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A Novel Mouse Model to Study B Cell Tolerance in the Gut Associated Lymphoid Tissues

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Cindi Chen

Committee in Charge:

Professor Michael David, Chair
Professor Peter Ernst
Professor Cornelius Murre
Professor Robert C. Rickert

2016

The Thesis of Cindi Chen is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2016

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ABSTRACT OF THE THESIS

A Novel Mouse Model to Study B Cell Tolerance in the Gut Associated Lymphoid Tissues

by

Cindi Chen

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Professor Michael David, Chair

To maintain tolerance, auto-reactive B cells are deleted at different stages of development, in the bone marrow and in the periphery. The gut environment imposes unique challenges for B cells due to the need to differentiate between self-tissues, food antigens and commensal bacteria. To study B cell tolerance in the gut associated lymphoid tissues (GALT), we developed a novel mouse model in which a neo-self antigen, membrane bound duck egg lysozyme (mDEL), is specifically expressed in the

gut epithelium. The expression of mDEL in the gut was uniform and did not affect the microbiota. In this mouse model, auto-reactive B cells are deleted in the Peyer's Patches (PP); however the population of lysozyme-specific B cells remained unaltered in the spleen. Lysozyme-specific B cells from the PP showed decreased IgM expression and increased expression of the activation marker, CD86. However, the cells did not enter the germinal center. Furthermore, mDEL expression in the gut did not induce production of lysozyme-specific IgM, IgG and IgA in the serum, suggesting that lysozyme-specific B cells did not differentiate into plasma cells upon antigen encounter. To study the survival of mature auto-reactive B cells, lysozyme-specific B cells were adoptively transferred into mice. We found the frequency of lysozyme-specific B cells to be reduced in both the PLNs and the PP if mDEL was expressed in the gut epithelium, suggesting that mature autoreactive B cells are deleted. Future experiments are aimed at analyzing the mechanisms by which B cell tolerance is maintained in the gut.

I. INTRODUCTION

The Immune System

The immune system is unique in its ability to discriminate between self and non-self. A failure to respond appropriately can lead to immune deficiencies or autoimmunity. The immune system is generally divided into two categories: innate and adaptive. While the innate immune system recognizes common structures expressed by a wide range of pathogens, the adaptive immune system recognizes specific epitopes.

B cell Development in the Bone Marrow and Central Tolerance

B cells are an essential part of the adaptive immune response and develop from common lymphoid progenitor (CLP) cells, which are derived from hematopoietic stem cells (HSC). Once the CLPs commit to the B cell lineage, the pro-B cells start to rearrange the immunoglobulin heavy chain locus. Successful rearrangement of the heavy chain gene segments leads to the formation of a μ heavy chain. In many cases, the initial rearrangement on the first chromosome is nonproductive and a subsequent rearrangement in the second chromosome is necessary. If unsuccessful in producing a μ heavy chain, the pro-B cells are eliminated. This is known as the first checkpoint of B cell tolerance. Once a productive heavy chain is formed, it is paired with a surrogate light chain to form the pre-BCR, which is expressed on the surface of pre-B cells and signals the cell of a successful rearrangement. However, successful rearrangement of the two heavy chain alleles can lead to the production of two different BCRs, thus allelic exclusion occurs, in which only one of the two alleles is expressed in a cell. During the transition from pro-B cell to pre-B cells, the cells undergo cell division to expand the population before light chain rearrangement occurs. Multiple rearrangements of the light chain locus in one allele

can occur before rearrangement of the second allele. Allelic exclusion of the light chain loci also occurs. Successful rearrangement of the light chain locus is increased since two types of light chain are possible: κ or λ. Rearrangement of the κ light chain locus generally occurs before the λ locus. The two types of possible light chain makes it necessary for there to be isotypic exclusion, so only one type of light chain is expressed. Successful rearrangement of the light chain and its pairing with the heavy chain forms the B cell receptor (BCR) and the expression of the IgM on the surface marks the transition of the pre-B cell to an immature B cell.

The BCR is then tested for autoreactivity. If the BCR does not bind to self-Ag, the immature B cell migrates to the periphery. However, if it binds to self-Ag, several cellular outcomes can occur. The fate of the autoreactive B cells is essentially dependent on the strength Ag binding to the BCR. High avidity interactions between the BCR and self-Ag, such as in the case of highly expressed membrane bound Ag (multivalent Ags), leads to BCR crosslinking and a strengthening of the BCR signal, resulting in two potential outcomes. The auto-reactive B cells can undergo clonal deletion or receptor editing. Clonal deletion leads to the death of the auto-reactive cell by apoptosis, whereas receptor editing leads to the arrest of B cell development and continued light chain rearrangement. Low avidity interactions, on the other hand, such as the case with certain soluble Ag, enable the auto-reactive B cell to migrate to the periphery; however, the cells are anergic. Anergic B cells are unresponsive to Ag, have decreased expression of surface IgM and have a shortened lifespan [1]. B cells with BCRs binding to monovalent, low affinity or inaccessible Ags migrate to the periphery, but are immunologically ignorant. That is, they do not respond to self-Ag except under certain conditions. The selection

against auto-reactive B cells and for B cells tolerant to self-Ag in the bone marrow is known as central tolerance.

Of the immature B cells, approximately 50-75% have an auto-reactive BCR [2, 3]. After selection in the bone marrow, immature B cells leave the bone marrow to finish maturation in the spleen. Of the transitional and naïve mature B cell pool, 20-40% of the cells are still auto-reactive [2, 3]. This highlights the limits of central tolerance in ensuring that auto-reactive B cells do not enter the periphery and raises the question of how tolerance is maintained in the periphery.

B cell Development in the Periphery and Peripheral Tolerance

Once the naïve B cells leave the bone marrow, they are known as transitional B cells. Approximately $2x10^7$ immature B cells are formed daily in the bone marrow, of which 10% enter the periphery and 10-30% of these continue on to become immuno-competent [4, 5].

Transitional B cells can be divided into 3 different subsets, which can be distinguished by the expression of certain developmental markers. T1 B cells are known as early transitional B cells and do not have the ability to recirculate, which is why they are only found in the bone marrow and spleen. They are characterized by $IgM^{hi}D24^{hi}CD21^{-}IgD^{-}CD23^{-}AA4.1^{hi}$ expression. If T1 B cells are autoreactive and encounter a strong BCR signal, they are eliminated. If the T1 B cells are not autoreactive, they become T2 B cells, which are also known as late transitional B cells and usually found in the follicle of the spleen. T2 B cells have gained the ability to recirculate [6]. T2 B cells are characterized by $IgM^{hi}CD24^{hi}CD21^{+}IgD^{+}CD23^{+}AA4.1^{hi}$ expression. When stimulated through their BCR, T2 B cells can either become anergic when an auto-Ag is

encountered or proliferate and differentiate into mature B cells when a non-self Ag is encountered. However, the exact mechanism of how T2 cells are selected into the mature B cell pool is incompletely understood [6]. The third subset transitional B cells in the periphery are the T3 B cells. T3 B cells were originally thought to be a part of the linear development pathway from immature to mature B cells; however, it has been discovered that while T2 B cells can develop into T3 B cells, T3 B cells cannot develop into mature B cells [7]. Additionally, it has been found that T3 B cells can also develop from mature auto-reactive B cells that are found in the lymph nodes and peripheral blood [8]. They have now been found to be a special self-reactive anergic B cell subset that is different from the traditional transitional B cell subsets; thus they have also been called An1 B cells [9]. Anergy refers to the functional unresponsiveness of an immune cell to a particular Ag and is a method by which B cells in the periphery maintain tolerance. While the mechanism of anergy is unknown, there are certain characteristics that aids in the identification of anergic cells, such as having a short lifespan and low surface expression of IgM. It has been shown that maintenance of anergy may require the BCR to be constantly occupied [10].

The elimination of auto-reactive B cells in the periphery, including transitional B cells, is the second tolerance checkpoint and is known as peripheral tolerance. There are multiple checkpoints during the development of the immature transitional B cells to mature B cells and a significant decrease from T1 to T2 and from the T2 to T3 transitional stage has been found [11]. The elimination of auto-reactive transitional B cells by follicular dendritic cells (FDCs) displaying self-antigen has been shown to be a possible mechanism by which B cell tolerance to self tissue is maintained[12]. FDCs

have the potential to sequester and display self-Ag, which can lead to the BCR engagement of autoreactive B cells to self-Ag and leading to the initiation apoptosis, which is another method by which tolerance can be maintained.

Naïve mature B cells can be divided into two subsets: marginal zone (MZ) B cells and follicular (FO) B cells. Marginal zone B cells cannot recirculate and are mainly located along the marginal sinuses, outside the B cell follicles and are known as innate-like antibody producing lymphocytes [13]. There are two types of Ag recognized by B cells: Thymus independent (TI) or Thymus dependent (TD) Ags. B cells that recognize TI Ag do not require T cell help to induce an Ab response. They induce a nonspecific and polyclonal response or activate complement, but cannot induce memory. B cells that respond to TD antigens require T cell help and give a strong and specific Ab response and can induce memory. Due to their proximity to the marginal sinus, MZ B cells are among the first cells to encounter antigen. They have been shown to often express polyreactive BCRs and play an important role in responding to TI antigens. FO B cells, on the other hand, make up the majority of B cells and have the ability to recirculate between secondary lymphoid organs (SLOs). They are mainly located in the B cell follicle, which is located next to T cell zones and includes follicular dendritic cells (FDCs). FO B cells are preferentially activated by TD Ag and tend to express monoreactive BCRs.

During a TI immune response, B cells are activated by antigen presented by macrophages or DCs cells. The activated B cells can differentiate into plasma cells and secrete low affinity antibodies mainly of the IgM or IgG3 isotype. However, no memory is formed. During a TD immune response, activated B cells migrate to the border of the T

cell zone and present Ag to T cells within a MHCII-Ag complex. If a T cell recognizes the respective antigen, B cells receive co-stimulatory signals and can either differentiate into plasma cells secreting low affinity antibodies or return to the B cell follicle and form a germinal center. In the germinal center, B cells proliferate, and undergo class switch recombination (CSR) and somatic hyper mutation (SHM). CSR refers to the switching of the constant region heavy chain to one with a different isotype, resulting in clonal expansion. This allows for the production of Abs with different isotypes and effector functions but does not change antigen specificity. SHM, on the other hand, refers to the process in which the variable region of the Ig genes are mutated via single base pair substitutions, insertions or deletions leading to the production of antibodies with higher affinity or changes in Ag specificity, resulting in affinity maturation. The final result of the germinal center reaction is the formation of plasma cells secreting high affinity antibodies and the generation of memory B cells.

Autoreactive B cells are generally excluded from the GC in what is known as follicular exclusion and tend to accumulate in the outer periarteriolar lymphatic sheath (PALS) when in competition with non-autoreactive B cells [14]. However, when undergoing SHM in the GC, B cells that were previously not autoreactive can become autoreactive, although they are usually deleted when self Ag is encountered [15]. A break in tolerance in the germinal center is observed in patients with SLE [16].

The Gut and Gut Associated Lymphoid Tissues

The gut is the largest mucosal surface in the human body with an average surface area of 100 m^2 containing 10^{14} commensal bacteria [17], two times more cells than the human body. It is one of the most densely populated microbial habitats. The gut is also

the location of 70% of the body's immune cells, which emphasizes its importance in affecting the host immune response. Together, the regulation of interactions between the intestinal epithelial cells (IECs) and gut associated lymphoid tissues (GALTs), the microbiota and host immune system maintains gut homeostasis. Gut homeostasis is the balance between immune regulation, the structure of the gut epithelium and their response to commensal or pathogenic bacteria and other gut Ags.

Gut homeostasis is maintained when food antigens, commensal bacteria and self-tissues are not recognized by the host immune system. The IECs create a physical barrier that separates the lumen containing food and commensal bacteria from the host tissue. In addition to serving as a physical barrier, IECs also secrete anti-microbial peptides such as lysozymes and mucins into the mucosa, creating a selective layer for certain microbes and reducing the amount of harmful pathogens that can reach the IECs [18]. Multiple gut associated lymphoid tissues (GALT) also play a crucial role in maintaining gut homeostasis, such as the isolated lymphoid follicles (ILFs), Peyer's Patches (PP) and the mesenteric lymphnodes (MLNs). The ILFs are small microscopic structures that line the anti-mesenteric wall of the mouse small intestine and consist of a large B cell area and can form germinal centers [19]. ILFs have been found to be potential sites for the development of intestinal IgA responses that changes based on the intestinal microbiota and are potentially formed in response to mucosal challenges [20].

The PPs are macroscopic structures containing an aggregate of lymphoid follicles that are similar to ILFs. PPs consist of three distinct zones: the GC, subepithelial dome (SED) and follicle associated epithelium (FAE)[21]. Unlike the spleen and peripheral lymph nodes (PLN), PPs contain GCs even without immunizations, making it an

important site for maintaining gut homeostasis. The SED contains B cells, T cells, DCs and macrophages; and it has recently been shown that prolonged interaction of B cells with DCs from the SED is required for B cells to class switch to IgA[22]. DCs in the PP are functionally different from those in the spleen and seem to preferentially activate a Th-2 anti-inflammatory response by inducing IL-10 and IL-4 secretion, cytokines that are important in the maintenance of tolerance [23]. The FAE contains M cells, which are specialized epithelial cells that are located on the apical side of the ILFs and PPs and function as transporters of luminal antigens to the GALT [24]. They express IgA receptors that allow for the capture and transport of bacteria targeted by IgA to be processed in the PPs and interact with B cells or DCs [25].

The MLNs are draining lymphnodes of the GALTs and provide a unique microenvironment to immune cells from the GALT to interact with both, systemic Ags as well as gut-derived Ags. DCs can exit the PP and LP to enter the MLNs, where they can present Ag to T cells [26]. The lack of systemic immune response to gut-derived Ags is due to the inability of DCs to enter systemic circulation via the MLN, indicating the important role that the MLN has in preventing a systemic response to gut bacteria [27].

