AN ABSTRACT OF THE THESIS OF

Hyekyoung Hannah You for the degree of Master of Science in Comparative Health Sciences presented on June 21,2019.

Title: Establishing a Humanized Microbiota Mouse Model of CVID-Enteropathy

Abstract approved:

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Natalia Shulzhenko

Common variable immunodeficiency (CVID) is the most common primary immunodeficiency that is characterized by low immunoglobulin levels in the body. CVID is indicated by failure of b cell differentiation and decrease production of immunoglobulins such as IgG and at least one of IgA or IgM. CVID patients with enteropathy (E-CVID) show a decline in already low levels of intestinal IgA that distinguishes from other CVID patients. Previous studies from our lab showed that absence of IgA in the small intestine determine the factor of CVID enteropathy. We also found that microbiota is important for the development of E-CVID. Due to the importance of the gut microbiota in the E-CVID, it is important to establish an animal model which is not only genetically similar to E-CVID patients but also similar in terms of microbiota. We have established a humanized microbiota mouse (HMM) model to investigate phenotypic traits, cellular and molecular features, and characteristics of E-CVID patients in HMM. Results showed similar phenotypic traits, gene expression and intestinal immunity and lymphocyte population in HMM and in E-CVID patients. HMM model has potential application of understanding the mechanism and function of host to microbe interaction.

Establishing a Humanized Microbiota Mouse Model of CVID-Enteropathy

by
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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Hyekyoung Hannah You, Author

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Introduction

Common Variable Immunodeficiency (CVID)

Dysregulated immune responses play a crucial role in several chronic enteropathies. Common variable immunodeficiency (CVID) is the most common primary immunodeficiency and is a genetically heterogeneous disease characterized by low immunoglobulin levels (Leone et al., 2018). The prevalence of CVID varies widely, ranging from 3.8/100,000 in Denmark to 0.6/100,000 in Spain (Selenius et al., 2017). The pathogenesis of CVID has not been delineated clearly, however mutations in several genes associated with B-cell development, including autosomal-recessive mutations in BAFF-R, CD20, CD19, CD81, CD21, and inducible costimulator (ICOS), have been found in a small subset of CVID patients (Agarwal and Mayer, 2013). CVID is characterized by significant reduction of blood immunoglobulins of at least two classes and is associated with several complications such as chronic infections, lymphoproliferation and enteropathy. The latter occurs in about one third of CVID patients, and is presented by malabsorption syndrome and chronic diarrhea, which primarily affects the small intestine (Uzzan et al., 2016). The major clinical hallmark of this condition is fat malabsorption due to villous atrophy and dysfunction of the absorptive epithelium. A common treatment option of CVID is monthly infusions of IgG to restore blood antibody levels, yet this treatment is not effective in treating enteropathy-associated CVID (E-CVID). Currently there is no standard therapy that can consistently improve gut health in these patients and cure the disease.

Immune defects in CVID

Immune cells are involved in metabolism metabolic disorders. Understanding the roles of these immune cells are vital in figuring out the cause of the molecular defect, which triggers an imbalance between immune and metabolic programs within intestinal cells. We have previously shown that this imbalance is correlated to

immunodeficiency-associated malabsorption in both a CVID animal model as well as patients with immunodeficiency (Shulzhenko et al., 2011). Malabsorption is the consequence of a deviated interaction between gut microbiota and epithelial cells that leads to an increased immune response in the intestinal epithelium. A hallmark feature of patients with CVID enteropathy (E-CVID) that distinguishes these patients from other CVID patients is a decline in already low levels of intestinal IgA (Shulzhenko et al., 2018).

The main properties of IgA are preventing toxins and pathogens embodiment in the gut and protecting intestinal epithelium (Benckert et al., 2011; Burns et al., 2018; Gutzeit et al., 2014; Moor et al., 2017). CVID patients have a wide range of clinical manifestations, including autoimmune disease, granulomatous/lymphoid infiltrative disease, and increased incidence of malignancy (Agarwal and Mayer, 2013). All CVID patients have profoundly decreased levels of IgA and IgG, and about 50% of them also lack IgM. They also have poor or absent antibody production to protein and carbohydrate vaccines, such as tetanus and/or diphtheria toxoids (Choi et al., 2014). In CVID patients, circulation of mature B cells occurs, but plasma cell differentiation and antibody production processes are impaired and memory B cells may also be absent (Sava and Deleanu, 2017). Most CVID cases are related to intrinsic B cell defects: impaired isotype switching or somatic hypermutation, defective activation of BCR signaling, impaired DNA repair, abnormal protein tyrosine phosphorylation, or upregulated Fas expression.Homozygous deletions of the *ICOS* gene, which is essential for isotype switching, can result in CVID. For B cell activation, mutations of the *CD19* gene encodes a protein regulating the signal transduction. Although the relationship is not yet clear, susceptibility to CVID has also been associated with heterozygous mutations in the BAFF receptor, TACI. BAFF is a cytokine that is essential for B cell development. Individuals with CVID usually have normal numbers of surface Ig-bearing B-cell precursors, but the cells are unable to differentiate into Ig-secreting cells due to the mutation of the BAFF receptor. This defect is accompanied by variable reductions in the secretion of IgA and IgG in most patients, as well as IgM and IgE in many, resulting in reductions in

serum Ig levels and defective antigen-specific antibody responses. Cell-mediated immunity is usually normal in CVID patients. However, a subset of patients display T-cell abnormalities, which includes impaired T-cell receptor and CD28 signaling, reduced expression of activation molecules, reduced Th2 cytokine secretion, enhanced apoptosis, and deficient inducible co-stimulator molecule expression. These abnormalities lead to a reduction in T-cell activation and proliferation. The role of T cells in regulating B-cell function shows that normalization of reduced Ig levels and impaired antibody responses in CVID patients after depleting theT cells allowed restoration of B-cell function.(Elson III and Smith, 2008).

B cells undergo somatic hypermutation (SHM) and class switch recombination (CSR) in the germinal center of gut-associated lymphoid follicles. Both SHM and CSR require activation-induced cytidine deaminase (AID), which is a B-cell specific enzyme that is highly expressed in the GC. As SHM introduces point mutations in the recombined V(D)J exons that encode the antigen-binding V regions of Igs, structural changes are generated that promote selection of B cells expressing high-affinity Ig variants by antigens. CSR, in contrast, alters the effector function of Igs without changing their antigen specificity by replacing μ and Cδ exons encoding IgM and IgD (two antibody isotypes expressed by naïve B cells) with Cγ, Cα, or Cε exons encoding IgG, IgA, and IgE, respectively (Gutzeit et al., 2014).

Intestinal immunity, Gut Microbiota and CVID

Intestinal B cells go through class switching to IgA and affinity maturation within organized follicular structures associated with the gut-associated lymphoid tissue (GALT). Affinity matured and IgA class-switched B cells, emerging from intestinal follicles, enter the general circulation and then home to the intestinal lamina propria (LP), an effector site that fosters the differentiation of IgA-secreting plasma cells. These plasma cells cooperate with intestinal epithelial cells (IECs) to release SIgA onto the mucosal surface.

