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EPITHELIAL GATA4 REGULATES BACTERIAL COLONIZATION AND REGIONALIZATION OF INTESTINAL IMMUNITY IN HOMEOSTASIS AND DISEASE

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ABSTRACT

The gastrointestinal tract is regionally compartmentalized to coordinate digestion and metabolism of nutrients. This metabolic compartmentalization impacts host regional tissue immunity and bacterial communities. However, the mechanisms underlying intestinal regionalization and its impact on the mucosal immune response in homeostasis and disease are unclear. We hypothesized that intestinal epithelial cells coordinate regional metabolic and immune functions through the transcription factor GATA4. We generated conditional GATA4 deficient mice in intestinal epithelial cells to test this hypothesis. We found that GATA4 controlled the regionalization of lipid metabolism and inflammatory T cell responses in the small intestine. In the absence of GATA4, *Segmented filamentous bacteria* changed its regionalization to colonize the jejunum and drive IFNγ and IL17 T cell responses. We observed that GATA4 controlled levels of luminal IgA through regulating retinoic acid metabolism in intestinal epithelial cells. The increased capacity of the proximal intestine to produce luminal IgA in wild type mice protects the epithelium from bacteria adhesion by *Segmented filamentous bacteria* and limits inflammatory immune responses. Finally, we tested the role of GATA4 deficiency and intestinal regionalization in the host response to pathogen infection or human autoimmune disease. We observed that the GATA4 controlled the regionalization of *Citrobacter rodentium* colonization and survival from the infection. In the context of human disease, we observed a loss of GATA4 in active celiac patients coupled with higher IL17 signaling and decreased retinol metabolism. The presence of Actinobacillus in active celiac patients was associated with lower GATA4 expression, higher IL17 signaling, and increased tissue destruction. These data identify GATA4 as critical regulator of regional metabolism, bacterial colonization, and the host immune response.

1. INTRODUCTION

1.1 Anatomy and physiology of the gastrointestinal tract

The lower digestive tract is composed of the small and large intestine. The small intestine is further divided, from proximal to distal, into the duodenum, jejunum, and ileum. The small intestine is optimized for the digestion and absorption of nutrients. To accomplish this task, the intestine maximizes surface area by forming long circular folds called plicae circulares, and fingerlike projections termed villi. The absorptive capacity of the intestine decreases from the duodenum to the ileum and this is marked by decreased intestinal folds, shorter villi, and decreased expression of specific transporters (Porteous, 1979). Between each villi are intestinal crypts, which house specialized secretory intestinal epithelial cells (IECs) called Paneth cells. The large intestine starts at the cecum and proceeds through the ascending, transverse, descending colon and terminates at the rectum. The large intestine is not involved in the reabsorption of dietary nutrients but instead reabsorbs H_20 and Na^+ ions and takes up key metabolites, such as short chain fatty acids, produced by the intestinal microbiota.

The intestine is divided into 4 layers the mucosa, submucosa, muscularis, and serosa. The mucosa is further composed of the epithelium, the lamina propria, and the muscularis mucosae. The epithelial cells are simple columnar and are both secretory and absorptive. The absorptive epithelial cells, enterocytes or colonocytes, are lined with microvilli termed the brush border which express specific enzymes needed to further breakdown carbohydrates, lipids, and proteins. The epithelial cells of the colon, colonocytes, no longer contain microvilli and lack the digestive brush border enzymes found in the small intestine. IECs contain diverse specialized cells which will be reviewed in more detail in section 1.2. Underlying the basement membrane of the IEC layer is a supportive connective tissue layer termed the lamina propria (LP). The lamina propria

houses stromal cells such as fibroblasts and various immune cell populations. The LP is also highly vascularized and contains a network of capillaries, which are important for the diffusion of nutrients and oxygen, and lacteals, which transport lymph and lipids which are packaged into chylomicrons (Mowat and Agace, 2014). Beneath the lamina propria is the muscularis mucosae which is a thin layer of smooth muscle. The submucosa consists of dense connective tissue and houses a collection of nerve fibers termed the submucosal plexus. The muscularis is made up of two muscle layers, an inner circular layer and an outer longitudinal layer. In between the two muscle layers is another collection of nerves called the myenteric plexus. These nerves are collectively termed the enteric nervous system and cause peristalsis, or contraction of the muscle layers which moves the food along the intestinal tract. The final layer, the serosa, is a connective tissue layer surrounded by simple squamous epithelial cells.

