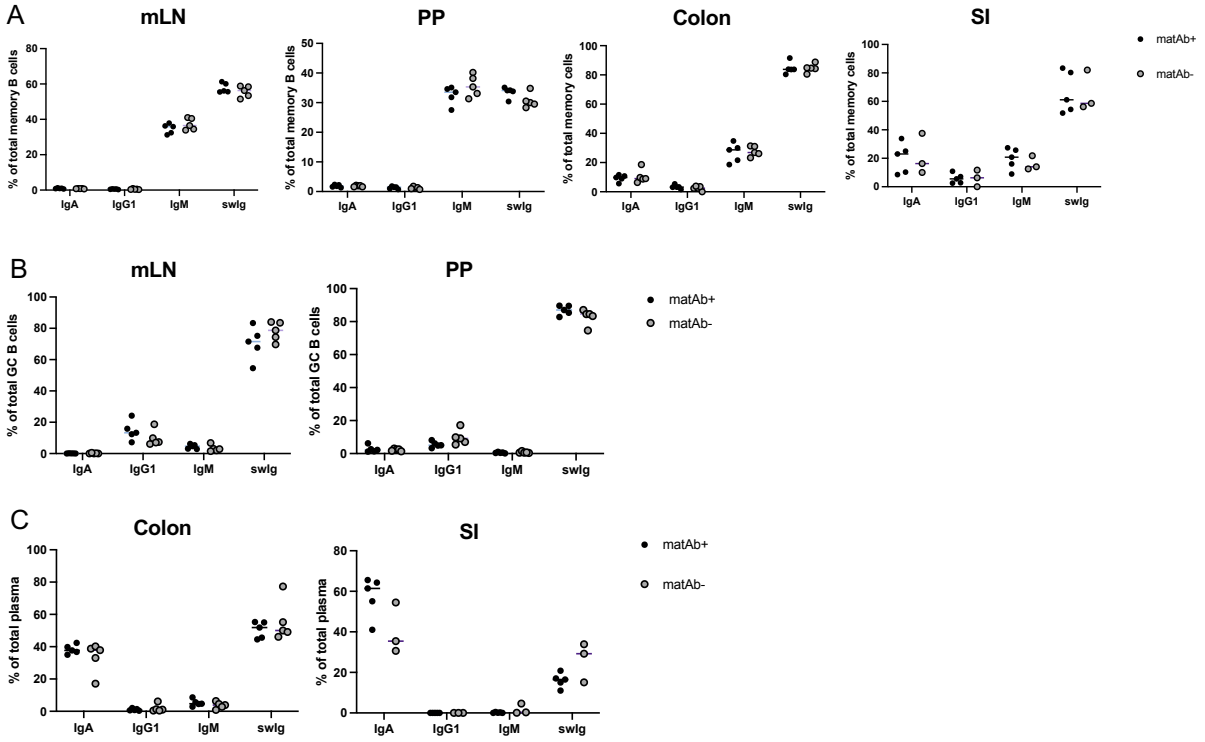


Figure 3.3 **Distribution of antibody isotypes in memory, germinal center, and plasma B cell.** Offspring that either received or did not receive maternal antibodies were analyzed at 7 weeks of age for distribution of IgA, IgG1, IgM and other swIg in B220+CD38+IgD-CD73+ memory B cells (A), B220+GI7+ germinal center B cells (B), and B220-TACI+ plasma cells (C) at the indicated tissues. Data representative of 3 independent experiments with n>=5/group. Bars indicate median. Significance was determined by unpaired t-tests.

A

Colon

Experiment	PB	Plasma cells	GC	Memory	Act. Precursor
1	n/a	n/a	n/a	n/a	n/a
2	nd	nd	nd	nd	nd
3	nd	nd	nd	nd	nd
4	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd

SI

Experiment	PB	Plasma cells	GC	Memory	Act. Precursor
1	n/a	n/a	n/a	n/a	n/a
2	n/a	n/a	n/a	n/a	n/a
3	nd	nd	nd	increased	nd
4	increased	nd	nd	nd	nd
5	nd	nd	nd	nd	nd

mLN

Experiment	PB	Plasma cells	GC	Memory	Act. Precursor
1	nd	nd	decreased	nd	nd
2	nd	nd	nd	nd	nd
3	n/a	n/a	n/a	n/a	n/a
4	n/a	n/a	n/a	n/a	n/a
5	nd	nd	nd	nd	nd

PP

Experiment	PB	Plasma cells	GC	Memory	Act. Precursor
1	nd	nd	decreased	nd	nd
2	nd	nd	nd	nd	nd
3	n/a	n/a	n/a	n/a	n/a
4	n/a	n/a	n/a	n/a	n/a
5	nd	nd	nd	nd	nd

Key
- Increased in matAb-
- Decreased in matAb-
- No difference (nd)
- No data (n/a)

B

Colon

	Memory B cells				GC B cells				Plasma cells			
Experiment	IgA	IgG1	IgM	swIg	IgA	IgG1	IgM	swIg	IgA	IgG1	IgM	swIg
1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2	nd	nd	nd	nd	n/a	n/a	n/a	n/a	nd	nd	nd	nd
3	nd	nd	nd	nd	n/a	n/a	n/a	n/a	nd	nd	nd	nd
4	nd	nd	nd	nd	n/a	n/a	n/a	n/a	decreased	nd	nd	increased
5	nd	nd	nd	nd	n/a	n/a	n/a	n/a	nd	nd	nd	nd

SI

	Memory B cells				GC B cells				Plasma cells			
Experiment	IgA	IgG1	IgM	swIg	IgA	IgG1	IgM	swIg	IgA	IgG1	IgM	swIg
1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
3	nd	nd	nd	nd	n/a	n/a	n/a	n/a	nd	nd	nd	nd
4	nd	nd	nd	nd	n/a	n/a	n/a	n/a	nd	nd	nd	nd
5	nd	nd	nd	nd	n/a	n/a	n/a	n/a	nd	nd	nd	nd

mLN

	Memory B cells				GC B cells				Plasma cells			
Experiment	IgA	IgG1	IgM	swIg	IgA	IgG1	IgM	swIg	IgA	IgG1	IgM	swIg
1	nd	nd	nd	nd	nd	nd	nd	nd	n/a	n/a	n/a	n/a
2	nd	increased	nd	nd	nd	nd	nd	nd	n/a	n/a	n/a	n/a
3	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
5	increased	nd	nd	decreased	nd	nd	nd	nd	n/a	n/a	n/a	n/a

PP

	Memory B cells				GC B cells				Plasma cells			
Experiment	IgA	IgG1	IgM	swIg	IgA	IgG1	IgM	swIg	IgA	IgG1	IgM	swIg
1	nd	nd	nd	nd	nd	nd	nd	nd	n/a	n/a	n/a	n/a
2	nd	nd	nd	nd	nd	nd	nd	nd	n/a	n/a	n/a	n/a
3	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
5	nd	nd	nd	nd	nd	nd	nd	nd	n/a	n/a	n/a	n/a

Key
- Increased in matAb-
- Decreased in matAb-
- No difference (nd)
- No data (n/a)

Table 3.1 **Summary of all B cell experiments.** Accumulation of B220+TACI+ plasmablasts, B220-TACI+ plasma cells, B220+GI7+ germinal center B cells, B220+CD38+IgD-CD73+ memory B cells, and B220+CD38+GI7- activated precursor B cells out of CD45+, live singlets in offspring that received or did not receive maternal antibodies summarized in (A). Distribution of IgA, IgG1, IgM and other swIg memory B cells, germinal center, or plasma cells in offspring that received or did not receive maternal antibodies summarized in (B). Red boxes indicate increased accumulation of subset in matAb- mice compared to matAb+ mice. Blue boxes indicate decreased accumulation of subsets in matAb- mice compared to matAb+ mice. White boxes indicate no difference between the groups. Gray boxes indicate no data from a particular experiment. Differences were determined by comparing medians. Mice were all ~7 weeks of age at analysis.

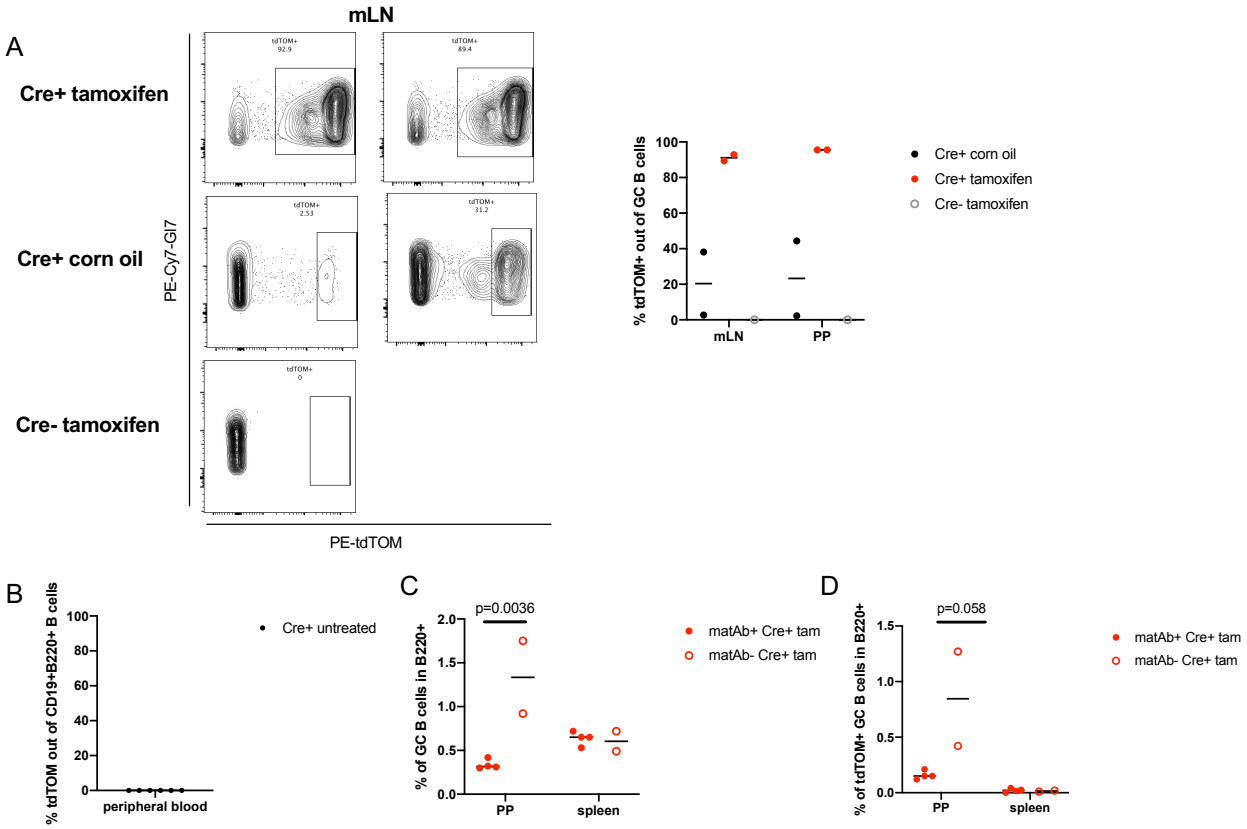


Figure 3.4 **Validation of S1PR2-Cre-tdTOM system.** A) Mice were given 7.5mg of tamoxifen dissolved in 100uL of corn oil or corn oil control alone via oral gavage at day 21 and 23 of life. mLN and PP were harvested two days later. Representative flow plots (left) and enumeration (right) of tdTOM staining within CD38-GL7+ germinal center B cells in different treatment groups, as indicated. Cells were pre-gated on B220+CD19+CD38-GL7+ (left). B) tdTOM expression of CD19+B220+ B cells in the peripheral blood of 7-13 week old S1PR2-Cre+-tdTOM untreated mice. C-D) Mice were given 5mg of tamoxifen dissolved in 100uL of corn oil or corn oil control alone at day d22 and d23 of life. Tissues were harvested 13 weeks later (week 16 of life). Lin gate include CD4, CD8, Ly6G, SiglecF, and XCR1. C) Frequency of all GC B cells in the PP and mLN of matAb+ or – Cre+ mice treated with tamoxifen at the indicated locations. D) Frequency of tdTOM GC B cells in the PP and mLN of matAb+ or – Cre+ mice treated with tamoxifen at the indicated locations. Bars indicate median. Significance was determined by unpaired t-test. All mice were on acidified water.