While the GALT is important as inductive sites for IgA class switching, the lamina propria (LP) is another proposed site in which IgA class switching can occur, although there have been conflicting results. While there has been evidence to suggest that IgA class switching can occur in the LP[28], there has also been evidence that suggests otherwise [29].

IgA mediates gut homeostasis in several ways [30]. SIgA can block pathogens and toxins from binding to receptors on the IEC surface, effectively entrapping them in

the mucus, which can have antibacterial properties and facilitates its clearance from the gut [31]. Furthermore, IgA also has an important role in maintaining homeostasis of the gut microbiota [33]. It has been suggested that SIgA from breast milk helps in the formation of a newborn's microbiota, which in turn influences the development of the GALTs [34]. IgA has been found to mainly target commensal bacteria in the small intestine via a TI IgA response [32]. A breach in gut homeostasis and tolerance, such as seen in IBD lesions, sees a shift in homeostasis maintaining IgA to pro-inflammatory complement activating IgG [35].

The gut microbiota play a crucial role in determining the immune response during healthy and diseased states [36, 37]. In healthy individuals, commensal bacteria induce tolerance mechanisms in an anti-inflammatory manner via the production of the anti-inflammatory cytokine IL-10 by Tregs [38]. Germ free mice have given insights on the importance of the gut microbiota in immune responses and development of the GALT [39]. It has also been shown that the lack of a diverse microbiota prevents effective protection when infection does occur [36]. Certain strains of bacteria can also induce the production of IL-10 by Tregs and potentially protect against colitis [40]. Notably, the role of Tregs has become increasingly important in the maintenance of gut homeostasis. Tregs have been shown to inhibit inflammatory responses to commensal bacteria and their loss has been shown to lead to autoimmunity and inflammation [41, 42].

Oral Tolerance and Tolerance to Commensal Bacteria

The gut is a site where commensal bacteria and dietary Ags are constantly present and in close contact with the host mucosal immune system, making it necessary for the body to recognize food Ags and commensal bacteria as 'self'. Oral tolerance is a unique

part of peripheral tolerance that focuses on the maintenance of immune unresponsiveness towards orally administered Ags. Breaks in oral tolerance can lead to local and systemic immune responses. The role of B cells in oral tolerance is not well understood.

The recognition of commensal bacteria is distinctly different from oral tolerance as breaks in tolerance to commensal bacteria have been shown to favor local immune responses [43].

A breach in the system is thought to play a role in the development of food allergies, celiac disease, and inflammatory bowel disease (IBD), such as Crohn's Disease (CD) and ulcerative colitis (UC). The MLN has been shown to be of critical importance in the maintenance of oral tolerance and is the main location in which T cells are tolerized to oral Ag [44, 45]. There is also a potential role for DCs located in the LP, where orally administered Ags have been discovered post feeding [46] and in which DCs loaded with the feed Ag have been observed[47]. The DCs can migrate to the MLN to tolerize the T cells and prevent a break in oral tolerance.

Inflammatory Bowel Disease

IBD is a broad term used to describe two chronic inflammatory intestinal disorders: Crohn's disease (CD) and ulcerative colitis (UC). CD involves discontinuous inflammation of the ileum and colon, while UC involves inflammation of the rectum to the colon in a continuous manner. The exact cause of IBD is not fully understood yet; however a variety of factors have been found to contribute to the development or pathogenesis of IBD such as genetic predisposition or environmental factors, suggesting that inappropriate inflammatory responses in the gut in genetically susceptible individuals may result in IBD.

As mentioned, the microbiota play a crucial role in maintaining gut homeostasis. A change in the microbiome has been seen in patients with IBD and the presence of certain bacteria exacerbates inflammation while others alleviate inflammation [48]. Patients with IBD also see a decrease in the diversity of their gut microbiota [49].

In the dextran sodium sulfate (DSS) inducible colitis model, the lack of B cells leads to a worsened disease outcome, while disease was attenuated with B cell transfer [50], suggesting a role for B cells in maintaining and regulating the severity of disease. Oral administration of DSS in drinking water induces inflammation and damages the epithelial lining, allowing for the passage of intestinal contents. B cells have also been shown to be important in regulating the proliferation of Tregs, which in turn regulates B cell differentiation to IgA secreting plasma cells [50]. However, the production of autoantibodies against intestinal epithelia-associated Ags, such as hTM5, was observed in patients with UC and is pathogenic in patients with UC [51, 52], implicating B cells in both the prevention and pathology of UC. Depletion of B cells in other autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid rheumatoid arthritis (RA), have lead to improved disease outcome [53, 54]. However, CD20 depletion with ritixumab has not led to significant improvement of disease pathology in IBD patients [55].

Different Mouse Models for Peripheral Tolerance and IBD

To date, several mouse models have been created to study different aspects of B cell tolerance. Models specifically using hen egg lysozyme (HEL) as a novel Ag have been generated and well studied. One such model is the soluble HEL Tg mice (ML5). It was crossed to HEL Ig Tg mice (MD4), in which 90% of the B cells are specific for HEL,

but cannot undergo CSR, to generate MD4 x ML5 mice. The MD4 x ML5 mice was used to study peripheral tolerance of self-reactive B cells showing that soluble Ag causes anergy in self-reactive B cells[1]. ML5 mice have also been crossed to SW_{HEL} mice, which similar to MD4 mice, have B cells that are specific for HEL, but unlike MD4 mice, SW_{HEL} B cells have the ability to undergo CSR and make up 40-60% of the B cell repertoire [56]. HEL-specific B cells from ML5 x SW_{HEL} mice are anergic with an immature phenotype[56]. Models in which the antigen is expressed in a membrane bound form have also been created to analyze how multivalent self-antigen induces tolerance. In immunoglobulin transgenic mice with B cells specific for MHC-I Ag, self-reactive B cells are deleted when membrane bound self-Ag is encountered in the periphery [57]. This was further confirmed in mice systemically expressing mHEL [58]. Additionally, tissue specific Ags have also been generated. Thyroid specific mHEL expression did not lead to B cell deletion or anergy, which is thought to be due to the inacessability of the Ag. In another model, liver specifc Ag was found to induce apoptosis rather than anergy [59].

There are 5 groups that experimental IBD models are classified in: chemically induced, cell transfer, spontaneous, congenital and genetically engineered [60]. Of the chemically induced models, treatment with dextran sodium sulfate (DSS) is one of the most commonly used to study IBD due to its cost effectiveness, being easy to control the severity of disease based on how much DSS is given, its ability to be used as a chronic inflammation model, and the ability to study the healing process after induction of damage [61]. However, there are some disadvantages when using DSS treatment as a model for IBD: The severity and location of intestinal inflammation varies from mouse to

mouse, making it difficult to assess damage, protocols vary in dose and treatment period. While a role for immune cells in IBD pathogenesis in patients is widely accepted, the chemical induction of IBD using DSS does not require the presence of T or B cells, as seen in Rag KO mice [62]. However, we have chosen the DSS model as it allows us to study the contribution of B cells to disease severity after tissue damage has been initiated.

Currently, there are no mouse models for the study of peripheral B cell tolerance against membrane associated Ag in the GALT.

Designing the mDEL Mouse Model

Irene W. Yau, Ph.D, a former graduate student in our laboratory, generated the mouse model that will be the focus of this thesis. She created the transgenic $mDEL^{loxp}$ mice, whose cells expresses membrane bound duck egg lysozyme (mDEL). It was then crossed to $CD21^{cre}$ to form the line $CD21^{cre}mDEL^{loxp}$, so that mDEL was specifically expressed on the surface of mature B cells and radioresistant FDCs. Cre mediated inversion of the transgenic locus leads to the fixed expression of mDEL. The mice were irradiated and reconstituted with cells from anti-HEL (SW_{HEL}) mice to create a mouse model to address selection of autoreactive B cells by Ag on FDCs [12]. SW_{HEL} mice are V_H knockin and V_K transgenic mice, with a V_H knockin in the IgH gene and transgenic for the light chain [56]. SW_{HEL} B cells are described as B cells that are heterozygous for both heavy and light chain transgenes. 40-60% of the B cells in SW_{HEL} mice are specific for the HEL Ag and are able to undergo CSR. SW_{HEL} mice were chosen due to their ability to undergo CSR and because SW_{HEL} B cells bind to DEL with a lower affinity than HEL, which mimics what occurs physiologically. The expression of membrane bound self-Ag on FDCs led to the elimination of autoreactive B cells at the transitional

stage. While some autoreactive B cells escape this checkpoint, they are short lived and are unable to recruit T cell help [12].

To study peripheral B cell tolerance to gut associated Ags, $mDEL^{loxp}$ mice were crossed to $Villin^{cre}$ mice to generate $Villin^{cre}mDEL^{loxp}$ mice. These mice specifically express mDEL on the surface of gut epithelial cells. Control ($Villin^{cre+}$ or $mDEL^{loxp}$) and $Villin^{cre+}mDEL^{loxp}$ mice were then either irradiated and reconstituted with bone marrow from SW_{HEL} mice or crossed to SW_{HEL} mice, generating reconstituted (R) control and $RVillin^{cre+}mDEL^{loxp}$ or intact control and $Villin^{cre+}mDEL^{loxp}SW_{HEL}$ mice, respectively.

This mouse model uniquely allows for the study of peripheral B cell tolerance to membrane associated Ags in the GALT. It can be used to study how B cell tolerance is maintained and broken in the GALT and will give insights on the role of B cells in IBD; something that has been contested in different IBD mouse models.

II. MATERIALS AND METHODS

Mice

Villin^{cre+}mDEL^{loxp} or control mice and SW_{HEL} or SW_{HEL} x BAFF^{Tg} and Villin^{cre}mDEL^{loxp}SW_{HEL} or control mice and CD45.1 and C57B/6 mice were used in the various experiments. To generate the reconstituted Villin^{cre}mDEL^{loxp}SW_{HEL} mice, Villin^{cre}mDEL^{loxp} mice were lethally irradiated (10Gy) and reconstituted via iv injection, performed by vivarium staff of Sanford Burnham Prebys Medical Discovery Institute, with ~$7x10^6$ bone marrow cells from SW_{HEL} mice. The mice were given antibiotics (5 mL of Sulfamethalazone in autoclaved water) for 4 weeks and regular water for another 4 weeks before use. For the mice used in the adoptive transfer studies, Villin^{cre}mDEL^{loxp} mice were given SW_{HEL} B cells. All animals were treated in accordance with the eithical standards set and approved by Sanford Burnham Preby's Medical Discovery Institute's Institutional Animal Care and Use Committee. The mice were euthanized with CO_2 and blood and organs were collected. The animals were treated according to Sanford Burnham Preby's Medical Discovery Institute IACUC.

Intestinal Epithelial Cell Isolation

The intestine was removed and transferred into cold PBS. Intestinal contents were removed and the intestine was cut open longitudinally and washed in cold PBS. The tissue was then placed into a tube containing pre-warmed PBS containing 1mM DTT and shaken for 10 minutes at 180 rpm and 37°C and then washed with PBS. The tissue was removed and incubated in HBSS supplemented with 1.5 mM EDTA for 15 minutes at 180 rpm and 37°C. Tubes containing the tissue were vortexed for 1 minute and tissue was removed. Intestinal epithelial cells were centrifuged down to a pellet and resuspended in

1 mL of PBS. Cells were then filtered and either stained for flow cytometry or used for the lysozyme activity assay.

Transfection of HEK293T cells

HEK293T cells were either transfected with an empty MSCV-P2GM plasmid or MSCV-P2GM mDEL plasmid. 3ug of plasmid was added to 18ul of PEI and 172ul of DMEM and mixed gently. Plasmid was incubated at room temperature for 20 minutes before the addition of 2 mL of complete DMEM (10% FBS, 2mM L-glutamine, 100U/mL penicillin, and 100ug/mL streptomycin). HE293 T cells were incubated plasmid for 2 days. Additional complete DMEM was added after overnight incubation and whole media changed 2 days post transfection. Cells were analyzed by flow on the third day by flow to check transfection efficiency.

Lysozyme Activity Assay

To analyze lysozyme activity, the EnzChek® Lysozyme Assay Kit (Molecular Probes) was used according to manufacturer's instructions, with modifications for testing on cells. Transfected HEK293T cells, isolated IECs and whole intestine sections were used for the assay. $5x10^4$ mDEL expressing cells or 8 mm sections of the small intestine of $Villin^{cre+}mDEL^{loxp}$ mice were used. Fluorescence was measured using a fluorescence microplate reader with a fluorescein filter.

MACS

B cells were negatively selected for using α-CD43 beads (Miltenyi Biotec) according to manufacturer's instructions.

Stimulating primary B cells in culture

B cells were MACS sorted. Cells were counted and put into 24 well plate and

incubated with varying concentrations of lysozyme or diluted serum in complete RPMI for 3 hours or overnight.

gDNA extraction

gDNA was extracted from fecal pellets collected from mice using a Qiagen QIAamp® Fast DNA Stool Mini Kit according to manufacturer's instructions with adjustments. Stool was collected into 2mL microcentrifuge tubes (Eppendorf). 1mL InhibitEX Buffer was added to each stool sample and vortexed until stool sample was homogenized. The suspension was heated to 95°C for 10-15 minutes and vortexed for 15 seconds. Sample was centriguged for 1 min and 600ul of supernatant was added to 25ul of ProteinaseK. 600ul of Buffer AL was added to the sample and vortexed for 15 seconds before it was incubated at 70°C for 10 minutes. 600ul of ethanol was added to the lysate and mixed by vortexing. Lysate was added to the QIAamp spin column and centrifuged for 1 minute. Filtrate was discarded. Once all sample has been loaded onto column, 500ul Buffer AW1 was added to the column and centrifuged for 1 minute. The column was then washed with 500ul Buffer AW2 and centrifuged for 3 minutes. Column was centrifuged for 3 minutes and allowed to dry for 5 minutes at room temperature. To elute gDNA, 200ul of Buffer ATE was added to the column membrane and incubated for 2 minutes at room temperature before elution by centrifugation for 1 minute. Samples were stored at -20°C.