Several studies have shown that IgA-secreting plasma cells arise from either newly activated naïve B cells or previously selected memory B cells that become re-

activated by antigen. In general, the intestinal IgA repertoire is comprised of high-frequency clones that recognize highly prevalent and stable components of our microbiota, and low-frequency clones, which may reflect adaptive adjustments to minor changes in the microbiota or exposure to pathogens (Gutzeit et al., 2014; Pabst, 2012). After postnatal gut colonization by bacteria, the gut IgA repertoire becomes progressively more diverse through the introduction of additional mutations in highly expanded B-cell clones and the generation of new mutated B-cell clones. Commensals provide some of the signals required for the induction of mutated plasma cells. Remarkably, commensals can also provide checkpoint signals to remove autoreactivity from systemic B cells. Studies have shown that different probiotic strains are capable of enhancing mucosal IgA in mice. This capacity of probiotics has also been linked to protection against gut pathogens, such as *Acinetobacter baumannii* (Burns et al., 2018).

The immune system has developed strategies to maintain a homeostatic relationship with the resident microbiota. IgA as the most dominant immunoglobulin isotype at the mucosal surface of the intestine, is central in this relationship (Macpherson et al., 2015). While early studies implicated some pathogens, such as *Giardia*, as a potential cause of CVID enteropathy, in the past decade most patients with enteropathy are free of *Giardia* infection and other common pathogens. Recent studies report a role for IgA in shaping large numbers of the intestinal microbiota and exploit strategies to characterize IgA-binding for their inflammatory potential, in addition to control of pathogens (Macpherson et al., 2015). As underlined by several studies, the development of the complex microbial assemblage within a specific ecological niche reaches a, so-called, climax status represented by the establishment of a balanced equilibrium of its microbial components.

Numerous factors are known to cause shifts in the composition of the microbiota, thereby disrupting this microbial homeostasis and causing a dysbiosis state. Dysbiosis is typically associated with having a negative impact on host health with long-term consequences, being associated with various disorders or diseases, such as obesity, diabetes, inflammatory bowel disease (IBD), and metabolic

syndrome. However, there are still substantial knowledge gaps related to the associated mechanisms of action. Around 100 trillion microorganisms live in the GI tract and play an important role in host health and immunity. Commensal bacteria compete for food and nutrients against pathogenic or pathobiont microorganisms and provide metabolic capacity to digest food products by generating important compounds or by assisting other microorganisms with supportive roles (Konjar et al., 2017). The small intestine, part of the gastrointestinal tract, has an essential role in digestion and absorption of nutrients. The intestinal epithelia is a single layer cell on the surface that separates from the host. The luminal layer of the small intestine forms a physical barrier between the host and the environment and offers protection against pathogens to avoid tissue damage. This organization? all requires an effective immune system. If there is an inappropriate immune responses to the antigens, pathologic diseases such as inflammatory bowel disease may occur (Couter and Surana, 2016; Konjar et al., 2017). The GI tract represents the largest immune organ in the body and contains more than 70% of all antibody-secreting cells (Pabst, 2012). The small intestine immune system has lamina propria (LP), intraepithelial layer (IE), and Peyer's patches. Each compartment consists of distinct lymphocytes.

The GI tract is the largest immune organ in the body and contains over 70% of all antibody-secreting cells. The small intestine in particular, is formed into three parts which are the lamina propria(LP), the intraepithelial layer, and Peyer's patches. Each contains a distinct group of lymphocytes. The LP contains ~20% of B cells while the remaining contains ~70% of TCRαβ+ T cells . intraepithelial lymphocytes (IEL) contain very few B cells with more TCRγδ+ T cells than TCRαβ+ T cells. Peyer's patches contain ~80% of B cells. Each group has slightly distinct functions to protect the host from pathogens. (Couter and Surana, 2016). Several studies have shown that microbiota are essential for the development of the intestinal immune system. As different types of diseases occur in different location of the intestine, the immune system of the intestine affects the immune responses in anatomically distant sites. (Couter and Surana, 2016). The combination of intestinal epithelial lymphocytes and lamina propria lymphocytes contain the largest population of T

cells, plasma cells, and macrophages in the body (Mowat and Agace, 2014). IELs secrete, and respond to, various cytokines and express molecules that can directly interact with other lymphocytes.

In some CVID cases there are defects in T cells; abnormalities in the interactions between B and T helper (Th) cells or between Th cells and APCs.

The immune system in the gut plays a role as a surveillance network; it makes sure gut homeostasis is not disturbed and also works on repairing any damaged tissue. The immune system also activates innate immune cells and adaptive immune cells (Arora and Bäckhed, 2016; Fagarasan et al., 2010; Gutzeit et al., 2014; Ventura et al., 2018). The innate system includes intestinal epithelial cells and classical immune cells, which include macrophages, monocytes, and dendritic cells. These cells migrate from the bone marrow to the gut. When there is an infection, the antigen presenting cells and lymphocytes interact in specialized structures (lymphoid follicles and Peyer's patches) and initiate proinflammatory responses. T lymphocytes are able to recognize antigens by T cell receptors attached to their surface (Konjar et al., 2017). When an inflammatory environment is created by the innate response, adaptive immunity responds by stimulating and expanding the B cells and T cells (αβ T cells, and γδ T cells). They then differentiate into cells with variety of functions that can go through immunological challenges. When eliminating pathogens, adaptive cells create memory cells. The memory cells then provide fast and protective immune responses when the same infection occurs. The adaptive responses control and protect from pathogens and form memory cells that can protect the host from re-infection (Pennock et al., 2013).

CVID is driven by T lymphocytes and it has been characterized by increases in pro-inflammatory cytokines such as IFNγ. Our laboratory has previously shown that a B-cell deficient mouse model has similar characteristics to E-CVID patients. When B cells are absent, the gut microbiota can induce interferon-related immune responses in the epithelium (Shulzhenko et al., 2011).

Humanized Microbiota Mice (HMM)

Fecal Microbiota Transplantation (FMT) is administering fecal matter from a donor into a recipient in order to change the composition of the microbiota. FMT has been clinically used to treat *Clostridium difficile* infection (CDI), inflammatory bowel disease (IBD), and several other (intestinal) diseases (Gupta et al., 2016). FMT treatment for recurrent *Clostridium difficile* infection (rCDI) has greater than a 90% success rate (Drekonja et al., 2015; Ubeda et al., 2010). A mouse model also showed that mice colonized with vancomycin-resistant *Enterococcus faecium*(VRE) eliminated infection by pathogenic microorganisms (Ubeda et al., 2013). Mouse models are widely used in gut microbiota research in order to understand the function and role of the gut microbiota in disease. Diseases associated with alterations in gut microbiota includes type 2 diabetes (Baothman et al., 2016; Brunkwall and Orho-Melander, 2017), obesity (Baothman et al., 2016), inflammatory bowel disease (IBD) (Manichanh et al., 2012) , autoimmune diseases, and CVID. The Humanized Microbiota Mouse (HMM) model is used to establish a human fecal microbiota in germ free (GF) mice through fecal microbiota transplantation (FMT). This model exams the phenotypes of GF mice colonized with patients' fecal microbiota to understand gut microbiome, cellular, and molecular interactions. Since several studies show that colonizing mice with human(donor) microbiota recapitulate phenotypic aspects from the donor, it is important to reproduce this aspect in mice to gap the bridge between mice and humans (Arrieta et al., 2016) Le Bastard et al., reported that FMT administration to patients with dysbiosis from antibiotic and chemotherapy may reduce the risk of complications from the disease. They showed that having a diverse and healthy microbial ecosystem protects and maintains the gut barrier from pathogenic microorganisms to translocate into the gut using the HMM model. (Le Bastard et al., 2018) Humanized microbiota mice (HMM) are used to understand the function and mechanism of host=microbe interaction by altering gut microbiota composition of germ free (GF) mice that has a specific genetic background knocked out (KO). This model is important because it allows us to perform experiments that

cannot be done in humans to prove how gut bacteria influence host metabolism (Bäckhed et al., 2007).

