The Role of Enteric RORγt+ Regulatory T Cells in Altering Systemic Vaccine Responses Following Oral Antigen Exposure

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Abstract

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Many promising vaccine candidates and licensed vaccines lead to variable immune responses within humans and can fail to elicit protection against critical pathogens. Recent studies suggest that environmental exposures in the gastrointestinal (GI) tract could contribute to a reduction in vaccine efficacy via immune tolerance at this site. Tolerogenic mechanisms in the GI tract are partly achieved by the characteristic high abundance of regulatory T cells (Tregs). Subsets of regulatory T cells have been recently described; specifically, RORγt+ FoxP3+ Tregs could contribute to tolerating orally acquired antigens. This subset relies on the presence of commensal bacteria for development and has been described in regulating Type 2 associated

immune responses. Currently, it is unclear which Treg subsets control systemic vaccine responses following oral antigen pre-exposure.

In this study, we implemented a conditional RORγt+ Tregs knock-out (cKO) mouse model to examine the role of this subset in the suppression of systemic antibody titers after oral exposure to vaccine antigen. Following oral exposure to the model antigen ovalbumin (OVA) prior to immunization, we found similar induction of vaccine-induced antibody responses in mice lacking RORγt+ Tregs compared to sufficient controls. cKO mice exhibit higher frequencies of IL-33R+ and GATA3+ Tregs at baseline in the gut, suggesting that there is possible compensation from other subsets upon loss of expression of RORγt+ in Tregs. By utilizing a variety of adjuvants in our model, we have determined that vaccine-specific IgG1 is suppressed following oral antigen exposure, but not IgG2c, regardless of adjuvant strength or skew. In RORγt+ Treg cKO mice, use of different adjuvants led to similar findings; no differences were observed in the maintenance of tolerance to systemic OVA vaccination in cKO mice upon use of various adjuvants. Methods were explored to understand antigen-specific CD4+ response upon oral exposure prior to systemic vaccination; adoptive transfer of OVA-specific OT-II cells will continue to be explored in future studies. Taken together, our data suggest that RORγt+ Tregs are not solely responsible for controlling tolerance to oral antigens in a murine model of tolerance to a systemic vaccine.

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ABBREVIATIONS

Ad – adenovirus

AIM – activation-induced markers

Aire – autoimmune regulator

ALDH – aldehyde dehydrogenase

APC – antigen presenting cell

AS – adjuvant system

BCG - Bacille Calmette-Guérin

BMI – body mass index

CD – cluster of differentiation

CFA – Complete Freund's Adjuvant

cKO – conditional knock-out

cLP – colonic lamina propria

CMV – cytomegalovirus

COVID-19 – Coronavirus disease 2019

CTLA-4 – cytotoxic T-lymphocyte-associated protein 4

DAMPs – danger-associated molecular patterns

Das – Dasatinib

DC – dendritic cell

DNA – deoxyribonucleic acid

DOL – days of life

DSS – dextran sodium sulfate

DT – diphtheria toxin

EBV – Epstein-Barr virus

FBS – fetal bovine serum

Fc – crystallizable fragment

FoxP3 – forkhead box P3

FT – flow through

GALT – gut-associated lymphoid tissue

GAPs – goblet cell-associated antigen passages

GC – germinal center

GF – germ-free

GI – gastrointestinal

GITR – Glucocorticoid-induced TNF receptor-related protein

gp – glycoprotein

hClip – human CAP-Gly domain containing linker protein

hDTR – human diphtheria toxin receptor

HIV – human immunodeficiency virus

HLA – human leukocyte antigens

HVTN – HIV Vaccine Trials Network

IBD – inflammatory bowel disease

ICS – intracellular cytokine staining

IEL – intraepithelial lymphocyte

IFN – interferon

Ig – immunoglobulin

IL – interleukin

ILC – innate lymphoid cell

i.p. – intraperitoneal

IPEX – Immune dysregulation, polyendocrinopathy, enteropathy, X-linked

LAG-3 – lymphocyte activation gene-3

MAIT – Mucosal-associated invariant T

MHC – major histocompatibility complex

MLN – mesenteric lymph node

MPL – monophosphoryl lipid A

mRNA – messenger ribonucleic acid

MR1 – MHC-I related molecule 1

MyD88 – myeloid differentiation primary response 88

neg – negative control

NLRP3 – Nod-like receptor family pyrin domain containing 3

Nod2 – nucleotide-binding oligomerization domain containing 2

Nrp1 – neuropilin 1

OPV – oral polio vaccine

OVA – Ovalbumin

PAMP – pathogen-associated molecular pattern

PBS – Phosphate-Buffered Saline

pos – positive control

PP – Peyer's patches

pTreg – peripherally-derived Treg

RAG-1 – recombination activating gene 1

RAR - retinoic acid receptor

RA – retinoic acid

RIG-I – retinoic acid-inducible gene-I

RORγt – RAR-related orphan receptor gamma

SARS-CoV-2 – severe acute respiratory syndrome coronavirus 2

SCFA – short-chain fatty acids

SFB – segmented filamentous bacteria

siLP – small intestine lamina propria

SNP – single nucleotide polymorphism

SPF – Specific-pathogen free

Tconv – conventional T cell

TCR – T cell receptor

T_{FH} – T follicular helper

TGF-β – Transforming growth factor beta

T_H – T-helper

TIV – trivalent influenza vaccine

TLR – toll-like receptor

TNF – tumor necrosis factor

Treg – regulatory T cell

T_{RM} – T resident memory

TT – tetanus toxoid

tTreg – thymically-derived Treg

VLP – virus-like particle

WT – wild-type

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Chapter 1. INTRODUCTION

1.1 VACCINES

Globally, it is essential to create effective vaccines to reduce the spread of pathogens. While research in vaccinology has advanced tremendously since first conception by Edward Jenner in the 1790s, this remains an area of study with many open questions[1]. The "big three" infectious diseases, human immunodeficiency virus (HIV), malaria, and tuberculosis, have had vaccine candidates or licensed vaccines with mixed success, leaving large opportunity for efficacy improvement[2]. A vaccine elicits protection from potentially harmful infections by conditioning the immune system to respond properly upon pathogen detection[3]. By prior exposure to an infectious agent or elements of the infectious agent, our immune system creates a memory response to better position itself upon infection by that same bacterium, virus, or parasite. The main mechanism utilized by many vaccines is creation of antigen-specific B cells that produce antibodies that can contribute to protection. The T cell response is also important, as helper T cell subsets contribute to activation of antibody-producing B cells[4]. It is essential to understand the immune response that follows upon immunization and what factors contribute to efficacy, or what may be hindering a successful and productive response.

1.1.1 *Heterogeneity in Vaccine Efficacy*

Multiple factors contribute to successful vaccines and immune responses upon vaccination. Recently, amid the COVID-19 pandemic, vaccine skepticism and hesitancy increasingly became a problem, as misinformation was widespread[5]. The belief of well-trusted sources and advocacy for science in a way previously unheard of became key to participation in

vaccine efforts to reduce the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Looking forward to future vaccine research against potential pandemics, it is critical to understand how both social, along with basic scientific factors, contribute to successful vaccine campaigns.

Aside from acceptance of vaccines, factors including lifestyle, microbiome, pre-exposure to infections, host genetics, vaccine factors (type of vaccine, adjuvant, timing), and more contribute to vaccine efficacy[4,6-8]. A current area of increasing study in the field is the heterogeneous effect vaccines have on individuals and what factors contribute to differential outcomes[9,10]. Differences in behavior, including diet, exercise, and sleep have been linked to vaccine success[6]. Both a high body mass index (BMI) and malnourishment have been correlated with a reduction in antibody responses to the hepatitis B vaccine and others[11,12]. One study also reported that the trivalent influenza vaccine (TIV) is more effective in adults over the age of 62 that exercise three or more times a week compared to sedentary counterparts, indicating that an active lifestyle could be beneficial in vaccine-induced immunity[13]. Sleep deprivation has also been linked to reduced antibody titers upon hepatitis A and hepatitis B vaccination[14,15]. Other behavioral factors that may contribute to vaccine efficacy are alcohol consumption, stress levels, physical environment, and smoking[6].

Genetics, age, and existing immunity can also play a role in determining outcomes upon vaccination[7,9]. Polymorphisms within immune response genes including those that encode for human leukocyte antigens (HLA), cytokines, and cell surface receptors play a role in controlling downstream responses after immunization[16,17]. In a study of measles-specific vaccine-induced immunity, single nucleotide polymorphisms (SNPs) within the genes that encode toll like receptors 2 and 4 (TLR2 and TLR4) were associated with increased or decreased antibody levels,

respectively[18]. Epigenetic factors also contribute to heterogeneity; females exhibit increased expression of TLR7 in B cells due to reduced deoxyribonucleic acid (DNA) methylation than males upon influenza vaccination in mice, leading to a more effective response[19]. Biological sex is also relevant, as a meta-analysis suggests that COVID-19 vaccines are more effective in men than in women[20]. Both infants and elderly populations have altered vaccine-induced immunity compared to adults[6]. Though some vaccines, like Bacille Calmette-Guérin (BCG), are effective when given to infants, others are less effective due to interference by maternal passively transferred antibodies[21]. The immature immune system of infants is often not able to recognize recombinant vaccines unless they are conjugated[22]. Elderly people also have reduced responses to certain vaccines including hepatitis A and B and the influenza vaccine[6]. Individuals over the age of 80 also have a reduction in antibody production compared to younger groups to the SARS-CoV-2 vaccine[7,23]. Vaccine factors and pre-existing immunity also contribute to efficacy. One study suggests that children infected with Epstein-Barr virus (EBV) respond poorly to measles vaccination compared to uninfected, however, those that are co-infected with EBV and cytomegalovirus (CMV) show typical vaccine responses[24]. Factors including vaccine dose, schedule, adjuvant, route, and site also contribute to varying outcomes, suggesting that each of these needs to be optimized for maximal efficacy[6].

Finally, the gut microbiome has also been linked to vaccination outcomes[24-25-32]; this will be discussed in detail in section 1.2.3. In summary, understanding what factors contribute to vaccine efficacy and how these could be leveraged to create more effective vaccines for potentially harmful pathogens is essential for improving human health.

1.1.2 *Types of Vaccines*

The success of vaccines in humans depends on many factors mentioned above, including vaccine design and the type of product that is used to stimulate the immune system. Some types include live attenuated pathogens, heat killed or inactivated pathogens, purified pathogen proteins or polysaccharides, toxoid, virus-like particles (VLPs), viral vectors, and most recently, nucleic acid-based vaccines[1,33]. Each of these have been used in clinical trials or are in currently licensed vaccines; pros and cons exist for each type. Understanding the potential pitfalls for each is critical for vaccine design.

Live-attenuated vaccines were the first type widely used in humans during the smallpox eradication efforts that began in the late 18th century[1]. These vaccines are comprised of a living organism that has been attenuated to prevent active disease but is able to trigger an immune response upon primary exposure. Thus, during secondary exposure to the pathogen itself, the immune system can leverage its memory against the organism or virus that was developed upon the previous vaccine event[34]. Killed or inactivated vaccines were developed next by utilizing heat or chemicals to prevent the organism from replicating or causing infection, while maintaining the structural components that stimulate an immune reaction upon immunization[1]. Because the strong response elicited by a live-attenuated vaccine has the potential to cause unintentional harm and pathology, inactivated vaccines are useful for individuals with suppressed immune systems[34]. Inactivated vaccines were used in the late 19th century to prevent cholera, typhoid, and the plague[1].

Subunit or protein vaccines were the next approach utilized for developing vaccines against hepatitis A and B, influenza, shingles, and many others since they were first administered the 1920s[1]. These are created by administering either a partial or full protein, typically found on

the surface of the pathogen. Exposure to these surface antigens allows for development of antibodies that can bind to and neutralize the infection upon subsequent exposure to the pathogen. Toxoids can also be used as the antigen given in a vaccine, as is the case for diphtheria and tetanus vaccines[1]. Adjuvants are immunostimulatory substances that enhance the immune response when administered along with a vaccine antigen[35]. These can be important for developing memory responses upon vaccination and are described further in section 1.1.3. These approaches have evolved over the past 100 years and are some of the most common vaccines in development, though in some cases, they face the challenge of providing enough immunogenicity to create a strong and lasting response over time.

VLPs are a non-infectious nanoparticle of assembled proteins that are synthesized from pathogens[36]. Because these are engineered particles and can be designed to contain multiple replicates of the same protein, they can be more immunogenic than conventional subunit vaccines. It is also safe and fast to produce VLPs, making them a promising technology for future candidates[36].

Viral vector vaccines utilize a well-characterized attenuated or replication-impaired virus, such as adenoviruses (Ad)[37]. This platform functions by administration of such viruses that have been genetically engineered to encode key antigens from pathogens[38]. Expression of these genes and exposure to the host will allow for immune responses toward the pathogen protein, leading to generation of memory T and B cell responses. Ad vector-based vaccines do not require an adjuvant, as the virus itself is able to stimulate innate immune pathways including TLRs and the retinoic acid-inducible gene-I (RIG-I) pathways. Ad vectors have been used in trials for HIV vaccines and are also used in vaccines for Ebola and SARS-CoV-2[37].

Nucleic acid vaccines, including the messenger ribonucleic acid (mRNA) platform used in the COVID-19 vaccines produced by Pfizer and Moderna, are now widely accepted as successful methods of delivery[33]. During the COVID-19 pandemic, it was paramount to develop a vaccine as quickly as possible to reduce spread of infection, especially for aged or immunocompromised individuals at high risk of increased morbidity. RNA vaccines can directly carry sequences that code for pathogen antigens to antigen-presenting cells (APCs)[39]. Once internalized, the mRNA can be translated within the cytoplasm[33]. These proteins can be processed and presented on the APC surface, leading to T cell activation. Previous work by the Weissman group paved the way for such vaccines; in 2005, the group reported that mRNA could be used as a therapeutic[40]. They reported that bacterial and mitochondrial RNA was able to stimulate dendritic cells (DCs) and TLR-expressing cells to secrete cytokines and activate the immune system. This immunogenicity, however, has been reported to be reduced compared to other vaccine platform, as it is now evident that the COVID-19 mRNA-based vaccines wane relatively quickly compared to vaccines for other viruses in use[33].

Though these vaccines were able to provide a quick solution to the pandemic and received Emergency Use Authorization in the US in under one year, which was a record breaking time, further research is necessary to establish how mRNA vaccines can lead to lasting protection[41]. DNA vaccines have also been studied for their potential to prevent infection and disease. These utilize a similar mechanism of action to mRNA vaccines, though the DNA can also be expressed by somatic cells and presented to CD8 T cells[33]. Thus, multiple approaches to create safe and effective vaccines are currently underway globally. By examining correlates of protection from vaccine trials and utilizing novel methods of vaccine delivery systems, we

increase the potential for developing solutions to potentially harmful pathogens, both existing and emerging.

1.1.3 *Adjuvants*

Adjuvants are substances that enhance the immune reaction upon antigen exposure in a vaccine[35,42,43]. Though adjuvants have been utilized in many licensed vaccines and candidates undergoing trials for almost 100 years, there remains a knowledge gap in understanding precise mechanisms of action and what components of an adjuvant contribute to enhanced vaccine efficacy[44]. An adjuvant is necessary for achieving protection from pathogens in many cases, especially for subunit vaccines where exposure to a protein alone without stimulation of the innate immune system is ineffective[35]. To mount an effective immune response upon vaccination, multiple signals are necessary. Signal 1 is provided by a given antigen to the pathogen itself, which can be in the form of a protein (as in subunit or protein vaccines), or a region that encodes the given protein (as in viral vector-based, or nucleic acid vaccines)[34]. The second signal stimulates the innate immune response and informs the host that it needs to respond to the given antigen. Signal 2, which can occur by utilization of adjuvants during vaccination, indicates that the given antigen is foreign and potentially dangerous, and that the host needs to respond[39]. While some vaccines can achieve protection from infection or disease in the absence of an added adjuvant, it is critical to understand how these substances can act to enhance vaccine responses and in which scenarios they are necessary. Further research on how adjuvants enhance vaccine responses and how they could potentially be improved is critical to the field.

First utilized in 1926, alum is historically one of the most widely used adjuvants for vaccines in humans[44,45]. Alum is an insoluble aluminum salt that was the only adjuvant used in licensed vaccines for nearly 70 years until the emergence of oil-in-water emulsions[42]. Vaccines that protect against diseases including hepatitis B, pertussis, pneumococcus, diphtheria, tetanus, meningococcus, and human papilloma virus (HPV) have used alum as an adjuvant in formulation[42,46,47]. Other aluminum salts have also been tested as adjuvants with varying success compared to alum[44]. Alum was originally classified as aluminum hydroxide and aluminum sulfate, though aluminum phosphate is now one of the main components of commercially available vaccines[43].

Alum preferentially skews the immune response toward Type 2 induced immunity by leading to an increase of T helper 2 (Th2) cells, immunoglobulin G1 (IgG1) and IgE, in contrast to other adjuvants such as Complete Freund's Adjuvant (CFA), which skews toward Th1 and IgG2a in studies using BALB/c mice[48]. The mechanism of action of alum has been studied over time; once thought to involve TLR signaling, it has been established to be independent of myeloid differentiation primary response 88 (MyD88) and TLRs[49]. It is thought that alum may activate the Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome pathway, though some data is contradictory to this point[50,51]. Alum is also able to enhance the immune system by causing local tissue damage at the site of injection and activating inflammatory DCs[52]. This occurs via upregulation of major histocompatibility complex class II (MHCII), CD40 and CD86 on DCs[44,53]. Damage to the local tissue also recruits neutrophils to the site, where they are able to release extracellular traps comprised of chromatin[54]. Though the exact role extracellular DNA plays in enhancing the activity of alum is not known, it could be an important factor in how the adjuvant functions[42].

Another type of adjuvant that has become more prominent since the 1990s is oil-in-water emulsions including MF59 developed by Novartis or the related reagent Addavax[55]. MF59 has been used in seasonal influenza vaccines in Europe and has been used in 100 million people as of 2021[42]. In the influenza vaccine, MF59 contributes to the development of virus specific antibodies, as well as memory T and B cell populations, leading to greater likelihood of protection upon challenge[56]. Squalene emulsion-based adjuvants function by rapidly recruiting CD11b+ cells to the injection site at a greater rate compared to alum[57]. Many studies have contradicted previously reported results, and the exact mechanism of action of MF59 and related adjuvants remains uncertain, though recent work suggests that CD8 T cells are triggered upon immunization[55]. MHCII expression is also required for the action of MF59, suggesting that CD4 T cells are likely also involved in immune activation[56].

Adjuvant systems including AS01 are comprised of multiple immunostimulatory compounds that can act synergistically to elicit a strong immune response[58]. Currently, AS01 is used in the malaria vaccine RTS,S, as well as the licensed shingles vaccine, Shingrix, recommended for older adults in the United States[42,59]. AS01 contains 3-O-desacyl-4'-monophosphoryl lipid A (MPL) isolated from *Salmonella minnesota* along with a saponin QS-21, a plant extract derived from tree bark of *Quillaja saponaria*[58]. AS01 also contains a liposome to deliver the components along with the vaccine antigen[42]. This adjuvant is a TLR4 agonist, as MPL acts to stimulate this pathway, while QS-21 activates caspase-1. These two pathways work synergistically to elicit the innate reaction necessary for adjuvant function, as neither component alone leads to as potent of a response. This indicates the potential for multiple adjuvant systems to act together for increased adaptive immune responses upon vaccination and could be relevant for development of novel adjuvants[43,58].

This is not an exhaustive review of all adjuvants currently being used in vaccines; adjuvants including CpG, AS04, poly I:C, and others contribute to the success of licensed vaccines, with more in development. While each of these adjuvants discussed contribute to enhanced vaccine efficacy and are currently used in licensed vaccines, it is still unclear how some of these function to achieve protection, making it clear that a deeper understanding of the modes of action of these adjuvants could pave the way for more rational vaccine design[42]. Emerging evidence points to the importance of danger-associated molecular patterns (DAMPs) and their potential role in stimulation of the innate immune system during vaccination with an adjuvant[60]. There is currently much research on how adjuvants can be enhanced and utilized to improve vaccine strategies that will be used in clinical settings.

1.1.4 *Tolerance to Vaccines*

While many licensed vaccines and vaccine candidates show varied levels of efficacy, the factors that contribute to protection from disease are incompletely understood. Vaccines for HIV, BCG and rotavirus have all been reported to be affected by previous exposures through the oral route, though likely, various mechanisms affect subsequent immune responses.

Since the virus was reported in the early 1980s, efforts for developing an HIV vaccine have been underway with varying levels of success[61]. HIV remains one of the largest contributors to the global burden of disease among infectious agents with 38.4 million people living with HIV in 2021[62]. While there are successful prophylactic strategies in place to reduce the spread of the disease, vaccination remains as the most effective method for reducing the spread of this virus[63,64]. To date, the most effective vaccine trial for HIV is the RV144 trial, which was demonstrated to have ~31% efficacy on the population examined in Thailand[65].

Vaccine-induced heterogeneity partially explains the differential protection among individuals[10]. This study led to several findings essential to moving the HIV vaccine field forward, including an understanding of the correlates of protection. These include high levels of circulating envelop-specific IgG, specifically for the V1V2 region of the glycoprotein 120 (gp120) protein[66]. While current efforts are underway to lead to a more efficacious vaccine, examining previous trials and factors that contributed to ineffective responses are essential for moving the field forward.

Analysis of other vaccine trials as well as natural infection suggests that antibodies specific for another HIV envelop protein, gp41, are non-neutralizing and do not control viremia[67]. In contrast to the protection that can be achieved via antibodies specific for gp120, the gp41 response that is elicited early in infection (within the first two weeks) is frequently polyreactive. Multiple studies have focused on these non-protective responses against gp41, and what factors could be contributing to the initial expansion of B cell populations that produce these antibodies[31,67-70]. Analysis of gp41 specific responses points toward cross reactivity between antibodies specific for the HIV-1 envelop protein and gut microbiota[69]. Prior to the onset of HIV-1 infection, there exists a population of memory B cells in the intestine that is specific to commensal antigens[68]. These studies found that among terminal ileum B cells that were specific for HIV-1 gp41, 82% were also reactive to commensal microbes[68]. Upon exposure to the virus, the gp41-specific response can be triggered by the baseline presence of these cross-reactive commensal-specific cells, leading to a non-protective response against the virus. This is known as the diversion hypothesis, as it suggests one explanation on how pre-exposure to certain gut commensals could impact downstream non-productive responses upon subsequent immunization. Follow up studies examined a HIV-1 DNA prime-recombinant Adenovirus Type 5 (rAd5) vaccine candidate, which did not lead to protection against HIV-1 acquisition[69].

Sequencing of the resulting immunoglobulin heavy-chair repertoire suggests that antibodies specific for intestinal microbiota developed upon immunization. Specifically, the authors found that 47% of gp41 specific antibodies were also able to bind to *E. coli* RNA polymerase[69]. Sequence homology between the two proteins may contribute to this effect.

Subsequent studies from our group have followed up on the hypothesis that prior to vaccination, pre-existing antibody repertoires could be specific for the gp41 protein[31,70]. By assessing microbiota composition of individuals in a HIV vaccine trial (HVTN 096) and gp41 IgG binding, a study from our group was able to establish correlations between commensal clusters and correlates of vaccine protection[31]. The HVTN 096 trial utilized a gp120 protein in combination with a DNA plasmid or vaccina virus containing DNA that encoded Env, Pol, Gag and Nef from HIV-1. Here, rectal swabs were collected from 21 participants at multiple timepoints to assess correlations between microbiota and responses upon vaccination[31]. Sequencing analysis of microbiota communities in conjunction with vaccine-induced immune responses found that specific groups of commensals correlated with specific outcomes upon vaccination. For example, one cluster of families was associated with low gp41 non-neutralizing IgG titers and high gp120 IgG titers at 6.5 months after vaccination, while another group of bacteria was associated with high gp41 and low gp120-specific titers. This study also observed that the richness in community structure was correlated with the magnitude of immunogenicity to the vaccine[31]. A follow up study examined 12 HVTN vaccine trials to determine if microbiota composition affects baseline gp41 titers and how this controlled the subsequent response to the vaccines tested in each trial[70]. In individuals with no known exposure to HIV-1 or related vaccines, we found positive associations between anti-gp41 IgG at baseline and taxa in the *Eubacteriales* order; this did not affect the ability of participants to produce gp120 specific

antibodies upon immunization[70]. These data indicate that microbiota community structure can influence outcomes upon vaccination.

Vaccines developed for other infectious diseases including tuberculosis are also affected by the gut microbiota or oral exposure prior to immunization[29,71]. Most recently, pre-existing antibodies that are specific for gut commensals and cross-react with SARS-CoV-2 have been described and could affect immunity to the virus[72]. BCG was developed in the 1920s to protect against tuberculosis and is one of the first licensed vaccines[73]. One study examined the effect of pre-exposure to environmental mycobacteria on subsequent vaccine response to BCG in C57BL/6 mice[71]. In these experiments, authors administered *Mycobacteria avium* to mice orally over a 4-week period. After one week, mice received an intradermal BCG vaccination, and assessed for vaccine responses. Mice that received oral M. avium prior to immunization exhibited higher numbers of forkhead box P3 (FoxP3) expressing regulatory T cells (Tregs) in the spleen and lungs. This group also had a higher burden of the *M. avium* CFU upon challenge, indicating a less effective response to the vaccine. Tolerance to the intradermal BCG vaccine was overcome by altering the route of vaccination; pulmonary BCG was not affected by pre-exposure to *M. avium* prior to immunization[71]. These data support previous findings that pre-exposure or antigens similar to that in the vaccine are able to suppress downstream immune responses.

Oral vaccines also present a challenge when designing candidates that can stimulate the immune system and overcome tolerance, while avoiding immunopathology[74]. Factors including dose and presence of an adjuvant are critical to consider when designing oral vaccines, as too large of a dose can stimulate tolerance, and lack of danger signals will not trigger a response[75,76]. These hurdles present an open area of research to find strategies to overcome immune tolerance

that occurs in the gut to stimulate an appropriate immune response upon vaccination. Understanding the underlying biological mechanisms that occur in the gut and in tolerance is critical for making progress for effective vaccines and reducing the spread of infectious diseases globally.

1.2 The Gut Immune System

In the intestine, it is critical that the local immune system protect the host from invading pathogens while avoiding inappropriate inflammation to innocuous substances, like dietary proteins and the microbiome[34]. Many aspects of the gut, including immune cell subsets, are critical in distinguishing between harmful or harmless exposures at this site. Structural elements provide niches for specific cell. Signals from the gut microbiota to immune cells also shape the landscape at this site. Here, these will be discussed with an emphasis on how these contribute to shaping local regulatory T cell (Treg) responses that could affect the systemic immune system.

1.2.1 *Structure*

Along the gastrointestinal (GI) tract, multiple structural components contribute to barrier integrity and allow for nutrient absorption for the host (Figure 1.2.1.1). The full GI tract consists of the oral cavity, esophagus, stomach, and the small and large intestines, each with unique anatomy to allow for the appropriate function[77]. In humans, the small intestine is typically 3-5 meters long, containing multiple segments: the duodenum, jejunum and ileum[78]. At this site, villi, or folds in the intestine, increase the surface area, allowing for maximal nutrient reabsorption from the diet[79]. Epithelial cells are coated with a layer of mucus, which adds an additional barrier to commensal microbiota in the lumen. On the basolateral (or internal) side of

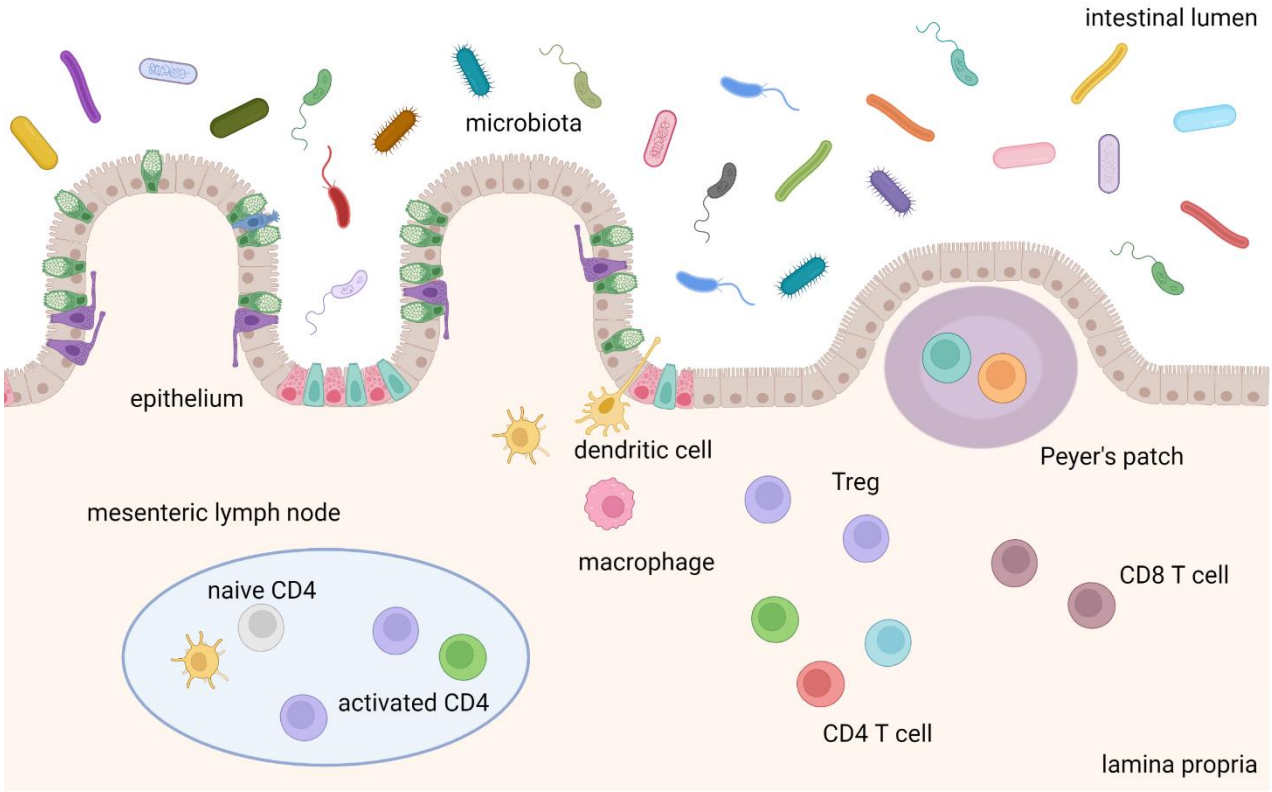

Figure 1.2.1.1 – Representation of the cells in the gut-associated lymphoid tissue.