Flow Cytometry Staining

Spleen, PLN, MLN and PP were isolated from mice euthanized with $CO_{2.}$ Organs were put into 10% FBS in PBS 1x106 cells were stained with 100μl of staining solution (1:100 Ab unless otherwise mentioned in FACS buffer) for 25 minutes washed and

stained with secondary and washed. Abs used from eBioscience: anti- B220 (RA3-6B2), -CD86 (B7-2), -IgM(11/41), -CD69(H1.2F3), -CD95/Fas (J02) and -GL7 conjugated to APC, APC780, PE, FITC, PeCy7 or PerCp Cy5.5. To stain for HEL binding B cells, HEL bio (GeneTex) was used and revealed by using streptavidin-PerCpCy5.5, PECy7 or PE. Alternatively, cells were incubated with soluble HEL followed by anti-HELbio or anti-HEL (Rockland) and subsequently stained with either streptavidin or donkey anti-Rabbit FITC (Jackson), respectively. Live cells were identified using forward and side scatter. Data was with a BD FACS CANTO using the FACS DIVA software (BD Biosciences) and data was analyzed using FlowJo (Treestar).

ELISA

Each well in a 96 well high binding plates was coated with 50 μl of 10 μg/mL lysozyme solution and incubated in humidity chamber overnight at 4°C. Each wash consisted of washing with DI water 3x and each incubation occurred at 37°C for 2 hours in a humidity chamber unless otherwise indicated. Plates were blocked with 200 μl of 5% BSA in PBS, incubated then washed. 50 μl of diluted samples were then added to each well with the highest concentration being 1:10 of serum and serially diluted 1:3 and incubated and washed. Samples were diluted with dilution buffer (0.5% BSA in PBS). 50 μl of α-IgM AP, α-IgG AP and α-IgA AP (diluted 1:1000 in dilution buffer) were then added to the plates and incubated, then washed. 100 μl of phosphate substrate in PNPP buffer was then added to each well and incubated at room temperature in the dark. Plates were read when standard curves plateaued (usually when OD405nm at 2.5 to 3).

Adoptive Transfer

The spleens from SW_{HEL} mice were removed and a single cell suspension was made. The cells were centrifuged at 1350 rpm for 5 minutes at 4°C. The supernatant was removed and the cells were re-suspended in 500 μl ACK and incubated on ice for 5 minutes to lyse the red blood cells. The cells were washed with PBS and resuspended in concentration of $1x10^8$ cells/ mL MACS buffer.10μl of α-CD43 beads/ $1x10^7$ cells were added to cell suspension and incubated for 15 minutes at 4°C before being washed and resuspended in 1 mL of MACS buffer and ran through a washed LS column.

Data Analysis

All flow cytometry data was collected with BD FACS DIVA and analyzed using FlowJo Version 8.8.7. ELISA data was analyzed with Microsoft Office Excel and Prism 6 and unpaired student t test used for statistics.

III. RESULTS

Chapter 1: Verifying the Model

The Villin^{cre+}mDEL^{loxp}SW$_{HEL}$ system provides a unique model for studying peripheral tolerance in the GALT and potentially as a model for IBD and the role of B cells in these instances. In the first part of the results section potential technical issues of the model will be addressed such as: tissue specific antigen expression, potential release of the antigen into the circulation, lysozyme activity of mDEL.

1.1 mDEL expression in the gut

To ensure that DEL is expressed uniformly in the intestinal epithelium of Villin^{cre+}mDEL^{loxp} mice, cells from the intestine of control and experimental mice were stained and analyzed for DEL via flow cytometry. The small intestine was separated into two sections and IECs were isolated. Two controls were used to ensure that the expression of mDEL required the presence of both the mDEL transgene and cre promoter. The cells from the two sections of small intestine from Villin^{cre+}mDEL^{loxp} mice exhibited an increase in DEL expression compared to control mice and had similar expression of mDEL (**Fig 1**). Expression of cre inverts the Thy 1.1 gene; therefore mice lacking cre are able to express Thy 1.1 on the surface of its cells. As per the mouse model , Villin^{cre-}mDEL^{loxp} mice had an increase in surface expression of Thy 1.1 compared to Villin^{cre+}mDEL and Villin^{cre}mDEL^{loxp} mice (**Fig 1**). In conclusion, we have verified that mDEL is expressed in the gut epithelium from Villin^{cre+}mDEL^{loxp} mice and that expression is not restricted to a specific region of the gut. Thus, B cells residing in the GALT have the opportunity to interact with DEL irrespective of their localization.

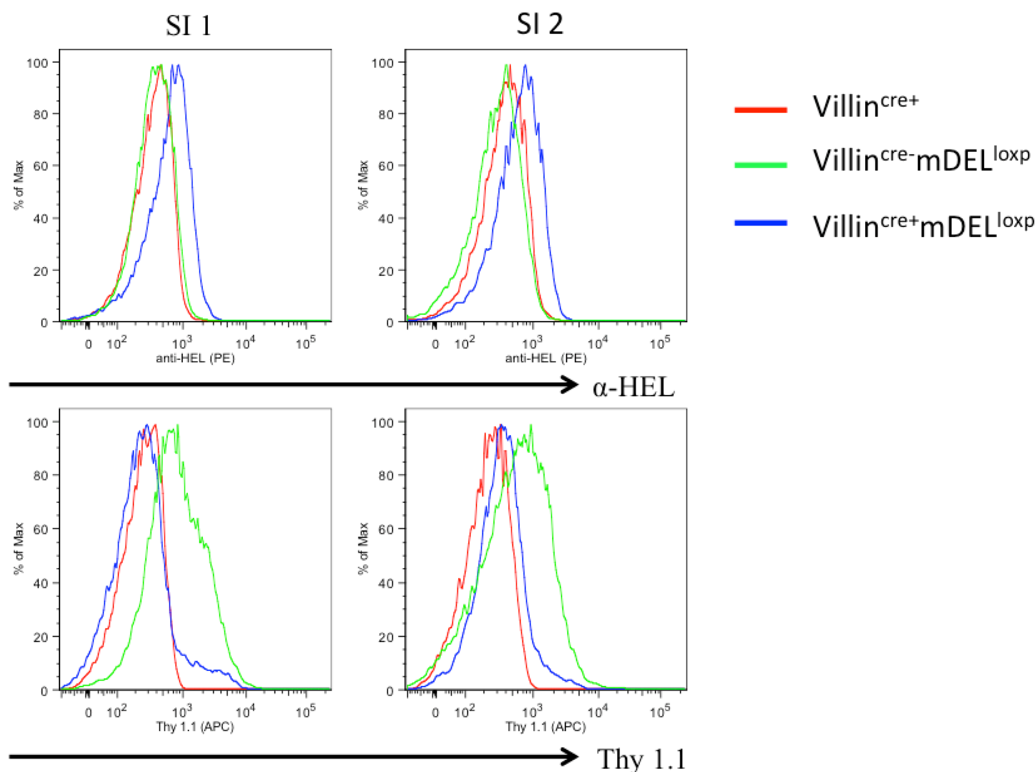


Figure 1: mDEL is expressed uniformly in the gut epithelium. IECs were isolated from the first and second half of the small intestine of control and Villin^{cre}mDEL^{loxp} mice and stained with Rabbit α-HEL/ α-Rabbit to detect the presence of mDEL. Successful cre-mediated inversion led to the deletion of Thy 1.1 and the expression of mDEL.

1.2 Cleavage of mDEL

IECs are known to have a high turnover rate; thus it is possible that mDEL could be released in substantial amounts from dying IECs, enter the circulation and act as a systemic rather than a local antigen in Villin^{cre+}mDEL^{loxp} mice. To rule out this possibility a lysozyme assay was performed via flow cytometry.

First, to determine the amount of lysozyme that SW$_{HEL}$ B cells can detect, freshly isolated splenic cells from SW$_{HEL}$ mice were stained with different concentrations of lysozyme and analyzed by flow. HEL-binding by SW$_{HEL}$ B cells was no longer detected when stained with a concentration of 2-20 pg lysozyme/ µl (**Fig. 2a**). To determine

whether the amount of lysozyme in the serum of $Villin^{cre+}mDEL^{loxp}$ mice is enough to stain for SW_{HEL} B cells, 1:10 diluted serum from control or $Villin^{cre+}mDEL^{loxp}$ mice was added to the cells. SW_{HEL} B cells were not detected, implying that the concentration of lysozyme in serum is less than 200pg lysozyme/μl (**Fig. 2a**).

SWHEL B cells react to the presence of lysozyme through the downmodulation of their HEL-specific BCR. Thus, BCR downregulation on SW_{HEL} B cells can be used to test whether lysozyme is present in a sample. To determine the concentration of lysozyme at which the HEL-binding BCRs become down-modulated, different concentrations of HEL were added into wells containing 5×10^5 SW_{HEL} B cells. To determine the concentration of lysozyme that is necessary to activate SW_{HEL} B cells, B cells from SW_{HEL} were incubated with different concentrations of lysozyme or serum from control or $Villin^{cre+}mDEL^{loxp}$ mice. After 3 hours of incubation with either lysozyme or diluted serum, a down modulation of the HEL binding BCR in SW_{HEL} B cells was observed in cells incubated with a lysozyme concentration of 2pg/μl or greater; however, the HEL binding BCR was comparable between PBS treated and serum treated SW_{HEL} B cells, indicating that serum levels of lysozyme was lower than 20pg/μl (**Fig. 2b**). After overnight incubation, B cells from SW_{HEL} mice were activated, as indicated by an increase in the percent of B cells that were positive for CD86 and CD69, when incubated with lysozyme concentrations of 2 pg/μl or greater; however, B cells from SW_{HEL} mice treated with diluted serum were not activated, suggesting that serum lysozyme concentrations were less than 20 pg/μl (**Fig. 2c**). Overall, this suggests that the presence of mDEL in the gut does not inadvertently cause it to become a systemic antigen that can impact tolerance at distant sites.

a.

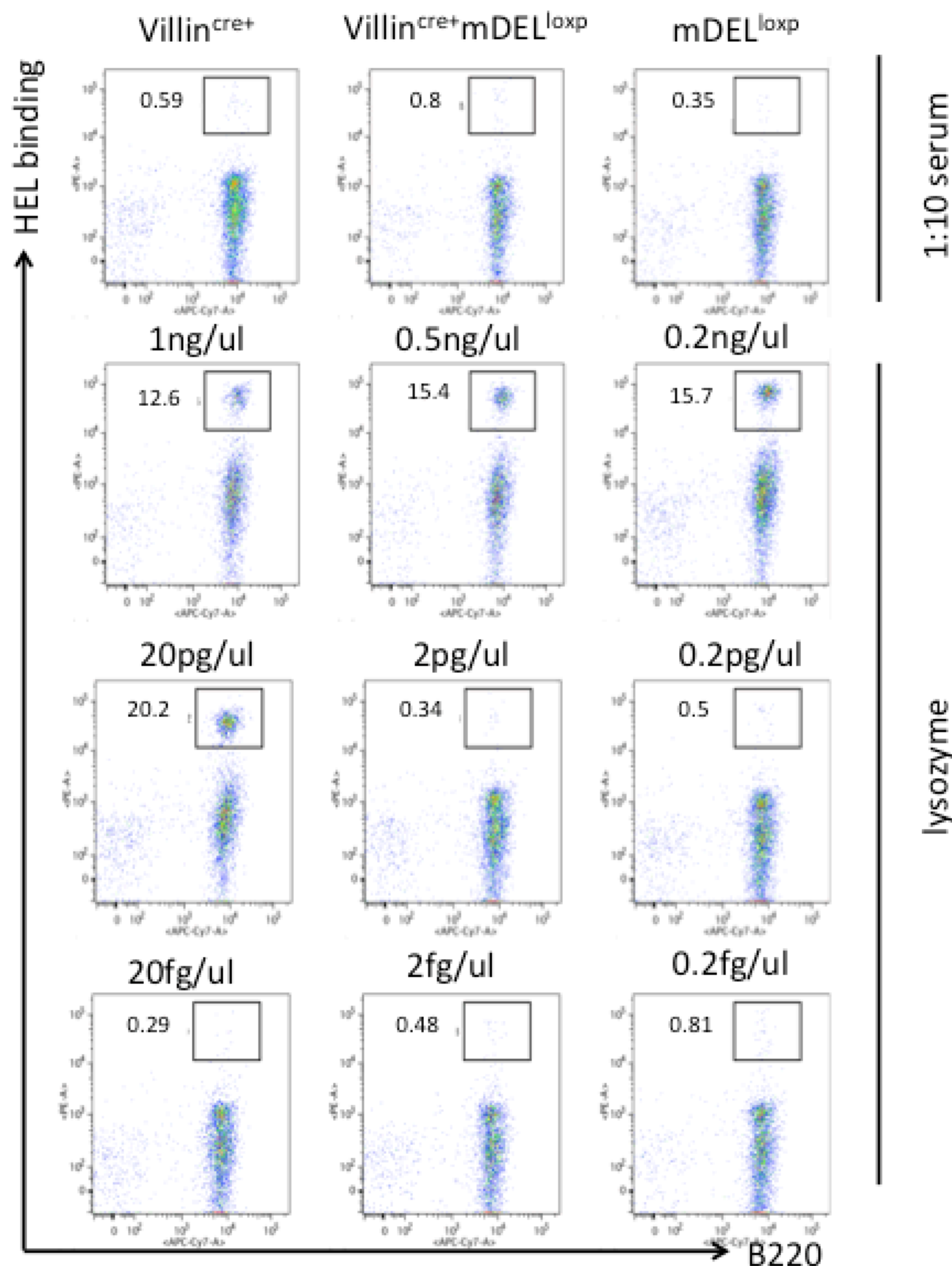
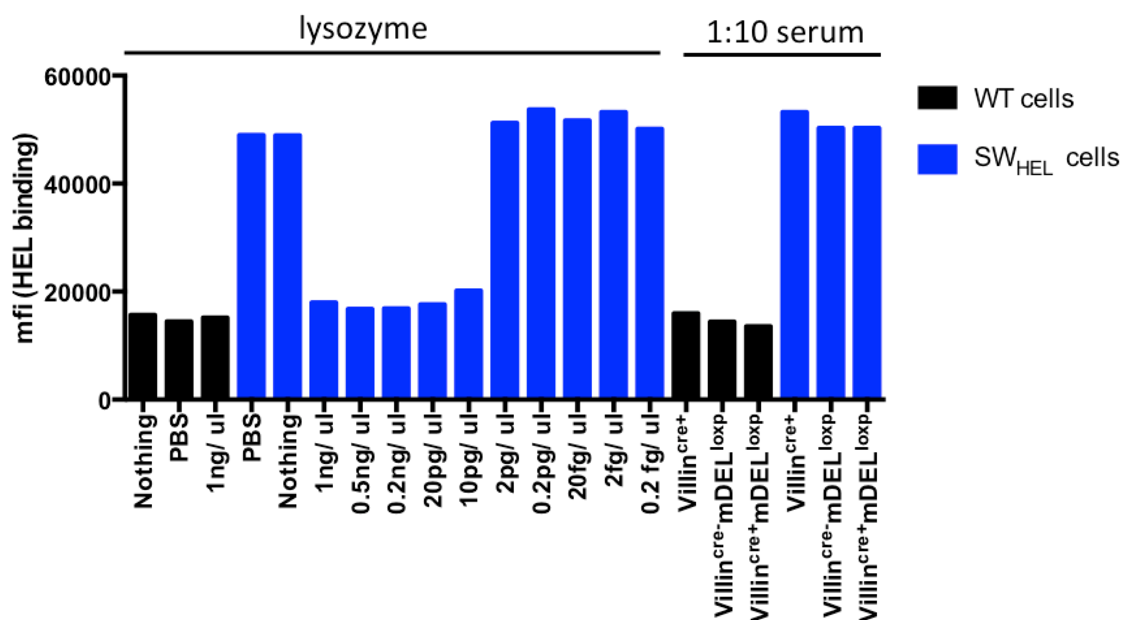