AIMS

1. Establish a humanized microbiota mouse (HMM) model of E-CVID
2. Investigate phenotypic traits, cellular and molecular features, and characteristics of E-CVID patients in HMM.

Materials and Methods

Mice

Germ-free B10.A-μMT mice and controls were re-derived and maintained at Taconic farms Gnotobiotic Center. Mice breeding was held at the Laboratory Animal Resources Center (LARC). Pups were weaned 21 days postnatal where they were separated by sex. During the weaning process, 0.2 cm of the ear tissue was collected for genotyping. After separation, mice were paired by their genotype (Het and KO). Mice used in the experiments were of both genders and 1–6 months old. The study was approved by the Oregon State University Institutional Animal Care and Use Committee (IACUC) and experimental procedures were carried out in accordance with protocols approved by IACUC. Mice were housed in pairs (Het and KO) at the Laboratory Animal Resource Center at Oregon State University and maintained under a 12-hour light/12-hour dark cycle. For all colonization studies, mice were maintained with autoclaved supplies, food (5010, Research Diets) and water.

Human microbiota sample

Human microbiota samples were obtained from E-CVID patients. Patient and microbiota information can be found in our previous paper (Shulzhenko et al., 2018).

Human microbiota Colonization

GF mice were colonized with human stool inoculum two times, three days apart. 100 μL of Inoculums were colonized to mice via oral gavage. Pairs were single cages two weeks post colonization. Stools were collected and weights were measured every week to check bacterial quantity and animal health.

Tissue Collection

Mice were harvested immediately after euthanization by cervical dislocation followed by cardiac puncture. Surgical scissors and forceps were used for tissue collection. All tissues were stored at -80°C until analysis.

RNA extraction and cDNA synthesis

10-50 mg of jejunum was homogenized with OMNI Bead Ruptor and 2.8 mm ceramic beads (OMNI International) in RLT buffer followed by Qiashredder and RNeasy kit. Total RNA concentration was quantified using the Qubit RNA BR Assay Kit (Life Technologies). Complementary DNA was prepared using qScript reverse transcription kit (Bio-Rad) under the recommended protocol. cDNA samples were stored at -20°C until used for real time PCR.

Gene expression and Data analysis

10 μL of the RT-qPCR reaction mix, comprised of 20 ng cDNA, 5μL PerfeCTA SYBR mix (QuantaBio), 3.8 μL of water, and 0.2 μL of forward and reverse primers , were plated into each well of the 96-well plate. Five gene specific primers were used, each primer per plate, with primer sequences shown in Table 1.

Samples were amplified on a StepOne Plus Real Time PCR system and software (Applied Biosystems) under the following conditions: 10 minutes at 95°C (hot start), 40 cycles of 15 seconds at 95°C and 1 minute at 58.8°C (fluorescence data collection), and hold at 10°C until samples are removed. RNA Polymerase II Subunit C (Polr2c) was used as housekeeping genes. Statistical analyses were performed with GraphPad Prism Version 7.0. Paired *t*-test was used to compare two groups. A *p*-

value of 0.05 was considered significant unless indicated otherwise are denoted as follows: *0.05>p>0.005, **0.005>p)0.0005, ***0.0005>p>0.00005.

Table 1. Specific primer sequences for RT-qPCR run

Primer name	Sequences
Ubd F SYBR	CCAATGGCGGTTAATGACCTT
Ubd R SYBR	TTTCGATGGGGCTTGAGGATT
Mouse Igha F SYBR	ACTCTAACGCCGTCCAAGAATTG
Mouse Igha R SYBR	CATCTGAACCCAGGAGCAGG
Mouse Cxcl9 F SYBR	TCCTTTTGGGCATCATCTTCC
Mouse Cxcl9 R SYBR	TTTGTAGTGGATCGTGCCTCG
Mouse Zbp1 F SYBR	CAAGTCTCTCCGACTCCTTGC
Mouse Zbp1 R SYBR	ACTTGGTTGAGCTCCCTCTT
Mouse Polr2c F SYBR	CTCACCGAAGAGAACGTCAAG
Mouse Polr2c R SYBR	TCGATGGCTATTATGGGCACC

Isolation of LPL and IEL

IEL and LPL were isolated by using a modification of the method of Shulzhenko and coworkers (Shulzhenko et al., 2011). After removal of Peyer's patches (PP) ~10 cm of the harvested jejunum was opened longitudinally, rinsed twice with ice-cold HBSS and cut into 5 mm pieces into HBSS with 0.2% BSA and 5 mM EDTA. The tissue was incubated at 37 ° C for 30 min under a magnetic stirring (200 rpm), after which the cell suspension was filtered and washed.

Antibodies used in Flow cytometric Staining

Fc receptors were blocked with rat IgG (Jackson ImmunoResearch) and stained with the following antibodies: Viability APC 780, CD45(30-F11), CD4 (RM4-5), CD8 (53-6.7), TCRb (H57-597), TCRgd (ebioGL3), CD11c (n418) , CD11b (M1/70) , CD103 (m290), CX3CR1 (sA011f11). Cells were fixed and permeabilized using the Foxp3 Fixation/Permeabilization buffer (eBioscience). Data were acquired on a Cytoflex flow cytometer (Beckman Coulter). The use of CD45 and viability dye clearly distinguishes live immune cells from non-immune cells and dead cells. Data were compensated and analyzed using FlowJo (Treestar) software. Single staining and fluorescence minus one controls were used for setting gates.

Table 2. Lymphocyte antibodies and clones for cell staining

Myeloid- LPL		Clone	**T cells - IEL**		Clone
Viability	APC 780		Viability	APC 780	
CD 45	PECy7	30-F11	CD 45	BV 605	30-f11
CD11c	BV 510	n418	CD4	BV 510	RM4-5
CD11b	AlexaFluora 700	M1/70	CD8	PE	53-6.7
MHC II	FITC	2G9	TCRb	APC	H57-597
CD103	BV605	m290	TCRgd	PE Cy7	ebioGL3
CX3CR1	450	sA011f11			

Result

Phenotypic trait in HMM Mice

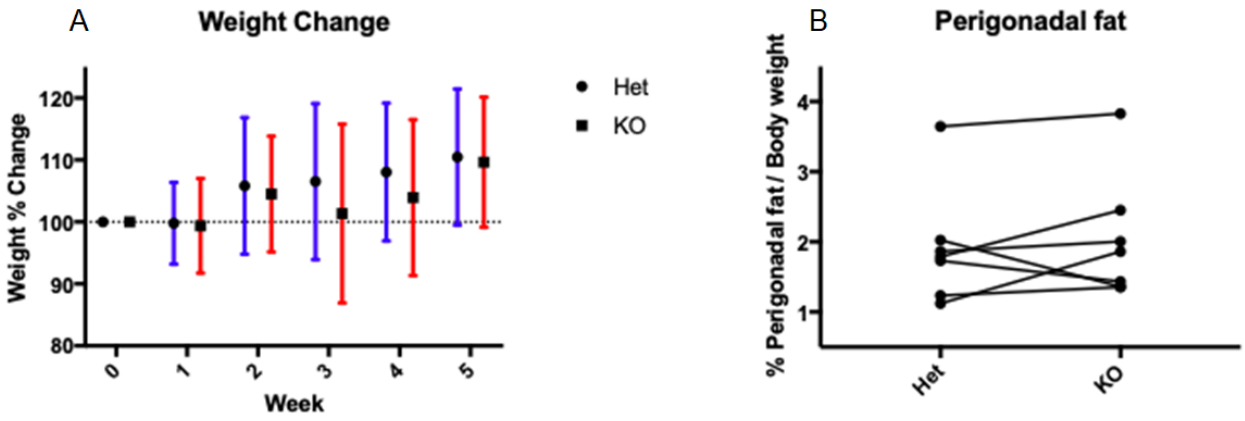

Fig 1. Phenotypic traits in HMM mice. Weight change (A) and proportion of perigonadal fat (gram) in relation to total body weight (gram) in KO (B)