Each region of the intestine performs a unique physiologic function for the host. The duodenum accepts chyme from the stomach and mixes this with bile produced by the liver to emulsify lipids, and pancreatic enzymes which facilitate the breakdown of macromolecules. Also in the duodenum stomach acid is neutralized with bicarbonate to produce an alkaline solution. The content is passed from the duodenum to the jejunum where the majority of dietary nutrients are absorbed. The jejunum expresses specific transporters, *NPC1L1*, *CD36*, *SCARB1* which are involved in the active transport of dietary lipids (Altmann et al., 2004). This region is also important for the uptake of lipid soluble vitamins, such as vitamin A and D. The jejunum uniquely expresses the brush border enzyme lactase which breaks down lactose into galactose and glucose (Nichols et al., 1992). Nutrient absorption continues in the ileum, however the ileum is further specialized for the reabsorption of bile acids and vitamin B12. The ileum uniquely expresses the active transporter *SLC10a2*, which is the primary active transporter of bile acids

from the lumen into the IECs (Wong et al., 1994). Once bile is in the IECs it is sequestered by FABP6 and passed off to the transcription factor FXR. Bile uptake induces the expression of the hormone *FGF15* by FXR to feedback on the liver to determine the amount of bile acids the host needs to produce (Gonzalez, 2012). This process is called the enterohepatic circulation of bile acids and ileal epithelial cells are critical in controlling this process. The majority of dietary nutrient absorption ceases after the ileum. The colon is involved in the reabsorption of H_20, Na^+ ions , and vitamin K from the content. In addition, the colon houses the highest density of microbes for the host. These microbes play a key role in producing key metabolites such as butyrate which is an essential energy source for colonocytes and has immunomodulatory capabilities (Parada Venegas et al., 2019). In addition the bacteria deconjugate primary bile acids to secondary bile acids which prevent the colonization of specific pathogens and modify immune cell intrinsic functions (Wahlström et al., 2016).

1.2 Functional diversity of intestinal epithelial cells

The IECs are composed of numerous specialized cell types which perform unique functions: enterocytes, goblet cells, Paneth cells, Tuft cells, enteroendocrine cells, M cells, and stem cells. The dominant IEC are the simple columnar absorptive enterocytes. The primary function of the enterocytes is digestion and absorption of nutrients. These cells contain specialized microvilli and brush border enzymes which break down disaccharides and dipeptides into monosaccharides, and amino acids which are further absorbed into the cell by active or passive transporters. Along the axis of the intestine, enterocytes express unique metabolic pathways which facilitate regional metabolic differences (Thompson et al., 2018). A key transcription factor GATA4 controls regional metabolic genes between the jejunum and ileum and will be reviewed in more detail in section 1.4. In addition to metabolic functions, enterocytes

are critical for communicating with the intestinal microbes and tissue immune cells. Enterocytes express various pattern recognition receptors which allow the cells to sense conserved microbial patterns, such as toll like receptors, nod like receptors, c-type lectins, and RIG-I receptors (Fukata and Arditi, 2013). These receptors are critical for sensing and responding to the commensal microbes to maintain homeostasis. In addition IECs can coordinate with the adaptive immune system through expression of stress ligands (MICA/B) or MHCII. These molecules are upregulated on IECs following infection or stress and can activate the adaptive immune response by presenting antigens to activate CD4+ T cells in the case of MHCII, or stress ligands which signal to licensed CD8 IELs to drive cytolysis in an NKG2D manner (Koyama et al., 2019; Meresse et al., 2004; Meresse et al., 2006).