The intestinal lumen and microbiota are separated from the internal lamina propria by the epithelial barrier. Upon antigen uptake by dendritic cells, these cells can traffic to the mesenteric lymph nodes, where antigen can be presented to naïve CD4 T cells. Once these cells are activated, they can traffic back to the lamina propria. T cells are necessary in the gut to prevent infection from potential invading pathogens through the GI tract, while regulatory responses are necessary to control inappropriate inflammation to innocuous exposures at this site.

the epithelial layer lies the lamina propria; this connective tissue helps to maintain the structure of this site and contains immune cell populations that are critical for immunosurveillance and tolerance in the gut[80]. Local structures in the mucosa known as Peyer's patches (PP) contain follicles and germinal center (GC) B cells that contribute to intestinal immunity. Lastly, the mesenteric lymph nodes (MLN) drain this site and contain important immune populations[34].

Within the epithelial layer, multiple cell types are present, including enterocytes, goblet cells, Paneth cells, M cells, and enteroendocrine cells[79,81]. Stem cells contained within crypts allow for differentiation of continuous renewal of these cells[82]. Enterocytes are the major cell type in the intestinal epithelial layer; these epithelial cells help absorb nutrients including ions, water, sugars, peptides, and lipids[79]. These cells are also able to secrete IgA into the lumen. Goblet cells are the main mucus producers in the intestine via production of the secretory mucin glycoprotein MUC2. Impaired MUC2 leads to a greater susceptibility of the host to colitis upon treatment with dextran sodium sulfate (DSS)[83]. Mucus produced by goblet cells in the intestine also provides an additional barrier between commensal bacteria and the epithelial layer; the mucus layer acts as the first innate defense system encountered by pathogens that invade via the GI tract[84]. Mucins, or the gel-like molecules form a barrier to create mucus, can multimerize and act to trap bacteria. These mucins can also provide additional protection by trapping other antimicrobial agents, including defensins and IgA[84]. Multimerization of these proteins leads to a dense layer of mucus on the luminal side of the intestinal tract, though some pathogens have evolved mechanisms to penetrate this layer and invade host cells.

Granules produced in Paneth cells that contain defensins, lysozyme and other antimicrobial peptides are released at the apical surface into the lumen[85]. These play a crucial role in host defense against invading pathogens, though can also be released at steady-state in the absence of infection[86]. M cells are a type of antigen-delivery cells, where they undergo a process called transcytosis to transport molecules from the lumen to the lamina propria[87]. Hormones that regulate gut function are secreted by enteroendocrine cells and regulate some of the processes carried out by other cell types[88]. Each of these cell types provide the host with the ability to

acquire essential nutrients to maintain critical functions while contributing to barrier function at this site.

The small intestine lamina propria (siLP) also contains cells that contribute to protecting the host from invading pathogens, while balancing tolerogenic mechanisms that are specific for innocuous encounters at this site. This compartment includes effector T cells and Tregs, along with many other immune cell populations[34]. This layer is surrounded by smooth muscle fibers that provide structure to this site[89]. The site also contains a blood supply and connects to the broader circulatory network. Further discussion of immune cells in the siLP are discussed in the following section.

1.2.2 *The GALT Immune System*

The mucosal immune system in the gut, known as the gut-associated lymphoid tissue (GALT) is critical for differentiating between innocuous environmental exposures from potential pathogens. Multiple immune cell populations, including T and B cells, DCs, macrophages, innate lymphoid cells (ILCs), and others are important for regulating this balance[34]. Here, emphasis will be placed on discussion of CD4+ regulatory T cells and subsets of these cells, which are essential for maintenance of homeostasis both at this site and systemically.

1.2.2.1 T Cells

Within the T cell compartment, both CD4 and CD8 T cells are critical in providing protection from invading pathogens and maintaining gut homeostasis. CD4+ T cells, also known as T helper cells, play an important role in the adaptive immune system by helping activate B cells, maintaining immune memory, and releasing cytokines to control surrounding cells, among other functions[90].

Progenitor cells originate in the bone marrow where they must travel to the thymus for development and maturation into T cells before being released into circulation[34]. Activation of CD4+ T cells requires antigen presentation via MHCII, expressed on APCs including DCs[91]. Upon sampling of their local environment, DCs take up antigen, process these for presentation into small fragments, and load the peptide on to MHCII where the complex is transported to the cell surface[92]. Once the DC encounters the appropriate naïve CD4+ T cell that contains a TCR that recognizes the MHCII-peptide complex, it acts as the first signal to trigger activation of the T cell. Activated, mature DCs also express co-stimulatory molecules CD80 and CD86, which bind to CD28 on the CD4 cell surface and act as the second signal. DCs must first be activated by encountering pathogen-associated molecular patterns (PAMPs), along with receiving signals from cytokines secreted by surrounding tissue or innate immune cells[93]. Finally, the surrounding environment and cytokines present are necessary for T cell differentiation[92]. These cytokines help to determine the downstream T-helper (Th) response that is initiated, which impact the effect these cells have on the immune system. This has been referred to as signal 3. Each of these signals prevents inappropriate inflammation when there is no threat of invading pathogens.

The major subsets of CD4+ T cells include Th1, Th2, Th17, Tregs, and T follicular helper cells (T_{FH})[92]. These T cells have associated hallmark transcription factors that can be used to differentiate these populations; Th1 express T-bet, Th2 cells express GATA3, Th17 express retinoic acid receptor (RAR)-related orphan receptor gamma (RORγt), and Tregs express FoxP3. T-helper populations are also characterized by the cytokines they secrete; in general, Th1 cells produce interferon γ (IFNγ), whereas Th2 cells produce interleukin-4 (IL-4), IL-5, and/or IL-13. Th17 cells produce IL-17A and IL-17F, as well as IL-22, and Tregs can secrete IL-10 and transforming growth factor beta (TGF-β). Another subset of CD4+ T cells are T_{FH}, which are

primarily found in GCs where they are pivotal in providing help to GC B cells. T_{FH} cells express CXCR5 and PD-1, along with the transcription factor Bcl6 and produce IL-21[34]. The type of response elicited and subsequent cytokine response also determines the skew of B cells and subclasses of antibodies they produce; IFNγ produced by Th1 cells gives rise to IgG2 in humans or IgG2a/c in mice, while IL-4 is secreted by Th2 CD4+ cells and elicits IgG1[94].

CD8 T cells are also found in the GALT, though at lower frequencies than CD4+ cells in mice. Resident memory populations (T_{RM}), which express CD69 and CD103, are present in the intestinal epithelium and lamina propria compartments[95,96]. In the intestine, intraepithelial lymphocytes (IELs) are CD8+ T cells, where their residence between gut epithelial cells positions this population to protect against potential infections through the GI route[97]. Subsets of CD8+ T cells include mucosal-associated invariant T (MAIT) cells, which are innate-like cells that express semi-invariant T cell receptors (TCRs)[98]. MAIT cells recognize bacterial metabolites presented by APCs on MHC-I related molecule (MR1). These cells develop in the thymus, but are important in establishing homeostasis with the gut microbiota, where they comprise ~1-3% of the T cell compartment[98]. Together, T cells in the gut are critical for balancing protective responses against invading pathogens and suppression of innocuous encounters from dietary antigens and commensals.

1.2.2.2 Regulatory T Cells

Tregs are critical in maintaining balance to the immune system[99]. The hallmark transcription factor, FoxP3 is essential for the function of these cells[100,101]. Mice deficient of FoxP3 die by 3-4 weeks of age due to immune dysfunction[102]. When mutations are present in the *FOXP3* gene, immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome develops in humans, which is characterized by multiple autoimmune disorders[103]. In

mice, a truncated *FOXP3* protein causes a similar condition known as "scurfy". Because FoxP3 is an X-linked gene, these conditions are more likely to affect males[103]. Tregs that localize to the gut express α4β7 integrin and CCR9[104]. Tregs also express high levels of CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Glucocorticoid-induced TNF receptor-related protein (GITR), and lymphocyte activation gene-3 (LAG-3), among other markers expressed by CD4+ T cells[99,105]. These cells act via multiple mechanisms to keep inflammatory responses in check, four of the most important being inhibitory cytokines secreted by Tregs (IL-10 and TGF-β), metabolic disruption by IL-2 cytokine deprivation or adenosine mediated immunosuppression, cytolysis via granzymes and perforin, and targeting and inhibition of DCs[105].

Tregs can be activated both in the thymus and the periphery, leading to different subsets: thymically-derived Tregs (tTregs) and peripherally-derived Tregs (pTregs)[106]. In the thymus, a population of $CD25^{hi}$CD4+ T cells can induce FoxP3 expression in cells that have TCRs that have a relatively strong affinity for self-antigens[107,108]. In general, tTregs have TCRs that favor self antigens, while pTregs encompass a TCR repertoire that favors binding to microbial and environmental antigens (Figure 1.2.2.1)[109]. pTregs develop outside of the thymus, where they circulate as naïve CD4+ T cells prior to antigen exposure[106]. This Treg subset can be induced *in vitro* by providing TGF-β stimulation. *In vivo*, both pTregs and tTregs are able to suppress effector responses and are essential for controlling immune homeostasis[110]. pTreg subsets have been shown to control Th2 effector responses, whereas tTregs are involved in tissue repair during inflammation[111,112]. The varied role of these Treg subsets may also be controlled by which transcription factors they expressed; these are discussed in the following section.

1.2.2.3 Peripherally-derived Regulatory T Cells

Within FoxP3+ Treg cells, there are subsets that express transcription factors associated with the T conventional (Tconv) compartment[113]. RORγt+ Tregs are thought to be peripherally-derived and are found in high frequencies in the siLP and colonic lamina propria (cLP)[114,115].

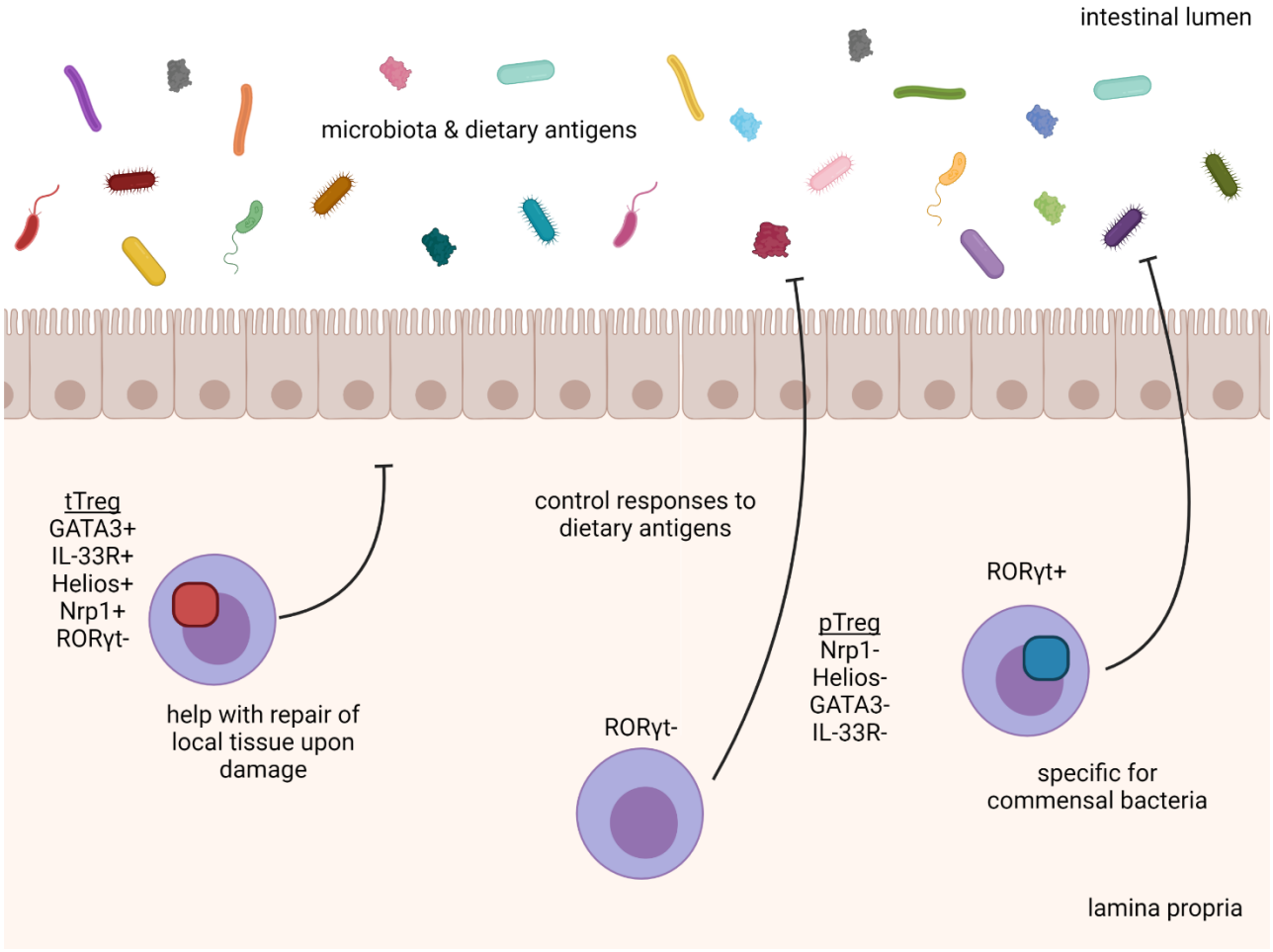

Figure 1.2.2.1 – Subsets of regulatory T cells in the small intestine

These sites encounter a large degree of environmental exposures to commensal organisms present in the gut. RORγt+ Tregs are also largely absent in germ-free (GF) mice, where lack of exposure to microbiota leads to intestinal inflammation and dysregulation. Early in life prior to diversity in microbiota composition, this subset is also largely absent, only appearing near the

time of weaning around 15-25 days of life (DOL)[114,116]. The TCR repertoire of RORγt+ Tregs is distinct from other subsets, as it favors TCRs specific for bacterial antigens[117]. Expression of RORγt+ in both Tconv and Tregs is dependent on the Stat3 mediated IL-6 and IL-23 pathways[118-120]. The transcription factor c-MAF is also important for the formation of the RORγt+ Treg population, as conditional knock-out of c-MAF leads to a reduction in the frequency of these cells in the cLP of mice[121].

RORγt+ Tregs are largely peripherally-derived, as evidenced by the TCR repertoire of these cells that can be specific for bacterial proteins[114,120]. Expression of RORγt+ is largely absent from Tregs that express GATA3, neuropilin 1 (Nrp1), IL-33R (or ST2), or Helios[114,120-123]. RORγt is a nuclear hormone receptor that was first identified as the regulator of Th17 cells in 2003, and later described in FoxP3+ Tregs in 2008[124-126]. RORγt+ Tregs have been found to share more similarity to RORγt- Tregs than Th17 Tconv cells, as they do not produce IL-17, can suppress CD4+ function *in vitro,* express high levels of CTLA-4, and produce IL-10[113,120,126]. The RORγt+ Treg subset drive suppression of Th17 cells but have also been shown to suppress Th2 responses in the gut[117,120,121]. Similarly, the Rudensky lab reported that blocking the differentiation of pTregs led to Th2 associated pathology in the gut, including allergic inflammation[111]. By utilizing a conditional knock-out (cKO) model of RORγt+ expression within Tregs ($FoxP3^{cre}$ $Rorc^{fl/fl}$), the Eberl group reported that these mice had increased levels of Type 2 cytokines IL-4 and IL-5 in the colons of mice treated with oxazolone-treated mice to induce colitis compared to wild-type (WT) controls[120].

Microbial stimulation by gut commensals contributes to the formation of RORγt+ Tregs, though the precise mechanism of the development of this subset remains unclear. It has been reported that the vitamin A metabolic retinoic acid (RA) contributes to the generation of RORγt+

Tregs *in vitro*[127]. RA and subsequent TGF-β also limits the IL-6 mediated generation of Th17 cells, suggesting an existing axis between these populations[113,128]. A study from the Benoist group examined how certain commensals differentially contribute to the RORγt+ Treg population by utilizing a gnotobiotic model of monocolonization[114]. They reported that in the cLP, colonization by multiple phyla including *Firmicutes, Bacteroidetes, Fusobacteria,* and others were able to induce RORγt+ Tregs in GF mice two weeks after colonization, though specific-pathogen free (SPF) mice with a diversity of commensals showed the highest frequency of this subset among FoxP3+ cells. RORγt+ Tregs have recently been implicated in their role in controlling oral vaccine responses[129]. By utilizing an inducible cKO model of these cells, the Hand group reported that RORγt+ Tregs suppress oral vaccine response in a model of environmental enteric dysfunction. This study suggests that this gut-associated subset is critical in modifying vaccine response, but whether RORγt+ Tregs controls tolerance to a systemic vaccine remains unknown[129].

Recently, multiple studies have highlighted how various MHCII+ RORγt+ APC populations in the gut may contribute to the formation of RORγt+ Tregs[120,130-133]. Previously, CD103+ DCs have been reported to induce pTreg differentiation, a population that is generally defined as RORγt+ FoxP3+ cells[134,135]. Work from the Littman lab suggest that two populations that are CD11c+ MHCII+ RORγt+, ILC3s and autoimmune regulator+ (Aire+) Janus cells, mediate antigen presentation[131]. This paper also concludes that these populations can produce TGF-β1 that leads to differentiation of RORγt+ Tregs in the gut. This work relies on adoptive transfer of *H. hepaticus*-specific T cells and colonization of this bacteria in mice of various genetic backgrounds, which could contribute to differences in findings between the studies[131,133]. Work from the Sonnenberg group utilized single cell RNA sequencing to find that $Rorc^{cre}$ H2-

$Ab^{fl/fl}$ mice had a reduction of RORγt+ Tregs, suggesting that an MHCII+ RORγt+ population is responsible for the induction of this Treg subset[130]. This group concluded that either ILC3 or a population of Aire expressing extrathymic cells are responsible for RORγt+ Treg differentiation. Further experiments disrupted either Aire or RORγt+ on the extrathymic population and did not observe differences in RORγt+ Tregs, suggesting that ILC3s are sufficient for differentiation of this Treg subset[130]. Lastly, the Brown group utilized a model of inducible manipulation of RORγt+ in MHCII+ cells by introducing a tamoxifen-dependent site to $Rorc^{cre}$ mice[132]. They reported that disruption of RORγt+ in MHCII+ cells did not lead to changes in RORγt+ Treg cells, suggesting that anther RORγt+ APC subset may be involved, which they refer to as Thetis cells. Thetis cells were characterized as expressing transcription factors similar to epithelial cells and DC, suggesting a hybrid function of the two cell types[133]. This group also confirmed that conditional knock-out of RORγt from MHCII+ cells led to a reduction in RORγt+ Treg subset. Upon inducible knock-out of RORγt+ MHCII+ in adult mice, the pTreg compartment was not altered, indicating that the APC subset responsible for differentiation may not be required for maintenance[132]. These studies point toward the need for continued research in this area to gain a more complete understanding of pTregs, what cells are required for their development, and how they may play a role in health and disease.

Another subset of pTregs is induced by dietary antigens and does not express RORγt, Nrp1, T-bet, or Helios[122]. Though not many studies have focused on this subset, the Surh group reported on this subset in 2016 by utilizing a model of restricting exposure from dietary antigens in GF mice[122]. They report that dietary antigens induce the development of most pTregs, though these are distinguishable from microbial-specific pTreg cells which are RORγt+. Dietary antigen including ovalbumin (OVA), are able to induce RORγt+ Tregs from naïve OVA-specific OT-II

CD4+ T cells, though OT-II cells expand further in the absence of any other dietarily acquired antitgens[122,136].

1.2.2.4 Thymically-derived Regulatory T Cells

Thymically-derived Tregs (tTregs) comprise a separate subset of cells which can express GATA3, Helios, Nrp1, or IL-33R; these are often co-expressed on the same FoxP3+ Treg[123,137-139]. This subset is also found at a relatively high frequency in the small intestine lamina propria, but the function and origin differ from RORγt+ Tregs. Many tTregs that reside in mucosal barrier sites including the gut and skin express the transcription factor GATA3[137]. Expression of GATA3 in Tregs is regulated by IL-2 and TCR stimulation, and expression limits Treg polarization towards effector cells and helps maintain suppressive functions[137]. This subset does not depend on microbial stimulation for development but is markedly reduced in mice that lack antigen exposure within their diets[122]. Deletion of GATA3 within Tregs leads to development of inflammatory disorders within mice, indicating that this subset may control Type 2 immunity[139]. Because GATA3 interacts directly with FoxP3, the control of this subset could also be less specific to Th2 cells and more important in mediating overall suppression of effector cells[140]. These cells are also able to respond to alarmins via IL-33R[99]. The cytokine IL-33 is secreted by epithelial cells at barrier sites like the intestine and functions as an alarmin, signaling danger to the immune system upon tissue damage[141]. IL-33R, also known as ST2, is expressed on Tregs in the colon where it enhances TGF-β mediated expansion of Tregs[138]. IL-33 acts with IL-2 to enhance expression of GATA3 within Tregs, which contributes to co-expression of IL-33R on the same subset of cells[138]. In a model of environmental allergen exposure, IL-33R+ Tregs have

been shown to suppress γδ T cells that produce IL-17 in the lung, suggesting their role in regulation of innate signaling[142].

Helios expression is upregulated in tTreg populations compared to pTregs, though some studies suggest Helios is also expressed in pTregs[143-145]. Helios+ Tregs have a slightly higher suppressive capacity and have varied TCR repertoires than Helios- populations[146]. This can be co-expressed with GATA3, IL-33R, and Nrp1+ Tregs on tTreg subsets that have preferential specificity for self-antigens[129,147]. Nrp1 expression within Tregs is under control of TGF-β and these cells are present in inflammatory settings like autoimmune encephalomyelitis[147]. Together, these cells typically do not express RORγt+ are functionally distinct, as they likely play differing roles in the Treg compartment.

A subset of Tregs also express Th1 associated transcription factor T-bet and are able to inhibit Th1 effector responses[148]. In an inflammatory setting, Tregs upregulate T-bet, while deletion of the gene that encodes T-bet, *Tbx21,* along with *Gata3* lead to autoimmune-like symptoms in mice early in life[149,150]. In the gut, T-bet+ Tregs are required for the induction of colitis[151]. In patients with inflammatory bowel disease (IBD), Tregs express T-bet and IFNγ, suggesting their role in controlling Type 1 inflammatory responses at this site[151]. While Tregs are known to be essential for maintaining homeostasis and to prevent autoimmunity, their role in systemic vaccination upon oral antigen exposure is not well characterized. Whether a specific Treg subset is involved in this process remains an open question that my work has aimed to answer.

1.2.2.5 Other Immune Cells in the GALT

T cells are a critical piece of the GALT immune system, but other cells reside in this compartment as well that are essential for providing protection from infectious through the GI

tract. B cells, DCs, macrophages, ILCs and other cell subsets contribute to the landscape at this site, each of which play specific roles in shaping effective local immune responses.

B cells are key players in mediating the antibody response to invading pathogens and upon vaccination. Though B cells that produce IgG are the most common circulating in the blood, and are present at the highest frequencies systemically, IgA is the most prevalent and crucial in the gut[152]. At this site, plasma cells that secrete IgA are critical in regulating bacterial populations in the intestinal lumen[153]. There are few B cells in the lamina propria, though the cells that are present are mostly plasma cells that secrete IgA[154]. Within the gut, many B cells also reside in GCs, where they interact with DCs, T_{FH}, and macrophages and leads to expansion of specific B cell clones[152].

Dendritic cells that reside in the gut are also essential in controlling the response to pathogens and are involved in oral tolerance. DCs are professional APCs that are able to present antigen to a T cell via MHC molecules[34]. CD4+ T cells including Tregs are activated by MHCII on DCs. Within the DC population, there are subsets of these cells that perform different functions, similar to the heterogeneity observed in CD4+ T cell and Treg populations[155]. Among DCs, classical DCs are primarily responsible for priming T cell responses, whereas plasmacytoid DCs can produce type I interferons[156]. Surface markers also help define various subsets, with CD103 and CD11b as defining markers of various gut DC populations; high frequencies of CD103+ CD11b+ DCs are present in the small intestine[157]. These give rise to varied T cell populations; CD103+ aldehyde dehydrogenase+ (ALDH+) leads to pTreg differentiation from naïve CD4+ T cells in the presence of RA[134,135,158,159]. The type of CD4+ response stimulated by DC populations also depends on the surrounding cytokine environment[160].

DCs must sample the lumen for antigens in order to present peptides to T cells and facilitate tolerance toward the microbiota and dietary antigens; this occurs primarily via three methods[159]. First, goblet cells deliver small molecular weight soluble antigens to tolerogenic CD103+ DCs[161]. A subset of CX3CR1+ phagocytotic macrophages can directly pass antigen to CD103+ DCs[162]. Lastly, DCs utilize their finger-like projections, or dendrites, to sample the lumen directly by passing through the epithelial layer[163].

Another subset that is present in the GALT is ILC populations. ILCs are innate cells that play a critical role in host defense and have subsets homologous to CD4+ T cell subsets, expressing transcription factors and cytokines associated with T-helper cells[164]. Like DC subsets, differentiation of ILC subsets in the gut is affected by the proximity to commensal bacteria and their metabolites; the presence of RA in the gut favors ILC3 phenotypes, which express RORγt+, over ILC2[165]. Recently, a subset of ILC3s that are Aire+ and have features similar to APC populations have been characterized[166]. These cells are found in the gut-draining lymph nodes and could be involved in promotion of pTreg populations[130]. While this is not an exhaustive review of every cell in the gut, those discussed comprise populations that contribute to the maintenance of tolerance where it is especially critical at the gut mucosal barrier.

1.2.3 *The Microbiome*

Within the gut lumen, the microbiome, comprising bacteria, viruses, fungi, and archaea, influence health and disease[167]. Heterogeneity exists between individuals depending on many factors; behavioral habits, exposure to diseases, and genetics all contribute to changes in composition, which fluctuates day to day. Various microbes are known to influence the host

immune system and can have implications for how individuals respond to infections and respond upon vaccination[4,26,28-30,168,169].

The composition of the microbiome in humans depends on multiple factors, including growth rate of specific strains and changes to microbial genes[170,171]. In general, high diversity in the microbiome community structure is indicative of a healthy gut, as disproportionate colonization by an individual species occurs in disease or during dysregulation. People with a less diverse microbiome showed greater inflammation and were at higher risk for obesity compared to groups with higher diversity in one study[172]. During development, infants are generally exposed to microbiota at birth, though the method of delivery alters this exposure, as babies born by cesarian section have delayed diversity in microbiota species[173]. There is evidence that this early life dysbiosis can lead to a greater risk of developing allergies, though the precise mechanisms of this remain unclear[174]. In recent years, the effect of gut commensals on the immune system, both local and systemic, has become an increasing area of study.

Though an emphasis on how the microbiome controls human health has only been appreciated in the past decade, many groups have since focused on studying how commensals shape the immune environment in the intestine. Studies from GF mice have been informative as to the role of commensals in establishing various immune populations, as in the absence of commensals, GF mice have elevated IgE levels, a reduction in IELs, and few RORγt+ Tregs[114,116,175]. Use of oral antibiotics alter the phenotype of colonic CD4+ T cells and invariant natural killer T cells, skewing these towards pro-inflammatory phenotypes[176]. Conversely, restoration of microbial communities by fecal microbiota transplant with the gram-negative bacteria *Lactobacillus johnsonii* following antibiotic administration can lead to increased CD4+ numbers and IL-10 secretion[177]. It is also clear that there is a window of opportunity in which

rescuing GF mice can lead to protection from dysregulated immunity early in life[178]. Upon colonization of GF mice early in life, immunoregulation can be restored and elevated IgE levels can return to baseline, but only if colonization occurs near the time of weaning[116,179].

In the siLP, certain T cell populations are driven by specific commensals. Th17 cells arise upon colonization of GF mice with segmented filamentous bacteria (SFB)[180]. Other studies have also reported that other species from the human microbiome drive Th17 responses in mice[181]. Similarly, the development of Tregs is driven by commensals, as multiple *Clostrida* species drive pTreg differentiation[182,183]. Short-chain fatty acids (SCFAs) produced by commensals can directly drive differentiation of Tregs in the appropriate environments[184,185]. Butyrate, an SCFA, can specifically drive Treg extrathymic generation of Tregs[186,187]. Certain SCFAs can also drive effector phenotypes including Th1 and Th17, depending on the cytokine milieu[184]. SCFAs, however, do not have a direct effect on RORγt+ Treg populations[114]. Microbiota also drive Treg development in the MLN and PP, though at these sites, this was not dependent on TLR stimulation, as MyD88 was dispensable for this effect[188]. Another study, however, reports that MyD88 signaling and TLR stimulation by commensals was critical for Treg accumulation in the gut LP, suggesting various pathways may be involved at distinct sites[189]. RA is also able to prime DCs to express CD103, where it induces IL-10 producing Tregs in the gut[128,134,135,190]. These studies and others indicate that the gut microbiota affects intestinal lymphocyte populations, and may play a role in controlling systemic responses, which can affect the immune system prior to vaccination.

The gut microbiome has been implicated in altering outcomes upon vaccination[25,26,28,30,32]. As previously discussed, heterogeneity between individuals contributes to differential vaccine outcomes and variation in efficacy. The contribution of microbial

communities to this heterogeneity could partially explain why some vaccines fail to elicit protective responses[31]. Microbiota may act as adjuvants to stimulate pattern recognition receptors that act to stimulate the immune system, leading to more effective vaccine responses[28]. Studies of the seasonal influenza vaccine in GF mice reveal the contribution of the microbiota to initiating antibody responses, as GF mice did not elicit antibody titers upon vaccination[191]. This was rescued upon colonization with flagellated bacteria, suggesting the role of TLR5 in mediating this process. In mice, cholera toxin adjuvanticity is enhanced by nucleotide-binding oligomerization domain containing 2 (Nod2) sensing of bacteria in the nasal cavity, indicating the role of microbiota on altering vaccination in mucosal routes[192].

Clinical studies have also linked microbiota to vaccination outcomes. In a study of infants in Bangladesh, 16S sequencing was performed early in life at multiple timepoints and examined in relation to response to BCG, tetanus toxoid (TT), hepatitis B, and oral polio virus (OPV) vaccines[27]. This work suggests that certain phyla are associated with protective vaccine responses. *Actinobacteria* was associated with T cell responses to BCG, TT and OPV, whereas *Enterobacteriales*, *Pseudomonadales*, and *Clostridiales* were associated with lower vaccine responses. Another study examined the oral typhoid vaccine and observed greater antigen-specific cellular responses in individuals with more diverse and complex microbial communities[193]. Rotavirus vaccine outcomes have also been linked to microbiota in clinical settings[29]. Upon use of the antibiotic vancomycin, anti-rotavirus IgA was increased at Day 7 after immunization; this was associated with an expansion of *Prevotellaceaea* at this timepoint.