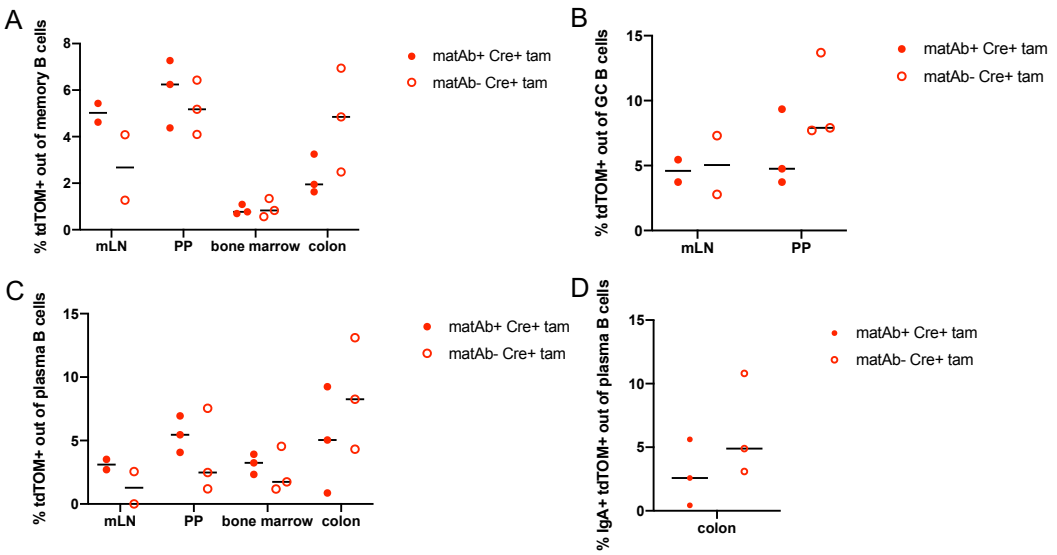


Figure 3.5 **Fate of GC B cells activated at the time of weaning.** Mice are treated with tamoxifen or corn oil as described in Figure 3.4 C-D and tissues harvested 13 weeks later (week 16 of life). Accumulation of tdTOM+ cells in B220+CD38+IgD- memory B cells (A), CD38-GI7+ GC B cells (B), and B220-TACI+ or CD138+ plasma cell in matAb+ Cre+ (solid red circle) vs matAb- Cre+ (open red circle) mice treated with tamoxifen at the indicated locations. Black shapes indicate background tdTOM expression. D) Frequency of IgA+tdTOM+ cells out of plasma cells in the colon. Significance was determined by unpaired t-test. All mice were on acidified water.

Reagent	Clone	Dilution	Vendor	Catalog #
IgM-biotin	II/41	1:150	BD	553436
IgG1-biotin	A85-1	1:200	BD	553441
IgG2b-biotin	R12-13	1:200	BD	550333
IgG2c-biotin	polyclonal	1:200	Jackson ImmunoResearch	115-065-208
IgG3-biotin	R40-82	1:200	BD	553401
IgA-biotin	11-44-2	1:200	Southern Biotech	1165-08

Table 3.2 **Antibodies for mFLOW.**

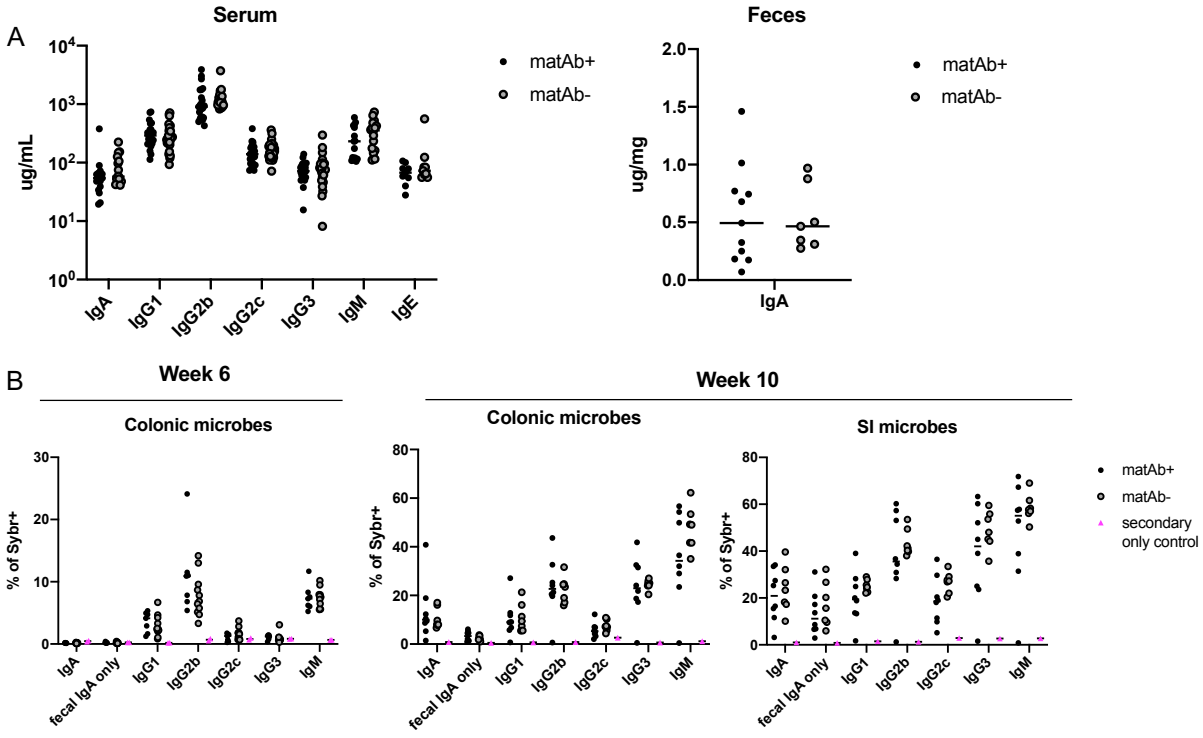


Figure 3.6 **Antibody responses to own intestinal microbes**. A) Quantification of serum (left) or feces (right) antibody isotypes via ELISA of matAb+ (black circles) or matAb- (gray circle) offspring at 7 weeks of age. Data combined from 2-3 independent experiments. B) mFLOW analysis of microbes isolated from the proximal colon or SI (terminal ileum) bound by indicated isotypes from own serum at week 6 (left panel) and week 10 (right panel) of life in matAb+ (black circles) or matAb- (gray circle). Background binding of secondary antibody to microbes is indicated in pink triangles. Data representative of two independent experiments. Bars indicate median. Significance was determined by unpaired t-tests.

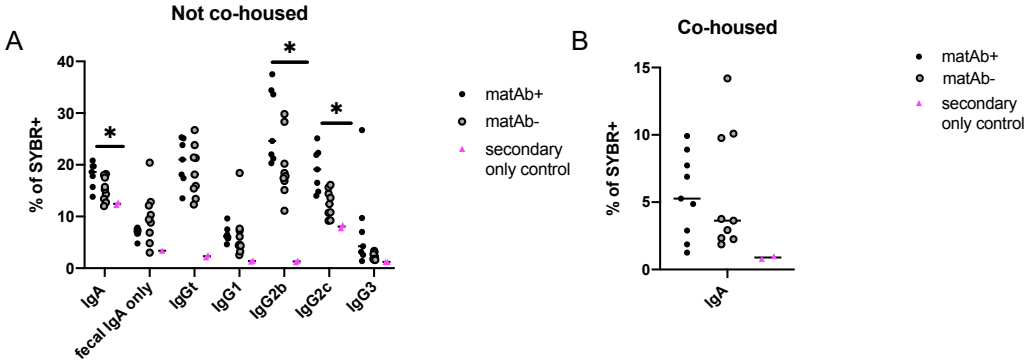


Figure 3.7 **Co-housing changes IgA responses to early life microbes.** Mflow analysis of colonic microbes isolated from muMT-/- 18-day old neonate bound by indicated isotypes from the serum of 6-week old matAb+ (black circles) or matAb- (gray circle) offspring in the context of housing with its own littermates at weaning A) or co-housing B). Background binding of secondary antibody to microbes is indicated in pink triangles. Bars indicate median. Significance was determined by unpaired t-tests. * $p<0.05$.

Chapter IV

Influence of maternal antibodies on offspring intestinal microbiota

4.1 Introduction

The intestinal commensal microbiota is vital to the health and development of the host by enhancing digestion, promoting immune development, and resisting infection. Antibodies are crucial to regulating interactions with our commensal microbiota. Numerous studies in both mice and humans have demonstrated that deficiencies in antibody production strongly correlate with alterations in intestinal homeostasis and microbiota dysbiosis (summarized in Chapter 1 and references (175-177)).

Extensive research has been done investigating the impact of TD immune responses, including Tfh cells and TD antibodies in microbiota composition and function. B and T cell-deficient mice have decreased microbiota diversity (177). In a mouse model that results in decreased antibody somatic hypermutation, a hallmark of T cell help, mice harbored a microbiota that was significantly less diverse than control animals (178). TD IgA antibodies have been shown to preferentially bind 'border dweller' bacteria that reside proximal to the intestinal epithelium, such as segmented filamentous bacteria (SFB), Mucispirillum, Prevotella, and *Helicobacter* species (93, 99-101). Recently, it was shown that mice deficient in Tfh cells (via Pdcd1 KO) exhibited dysbiosis exemplified by an outgrowth of certain bacteria associated with pathological outcomes (179). Conversely, mutations that result in the increase of Tfh cells (via P2rx7 KO animals) resulted in an increased abundance of commensal microbes associated with obesity (180, 181). Collectively, these findings demonstrate that the proper regulation of Tfh and TD antibody responses is essential for maintaining intestinal health.

As summarized in Chapter 1, mice deficient in maternal antibodies mount an elevated microbiota-dependent Tfh and GC B cell response in gut-associated tissues

during the weaning transition. Given the microbiota-dependent nature of the response, coupled with the importance of Tfh cells and TD antibody responses in regulating host-microbe interactions, we hypothesized that this dysregulated adaptive immune response altered the gut microbiota. To address this hypothesis, we assessed whether the composition or localization of the intestinal microbiota was altered in mice that received or lacked maternal antibodies. Additionally, we tested whether the lack of maternal antibodies changed the functional capacity of the microbiota. For these latter studies, we tested host susceptibility to infection with enteric pathogens *Salmonella typhimurium* and *Citrobacter rodentium,* which must compete with the gut microbiota to successfully colonize the host. We also assayed intestinal transit time, which is influenced by microbial metabolite production and is thus a useful method for assaying microbiota function.

We found that the overall abundance, composition, and diversity of the intestinal microbiota was equivalent in adult offspring that received or lacked maternal antibodies. Additionally, we found that maternal antibody-sufficient and -deficient offspring were equivalently susceptible to infection with *S. typhimurium* and *C. rodentium* at 6 weeks of age. Finally, did we observe differences in intestinal transit time between the two groups.

4.2 Materials and Methods

Animals

C57Bl/6 (B6), and muMT$^{-/-}$ mice are from Jackson Labs. 7-week-old offspring that received maternal antibodies (offspring of muMT$^{-/-}$ dam x B6 sire) or did not receive maternal antibodies (offspring of muMT$^{+/-}$ dam x B6 sire) were used unless otherwise specified. Mice strains were maintained under SPF conditions at the Fred Hutch to normalize the microbiota as much as possible. For experiments comparing two or more groups, mice from each experimental category were co-housed. For breeding of littermate or cohoused dams, females were separated into individual cages and bred for 2–3 nights; dams were then "re-cohoused" for approximately 18 days. Pregnant females were separated and housed individually until parturition. All mice were maintained on non-acidified water unless specified. All animal experiments were approved by Fred Hutch IACUC, and the study was conducted in strict compliance with the PHS Policy on Humane Care and Use of Laboratory Animals.

16s qPCR

For wall-associated microbes, 2cm of the terminal ileum or proximal colon were dissected, flushed with 5mL sterile PBS to remove non-adherent microbes, and then cut open longitudinally. For lumen samples, one fecal pellet from the terminal ileum or proximal colon was collected. Samples were flash-frozen in liquid nitrogen and stored at -80C until DNA extraction. DNA was extracted using the PowerFecal Pro kit (Qiagen) per manufacturer's directions. Extracted DNA was quantified using a Nanodrop spectrophotometer (Thermo) and then stored at -80C until quantification with qPCR.

qPCR was run using SYBR Green (Thermo Cat# A25742). Reactions were done in 384 well plates: 7.5uL SYBR Green, 1.5uL primer mix at 100nM, 50ng DNA, and water to bring volume up to 15uL. A quantitative real-time PCR was set up with a standard curve. The standard curve was made with plasmid DNA containing one copy of the 16S gene and linearized with digestion enzyme prior to use. Standards were run in duplicate. Curve was started at 1e7 copies and serially diluted 1:4 across the plate. The 16s gene was amplified using 16s F (ACT-CCT-ACG-GGA-GGC-AGC-AGT) and 16s R (5'- ATT-ACC-GCG-GCT-GCT-GGC-3'). Gapdh gene was amplified using Gapdh1F 2018 F (TGTAGTAGCAGCCCCTTCCA) and Gapdh1R 2167R (TTTCTGGAGGATGGAACGGG). Rps17 gene was amplified using Rps17F 606 F (TATGGCCGTGTTGTGCAAAA) and Rps17R 755 R (CACACAGACACACACACAGC). qPCR product was run on a 2% agarose gel to confirm amplification of correct size, ~200bp. Ct of the 16s gene was normalized to eukaryotic DNA via quantification of gapdh and rps17 before interpolating with the 16s standard curve for concentration. The standard curve was fit to Sigmoidal, 4PL, x is log (copies).