Tuft cells, enteroendocrine cells, and M cells constitute more rare epithelial lineages. Tuft cells are chemosensory epithelial cells which produce IL25, in response to succinate, which induces an ILC2 IL13 circuit in the lamina propria (Nadjsombati et al., 2018; Schneider et al., 2018; von Moltke et al., 2016). Enteroendocrine cells, are hormone secreting cells which are able to sense and respond to nutrient uptake and regulate digestion, absorption, insulin secretion, and appetite through the production of GLP1/2, PYY, and CCK (Gribble and Reimann, 2019; Worthington et al., 2018). M cells are specialized epithelial cells found overlaying the epithelial dome of Peyer's patches (PP). These cells allow for the transcytosis of luminal antigens into the subepithelial dome which are picked up by resident dendritic cells and presented to native T cells and B cells to initiate adaptive immune responses in the intestine (Jang et al., 2004; Mabbott et al., 2013; Rescigno et al., 2001). PPs are part of the gastrointestinal-associated lymphoid tissue (GALT) and one of the key inductive sites of adaptive immune response. M cells are thus critical for the formation of these lymphoid structures, the sampling of luminal antigens, and initiation of

these responses (Mabbott et al., 2013). PPs and M cells are only found in the small intestine and are absent from the colon.

1.3 Intestinal epithelial cells as a barrier

The segregation of commensal bacteria with the host is a key function of intestinal epithelial cells. This allows the host to concentrate its efforts towards metabolic functions and limit inflammatory responses. Specialized epithelial cells such as goblet cells and Paneth cells along with intraepithelial lymphocytes each play a role in maintaining this barrier. Interspersed among enterocytes are goblet cells which express *MUC2* and secrete mucin glycoproteins. Mucus is a hydrated gel which is an important component of the epithelial barrier that increases distally from the duodenum to the colon. The goblet cells are more numerous in the colon than the proximal small intestine and produce a thick mucus layer which spatially segregates commensal microbes from the host tissue. Unlike the small intestine, the colon contains two thick mucus layers the inner layer which contains a high density of MUC2, the core mucin glycoprotein, and prevents most microbes from penetrating this layer (Johansson et al., 2011; Johansson et al., 2008). The outer layer is much less dense than the inner. Microbes are able to attach to mucins in the outer layer, and metabolize these components for nutrients (Johansson et al., 2013). The production of mucus is both constitutive and induced by both commensal microbes and pathogens. Goblet cells express pattern recognition receptors which sense microbial components and respond by secreting mucus to maintain the barrier (Arike et al., 2020). Germ free mice, which are devoid of bacteria, express a much smaller less dense mucus layer which is induced following microbial colonization (Johansson et al., 2015). In addition, mice that are deficient in MUC2 develop intestinal inflammation and an ablation of the mucus

layer is observed in inflammatory bowel disease (Johansson et al., 2013; Van der Sluis et al., 2006).

Paneth cells are a specialized secretory epithelial cell found at the base of intestinal crypts. These cells are important in secreting high levels of antimicrobial peptides (AMPs) such as, lysozyme, alpha defensins, and RegIIIγ, which limit bacterial interaction with host tissue (Bevins and Salzman, 2011). Deletion of RegIIIγ in mice leads to a loss of separation of microbiota with host tissue and an increased activation of adaptive immune responses (Vaishnava et al., 2011). In addition, DEFA5 transgenic mice which overexpress a particular alpha defensin is sufficient to prevent the colonization and attachment of particular adherent microbes such as SFB and the induction of Th17 cells (Salzman et al., 2010).