The use of gnotobiotic mouse models to understand these systems is imperative, as these allow for manipulation of single variables to gain insights as to the role of specific taxa on controlling vaccination[10]. Monocolonization of a single commensal to examine the effects on the

immune system and potential to alter vaccine responses is also an important tool in this research[194]. The increasing accessibility of next generation sequencing and bioinformatic tools also allow for more accurate characterization of commensals, allowing for a deeper understanding of the effect of microbiota on vaccine responses[32]. The precise link between gut commensal organisms and the maintenance of homeostasis (lack of allergic responses, strong gut barrier function, and development of appropriate immune responses upon immunization) remains an open an open area of research, as many aspects are incompletely understood.

1.3 TOLERANCE

Broadly, immunological tolerance is the lack of response, or regulated unresponsiveness to specific antigens, including self[195]. Tolerance can be divided into central and peripheral, where central tolerance prevents self-reactive T and B cells from developing, and peripheral tolerance will induce anergy or T cell mediated regulation by Tregs to control inappropriate responses[34]. The break of tolerance can lead to autoimmune diseases and allergy; understanding the mechanisms by which tolerance is maintained and how this affects systemic vaccination is critical for developing vaccines and anti-immune therapeutics.

1.3.1 *T Cell Tolerance*

The immune system, while necessary for preventing harmful infections, is also poised to be self-destructive if proper mechanisms are not in place. Autoimmune reactions occur when these systems fail; this was first described by Paul Ehrlich in the early 20th century when he described autoreactive antibodies against self-antigens, which was termed “horror

autotoxicus"[196]. Since then, mechanisms of immune tolerance, and specifically T cell tolerance have been characterized.

Central tolerance, or selection against self-reactive cells during development, is the first step in preventing inappropriate reactions[197,198]. In the thymus, T cells undergo selection of TCR affinity to MHC-self-peptide complexes to determine if the T cell is able to bind to self-antigens[197]. Any T cells that have no reactivity to self-antigen will die by neglect, whereas those that bind too strongly will undergo negative selection by clonal deletion[199,200]. In the thymus, antigens that mimic self will be expressed and presented by medullary epithelial cells[201]. These are loaded on to MHCII and presented to T cells for selection in the thymus. A subset of Tregs is also derived in the thymus; CD4 T cells that have TCRs that bind more strongly to self-antigens will be skewed toward Treg development (tTreg) and upregulate FoxP3, known as clonal diversion[202,203].

Upon selection of TCRs that have low-affinity for self, known as the positive selection process, cells develop and can traffic to the periphery. Though central tolerance is highly regulated, not all self-reactive cells are eliminated, promoting the need for peripheral mechanisms of tolerance[197]. Anergy, deletion via apoptosis, ignorance, and Tregs all contribute to peripheral tolerance. Anergy is the active process of T cell repression by inhibition of TCR signaling and IL-2 expression[197,204]. This is due to absence of co-stimulation of CD28 on T cells, which leads to suppression of progression through the cell-cycle and effector functions[205]. Deletion of T cells that may cause inappropriate responses is initiated by stromal cells in the lymph node, including follicular DCs, lymphatic endothelial cells, and others that express tissue-specific antigens[197,206]. This is achieved by both Fas- and Bim-mediated apoptosis and is initiated upon stimulation of self-reactive T cells. Another mechanism of peripheral tolerance is

ignorance, or the idea that there is a pool of naïve T cells that is not activated and cannot mature to achieve full effector function[207]. Ignorant T cells could arise from lack of antigen presence, low affinity between TCR and MHC, or low affinity between peptide and MHC, resulting in lack of binding and T still activation[208-210]. Together, these mechanisms, along with regulatory T cells, work to prevent inappropriate inflammation due to the development of self-reactive T cells.

Tregs, described above in the context of the GALT immune system, are also essential in preventing autoimmunity and the maintenance of tolerance. It has been observed that highly self-reactive TCRs skew toward Treg development in the thymus[211]. Within the Treg population, the relative affinity for MHCII and TCR leads to differences in function[212]. For example, $GITR^{lo}PD\text{-}1^{lo}CD25^{lo}$ Tregs with a reduced self-affinity compared to counterparts that express these three markers at a higher level showed an increased ability to promote the conversion of CD4+ T cells to pTregs and control colitis. By using mice that express the human diphtheria toxin receptor (DTR) downstream of the FoxP3 promoter ($FoxP3^{DTR}$) and administration of diphtheria toxin to deplete Tregs, adoptive transfer of naïve T cells leads to upregulation of FoxP3 and expansion of the pTreg population, suggesting the critical role of these cells[213,214]. Therefore, Tregs are necessary to prevent autoreactivity and keep the immune system in check.

Autoimmunity is broadly characterized as the breakdown of tolerance, as most autoimmune disorders result from self-reactive immune cells that are uncontrolled. In T cells, mutations in CTLA4 have been linked with increased risk of autoimmune diseases like type I diabetes and Graves' disease[215]. T cell dysfunction is also associated with rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and atherosclerotic cardiovascular disease[216]. Such diseases can involve a break in tolerance by a variety of mechanisms, some of which include disruption in co-stimulation and the IL-2 pathway[217]. Understanding how tolerance is controlled

and what mechanisms lead to the break in tolerance will contribute to progress towards developing therapeutics for autoimmune diseases and allergy.

1.3.2 *Oral Tolerance & Allergy*

Oral tolerance is the state of systemic unresponsiveness following prior exposure of an antigen through the gastrointestinal tract[218,219]. This response is necessary for avoiding inappropriate inflammation to innocuous exposures like food, commensal or environmental exposures encountered orally. To study these tolerogenic mechanisms, mouse experimental models typically involve oral antigen exposure, usually through oral gavage or via drinking water, followed by immunization with the same antigen[136]. To study allergic responses, this can be followed by several repetitive oral exposures to the same antigen following immunization and analysis of inflammation, including diarrhea production and ear or footpad swelling following local challenge[136,220,221]. Tolerance to oral antigens was first described in 1911 by H.G. Wells and T.B. Osborne[222]. Using a guinea pig model, the authors reported that anaphylaxis could not be achieved to antigens found in large quantities in their diets. Since this, countless studies have explored the underlying mechanisms behind this phenomenon and how it relates to allergy and hyporesponsiveness upon vaccination.

Allergic diseases are increasing in prevalence and affect roughly 10% of the population[223]. Food allergies arise upon inappropriate inflammation to a typically innocuous dietary antigen and involves the production of IgE and cellular responses[218]. One recently approved treatment for severe peanut allergies is oral immunotherapy, or the delivery of small amounts of the target antigen to desensitize the immune system and reduce subsequent IgE-mediated responses[224]. This therapy is currently underutilized by allergists, which could be due

to an incomplete understanding of mechanisms that mediate oral tolerance. Allergic reactions are mediated by antigen-specific IgE, mast cells, eosinophils and basophils, all associated with Type 2 immunity[225]. Class switching to IgE occurs in the GI tract of individuals with food allergies[226]. Upon antigen binding to IgE, crystallizable fragment (Fc)-mediated binding to mast cells activates the release of histamines from these cells, triggering allergic reactions[227].

Risk factors for developing allergies include diet, environment, microbiota composition, exposure and route of food exposures[223]. Dietary fiber along with vitamin A has been linked to increased tolerogenic DCs and Treg differentiation, leading to suppression of food allergy in mice[228]. This effect was dependent on microbiota composition and presence of SCFAs, which have been previously reported to enhance Treg differentiation[184-186]. Commensal bacteria have also been shown to be associated with protection from food allergy, as food sensitization is heightened in mice treated with antibiotics or unexposed to bacteria[229]. The hygiene hypothesis also supports the idea that increased exposure to microbial diversity early in life leads to subsequent protection from development of allergies and asthma[230]. Antibiotic use has also been linked to the development of atopic disease[231-233]. The use of antibiotics in children early in life leads to a less diverse microbiota composition and a reduction in stability in populations of commensals compared to untreated children[231]. A study examining a birth cohort of nearly 150,000 children found that exposure to antibiotics in infancy was correlated with a heightened risk for development of asthma through adolescence[233]. Similarly, a study in twins concluded that use of antibiotics, particularly, those prescribed for respiratory infections, was associated with an increased risk of asthma[232]. Thus, various exposures via the GI tract contribute to allergic diseases and play a role in mediating oral tolerance.

Much of the previous work on tolerance to an orally acquired antigen prior to vaccination focuses on subsequent development of allergic responses. Thus, it remains currently understudied how factors of a systemic vaccine itself may alter the type of tolerance that is induced. Previous studies have examined the effect on microbiota composition and the maintenance of oral tolerance[234-237]. An early study in this field reported that tolerance is maintained in GF mice in the absence of microbiota, but it was found to last for a shorter duration compared to conventional mice[234]. Here, mice were fed OVA orally prior to immunization with CFA. After the third dose, the GF mouse group that received OVA gavage developed OVA-specific antibodies, whereas the conventional group did not, indicating a break in tolerance over time in the absence of microbiota[234]. This was also observed in a follow up study using alum in the vaccine[235]. Other groups have reported more severe impairment of oral tolerance in GF or gnotobiotic conditions (monocolonization with *Clostridium perfringens* or *Staphylococcus aureus*), suggesting that lack of exposure to microbes leads to a break in tolerance upon oral exposure prior to vaccination[236]. This study also reported that a loss in tolerance was associated with a reduction in T cells in PP, suggesting these cells may play a role in establishing tolerance[236]. There are, however, controversial findings from various experiments and lab groups in this area. Following these findings, another group reported that tolerance remains intact in GF mice given either a high or low dose of OVA prior to immunization[237]. While this is still an active area of research, the mechanisms that control oral tolerance have been explored and at least in part, involve regulatory T cells[136].

1.3.3 *Mechanisms of Oral Tolerance*

Multiple aspects of the gut immune system are likely involved in the maintenance of oral tolerance. It is thought that CD4+ T cells that reside in the gut which express α4β7 integrin and CCR9 are required for induction of oral tolerance[104,238]. Antigens are acquired by CD103+ DCs, which can travel to the MLN and present these antigens to naïve T cells, which are able to differentiate into pTregs[239]. These Tregs can then exit the lymph node to migrate to the siLP, where they reside and expand.

To induce oral tolerance, antigens from the intestinal lumen must be taken up by APCs and presented to T cells. This is primarily performed by CD103+ DCs, previously discussed in the context of cell subsets in the GALT[161]. Goblet cell associated-antigen passages (GAPs) can transport low molecular weight antigens to these DCs in the siLP[240]. Recently, it was reported that these GAPs support oral tolerance by facilitating the maintenance of pre-existing pTregs[220]. In the absence of goblet cells, oral tolerance is impaired as measured by footpad challenge following oral OVA exposure. It also evident that CD103- CX3CR1+ macrophages send protrusions to the lumen to acquire antigens, which can be transferred via gap junctions to CD103+ DCs[162]; these cells can also contribute to the establishment of oral tolerance. The Pabst group reported that CX3CR1 was required for tolerance, as a complete knock-out of these gene resulted in ear swelling comparable to the unexposed group[136]. Though monocyte derived APCs are dispensable, classical DCs are required for tolerance and pTreg induction, as measured by ear swelling and OVA-specific IgG1 upon oral exposure, immunization, and challenge[241]. This study reported that migratory CD11b- DCs were efficient at differentiation of pTregs, though this subset was not required for tolerance, indicating others may be involved[241].

In the lymph nodes, TGF-β is required to convert naïve T cells into pTregs, and also depends on the presence of RA[134]. While it had been previously established that oral tolerance takes place primarily in the small intestine, a recent study compartmentalized individual gut draining lymph nodes and characterized these for their potential to generate tolerogenic responses[242]. In the lymph nodes associated with the proximal small intestine, there was an increased frequency of Tregs, but a reduced frequency in RORγt+ Tregs compared to the distal lymph nodes and those that drain the colon. This suggests that various segments of the intestine and associated LNs may differentially regulate tolerance[242].

CD4+ T cells have been implicated in oral tolerance and shown to be critical by both depletion and adoptive transfer of these cells[238,243]. Depletion of FoxP3+ Tregs using the DT system leads to a break in oral tolerance, as measured by mice with dysregulated GI symptoms (diarrhea production) following oral OVA administration, OVA + alum immunization, and orally challenged[136]. One study highlights the role of various Treg subsets in mediating tolerance by using a mouse model that lacks tTregs; the mice used were on the BALB/c background and contain influenza-specific B cells, OVA-specific T cells, and are recombination activating gene 1 (RAG-1) knockouts, leading to monoclonal populations among both T and B cells[244]. These mice lack tTregs and skew towards Th2 phenotypes upon immunization or respiratory challenge. In the absence of tTregs, oral tolerance is maintained, suggesting that pTregs may contribute to this function[244]. These experiments utilized a transgenic mouse model that lacks tTregs, though these animals generate pTregs following oral antigen exposure. Recently, CD4+ T cells have been reported to change during active tolerance or in allergy[245]. During tolerance, more Tregs are recruited to the epithelium, whereas an allergic model using cholera toxin favors recruitment of

pro-inflammatory IL-17 producing cells. This suggests that different dietary signals may trigger distinct pathways and downstream responses in the gut[245].

In the context of food allergy, RORγt+ Treg were able to protect from allergic responses in a MyD88 dependent manner; this suggests that this subset may play a role in tolerance via the oral route[246]. It has also been reported that the dose of oral exposure affects tolerance mechanisms, as low dose exposures lead to Treg induction, whereas high doses trigger anergy and deletion[247]. Recent findings suggest that immunotherapy by expansion of anergic memory cells may be a treatment for allergy[248]. This occurred in the absence of antigen-specific Treg expansion. While these studies highlight differential tolerogenic mechanism that are utilized in various contexts, there exists a knowledge gap in how tolerance is maintained in various settings. Whether Treg subsets, including RORγt+ Tregs, control oral tolerance to a systemic vaccine remains unclear.

1.4 Open Questions & Thesis Goals

The main focus of this work is to understand if RORγt+ Tregs contribute to the maintenance of tolerance to systemic vaccination following oral antigen exposure. Prior to these studies, the literature suggests that these cells, which are found in abundance in the small intestine, help to maintain tolerance to microbiota at this site[114]. The small intestine is the likely site of oral tolerance[242]. While Tregs have been found to be critical for tolerance to systemic vaccines[136], it is unclear whether certain Treg subsets contribute to this effect. Previous studies also show that RORγt+ Tregs can control Type 2 immunity, and we and other groups have observed that following oral antigen exposure, Type 2 immune responses are differentially

suppressed over Type 1[120,244]. For these reasons, we pursued a cKO model of RORγt+ Tregs to explore the function of these cells in oral tolerance to a systemic vaccine.

Another aspect of my studies focused on the effects of various adjuvants on tolerogenic responses. Prior to this study, few groups have reported on the differences between adjuvants in the setting of oral tolerance. While we explored how adjuvants with differing skews and mechanisms of action can affect a model of oral tolerance, it is critical to understand more about how each adjuvant functions *in vivo*. Many open questions remain on which adjuvant contributes to maximal vaccine efficacy in certain settings.

Following these studies, it will be critical to understand how environmental exposures via commensals or dietary antigens affect local T cell polarization and Tregs that could alter vaccine outcomes. While reports in the HIV field have begun to understand this effect, outstanding work for other vaccines could be informed by such concepts. Ultimately, it will be critical to understand what factors contribute to inducing tolerance to vaccines to create more effective vaccines for infectious diseases that affect humans globally.

Chapter 2. ORAL TOLERANCE TO SYSTEMIC VACCINATION REMAINS INTACT IN THE ABSENCE OF RORγt EXPRESSION IN REGULATORY T CELLS

2.1 INTRODUCTION

Vaccine development is critical to effectively control existing and emerging pathogens that pose a threat to humans. Upon immunization, it is imperative that the immunogen generates an effective immune response to protect from future infection[31]. The desired immune response is dependent on the type of pathogen, though generating memory T and B cell populations is generally beneficial to the host. Heterogeneous responses to vaccines have been reported, and numerous studies have attempted to explain why populations respond differentially to a particular vaccine. For example, polymorphisms in toll-like receptor genes and downstream pathways have been correlated with differential immunity to the measles vaccine[18]. Here, we explore how pre-existing immunity in the gut could impact subsequent systemic vaccination[30]. Recently, evidence from clinical vaccine trials suggest that effective immune responses may be diverted due to previous exposure to antigens, specifically components of gut microbiota, which are structurally similar to those within the vaccine and lead to a non-protective immune response or immunological tolerance[69,71]. Analysis of clinical trials of HIV vaccine candidates suggest a shared amino acid sequence between the envelope protein gp41 and proteins derived from *E. coli* resident to the GI tract[10,67-70]. Similarly, evidence suggests that the BCG vaccine used to prevent tuberculosis is less effective after exposure to environmental mycobacterium, suggesting diversion from building effective immunity against the pathogen due to pre-exposure of homologous antigens[71]. In a randomized-control trial of the rotavirus vaccine, oral administration

of the antibiotic vancomycin and the resulting expansion of proteobacteria among gut commensals was correlated with increased anti-rotavirus IgA titers and shedding by Day 7 following boost[29]. Understanding the mechanisms by which vaccine responses are inhibited by previous oral antigen exposure is a significant objective that will inform efforts to maximize vaccine immunogenicity and restore protection across individuals.

The GALT is a site where regulatory mechanisms are critical to prevent inappropriate inflammation in response to innocuous environmental exposures to dietary antigens or commensal microbes[123]. Upon systemic exposure to antigens previously introduced in the gut, a reduction in antibody titers and antigen-specific cellular responses develop compared to antigens that are naïve to this site[235]; this is known as oral tolerance. The underlying mechanism of oral tolerance has been studied in depth and involves the passage of antigens through goblet-cell associated antigen passages[220] and processing by tolerogenic dendritic cells in the lamina propria for presentation to naïve T cells in the MLN[241,242]. These T cells, which can differentiate into Tregs, can then home back to the lamina propria where they are critical for inhibiting potent immune responses against the antigen upon subsequent exposure[136]. The role of these Treg subsets in shaping the local environment in the gut and the effect on systemic vaccine responses following oral antigen exposure remains unknown.

At homeostasis, inflammation is controlled by various regulatory mechanisms, including through Tregs, which express FoxP3[105,123]. This subset of CD4+ T cells is positioned to reduce effector responses of other CD4+ T-helper subsets. Tregs act to suppress inappropriate inflammation, reduce immunopathology, and are especially pertinent at mucosal barrier surfaces like the gut, where tolerance to commensals is critical for symbiosis. Subsets of Tregs can arise

either in the thymus or can be induced in the periphery[244]; each of these have unique T-cell receptor (TCR) repertoires depending on their origin[249,250]. Transcription factors that are typically associated with CD4+ T-helper subsets can also be expressed in Tregs. For example, T-bet is associated with Th1 responses, GATA3 with Th2, and RORγt that is expressed in Th17 cells; each of these transcription factors can be expressed in FoxP3+ Tregs and have been studied for their suppressive effects on local and systemic responses[113,117,126]. Subsets of Tregs have been shown to specifically reduce the function of certain types of effector responses[114,120,142]. For example, T-bet+ Tregs can inhibit Th1 responses, and GATA3+ Tregs help control inflammatory disorders in mice[139,149].

RORγt+ Tregs are found in high numbers in the GALT and are typically peripherally-derived with unique TCRs enriched for specificity to microbial antigens[109,133,246,251]. Recent work suggests that development of this subset is dependent on MHCII+ RORγt+ antigen-presenting cells[130-133]. Notably, specific commensals have been associated with RORγt+ Treg frequencies in the colon[114]. This subset has also been reported to suppress Type 2 responses, as mice lacking these cells have elevated total IgE titers and increased frequencies of CD4+ GATA3+ Tconv and Tregs[120,252]. Recent work also suggests that RORγt+ Tregs may inhibit Th1 and Th17 responses in some models with the same specificity[115]. This subset has also been implicated in suppressing oral vaccine responses[129]; however, the role of RORγt+ Tregs in affecting systemic vaccine responses is uncharacterized.

Our studies have aimed to define the role of RORγt+ Tregs in altering systemic vaccine responses following oral antigen exposure to the same antigen. GF mice display hyper-IgE syndrome[116] and show markedly reduced RORγt+ Tregs in the siLP and colon compared to

conventionally raised SPF mice. This highlights the necessity of microbial exposure to the development of RORγt+ Tregs[114]. Notably, GF mice with reduced levels of RORγt+ Tregs also display altered tolerogenic responses upon oral gavage prior to vaccination. GF mice exposed to the model antigen ovalbumin (OVA) prior to OVA vaccination displayed increased OVA-IgG titers compared to SPF mice, indicating a break in tolerance[122,235]. Because tolerance is maintained in the absence of thymically-derived Tregs[244], it is reasonable to hypothesize that a peripherally-derived Treg subset, which can express RORγt, may be involved in controlling responses upon oral exposure prior to immunization.

Many studies examining oral tolerance have largely focused on how altering the oral antigen dose or timing of the response impacts peripheral tolerance. Our study addresses key questions on the cellular mechanisms of oral tolerance and systemic vaccination and whether this is impacted by the adjuvant used during vaccination. We have utilized a conditional knock-out mouse model of Rorcfl/fl FoxP3cre to characterize the role of RORγt+ Tregs in altering systemic vaccine responses with multiple adjuvants following oral pre-exposure to OVA. The selection of adjuvants used in a vaccine is critical for eliciting an appropriate response. Use of strong adjuvants in mice have been reported to overcome peripheral anergy[253], and have also been shown to boost frequencies of RORγt+ Tregs in draining lymph nodes above baseline[115]. Our study utilizes a variety of adjuvants in a model of oral tolerance to a systemic immunization to characterize how different adjuvants may alter vaccine responses. We propose that suppression of vaccine responses following oral exposure is limited to Type 2 immunity regardless of adjuvant, and that oral tolerance remains intact in the absence of RORγt expression within regulatory T cells.

2.2 RESULTS

2.2.1 *Oral administration of antigen prior to vaccination with alum suppresses Type 2 immune responses.*

To interrogate the effects of enteric antigen pre-exposure on a systemic vaccine response, C57BL/6 mice were exposed to 1 mg of OVA by oral gavage for four consecutive days, ending one week prior to intraperitoneal (i.p.) vaccination with 10 ug OVA formulated with alum (Figure 2.2.1.1A). Control groups received only phosphate-buffered saline (PBS) by oral gavage prior to immunization with the same vaccine. Both groups were boosted on Day 21 and assessed one week later for systemic and local immune responses. Our findings support existing work[244] that OVA exposure prior to OVA vaccination suppresses total serum OVA-IgG titers (Figure 2.2.1.2A). This was not observed at Day 14 following prime alone (Figure 2.2.1.2B), but groups differed significantly following the boost. Using alum as an adjuvant in our tolerance model, we observed specific suppression of IgG1 over time (Figure 2.2.1.1B, C). Upon splenocyte re-stimulation for 72 hours with OVA protein, secreted cytokine production from culture supernatant was assessed by ELISA. We observed that mice that received OVA by gavage had a reduction of IL-4 secretion compared to PBS pre-treated groups (Figure 2.2.1.1B). Though oral administration of vaccine antigen prior to immunization has been previously described, we noticed this effect was specific for Type 2-associated immune responses. Suppression of both OVA-IgG2c and IFNγ upon OVA re-stimulation of splenocytes was not observed for OVA gavage groups (Figure 2.2.1.1D).

The ratio of IgG1 to IgG2c is commonly used as an indicator of skew induced immunity upon immunization. Here, we observe a higher IgG1:IgG2c ratio in mice treated with PBS prior

to systemic vaccination compared to mice pre-exposed to OVA (Figure 2.2.1.1E). This indicates that oral exposure to vaccine antigen specifically suppresses Type 2 induced immunity in our model. Additionally, we examined T-independent subclass OVA-IgG2b. Here, groups exposed to OVA prior to immunization had a significant increase in OVA-IgG2b compared to PBS treated groups; however, the overall antibodies of this subclass were relatively low compared to IgG1 and IgG2c and therefore contributes little to the total antigen-specific IgG response (Figure 2.2.1.2C). OVA-IgE titers were also suppressed following oral OVA exposure prior to immunization, in line with specific suppression of Type 2-mediated immune responses, though titers were overall relatively low (Figure 2.2.1.2D). There were also no differences in T follicular helper (T_{FH}) subsets (CXCR5+ PD-1+ CD4+) in splenocytes from both groups (Figure 2.2.1.2E).

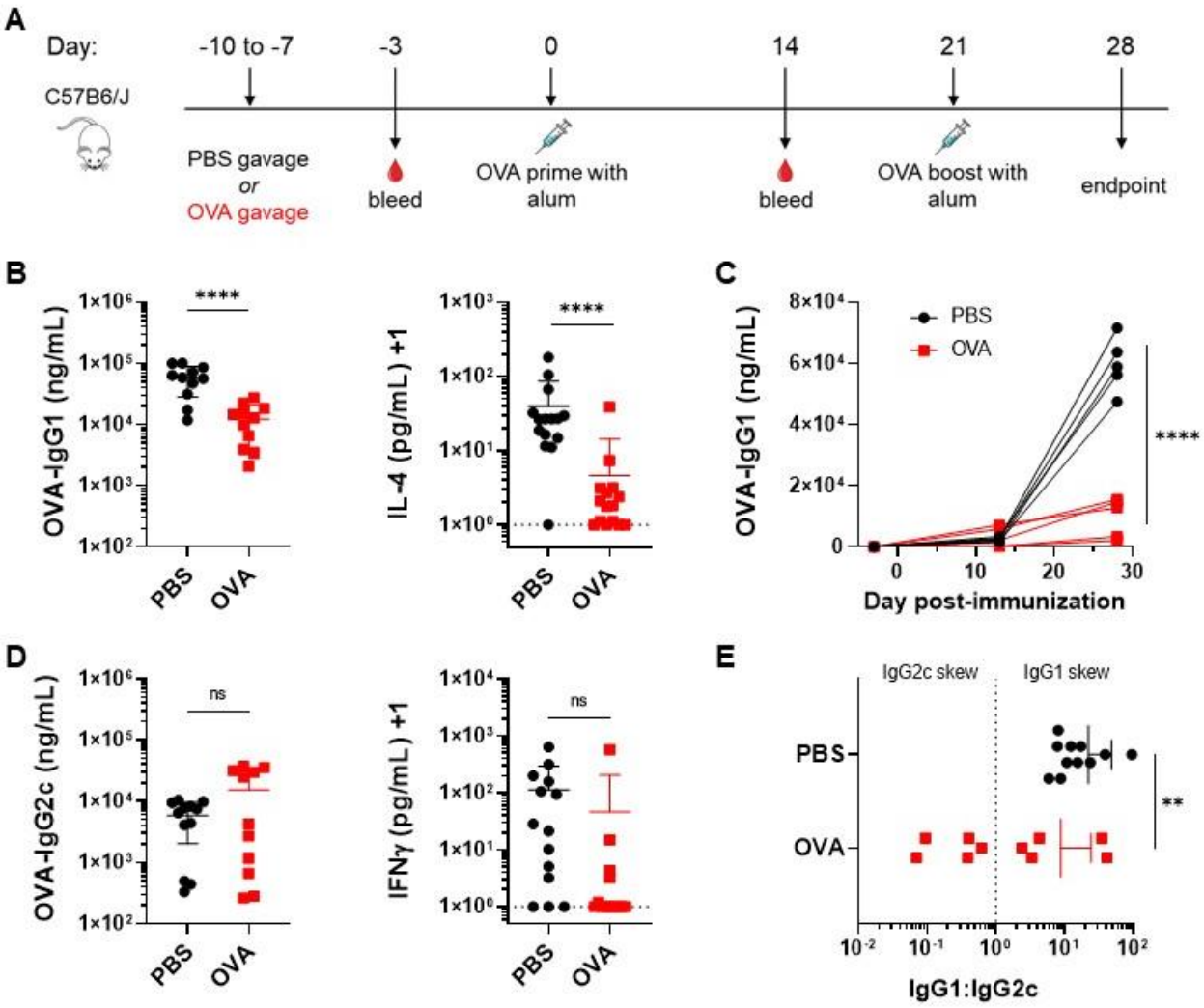

Figure 2.2.1.1 – Oral administration of antigen prior to vaccination with alum suppresses Type 2 immune responses.

Analysis of endpoint (Day 28) of mice gavaged with PBS (black) or OVA (red) prior to immunization with alum. **A)** Experimental timeline **B)** OVA-IgG1 titers and secreted IL-4 upon OVA-re-stimulation of splenocytes after 72 hours. **C)** OVA-IgG1 from an individual experiment over time, representative of 3 independent trials. **D)** OVA-IgG2c titers and secreted IFNγ upon OVA-re-stimulation of splenocytes after 72 hours. **E)** Ratio of IgG1:IgG2c titers for individual mice. Each data point represents an individual mouse. Data pooled from 3 independent experiments. Unpaired t tests were used for statistical analysis.

To explore mechanisms of Type 2-specific suppression of vaccine induced immunity following oral antigen exposure, we turned to regulatory T cell subsets. Previous work suggests that Tregs are critical in the maintenance of oral tolerance[136,244], and data indicate that Tregs expressing the transcription factor RORγt+ could specifically suppress Type 2 immunity[120]. Given that these cells arise from bacterial exposure and are highly prevalent in the siLP[130], the likely site of oral tolerance induction[220,242], we hypothesized that mice orally exposed to OVA prior to systemic immunization could directly alter Treg phenotypes. Though RORγt+ Tregs are typically found in low frequencies systemically, an increased frequency of these cells was observed in the spleens of groups that received OVA prior to immunization compared to PBS treated groups (Figures 2.2.1.3, 2.2.1.4). However, OVA gavaged mice exhibited no change in FoxP3+ Treg frequencies in the MLN or siLP (Figure 2.2.1.4). We examined CD4+ GATA3+ FoxP3- T conventional Th2 cells and found that in agreement with our serum antibody and IL-4 data (Figure 2.2.1.1B), Th2 responses in the spleen were suppressed in OVA gavaged groups (Figure 2.2.1.4). Surprisingly, in the MLN and siLP, no differences in Th2 cells, RORγt+ Tregs (Figure 2.2.1.4), or other Treg subsets examined (GATA3+ Tregs, IL-33R+ Tregs, data not shown) were observed. This indicates that pre-exposure to vaccine antigen prior to systemic immunization using alum contributes to modulation of systemic immune responses, but not detectable changes in T-cell subsets in the GALT.