Figure 2: Serum from Villin^{cre}mDEL^{loxp} mice does not contain cleaved DEL. B cells from either control or SW_{HEL} mice were isolated and were **a)** freshly stained for HEL binding or incubated in culture with either soluble HEL or serum and we looked at **b)** the mfi for HEL binding 3 hours post stimulation and **c)** activation markers 24 hours post stimulation. Results representative of 2 experiments.

b.



c.

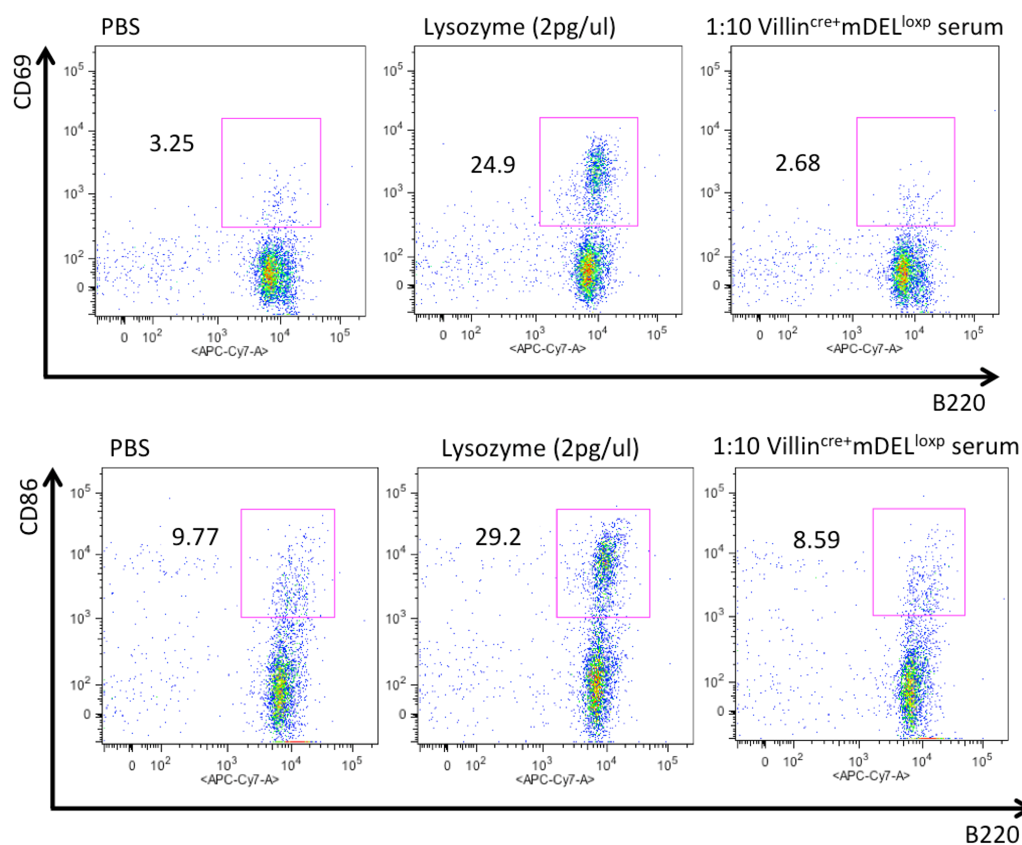


Figure 2: Serum from Villin^{cre}mDEL^{loxp} mice does not contain cleaved DEL, continued

1.3 Lysozyme Activity

DEL used in our mouse model has a largely unaltered amino acid sequence of normal duck egg lysozyme, and could therefore display antibacterial activity and affect the composition of the gut microbiome. To determine whether membrane bound DEL possesses lysozyme activity, we used the EnzChek Lysozyme Assay Kit (Molecular Probes). IECs from Villin^{cre+}mDEL^{loxp} mice were isolated and lysozyme activity was assessed to determine whether mDEL shows lysozyme activity. Isolated IECs from control and Villin^{cre+}mDEL^{loxp} mice had similar levels of lysozyme activity (**Fig. 3a**). To ensure that the method of isolating the IECs did not damage the activity of mDEL, the lysozyme activity of PBS washed intestinal tissue was analyzed from control and Villin^{cre+}mDEL^{loxp} mice and shown to have similar levels of activity in (**Fig. 3b**), indicating that any activity detected by the kit was due to endogenous mouse lysozyme produced by IECs in the gut and that mDEL expression does not increase the lysozyme activity in IECs and tissue.

Furthermore, to ensure that endogenous lysozyme activity in the gut did not obscure the lysozyme activity of mDEL, HEK 293T cells were transfected with MSCV-P2GM mDEL. The HEK cells were successfully transfected with 9.3% of the cells being positive for mDEL. $5x10^4$ mDEL+ cells were used in the lysozyme activity assay, showing lysozyme activity below detection levels (**Fig. 3c**). This suggests that DEL is not catalytically active in its membrane-bound form.

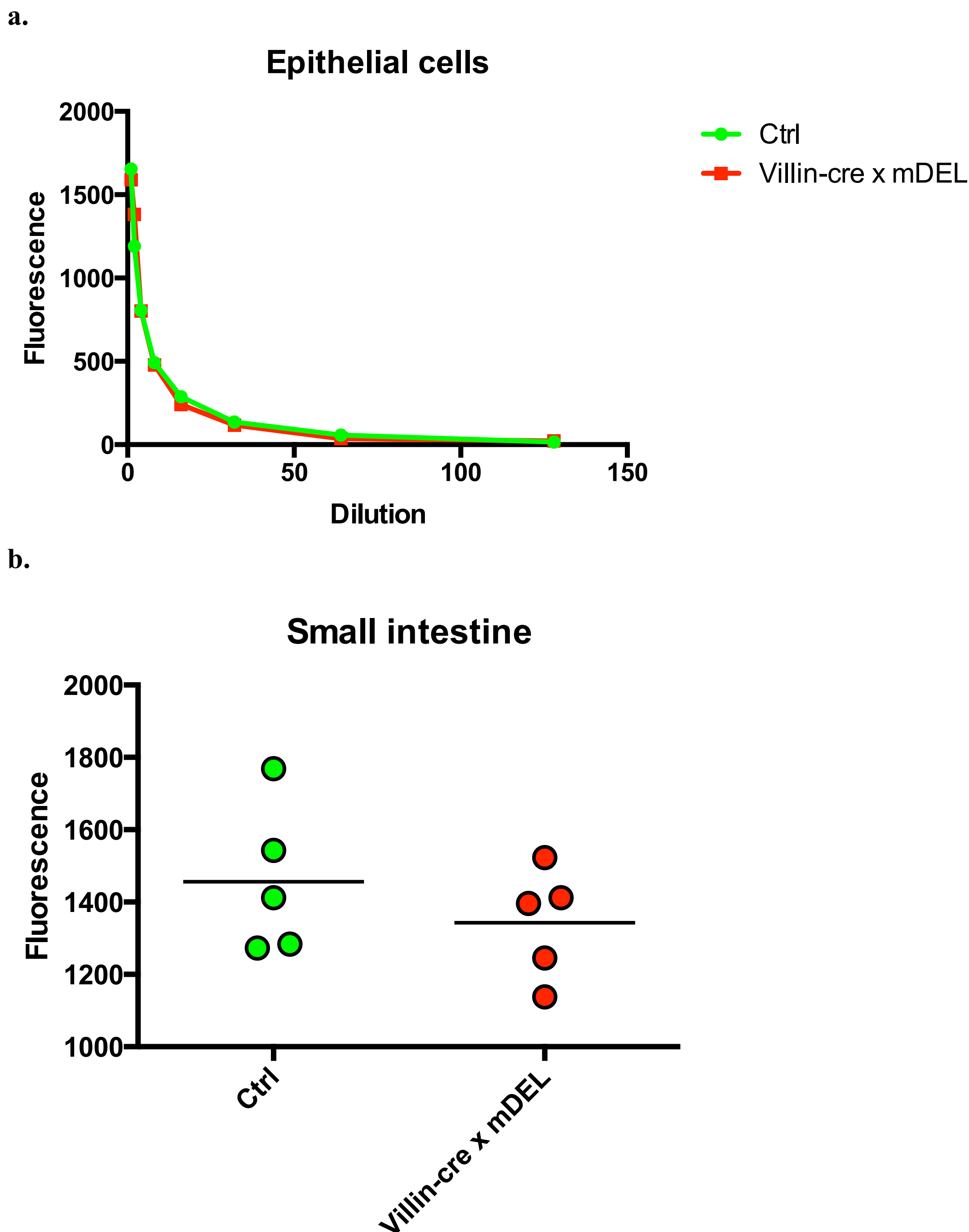


Figure 3: Tissue and isolated IECs from Villin^{cre+}mDEL^{loxp} mice have similar lysozyme activity to control mice. Lysozyme activity of **a)** IECS, **b)** 8mm intestinal tissue and **c)** HEK 293 T cells transfected with MSCV-P2GM mDEL vector. Lysozyme activity was tested using the EnzCheck Lysozyme Activity Assay Kit (Life Technologies). $5x10^4$ cells were put into each well with subsequent 1:2 serial dilutions.

c.

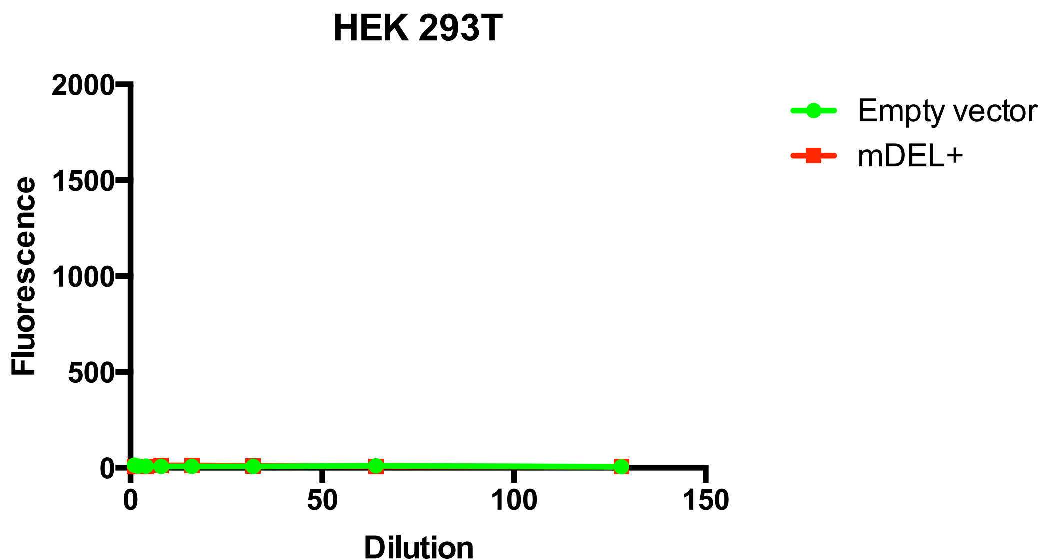


Figure 3: Tissue and isolated IECs from Villin^{cre+}mDEL^{loxp} mice have similar lysozyme activity to control mice, continued

1.4 Microbiome

Lysozymes are known for their potential as antibiotics and the catalytic activity of lysozymes has been shown to not be the only factor in determining their bactericidal abilities [63]. Since in our mouse model lysozyme is expressed in the gut epithelium, it is important to determine whether the microbiome is affected by the presence of mDEL. To this end, genomic DNA (gDNA) from bacteria present in feces of chimeric and non-chimeric control and from Villin^{cre+}mDEL^{loxp} SW$_{HEL}$ mice was extracted and sequenced. Chimeric and non-chimeric control and Villin^{cre}mDEL^{loxp}SW$_{HEL}$ mice had a similar distribution of microbes in their fecal microbiome (**Fig. 4a**), showing that the expression of mDEL in the gut does not affect the microbiome.