Gene expression in the jejunum of HMM Mice

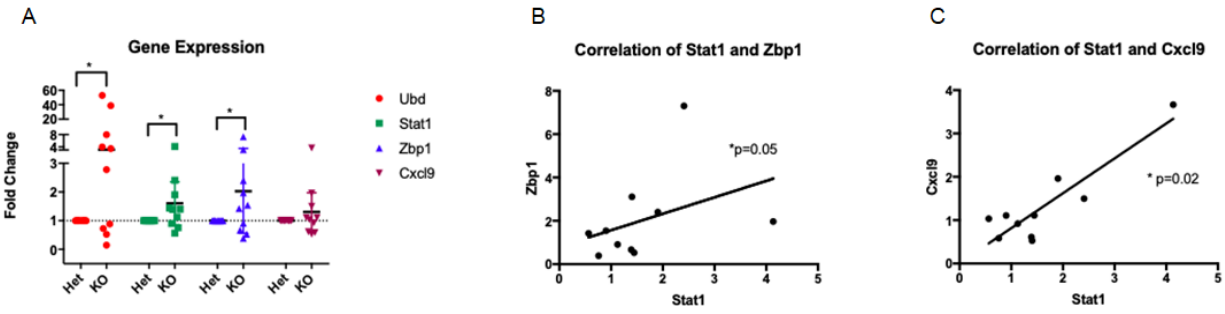

Fig 2. Het and KO Gene expression in jejunum. (A) Expression of the IFN-γ-regulated genes, Ubd, Stat1, Zbp1, and Cxcl9 in the jejunum of Het and KO mice was measured by quantitative real-time PCR (q-RT-PCR). The line represents the median of the fold change values. Fold change was measured in comparison to Het. (B) Correlation of Stat1 and Zbp1 gene expression in KO (C)Correlation of Stat and Cxcl9 gene expression in KO

Previous studies have shown that the intestinal epithelium of B cell deficient mice upregulate IFN-inducible immune response pathways in response to microbes (Shulzhenko et al., 2011). We have used *Ubd, Stat1, Zbp1* and *Cxcl9* genes that are a part of IFN-inducible immune pathways. STAT1 promote IFN-alpha and IFN-beta signaling pathway, which is an important defense mechanism against bacteria and viruses (Yamazaki et al., 2014). Our results show that the gene expression of *Stat1* was higher in the KO jejunum. Z-DNA binding protein 1, otherwise known as Zbp1, is an IFN-inducible protein that harbors nucleic acid binding domains for left handed helix (Z form) (Kuriakose and Kanneganti, 2018). Zbp1 is known to be an essential regulator of cell death and inflammation. Our results show that there was a higher fold change of *Zbp1* in the KO mice compared to that of the Het. Ubd is a ubiquitin-like protein modifier that is also induced by the pro-inflammatory cytokines interleukin (IL)-1β and interferon (IFN)-γ (Brozzi et al., 2016). Our results show that Ubd expression is higher in the KO group. CXCL9 is a chemokine that binds to common receptor, CXCR3 and are known to play a part in T cell mediated immunity. Carter et al., found that CXCL9 was induced when mice were treated with (IFN)-γ. They showed that (IFN)-γ is a mediator of CXCL9 (Carter et al., 2007). Our result shows that *CXCL9* gene expression is higher, but not significant, in the KO mice. Overall, HMM KO mice have more expression in genes *Ubd, Stat1, and Zbp1*. Also, *Stat1*, *Zbp1*, and *Cxcl9* were significantly correlated to each other. This may imply that without B cells, genes are able to upregulate immune pathways that are essential for inducing IFN.

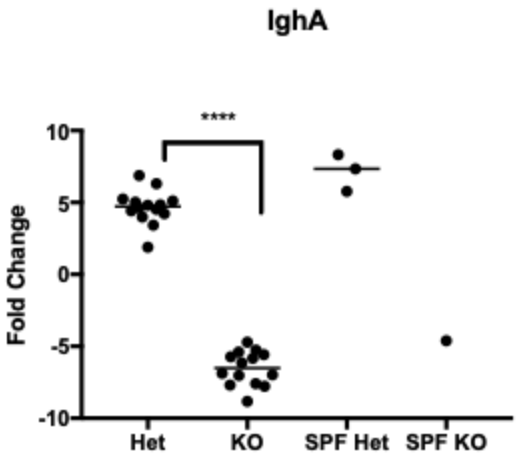

Fig 3. IgA gene expression in humanized microbiota mouse groups (Het and KO) and SPF (Het and KO). Line represents the median of the fold change values.

In order to show that mice colonized with human microbes have the same characteristics of IgA level similar to CVID patients, we looked at gene expression of mice jejunum to see the IgA expression in our experimental groups. As described above, the Het group had significantly higher expression of IgA compared to that of the KO group. When comparing the data between Het and SPF mice (no mutation in B cell development), it shows that the expression levels are similar between the two. Colonizing mice with human microbiota did not change or shift the IgA levels. This finding shows that the HMM depicts both human and mouse characteristics of CVID.

FACS Analysis

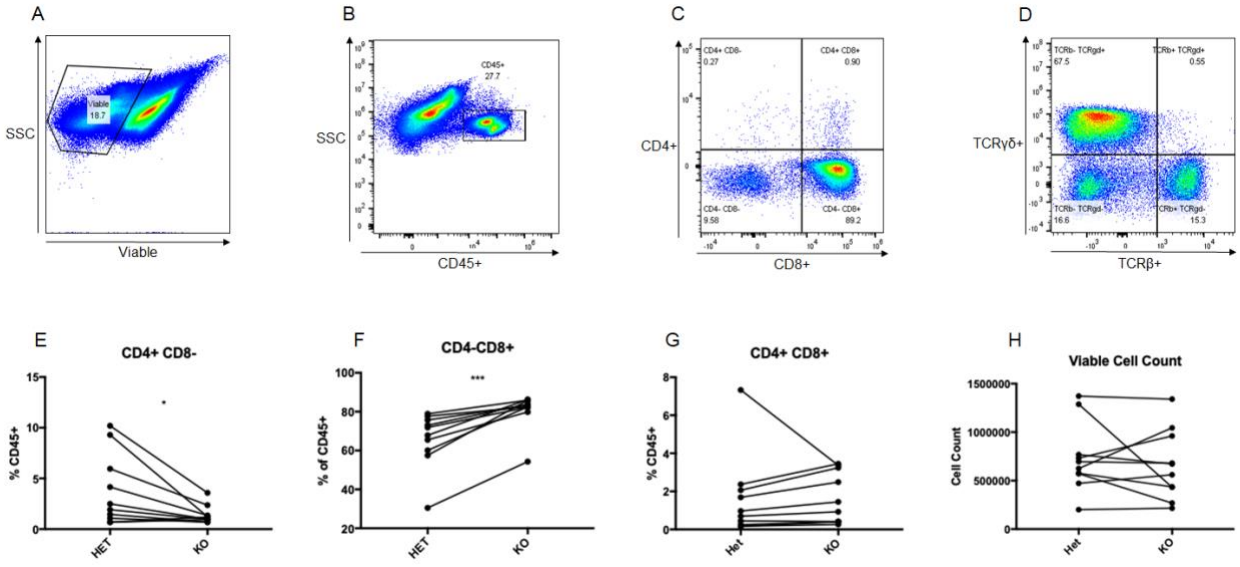

Fig 4. CD4 and CD8 population from % of CD45+ (A)-(D) Representative gating strategy for T cells in IEL small intestine (SI): Selection of viability gate based on SSC-A versus APC 750-A. After selection of viable cells, select CD45+ in a CD45+ vs SSC-A plot. After selection of CD45+, select CD4 and CD8 in a CD4+ vs. CD8+ plot. After selection of CD4 and CD8, select TCR in a TCRβ+ vs. TCRγδ+ plot. (E) CD4+CD8- population from %CD45+ (F) CD4-CD8+ population from %CD45+ (G) CD4+CD8+ population from %CD45+ (H) Absolute number of viable cells in IEL