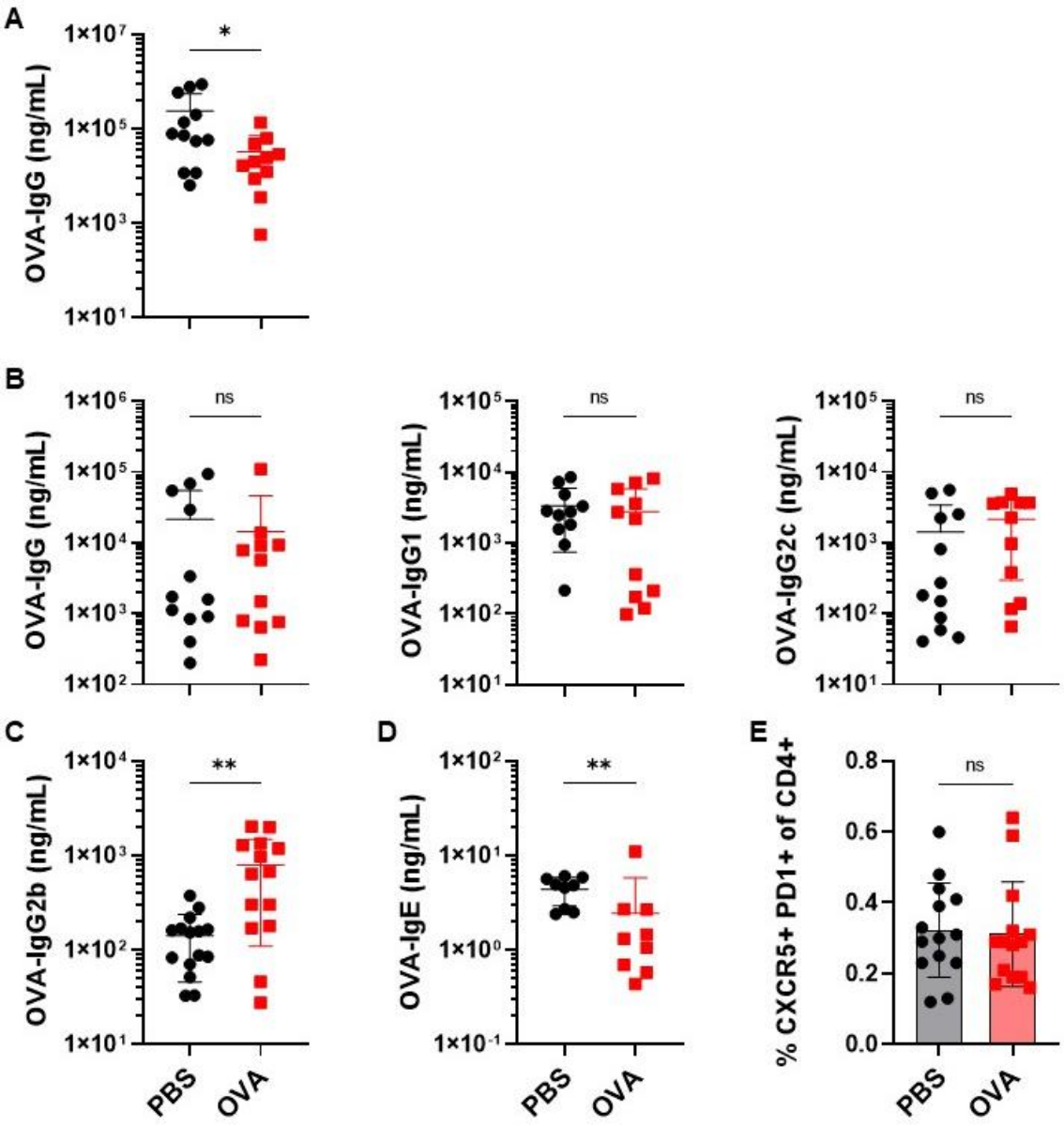

Figure 2.2.1.2 – Oral administration of antigen prior to vaccination with alum: IgG, Day 14, other isotypes, and T-follicular helper cells.

Analysis of antibody titers and cellular responses for experiments described in Figure 2.2.1.1. **A)** Endpoint OVA-IgG titers. **B)** OVA-IgG, IgG1 and IgG2c at Day 14 following immunization after oral antigen pre-exposure. **C)** Endpoint OVA-IgG2b titers. **D)** Endpoint OVA-IgE titers. **E)** T_{FH} cells from spleens of mice, pre-gated on lymphocytes, singlets, live, CD3+, CD45+, CD4+, CD8- prior to CXCR5+ PD-1 gating (representative plot in Figure 2.2.3.1). Data pooled from 3 independent experiments. Unpaired t tests were used for statistical analysis.

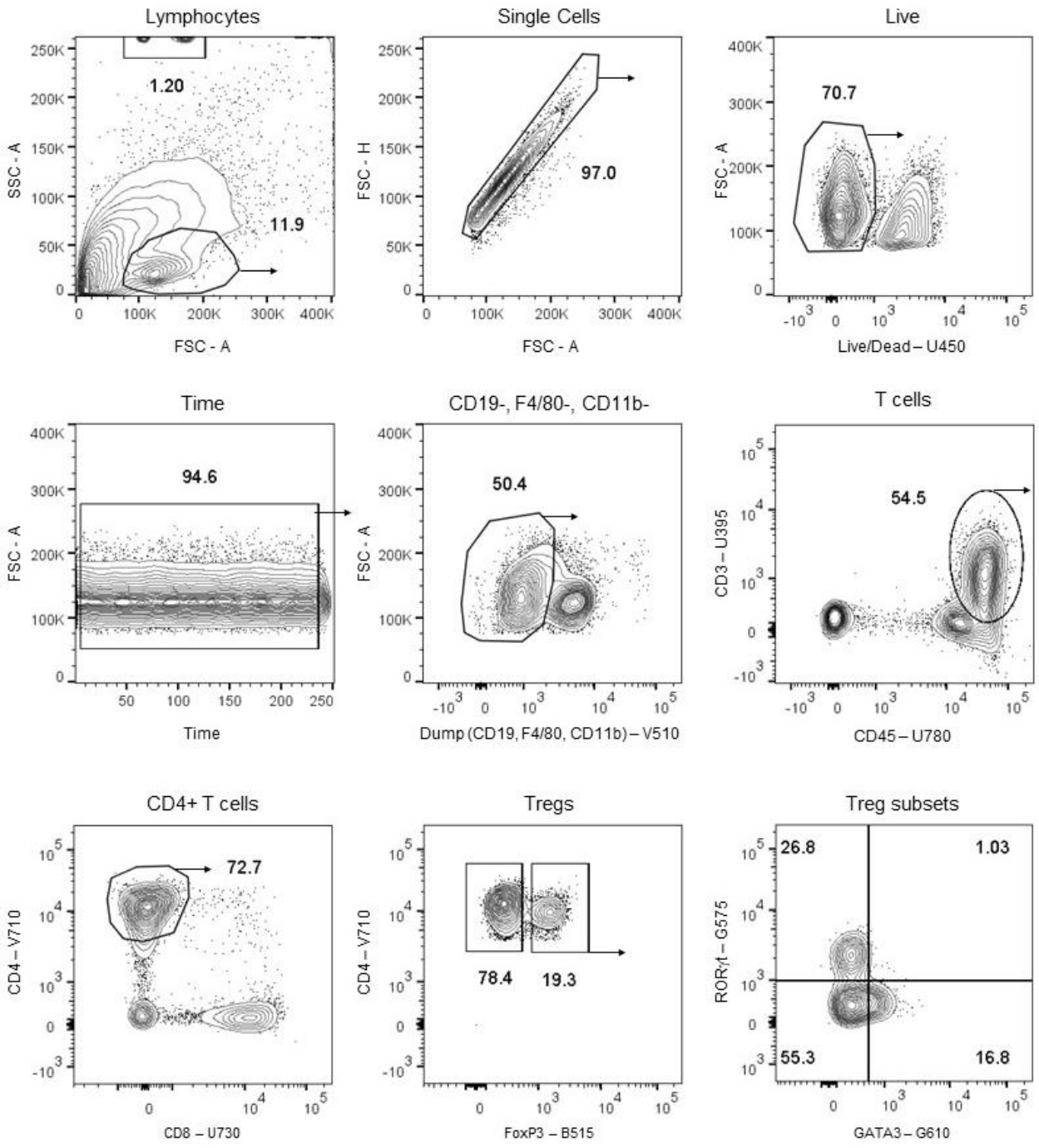

Figure 2.2.1.3 – Flow cytometry gating.

Gating strategy used to identify CD4+ T cells and FoxP3+ Treg subsets. Beads to assess total cell counts are also gated in the first panel (FSC-A v SSC-A).

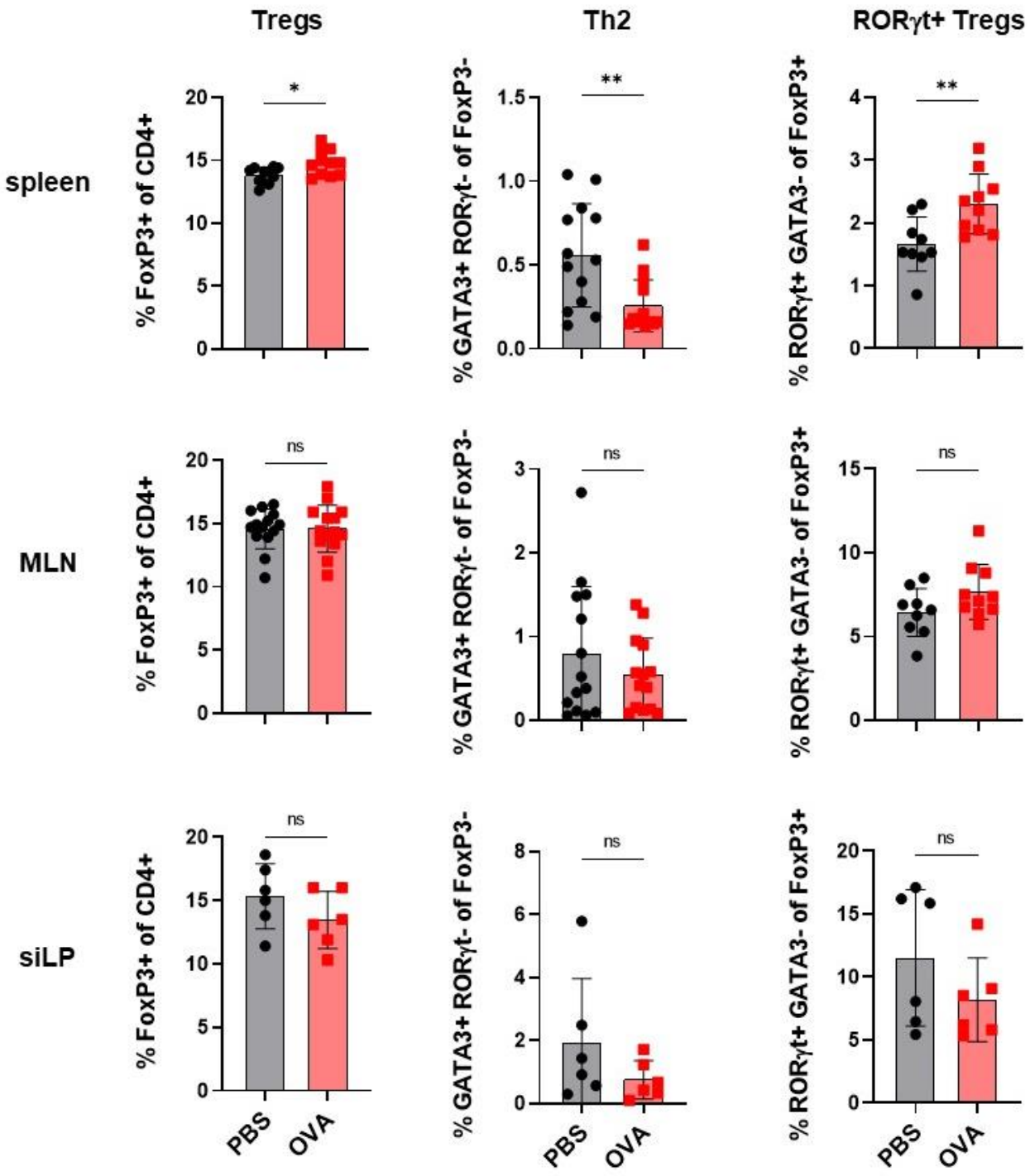

Figure 2.2.1.4 – Oral exposure to antigen prior to vaccination with alum: CD4+ responses.

CD4+ frequencies of indicated subset in spleen, MLN, and siLP for experiment defined in Figure 2.2.1.1A. Gated using strategy outlined in Figure 2.2.1.3. Data pooled from 3 independent experiments. Unpaired t tests were used for statistical analysis.

2.2.2 *RORγt+ Tregs frequencies increase upon exposure to the gut microbiota and upon immunization*

At steady-state in the siLP, pTregs, including those that express RORγt+, maintain gut barrier integrity and mediate effector responses. These cells also contribute to tolerance against gut commensal microbes and controlling homeostatic interactions against environmentally acquired antigens. Additionally, these cells have been observed to be largely absent in GF mice, indicating the necessity of commensal stimulation for the development of this subset[114]. We next set out to define the optimal strategy for oral exposure prior to immunization in GF and SPF mice. To this end, we characterized frequencies of Tregs and various subsets early in life. We assessed the baseline frequencies of SPF and GF mice over time before (DOL 12-15), during (DOL 20-22), and after the weaning period (DOL 26-29), as well as in adulthood (DOL 75-85). We observed that though there were minor differences in abundance of total FoxP3+ cells between groups at each timepoint, there were no consistently significant differences between Treg frequencies in spleen, MLN, siLP or colonic lamina propria (cLP) (Figure 2.2.2.1A). Despite similarities of Treg frequencies, we saw a significant increase of RORγt+ FoxP3+ Treg frequencies in the spleen, MLN, siLP, and cLP in adult SPF mice compared to GF counterparts of the same age (Figure 2.2.2.1B). Similar trends were observed with cell numbers (Figure 2.2.2.2). Recently, it has been described that the RORγt+ Tregs require MHCII expression on ILC3s for development[130]. Early in life, prior to diverse exposures to commensal microbes, the RORγt+ Treg compartment is not developed in SPF mice. We observed no differences in this subset between SPF and GF mice early in life prior to weaning. Our data confirm that development of RORγt+ Tregs occurs within the first few weeks of life and leads to establishment of this subset in adult mice. Notably, we did not observe differences in other Treg

subsets including T-bet+ RORγt- FoxP3+ or GATA3+ RORγt- FoxP3+ cells between adult SPF and GF mice (Figure 2.2.2.2). Together, these data indicate that RORγt+ Treg frequencies increase during the post-weaning period into adulthood and are increased in SPF compared to GF settings.

To determine if immunization also alters RORγt+ Treg population dynamics, we assessed this subset upon immunization with various adjuvants (Figure 2.2.2.1C). In naïve mice, frequencies of RORγt+ Tregs locally in MLN and siLP were 9% and 15% respectively, consistent with previous reports[114] (Figure 2.2.2.1E). Upon immunization with alum, frequencies of these cells remained relatively unaffected in each tissue. One report using a CFA vaccine suggests that use of a more potent adjuvant may lead to expansion of RORγt+ Treg frequencies in gut associated tissues[115]. When Addavax, AS01 and Lit4Q were used to adjuvant OVA immunization, an increase of RORγt+ Tregs was observed in the MLN and siLP (Figure 2.2.2.1D, E). Therefore, proportions of RORγt+ Tregs in gut tissue are increased in C57BL/6 mice immunized with strong immunostimulatory adjuvants, though these cell proportions are not affected by oral antigen pre-exposure (Figure 2.2.1.4).

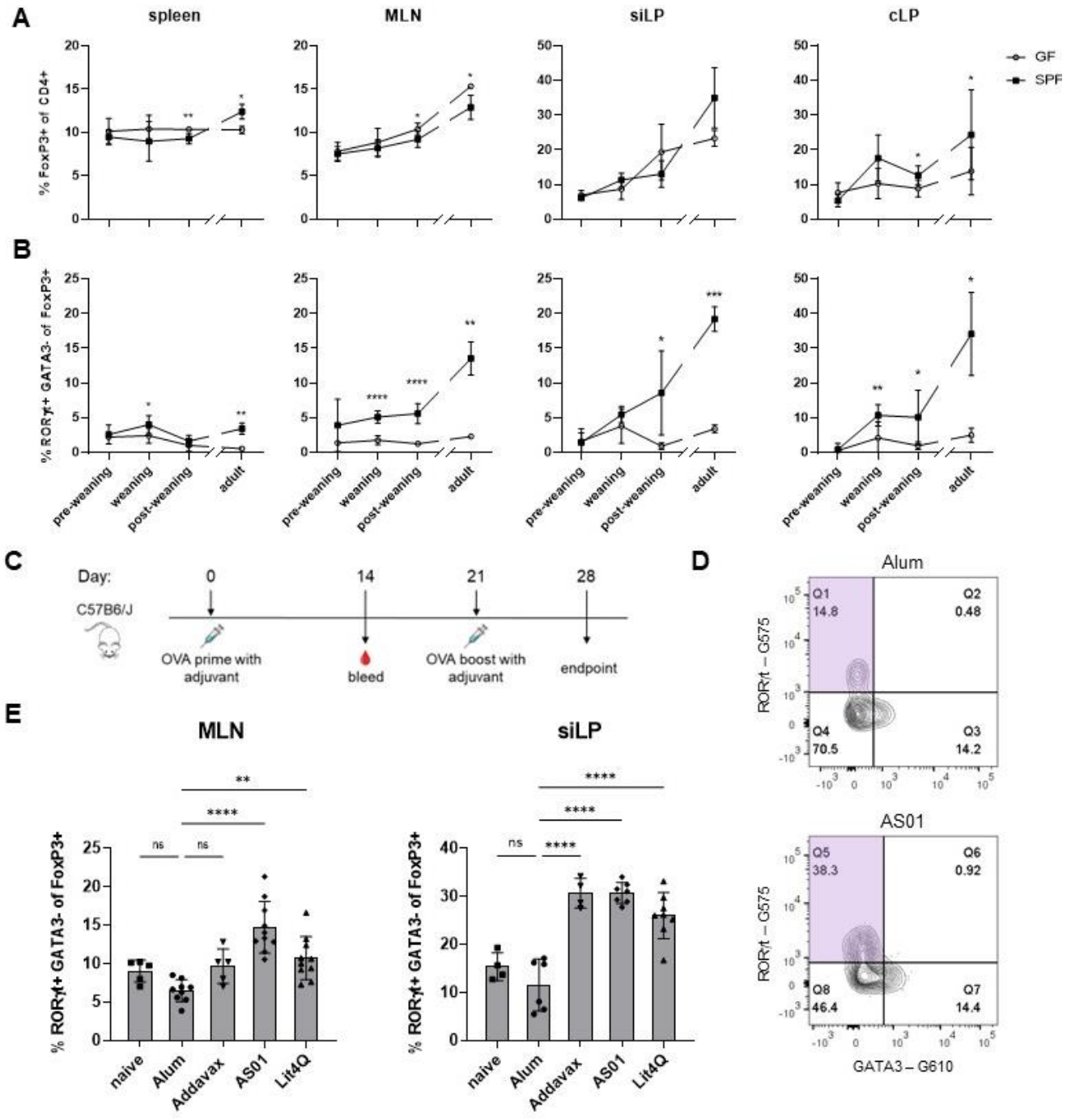

Figure 2.2.2.1 – RORγt+ Tregs frequencies increase upon exposure to the gut microbiota and upon immunization.

A) Frequencies of FoxP3+ Tregs and **B)** RORγt+ Tregs in GF and SPF mice over time in various tissues. For time course experiments, data pooled from 10 independent experiments of mice at

various ages. n = 5-10 for each group at each timepoint early in life and n = 3 for adult mice. **C)** Experimental timeline for remaining figure panels. **D)** Representative gating of RORγt+ Tregs upon immunization with Alum (top) or AS01 (bottom). **E)** Frequencies of RORγt+ Tregs in mice immunized with indicated adjuvant. Data pooled from 9 independent experiments; each adjuvant was tested in 2-3 independent experiments apart from the naïve mouse group. Unpaired t tests were used for statistical analysis.

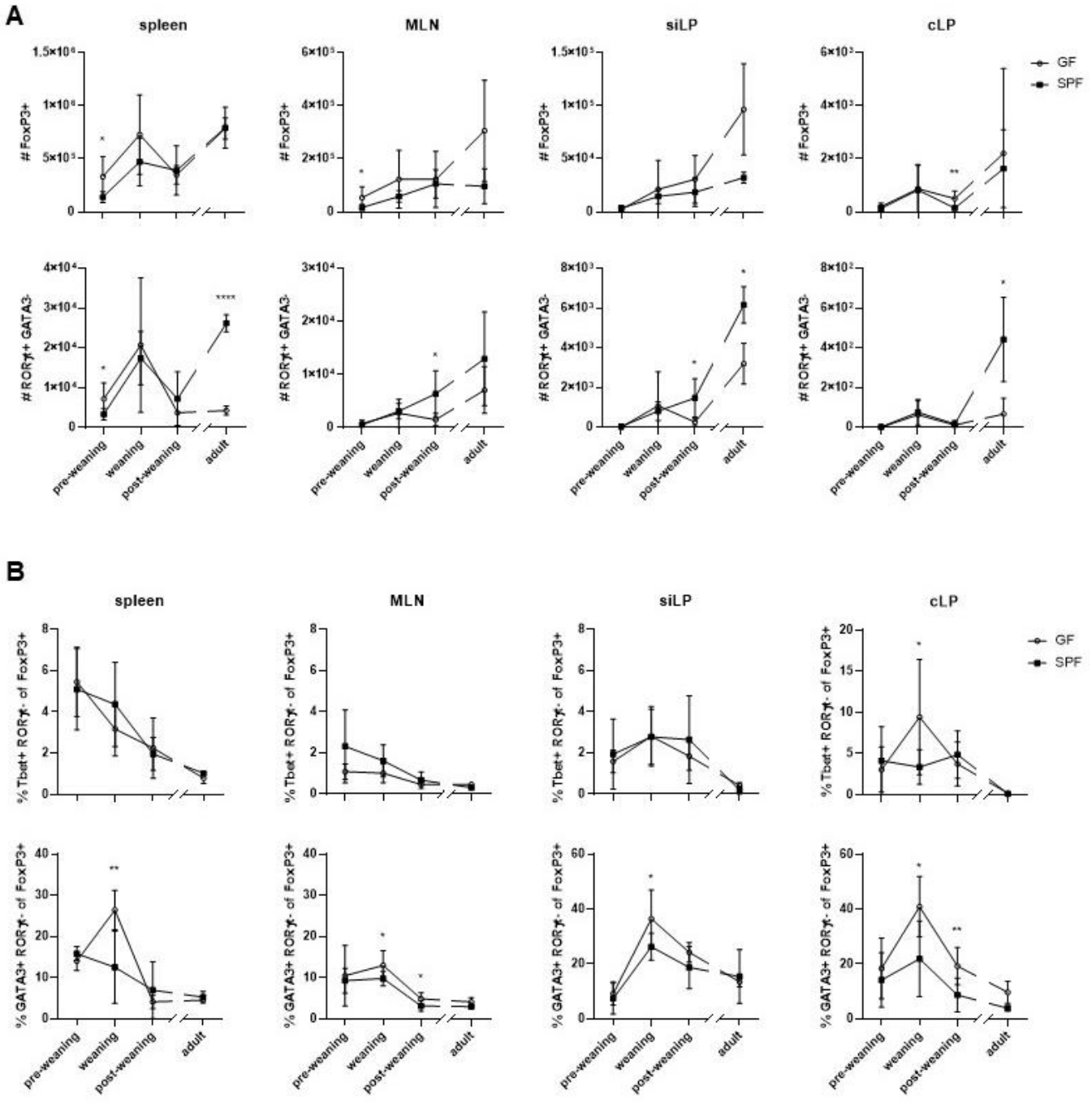

Figure 2.2.2.2 – GF vs SPF profiling early in life: cell counts and various Treg subsets.

A) Counts of FoxP3+ Tregs and RORγt+ Tregs corresponding to frequencies reported in Figure 2.2.2.1A. **B)** Frequencies of T-bet+ and GATA3+ Tregs in GF and SPF mice over time in various tissues. For time course experiments, data pooled from 10 independent experiments of

mice at various ages. n = 5-10 for each group at each timepoint early in life and n = 3 for adult mice.

2.2.3 *Compensatory increase in GATA3+ and IL-33R+ subsets in mice lacking RORγt expression in Tregs*

To examine the role of RORγt Tregs in mediating tolerance to a systemic vaccine following oral exposure, we developed a $Rorc^{fl/fl}$ $FoxP3^{cre}$ cKO line of C57BL/6 mice housed in specific pathogen-free conditions. As previously described[120], these mice that lack RORγt+ expression specifically in FoxP3+ Tregs and have imbalanced immune cell populations in the GALT where these cells are normally present at increased frequencies compared to the spleen. To further investigate these findings, we compared immune cell populations and baseline immunoglobulins across various tissues of $Rorc^{fl/fl}$ $FoxP3^{cre}$ cKO mice and $Rorc^{+/+}$ $FoxP3^{cre}$ controls. We examined serum IgE levels in cKO lines and control mice and did not detect any differences between groups, and though in contrast to previously reported findings[120], this is likely due to a high baseline antibody titer in the $Rorc^{+/+}$ $FoxP3^{cre}$ control line compared to WT B6 mice (Figure 2.2.3.1A). As expected, we found a significant reduction in the frequencies of RORγt+ Tregs in $Rorc^{fl/fl}$ $FoxP3^{cre}$ cKO mice compared to controls (Figure 2.2.3.2A, B). No significant differences were observed in FoxP3+ frequencies among all CD4+ in the siLP, though frequencies of Tregs were increased in the MLN in cKO mice. Frequencies of GATA3+ RORγt- FoxP3+ Tregs and IL-33R+ RORγt- FoxP3+ cells were increased in $Rorc^{fl/fl}$ $FoxP3^{cre}$ cKO mice compared to $Rorc^{+/+}$ $FoxP3^{cre}$ control mice in both the MLN and siLP (Figure 2.2.3.2A). Effects of this cre-lox system were specific to FoxP3+ Tregs, as there were no differences in frequencies of RORγt+ FoxP3- Th17 cells (Figure 2.2.3.2C, D). cKO mice also had increased proportions of Th2 (GATA3+ RORγt- FoxP3-) cells compared to controls in local

tissues (Figure 2.2.3.2C, D). Similar trends were observed in total cell numbers; there was a significant increase in GATA3+ RORγt- FoxP3- Th2 cells in the siLP of cKO mice, no differences in Treg cell numbers, and greater numbers of GATA3+ RORγt- FoxP3+ Tregs and IL-33R+ RORγt- FoxP3+ in the MLN and siLP (Figure 2.2.3.3). In the spleen, we observed a slight increase in FoxP3+ frequencies overall, but similar patterns in Th2, GATA3+ RORγt- FoxP3+ and IL-33R+ RORγt- FoxP3+ subsets (Figure 2.2.3.2E). At baseline, there were no differences in the T_{FH} compartment between cKO and controls in the spleen, MLN, or siLP (Figure 2.2.3.1B, C). Taken together, these data support the role of RORγt+ Tregs in controlling Type 2-associated immunity in mucosal tissues.

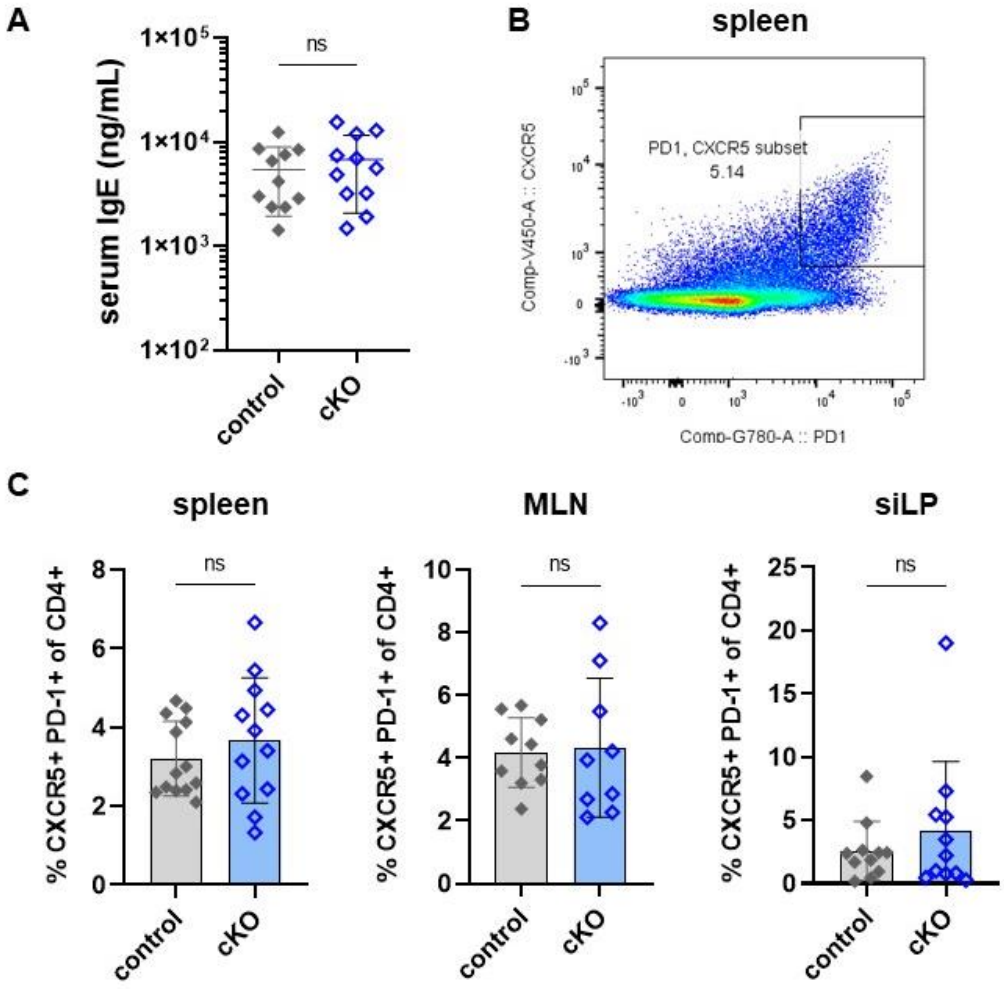

Figure 2.2.3.1 – Phenotyping of mice lacking RORγt expression in Tregs: IgE and T_{FH} cells.