16s sequencing

Samples were collected and sent to Zymo Research for 16s sequencing. For wall-associated microbes, 2cm of the terminal ileum or proximal colon were dissected and flushed with PBS to remove non-adherent microbes. For lumen samples, one fecal pellet from the terminal ileum or proximal colon were collected. Samples were immediately transferred to -80C freezer for storage and subsequently shipped on dry ice.

In brief, DNA extraction was performed using the ZymoBIOMICs-96 MagBead DNA Kit (Zymo Research), and samples were prepped for targeted sequencing with the Quick-16S NIG Library Prep Kit (Zymo Research) using the V3-V4 primer set. PCRs were performed. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned up with the Select-a-Size DNA Clean & Concentrator (Zymo Research), then quantified with TapeStation (Agilent Technologies) and Qubit (Thermo Fisher Scientific). ZymoBIOMICS Microbial Community DNA standard was used as a positive control for the targeted preparation, and negative blank extraction and library preparation controls were included to asses the level of microbial contamination. The final library was sequenced on Illumina MiSeq with a v3 reagent kit (600 cycles). The sequencing was performed with 10% PhiX spike-in.

16s analysis

Processed reads were input into MaLiAmPi (182), a Nextflow workflow. Amplicon Sequence Variants (ASVs) were obtained using DADA2 (183) which is in the MaLiAmPi pipeline. Chimeric sequences were also removed with the Dada2 pipeline. Taxonomy assignment was performed using MaLiAmPi, which used phylogenetic placement to estimate the taxonomic classification of the ASVs. Taxonomy was assigned using the AFR database, publicly available at: https://zenodo.org/record/6876634#.Y-LLSOzMKik. Alpha diversity estimates were generated by MaLiAmPi, and beta diversity was calculated using the R package “vegan” (184).

Processed reads were also analyzed in parallel by Zymo Research using similar methods. In brief, unique amplicon sequences were inferred from raw reads using the Dada2 pipeline (183). Chimeric sequences were also removed with the Dada2 pipeline. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1. Taxonomy was assigned with the Zymo Research Database, a 16S database that is internally designed and curated, as a reference. Composition visualization, alpha-diversity, and beta-diversity analyses were performed with Qiime v.1.9.1 (185). Heatmap analysis were performed with internal scripts.

Infection

For *S. typhimurium* infections, mice were fasted overnight in clean cages with unlimited access to water. *Salmonella enterica* serovar Typhimurium SL1344 (DMSZ) with a streptomycin resistance cassette was used. *S. typhimurium* was grown aerobically in LB broth (VWR) with 50ug/mL of streptomycin aerobically at 37C for 17 hours exactly at 225rpm. Mice were oral gavaged 2e7 CFU in 200uL PBS. For *C. rodentium* infections, mice were fasted for 6 hours in clean cages with unlimited access to water. *Citrobacter rodentium* strain DBS100 (ATCC Cat #51459) was used. An overnight culture of *C. rodentium* grown in LB broth (VWR) was back diluted in LB to an OD=.1 and grown again aerobically at 225rpm until OD=1-1.5. CFU was calculated using log(CFU)=.393(OD)+8.01. Mice were gavaged 4e10 CFU in 200uL PBS. For calculation of colony forming units (CFU) in the PP, ileum (distal 5cm without Peyer's patches), colon (proximal 5cm), spleen, liver, cecum, and feces, samples were harvested aseptically into PBS and dissociated using a Polytron PT2100 homogenizer (Kinematica). Dilutions were

plated in duplicate onto LB agar with 50 ug/ml streptomycin for *S. typhimurium* or MacConkey agar for *C. rodentium*. Plates were grown under aerobic culture conditions at 37C for 17 hours before enumeration of colonies. Additionally, Peyer's patches were treated with 100 uM gentamicin for 1 hr at 37°C to remove extracellular bacteria and washed twice with PBS before homogenizing and plating.

Intestinal transit time assay

Mice were gavaged orally with 150uL per mouse of a sterilized 6% (w/v) solution of carmine red (Sigma-Aldrich) in 0.5% methylcellulose (Sigma-Aldrich) (186). Fecal output was monitored every 30 min. The time from gavage to the appearance of bright red dye was recorded as the whole gut transit time.

Statistical analysis

Statistical analyses were performed with Prism software version 9.1 (GraphPad Software). Parametric data were assessed with unpaired t-tests. Nonparametric data were assessed with Mann Whitney tests. Significance was defined by a p-value less than .05.

4.3 Results

Experimental set up

The same breeding and housing strategy were utilized as described in Chapter 3. As it relates for Chapter 4, we were able to collect longitudinal samples to study the impact of maternal antibodies on gut microbiota function throughout life. However, this was not feasible for all studies. Following activation, GC-derived TD antibodies reach high titers approximately 21 days after initial antigen encounter (172). As we had previously shown that mice lacking maternal antibodies harbored increased numbers of GC B cells at 25 days of age, we hypothesized that the peak of the TD antibody response and thus any changes in gut immunity induced by this immune dysregulation would become apparent at 6-7 weeks of life. Thus, for experiments in which longitudinal samples could not be obtained, we focused our analysis on 7-week-old offspring.

Equivalent abundance of intestinal wall-associated microbes in offspring that received or did not receive maternal antibodies

In the absence of maternal antibodies, offspring mount an increased GC B cell response that is dependent on colonization by the commensal microbiota. Previous work has shown that mucosal TD-dependent GC B cells produce of high-affinity, highly specific antibodies that preferentially bind to 'border dweller' bacteria residing proximal to the intestinal epithelium, such as segmented filamentous bacteria (SFB), Prevotella, and *Helicobacter* species (93, 99-101). Production of these TD antibodies have been shown to limit the accumulation of these species at the intestinal barrier. As such, we hypothesized that the absence of maternal antibodies leads to increased targeting of

'border dweller' bacteria, resulting in reduced accumulation of microbes that reside in close proximity to the intestinal wall.

To address this hypothesis, we isolated the terminal ileum and proximal colon samples from matAb+ and matAb- offspring, flushed with PBS to remove non-adherent microbes, and used qPCR of the 16s rRNA gene to assay for microbial abundance. We performed this assay on experimental cohorts at week 3, week 5, and week 11 of age (Figure 4.1A). We found no statistically significant difference in the quantity of the wall-associated microbes between matAb+ and matAb- offspring at any of the time points. We did observe a trend of increased colonic wall-associated microbes in the absence of maternal antibodies at week 3. Because maternal antibodies are present in the infant intestines at this time (from our unpublished observations, the washout period of maternal antibodies is 2-3 days), this result suggests that maternal antibodies function to impair the associated of microbes with the colonic epithelium during the weaning transition. At 6 weeks of age, we observed no difference in the abundance of wall-associated microbes between matAb+ and matAb- mice in either tissue site. However, we observed a trend toward reduced 16S levels in the small intestinal wall samples from matAb- compared to matAb+ mice at 11 weeks of age. This result is consistent with our original hypothesis and suggests that the absence of maternal antibodies leads to alterations in gut microbiota localization. However, the appearance of these phenotypes may take time to develop. Further work to expand upon these findings is warranted.

Similar gut microbiota composition in adult offspring that received or did not receive maternal antibodies

To understand if the absence of maternal antibodies and the subsequent rise in adaptive immune response alters the composition of the microbiota, we performed deep sequencing of the 16s rRNA gene from wall and luminal samples taken from the SI and colon of co-housed matAb+ and matAb- offspring at 7 weeks of age. We included samples from four litters in our analysis, two litters were progeny of an antibody-sufficient dam and two litters were progeny of an antibody-deficient dam. We performed unsupervised hierarchical clustering of ASVs from samples and analyzed each tissue site separately. The 50 most abundant species are shown in Figure 4.1B. Samples from the small intestine, for the most part, grouped together by housing. In other words, the microbiota composition was most similar amongst cage mates. However, in the colon, there was no apparent segregation of samples. Notably, we did not observe clustering of samples based on maternal antibody status in any tissue site sampled. Correlating with these results, non-metric multidimensional scaling (NMDS) analysis, a type of beta-diversity analysis, revealed that matAb+ and matAb- samples grouped together (Figure 4.1C). Finally, species richness and diversity, assessed via Shannon diversity index, did not reveal significant differences between matAb+ and matAb- offspring (Figure 4.1D). Cumulatively, these results indicate that the absence of maternal antibodies in early life does not substantially alter the overall composition and diversity of the gut microbiota later in life.

Equivalent susceptibility to small intestinal pathogen *S. typhimurium* in offspring that received or did not receive maternal antibodies

The composition, gene expression, microbial metabolite production, and niche occupancy of the commensal microbiota can all influence colonization resistance to the enteric bacterial pathogen *Salmonella* typhimurium (187-189). For example, the Enterobacteriace family (188), specific members of Bacteroidales (190), and the microbe SFB (191) have all been found to antagonize infection by *S. typhimurium* via different mechanisms. Germ-free mice and animals with a reduced microbiota diversity have increased susceptibility to *S. typhimurium* (192, 193). Butyrate, a microbial metabolite, has been shown to inhibit the growth of *S. typhimurium* (194-196). Thus, while we found no differences in the overall abundance or composition of the intestinal microbiota between matAb+ and matAb- offspring, we do not know the relative contributions of individual species or potential changes in the gene expression. Investigating the susceptibility of matAb+ and matAb- offspring to *S. typhimurium* is a good strategy to probe alterations in the functional capacity of the gut microbiota.

To explore how maternal antibodies impact colonization resistance, we first determined the dose of *S. typhimurium* in which 50% of animals were infected, which was 2e7 CFU in our mouse colony (Figure 4.2A). We then infected matAb+ and matAb- offspring at 7 weeks of age with 2e7 CFU, which gave us a dynamic range to test colonization resistance of *S. typhimurium*. We harvested organs at 1- and 5- days post infection for CFU analysis and cytokine profiling, respectively (Figure 4.2B). *S. typhimurium* gains access to host tissue by infecting Peyer's patches and small intestinal epithelial cells (191), so we assessed CFU in the Peyer's patches, ileum, and feces. We found no significant difference in *S. typhimurium* loads at these sites (Figure 4.2C). We next performed a longitudinal analysis of *S. typhimurium* fecal loads, as decreased

shedding of *S. typhimurium* over time is indicative of colonization resistance. We found that matAb+ and matAb- offspring harbored similar *S. typhimurium* CFU in feces throughout the timepoints assayed (Figure 4.2D). While all infected animals lost weight throughout the course of the experiment, the degree of weight loss was equivalent between the two groups (Figure 4.2E). Finally, we assessed *S. typhimurium* loads in the spleen and liver at 5 days post-infection, which indicates how well the host can resist *S. typhimurium* translocation or clearance (Figure 4.2F). While not statistically significant, we did observe a trend of decreased bacterial load in the liver of matAb- offspring, indicating that matAb- offspring were better able to control systemic infection by *S. typhimurium*. The high variability of bacteria recovered in the different organs aligns with other studies of *S. typhimurium* (188, 191). Thus, detecting subtle changes in CFU using this infection model requires the use of numerous animals in each experimental group, more than what was used for our studies.

We quantified levels of 13 cytokines and chemokines involved in immune signaling at 5 days-post *S. typhimurium* infection by cytokine bead array. There were no differences in cytokine production 5 days-post *S. typhimurium* in females but decreased levels of IFN-gamma and increased levels of IL-23 in matAb- males compared to matAb+ males. Further investigations are needed to determine the significance of these subtle responses in males.

Overall, there seemed to be no differences in the susceptibility of matAb+ vs matAb- offspring to S. typhimurium.

Equivalent susceptibility to colonic pathogen *C. rodentium* in offspring that received or did not receive maternal antibodies

S. typhimurium has tropism for the SI, which has a differing microbiome composition compared to the colon (197). To probe potential changes in the functional capacity of the colonic microbiota, we infected co-housed matAb+ and matAb- offspring at 7 weeks of age with the colonic pathogen *Citrobacter rodentium (C. rodentium)*. Similar to *S. typhimurium,* colonization resistance to *C. rodentium* is dependent on specific functions of the microbiota and the host immune system (198, 199). We harvested organs 7-days post infection and performed CFU analysis (Figure 4.3A). We observed equivalent CFU of *C. rodentium* in the feces, colon, spleen, and liver between matAb+ and matAb- offspring (Figure 4.3B).

We also assessed whether housing status affected susceptibility to *C. rodentium* with the rationale that co-housing may be equilibrating the composition of the microbiota and mask any potential differences in susceptibly to C. rodentium. We observed equivalent *C. rodentium* bacterial loads in the feces, colon, spleen, and liver in offspring that were housed with their own littermates at weaning (Figure 4.3C). Overall, our results suggest that the absence of maternal antibodies does not impact susceptibility of 7-week-old offspring to *C. rodentium*, and that this effect is independent of housing status.