As the mouse model will be used in the study of oral tolerance, it is also important for us to determine whether the oral administration of HEL will affect the gut microbiome. HEL can potentially disrupt gut homeostasis due to its effects on certain

bacteria, specifically gram-negative bacteria, which can affect the frequency of certain commensal bacteria in the gut as well as lead to the release of bacterial contents that can change the gut environment. This effect will make it difficult to interpret results between mice given PBS and mice given HEL. If mice given HEL have an altered microbiome, it can potentially become difficult to determine whether results were due to the presence of HEL acting as a oral Ag and not as an antimicrobial agent or due to the altered microbiome. To this end, CD45.1 mice were gavaged with either PBS or HEL (0.25mg and 25mg) and the gut microbiome was observed over a two-week period. We observed that the variations in the gut microbiota were consistent between the PBS treated and HEL treated mice (**Fig. 4b**), thus the acute treatment with HEL does not lead to significant changes of the microbiome.

a.

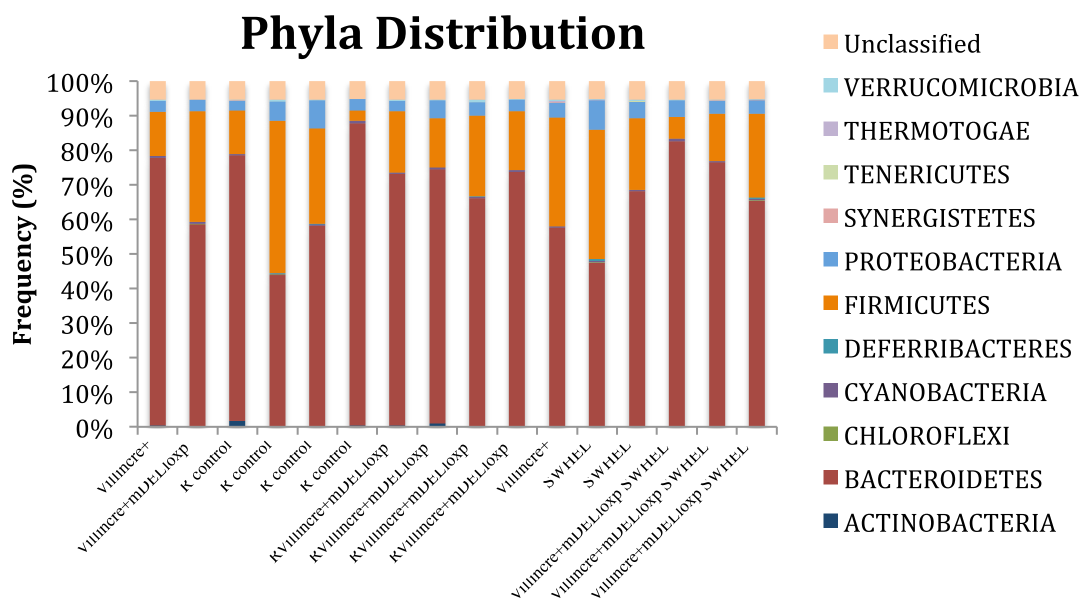


Figure 4: Microbiome comparison. a) Phyla distribution chimeric and non-chimeric control and Villin^{cre+}mDEL^{loxp}SW$_{HEL}$ mice. b) Phyla distribution of mice gavaged with PBS or HEL (0.25mg or 25mg) over a 2 week period (3,7 and 14 days post treatment). Feces of mice were collected and gDNA extracted with the Qiagen Stool gDNA extraction kit and sequenced. Analysis performed by Lisa Elman.

b.

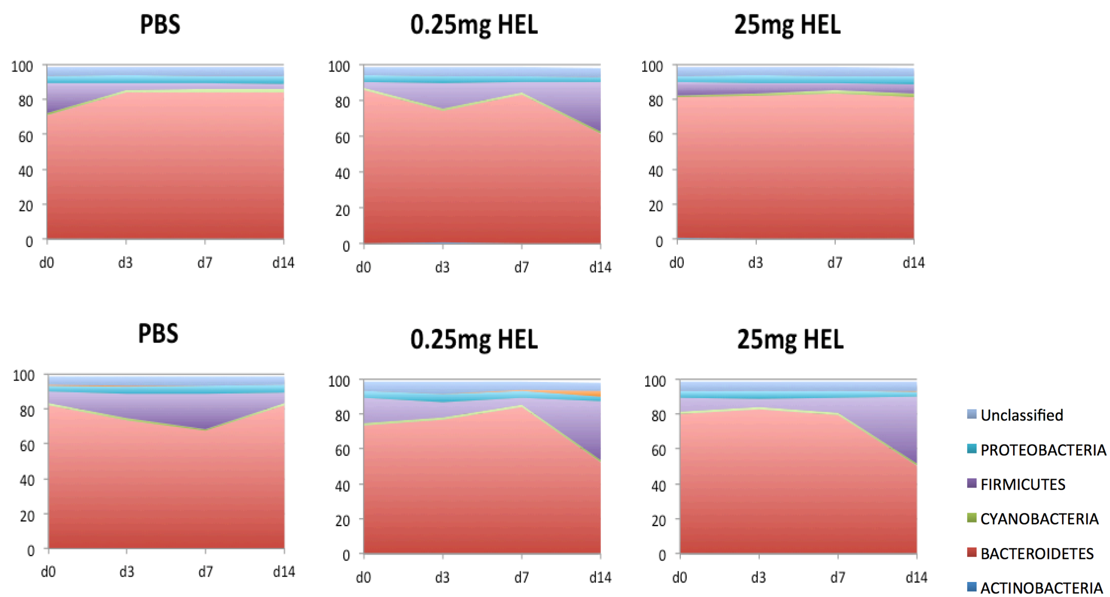


Figure 4: Microbiome comparison, continued

Chapter 2: Peripheral Tolerance

Peripheral tolerance is important in ensuring that potentially auto-reactive B cells in the peripheral B cell pool do not become activated and cause tissue destruction when encountering self-antigen. To study peripheral tolerance in our novel mouse model, we first analyzed the mice under normal conditions to investigate how peripheral tolerance is maintained.

2.1 Comparing Reconstituted and Intact Mice Under Normal Conditions

Two versions of the Villin^{cre}mDEL^{loxp}SW$_{HEL}$ mice were generated: a chimeric and intact version. The chimeric mice are Villin^{cre+}mDEL^{loxp} mice that were lethally irradiated and reconstituted with bone marrow from SW$_{HEL}$ mice and will be referred to as reconstituted control or RVillin^{cre+}mDEL^{loxp} mice. The intact mice will be referred to as control or Villin^{cre}mDEL^{loxp}SW$_{HEL}$ mice. The two versions were generated due to the difficulties in obtaining a large enough group of intact mice with the experimental genotype.

Under normal conditions, RVillin^{cre+}mDEL^{loxp} mice showed a decrease in the percentage of HEL binding B cells in the PLN, and PP compared to reconstituted control mice (**Fig. 5a, b**). While not statistically significant, the percentage of HEL binding B cells in the MLN are also consistently lower in the RVillin^{cre+}mDEL^{loxp} mice. This suggests that the HEL binding B cells are being eliminated. The binding of HEL to the HEL BCR leads to the internalization of the HEL BCR due to the strong interaction between the Ag and BCR. HEL binding in the spleen and the PLN were comparable between the control and Villin^{cre+}mDLE^{loxp}SW$_{HEL}$ chimeric mice (**Fig. 5c**), indicating that

the SW_{HEL} B cells in the spleen do not come into contact with the self-antigen, mDEL. On the other hand, HEL binding B cells in the MLN and PP showed a down-modulation of the of HEL binding BCR as determined by staining for HEL binding and IgM expression (**Fig. 5c**), suggesting that the B cells in the MLN and PP encountered the self-antigen expressed in the gut epithelium. HEL-binding B cells also showed an increase in the activation marker CD86 and a slight increase in CD69 in the MLN and PP, but not in the spleen and PLN (**Fig. 5d**); however, HEL binding B cells do not seem to enter the germinal center, as indicated by the lack of GL7+ HEL-binding B cells (**Fig. 5e**). Although activated, HEL-binding B cells in this mouse model do not produce significant levels of HEL specific antibodies as determined by ELISA (**Fig. 5f**).

In contrast, the Villin^{cre+}mDEL^{loxp}SW_{HEL} mice showed a slightly different phenotype than the RVillin^{cre+}mDEL^{loxp} mice. Villin^{cre+}mDEL^{loxp}SW_{HEL} mice had a decrease in the percentage of HEL binding B cells in the spleen compared to the reconstituted mice (**Fig. 6a**). When comparing the HEL binding B cells in the spleen and PP of chimeric and non-chimeric mice, we noticed that the intact mice showed a similar phenotype in terms of HEL binding and the expression of activation markers CD86 and CD69 (**Fig. 6b**). This leads to the question of why this difference was occurring.

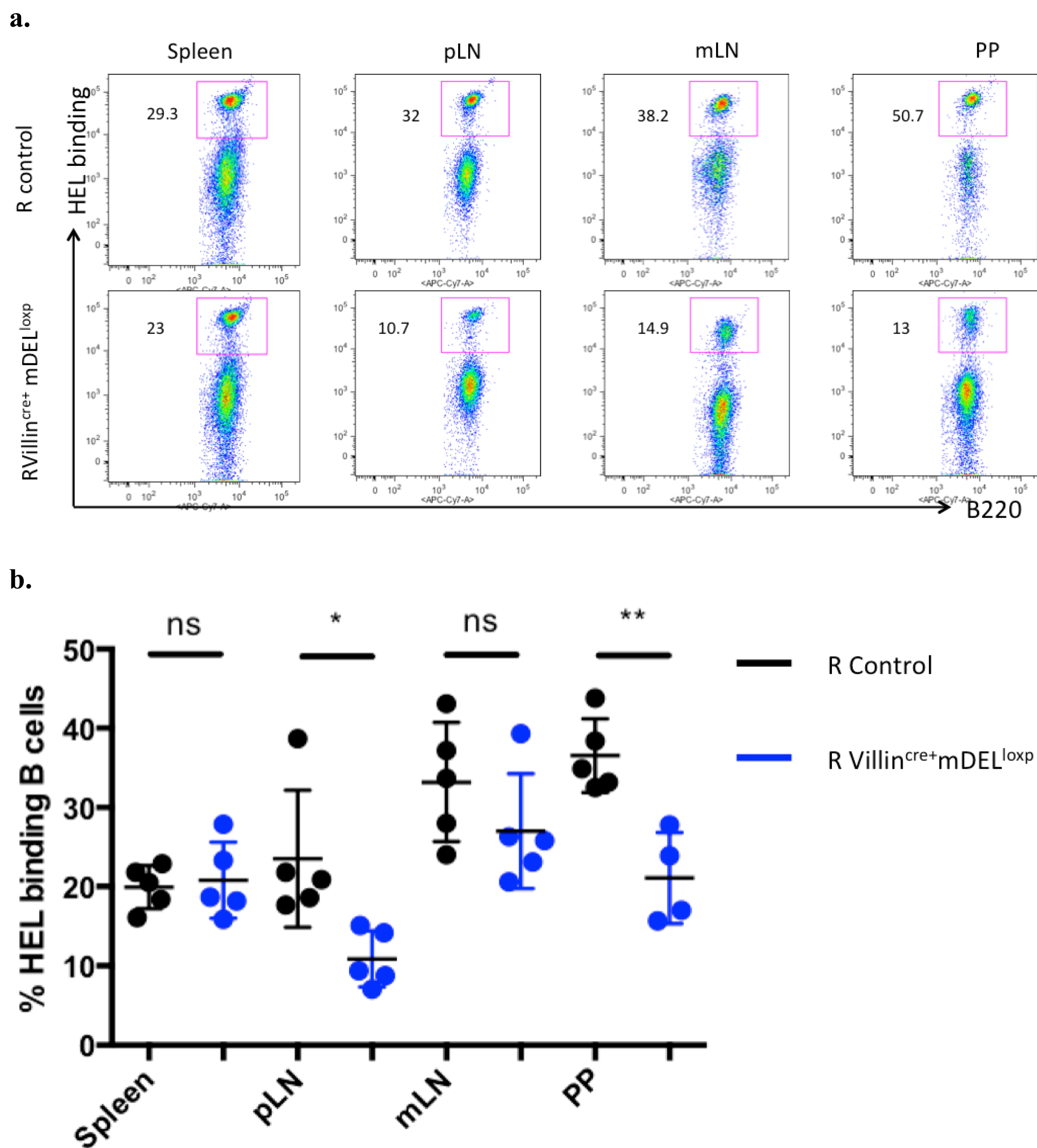


Figure 5: Chimeric mice phenotype. The phenotype of HEL binding B cells were analyzed via flow for: **a)** % HEL binding B cells in spleen, PLN, MLN and PP, **b)** surface expression of HEL binding BCR and IgM in Spl, PLN, MLN and PP, **c)** activation markers and **d)** germinal center B cells. **e)** Serum levels of IgM, IgG and IgA via ELISA. Data shown is representative of 4 experiments (n=2-10). * $P \leq 0.05$, ** $P \leq 0.01$ using unpaired student t test.