Analysis of $Rorc^{+/+}$ $FoxP3^{cre}$ (control, gray) or $Rorc^{fl/fl}$ $FoxP3^{cre}$ (cKO, blue) mice at steady-state. **A)** total serum IgE titers at baseline. **B)** Representative gating of CXCR5+ PD-1+ T_{FH} cells pre-gated on lymphocytes, singlets, live, CD3+, CD45+, CD4+, CD8-. **C)** T_{FH} frequencies from indicated tissue in cKO or control mice. Data pooled from 3 independent experiments. Unpaired t tests were used for statistical analysis.

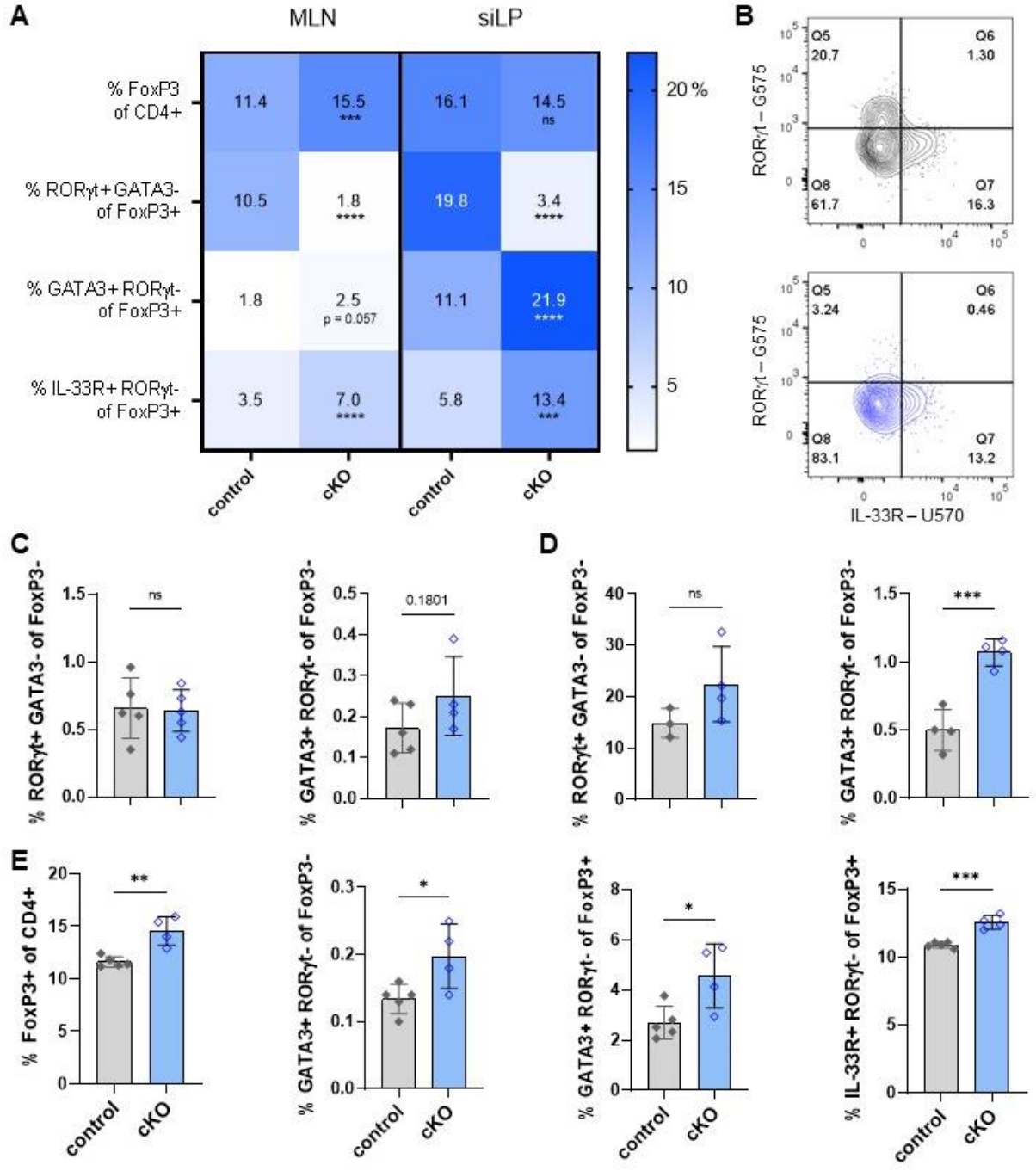

Figure 2.2.3.2 – Compensatory increase in GATA3+ and IL-33R+ subsets in mice lacking RORγt expression in Tregs.

A) Frequencies indicated Treg or Treg subset from Rorc$^{+/+}$ FoxP3^{cre} (control) or Rorc$^{fl/fl}$ FoxP3^{cre} (cKO) mice in MLN or siLP at steady-state. **B)** Representative gating of RORγt+ Treg

population from siLP of control (gray) or cKO (blue) mice. **C)** Frequencies of indicated Tconv subsets in MLN or **D)** siLP from control (gray) or cKO (blue) mice. **E)** Frequencies of indicated Treg or Tconv cell from spleens of control or cKO mice. Data representative of 3 independent experiments. Unpaired t tests were used for statistical analysis.

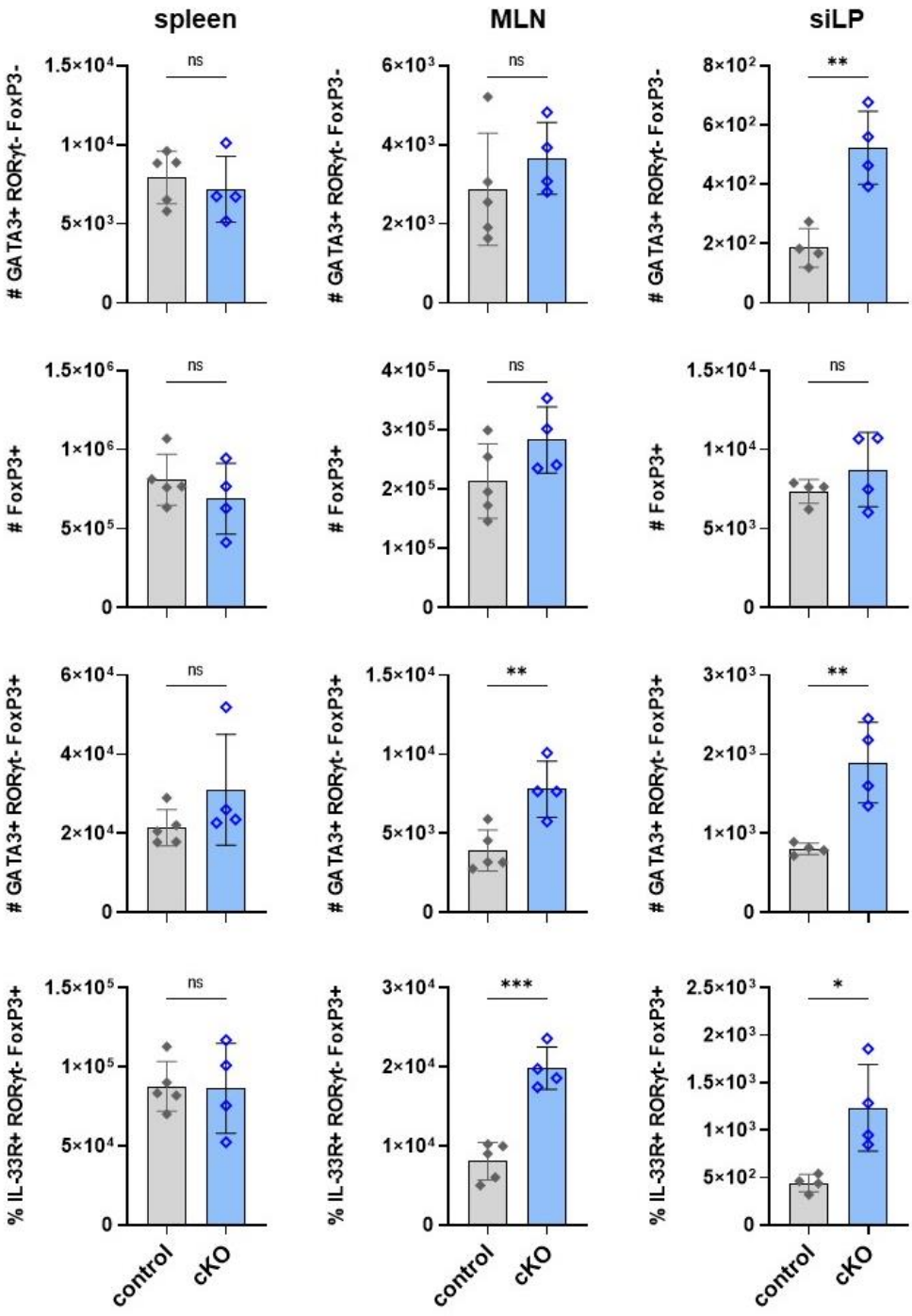

Figure 2.2.3.3 – Counts of Tregs, GATA3+ Tconv and Tregs, and IL-33R+ Tregs from Rorc$^{fl/fl}$ FoxP3^{cre} cKO and control mice.

Total cell counts of indicated Treg or Treg subset from Rorc$^{+/+}$ FoxP3^{cre} (control, gray) or Rorc$^{fl/fl}$ FoxP3^{cre} (cKO, blue) mice in spleen, MLN or siLP at steady-state. Data representative of 3 independent experiments. Unpaired t tests were used for statistical analysis.

2.2.4 *Tolerance remains intact in mice lacking RORγt expression in Tregs upon immunization with alum.*

To examine the role of RORγt+ Tregs in oral tolerance to systemic vaccine responses, $Rorc^{fl/fl}$ $FoxP3^{cre}$ cKO mice and $Rorc^{+/+}$ $FoxP3^{cre}$ controls were used in our immunization model using alum adjuvant. First, cKO and control mice were assessed for differences in vaccination (OVA + alum) without oral antigen pre-exposure (Figure 2.2.4.1). Both groups were able to produce anti-OVA-IgG and subclasses at comparable levels, indicating that lack of expression of RORγt within Tregs does not contribute to differences in vaccine response. Next, both cKO and control mice received OVA or PBS by oral gavage for 4 consecutive days ending one week prior to i.p. immunization with OVA formulated with alum (Figure 2.2.4.2A). We hypothesized that if RORγt+ Tregs were necessary in regulating oral tolerance to a systemic vaccine, then cKO mice pre-exposed to OVA would have comparable antibody titers, specifically OVA-IgG1, to cKO mice that received PBS. To our surprise, we observed that $Rorc^{fl/fl}$ $FoxP3^{cre}$ cKO mice exposed to OVA maintained tolerance as measured by suppression of OVA-IgG1 and had antibody titers comparable to controls given OVA prior to immunization (Figure 2.2.4.2B). Upon OVA re-stimulation of splenocytes and analysis of secreted cytokines, there was suppression of IL-4 production in both $Rorc^{fl/fl}$ $FoxP3^{cre}$ cKO mice exposed to OVA prior to immunization compared to PBS treated controls (Figure 2.2.4.2B). All $Rorc^{+/+}$ $FoxP3^{cre}$ controls also maintained tolerance as measured by suppression of IgG1 and IL-4 production in groups that received OVA gavage prior to immunization (Figure 2.2.4.2B). OVA-IgG2c titers and IFNγ production after OVA re-stimulation were comparable among all groups (Figure 2.2.4.2C), consistent with previous findings in WT mice (Figure 2.2.1.1D). In sum, we find that RORγt expression in Tregs is not necessary for the maintenance of oral tolerance to systemic vaccination using alum adjuvant.

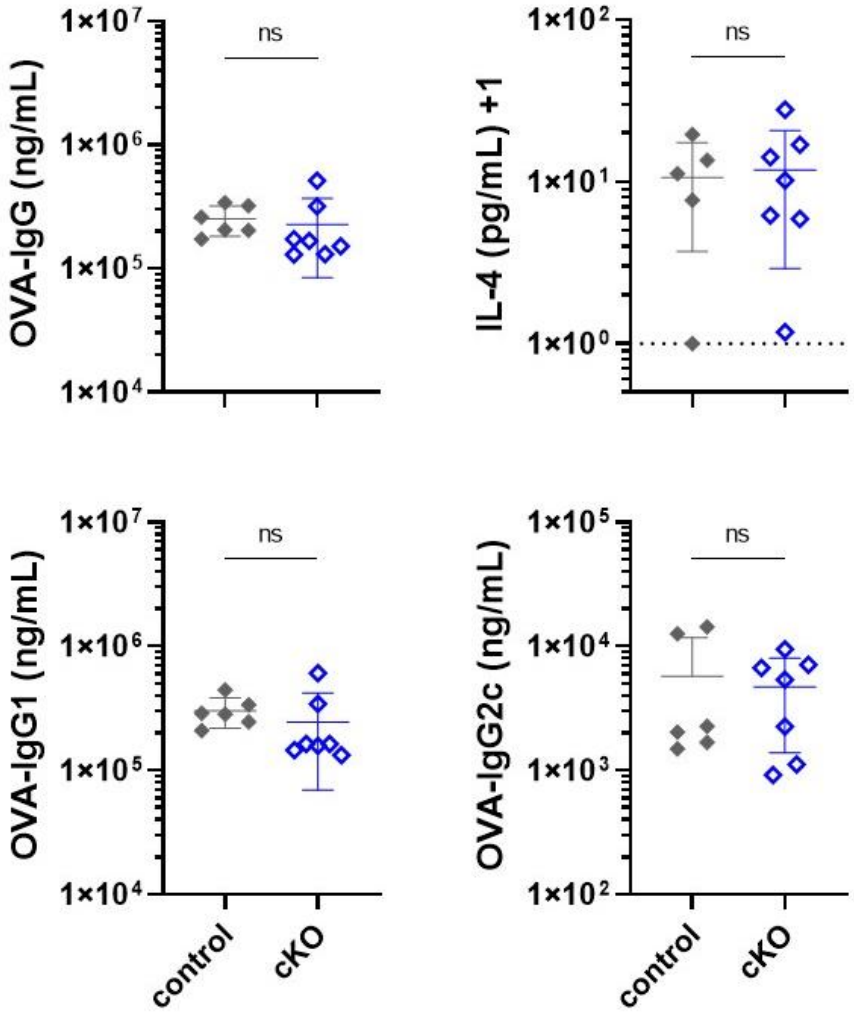

Figure 2.2.4.1 – No differences of immunization with alum alone on mice lacking RORγt+ Tregs compared to controls.

$Rorc^{+/+}$ $FoxP3^{cre}$ (control, gray) or $Rorc^{fl/fl}$ $FoxP3^{cre}$ (cKO, blue) mice were immunized with OVA+ alum at Day 0, boosted at Day 21, and assessed at Day 28 for serum antibody titers (OVA-IgG, IgG1 and IgG2c) and cytokine secretion of IL-4 after 72-hour stimulation of splenocytes. Data pooled from 2 independent experiments. Unpaired t tests were used for statistical analysis.

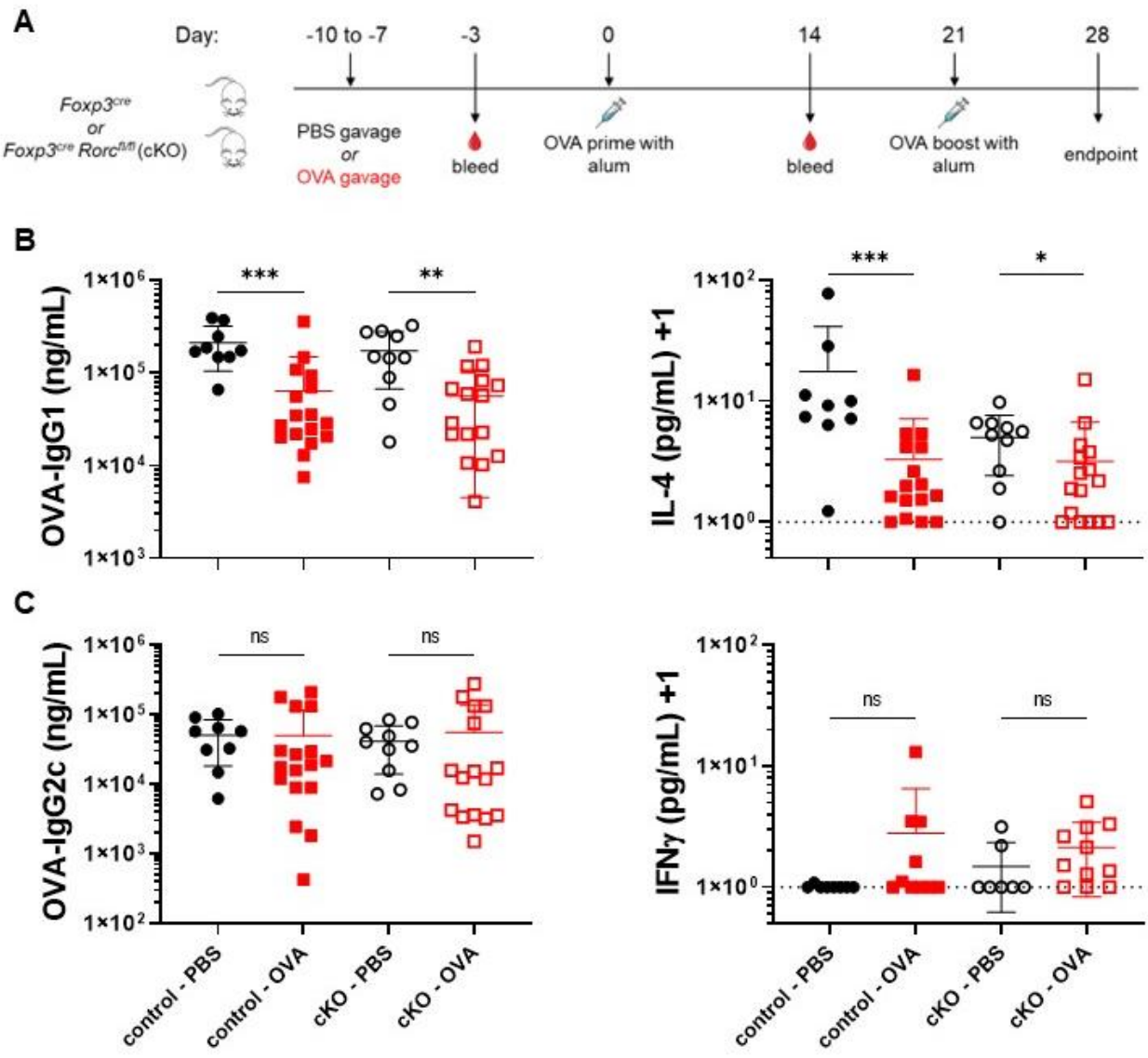

Figure 2.2.4.2 – Tolerance remains intact in mice lacking RORγt expression in Tregs upon immunization with alum.

Analysis of endpoint (Day 28) of mice gavaged with PBS (black) or OVA (red) from control mice (closed symbols) or cKO mice (open symbols). **A)** Experimental timeline. **B)** OVA-IgG1 titers and secreted IL-4 upon OVA-re-stimulation of splenocytes after 72 hours. **C)** OVA-IgG2c titers and secreted IFNγ upon OVA-re-stimulation of splenocytes after 72 hours. Data pooled from 3-4 independent experiments. Unpaired t tests were used for statistical analysis.

2.2.5 *Suppression of Type 2 responses maintained upon oral exposure prior to vaccination with various adjuvants.*

Though alum, the first licensed adjuvant used clinically, was used for many original studies of tolerance, stronger immune stimuli are now used in the development of novel vaccine candidates[42]. Despite their use in licensed vaccines for seasonal influenzas and shingles, the mechanisms of action of adjuvants like Addavax, similar to MF59, and AS01 are incompletely understood. Characterization of how antigen pre-exposure affects immune suppression upon vaccination with these adjuvants has not been examined prior to this study.

When using alum, Type 2 immune responses were suppressed upon oral exposure to the vaccine antigen (Figure 2.2.1.1). Alum induces Th2 cells and facilitates antigen-specific antibody production via NLRP3 activation[44,50]. The Type 2 skewing of alum and subsequent suppression of Type 2 immune response upon oral exposure led us to question whether this would be the case when stronger adjuvants or those that elicit a balanced Type 1 and 2 response were used. To this end, we used Addavax, AS01, and Lit4Q in our model of oral tolerance. Addavax is associated with increased T_{FH} formation and acts through NLRP3 independent pathways by recruiting CD11b+ DCs that stimulate both Th1 and Th2 responses[55,57]. AS01, which is comprised of 3D-MPL that signals through TLR-4 and QS-21 which induces caspase-1 activation, elicits a balanced Th1 and Th2 response when used in a vaccine[42,58]. The novel compound Lit4Q also contains a TLR-4 agonist in association with QS-21 and is expected to lead to comparable responses to AS01 *in vivo* when given at the same dose. To assess the skew of each of these adjuvants varied responses, we compared IgG1:IgG2c titers in mice after OVA immunization in the absence of oral OVA pre-exposure. While immunization with alum,

Addavax, and AS01 led to a skew toward OVA-specific IgG1, Lit4Q lead to a skew toward IgG2c production, indicating differences in adjuvant mechanism of action (Figure 2.2.5.1A).

Using each of these adjuvants with OVA in our tolerance model in C57BL/6 mice (Figure 2.2.1.1A), we observed suppression of OVA-IgG1, but not OVA-IgG2c, in mice that received OVA gavage prior to immunization compared to PBS gavaged controls regardless of adjuvant strength or skew (Figure 2.2.5.1B, C). Overall, both AS01 and Lit4Q adjuvanted vaccines led to greater production of IgG1 and IgG2c antibody titers compared to other adjuvants tested. Similarly, we observed that mice gavaged with OVA prior to immunization with Addavax and Lit4Q led to suppression of IL-4 upon OVA re-stimulation of splenocytes. Mice immunized with AS01 had higher overall IL-4 production and no differences between groups. Because AS01 elicited the greatest IgG1 response among those examined, the OVA re-stimulation of splenocytes may have led to higher overall IL-4 production that was not able to be suppressed by pre-exposure to vaccine antigen. No differences were observed in IFNγ production upon OVA re-stimulation, indicating that all the adjuvants suppressed only Type 2, and not Type 1 mediating immune responses upon immunization following oral antigen exposure (Figure 2.2.5.1C). Consistent with our findings using alum, there were no observable impact of oral OVA exposure on Treg or RORγt+ Treg frequencies in the MLN and siLP compared to mice given PBS prior to immunization (Figure 2.2.5.2). There were no differences between OVA gavage and PBS gavage on the T_{FH} compartments of spleen, MLN, and siLP, regardless of adjuvant (Figure 2.2.5.3). These data indicate that suppression of Type 2 vaccine-induced immunity is not specific to adjuvants such as alum that skew towards Type 2 but is consistent for a variety of adjuvants examined in our model.

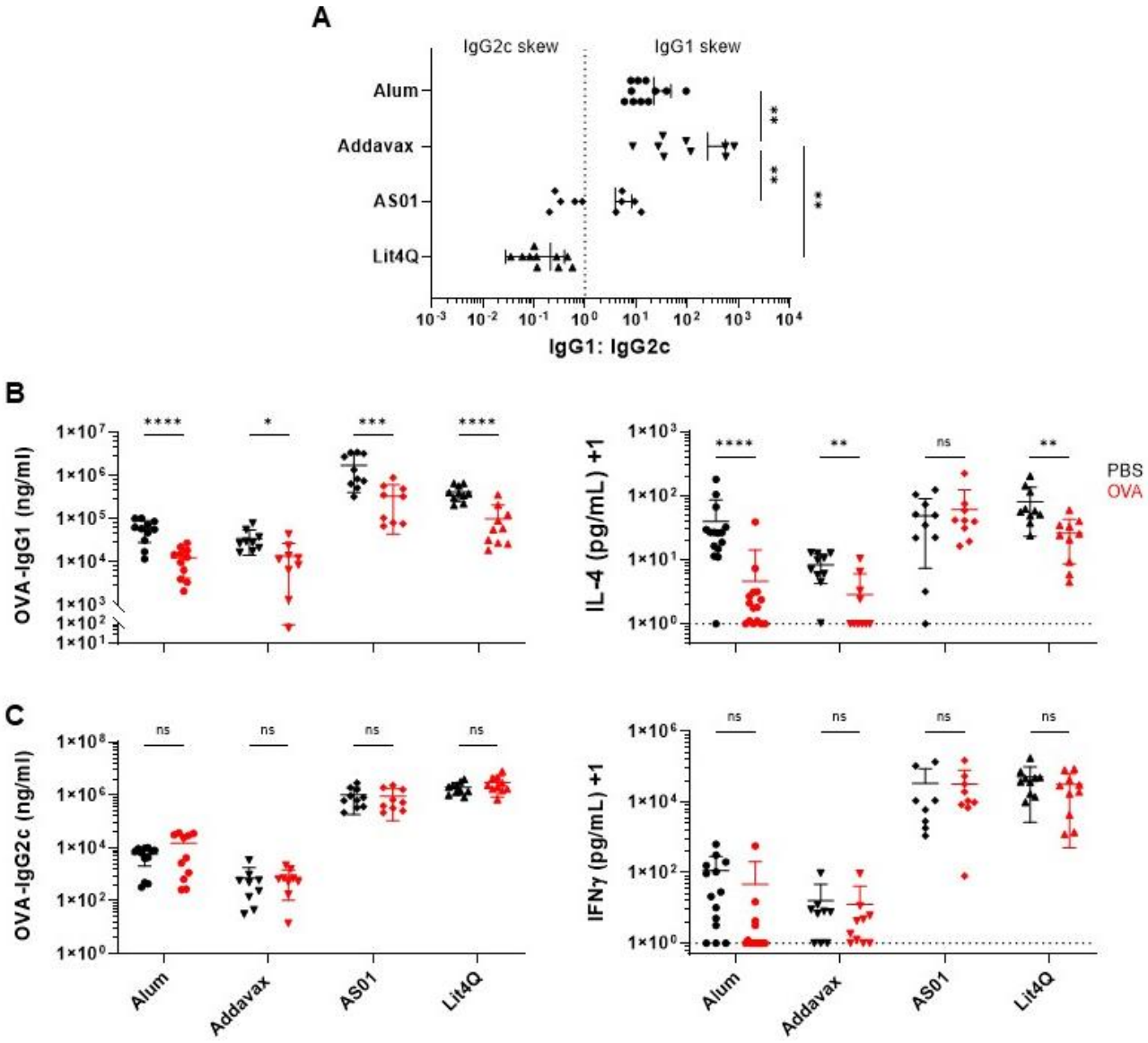

Figure 2.2.5.1 – Suppression of Type 2 responses maintained upon oral exposure prior to vaccination with various adjuvants.

Experimental timeline from Figure 2.2.1.1A was used with various adjuvants. **A)** Ratio of IgG1:IgG2c titers for individual mice. **B)** OVA-IgG1 titers and secreted IL-4 upon OVA-re-stimulation of splenocytes after 72 hours. **C)** OVA-IgG2c titers and secreted IFNγ upon OVA-re-stimulation of splenocytes after 72 hours. For each adjuvant, data pooled from 2-3 independent experiments. Unpaired t tests were used for statistical analysis.

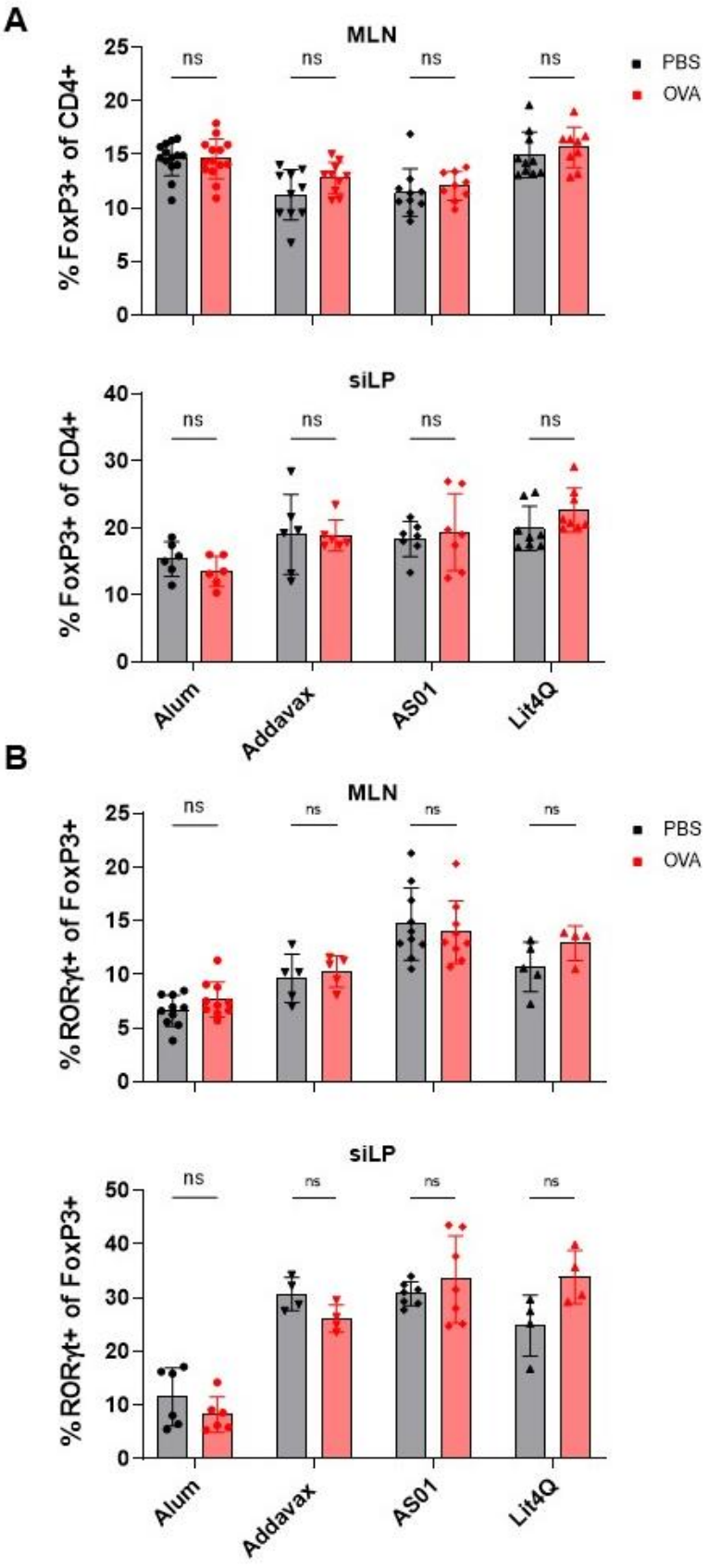
A
MLN
% FoxP3+ of CD4+
ns
PBS
OVA
siLP
Alum
Addavax
AS01
Lit4Q
B
% RORγt+ of FoxP3+

Figure 2.2.5.2 – No effect of oral exposure to OVA on frequencies of Tregs or RORγt+ Tregs in MLN and siLP.