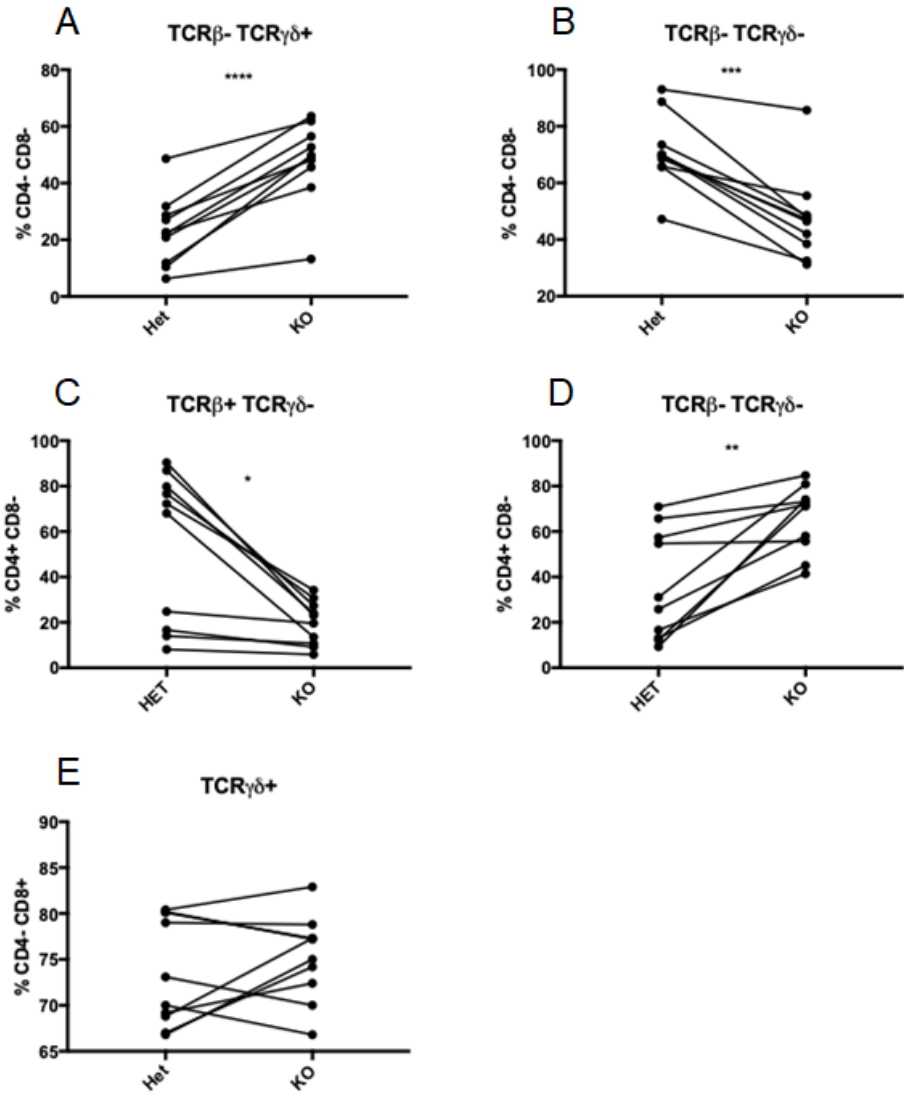

Fig 5. population of TCRβ and TCRγδ (A) TCRγδ+ population of %CD4-CD8- (B) TCRβ- and TCRγδ- population of %CD4-CD8- (C) TCRβ+ and TCRγδ- population from %CD4+CD8- (D)TCRβ- and TCRγδ- population from %CD4+CD8- (E) TCRβ- and TCRγδ+ population from %CD4-CD8+.

Intraepithelial cell population in BcKO Small intestine

Previous studies have shown that there is an increased proportion of CD8+ T lymphocytes in the gut of B-cell KO mice (Bateman et al., 2012; Roberts and Hayday, 1996; Shulzhenko et al., 2011). Similar to this result, ours show that there is

an increased population of CD8+ T lymphocytes in the gut of B-cell KO mice after colonized with human microbes.

Majority of T lymphocytes express a TCR composed of αβ or γδ. IELs consist mostly of gd T cells. (Roberts and Hayday, 1996). This is a unique characteristic of the epithelial immunity. Our data also shows that out of CD8+, 73% and 75% are TCRgd of BcHet and BcKO, respectively. It is similar to several papers showing that TCRgd+ is the majority of T lymphocytes, including CD8+, CD4+, CD4+CD8+ and CD4-CD8- (double negative).

Most of the population was CD4-CD8+, being 65% in Het and 85% in Ko. The next highest population was CD4-CD8- being 29 and 15%, respectively. CD4+CD8- consisted of 4% and 1% of CD45+ and CD4+CD8+ were both 1.5% of CD45+.Robers and Hayday found that in CVID patients, total and naive CD4+ CD45+ T cells were reduced (Roberts and Hayday, 1996). This is similar to our result that the population of CD4+ was lower in KO compared to their pairs (Het).