Equivalent intestinal transit time in offspring that received or did not receive maternal antibodies

Microbiome-encoded bile acids can alter intestinal transit time (186). As a final measure of the functional capacity of the microbiota, we assessed the intestinal transit

time in offspring that received or did not receive maternal antibodies. Intestinal transit time is partially determined by length of the small intestine; we saw no differences in SI length between matAb+ and matAb- offspring (Figure 4.4A). However, even in animals with the same SI length, differences in microbiota function, such as bile acid production, can alter transit time. To assess intestinal transit time, we gavaged mice with a dye and recorded the time when the dye was visible in the feces (Figure 4.4B). We observed no differences in intestinal transit time overall (Figure 4.4C) nor when data was stratified by sex (Figure 4.4D). This data suggests that maternal antibodies do not substantially alter intestinal transit time in offspring, at least at the 7-week timepoint.

4.4 Discussion

In summary, we found no evidence for maternal antibodies in impacting the abundance, composition, and diversity of the intestinal microbiota in offspring later in life. Additionally, we did not observe changes in the functional capacity of the intestinal microbiota by way of colonization resistance to small intestinal pathogen *S. typhimurium* or colonic pathogen *C. rodentium* or via intestinal transit time. In-depth analysis of the abundance of specific wall-associated microbes and the relative composition of select microbes, such as such as SFB, *Helicobacter* spp, from our sequencing analysis are ongoing.

There are several limitations to our study. First, we focused the majority of our analysis on offspring at approximately 6-7 weeks of age with the premise that the TD antibody response peaks 21 days after the initial antigen encounter and would thus have an impact on intestinal homeostasis at this timepoint (and potentially beyond). Additionally, the altered GC B cell response in matAb- offspring at d25 was dependent on colonization by the microbiota at the time of weaning. Though the microbiota at the time of weaning differs from the microbiota at week 6-7, there are some conserved species (90), which we hypothesized would drive a lasting TD antibody response and may subsequently be impacted by this response. While we performed a time course study of bacterial wall abundance in different intestinal tissue sites, we did not assess whether the microbiota composition and diversity changes over time, nor did we perform a time course to measure susceptibility to enteric infection with *S. typhimurium* or *C. rodentium*. Thus, if there is a narrow window in which the offspring have differing microbiota composition or function, we would have missed that by focusing on offspring at week 7.

Additionally, it is formally possible that the GC B cell response observed on day 25 does not receive appropriate survival signals and that the TD antibodies generated from this response are replaced throughout development. Indeed, the model that TD antibodies reach high titers at approximately 3 weeks-post B cell activation and can be maintained for long periods of time are largely derived from immunization studies done in adults (172). Additionally, though some species are maintained for long-periods of time, the composition of the microbiota does vary drastically from weaning to adulthood (200), and the relevant microbial species that instigate the dysregulated TD GC responses in matAb- mice may not persist to feed a prolonged response. Recently, a method to fate-track GC derived antibodies was developed (201). Further investigations using this method look at the longevity of the TD antibody response and the specificity of these antibodies will be informative.

Second, while we chose our co-housing strategy to equilibrate any differences in the microbiota between matAb+ and matAb- offspring, this strategy could have masked subtle differences between experimental groups due to constant exchange of the microbiota via coprophagy. To address this, future studies performing similar analyses on matAb+ and matAb- mice singly housed following weaning can be conducted. Of note, I attempted a variation of this by housing matAb+ littermates apart from matAb- littermates at the time of weaning and assessing susceptibility to *C. rodentium* at week 7 of age (Figure 4.3C). However, I did not observe significant differences in bacterial loads in all sites analyzed between the two groups, suggesting that the absence of maternal antibodies does not largely impact susceptibility to *C. rodentium*, even when 'microbiota-mixing' is prevented.

It is possible that the functional capacity of the microbiota is altered by maternal antibodies, but not in a way that influences colonization resistance to S. typhimurium or C. rodentium. To further test how maternal antibodies impact microbiota function we could perform studies analyzing transcriptomics, metabolite profiling, and response to infection by other intestinal pathogens between matAb+ and matAb- offspring (202, 203).

Finally, we have been working off the hypothesis that the elevated early life Tfh-GC B cell response generated in the absence of maternal antibodies results in altered gut homeostasis. However, this response could be a compensatory response to restore homeostasis in the absence of maternal antibodies. Studies to assess the composition and function of the microbiota in maternal antibody deficient offspring that cannot mount this transient TD immune response, either through genetic models or in vivo blocking methods, could address this. I elaborate on this idea more in the final chapter.

4.5 Figures

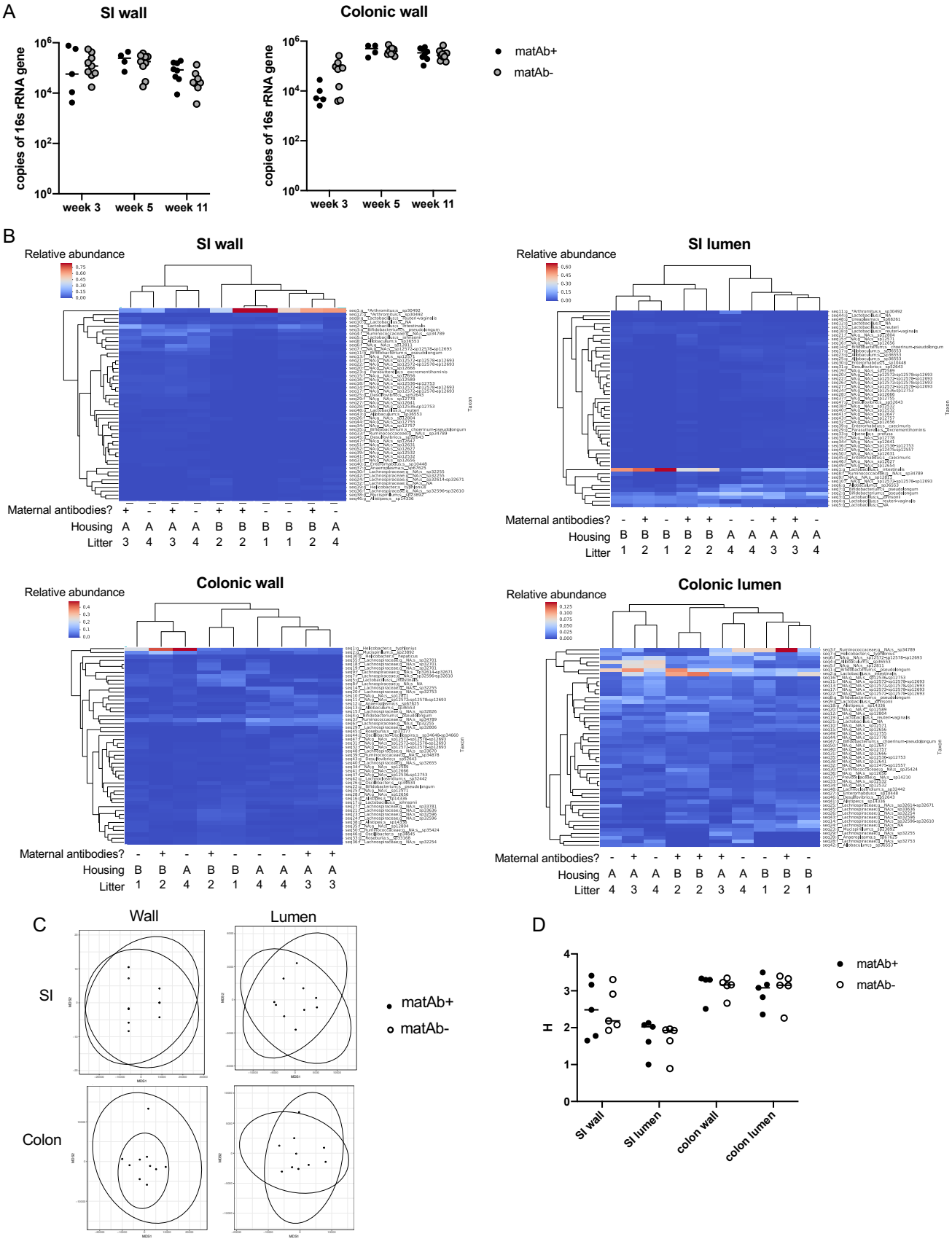
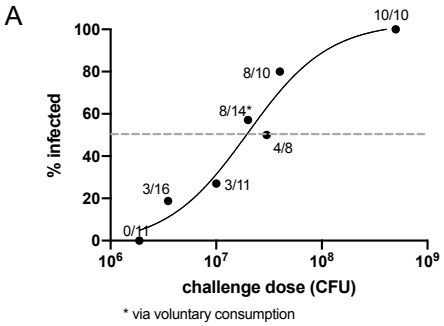
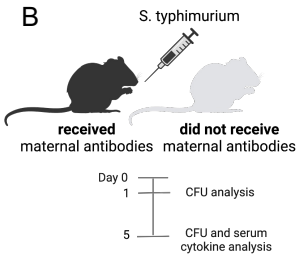
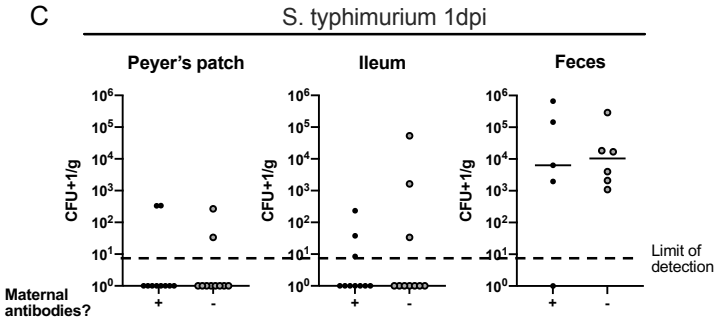

A
SI wall
Colonic wall
copies of 16s rRNA gene
week 3
week 5
week 11
matAb+
matAb-
B
SI wall
SI lumen
Colonic wall
Colonic lumen
Relative abundance
Maternal antibodies?
Housing
Litter
C
Wall
Lumen
SI
Colon
matAb+
matAb-
D
H
SI wall
SI lumen
colon wall
colon lumen
matAb+
matAb-

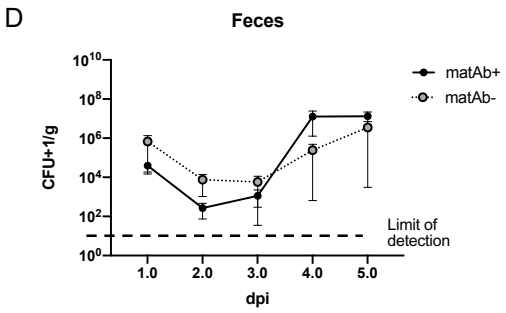
Figure 4.1 **Microbial abundance and composition in offspring that received or did not receive maternal antibodies.** A) Quantity of the bacterial 16s rRNA gene in offspring that received or did not receive maternal antibodies at the indicated timepoints. Terminal ileum or proximal colon samples were harvested, flushed with PBS to remove non-adherent microbes, and lysed for DNA extraction and qPCR. Samples were normalized to eukaryotic house keeping genes gapdh and rps17. Mice were on acidified water. B) Unsupervised hierarchical clustering of ASVs from V3-V4 segment of the 16s gene from microbes in offspring that received or did not receive maternal antibodies at the indicated locations. Heatmap shows the microbial composition of samples at the ASV level with the top fifty most abundant ASVs identified. Housing and litter information is as noted. For wall associated microbes, terminal ileum or proximal colon samples were harvested and flushed with PBS to remove non-adherent microbes. For lumen samples, feces from the terminal ileum or proximal colon were collected. C) NDMS plots of microbes from offspring that received (black circles) or did not receive (open circles) maternal antibodies at the indicated locations. D). Shannon diversity of microbes from offspring that received (black circles) or did not receive (open circles) maternal antibodies at the indicated locations. B-D) Data representative of two independent experiments with n>=4/group. Statistics: A, D) Unpaired t-test.