Experimental timeline from Figure 2.2.1.1A was used with various adjuvants. **A)** Frequencies of FoxP3+ Tregs or **B)** RORγt+ Tregs pre-gated on lymphocytes, singlets, live, CD3+, CD45+, CD4+, CD8- of mice gavaged with OVA or PBS prior to immunization. For each adjuvant, data pooled from 2-3 independent experiments. Unpaired t tests were used for statistical analysis.

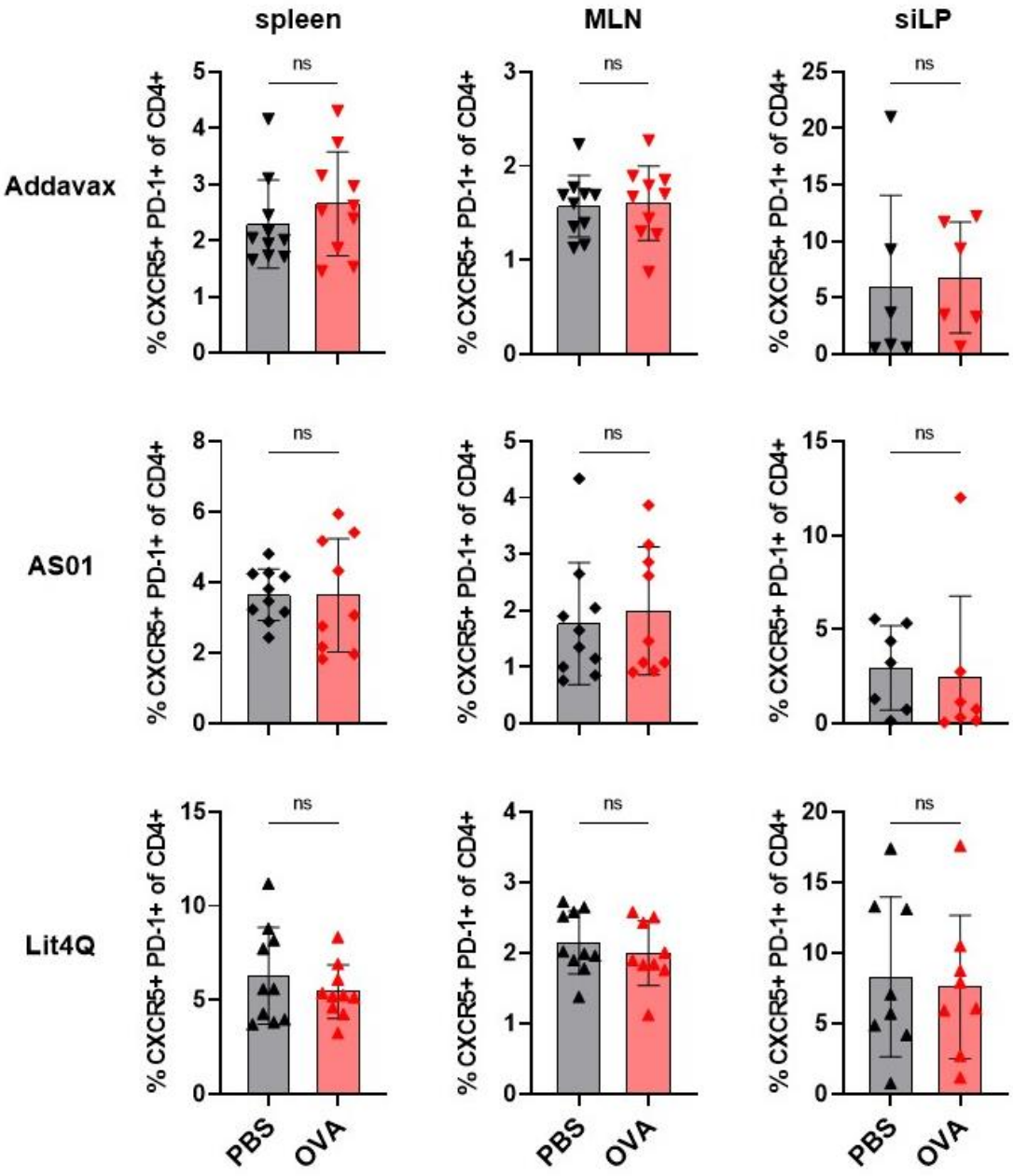

Figure 2.2.5.3 – T_{FH} cells are unchanged between OVA and PBS exposed groups prior to immunization with various adjuvants.

T_{FH} frequencies from indicated tissue from indicated tissue of mice exposed to OVA or PBS prior to immunization with Addavax, AS01 or Lit4Q. of CXCR5+ PD-1+ T_{FH} cells pre-gated on lymphocytes, singlets, live, CD3+, CD45+, CD4+, CD8-. For each adjuvant, data pooled from 2 independent experiments. Unpaired t tests were used for statistical analysis.

2.2.6 *Tolerance maintained in mice lacking RORγt expression in Tregs with Lit4Q adjuvanted immunization.*

After testing multiple adjuvants in our tolerance model, Lit4Q was chosen for use in the conditional knock-out model of RORγt+ Tregs to examine whether use of different adjuvants would be sufficient for breaking tolerance in a systemic vaccine. Our previous data indicated that use of Lit4Q led to an increased frequency of RORγt+ Tregs compared to immunization with alum in both the MLN and siLP (Figure 2.2.2.1E) and favored IgG2c production over IgG1 (Figure 2.2.5.1A-C). Therefore, we decided to investigate if immunization with Lit4Q be sufficient for recovery of IgG1 upon oral OVA exposure in mice lacking RORγt expression in Tregs. We hypothesized that tolerance could be broken in mice lacking RORγt+ expression in Tregs when orally exposed to the vaccine antigen prior to vaccination with Lit4Q. To test this, a similar model to Figure 2.2.4.2A was utilized but using a Lit4Q adjuvanted OVA vaccine. Similar to alum-adjuvanted immunization, we observed that upon use of Lit4Q, OVA-IgG1 was suppressed in both $Rorc^{fl/fl}$ $FoxP3^{cre}$ cKO and $Rorc^{+/+}$ $FoxP3^{cre}$ control mice gavaged with OVA prior to immunization (Figure 2.2.6.1A). Upon OVA re-stimulation of splenocytes, OVA gavaged groups had a trend towards suppression of IL-4 production, though these did not reach the threshold for statistical significance (Figure 2.2.6.1A). In line with our findings using alum (Figure 2.2.4.2C) and in WT C57BL/6 mice (Figure 2.2.5.1C), there were no differences in OVA-IgG2c production or IFNγ upon OVA re-stimulation between mice of different genotypes or groups pre-exposed to oral OVA compared to PBS (Figure 2.2.6.1B). This indicates that regardless of adjuvant strength of skew, RORγt expression within Tregs is dispensable for tolerance to a systemic vaccine following oral antigen exposure.

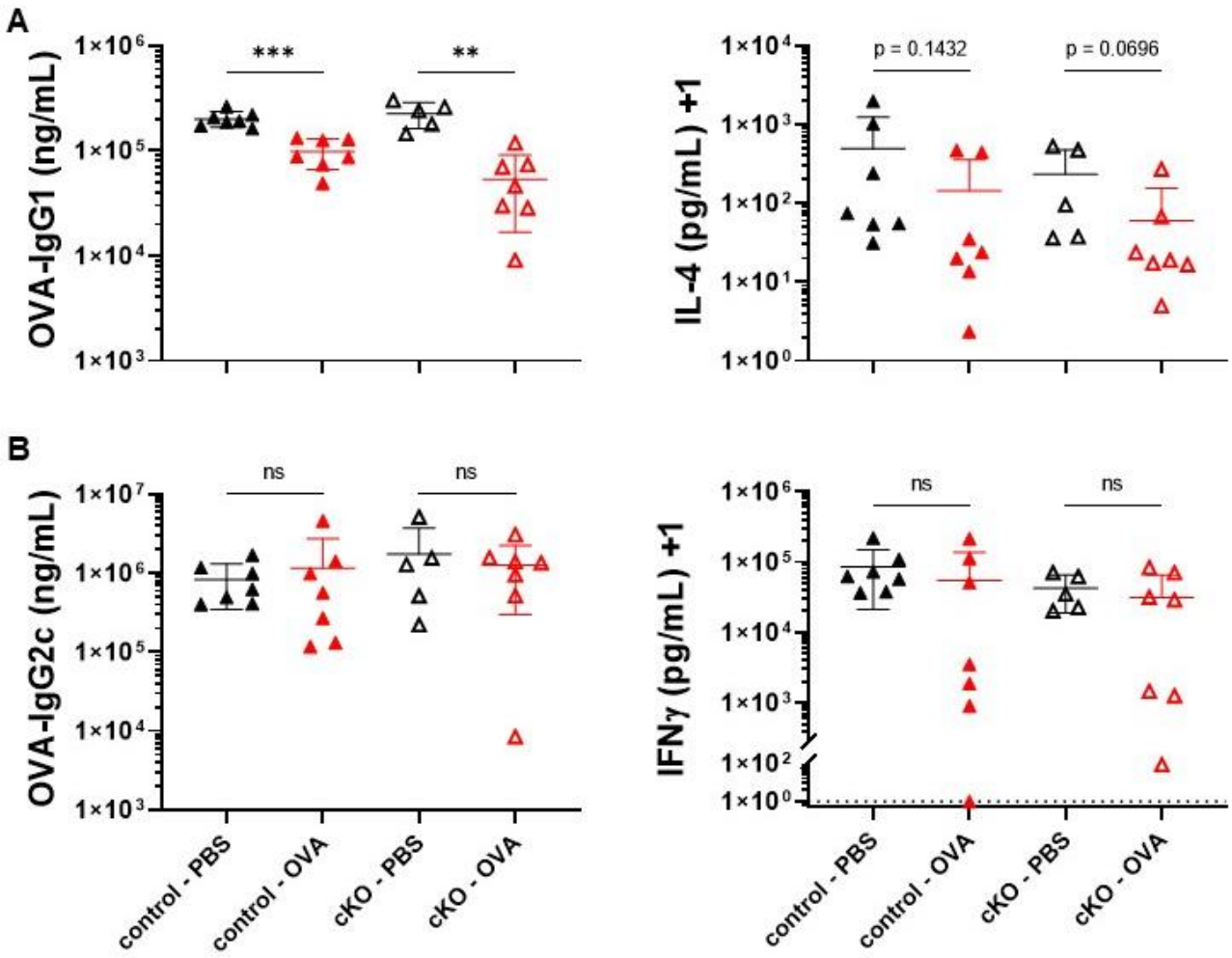

Figure 2.2.6.1 – Tolerance maintained in mice lacking RORγt expression in Tregs with Lit4Q adjuvanted immunization.

Experimental timeline from Figure 2.2.4.2A was used with Lit4Q as adjuvant in immunization. Control mice (closed symbols) or cKO mice (open symbols) were gavaged with PBS (black) or OVA (red) prior to immunization. **A)** OVA-IgG1 titers and secreted IL-4 upon OVA-re-stimulation of splenocytes after 72 hours. **B)** OVA-IgG2c titers and secreted IFNγ upon OVA-re-stimulation of splenocytes after 72 hours. Data pooled from 2 independent experiments. Unpaired t tests were used for statistical analysis.

2.3 DISCUSSION

Our data indicate that RORγt expression in Tregs is dispensable for controlling oral tolerance to systemic vaccination. Mice that were exposed to OVA prior to immunization displayed a reduction in OVA-IgG1 and IL-4 production upon re-stimulation, however, we did not observe suppression of Type 1 immunity, including OVA-IgG2c or IFNγ production. We then characterized RORγt+ Treg frequencies in multiple settings; in early-life, no differences in this population were observed between GF and SPF mice, but as this subset develops upon commensal exposure in SPF mice, there was a significant increase in adult mice. Upon use of TLR4 agonists like AS01 and Lit4Q, frequencies of RORγt+ Tregs were increased compared to naïve groups and use of alum. To dissect the role of this subset further, we used a conditional knock-out model ($Rorc^{fl/fl}$ $FoxP3^{cre}$) and observed a local increase of Th2 cells, along with GATA3+ and IL-33R+ Treg subsets. In these cKO mice, tolerance to alum immunization was maintained following oral OVA exposure, indicating that expression of the transcription factor RORγt was dispensable within Tregs for mediating the function of suppressing systemic antibody titers. To test whether this was true in other settings, we utilized Lit4Q, an adjuvant which skews toward OVA-IgG2c production and leads to development of an increased antibody titers compared to immunization with alum. Oral OVA exposure prior to OVA + Lit4Q immunization led to similar suppression of Type 2 immunity (IgG1 and IL-4 production). In mice lacking RORγt expression within Tregs, immunization with Lit4Q lead to similar maintenance of tolerance, indicating that the type of adjuvant used likely does not determine how systemic immunization is controlled upon prior oral exposure. Here, we found that tolerance to systemic vaccination following oral antigen pre-exposure remains intact in the absence of RORγt expression within Tregs, regardless of adjuvant used in the immunization.

In our model, we disrupt RORγt expression within Tregs, though these pTregs, albeit lacking RORγt, are still present in local tissues. It is possible that genes controlled by RORγt are non-essential for the maintenance of tolerance to systemic vaccination, though the presence of the Treg is still required. While various genes are upregulated in colonic Tregs[114], which consist of ~40% RORγt+ Tregs, the distinct role this transcription factor and downstream pathways play in the phenotype of the cell has not been well characterized. Sequencing experiments in various settings will be critical in understanding how these cells function. Previous work has also characterized the role of RORγt+ Tregs in suppressing oral vaccine response in environmental enteric dysfunction[129]. Though our work found that vaccine-specific T cells and IgA, both of which were found to be altered in the absence of RORγt+ Tregs, the consequences on systemic vaccination and in the context of non-dysregulated systems was not examined. Understanding how RORγt+ Tregs may function differently in oral vs. systemic vaccines will be essential for improving effective outcomes for vaccines in development.

By utilizing a conditional knock-out model, our cKO mice lacking RORγt expression in Tregs exhibit dysregulated local immunity, though reduction of this subset did not contribute to differences in Treg frequencies between groups in the siLP (Figure 2.2.3.2). Immunization with adjuvants like Lit4Q boosts RORγt+ Treg frequencies above baseline, similar to what has been previously reported for CFA[115]. Whether targeting cells in the local gut immune system could affect systemic vaccine responses is not known. By exposing mice to certain commensals, cells in the siLP can be skewed; segmented filamentous bacteria contributes to local Th17 induction[180], while *H. hepaticus* elicits RORγt+ Treg expansion at steady-state[121]. Addition of commensals from wild mice leads to a more accurate model of human environmental encounters[254]. Our model also introduces OVA as an innocuous, dietary exposure, while

expression of OVA in a commensal could contribute to innate signaling more representative of commensal exposures. Further investigation of how the addition of such perturbations to the system could impact systemic immunization is warranted.

We observed that lack of expression of RORγt in Tregs led to upregulation of other Treg subsets, specifically of GATA3+ Tregs in the MLN and siLP. GATA3+ Tregs are largely thymically-derived, and cKO of GATA3+ Tregs leads to development of inflammatory disorders in mice, indicating these cells control Type 2 immunity[114,139]. The increased frequency of these cells may compensate for the lack of RORγt+ expression, as we did observe an increased Treg frequency in the spleen and MLN of cKO mice compared to controls (Figure 2.2.3.2A, E). Specific deletion of GATA3+ within Tregs leads to inflammatory disorders and increased susceptibility to allergic reactions[122,139]. Studies suggest that dietary antigens induce pTregs in the siLP, but this population is distinct from pTregs induced by microbial antigens[122,123]. Whether these distinct subsets play a role in controlling tolerance to systemic vaccination is unclear. It also remains a possibility that Tregs that only express FoxP3 and no transcription factors associated with other T-helper subsets are critical for this response. These studies aim to understand cell subsets that mediate peripheral tolerance to oral antigen prior to systemic vaccination. Recent work has highlighted the necessity of MHCII+ RORγt+ APC subsets for formation of pTregs[130-132], though the precise mechanism by which this occurs is not well understood. Further work in characterizing the role of various APC populations in mediating oral tolerance to a systemic vaccine would provide critical insight to this system.

Our model utilizes primarily adult mice in the context of health, though further studies could focus on models of enteric dysfunction, such as was utilized to study of RORγt+ Tregs

during oral vaccination[129]. Similarly, examining various timepoints early in life and how RORγt+ Tregs develop during weaning would provide further insight to this system. Encounters with gut commensals early in life are critical for proper development of pTreg subsets and proper formation of the local immune system to tolerize innocuous interactions[179,252,255]. Variation in laboratory mouse models have also been reported to display altered RORγt+ Tregs frequencies at baseline[256]. By disrupting the state of health, altering the timing of exposure to OVA, or testing other strains, a greater understanding of tolerance upon systemic vaccination could be obtained.

In clinical settings, heterogeneous responses to vaccination between individuals results in difficulties predicting success[25]. Environmental exposures, including pre-existing immunity (prior vaccinations or infections), microbiota composition, genetics, and epigenetic factors each contribute to how an individual responds following immunization[26,32,257]. It is also apparent that components of the vaccine, including adjuvant, immunogen, and route of administration ultimately contribute to effectiveness. These factors must be understood to ensure increased vaccine efficacy and reduce the spread of harmful infections. Specifically, can the system be altered prior to vaccination to better position the immune system to respond? Ongoing studies from many groups aim to address these outstanding questions[4,8]. The scope of this work may extend to advance the understanding of the mechanisms involved in autoimmunity and allergy, both of which will be important for improving human health. We intend to contribute to elucidating mechanisms of how local signals from antigens encountered in the small intestine influence local and systemic immune responses that could be leveraged to improved vaccine efficacy.

2.4 METHODS

2.4.1 *Mice*

C57BL/6 mice were obtained from The Jackson Laboratory (strain 000664) and maintained in specific-pathogen free conditions at Fred Hutchinson Cancer Center. C57BL/6 GF mice were bred in-house at the University of Washington Gnotobiotic Animal Core and housed in GF conditions. FoxP3^{cre} mice on the C57BL/6 background contain a YFP at this locus[258] and were received from the Lund lab (Jackson Laboratory, strain 016959). Rorc$^{fl/fl}$ mice, also known as Rorc^{tm3Litt}/J were ordered from Jackson Laboratory[259] (strain 008771). All mice used in were males and were between 6-16 weeks old at the beginning of each experiment (except for early life experiments, Figure 2.2.2.1). Experiments were approved by the Institutional Animal Care and Use Committees, and all mice were euthanized following AVMA guidelines for CO_2 overdose.

2.4.2 *Mouse Tissue Collection & Processing*

Single-cell suspensions were prepared from mouse tissues. Spleens were homogenized and treated with ACK lysis buffer (Invitrogen; 501129751) to remove red blood cells. Lymphocytes were then washed and resuspended in PBS + 2% fetal bovine serum (FBS). MLNs were homogenized, washed, and resuspended in PBS + 2% FBS. To obtain lymphocytes from the small intestine lamina propria, intestines were removed from mice, then cleaned of remaining fat. Peyer's patches were removed and discarded. The tissue was then sectioned into 6 sections and cut lengthwise to remove intestinal contents. Segments were rinsed in PBS + 2% FBS and mucus was removed with tweezers, then rinsed again in clean PBS + 2% FBS and placed in a 50 mL conical containing no media. Following dissection, samples were resuspended with 10 mL of

Solution A (HBSS without calcium and magnesium + 4.2 mM sodium bicarbonate + 2% FBS, pH 7.4). Samples were vortexed for 20 seconds between washes and washed a total of three times with Solution A. Samples were then resuspended in Solution B (Solution A + 5 mM EDTA + 1 mM DTT) and incubated at 37°C while shaking for 15 minutes. Samples were then vortexed for 30 seconds and resuspended with 25 mL of Solution C (Solution A + 5 mM EDTA) and incubated at 37°C while shaking for 30 minutes. Intestinal fragments were vortexed for 30 seconds, washed in Solution A to remove EDTA, then resuspended in 5 mL of collagenase digestion media (HBSS containing Calcium and Magnesium + 4.2 mM sodium bicarbonate + 10 mM HEPES + 0.25 mg/mL Type-II collagenase + 1% FBS + 1000 Kunitz / mL of DNAse) for 30 to 45 minutes at 37°C while shaking. Following digestion, 5 mM EDTA was added to halt collagenase activity. Samples were passed through a 18g needle to mechanically disrupt tissue and passed through 100 mm filter, rinsed with cRPMI and passed through a 70 mm filter. Each tissue was then counted on a hemocytometer and normalized for flow cytometry staining.

2.4.3 *Ovalbumin Oral Gavage*

Endofit Ovalbumin (Invivogen, vac-pova-100) was reconstituted to 10 mg/mL per vendor instructions and stored at -20°C. Mice were treated with either 1 mg OVA or PBS by oral gavage for four consecutive days. Both groups received the final treatment with OVA or PBS one week prior to OVA immunization.

2.4.4 *Ovalbumin Immunization*

Mice were immunized with 10 ug of Endofit Ovalbumin diluted in PBS with adjuvant. Adjuvants doses & routes are as follows: 100 ug alum (Invivogen, Alhydrogel adjuvant 2%), 25

ul Addavax (Invivogen), both administered intraperitonially, or 4.5 ug AS01, 4.5 ug Lit4Q, both administered subcutaneously at base of tail.

2.4.5 *Cell Staining & Flow Cytometry*

Upon generating single cell suspension, cells were incubated on ice for 20 minutes with CD16/32 and live dead blue or aqua viability dye (Thermo Scientific L34962, L34966). The flow panels used include surface and intracellular antibodies: anti-CD3e (BD, clone 145-2C11, Brilliant Ultraviolet 395), anti-CD4 (BD, clone GK1.5, Brilliant Ultraviolet 496 or Invitrogen, clone RM4-5, PerCP-eFluor 710) anti-IL-33R (BD, clone U29-93, Brilliant Ultraviolet 563), anti-CD8a (BD, clone 53-6.7, Brilliant Ultraviolet 737), anti-CD45 (BD, clone 30-F11, Brilliant Ultraviolet 805), anti-CXCR5 (Biolegend or BD, clone L138D7 or 2G8, Brilliant Violet 421), anti-CD62L (Biolegend, clone MEL-14, Brilliant Violet 711), anti-CD44 (Biolegend, clone IM7, Alexa Fluor 700), anti-CD25 (Invitrogen, clone PC61.5, APC-eFluor 780), anti-PD-1 (Biolegend, 29F.1A12, PE/Cyanine7), anti-GATA3 (Invitrogen, clone TWAJ, PE-eFluor 610), anti-FoxP3 (Invitrogen, clone FJK-16s, APC or FITC), anti-RORγt (BD or Invitrogen, clone Q31-378 or B2D, Brilliant Violet 650 or PE), anti-CD19 (Biolegend, clone 6D5, Brilliant Violet 510), anti-F4/80 (Biolegend, clone BM8, Brilliant Violet 510), anti-CD11b (Biolegend, clone M1/70, Brilliant Violet 510). Intracellular proteins were detected using a FoxP3/transcription factor fixation/permeabilization reagent (Fisher Scientific 00-5523-00). Each sample was collected using the BD FACSymphany instrument from the HIV Vaccine Trials Network flow core. Cell counts were analyzed using AccuraCheck count beads (Life Technologies, PCB100) added to each sample prior to acquisition.

2.4.6 *ELISA for OVA-specific Immunoglobulins*

Mouse serum was heat inactivated at 56°C for 30 minutes prior to storage at 4°C. Plates were coated with 4 ug/mL OVA overnight in 0.1 M NaHCO3. Serum was diluted in milk buffer and incubated at 37°C for one hour on plates. Secondary antibodies specific for relevant Ig-subclass linked to HRP (SouthernBiotech 1033-05, BioLegend 405306, Thermo Fisher Scientific PA129288, M32407, A10551) were then incubated on plates for one hour. TMB (Invitrogen 00-4201-56) was used to develop plates and reaction was stopped with 2N H2SO4. Absorbance at 450 nm was read on a SpectraMax i3x plate reader.

2.4.7 *Re-stimulation of Splenocytes with OVA Protein*

Upon single cell suspension of splenocytes and normalizing counts, cells were plated on a 12-well flat bottom plate at 10^7 cells / mL and incubated for 72 hours at 37°C with cRPMI containing stimulation. Stimulation conditions include unstimulated media only control (used to subtract background signal), Endofit OVA (10 ug/mL), or positive control anti-CD3e (0.5 ug/ml, Invitrogen, clone 145-2C11) and anti-CD28 (0.25 ug/mL Invitrogen, clone 37.51). After incubation, supernatant was collected and frozen for analysis of cytokine secretion by ELISA. Th1/Th2 mouse uncoated ELISA kits from Invitrogen were used and kit instructions were followed to analyze secreted IL-4, IFNγ and IL-10.

2.4.8 *Statistical Analysis*

Data was log transformed for statistical testing on graphs that use a log scale. Unpaired t-tests were performed using GraphPad Prism 9 software analysis tool. Details of statistical tests provided in each figure legend. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.4.9 *Software*

Relevant programs were used for analysis and include GraphPad Prism 9 and FlowJo (version 10.6.0).

2.5 ACKNOWLEDGEMENTS

All co-authors on the manuscript submitted on this work were instrumental in the success of these experiments: Dr. Andrew Johnson, Kevin Hager, Dr. Jessica Graham, Phuong Van, Katelyn H. Lyn-Kew, Lakshmi Warrier, Irene Cruz Talavera, Dr. Jennifer M. Lund, and Dr. James G. Kublin. We thank PAI life sciences and Dr. Gray for providing Lit4Q and Drs. Koutsoukos and Lorin (GSK) for providing AS01. We thank the Lund lab for providing FoxP3^{cre} mice, as well as providing crucial support for these studies. In addition, we thank Dr. Martin Prlic and lab, as well as our collaborators Dr. Sam Minot, Dr. Andrew Fiore-Gartland and Dr. Koshlan Mayer-Blackwell for thoughtful discussions and their contributes to this research. We also thank Fred Hutchinson Cancer Center (FHCC) Comparative Medicine and the HIV Vaccine Trials Network flow cytometry core for support on this work. This work was funded by 1R01AI127100 (to JGK). The University of Washington ITHS TL1 training grant was also used to support this work (to NBP).

Chapter 3. ANTIGEN-SPECIFIC T CELL RESPONSES UPON ORAL ANTIGEN EXPOSURE PRIOR TO VACCINATION

3.1 INTRODUCTION

Understanding mechanisms of enhancing vaccine responses is critical for reducing the global burden of infectious diseases. As previously discussed, one method by which vaccine efficacy may be reduced is prior antigenic exposures through the oral route. While the role of Tregs, and specifically RORγt+ Tregs, has been explored in their ability to regulate antibody titers upon vaccination, along with cytokine production upon OVA re-stimulation, vaccine elicited T cell responses in this system have not been characterized. To improve understanding of tolerogenic responses to a systemic vaccine following oral antigen exposure, our goal was to track OVA-specific CD4+ cells. Multiple methods were tested for their ability to detect antigen-specific T cells, including MHCII tetramer staining and enrichment, intracellular cytokine staining (ICS), and adoptive transfer. Here, these will be discussed and assessed for their capability to appropriately determine antigen-specific T cell responses upon vaccination.

3.2 RESULTS

3.2.1 *MHCII tetramer to assess OVA-specific CD4+ Cells*

To understand the dynamics of antigen-specific cells following oral antigen exposure and vaccination, we utilized a combination of three OVA-specific MHCII tetramers[260]. These reagents have been reported to be specific for the CD4+ immunodominant epitope of the OVA

protein. To optimize staining conditions for the tetramers, OT-II mice were used; these mice contain TCRs specific for the 323-339 peptide of OVA, resulting in CD4+ cells that primarily recognize OVA[261]. C57BL/6 WT splenocytes were mixed with OT-II splenocytes and stained with the three tetramers (OVA 328-337, OVA 329-337, and OVA 325-335) at varied concentrations (2, 6, and 18 ug/mL), temperatures (4°C, 25°C, and 37°C), and for different lengths of time (30, 90, or 180 minutes). We observed the greatest separation between negative and positive tetramer population when cells were stained for 30 minutes, as longer staining led to greater background in the negative fraction (Figure 3.2.1.1A). Staining with 6 ug/mL of each tetramer led to separation of WT and OT-II cells, indicating this concentration is sufficient for designating antigen-specific cells. Upon staining at 25°C or 37°C, we observed problems with intracellular staining, making analysis of populations cells positive for transcription factors including RORγt and FoxP3 difficult to interpret (Figure 3.2.1.1B). For this reason, we stained cells on ice for future experiments.

One method of optimizing staining of tetramer populations when antigen-specific responses may be low or difficult to detect is by enriching for the tetramer positive cells using magnetic beads[262]. We utilized a similar protocol to determine if tetramer enrichment would lead to a clear separation between antigen-specific and non-specific cells. Cells were stained with tetramer or a negative control fraction received no tetramer, and were incubated at 37°C. This led to a reduction in FoxP3+ staining compared to cells that were kept on ice, indicating that incubation at a higher temperature interrupts intracellular staining (Figure 3.2.1.1B). The protein kinase inhibitor Dasatinib (Das) has also been reported to enhance the staining of MHC tetramers; this reagent was added to the tetramer staining and enrichment protocol to test if this would lead to the ability to greater distinguish between positive and negative cells[263]. At 37°C,

there was no difference in FoxP3+ cells between Das+ and Das- fractions (Figure 3.2.1.1B). Das was used in subsequent experiments.

In the siLP, utilizing the tetramer for staining led to high levels of background and non-specific positive populations when used in naïve WT mice (Figure 3.2.1.1C). This was consistent when tetramers were used at both 6 and 18 ug/mL. To improve this, a myeloid cell panel was utilized to determine if specific populations were binding to the tetramer and contributing the background staining (data not shown). Data indicating that siLP CD4+ populations could be cleaned up by including a "dump" channel in our staining panel, containing antibodies to gate out CD19+, F4/80+, and CD11b+ cells. To confirm that each of the three tetramers that had been used in the staining protocol were relevant in this context, each was utilized individually and in combination to stain siLP and splenocytes (Figure 3.2.1.1D). OT-II cells were mixed with WT cells from the same tissues, siLP or spleen, respectively, or WT cells alone were stained with each tetramer. For these experiments, the human CAP-Gly domain containing linker protein (hClip) tetramer was used as a negative control. Because cells used in these experiments come from mice, staining with the human Clip protein that does not share homology with mouse samples should be negative for hClip-specific cells. While combining all three tetramers led to the greatest frequency of tetramer positive cells among all CD4+, staining with tetramers 1 and 3 contributed to the most positive cells when used individually (Figure 3.2.1.1D). Optimization of staining for tetramer-positive populations using OT-II cells was then used when moving to *in vivo* models of OVA vaccination.