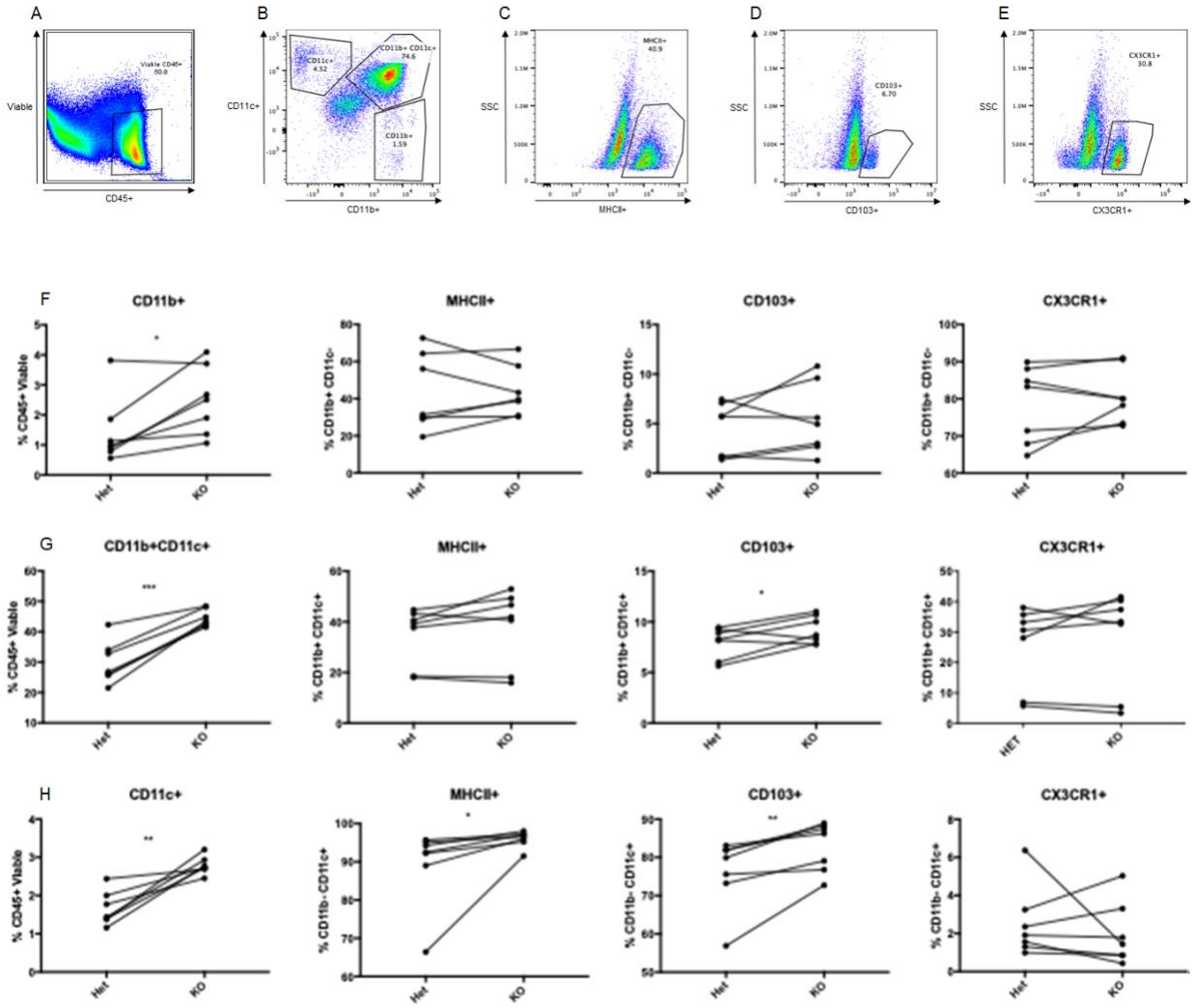

Fig 6. Myeloid cell population in Lamina Propria cells

A-E) Representative gating strategy for myeloid cells in LPL small intestine (SI): Selection of CD45+ viable gate based on CD45+ vs. viable plot (A). After selection of viable CD45+ viable cells, select CD11b+, CD11b+CD11c+, and CD11c+ in a CD11b vs. CD11c+ plot (B). After selection of either CD11b+, CD11b+CD11c+ or CD11c+, select MHCII+ in a MHCII+ vs. SSC-A plot (C); CD103+ in a CD103+ vs. SSC-A plot (D); CX3CR1+ in a CX3CR1+ vs. SSC-A plot (E). F-G) Myeloid population based on gating strategies

Myeloid cell population in BcKO small intestine

The myeloid cell population was measured in lamina propria cells. There was an increased population of CD11b+, CD11b+CD11c+ and CD11c+ in the KO group.

The average CD11b+CD11c+ population was seen in 30% of CD45+ in the Het while 44.5% was seen in the KO group. CX3CR1+ was seen in 26.5% of CD11b+ CD11c+ in the Het group while 30% was in the KO.

Population of MHCII+ was higher in the KO (38%) compared to the Het (35) % of CD11c, though the difference was not significant.

CD11c+ population was seen in 1.6% of CD45+ in Het and while 2.8% was seen in KO.

Population of CD103+ in CD11b- CD11c+ was higher in KO compared to that of Het, being 83% and 76%, respectively. Population of CD103+ in CD11b+ was higher in the KO, though not significant. MHCII+ was mostly seen in CD11b- CD11c+ showing 89.3% in Het and 96% in the KO.

DCs are important in the intestinal mucosa by promoting tolerance and immunity. They are known to sample and process luminal antigens extending transepithelial dendrites into the gut lumen and capture the antigens (Bogunovic et al., 2009; Denning et al., 2007; Gross et al., 2015). CX3CR1+ DCs initiate the host defense to intestinal pathogens and interact with T cells to mediate adaptive immunity and clean pathogens. DCs have the ability to move away from lamina propria to the epithelium, which give them multiple roles in maintaining gut immunity. Though once thought of as DCs, intestinal CX3CR1+ macrophages are residential and stable and are also of monocytic origin. Chemokine receptor CCR2 is expressed in monocytes to sense the inflammation-associated chemokine CCL1 for gut homing. This expression shows that monocyte is recruited by low-grade ongoing inflammation in the gut. Our data shows that there is a higher percentage of CX3CR1+ macrophages in the KO, though not significant. This may imply that without B cells, monocytic macrophages may be populated for gut homing. DCs in the small intestine lamina propria induce homing receptors, CCR9 and a4b7 integrin on T and IgA+ B cells. Blocking B cells, however, will inhibit accumulation of IgA producing plasma cells, which CCL25 or CCL28 are involved (Habtezion et al., 2016). Differentiation of Foxp3-expressing regulatory T cells are induced from these DC subsets and driven by vitamin A metabolite retinoic acid. CX3CR1+ DCs are closely related to the epithelial lining where CX3Cr1+ bind

fractalkine/CX3Cl1, which is expressed by intestinal epithelial and endothelial cells (Siddiqui et al., 2010). DCs and T cells are able to mediate adaptive immunity to clear pathogens. Niess and Adler showed that there are interactions between immunosuppressive and activating DCs and/or macrophages to maintaining intestinal homeostasis (Niess and Adler, 2010).

Discussion

In the gut, communication between the immune system and the gut microbiota is essential for each other. The immune system influences the composition of the microbiota while the gut microbiota directs the immune system and overall play a role in immune tolerance and homeostasis. When the immune function is not balanced, it may result in microbial translocation across the gut barrier, which leads to dysregulation of the systemic immune response. Inflammation in the gut may further induce gut leakiness which results in more translocation of gut microbiota and inflammation.

Presence of IgA in the gut lumen is also important in maintaining the microbiome in the gut. IgA can help fight pathogenic bacteria or pathobionts and keep the gut barrier intact by protecting against infections. CVID patients have low to no IgA and they are prone to more invasion of pathogenic bacteria and more invasive and proinflammatory species to proliferate. Microbial dysbiosis induce overgrowth of pro-inflammatory bacteria or inhibit anti-inflammatory bacteria which in result corrupt immune homeostasis (Berbers et al., 2017).

Jørgensen et al. found that levels of IgA from CVID patients were significantly reduced. They also looked into taxonomic profile between CVID patients and IBD patients and found that there was an increased microbial translocation in CVID patients. This shows that CVID immune dysregulation may change microbiome or the other way that microbiome may lead to dysregulation of the immune system. It is clear that there is a link between microbiome and immune dysregulation. (Jørgensen et al., 2016)

We have previously established a mouse model by creating a B cell deficient (KO) mice (Shulzhenko et al., 2011), by deleting the transmembrane domain of the IgM heavy chain to prevent B cell development of germ free (GF) mice. The study showed that without B cells, IgA secretion declines which is similar to E-CVID. We have also showed that a particular microbe, such as A. *baumannii*, may be responsible for inducing the phenotype of CVID (Shulzhenko et al., 2018). However, all of the previous mouse studies were done in either GF or Specific Pathogen Free (SPF) mice which had either no microbiota or GF mice colonized with mouse microbiota. Due to the importance of the gut microbiota in the E-CVID, it is essential to establish an animal model which is not only genetically similar to E-CVID patients but also similar in terms of microbiota.