In between intestinal epithelial cells but above the basement membrane reside intraepithelial lymphocytes (IELs). This heterogenous group of T cells are important for preserving the integrity of the epithelial barrier and restricting pathogens. These cells are divided into naturally occurring or induced IELs. The naturally occurring IELs are CD8αα+ or CD8αα- T cells that express either αβ or γδ TCR (Cheroutre et al., 2011). These cells populate the tissue early in life or in the absence of microbes (McDonald et al., 2018). The induced or adaptive IELs are CD4+ CD8αβ+ and contain the αβ TCR (Cheroutre et al., 2011). These cells are recruited into the tissue in response to infection or damage. IELs are non-circulating tissue resident effector cells that express cytolytic granules such as granzyme and perforin which lyse infected or damaged target cells (Hayday et al., 2001). They can also express NK receptors such as activating NKG2D or inhibitory NKG2A which recognize non classical MHCI molecules (Hüe et al., 2004; Meresse et al., 2004). Although these cells are critical for maintaining the barrier

uncontrolled activation of these cells can lead to tissue destruction as observed in celiac and Inflammatory bowel disease (Cheroutre, 2006; Jabri and Sollid, 2009).

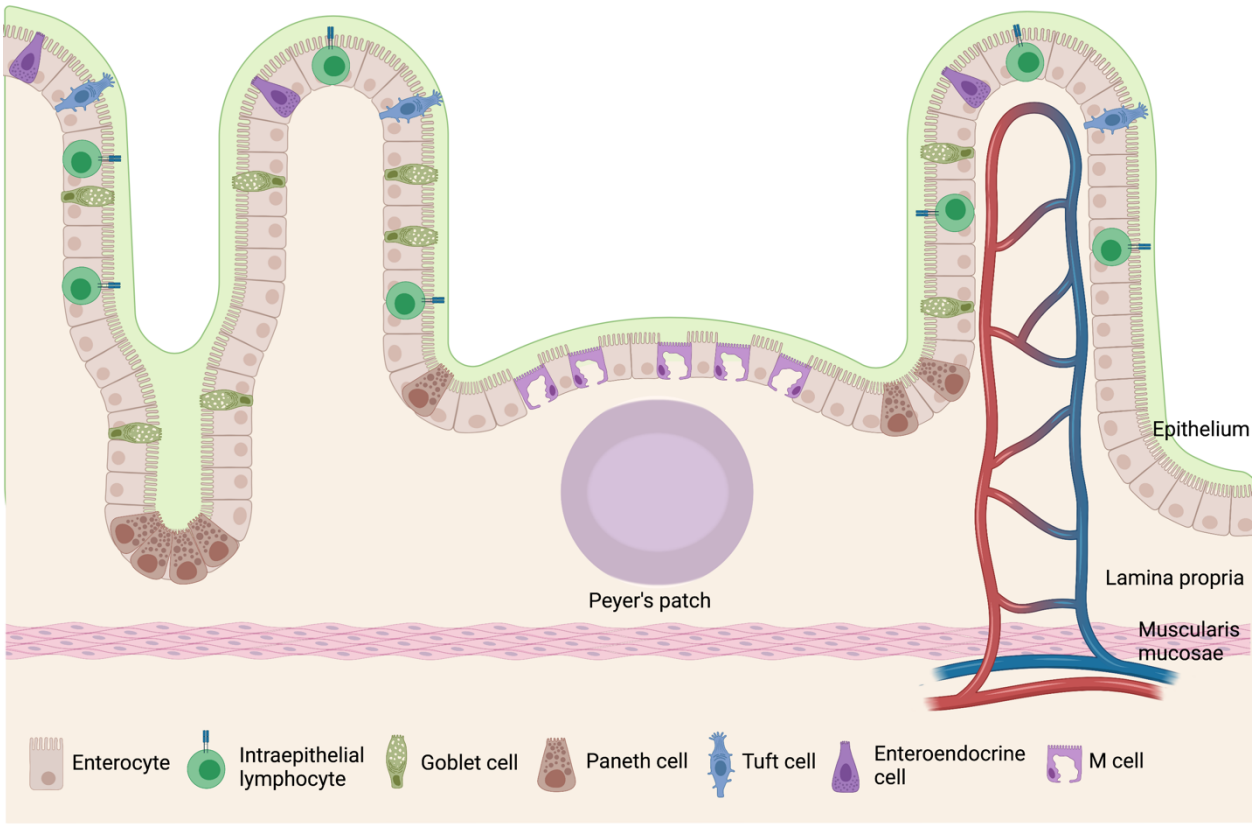

Figure 1. Intestinal epithelial cells

The mucosa of the intestine is divided into the epithelium, the lamina propria, and the muscularis mucosae. The epithelium is made up of numerous cell types. Enterocytes are the most numerous and the key cell type involved in digestion and absorption of nutrients. Goblet cells secrete mucus which acts as a physical barrier. Paneth cells are found at the base of cypts and secrete antimicrobial peptides which prevents bacteria from coming in close proximity with the host. Tuft cells are chemosensory cells and play a role in initiation of type II immune responses. Enteroendocrine cells secrete a variety of hormones which regulate secretion of digestive enzymes from the pancreas. M cells transcytose antigens from the lumen into the tissue where they are picked up by resident DCs in the PPs to initiate immune responses. Intraepithelial lymphocytes sit in between IECs and play an important role protecting the barrier from pathogen invasion.

1.4 Regionalization of intestinal epithelial cell function by the transcription factor GATA4