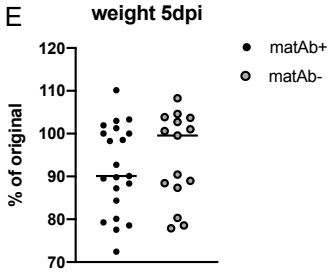


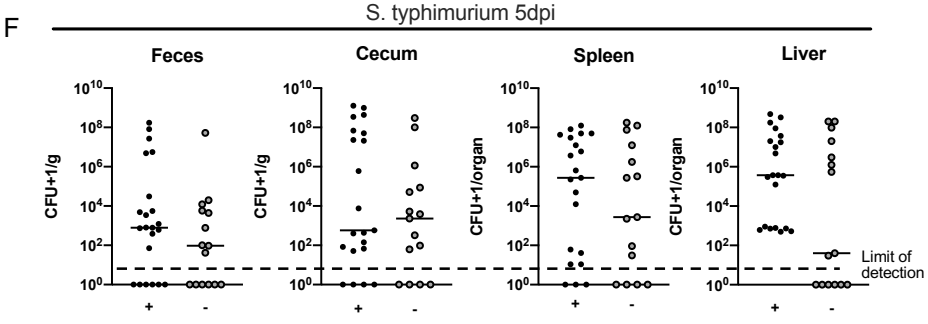


Infected with 2e7 CFU









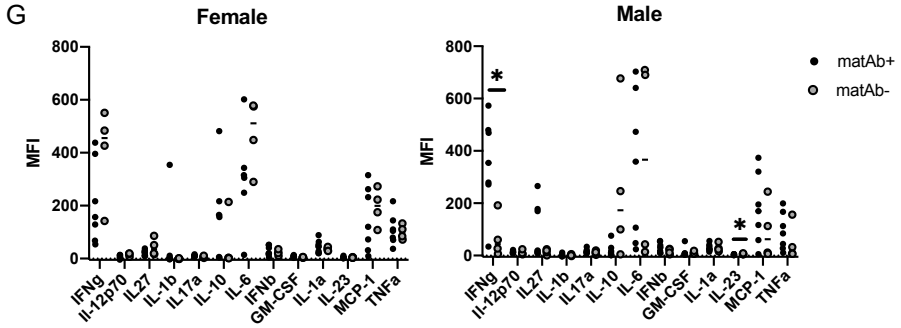


Figure 4.2 **Susceptibility to small intestinal pathogen S. typhimurium**. A) Infectivity curve of S. typhimurium in our mouse colony. Positive infection is indicated by >1 CFU recovered for spleen, liver, cecum or feces at 5dpi. Dashed line represents ID50 = 2e7 CFU. B) Experimental setup for C-G. Mice that received or did not receive maternal antibodies were infected with 2e7 CFU of S. typhimurium via oral gavage. Samples were harvested at 1- and 5-dpi for CFU and CFU with cytokine analysis, respectively. C) CFU of S. typhimurium in indicated organs 1dpi in offspring that received or did not receive maternal antibodies. CFU that fell below the the limit of detection (dashed horizontal line) is plotted on the x-axis. Bars on graph indicate median. Data combined from two independent experiments. D) CFU of S. typhimurium recovered in the feces from 1 to 5dpi of offspring that received or did not receive maternal. Dots indicate mean and error bars display SEM. E) Weight loss in mice infected with S, typhimurium. Bars on graph indicate median. F) CFU of S. typhimurium in indicated organs 5dpi in offspring that received or did not receive maternal antibodies. CFU that fell below the the limit of detection (dashed horizontal line) is plotted on the x-axis. Bars on graph indicate median. G) Cytokine and chemokine response 5dpi in maternal antibody sufficient or deficient offspring infected with S. typhimurium. Bars on graph indicated mean. D-G): Data representative of 3 independent experiments. Statistics: C, D, G) Mann Whitney test. F, G) Unpaired t-test. *$p<0.05$. All mice were on acidified water.

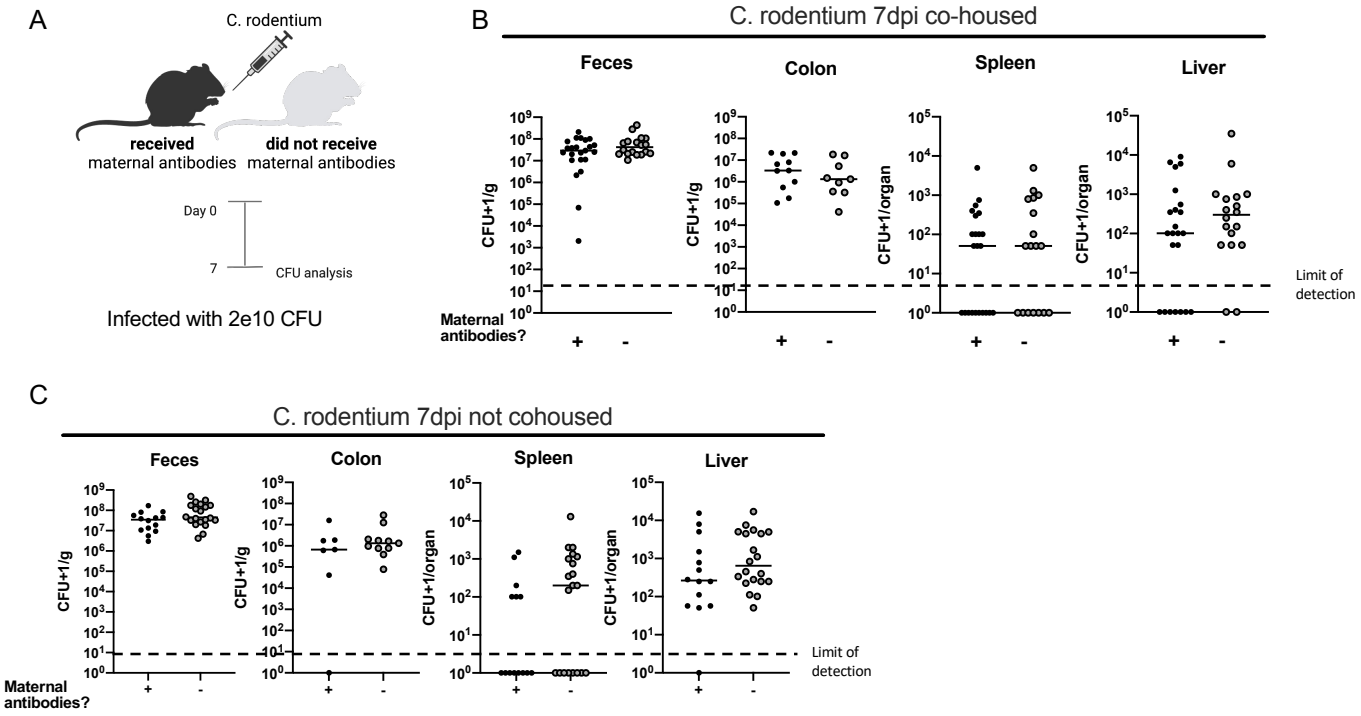


Figure 4.3 **Susceptibility to colonic pathogen C. rodentium.** A) Experimental setup for B-C. Mice that received or did not receive maternal antibodies were infected with 2e10 CFU of C. rodentium via oral gavage. Samples were harvested at 7 dpi for CFU analysis. B) CFU of C. rodentium in indicated organs 7dpi in offspring that received or did not receive maternal antibodies. CFU that fell below the the limit of detection (dashed horizontal line) is plotted on the x-axis. Bars on graph indicate median. Mice from different experimental groups were cohoused at the time of weaning through the end of this experiment. C) CFU of C. rodentium in indicated organs 7dpi in offspring that received or did not receive maternal antibodies. CFU that fell below the the limit of detection (dashed horizontal line) is plotted on the x-axis. Bars on graph indicate median. Mice were housed with its own littermates at the time of weaning through the end of this experiment. Mice were 6-9 weeks old at time of analysis. B-C) Data are combined from two independent experiments. Statistics: Mann Whitney test.

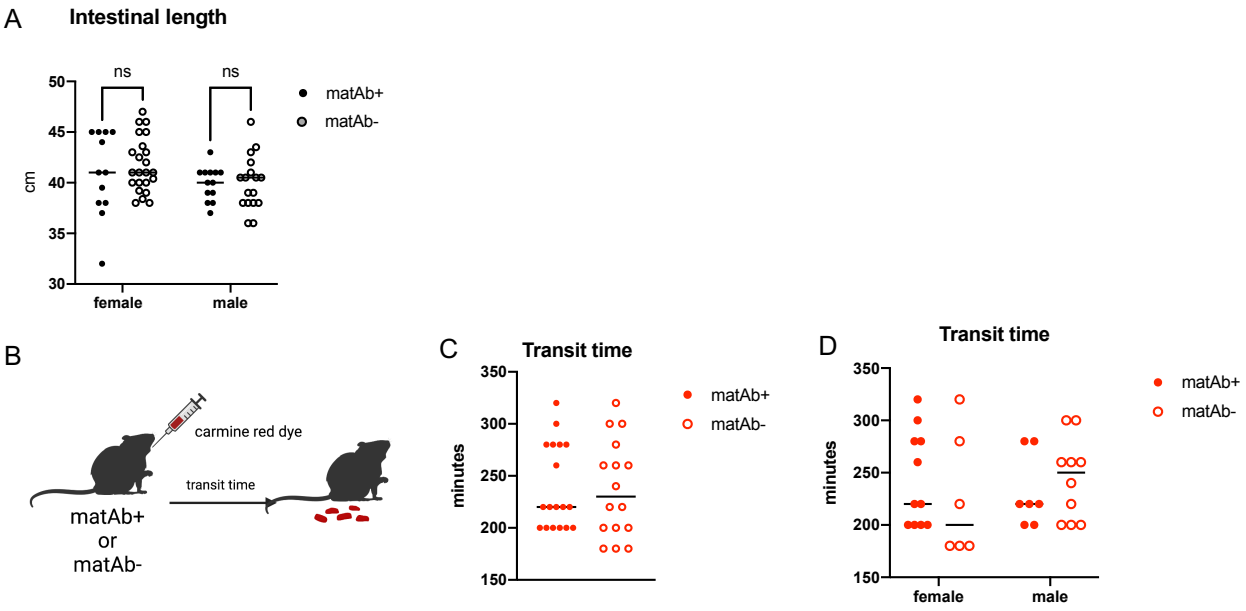


Figure 4.4 **Intestinal length and transit time.** A) Small intestinal length in offspring that received or did not receive maternal antibodies at 11 weeks of age. B) Intestinal transit time in offspring that received or did not receive maternal antibodies. Mice were gavaged with carmine red dye and time to observe a red fecal pellet was recorded. C) Intestinal transit time as in B) but segregated by sex. Data are combined from two independent experiments. Statistics: Unpaired t-test.

Chapter V
Comprehensive analysis of all immunoglobulin subclasses in human breast milk

This chapter is adapted from the following publication:

Wang, B, Schreder, MS, Sathyanarayana, S, Koch, MA. (2023) Comprehensive analysis of all immunoglobulin subclasses in human breast milk*. In preparation.*

5.1 Introduction

Breast milk contains many bioactive molecules, cytokines, chemokines, growth factors and hormones, and, importantly, antibodies (74). Unlike placentally transferred antibodies which play a dominant role in systemic immunity (204), breast milk antibodies coat the bacteria living in the gut lumen of neonates and mediate the initial host-microbiota interaction.

Antibodies are transmitted to the breast milk via receptors found in the mammary glands. The polymeric Ig receptor (pIgR) is responsible for the transfer of secretory IgA and IgM into the lumen of lactiferous ducts (46). Uniquely to IgA and IgM, the external domain of the pIgR, called the secretory component, remains attached to IgA and IgM and plays a crucial role in protecting these antibodies from cleavage by proteases. Accordingly, IgA and IgM concentrations are among the highest found in breast milk(205). In contrast, IgG is transferred through the neonatal Fc receptor (FcRn) (206). Additionally, there is evidence of paracellular transport of IgG as well. Mice deficient in the FcRn have low levels of IgG in breast milk (80). The mechanism of transfer of IgE to breast milk is not well defined. Though controversial (207), studies have shown that IgE can be transferred trans-placentally in conjunction with IgG via the FcRn (208), suggesting that a similar mechanism via FcRn transport could also exist in the mammary glands. While secreted IgD does exist at very low levels in the serum (209), there is no described pathway for which IgD could be transported to human breast milk. Thus, we left IgD out in our analysis.

The most well-studied subclass in human breast milk is IgA, which exists in two isoforms, IgA1 and IgA2. IgA plasma cells are first activated in mucosal lymph nodes like

the Peyer's patches and then home to the mammary glands following mucosal chemokine CCL28 gradient (39). Accordingly, SIgA is important for homeostasis towards gut microbes in many ways, such as excluding microbes from the intestinal epithelium, restricting growth of pathogens, and helping establish a healthy microbiota (70, 210). IgM coordinates with IgA to maintain a healthy microbiota. Humans with IgA deficiency generate IgM that is capable of trapping and driving microbial colonization though IgM targets a different subset of commensals and drives the expansion of more pathogenic microbes (175, 211). IgG comes in four isotypes, IgG1, IgG2, IgG3, and IgG4. Subclass switching of the IgG occurs in a one-way direction, beginning with IgG3 to IgG1, followed by IgG2 and IgG4 over time (212). These antibodies differ in their constant regions. Among the IgG subclasses, IgG1 and IgG3 antibodies are the most potent activators of complement. They are also highly efficient at binding a wide range of Fc receptors on immune cells, which engages effector functions, including phagocytosis, degranulation, etc. In contrast, IgG4 has been shown to induce an anti-inflammatory M2-like macrophage phenotype by inhibiting interferon gamma signaling (213). Considering these properties, it has been suggested that IgG4 can regulate the immune response and limit inflammation through competition with other antibodies (214). Human IgG1 has been shown to prevent allergic airway inflammation (215) and, in mice, to protect against enteric pathogens (76). In mice, breast milk IgG2b and IgG3 have been found to bind to commensal microbiota to maintain homeostasis (1).