Although commensals may contribute to the disease, it depends majorly on the host's genotype. HMM model is crucial for studying gene expression and mechanisms of CVID. The study focuses on systemic gene expression and metabolic pathways involved in the model. Knowing this, it is crucial to further develop this by establishing a humanized microbiota mice model. This will help understand the immune system and disease by deciphering the human microbiota in situations similar patients with CVID.

CD4-CD8+ population of CD45+ cells was significantly higher in HMM KO mice. This is similar to E-CVID patients, which showed infiltration of increased CD8+ in duodenal biopsies (Shulzhenko et al., 2018). Interestingly, there was a significant increase in CD4- CD8- DN (double negative) population in % TCRγδ cells of HMM KO. This shows that there are more development of unconventional T cells (Hayday and Gibbons, 2008)

CX3CR1+ macrophages tend to be higher in the KO group, though not significant. CX3CR1+ in LP cells are non-migratory population of cells and are unlikely to participate in the initiation of adaptive immune responses in intestinal draining lymph nodes (Schulz et al., 2009). CD103+CD11b+ lamina propria dendritic cells are close to the epithelial layer, which may play a part in sampling antigens. CD103 is a ligand for E-cadherin, which is an adhesion molecule expressed

by epithelial cells. This adhesion molecule may promote homing of CD103+CD11b+ LP DCs to the gut epithelia by sampling cell-associated antigens. Without CD103, CD11b+CX3CR1+ lamina propria may also acquire antigens directly through Peyer's Patches-independent M cells (Bogunovic et al., 2009). MHCII+ CD11c+ DCs are mainly located in the IEL and LPs. Bogunovic et al found that CD103+CD11b- DCs are enriched in PP, while they are not seen in the IELs. They also found that CD103+CD11c+DCs in lamina propria and in the intra-epithelial space of apical villi. Most of our CD103+ population were in CD11c+, with more population in the KO. Antigen presenting cells (APCs) are expressed by CD11b and CD11c; CD11c+CD11b- and CD11c+CD11b+ are DCs and CD11b+CD11c- are macrophages.

(Ohtani et al., 2012; Varol et al., 2009). CD11c+ DCs in the intestine have distinct origins where CD103+ DCs arise from common DC progenitor by pre-DC intermediate whereas CX3CR1+ DCS develop from Grl+ monocytes (Siddiqui et al., 2010). Siddiqui et al., showed that CD11c+ cells increased in colitic mice, which may contribute to disease pathogenesis as adoptive transfer of in vitro generated E-cadherin+ DCs into T cell-restored immunodeficient hosts led to an increased Th17 cell response and enhanced colitis. E-cadherin+ DCs express a number of features that may contribute to their pathogenic action in the gut. They express high amounts of a number of TLRs, making them poised to respond to microbial triggers. Upon activation, E-cadherin+ DCs produce large quantities of proinflammatory cytokines, such as IL-6, TNF-α, and IL-23 p19, that have previously been implicated in the pathogenesis of IBD. Persson et al., showed that CD103+CD11b- and CD103+CD11b+ classical DCs have dependent transcription factors. CD103+CD11b- subsets located in the small intestine are dependent on basic leucine zipper transcription factor ATF-like 3 (BAtf3), inhibitor of DNA-binding 2 (ID2), and IFN-regulatory factor 8 (IRF8). This shows that CD103+CD11b- population is linked to IRF8/Batf3-dependent lymphoid tissue resident CD8α+ cDCs. In the gastrointestinal immune system, M cells endocytose protein and peptide antigens in the intestinal epithelium and transport them to the underlying tissue, where they are

taken up by local dendritic cells and macrophages. DCs below the epithelium can also collect luminal antigens (Nagler-Anderson, 2001). Lamina propria lymphocytes that express CX3CR1 may significantly affect macrophage homeostasis and intestinal inflammation (Medina-Contreras et al., 2011). Bain et al., found 61 genes, including Cx3cr1, Csf1r and S100a4, to be higher in CD103-CD11b+ DCs. CD103+CD11b+ DCs are derived from conventional DC progenitors that mature locally after they arrive to the intestine. However, how the DCs are developed and environmental factors that play a part are still unknown. (Farache et al., 2013; Niess and Adler, 2010). CD11b+CD103+ DCS are important for generating and maintaining Th17 cells. Under inflammation, CD11b+CD103+ DCs are translocated into the epithelial layer to capture the antigen and then migrate to the tissue draining lymph node. Intestinal monocyte-derived CX3CR1 macrophages are resident nonmigratory cells that are needed for the maintenance of T regulatory cells. Inflammation recruits Ly6C+ monocytes from the blood circulation that become pro-inflammatory effector cells. Furthermore, Ly6C+ monocytes give under inflammatory conditions rise to migratory antigen presenting cells (Farache et al., 2013)

Farache et al., reported that mice, in a steady state, have a small population of CD103+ DCs that migrate between LP and intraepithelial compartments of the small intestine. They found that the motility of CD103+ DCs was greater than CX3CR1+ macrophages. Once the DCs enter the epithelium, CD103+CD11b+ DCs capture antigens. CX3CR1 macrophages, on the other hand, are able to uptake antigens into the intestinal LP. CD103+ DCs are essential for intestinal homeostasis and produce transforming growth factor (Treg), which then drive to the differentiation of IgA-secreting plasma cells (Farache et al., 2013) Muzaki et al.found that CD103+CD11b- DC subset can induce lymphocytes in colonic lamina propria and in epithelia to secrete IFN-γ that then can trigger a reversible early anti-inflammatory response in intestinal epithelial cells. (Muzaki et al., 2016). This shows that the CD103+CD11b− DC subset is able to repress inflammation via several epithelial interferon-γ (IFN-γ)-induced proteins.

Establishing a humanized microbiota mouse (HMM) model in enteropathy associated CVID is essential for understanding the phenotypic/clinical traits, cellular, and molecular expression in E-CVID patients. HMM showed a lower percentage of weight change in the KO, depicting similar results seen in E-CVID patients. Gene expression results showed that higher expression of IFN regulated genes was found in the KO group. This is similar to the results seen in E-CVID patients since they had an upregulation of immune response and inflammation. In the HMM gut immune population, we were able to see an increase of CD8+ population in the KO. This is similar to the results of E-CVID patients; they had an infiltration of CD8+ T lymphocytes in their duodenum. For future directions, examining how human microbiota from different E-CVID patients affects HMM will be essential to understand different mechanisms and functions of E-CVID development. Scrutinizing changes of microbiota at different time point of colonization, gene expression that is involved in IFN regulation, and gut immunity will allow us to understand host to microbe interaction and furthermore may help examine personalized HMM in E-CVID.

References

Agarwal, S., and Mayer, L. (2013). Diagnosis and Treatment of Gastrointestinal Disorders in Patients With Primary Immunodeficiency. Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc. *11*, 1050–1063.

Arora, T., and Bäckhed, F. (2016). The gut microbiota and metabolic disease: current understanding and future perspectives. J. Intern. Med. *280*, 339–349.

Baothman, O.A., Zamzami, M.A., Taher, I., Abubaker, J., and Abu-Farha, M. (2016). The role of Gut Microbiota in the development of obesity and Diabetes. Lipids Health Dis. *15*, 108.

Bateman, E. a. L., Ayers, L., Sadler, R., Lucas, M., Roberts, C., Woods, A., Packwood, K., Benckert, J., Schmolka, N., Kreschel, C., Zoller, M.J., Sturm, A., Wiedenmann, B., and Wardemann, H. (2011). The majority of intestinal IgA+ and IgG+ plasmablasts in the human gut are antigen-specific. J. Clin. Invest. *121*, 1946–1955.