Absorptive enterocytes are the most numerous IEC, critical for the digestion and absorption of nutrients. The gene expression profile of these cells change along the axis of the intestine to confer region specific metabolic functions for the host. Expression of region specific transcription factors (TF) induce unique gene signatures that define the spatial patterning of enterocytes. GATA4 is a zinc finger TFs which binds to the consensus nucleotide sequence (A/T)GATA(A/G) and is essential for regulating gene expression in proximal IECs (Molkentin, 2000). This TF is highly expressed in IECs of the duodenum and jejunum and is absent in the ileum and colon. It is expressed in enterocytes, Ki67+ stem cells, and Paneth cells and is absent in goblet cells or enteroendocrine cells (Bosse et al., 2006; Dusing and Wiginton, 2005). The role of GATA4 in the intestine was assessed using GATA4fl/fl villin-cre+ ($GATA4^{\Delta IEC}$) mice to conditionally delete GATA4 in IECs because global deletion of GATA4 was embryonically lethal. GATA4 is also expressed in cardiomyocytes and required for the development of the heart (Rivera-Feliciano et al., 2006). In the intestine, GATA4 controls the specification of IECs to become jejunal and inhibits the development of the ileum. It is both necessary and sufficient for regulating gene expression between the jejunum and ileum (Battle et al., 2008; Thompson et al., 2017). $GATA4^{\Delta IEC}$ mice lose expression of 53% of jejunal genes and gain expression of 47% of ileal genes in the jejunum (Battle et al., 2008). This loss of jejunal identity led to decreased expression of genes involved in lipid uptake and metabolism such as: *NPC1L1* (cholesterol transporter), *CD36* (fatty acid scavenger receptor), *SCARB1* (lipoprotein receptor), *FABP1*(cytoplasmic fatty acid binding protein), *SLC27a2* (fatty acid transporter) (Battle et al., 2008). The increased ileal genes in the jejunum of GATA4 deficient mice are involved in the enterohepatic circulation of bile acids such as *SLC10a2* (bile acid transporter), *FABP6* (cytosolic

bile acid binding protein), *NR1h4* (nuclear hormone transcription factor- FXR), *OSTα/β* (basolateral bile acid transporters) (Battle et al., 2008). Overexpressing GATA4 in the ileum with GATA4stopfloxed villin-cre mice led to a reduction of the ileal specific bile acid genes, just described, and an increase of jejunal transcripts *LCT*, *FABP1*, *IL33*, *CD36*. Therefore, GATA4 activates jejunal genes and represses ileal genes in the jejunum. CHIPseq of GATA4 in the jejunum revealed that GATA4 is equally bound to activated and repressed genes (Aronson et al., 2014). It represses the ileal program by inhibiting the acetylation of H3K27 and through coordinating with FOG cofactors (Aronson et al., 2014; Beuling et al., 2008).