To date, there is no comprehensive characterization of the relative frequencies of the subclasses of antibodies secreted into breast milk. While many studies have quantified the levels of IgA individually in breast milk, fewer have looked at the

concentration of IgG and its subclasses, and none to date have looked at the relative contributions of all subclasses combined in breast milk (205). Given the importance of these antibodies and their diverse functions in mucosal immunity, we sought to do a comprehensive profiling of the abundance of all seven antibody subclasses that can be secreted into human breast milk. We validated sandwich ELISAs for each subclass and quantified the antibody levels in 45 healthy lactating women from Seattle. We could not find commercially available reagents to detect IgG2 that did not cross-react with other isoforms of IgG. We found that IgA1 was the most abundant isotype found in breast milk, which is followed by IgA2 and IgM. We found detectable levels various subclasses of IgG in breast milk but the levels varied across individuals. We did not find detectable IgE levels. Finally, We found no correlation between the concentrations of any breast milk immunoglobulin subclasses and infant age, suggesting that breast milk antibody composition remains stable over time.

5.2 Materials and Methods

Participant Recruitment and Sample Collection

Primiparous healthy women pregnant and planning to breastfeed or currently breastfeeding and residing in or near Seattle, Washington, United States, were recruited using materials approved by the University of Washington Institutional Review Board. Recruitment took place over social media channels, through parenting groups, and via paper flyers. Breast milk samples (n = 45) were collected in March−October 2019. Participants were instructed to manually express breast milk, which was transferred into polypropylene jars precleaned with water, isopropyl alcohol, and methanol. The samples were retrieved from participants within 24 h after collection and stored at −4 °C until shipment to Fred Hutch, where they were stored at −80 °C until analysis. Participants also provided answers to a questionnaire to collect demographic data.

Sample analysis

Breast milk samples were thawed at room temperature and centrifuged at 300rpm for 25min to settle out the suspended cellular components. The supernatant was decanted into a new tube, and the lipid layer was removed by centrifuging at 3000rpm for 20min at 4C. Sandwich ELISAs were performed to quantitate the specific subclass of antibody. In brief, Nunc Hi Affinity ELISA plates were coated with subclass-specific capture antibodies at a concentration of 500-1000ng/mL overnight at 4C. Plates were then washed with wash buffer (.05% Tween in PBS) and blocked with 1% BSA in .05% Tween in PBS for 1 hr at 37C. Plates were then washed in wash buffer and incubated with subclass-specific standards serially diluted 1:2 across the plate and breast milk

samples, all plated in duplicate. Plates were incubated for 1.5 hours at 37C, washed, and incubated with a biotinylated secondary antibody at 500ng/mL for 1hr at room temperature. After washing, plates were incubated with SA-HRP (Biolegend) at 1:8000 for 20min at room temperature. Plates were developed using TMB (Pierce) for 5-10min in the dark. The reaction was stopped using 1N H2SO4. Absorbance at 450nm was measured using Agilent BioTek.

For complete list of antibodies and dilutions used, reference Table 5.1.

Quality control

Reagents to detect IgM, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, and IgE were checked for cross-reactivity to other subclasses by incubating with standards from the remaining seven subclasses. Data were corrected for background fluorescence by subtracting out PBS blanks on a per-plate basis. Averaged OD values from each set of replicates used in the interpolation of concentration and sample were repeated if the difference in OD value between the replicates was greater than 10% .

Data analysis

ELISA analyses were conducted in Prism 9 (GraphPad Software Inc). Samples below the lower limit of detection were given a value of halfway between 0 and the lower limit of detection. Correlation was determined via Pearson's correlation coefficient.

5.3 Results

Participant characteristics

The study cohort's demographic characteristics are shown in Table 1. This study included 45 lactating women. The median age of participants was 34 years old. There was a range of BMI; 60% of study participants were of normal BMI, 22% were considered overweight, and 13% were considered obese. 4% (2 individuals) did not have weight and height data recorded. The median day post-birth of sample collection was 198 days, with the interquartile range (IQR) of 98 days to 293 days. Two individuals did not have a date of sample collection recorded.

Reagent verification

We developed and verified an assay to detect all eight secreted human antibodies. To ensure accurate quantification of antibody subclasses in breast milk, we verified our ELISA reagents to ensure there was no cross-reactivity with other antibody subclasses. Each capture and secondary pair was tested against subclass-specific standards starting at 500ng/mL down to 18ng/mL. We verified that our reagents to detect human IgM, IgG1, IgG3, IgG4, IgA1, IgA2, and IgE were specific and did not cross-react to standards from other subclasses (Figure 1). However, all the reagents we found commercially available for human IgG2 displayed cross-reactivity towards other IgG subclasses. While several groups have analyzed IgG2 levels in breast milk(216), these studies assayed antigen-specific responses, and thus required only one reagent to specifically detect IgG2.

Immunoglobulin subclass distribution in breast milk

In alignment with studies from several groups summarized in this review here (205), IgA was the most abundant in breast milk, with the median (interquartile range; IQR) IgA1 concentration of 374.6 (182.9-587.7) ug/mL and the median IgA2 concentration of 238.5 (139.8-409.4) ug/mL (Figure 5.2A-C). IgM was the next abundant subclass in this cohort. The median concentration of IgM was 8.5ug/mL (IQR: 4.3-14.2) (Figure 5.2A-C). IgG3 and IgG4 were detected in all samples assayed and at similar levels. The median concentrations for IgG3 and IgG4 were both 0.66 ug/mL, with the IQR of IgG3 as .31-1.1 and IQR for IgG4 as .15-1.1 (Figure 5.2A-C). In contrast, IgG1 levels were the lowest of the detected IgG immunoglobulin isotypes and, varied widely between individuals, consistent with previous reports (205). The median IgG1 concentration was .008 ug/mL (IQR:.008-11.6) (Figure 5.2A-C). Out of 45 individuals, 26 individuals had IgG1 levels below the limit of detection of the assay.

IgE levels in the breast milk of the participants in this cohort were all below the limit of detection (data not shown), in alignment with other studies of IgE levels in the breast milk of healthy non-allergic mothers (217). In summary, the relative contribution of IgA, including IgA1 and IgA2, contributed to over 90% of the immunoglobulins that we measured in breast milk. IgM contributed around 8%, and the IgG subclasses, including IgG1, IgG3, and IgG4 contributed less than 1% (Figure 5.2B).

Breast milk antibody subclass remains stable over time.

The antibody composition in milk has been shown to vary between colostrum and "mature" milk (15). The median time of breast milk collection was 198 days post-birth (Table 1), with a range of 16-773 days. To determine if there were associations between

the stage of lactation and immunoglobulin subclasses past the colostrum period we performed correlation analysis between age of breast milk and antibody levels. We found no significant associations between the age of the breast milk and any of the seven isotypes we looked at, suggesting that the antibody composition of breast milk is is stable past the colostrum period (Figure 5.3).

5.4 Discussion

Breast milk antibodies are key to providing neonates protection from pathogens and maintaining homeostasis with gut microbes, particularly at a time when neonates cannot mount antibody responses themselves. Here, we report a comprehensive characterization of IgM, IgA1, IgA2, IgG1, IgG3, IgG4, and IgE levels in breast milk from healthy lactating women from the Seattle, WA area via ELISA. To our knowledge, this study is the first to assess the relative contributions of all secreted immunoglobulin subclasses in breast milk.

Like previous studies, we found that IgA was the most abundant subclass found in breast milk (205), followed by IgM and then IgG. Also similar to previous reports (205), the levels of IgG1 varied substantially across individuals; some had undetectable levels of IgG1, and others were found to have up to 43 ug/mL. Additionally, we found no apparent correlation between IgG1 levels and the stage of lactation, with IgG1 being undetected in samples taken early (ten days) or late (up to 2.5 years) past parturition. However, the interpretation of this result should be limited as we did not collect information about acute infection status among study participants. While the presence of allergen-specific IgE was found in the breastmilk of a small subset of atopic women with high serum IgE (217), we did not observe detectable quantities of breast milk IgE in our study participants.

Given the wide differences between colostrum vs. more mature milk(218), it was interesting to observe that the abundance of antibody subclasses remained stable throughout lactation. There are several caveats to this analysis. First, we do not have longitudinal data following individual women over time, which would be a better way to

quantify the abundance. Additionally, we did not adjust for age and other health measures like BMI, which have been found to be associated with differences in serum antibody levels (219, 220). These factors may be confounding variables that mask differences in antibody subclass concentrations over time. Finally, this study aimed to determine the absolute quantities of different subclasses of antibodies. While these are important measures, and we found that the abundance of these antibodies remains stable over time, it does not tell us about the affinity or avidity of these antibodies and how that may change over time.

In humans, breast milk antibodies coat the neonatal gut and generally do not enter systemic circulation (221). However, it is interesting to speculate on the function of these antibodies as breast milk contains immune cells, too (222), making it possible for these antibodies to engage Fc receptors with the cells in the breast milk.

The intestines are more permeable in premature infants. These infants are at increased risk of developing NEC, which results in the translocation of bacteria from the intestinal lumen into systemic circulation, causing sepsis. In the context of prematurity, breastfeeding reduces the risk of developing NEC compared to formula feeding (223). In a mouse NEC model, IgA antibodies were found to be associated with increased survival (43). Thus, understanding the relative contribution and importance of the different antibody subclasses is important for developing formula supplements to ensure healthy infant development.

In summary, we have comprehensively characterized the concentrations of IgM, IgA1, IgA2, IgG1, IgG3, IgG4, and IgE levels in breast milk from a cohort of healthy lactating women. This study will help inform future studies examining the functional

capacities of the different subclasses of breast milk antibodies and aid in the development of fortifying formula with bio-reactive factors like antibodies to ensure the healthy development of all neonates.

5.5 Acknowledgements

We thank the participants of this study.

5.6 Figures

Target	Purpose	Concentration	Vendor	Cat
IgM	Coat	500ng/mL	Southern Biotech	2020-01
IgG1	Coat	500ng/mL	Southern Biotech	9052-01
IgG3	Coat	500ng/mL	Southern Biotech	9210-01
IgG4	Coat	1000ng/mL	BD	555878
IgA1	Coat	500ng/mL	Southern Biotech	9130-01
IgA2	Coat	500ng/mL	Southern Biotech	9140-01
IgE	Coat	500ng/mL	BD	555894
IgM	Secondary	500ng/mL	BD	555781
IgG1	Secondary	500ng/mL	BD	555869
IgG3	Secondary	500ng/mL	Thermo	MH1532
IgG4	Secondary	500ng/mL	BD	555882
IgA1	Secondary	500ng/mL	Invitrogen	MA5-27921
IgA2	Secondary	500ng/mL	Invitrogen	MA5-27923
IgE	Secondary	500ng/mL	BD	555858
IgM	Standard	1000ng/mL	Southern Biotech	0158L-01
IgG1	Standard	500ng/mL	Southern Biotech	0151K-01
IgG2	Standard	1000ng/mL	Biolegend	403601
IgG3	Standard	1000ng/mL	Southern Biotech	0153L-01
IgG4	Standard	1000ng/mL	Biolegend	403702
IgA1	Standard	1000ng/mL	Bio-Rad	HCA189
IgA2	Standard	500ng/mL	Millipore	400110
IgE	Standard	1000ng/mL	Bio-Rad	HCA171

Table 5.1 **Reagents for human ELISAs.**

	Participants (n= 45)
Age years, median (IQR)	34 (31-37)
BMI	
underweight <=18.5	0 (0)
normal 18.5-25	27 (60)
overweight 25-30	10 (22)
obese >=30	6 (13)
missing	2 (4)
Sample collection, days post birth, median (IQR)*	198 (98-293)

Values are expressed as n (%) unless stated otherwise
*missing data from two participant