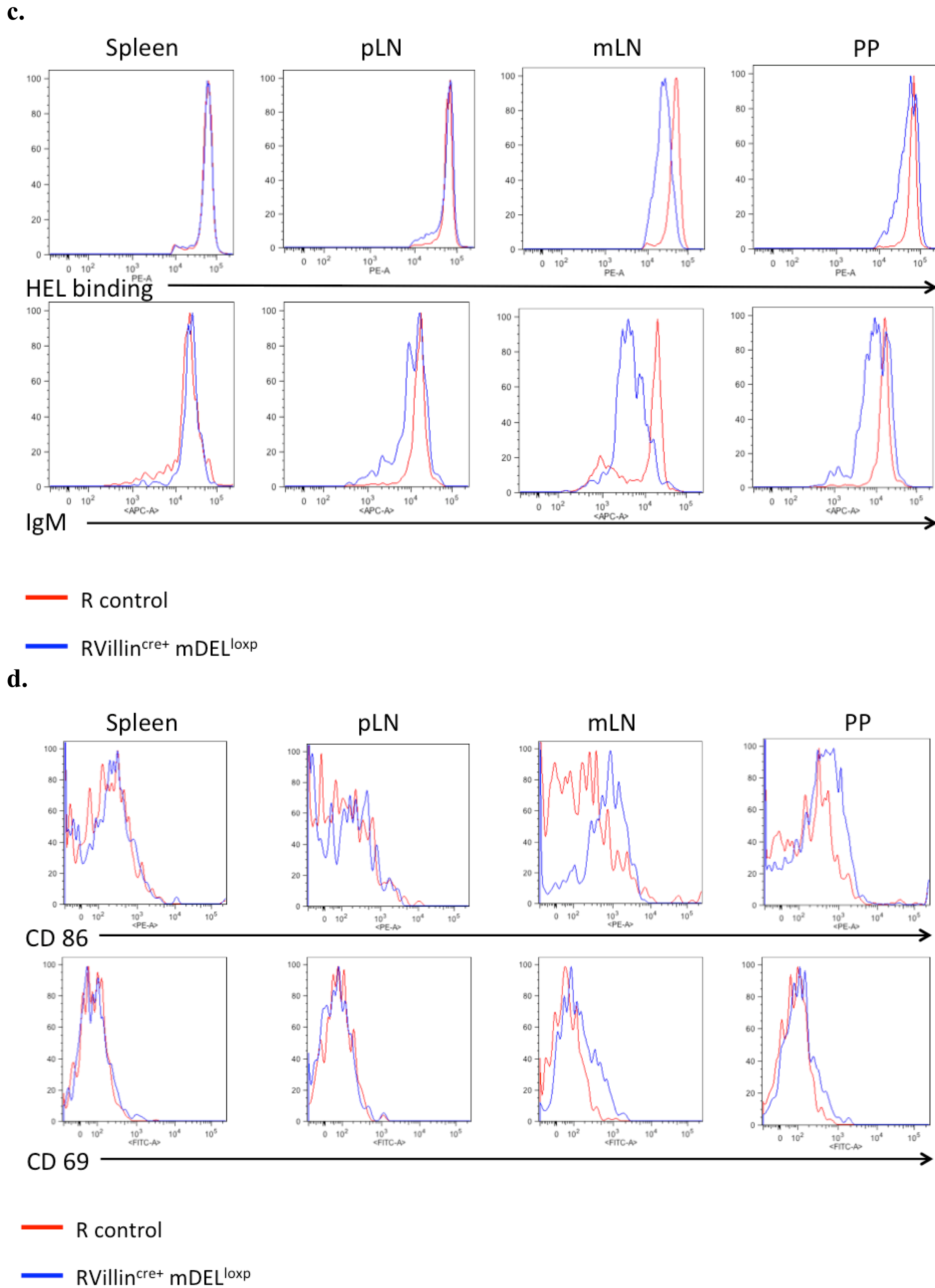
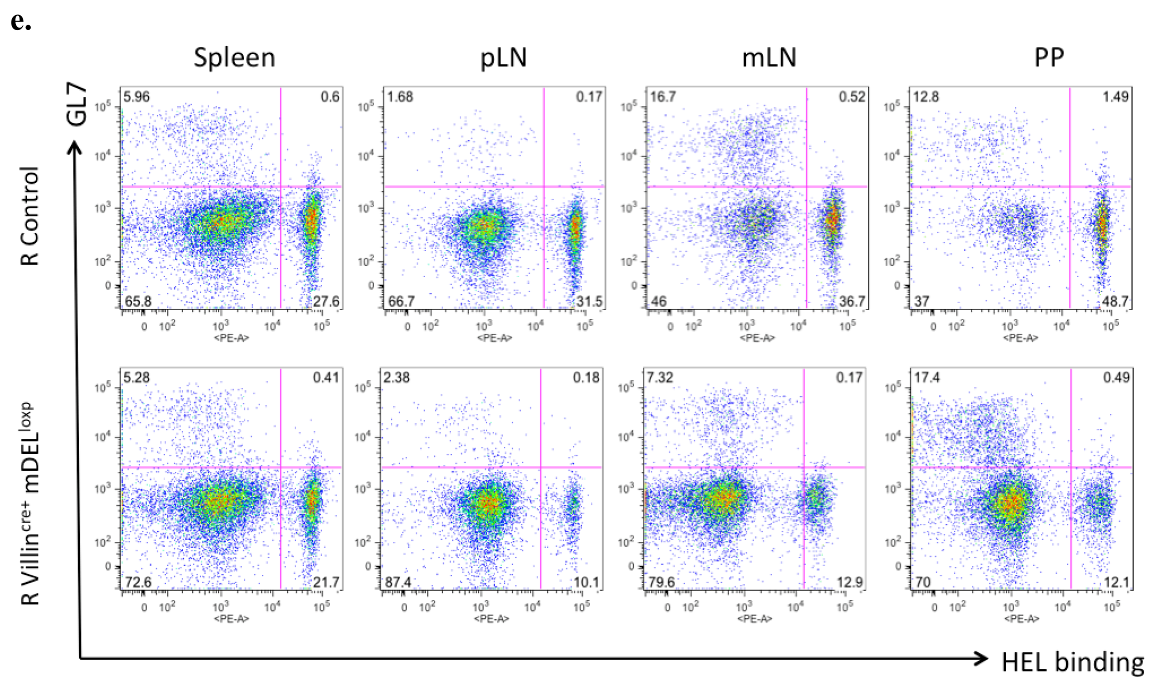


Figure 5: Chimeric mice phenotype, continued

e.



f.

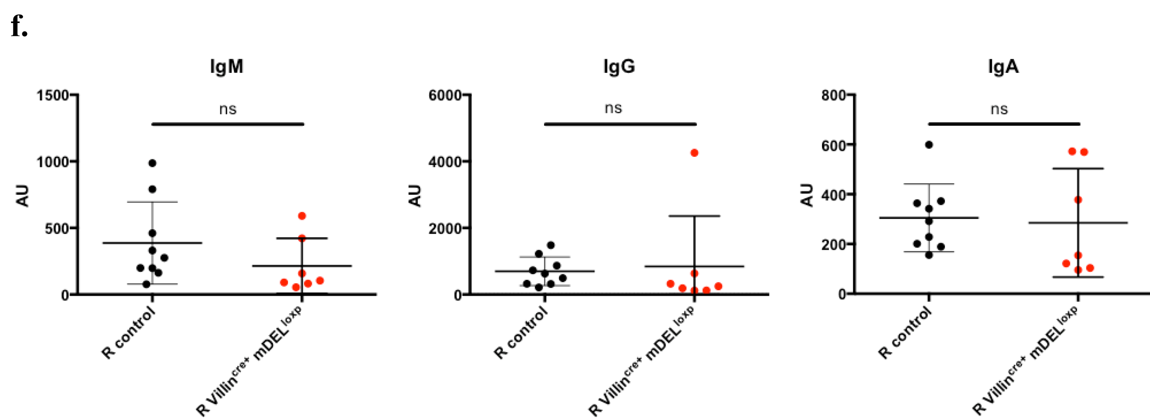


Figure 5 continued: Chimeric mice phenotype, continued

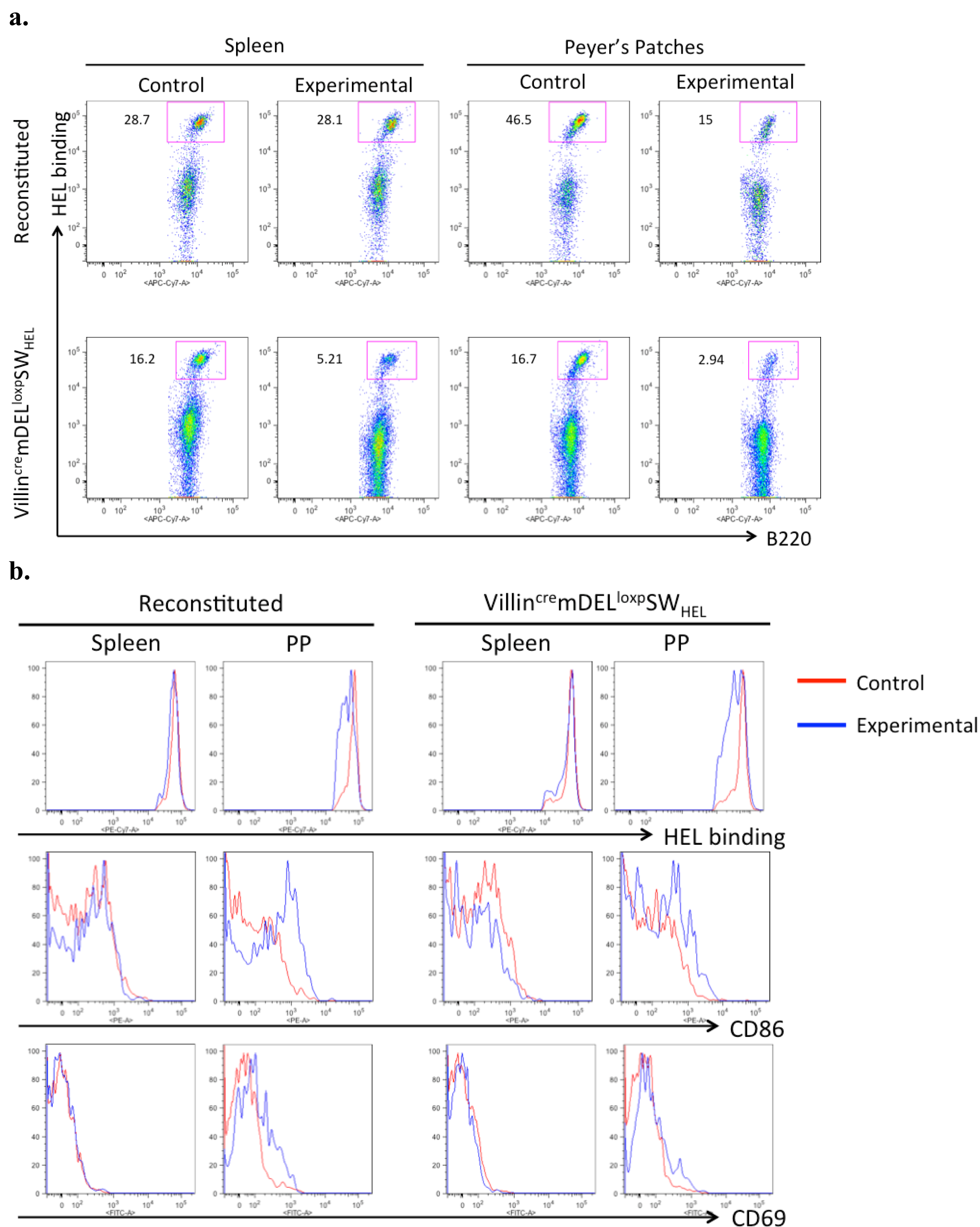


Figure 6: Comparing the phenotype of reconstituted and Villin^{cre}mDEL^{loxp}SW$_{HEL}$ mice. a) % HEL binding B cells in spleen and PP. **b)** HEL binding and activation markers. Data shown representative of 4 experiments in reconstituted mice (n=2-10 per experiment) and 2 experiments in Villin^{cre}mDEL^{loxpSW}$_{HEL}$ mice (n=4 per experiment).

2.2 SW_{HEL} Adoptive Transfer

To determine whether mature auto-specific B cells are deleted in Villin^{cre+}mDEL^{loxp} mice, B cells from the spleen of SW_{HEL} mice were sorted with MACS beads and labeled with the efluor-670 proliferation dye to track the adoptively transferred cells (**Fig. 7a**). Cells were adoptively transferred into control or Villin^{cre+}mDEL^{loxp} mice and analyzed 1, 3 and 5 days post transfer (**Fig. 7b-e**). We found that the percentage of HEL binding B cells decreased over time at a greater rate in the PLN and PP of the Villin^{cre+}mDEL^{loxp} mice compared to the control mice; however, this was not the case in the spleen and MLN (**Fig. 7b**). 1-day post transfer, the transferred HEL binding B cells in the Villin^{cre+}mDEL^{loxp} mice showed a downmodulation of the HEL binding BCR in the spleen, MLN and PP, but not in the PLN when compared to control mice lacking mDEL in the gut (**Fig.7c**). The HEL binding B cells in the Villin^{cre+}mDEL^{loxp} mice also showed an increased expression of the activation markers CD86 in the spleen, PLN, MLN and PP (**Fig. 7c**). Similarly, CD69 expression on HEL binding B cells was also increased in the Villin^{cre+}mDEL^{loxp} mice in the spleen, MLN and PP, but not in the PLN (**Fig. 7c**). This shows that the transferred HEL binding B cells encountered mDEL in the gut 1-day post transfer. HEL binding and CD86 was similar 3 and 5 days post transfer to 1 day post transfer; however, CD69 expression in the HEL binding B cells transferred into Villin^{cre+}mDEL^{loxp} mice 3 and 5 days post transfer were similar to those transferred into control mice (**Fig. 7d, e**). This indicated that mature HEL binding B cells recirculate and are possibly deleted when Ag is encountered.