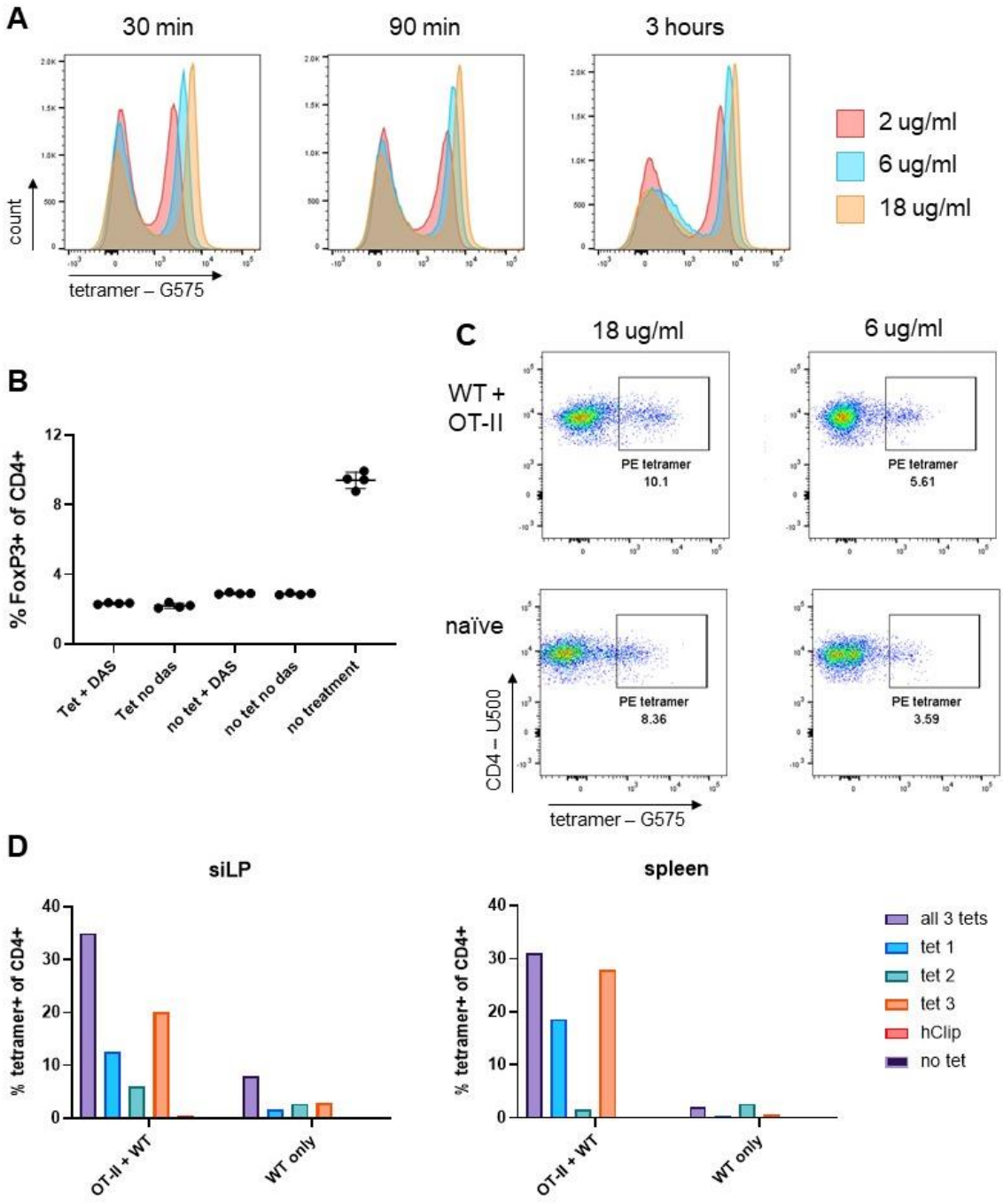

Figure 3.2.1.1 – Optimization of OVA MHCII tetramers using OT-II cells.

A) Histogram plots of tetramer+ cells from OT-II and WT splenocytes stained with MHCII OVA tetramers at various concentrations. Cells were pre-gated on live, CD4+ T cells as described in Figure 2.2.1.3. **B)** Frequency of Tregs from WT splenocytes stained with tetramer at 37°C with or without Das, or cells that were not stained with tetramer as a control. **C)** siLP samples (OT-II

mixed with WT, or samples form naïve mice), stained with tetramer at 18 or 6 ug/mL. Samples pre-gated on CD4+ cells as described in Figure 2.2.1.3. **D)** Frequency of tetramer+ cells pre-gated on CD4+ cells. Both siLP and spleen were stained with all three OVA tetramers in combination or individually (OVA 328-337, OVA 329-337, and OVA 325-335). Data from individual experiments.

To test if our optimized staining protocol and tetramer enrichment could be used to detect antigen-specific cells following vaccination, mice received an i.p. OVA immunization formulated with alum. After one week, splenocytes were assessed for OVA-specific CD4+ responses (Figure 3.2.1.2A). To control for positive and negative antigen-specific fractions, OT-II mice and naïve mice (unvaccinated C57BL/6) were utilized, along with staining of vaccinated mice with the hClip tetramer. As expected, OT-II splenocytes were positive for tetramer staining, and had the greatest frequency of positive cells when the tetramers were used at high concentrations (Figure 3.2.1.2B). hClip stained and naïve mice both had few positive cells, confirming that the negative controls were functioning properly. Surprisingly, splenocytes from mice immunized with OVA formulated with alum had few tetramer positive cells, even when the tetramers were used at very high concentrations (Figure 3.2.1.2B). Tetramer staining was greater in the hClip negative control sample than in the immunized group, indicating that while the tetramer staining conditions had been optimized for OT-II samples, this method was not effective for measuring antigen-specific CD4+ responses in our vaccination model.

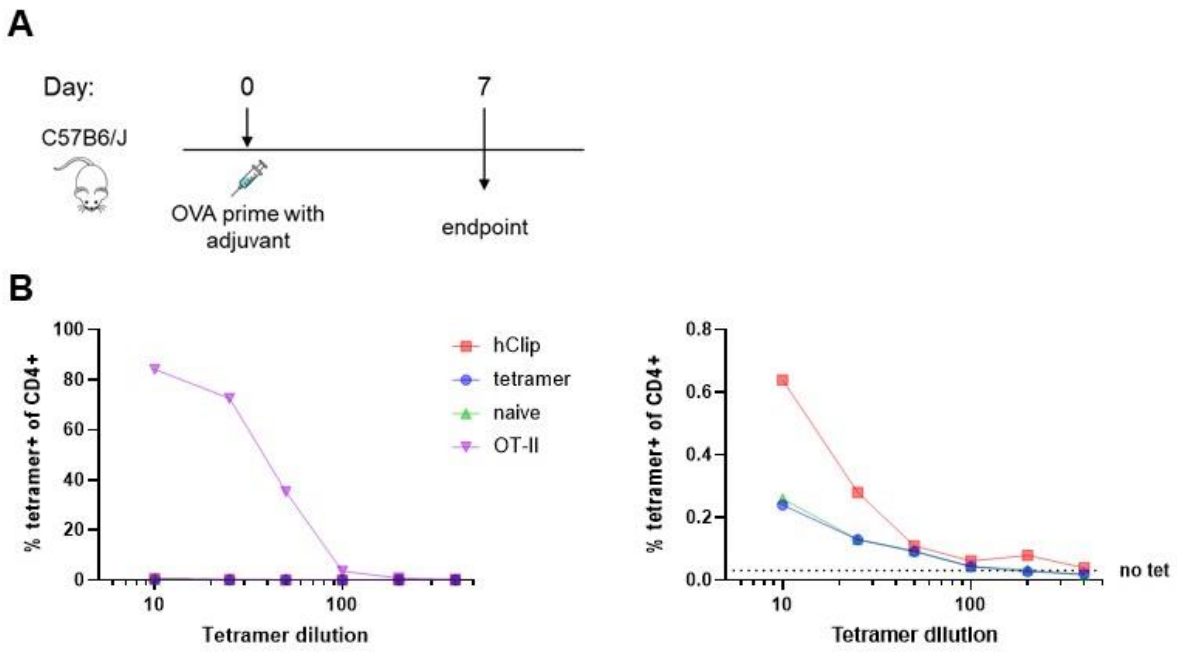

Figure 3.2.1.2 – Tetramer titration with OVA immunized mice.

A) Experimental timeline for results in **B)** Titration of MHCII tetramers used in combination on OT-II splenocytes (OT-II, purple), or immunized mice described in A (tetramer, blue). As negative controls, immunized mice were also stained with hClip (hClip, orange), or naïve mice were stained with the OVA tetramers (naïve, green). The right plot represents a zoomed-in version of the data plotted on the right to better interpret data near zero. Data from one experiment.

When optimizing tetramer staining conditions, OT-II mice were used; this transgenic model does not capture endogenous responses upon vaccination. One concern was that our vaccine regimen, 10 ug of OVA formulated with 100 ug of alum, given at prime and 3 weeks later at the boost, was not strong enough to generate a pool of antigen-specific CD4+ cells detectable by staining with the tetramers. To address this, modifications were made both to the dose of OVA given at the time of vaccination and the adjuvant used in the vaccine.

As discussed in Chapter 2, alum elicits fewer OVA-specific antibodies than adjuvants like AS01. Thus, we utilized Addavax, MF59, and AS01 along with alum to compare the frequencies of CD4+ tetramer-positive cells following the prime and boost immunization (Figure 3.2.1.3A). Notably, there were few differences in tetramer+ cells among CD4+ cells among all adjuvants tested, including alum, in the spleen, MLN, and siLP (Figure 3.2.1.3B). PBS immunized mice that did not receive an adjuvant were used as a negative control. Here, there were few differences in the frequency of tetramer+ cells and this group, indicating that the cells within the positive gates are likely background staining in each tissue. OT-II cells were used as a positive control for staining in the spleen and MLN; these samples were observed to be tetramer+ and significantly higher than cells from groups immunized with any adjuvant tested (Figure 3.2.1.3B). This indicates that altering the adjuvant used in immunization does not lead to more tetramer+ cells using optimized methods of staining.

Next, varying the OVA dose used in the vaccine formulated with alum was tested to see if this would increase the fraction of tetramer positive cells. Mice received either 10, 50, or 100 ug of OVA at both immunization time points and were assessed one week after the boost (Figure 3.2.1.3C). Analysis of tetramer+ cells in mice immunized with each dose were not different by both frequency and number of cells (Figure 3.2.1.3D). Here, tetramer-enrichment magnetic bead separation was used to differentiate between antigen-specific and non-specific populations more accurately. In the spleen, while enriched fractions were increased above the flow-through (FT) fractions, the frequency of tetramer+ cells were not affected by OVA dose received in the vaccine (Figure 3.2.1.3D). When assessing total numbers, both enriched fractions and total tetramer+ cells were not different between groups immunized with varying doses of OVA. Taken together with antibody titer data reported in Chapter 2, these results indicate that while the OVA

+ alum vaccine used can generate sufficient antibody responses, this vaccine regimen is not able to elicit OVA-specific CD4+ responses that are detectable by staining with MHCII tetramers. Multiple approaches were taken to further understand if frequencies could be detectable above negative controls, but either the vaccine procedure, tetramer staining conditions, or production of the tetramers are likely responsible for undetectable antigen-specific cellular responses. Due to these factors, we explored other methods of detecting antigen-specific responses following vaccination.

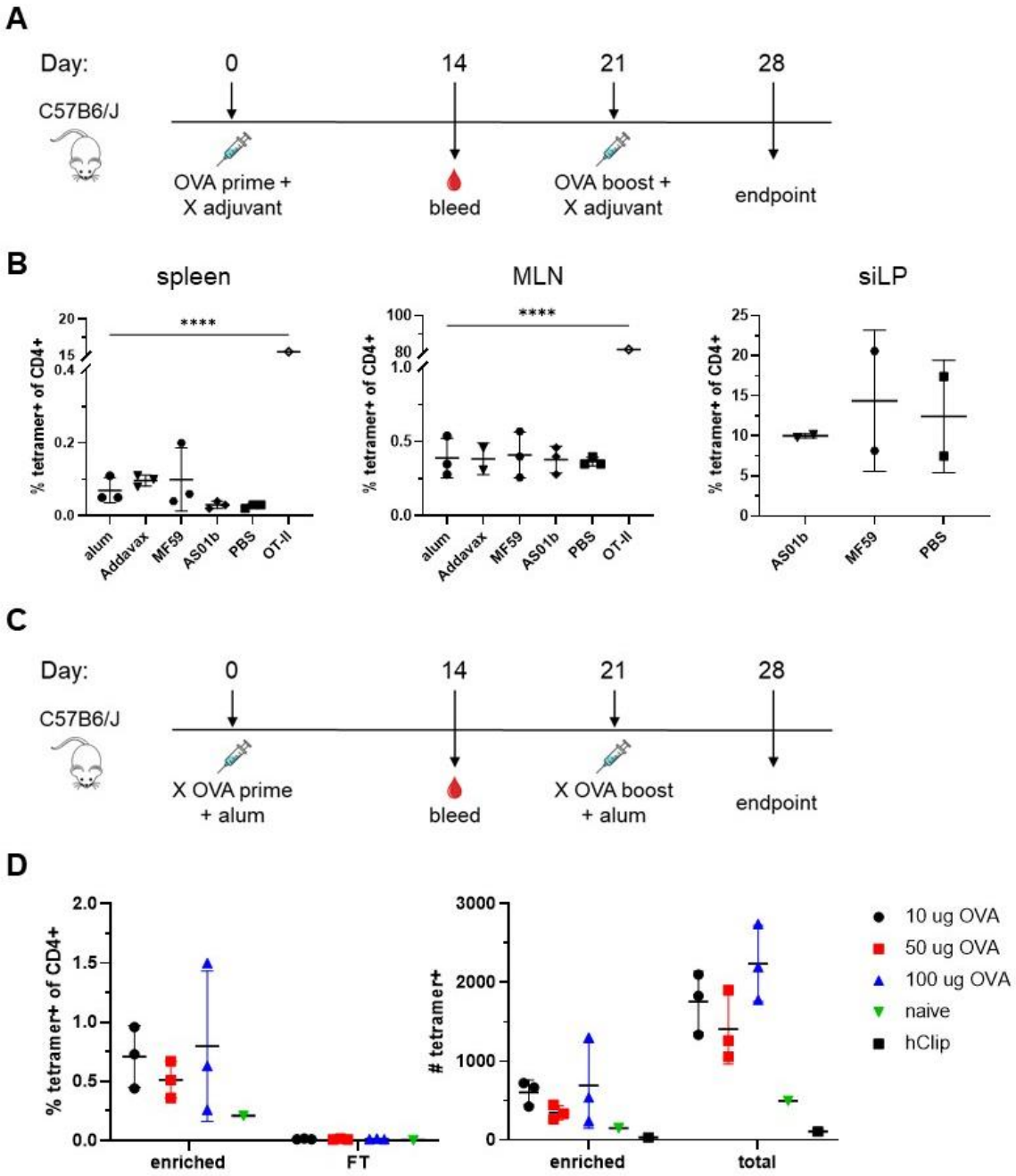

Figure 3.2.1.3 – Altering vaccine adjuvant or dose does not lead to enhanced tetramer staining.

A) Experimental timeline for data in **B)** Frequencies of tetramer+ cells pre-gated on CD4+ cells from spleen, MLN, or siLP as described in Figure 2.2.1.3. WT mice were immunized with OVA + indicated adjuvant or PBS only. OT-II mice were used as a positive control for tetramer+ cells. **C)** Experimental timeline for data in **D)** Frequencies or total numbers of tetramer+ cells from

splenocytes of WT mice immunized with indicated doses of OVA formulated with alum. Frequencies of tetramer enriched and flow through (FT) fractions plotted, whereas count data represented enriched samples and total cells (enriched + FT). hClip stained cells from immunized mice, as well as splenocytes from naïve mice, were used as negative controls. Data from individual experiments. One-way ANOVA was used for statistical analysis.

3.2.2 *Intracellular cytokine staining following splenocyte re-stimulation with OVA protein*

To explore other methods of detecting antigen-specific T cell responses following immunization, we utilized a flow cytometry panel for intracellular cytokine staining (ICS) following OVA re-stimulation. For this, we used splenocytes from an oral tolerance experiment described in Chapter 2.2.1 and Figure 2.2.1.1A. Splenocytes from mice gavaged with OVA or PBS prior to OVA immunization with alum were re-stimulated with either whole OVA protein or the CD4+ immunodominant epitope 323-339 (ISQAVHAAHAEINEAGR). After 24 hours at 37°C, cells were stained with a panel to assess intracellular cytokine production. Only cells with TCRs specific for OVA should respond to stimulation, and markers for CD4 T cells were used to assess various T cell subsets.

We found that upon examining production of IFNγ, tumor necrosis factor α (TNFα), IL-4, IL-10, and IL-17, all cytokines were at or below the limit of detection of our assay (Figure 3.2.2.1). This indicates that either antigen-specific CD4+ responses are low following vaccination, as suggested by the results using MHCII tetramers, or there were technical limitations with our assay. Positive and negative controls were included during stimulation, and for each cytokine examined, CD3/28 stimulation led to a positive fraction of CD4+ cells expressing cytokines above the unstimulated controls. This indicates that a 24-hour stimulation

may not be sufficient to detect the antigen-specific response. For this reason, we extended the re-stimulation to 72 hours and collected supernatant to test for secreted cytokines. While this method loses the ability to distinguish between which cell types are producing each cytokine, we are still able to detect an antigen-specific effect OVA has on bulk splenocytes. Results from these experiments are described in Chapter 2.

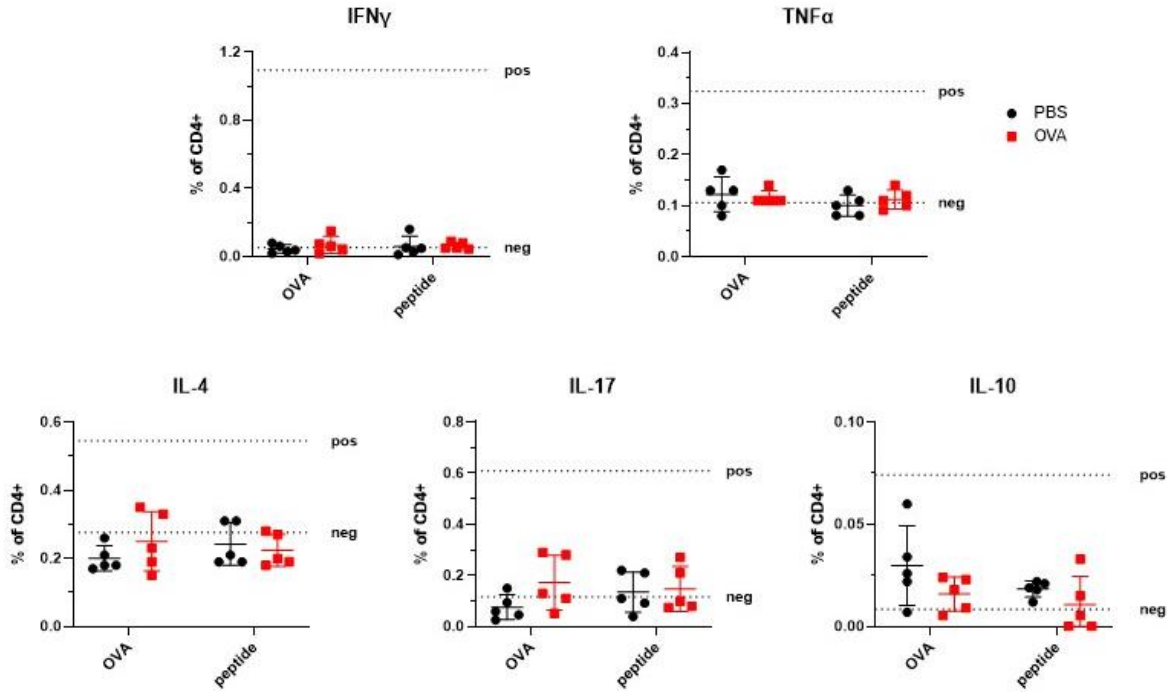

Figure 3.2.2.1 – Intracellular cytokine staining following OVA exposure prior to immunization.

Frequencies of cells expressing indicating cytokine among all CD4+ cells from splenocytes isolated from mice described in Figure 2.2.1.1. Cells were incubated with whole OVA protein or the immunodominant CD4 peptide of OVA (323-339) for 24 hours prior to staining for flow cytometry. Positive control (pos) dotted line represents the average of all samples stimulated with CD3/28, whereas the negative control (neg) line indicates background levels from matching unstimulated cells. Data representative of 3 independent experiments.

3.2.3 *Adoptive transfer of OT-II CD4+ cells into WT mice in model of oral tolerance*

Another commonly used method to detect antigen-specific cells in models of vaccination is adoptive transfer. By utilizing OT-II mice with congenic marker CD45.1, cells isolated form splenocytes can be transferred into mice on the CD45.2 background and are distinguishable from endogenous cells. Adoptive transfer of OT-II cells has previously been utilized to study oral tolerance to a systemic vaccination[136]. In this study, the Pabst group reported that adoptively transferred OT-II cells were able to differentiate into FoxP3+ Tregs following OVA gavage of 50 mg. While this dose is higher than our group uses for gavage, we wanted to test if cell transfer could help assess the behavior of OVA-specific CD4+ cells in our model, and specifically, if these cells could differentiate into RORγt+ Tregs.

Prior to utilizing this method in experiments to assess OVA-specific cells in our tolerance model, adoptive transfer was tested following vaccination alone and oral gavage alone in separate trials. First, 100,000 OT-II CD4+ CD45.1 splenocytes were isolating using a magnetic bead isolation kit and transferred into CD57BL/6 mice one day prior to OVA immunization with alum and assessed after 7 days. In both the spleen and siLP, mice that received OT-II cells prior to immunization exhibited ~1% of all CD4+ cells that contained transferred cells, indicated by CD45.1 (Figures 3.2.3.1A, B). Mice that received OVA immunization alone or no treatment (naïve) had very little background staining, suggesting this method can be used to assess OVA-specific response following immunization.

Next, varying amounts of OT-II CD4+ T cells were transferred into CD57BL/6 mice prior to oral OVA gavage. OT-II cells were transferred one day prior to the start of OVA gavage, which continued for 4 consecutive days. Mice were assessed one week after the last OVA dose.

We found that OVA gavage alone was not sufficient to expand OT-II CD4+ cells above the PBS gavage group, though the population of CD45.1 cells was still detectable 11 days after transfer (Figure 3.2.3.1C). In the spleen and siLP, mice that received OT-II cells and PBS gavage were not significantly different from groups that received OVA gavage. When comparing groups that received 1 million OT-II CD4+ cells with 100,000 cells prior to OVA gavage, there were not consistently higher levels of transferred cells following 11 days, suggesting that addition of more cells does not lead to conservation of this population following gavage alone (Figure 3.2.3.1C). This led us to proceed with 500,000 OT-II cells transferred for subsequent experiments.

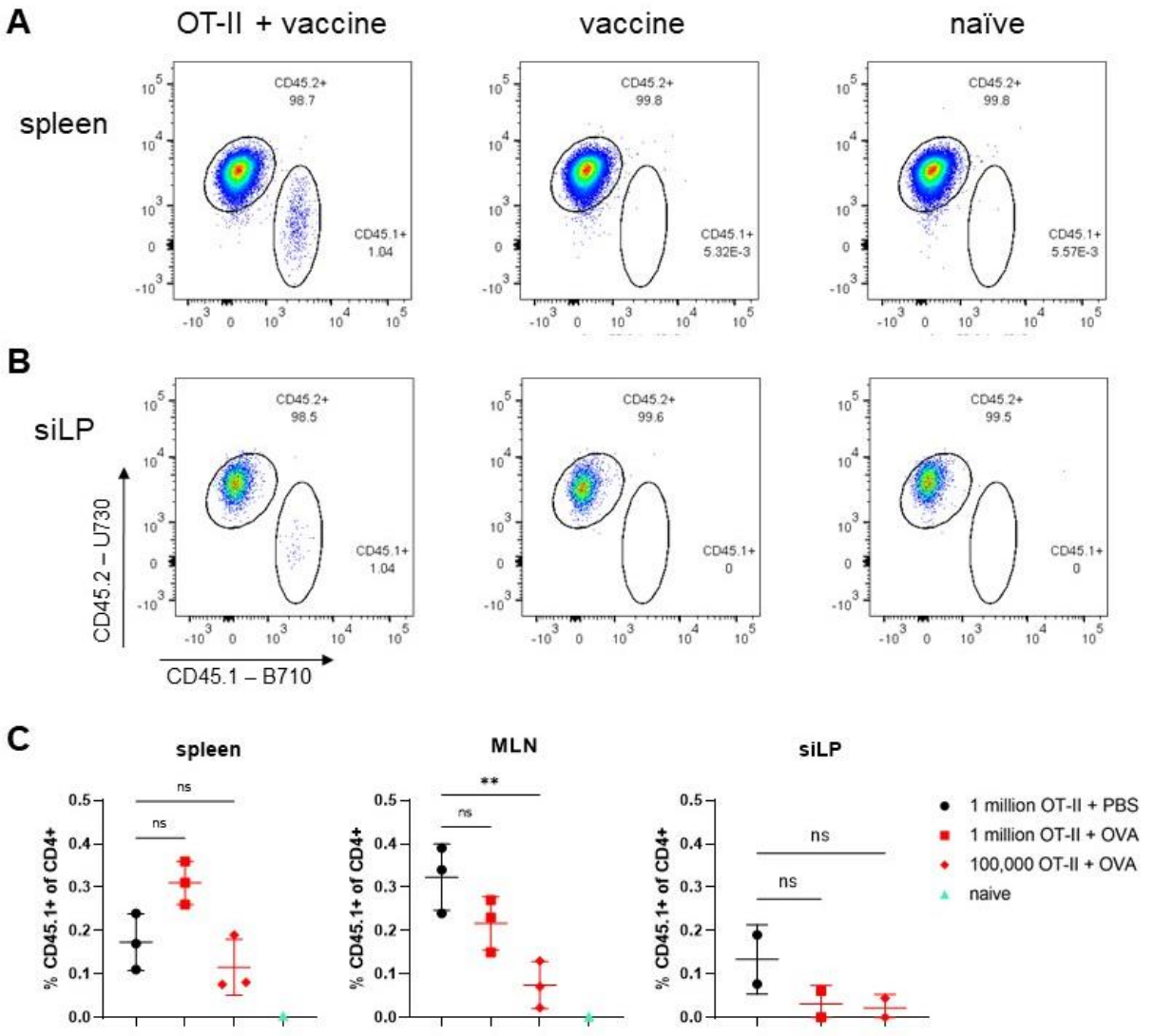

Figure 3.2.3.1 – Adoptive transfer of OT-II cells prior to immunization or oral gavage alone.

A) Representative flow cytometry plots of adoptively transferred OT-II cells pre-gated on CD4+ as described in Figure 2.2.1.3, indicated by CD45.1+ fraction from spleen or **B)** siLP of indicated mice. Groups received OT-II cells or did not receive cells one day prior to vaccination and analyzed one week after vaccination. **C)** Frequency of transferred OT-II cells, represented by CD45.1+ of all CD4+ from indicated tissue. Received varying amounts of OT-II CD4+ cells prior to gavage with either PBS or OVA the following day for four consecutive days. All groups were assessed one week after the last gavage. Data from individual experiments. One-way ANOVA was used for statistical analysis.

Following transfer of OT-II cells and exposure to OVA by either immunization or oral gavage, tolerance experiments were performed after transfer of 500,000 CD4+ OT-II cells (Figure 3.2.3.2A). Mice received CD45.1 cells one day prior to oral gavage with OVA or PBS for 4 consecutive days. After 1 week, mice received OVA immunization formulated with alum and were assessed 10 days later (Figure 3.2.3.2A). Unimmunized mice were used as controls; these groups received either OVA or PBS gavage at the same timepoints as immunized groups and were assessed at the same endpoint. As expected, groups that did not receive OVA + alum immunization led to the lack of expansion of transferred cells in both OVA and PBS gavage groups (Figure 3.2.3.2B). Upon OVA gavage prior to immunization, OT-II cells were undetectable in the spleen, MLN and siLP (Figure 3.2.3.2C). Mice that did not receive OVA gavage, however, had detectable OT-II cells by both frequency and cell counts at this timepoint (Figure 3.2.3.2D). These data were unexpected, and experiments were repeated using an AS01 adjuvanted vaccine and assessed 1 week after immunization, yielding similar results (data not shown). Consistently, mice that received OVA prior to immunization had no detectable OT-II cells that were initially transferred, while PBS gavaged groups maintained this population in each tissue examined. This suggests that OVA gavage following by immunization with multiple adjuvants leads to tolerogenic deletion of the OT-II population.

Interestingly, of the transferred CD45.1 cells detectable in the PBS gavage group, none of the OT-II cells differentiated into Tregs (Figure 3.2.3.2E). At baseline in OT-II mice, there are fewer Tregs than in WT mice (~5% in the spleen compared to ~10-15%, data not shown). Despite this discrepancy, the CD4+ isolation performed on OT-II splenocytes should have maintained the CD4+ Treg population upon transfer, though very few of the expanded OT-II cells in the spleen, MLN, or siLP expressed FoxP3+ following adoptive transfer and vaccination.

Further work is necessary to understand what mechanisms are at play in this system to lead to the deletion of all antigen-specific cells after OVA gavage and immunization. It was also unexpected that OT-II cells were Tregs, despite published reports of Treg differentiation among transferred cells following oral OVA exposure when given at a high dose[136]. Follow up experiments will characterize if antigen-specific cells control oral tolerance to a systemic vaccine.