Table 5.2 **Characteristics of study participants.**

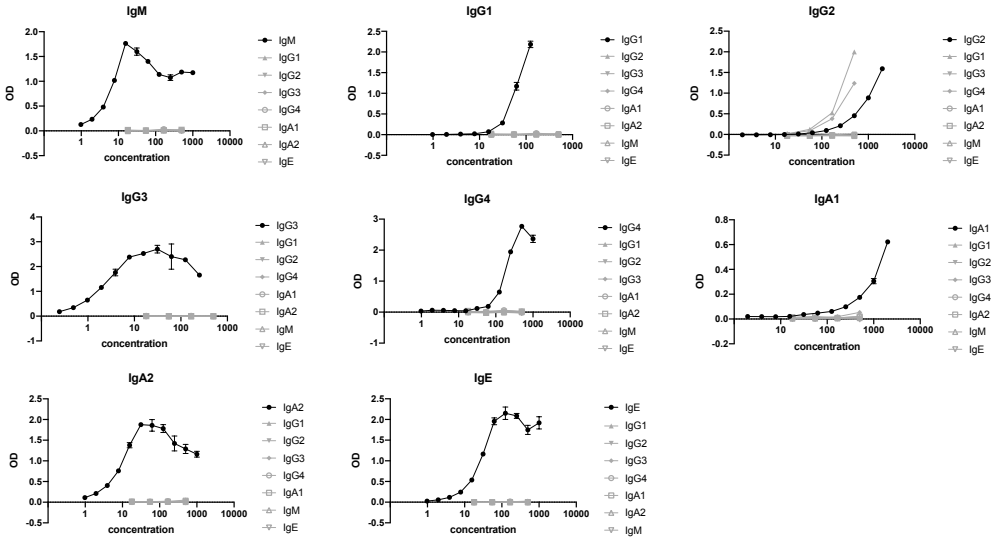
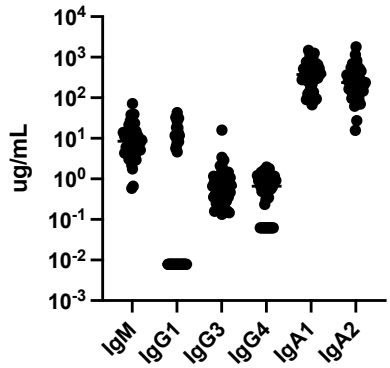
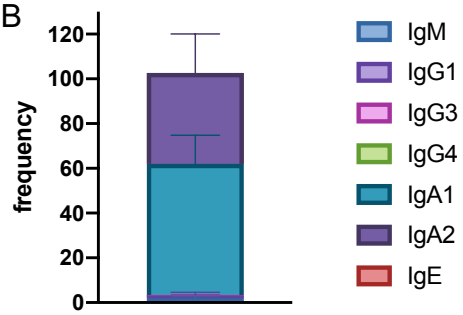


Figure 5.1 **Validation of reagents used in sandwich ELISAs to detect antibody concentration from human breastmilk.** Subclass specific coat and secondary antibodies to detect human IgM, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, and IgE were checked for cross reactivity by incubating with standards from other subclasses (gray). Standard curve is determined using subclass specific standard (black).

A



B



C

	IgM	IgG1	IgG3	IgG4	IgA1	IgA2
concentration ug/mL, median (IQR)	8.5 (4.3-14.3)	.008 (.008-11.6)	.66 (.31-1.1)	.66 (.15-1.1)	374.6 (182.9-587.7)	238.5 (139.8-409.4)

Figure 5.2: **Antibody concentrations found in breast milk**. A) Levels of IgM, IgG1, IgG3, IgG4, IgA1, IgA2 found in breast milk quantitated by ELISA. B) Contributions of different subclasses relative to the total sum of IgM, IgG1, IgG3, IgG4, IgA1, IgA2, and IgE. Bars indicated SEM. C) Median concentrations and the IQR of the different subclasses found in human breast milk. N=45.

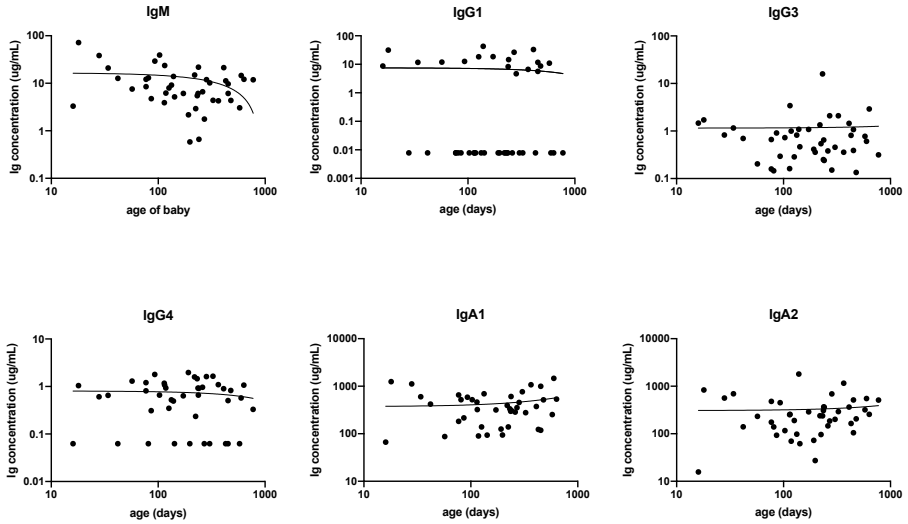


Figure 5.3: **Association between time of lactation and antibody concentration.** Time of lactation, indicated by days post birth is shown against the levels of antibody across the indicated subclasses.

Chapter VI

Conclusions and Future Directions

Breast milk is a key regulator of host-microbiome interactions in early life. Accumulating evidence links breastfeeding with a decreased risk of developing immune-mediated intestinal and metabolic diseases later in life (2-6). As such, current WHO guidelines recommend that mothers exclusively breastfeed for the first six months of life (8). However, this is not always possible for all women and children. While infant formulas are designed to meet the basic nutritional needs of growing infants, they lack certain components of human breast milk. Understanding which components and how these components of breast milk may influence long-term health can inform the development of treatments or supplements to formula to promote the healthy growth and development of neonates, particularly in infants who are not breastfed. Our lab discovered that at homeostasis, in the absence of infection, mice deficient in breast milk antibodies had increased commensal microbes in the mLN and mounted increased mucosal T cell-dependent immune responses in the gut-draining lymphoid tissues at the time of weaning (1). The central hypothesis of my dissertation is that perturbations in adaptive immunity, triggered by the absence of breastmilk antibodies, drive sustained impairments in intestinal immunity. To test this hypothesis, I performed a number of directed studies to evaluate the long-term consequences of maternal antibodies on mucosal immunity and the microbiota. Here I review the major findings of my work, the implications of my studies, and potential future avenues of research.

Here I review some of the major findings of my work and potential future avenues of research.

6.1 Summary

Due to the lifelong persistence of activated adaptive immune cells (121, 122, 167), I assessed whether the increased activation of GC B cells in mucosal lymphoid tissues found in the absence of maternal antibodies correlated with a persistent increase in memory and/or plasma B cells in the intestines. I developed a novel method to isolate and characterize intestinal B cell subsets. As discussed in chapter 2, one key advance of this work was to use cell surface expression of TACI to define gut plasma cells. Using TACI and other surface B cell markers, I comprehensively characterized the intestinal B cell subsets in offspring that received or did not receive maternal antibodies. I observed an equivalent accumulation of memory, GC, and plasma B cell in the intestines and intestinal-associated and lymphoid tissues between matAb+ and matAb- mice. However, when using a fate-tracking system to heritably label GC B cells that arose offspring during the weaning transition, I observed trends of increased early life plasma and memory B cells in the colonic lamina propria of adult mice that did not receive maternal antibodies compared to adult offspring that did receive maternal antibodies. Additionally, a larger proportion of these fate-mapped plasma cells expressed IgA expression, though this was not significant, likely due to this study being underpowered. Further studies are warranted to validate these intriguing findings. While we analyzed fate-mapped GC-derived B cells 13 weeks after labeling, a time course exploring the kinetics of this response could also shed light on how maternal antibodies shape mucosal B cell responses.

GC B cells can differentiate into long-lived antibody-producing plasma cells. Thus, we quantified levels of serum and fecal antibodies between matAb+ vs. matAb- offspring at 7 weeks of age. We observed equivalent titers of all antibody isotypes analyzed between experimental groups. Finally, using mFLOW, we found that the microbiota-reactive antibody response was largely similar between matAb+ and matAb- mice.

I also examined whether the absence of maternal antibodies resulted in durable changes in the composition and/or function of the intestinal microbiota. TD antibodies have been shown to limit the accumulation of wall-adherent microbes and bind to select species found in the commensal microbiota (96-98). We did not observe significant differences in the abundance of wall-associated microbes in the small intestine or the colon in offspring later in life, regardless of maternal antibody status. Furthermore, we did not detect substantial changes in the composition of the intestinal microbiota across different sites of the intestines between experimental groups. As the intestinal commensal microbiota functions to shield the host from infection by pathogenic microbes, we used several infection models to probe whether maternal antibodies were associated with durable changes in the functional capacity of the microbiota. We observed equivalent bacterial CFU in matAb+ and matAb- offspring in all organs tested following infection with the small intestinal pathogen *S. typhimurium* or the colonic pathogen *C. rodentium*. Finally, intestinal length and intestinal transit time of matAb+ or matAb- offspring were equivalent at 7 weeks of age. Further studies assessing the absolute and relative abundance of specific 'border dweller' microbes (like Helicobacter, SFB, Akkermansia) may provide additional insight regarding how maternal antibodies impact host-microbiota mutualism in offspring.

6.2 Future directions

While I did not observe substantial differences in matAb+ and matAb- mice in via the model systems used and experiments I performed, we should be cautious in interpreting these results to suggest that breast milk antibodies do not matter for host-microbiota mutualism. There are several limitations to my studies. While we hypothesize that 7 weeks was an appropriate time to look at consequences in the offspring, we do not formally know if the TD adaptive immune response in progeny persists to this time point. Additionally, while we hypothesize that the dysregulated GC B cell response generated in matAb- mice are to microbial antigens, we do not formally know the antigen-specificity of this response. Finally, it is possible that the absence of maternal antibodies impacts health outcomes not assessed by my studies, such as allergic or autoimmune responses in offspring (224-227). In the next section, I explore the potential future directions of this work.

Study the persistence of B cells activated in early life

We chose to study the immune and microbial responses in offspring at 7 weeks of age based on our hypothesis that the peak of the TD antibody response occurs three weeks after the onset of the GC reaction. In our study using a fate-mapping system, we analyzed labeled GC-derived B cells 13 weeks after initial activation, when offspring were 16 weeks old. We observed a trend toward increased accumulation of early-life GC-derived B cells in the colon of matAb- offspring. In future studies, it would be worthwhile to perform a time course to profile the kinetics of early life GC B cell accumulation in matAb+ and matAb- mice. For example, we might observe significant differences in the accumulation of fate-mapped B cells between experimental groups at week 10. Such a

finding would help inform complementary studies, for example, experiments to test microbiota function.

Identify the antigens activating the elevated Tfh and GC B cell response in matAb- offspring

While we have shown that the dysregulated Tfh and GC B cell responses observed in matAb- mice at weaning are dependent on colonization by commensal microbes, we do not know if this response is directed towards microbes and microbial antigens or, rather, directed towards non- microbial antigens, such as food antigens. In other words, microbes may be required to initiate TD immune responses (e.g., through innate immune activation), but they might not be the target of such responses. Understanding the antigens targeted by the dysregulated TD GC response in weanlings lacking maternal antibodies may inform future functional studies. For example, if the exaggerated GC B cell response evident in offspring lacking maternal antibodies is directed towards food antigens, we can explore whether these mice are more susceptible to developing food allergies.

There are several time and resource-intensive methods that could be used to determine the antigen-specificity of the GC B cells that arise in offspring lacking maternal antibodies. First, one can use a brute force approach and clone the BCR of the B cells found in the offspring in the presence or absence of maternal antibodies, produce protein, and determine its targets. Such an approach has been used by one group assessing IgA+ BCRs from the intestinal lamina propria (93) and has been used by another group to isolate broadly neutralizing antibodies to HIV isolated from a single patient (228). For our research, employing the S1PR2 approach and sorting out the tdTOM+ cells would enable

us to focus on the antigen-specificity of long-lived GC-derived B cells activated during the weaning period. Second, one could use gnotobiotic models of mice colonized with synthetic microbial communities such as the Altered Schaedler's Flora or oligoMM (229, 230). These communities are less complex than the microbiome of an SPF mouse, which narrows down the microbial antigens able to trigger immune dysregulation in offspring lacking maternal antibodies.

Once the antigen specificity of the aberrant GC B cells that arise in matAb- mice is defined, we could use these antigens as molecular "bait" to study the characteristics of the B cell responses over time and the mechanisms regulating their differentiation, accumulation, and persistence (231). Such knowledge would also guide future studies to understand the long-term impacts of the TD GC immune dysregulation arising in matAb- mice on host-microbiota mutualism.

Effect of maternal antibodies on oral tolerance and allergy

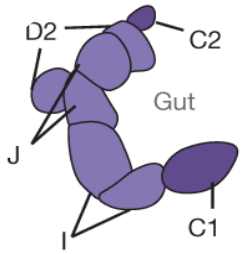


Figure 6.1. **Drainage of the nodes in the mesenteric lymph node (mLN)**.
D= duodenum; J= jejunum; I= ileum, C= colon. Figure adapted from (7).