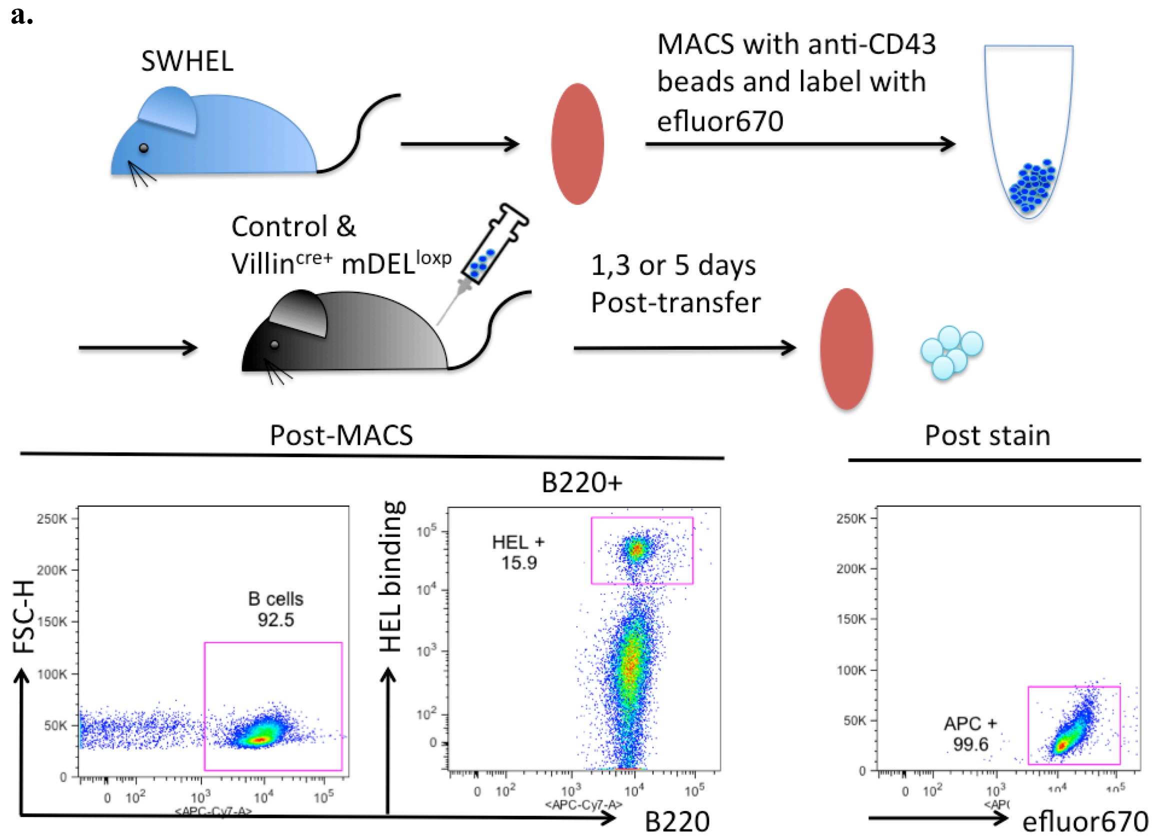


Figure 7: Adoptive transfer study. a) Experimental layout and percent HEL binding B cells post-MACS and post stain with efluor 670 **b)** % HEL binding B cells post transfer. HEL binding and activation markers **c)** 1 day post transfer, **d)** 3 days post transfer and **e)** 5 days post transfer. $7x10^6$ MACS sorted and efluor 670-labeled B cells from SW_{HEL} mice were transferred into control (n=6) or Villin^{cre+}mDEL^{loxp} mice (n=6).

b.

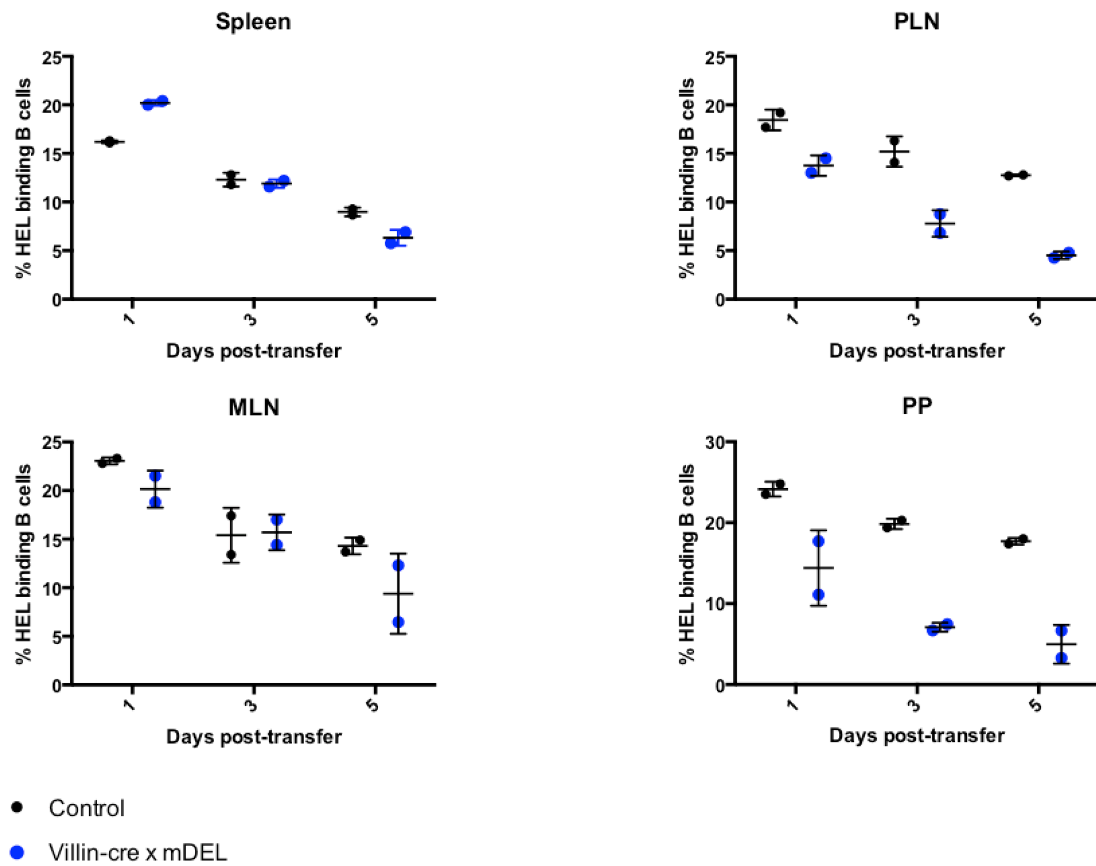
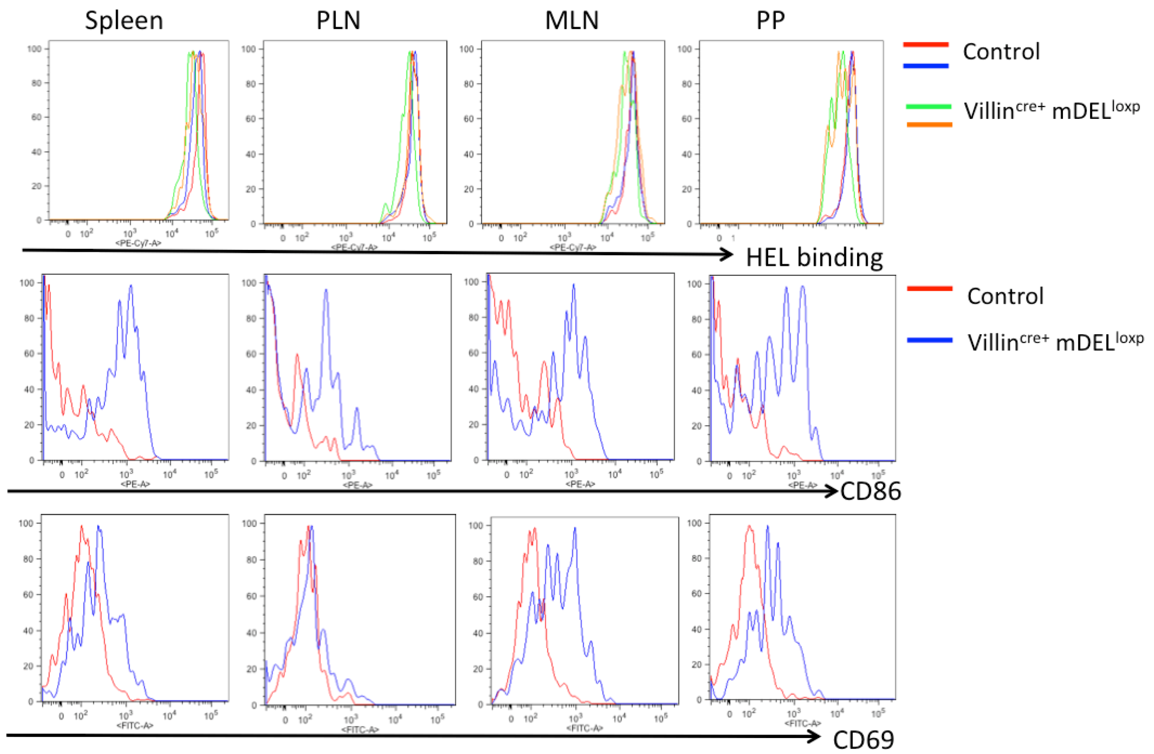


Figure 7: Adoptive transfer study, continued

c.



d.

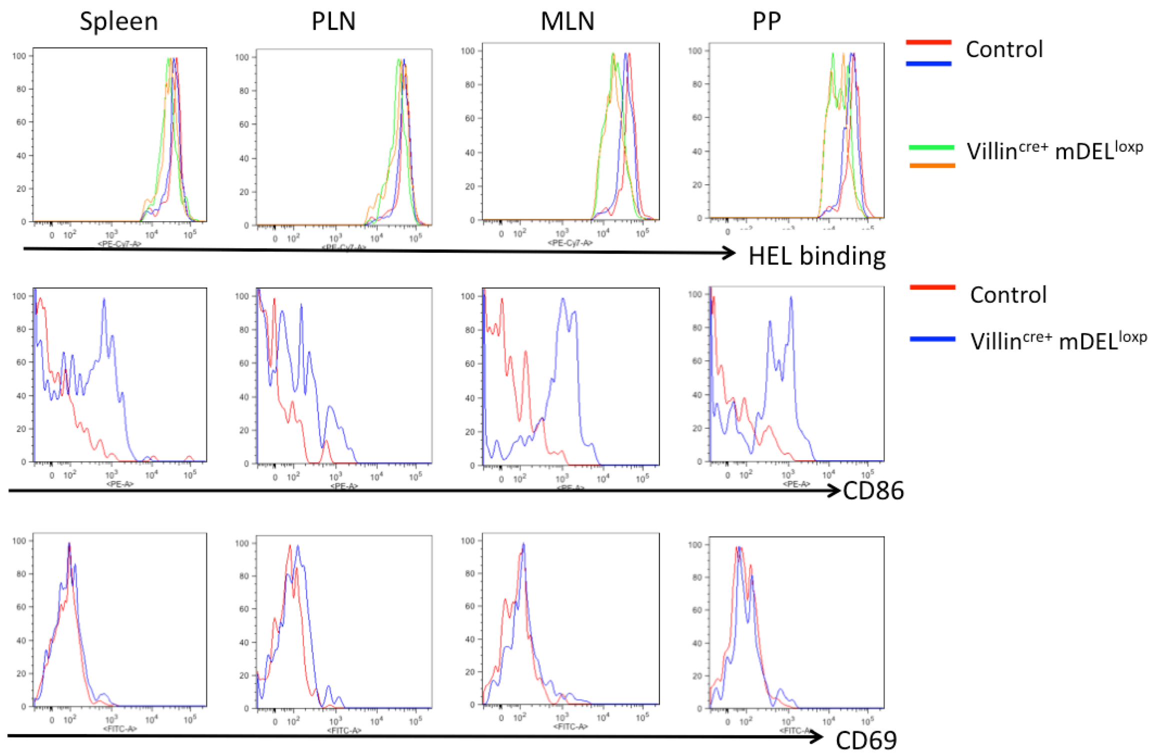


Figure 7: Adoptive transfer study, continued

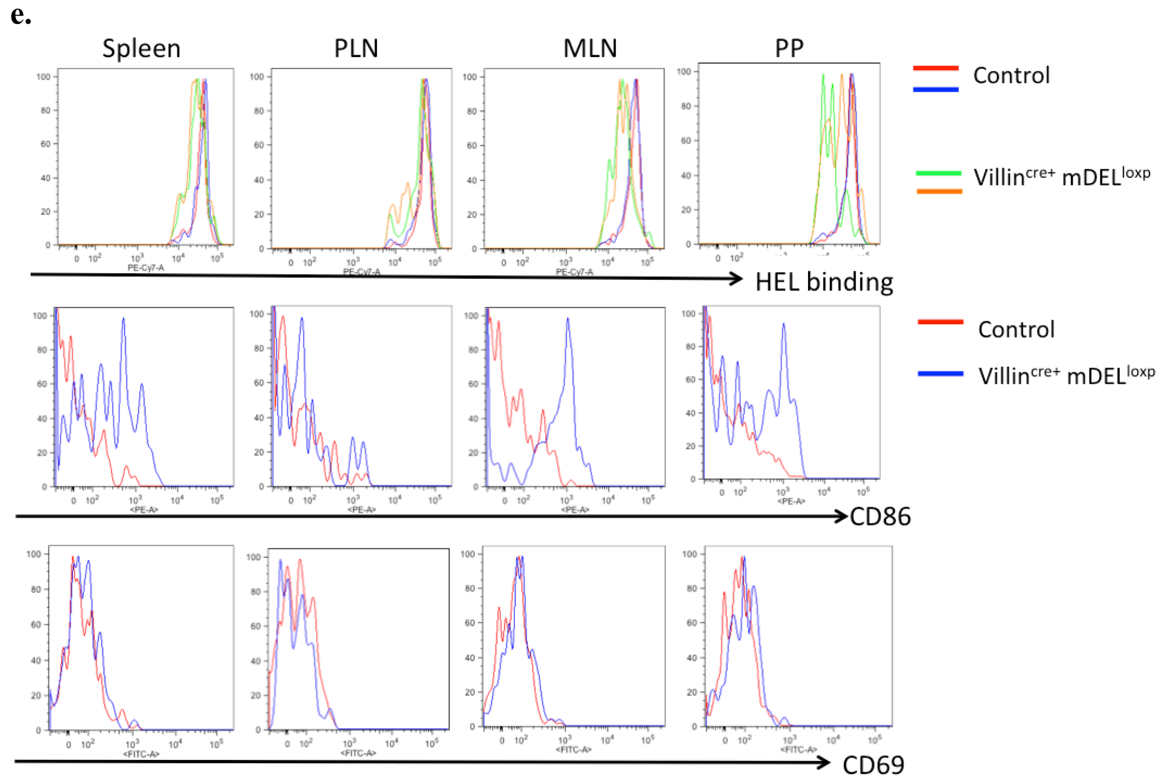


Figure 7: Adoptive transfer study, continued

IV. DISCUSSION AND FUTURE DIRECTIONS

Fate of Auto-reactive B cell

The mechanism by which B cells become tolerant to gut associated antigens and how tolerance can be breached is unknown. The impact of a breach in the intestinal epithelial barrier due to infection or IBD on tolerance is also unknown. Therefore, a mouse model in which a neo self Ag, mDEL, is expressed conditionally in the gut epithelium and contains autoreactive B cells that can recognize mDEL was generated. While there have been similar tolerance models with membrane bound expression of HEL in the thymus and liver [59, 64], there has yet to be one in the gut. The gut environment is unique for studying tolerance, because B cells have to be able to differentiate between commensal and pathogenic bacteria, food antigens, and self-tissue.