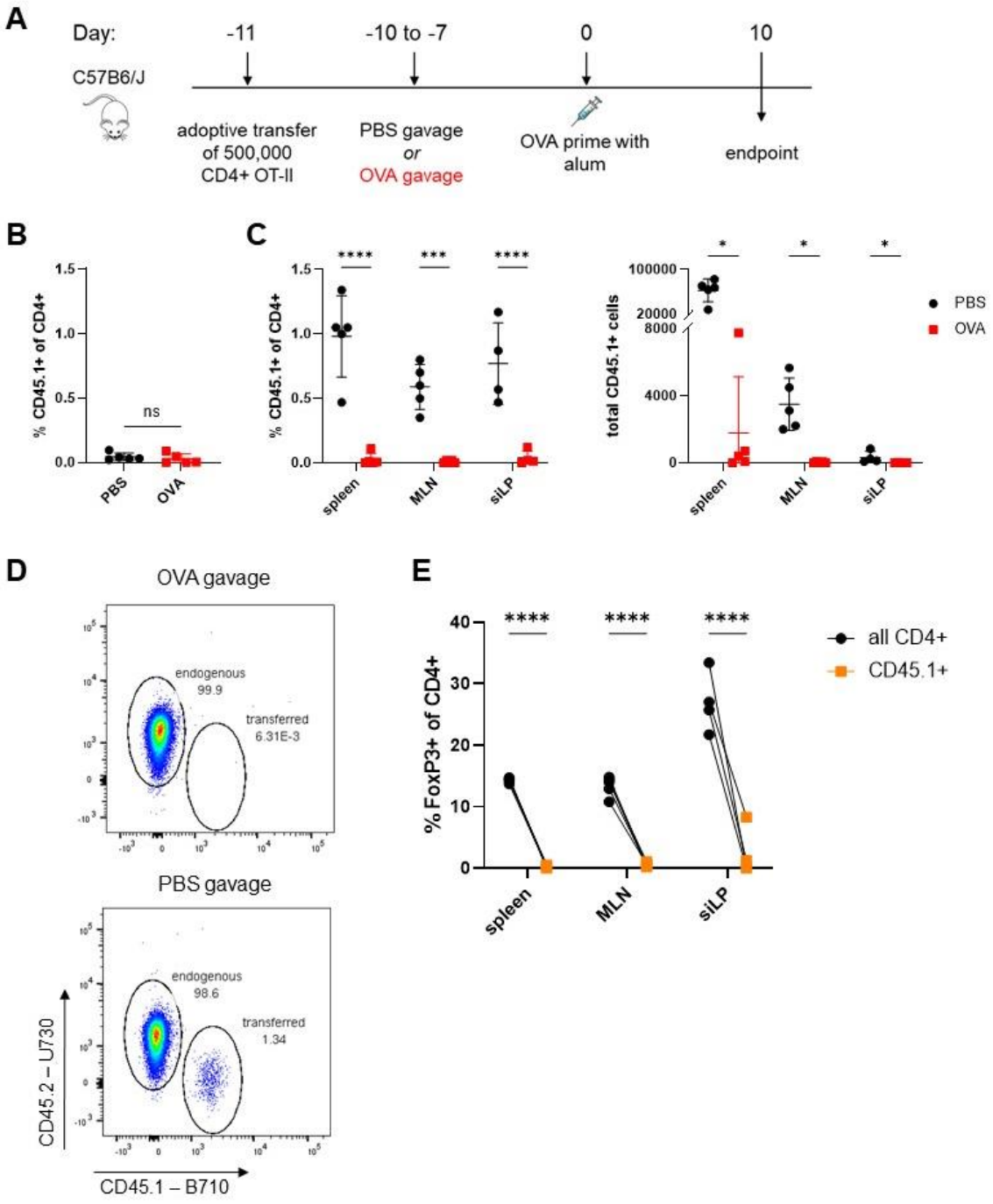

Figure 3.2.3.2 – Adoptive transfer of OT-II cells prior to OVA gavage and immunization leads to deletion of antigen-specific cells.

A) Experimental timeline used for data in figure panels C-E. **B)** Frequency of CD45.1+ OT-II cells pre-gated on CD4+ as indicated in Figure 2.2.1.3 in the spleen following adoptive transfer, oral exposure to OVA or PBS (unimmunized) and assessed at Day 10 described in panel A. **C)**

Frequencies and total cell count of CD45.1+ OT-II cells pre-gated on CD4+ cells in indicated tissue after adoptive transfer, exposure to OVA or PBS, and immunization. **D)** Representative flow plots of endogenous (CD45.2+) or adoptively transferred cells (CD45.1+) from mice that received OVA or PBS gavage. **E)** Frequency of FoxP3+ Tregs pre-gated on CD4+ cells of PBS exposed mice gated on either all CD4+ cells or OT-II cells. Data from one experiment, though similar results were obtained on Day 7 using AS01 in vaccination. Unpaired t tests were used for statistical analysis.

3.3 DISCUSSION

Through these studies, we have explored a number of methods to assess antigen-specific CD4+ T cell responses following oral antigen exposure and vaccination. While optimization of three MHCII CD4+ tetramers specific for the immunodominant epitope of OVA worked to separate populations in OT-II mice when used at high concentrations, these reagents were not able to distinguish between tetramer+ and negative cells in vaccinated mice. These results were supported by low levels of cytokine production via ICS upon re-stimulation with whole OVA protein and the CD4+ peptide. This suggests that using our vaccine regimen and experimental timeline, low levels of antigen-specific CD4+ cells are detectable in the spleen. Adoptive transfer of OT-II cells was also utilized; surprisingly, while PBS treated mice had detectable levels of transferred cells 10 days after immunization, mice gavaged with OVA prior to immunization had no detectable cells. These results are limiting because cells between two groups cannot be compared in the absence of OT-II cells in the OVA group. Further work aims to explore both the kinetics and mechanisms behind the deletion of transferred cells in these conditions.

In our studies, we initially used OT-II cells to optimize tetramer staining conditions and upon applying these to cells from immunized mice, found that our vaccination strategy yielded

low antigen-specific responses. By altering the adjuvant and OVA dose, we were not able to detect a higher frequency of tetramer+ cells. Future work could take advantage of highly immunostimulatory adjuvants, including Lit4Q, in combination with a higher OVA dose in both prime and boost, along with a third dose. Maximizing the potential for the vaccine to elicit T cell responses, along with titration with the tetramer and further optimization of staining conditions (lengthening staining time beyond 30 minutes or staining cells at room temperature) could lead to tetramer+ populations above the negative controls.

Our model tests whether exposure to OVA in an environmental or dietary exposure leads to oral tolerance to a systemic vaccine. Another method in which OVA could be initially seen by the immune system is in the context of a commensal or pathobiont by modeling exposure via the microbiome. Systems in the GI tract are poised to recognize and develop CD4+ responses specific for microbiota, so OVA exposure in this method has the potential to alter OVA-specific responses upon subsequent vaccination. This system may lead to a higher likelihood of detecting antigen-specific cells or cytokine production upon ICS.

Following high levels of exposure to the same antigen, tolerogenic mechanisms have evolved that aim to avoid immunopathology caused by highly activated effector cells. Deletion of these cells occurs via multiple mechanisms but may depend on the dose of exposure[247]. In our model of oral tolerance, 1 mg of OVA was used over 4 consecutive days. Other groups have utilized adoptive transfer of OT-II cells prior to OVA exposure, but in these studies, 50 mg of OVA was provided for two days[136,242]. Each of these studies found that this dose was able to lead to Treg differentiation of transferred cells, whereas these cells are undetectable in both OVA and PBS treated groups. These groups did not administer OVA vaccines following oral antigen

exposure, suggesting that either the gavage dose provided, or the immunization led to deletion of OT-II cells in OVA exposed mice in our experiments. Difference in mechanism that initiate the continued proliferation and differentiation of these cells is likely in play, and further studies are necessary to parse out the timing of the deletion of these cells.

Other assays may be the key to determining antigen-specific responses in our model. The activation-induced markers (AIM) assay that is frequently used in human vaccine trials takes advantage of the upregulation of specific markers on CD4+ T cells following activation[264]. These markers can be used to extrapolate which cells have been activated upon exposure to vaccine-antigen. Also, recent advancements to sequencing technologies could allow for further characterization of antigen-specific CD4+ responses in our system. Together, the methods described in our studies, along with currently unexplored techniques could be implemented to understand how antigen-specific CD4+ T cells, and specifically Tregs, contribute to oral tolerance to systemic vaccination.

3.4 METHODS

3.4.1 *Mice*

C57BL/6 mice were obtained from The Jackson Laboratory (strain 000664) and maintained in specific-pathogen free conditions at Fred Hutchinson Cancer Center. OT-II mice on the C57BL/6 background that contained the CD45.1 congenic marker were provided by the Koch lab. All mice used in were males and were between 6-16 weeks old at the beginning of

each experiment. Experiments were approved by the Institutional Animal Care and Use Committees, and all mice were euthanized following AVMA guidelines for CO_2 overdose.

3.4.2 *Mouse Tissue Collection & Processing*

Each tissue was used to prepared single-cell suspensions as described in Chapter 2.4.2.

3.4.3 *Ovalbumin Oral Gavage*

Endofit Ovalbumin (Invivogen, vac-pova-100) was reconstituted to 10 mg/mL per vendor instructions and stored at -20°C. Mice were treated with either 1 mg OVA or PBS by oral gavage for four consecutive days. Both groups received the final treatment with OVA or PBS one week prior to OVA immunization.

3.4.4 *Ovalbumin Immunization*

Mice were immunized with 10 ug of Endofit Ovalbumin diluted in PBS with adjuvant. Adjuvants doses & routes are as follows: 100 ug alum (Invivogen, Alhydrogel adjuvant 2%) administered intraperitonially, or 4.5 ug AS01 administered subcutaneously at base of tail.

3.4.5 *Adoptive transfer of CD4+ OT-II cells*

Spleens from OT-II CD45.1 mice were harvested and processed into a single-cell suspension. A CD4 isolation kit (STEMCELL Technologies 19852) was used to separate this population to be counted and transferred into CD45.2 recipients. Cells were transferred via tail vein injection in no more than 200 ul. Either 100,000, 500,000, or 1 million cells were transferred into recipient mice. Relevant mouse groups were exposed to OVA the following day, either by oral gavage or immunization.

3.4.6 *MHCII Tetramer Staining and Bead Enrichment*

Prior to surface stain, cells were stained with tetramer and in some experiments, enriched using magnetic beads. Cells were pre-incubated with a master mix containing Dasatinib (Fisher Scientific, NC0897653) at 0.5 uM, DNAse (100 ug/ml) and $MgCl_2$ (5 mM) for 30 minutes on ice. Tetramers were added to cells, which remained in solution with master mix for noted time and temperature, typically 30 minutes on ice. After tetramer staining, cells were washed and resuspended with anti-PE beads (Miltenyi Biotec, 130-048-801) and incubated for 25 minutes on ice. Samples were brought up to 4 mL with PBS + 2% FBS following incubation and run over magnetic columns until fully drained (FT fraction). Columns were then removed from magnets and rinsed with 5 mL of PBS + 2% FBS and cleared with a plunger (enriched fraction). $1/10^{th}$ of the FT fraction was then used for subsequent staining, whereas the entire enriched fraction was stained with surface and intracellular antibodies.

3.4.7 *Cell Staining & Flow Cytometry*

Upon generating single cell suspension, cells were incubated on ice for 20 minutes with CD16/32 and live dead blue or aqua viability dye (Thermo Scientific L34962, L34966). The flow panels used include surface and intracellular antibodies: anti-CD3e (BD, clone 145-2C11, Brilliant Ultraviolet 395 or Invitrogen, clone 17A2, Brilliant Ultraviolet 737), anti-CD4 (BD, clone GK1.5, Brilliant Ultraviolet 496 or Invitrogen, clone RM4-5, PerCP-eFluor 710) anti-CD8a (BD, clone 53-6.7, Brilliant Ultraviolet 737 or Brilliant Ultraviolet 395), anti-CD45 (BD, clone 30-F11, Brilliant Ultraviolet 805), anti-CXCR5 (Biolegend or BD, clone L138D7 or 2G8, Brilliant Violet 421), anti-CD62L (Biolegend, clone MEL-14, Brilliant Violet 711), anti-CD44 (Biolegend, clone IM7, Alexa Fluor 700), anti-CD25 (Invitrogen, clone PC61.5, APC-eFluor

780), anti-PD-1 (Biolegend, 29F.1A12, PE/Cyanine7), anti-GATA3 (Invitrogen, clone TWAJ, PE-eFluor 610), anti-FoxP3 (Invitrogen, clone FJK-16s, APC or FITC), anti-RORγt (BD or Invitrogen, clone Q31-378 or B2D, Brilliant Violet 650 or PE), anti-CD19 (Biolegend, clone 6D5, Brilliant Violet 510), anti-F4/80 (Biolegend, clone BM8, Brilliant Violet 510), anti-CD11b (Biolegend, clone M1/70, Brilliant Violet 510), anti-IFNγ (Biolegend, clone XMG1.2, Alexa Fluor 488), anti-TNFα (Biolegend, clone MP6-XT22, Pacific Blue), anti-IL-4 (Biolegend, clone 11B11, PE/Dazzle 594), anti-IL-10 (Biolegend, clone JES5-16E3, APC), and anti-IL-17 (Biolegend, clone TC11-18H10.1, PE). Intracellular proteins were detected using a Cytofix/Cytoperm (fixation /permeabilization) reagent kit (BD 554714). Each sample was collected using the BD FACSymphany instrument from the HIV Vaccine Trials Network flow core. Cell counts were analyzed using AccuraCheck count beads (Life Technologies, PCB100) added to each sample prior to acquisition.

3.4.8 *Re-stimulation of Splenocytes with OVA Protein*

Upon single cell suspension of splenocytes and normalizing counts, cells were plated on a 96-well round bottom plate at 10^7 cells / mL and incubated for 24 hours at 37°C with cRPMI containing stimulation. Stimulation conditions include unstimulated media only control (used to subtract background signal), Endofit OVA (10 ug/mL), CD4 OVA 323-339 peptide ISQAVHAAHAEINEAGR (1 ug/mL, EZBiolab C850W91), or positive control anti-CD3e (0.5 ug/mL, Invitrogen, clone 145-2C11) and anti-CD28 (0.25 ug/m Invitrogen, clone 37.51). Cells were stained with intracellular cytokine antibodies and prepared for flow cytometry.

3.4.9 *Statistical Analysis & Software*

Data was log transformed for statistical testing on graphs that use a log scale. Unpaired t-tests were performed using GraphPad Prism 9 software analysis tool. Details of statistical tests provided in each figure legend. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Relevant programs were used for analysis and include GraphPad Prism 9 and FlowJo (version 10.6.0).

3.5 ACKNOWLEDGEMENTS

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Chapter 4. CONCLUDING REMARKS

4.1 LIMITATIONS & IMPLICATIONS

Here, we explored the role of RORγt expression within Tregs and the role this cell subset plays in controlling oral tolerance to a systemic vaccine. The main finding of this work suggests that oral tolerance to systemic vaccines is maintained in the absence of RORγt in Tregs. Open questions remain on if expression of this transcription factor alone is dispensable, but the remaining pTregs subset is critical, or if other subsets are involved. RORγt+ Tregs have been described as having enhanced suppressive abilities, though their distinct role in the gut is unclear. Further work is necessary to understand which cells control oral tolerance to a systemic vaccine.

Some limitations of this work exist, though efforts were taken to minimize these when possible. Inherently when working with animal models, differences exist between the mouse and human immune systems. While our ultimate goal is to make scientific advances to improve human health, these models are important for understanding the basic mechanisms that may translate to humans. Also, our studies utilized primarily male mice. While some results suggest similar vaccine responses and immunophenotypes of cKO in female mice, similar studies could be performed in females to confirm this.

An outstanding question that we aimed to study was the antigen-specific T cell and Treg responses in our model, discussed in Chapter 3. By utilizing tetramer staining, ICS, and adoptive transfer methods, multiple approaches were taken to this end. While optimizing the tetramer presenting multiple challenges, our data suggest that either the tetramer is unable to detect antigen-specific CD4+ T cells or there are few OVA-specific T cells that exist upon oral gavage

and OVA vaccination in our model. Additional consultation with others who have used these reagents confirmed the challenges we experienced and may require more optimization of the tetramers to be of much utility to the field. As discussed, further work is also necessary to understand more about the effects of adoptive transfer in our system. We were surprised by the lack of detectable OT-II cells upon oral gavage and vaccination; further experiments will be planned to answer these outstanding questions.

This work can be used to draw conclusions about the mechanisms in the gut that are modulate systemic vaccine responses. While we have shown that RORγt expression in Tregs is dispensable for this effect, this work can be used to inform future studies examining the responsible subsets or cells. Implications from these findings could be important to subsequent work in vaccine trials. One approach to improving vaccine outcomes is introducing a pre- or pro-biotic near the time of vaccination to alter microbiota composition. Importantly, future studies should be performed to characterize how local changes in the gut prime the immune system to respond more effectively upon systemic vaccination. The potential of the gut microbiome to be harnessed to positively influence human health could be instrumental in reducing the spread of infectious diseases. As discussed, the diversion hypothesis states that pre-exposure to microbiota could negatively influence vaccine responses via tolerization of similar antigens. If microbiota composition could be influenced, there exists a possibility that these could be reduced prior to immunization. Though the evidence is not fully present that this will be possible, understanding how gut commensals or exposures through the gut influence immune cell populations will be critical for these outcomes. When we understand more about the cells that contribute to oral tolerance to a systemic vaccine, we will obtain a better idea of how therapeutics that influence the gut immune system could be used to improve vaccine responses in a clinical setting.

4.2 Future Directions

While this work answered one piece of an overall large question, many similar questions remain and should be explored in future studies. Interesting findings within the last year reported that RORγt+ MHCII+ populations led to the induction of RORγt+ Tregs. While this was reported during our studies, it will be important to consider how this effects our findings. Specifically, a cKO model of these cell populations may be a way to study the RORγt+ Treg compartment. If we are able to study a cell that is upstream of RORγt+ Tregs in a model of oral tolerance, key questions could be answered as to the cells responsible for maintenance of these responses. It will also be key to examine how other Treg subsets, including GATA3+ Tregs, could contribute to the system. Advancements in sequencing technology and imaging could be useful techniques to answer similar outstanding questions.

Another aspect of this work that would be interesting to understand is the effect of the microbiome on oral tolerance to a systemic vaccine. While this work has been carried out by others with mixed results, advancements in technologies like flow cytometry (the ability to assess many more markers than was possible in the early 2000s), and sequencing make this work more accessible. A future study to assess the role of various commensals on influencing the local immune environment and if these changes affect tolerance to a systemic vaccine will be informative. Since studies have reported on how different microbiota alter local T cell responses in the gut, it would be interesting to understand how this relates to systemic vaccine responses in gnotobiotic mouse models. Other future experiments could also examine the role of introducing OVA (or any vaccine antigen) within a commensal or pathobiont. Does the type of exposure

make a difference for inducing oral tolerance? Likely, differential signals of the innate immune system will influence how subsequent exposures are perceived.

Future work will also explore how introducing OT-II cells by adoptive transfer influences systemic vaccination following oral OVA exposure. Our data suggests that utilizing MHCII tetramers to measure the endogenous response presents many challenges, so the adoptive transfer system may provide an alternative that could be utilized to assess antigen-specific responses. Further experiments, including sequencing OT-II cells shortly after vaccination in mice that have either received oral OVA or PBS could be informative.

Many of the underlying hypotheses behind these studies branch from an interest in understanding how the microbiome contributes to vaccine responses. Recently, our group reported that CD8 T cell responses to a mRNA lipid nanoparticle vaccine are enhanced by the presence of microbiota compared to GF counterparts[265]. Some of the initial goals of my project was to understand how microbiota exposure contributes to baseline gp41 responses and led to non-productive responses to HIV vaccine candidates[70]. While the gp41 work led to more bioinformatics-based approaches, there are many questions that remain around this topic that could be carried into animal models.

While these studies utilized OVA as a model antigen, future studies could also make use of other models of infectious diseases for which we have effective vaccines to confirm these findings. This work stemmed out of curiosity around HIV-1 vaccines, so using similar experiments to assess vaccine responses for this virus or other infectious diseases will be useful for follow-up studies. Together, we hope that this work furthers the understanding of how oral exposure affects systemic vaccine response with the larger goal of improving health outcomes.

APPENDIX: TREG DEPLETION MODEL

One additional approach taken to explore the role of Tregs in mediating oral tolerance to systemic vaccination included a model of transient Treg depletion. This model utilizes expression of the human diphtheria toxin receptor (hDTR) in all FoxP3+ Tregs ($FoxP3^{DTR}$). Upon i.p. administration of diphtheria toxin (DT), Tregs are depleted systemically from mice[266]. Here, we utilized an experimental model described to examine whether oral tolerance was maintained in the absence of all Tregs (Figure 4.1A). Samples of blood were taken via the non-lethal submental route periodically following DT administration to examine frequencies of circulating Tregs. DT was used at 30 ug/kg for the initial dose at Day 0 and 10 ug/kg at the Day 1 timepoint. Because the chronic depletion of Tregs is fatal, $FoxP3^{DTR}$ mice can only be exposed to DT for short windows of time[102,266]. Upon examination of Treg frequencies 53 days after initial DT dose, Treg frequencies were not different in the spleen, MLN, siLP, or blood between mice that received OVA or PBS prior to vaccination (Figure 4.1B).

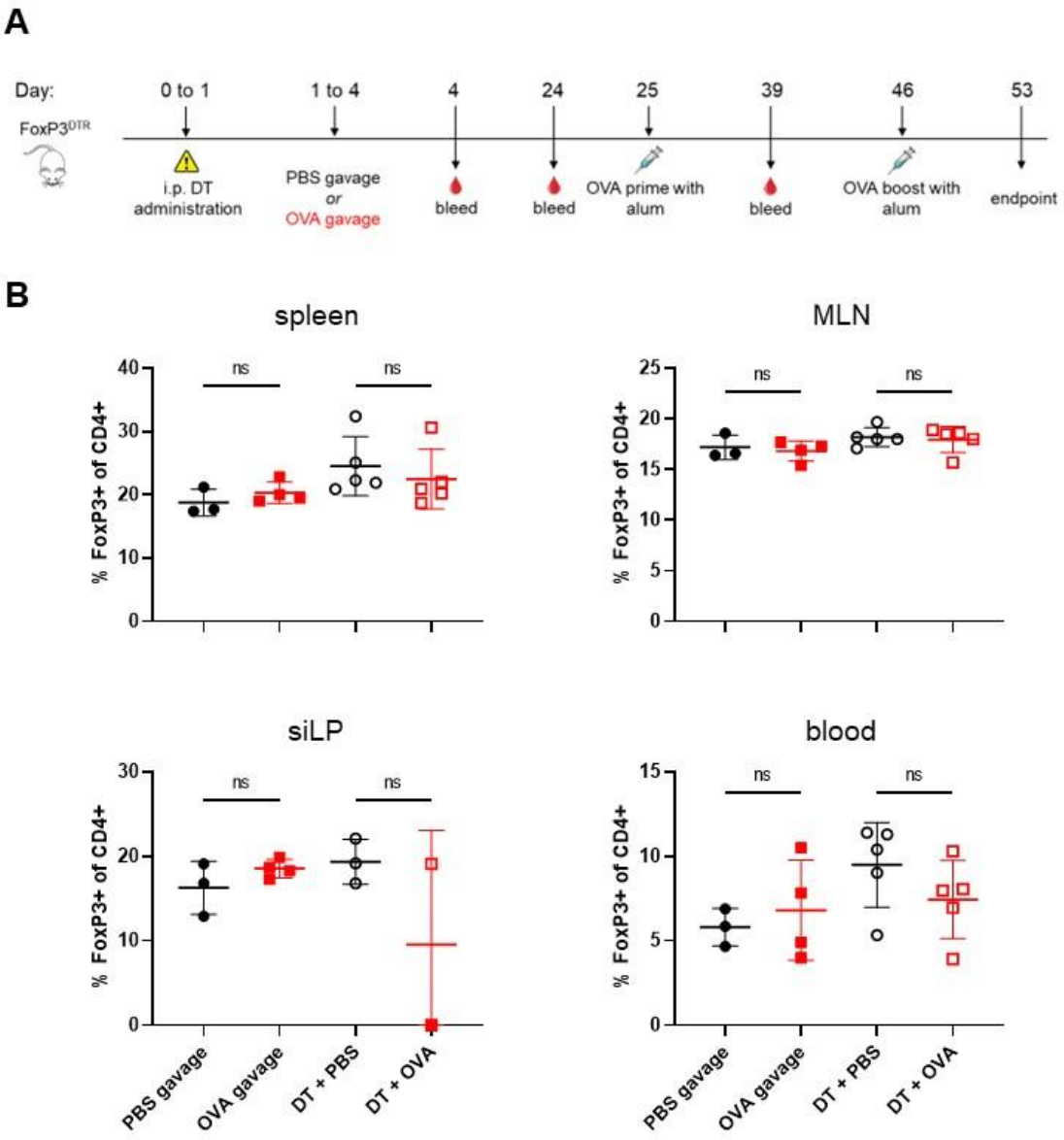

Figure Chapter 4.1 –Treg depletion model to assess oral tolerance to a systemic vaccine.

A) Experimental timeline for Treg depletion experiments. **B)** Frequencies of FoxP3+ Tregs in spleen, MLN, siLP, and blood, pre-gated on CD4+ cells described in Figure 2.2.1.3 from mice that received either PBS (black) or OVA (red) and PBS i.p. (closed symbols) or DT i.p. (open symbols. Data is representative of two independent experiments. One-way ANOVA was used for statistical analysis.

While the Treg frequencies were not different depending on gavage status, whether mice received DT prior to oral gavage affected Treg frequencies throughout the experiment. Following initial administration of DT, Treg frequencies were nearly completely absent from the blood of DT treated groups by Day 4 (Figure 4.2A, B). By Day 10, the Treg compartment had recovered to frequencies above baseline, increasing above PBS treated groups (Figures 4.2A, B). Mice were monitored throughout the course of the experiment and periodic samples were taken from the blood to examine Treg frequencies. At the endpoint of 53 days following first DT dose, Treg frequencies in mice that received DT had not returned to the same levels of PBS treated groups (Figure 4.2B). Although between Day 17 and Day 37, the frequencies of Tregs decreased, indicating that over time, depletion and subsequent re-population of the Treg compartment may stabilize, this data indicated that this model may not be appropriate for our studies (Figure 4.2C). Our goal was to mimic the cKO model of Treg depletion while keeping consistent experimental timelines. Because Treg frequencies did not return to baseline by the time we typically administer a systemic vaccine, this led to the concern that the resulting overshoot of Treg frequencies would affect vaccine responses. While this model has proven successful in other studies[267], we have concluded that this system will not provide us with a suitable method to answer the question we set out to understand due to the unresolved effects that follow systemic Tregs depletion.

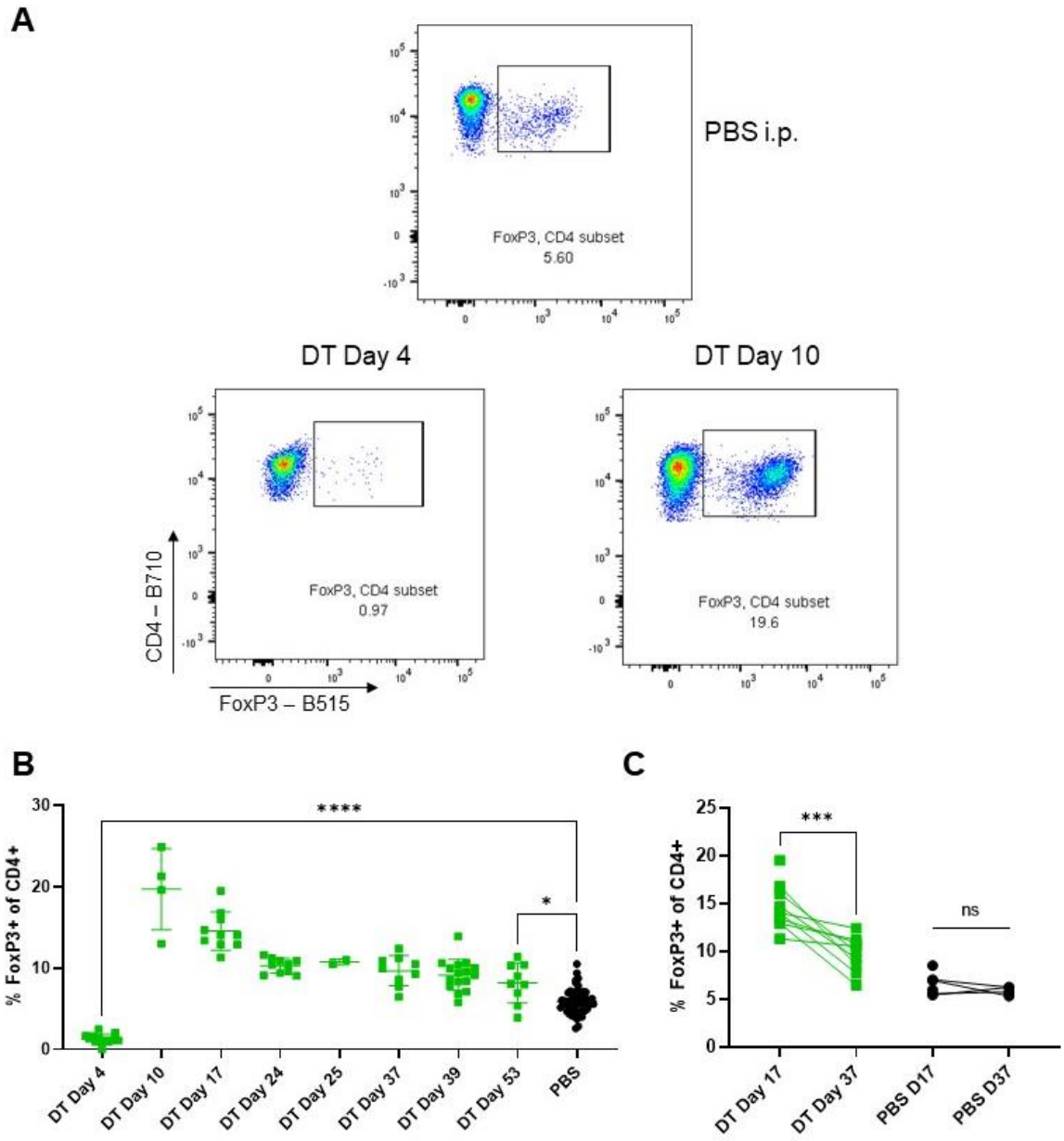

Figure Chapter 4.2 – DT administration leads to long-lasting increase in systemic Treg frequencies in FoxP3^{DTR} mice following recovery

A) Representative flow cytometry plots of Treg frequencies in mice that received PBS i.p. prior to oral gavage, or mice that received DT, examined at Day 4 or Day 10. **B)** Frequencies of FoxP3+ Tregs over time following DT administration (green) or PBS (black) pooled from three independent experiments or **C)** in one individual experiment. Some mice received OVA + alum vaccination 11 days after initial DT administration rather than 25 days, described in Figure 4.1A. One-way ANOVA was used for statistical analysis.

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VITA

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