Brunkwall, L., and Orho-Melander, M. (2017). The gut microbiome as a target for prevention and treatment of hyperglycaemia in type 2 diabetes: from current human evidence to future possibilities. Diabetologia *60*, 943–951.

Burden, J., Harrison, D., Kaenzig, N., et al. (2012). T cell phenotypes in patients with common variable immunodeficiency disorders: associations with clinical phenotypes in comparison with other groups with recurrent infections. Clin. Exp. Immunol. *170*, 202–211.

Burns, P., Oddi, S., Forzani, L., Tabacman, E., Reinheimer, J., and Vinderola, G. (2018). Variability in gut mucosal secretory IgA in mice along a working day. BMC Res. Notes *11*, 98.

Benckert, J., Schmolka, N., Kreschel, C., Zoller, M.J., Sturm, A., Wiedenmann, B., and Wardemann, H. (2011). The majority of intestinal IgA+ and IgG+ plasmablasts in the human gut are antigen-specific. J. Clin. Invest. *121*, 1946–1955.

Brozzi, F., Gerlo, S., Grieco, F.A., Juusola, M., Balhuizen, A., Lievens, S., Gysemans, C., Bugliani, M., Mathieu, C., Marchetti, P., et al. (2016). Ubiquitin D regulates IRE1α/JNK-dependent apoptosis in pancreatic beta cells. J. Biol. Chem. jbc.M115.704619.

Burns, P., Oddi, S., Forzani, L., Tabacman, E., Reinheimer, J., and Vinderola, G. (2018). Variability in gut mucosal secretory IgA in mice along a working day. BMC Res. Notes *11*, 98.

Carter, S.L., Müller, M., Manders, P.M., and Campbell, I.L. (2007). Induction of the genes for Cxcl9 and Cxcl10 is dependent on IFN-gamma but shows differential cellular expression in experimental autoimmune encephalomyelitis and by astrocytes and microglia in vitro. Glia *55*, 1728–1739.

Choi, S.W., Seo, K.W., Oh, J.-S., Lee, J.S., and Cha, H.J. (2014). Successful treatment of enteropathy associated with common variable immunodeficiency. Korean J. Intern. Med. *29*, 256–258.

Couter, C.J., and Surana, N.K. (2016). Isolation and Flow Cytometric Characterization of Murine Small Intestinal Lymphocytes. J. Vis. Exp. JoVE.

Elson III, C.O., and Smith, P.D. (2008). 74 - Immunologic disease of the gastrointestinal tract. In Clinical Immunology (Third Edition), R.R. Rich, T.A. Fleisher, W.T. Shearer, H.W. Schroeder, A.J. Frew, and C.M. Weyand, eds. (Edinburgh: Mosby), pp. 1099–1114.

Fagarasan, S., Kawamoto, S., Kanagawa, O., and Suzuki, K. (2010). Adaptive Immune Regulation in the Gut: T Cell–Dependent and T Cell–Independent IgA Synthesis. Annu. Rev. Immunol. *28*, 243–273.

Gutzeit, C., Magri, G., and Cerutti, A. (2014). Intestinal IgA production and its role in host-microbe interaction. Immunol. Rev. *260*, 76–85.

Hammarström L., Vorechovsky I., and Webster D. (2001). Selective IgA deficiency (SIgAD) and common variable immunodeficiency (CVID). Clin. Exp. Immunol. *120*, 225–231.

Hayday, A., and Gibbons, D. (2008). Brokering the peace: the origin of intestinal T cells. Mucosal Immunol. *1*, 172–174.

Konjar, Š., Ferreira, C., Blankenhaus, B., and Veldhoen, M. (2017). Intestinal Barrier Interactions with Specialized CD8 T Cells. Front. Immunol. *8*.

Kuriakose, T., and Kanneganti, T.-D. (2018). ZBP1: Innate sensor regulating cell death and inflammation. Trends Immunol. *39*, 123–134.

Leone, P., Vacca, A., Dammacco, F., and Racanelli, V. (2018). Common Variable Immunodeficiency and Gastric Malignancies. Int. J. Mol. Sci. *19*, 451.
Macpherson, A.J., Köller, Y., and McCoy, K.D. (2015). The bilateral responsiveness between intestinal microbes and IgA. Trends Immunol. *36*, 460–470.

Pabst, O. (2012). New concepts in the generation and functions of IgA. Nat. Rev. Immunol. *12*, 821–832.

Moor, K., Diard, M., Sellin, M.E., Felmy, B., Wotzka, S.Y., Toska, A., Bakkeren, E., Arnoldini, M., Bansept, F., Co, A.D., et al. (2017). High-avidity IgA protects the intestine by enchaining growing bacteria. Nature *544*, 498–502.

Muzaki, A.R.B.M., Tetlak, P., Sheng, J., Loh, S.C., Setiagani, Y.A., Poidinger, M., Zolezzi, F., Karjalainen, K., and Ruedl, C. (2016). Intestinal $CD103^{+}CD11b^{-}$ dendritic cells restrain colitis via IFN-γ-induced anti-inflammatory response in epithelial cells. Mucosal Immunol. *9*, 336–351.

Pennock, N.D., White, J.T., Cross, E.W., Cheney, E.E., Tamburini, B.A., and Kedl, R.M. (2013). T cell responses: naïve to memory and everything in between. Adv. Physiol. Educ. *37*, 273–283.

Roberts, S.J., and Hayday, A.C. (1996). T-cell aj3+ and yS3 deficient mice display abnormal but distinct phenotypes toward a natural, widespread infection of the intestinal epithelium. Proc Natl Acad Sci USA 6.

Sava, M.O., and Deleanu, D. (2017). Progression of Common Variable Immunodeficiency in Romanian Patients. J. Immunol. Tech. Infect. Dis. *2017*.

Shulzhenko, N., Morgun, A., Hsiao, W., Battle, M., Yao, M., Gavrilova, O., Orandle, M., Mayer, L., Macpherson, A.J., McCoy, K.D., et al. (2011). Crosstalk between B lymphocytes, microbiota and the intestinal epithelium governs immunity versus metabolism in the gut. Nat. Med. *17*, 1585–1593.

Shulzhenko, N., Dong, X., Vyshenska, D., Greer, R.L., Gurung, M., Vasquez-Perez, S., Peremyslova, E., Sosnovtsev, S., Quezado, M., Yao, M., et al. (2018). CVID enteropathy is characterized by exceeding low mucosal IgA levels and interferon-driven inflammation possibly related to the presence of a pathobiont. Clin. Immunol. *197*, 139–153.

Uzzan, M., Ko, H.M., Mehandru, S., and Cunningham-Rundles, C. (2016). Gastrointestinal Disorders Associated with Common Variable Immune Deficiency (CVID) and Chronic Granulomatous Disease (CGD). Curr. Gastroenterol. Rep. *18*, 17.

Ventura, M., O'Toole, P.W., Vos, W.M. de, and Sinderen, D. van (2018). Selected aspects of the human gut microbiota. Cell. Mol. Life Sci. *75*, 81–82.

Yamazaki, Y., Yamada, M., Kawai, T., Morio, T., Onodera, M., Ueki, M., Watanabe, N., Takada, H., Takezaki, S., Chida, N., et al. (2014). Two novel gain-of-function mutations of STAT1 responsible for chronic mucocutaneous candidiasis disease: impaired production of IL-17A and IL-22, and the presence of anti-IL-17F autoantibody. J. Immunol. Baltim. Md 1950 *193*, 4880–4887.