One of the main things that have to be done when proposing a new mouse model is to ensure that the mouse model does not intrinsically affect the results of proposed experiments and possibly confound conclusions. The gut epithelial cells rapidly turnover and thus can cause concern over its expression and the claim that it is a local Ag. The first goal was to determine whether mDEL was expressed in the gut and if expression was uniform. This ensures that our desired Ag is expressed in the proper location and is not affected by the high turnover rate of the IECs. mDEL expression was confirmed on isolated IECs of Villin^{cre}mDEL^{loxp} mice taken from different parts of the gut (**Fig. 1**). Additionally, there is no indication that mDEL is able to enter circulation to affect tolerance in unintended locations (**Fig. 2**), and so mDEL can be considered a local gut Ag affecting tolerance in the GALT.

The use of mDEL as a gut Ag can be questioned, as lysozymes are known to be antimicrobial, and can therefore have an affect on the composition of the gut microbiota. This is important because the composition of the gut microbiota has been implicated in determining and influencing the B cell pool in the periphery, such as B regulatory cells [36, 65, 66]. B regulatory cells play an important role in preventing autoimmunity by inhibiting inflammation via the production of IL-10, an anti-inflammatory cytokine, as well as regulating T cell dependent autoimmunity [67, 68]. mDEL was found to not be catalytically active (**Fig. 3**); however, lysozyme that have lost their catalytic ability have also been found to have potential antimicrobial properties [69]. Therefore, we examined the gut microbiota to determine whether the presence of mDEL affected its composition. The composition of the gut microbiota was not affected by presence of mDEL (**Fig.4**), indicating that mDEL's ability to act as an antimicrobial is not present. This ensures that the results from doing subsequent experiments are not influenced by an unintended change in the microbiota.

The expression of mDEL in the gut leads us to our interest in the PPs, which are the lymphoid organs along the antimesenteric side of the gut. When comparing the percentage of HEL binding B cells in RVillin^{cre+}mDEL^{loxp} mice to reconstituted control mice, no difference was observed in the spleen; however, there was a significant decrease in the percentage of HEL binding B cells in the PLNs and PP, but not in the MLNs (**Fig. 5a,b)**. We also observed a downmodulation of HEL binding and IgM in the MLNs and PPs of RVillin^{cre+}mDEL^{loxp} mice compared to the control (**Fig. 5c**). This indicates that the HEL binding B cells have encountered Ag in the GALT.

The HEL binding B cells remaining in the PP of the RVillin^{cre+}mDEL^{loxp} mice also showed an activated phenotype compared to reconstituted control mice (**Fig. 5d**). The upregulation of CD69 helps retain the HEL binding B cells in the lymph nodes and inhibits B cell migration by suppressing S1PR1[70, 71]. This can potentially mean that the autoreactive B cells that have encountered self-Ag to the GALT remain in the GALT and are unable to recirculate to the PLN and spleen. However, non-HEL binding B cells that do not become activated continue to recirculate, thus increasing the percentage of non-HEL binding B cells seen in the PLN and decreasing the percentage of autoreactive B cells in the PLN of RVillin^{cre+}mDEL^{loxp} mice.

However, in the spleen no decrease in the percentage of autoreactive B cells is seen, suggesting that it can be due to the HEL binding B cell population being repopulated by cells from the bone marrow. To study the turnover rate of HEL binding B cells, mice can be given BrdU, which labels proliferating cells. By looking at the ratio of BrdU labeled cells, we can potentially identify where the HEL binding B cells in mDEL expressing mice are eliminated. We can also look at the rate at which BrdU labeled cells are eliminated to determine the turnover rate of the B cells.

The upregulation of CD86 enables the HEL binding B cells to costimulate T cells [72]. An upregulation of CD86 is observed in the HEL binding B cell populations present in the MLN and PP (**Fig. 5d**). Since there is an upregulation of CD86, HEL binding B cells can potentially become GC B cells, especially in the PPs, where GCs are constantly present. However, HEL binding B cells were not in the GC (**Fig. 5e**). Furthermore, there was no significant difference between reconstituted control and RVillin^{cre+}mDEL^{loxp} mice in levels of HEL specific Abs in the serum and fecal extracts (**Fig. 5f**). The lack of

autoantibody production can also be due to the lack of T cell help, since mDEL is a TD Ag and is a weak T cell activator, since DEL was modified to prevent it from priming I-A_b restricted T cells (to prevent crossreactivity). Without T cell help, HEL binding B cells do not receive survival, proliferation or differentiation signals and thus we may see minimal auto-antibody production even with immunizations, much less under normal circumstances. They are also unable to initiate GC responses against TD Ag as seen in CD40 deficient mice[73] Additionally, the lack of T cell help to the activated HEL binding B cells in the GALT of RVillin^{cre+}mDEL^{loxp} mice may fate them to apoptosis, because activated B cells require interaction with IL-4 and CD40L to prevent Fas-mediated apoptosis [74]. This can be the reason why the percent of HEL binding B cells in the different organs are observed. Overall, this indicates that B cell tolerance is maintained in RVillin^{cre+}mDEL^{loxp} mice.

Breach of B cell tolerance can possibly occur if the auto-reactive Ba cells are able to recruit T cell help. Immunizing with HEL conjugated to SRBC has been suggested as a method for recruiting T cell help, since SRBC is a strong T cell activator[75]. We have previously shown that HEL binding B cells are able to induce OT-II T cells to proliferate *in vitro* in reconstituted control mice; however, proliferation was impaired in mDEL expressing mice, suggesting that mDEL expression on FDCs impairs the ability of autoreactive B cells to act as antigen presenting cells [12]. It would be interesting to see whether this similarly occurs when mDEL is expressed in the gut epithelium. Additionally, we can adoptively transfer OVA specific T cells labeled with proliferation dye from OT-II mice into reconstituted control and RVillin^{cre+}mDEL^{loxp} mice. We can then immunize with HEL conjugated to OVA. The adoptively transferred OT-II T cells

will provide a source of T cell help to the SW_{HEL} B cells and we would be able to determine whether autoreactive B cells are impaired as antigen presenting cells (APCs) *in vivo*. This will also allow us to see whether autoreactive B cells that receive T cell help can initiate a response.

Furthermore, from the adoptive transfer studies, we have seen that mature adoptively transferred HEL binding B cells are deleted at a faster rate in mice expressing mDEL in the gut than in control mice, (**Fig. 7**), suggesting that the turnover rate of B cells in the reconstituted mice may be greater than in our non-reconstituted mice. To test whether $RVillin^{cre+}mDEL^{loxp}$ mice have a greater turnover rate than $Villin^{cre+}mDEL^{loxp}SW_{HEL}$ mice, they can be given BrdU, as mentioned previously. A greater turnover rate in reconstituted mice would suggest that even though HEL binding B cells are deleted in the PP or MLN when encountering mDEL, this deletion is not enough to affect the HEL binding B cell population in the spleen, unlike in $Villin^{cre+}mDEL^{loxp}SW_{HEL}$ mice, whereas a slower turnover would mean that the bone marrow does not replenish the B cells in the spleen fast enough and so recirculation of the peripheral B cell pool with non- HEL binding B cells will be seen in the spleen.

The difference in the reconstituted and intact mouse model can be problematic, because this difference will have significance in determining the ideal model to use for studying B cell tolerance. However, these differences can be due to age, because younger mice have higher B cell turnover rates compared to older mice[76]. B cells from young mice are more easily tolerized than in older mice, which can explain why older mice and humans are more prone to autoimmunity [77, 78].

The MLN is also the location where DCs preferentially present Ag to T cells [79]. The importance of the MLN in T cell tolerance over the PP has been demonstrated in the inability of T cells to become tolerant to orally administered OVA when lacking MLNs; however, this was not the case in mice lacking PPs [80]. There may be a similar function for creating tolerant B cells in the MLN. The transport and display of gut self-Ag is not well understood; however, HEL binding B cells in the MLN show a downmodulation of the HEL binding BCR and IgM, suggesting that HEL binding B cells in the MLN have interacted with self-Ag. This can be potentially due to DCs from the PPs or LP, which can migrate from the GALTs to the MLN[81], but are unable to leave the MLN to enter systemic circulation. However, T cells that have been primed in the MLN are able to leave the MLN to the PLN, and was the source of antigen-specific T cells systemically [45].

Further understanding of where and when HEL binding B cells from control and experimental mice are eliminated and the mechanism by which this occurs can be done by analyzing apoptotic markers, such as cleaved caspase-3, annexin V and Bim at different developmental stages (transitional and mature) via flow cytometry. In other models of B cell tolerance to membrane-associated Ag, it has been discovered that auto-reactive B cells with high affinity receptors are deleted while those with low affinity receptors become anergic [82]. T1 B cells are not thought to enter lymphoid organs due to a lack of CD62L, a lymph node homing receptor, while T2 B cells do express CD62L but are normally found in the spleen [83]; however, transitional cells have recently been detected in the GALT of humans [84, 85], raising the possibility that transitional or transitional-like B cells may play a role in the GALTs. It has been shown in other studies

that the frequency of auto-reactive transitional B cells is diminished prior to entry into the naïve mature B cell pool [11]. Defects in this checkpoint have been found in patients with autoimmune disease, such as in SLE and RA [86, 87]. B cell tolerance in the gut may be similarly regulated and breaches in tolerance can lead to autoantibody production and potentially autoimmune disease. Indeed, it has been found that early B cell development can occur in the mouse intestinal LP in postnatal mice and seems to be influenced by the gut commensal microbes [88].

Conclusions and future prospects

The Villin^{cre+}mDEL^{loxp}SW$_{HEL}$ mouse model can also be used to analyze whether the survival of autoreactive B cells is enhanced in the presence of pro-survival molecules, such as BAFF or Bcl-2. The addition of BAFF in vivo has been shown to enable a greater percentage of T2 B cells to become mature B cells [89] and is required for the development of B cells [90]. Overexpression of BAFF has also been shown to rescue autoreactive B cells [91].

The RVillin^{cre+}mDEL^{loxp} mouse model can also be used to study how peripheral B cell tolerance against gut associated Ags is regulated and maintained, when under stress from the disturbances to gut homeostasis by bacterial enterotoxins or damage to the gut epithelium and when there is a breach in T cell tolerance. IBD is characterized by inflammation of the gut epithelium; however, the initial factor in causing the inflammation is unknown. The switched production of Ab from homeostatic maintaining IgA to IgG and the presence of autoantibodies in UC patients, suggests a role for B cells in IBD[35, 51]. This switch can be a potential initial effector of IBD or a secondary response resulting from damage to the gut epithelium. The mouse model allows us to

study whether inflammation triggers the activation of autoreactive B cells or whether autoantibodies cause inflammation.

To study how autoreactive peripheral B cells respond to a breach in the gut epithelial barrier, mice will be given 2.5% DSS water, which disrupts the gut epithelial barrier by permeablizing enterocytes. In patients with IBD, it is thought that a breach in the gut epithelium may lead to the release of commensal bacteria and other gut associated antigens, potentially leading to the activation of the adaptive immune response. This is seen by the increase in Abs against commensal bacteria [35] . It can also lead to the activation of autoreactive B cells, which can cause further destruction of the gut epithelium. This approach will help us determine whether the gut epithelium plays a role in influencing the selection of autoreactive B cells to gut associated Ags.

B cells in the gut must maintain tolerance to non-pathogenic Ags present in the gut lumen and respond to pathogenic Ags. Certain bacteria and viruses can generate enterotoxins that can disrupt the gut epithelial barrier. Enterotoxins are small, water-soluble molecules secreted in the gut lumen that are cytotoxic, leading to the formation of pores in cells. In the gut, this leads to the formation of a leaky lumen and causes secretory diarrhea. Enterotoxins, such as those generated by *Cholera* and *E. coli* have been used as strong mucosal adjuvants when orally administered with Ag. The nontoxic B subunit of E. coli heat labile enterotoxin (EtxB) has been shown to induce a weak Th-2 humoral response and break T cell tolerance when orally administered with Ag, making it the ideal enterotoxin for us to use as an adjuvant in our future studies of oral tolerance [92].

The mouse model designed is unique in its ability to be used to study peripheral B cell tolerance in the GALT, oral tolerance and IBD. Further characterization and use of

the model will reveal how B cells are tolerized against self-antigens that are expressed in the gut epithelium and how this tolerance can be broken. It will also reveal how oral administration of Ag affects autoreactive B cells and whether tolerance is maintained or broken and if this can have implications in creating oral vaccines. Furthermore, this will give greater insight on the role of B cells on IBD and whether autoimmunity plays a role in IBD.

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