The mLN is divided into several distinct nodes, each of which drains a different part of the intestine (Figure 6.1) (7). I dissected each node in the mLN and observed an increased accumulation of Tfh cells amongst all nodes in 25-day-old matAb- offspring compared to matAb+ offspring at day 25. Similarly, matAb- pups harbored increased frequencies and numbers of GC B cells across all nodes analyzed at d25 (Figure 6.2A).

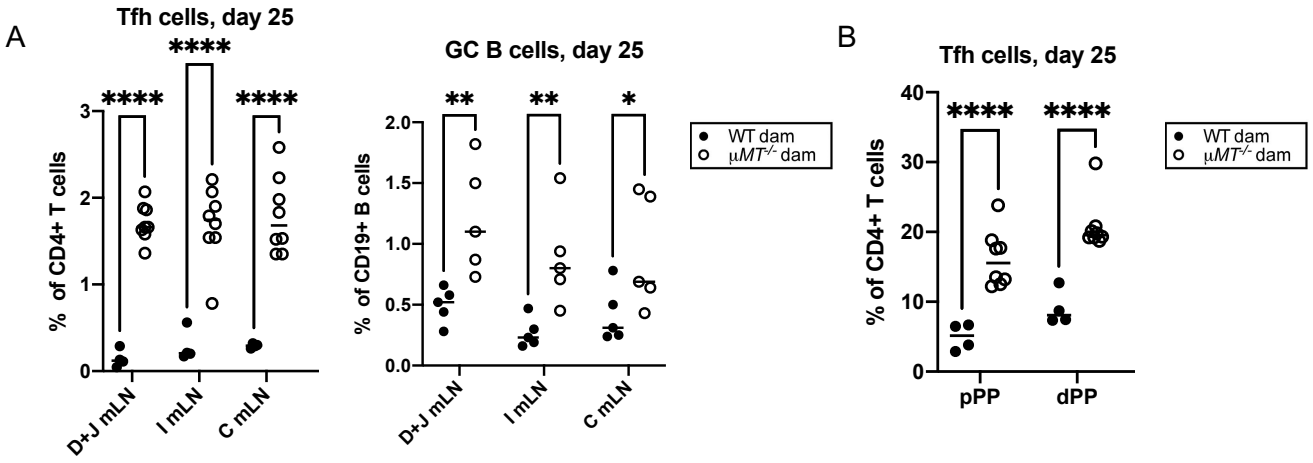


Figure 6.2. **Global activation of Tfh and GC B cells across the intestines.** A) Percentage of Tfh and GC B cells in the indicated parts of the mLN isolated from day 25 offspring born to indicated dams. D= duodenum; J= jejunum; I= ileum, C= colon. B) Percentage of Tfh cells in the indicated parts of the PP of d25 offspring born to indicated dams. pPP= PP within the proximal 5cm of the SI; dPP = PP within the distal 5cm of the SI. muMT$^{-/-}$ mice lack B cells. Data consists of 4-8 mice per group, statistical significance was determined using two-way ANOVA. * $p< 0.05$, ** $p<0.01$, **** $p<0.0001$.

I observed similar results in the Peyer's patches of matAb- mice, which contained increased frequencies and numbers of Tfh cells, whether they were proximal to the stomach or next to the colon (Figure 6.2B). While I did not analyze GC B cell responses in proximal vs. distal PP, we suspect that these tissues also harbor increased frequencies and numbers of these cells. The microbial level and the types of microbes differ in the small intestine compared to the colon. Additionally, the microbial composition differs across the length of the small and large intestines. The fact that we observed similar

expansion of Tfh and GC B cells in matAb- mice, regardless of intestinal location suggests that this response may not be directed against microbes but rather to other widespread antigens, such as food antigens. As we know, microbes are required for the T-dependent immune dysregulation in matAb- mice, we hypothesize that microbes are important for the initiation of altered Tfh and GC B cell responses, for example, by activating innate immune cells such as dendritic cells.

In line with this model, the lab has preliminary data indicating that offspring deficient in maternal antibodies generate elevated TD responses to food antigens. For these experiments, matAb+ and matAb- mice were given OVA-specific OTII CD4 T cells and fed ovalbumin via drinking water at day 18 through day 25 of age. Compared with control matAb+ mice, we observed an increased frequency of OVA-specific Tfh cells in matAb- mice 6-7 days post-OVA administration, which correlated with increased levels of ovalbumin-specific IgE in the ileum at day 43 of age. Currently the lab is working to first, reproduce these results in follow up studies, and second, assess whether there are long-term consequences resulting from the enhanced food-specific Tfh and IgE responses in matAb- mice. The lab is testing whether matAb- mice are more susceptible to diarrhea in an experimental oral allergen-induced diarrhea (232).

TD immune activation is a compensatory response to the absence of maternal antibodies

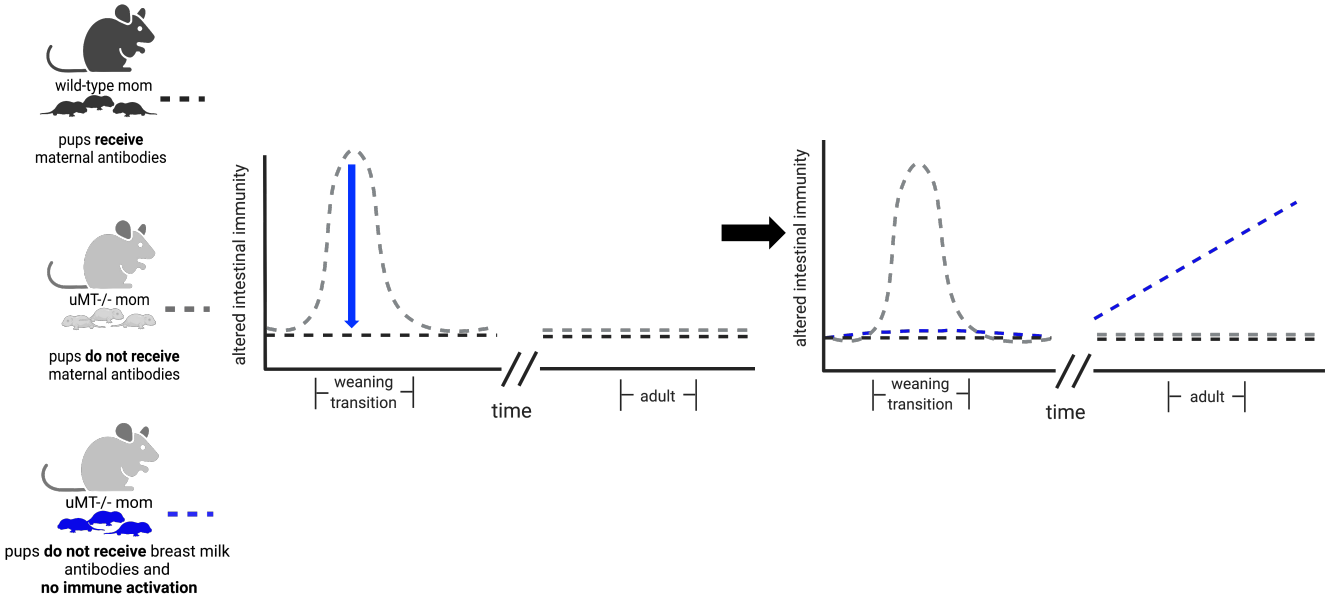


Figure 6.3. **Summary and future direction.** Summary: offspring that did not receive maternal antibodies (grey dashed line) exhibited altered intestinal immunity compared to offspring that received maternal antibodies (black dashed lines). However, both offspring exhibited no difference in intestinal immunity as adults. Future direction: the TD immune activation could be a compensatory response to the absence of maternal antibodies. If this is the case, offspring that do not receive maternal antibodies but cannot generated the TD immune response (blue dashed line) may show altered intestinal immunity as adults.

While we hypothesize that the aberrant TD immune response generated by mice lacking maternal antibodies results in long-term alterations in host-microbiota mutualism, it could also be possible that the TD immune activation functions as a compensatory mechanism to restore homeostasis in the absence of maternal antibodies (Figure 6.3). There are several ways to test this alternative hypothesis. One is to use systems to ablate or block the transient Tfh-GC B cell response in offspring that do not receive maternal antibodies and assess intestinal immunity later in life (Figure 6.4). Though not covered in the scope of this thesis, I did test several models designed to ablate the activation of Tfh

and GC B cells. First, we generated Bcl6f/f-dLck.Cre mice. Bcl6 is the master transcription factor for Tfh cells, and the distal Lck (dLck) promoter is expressed by all T cells (233). Thus, Tfh cell development should theoretically be ablated in Bcl6f/f-dLck.Cre mice. Unfortunately, during the course of generating these mice, we discovered through a collaborator that Tfh cell ablation was not 100% efficient in this mouse line. Instead, a few Tfh "escapees" were capable of expanding in unmanipulated mice and driving a robust GC reaction, particularly at mucosal sites like the PP, which exhibit continual GC activity. In fact, our collaborator found that the TD antibody response to SFB was unchanged in

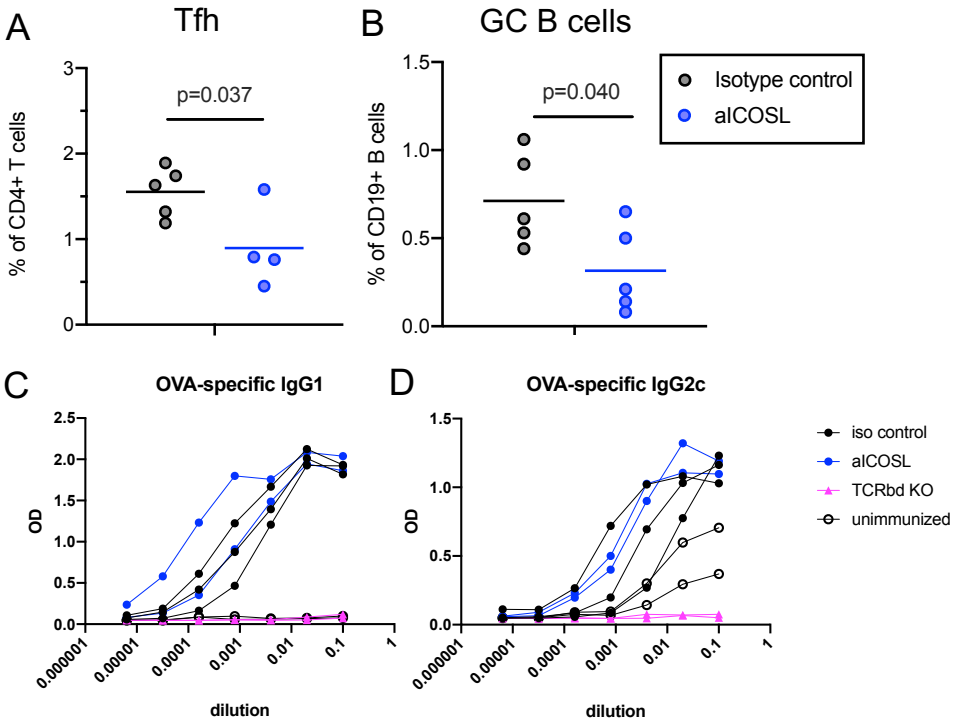


Figure 6.4: **ICOSL blockade on Tfh and GC B cells and antibody responses to NP-OVA**. A, B) Percentage of Tfh cells (A) and GC B cells (B) in the mLN of day 25 maternal antibody deficient mice injected with 75mg of aICOSL or isotype control antibodies retro-orbitally at day 18 and day 21 of life. C,D) Optical density (OD) of NP-OVA specific IgG1 (C) and IgG2c (D) antibodies in the serum 14 days after subcutaneous injection of 50ug of NP-OVA in 500uL of CFA at day 21 of age. Mice were treated with aICOSL as in A, B. TCRbd KO mice have no T cells. Data consists of 2-5 mice per group, statistical significance was determined using an unpaired student's t-test.

control Bcl6f/f mice compared to Bcl6f/f-dLck.Cre mice (unpublished observations). I also tested a blocking antibody specific to inducible T cell costimulatory ligand (ICOSL), which is a molecule found on CD4 T cells and is important for their differentiation into Tfh cells and the formation of GC B cells (234). While this model resulted in durable decreases in both the Tfh and GC B cell frequency and number (Figure 6.4A-B), it did not alter the TD antibody response to mice immunized with ovalbumin (Figure 6.4C-D). Thus, additional models should be investigated. Testing the CD40L blocking antibody could be of promise. Additionally, the lab has recently generated Bcl6f/f-CD4-Cre^{ERT2} mice which our collaborator has successfully used to selectively and efficiently ablate Tfh cells *in vivo*. This model has the additional benefit of enabling transient ablation of Tfh cells via tamoxifen-driven Cre activity, as opposed to lifelong ablation of these cells.

Although a small part of what is a vast field of study, my work proudly contributes to the collective understanding of how maternal antibodies impact intestinal homeostasis and health in offspring.

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