The impact of GATA4 deficiency on the host is a decrease in dietary fat and cholesterol absorption and a decrease of plasma cholesterol and phospholipids (Battle et al., 2008). In addition, the upregulation of *SLC10a2* in the proximal intestine leads to an increase transport of bile acids out of the lumen and into the tissue. There is a decrease of the total bile acid pool in the lumen of the jejunum and ileum and an increased ratio of tauro-β-muricholic acid to taurocholic acid (Beuling et al., 2010). There is also a shift in the architecture of the epithelium. The villi of GATA4$^{\Delta IEC}$ mice are both shorter and wider (Battle et al., 2008). In addition, there is an increase of goblet cells by Alcian blue staining and an increase in MUC2 expression (Battle et al., 2008; Bosse et al., 2006). There was no difference in the number of Paneth cells or enteroendocrine cells, however there was a change in regional enteroendocrine function (Bosse et al., 2006). Enteroendocrine cells lose expression of proximal hormones CCK and increase expression of distal hormones such as PYY in the jejunum (Bosse et al., 2006). Therefore, GATA4 is a critical transcription factor in controlling regional epithelial functions and defining the identity between the proximal and distal small intestine.

1.5 The intestinal microbiota shape local tissue immune responses

The gastrointestinal tract is colonized with trillions of microbes collectively termed the microbiota, which is a diverse group of bacteria, fungi, archaea, and viruses. The microbiota provides the host with numerous benefits such as regulation of immune responses, metabolism, and colonization resistance to pathogens. In return, the host provides the microbes a steady source of nutrients and immune protection from competing pathogens. Along the intestine, the bacteria range in abundance from 10^1-10^3 CFU/ml in the duodenum up to 10^{10}-10^{12} CFU/ml in the colon (Martinez-Guryn et al., 2019). The bacterial communities in the small intestine are distinct from those in the colon, and this is largely due to environmental differences. Compared to the colon, the small intestine has a lower pH, higher O_2 content, a faster transit time, higher levels of sIgA and AMPs. Distinct bacterial communities are found in both regions. For example, Lactobacillus and Enterobacteriaceae are primarily found in the small intestine while Bacteroides, Prevotella, Lachnospiraceae, and Ruminococcus are preferentially in the colon (Donaldson et al., 2016). Diet and genetics are two important factors which shape the microbiome composition. Animal based diets increase the abundance of *Bacteroides* at the expense of *Roseburia*, *Eubacterium rectale*, and *Ruminococcus bromii* which metabolize plant polysaccharides (David et al., 2014). Variants in genes such as *LCT*, *NOD2*, and *FUT2* for example can alter the microbiome composition and impact host physiology (Hall et al., 2017). Changes in microbiome composition leading to decreased diversity and expansion of pathobionts is called dysbiosis and is associated with numerous diseases (Levy et al., 2017).

An important contribution of the microbiota to the host is the development and maintenance of the immune response. Comparing specific pathogen free (SPF) mice to germ free (GF) mice, which are devoid of bacteria, revealed that the microbiota is key to driving the

development of the GALT and adaptive immune response. GF mice have a decreased number and size of PPs, and isolated lymphoid follicles (ILFs), in the intestine tissue. GF mice also have smaller mesenteric lymph nodes (MLNs), the lymph nodes which drain the intestine (Round and Mazmanian, 2009). In the intestine, GF mice have reduced IEL CD4 and CD8 T cells, lamina propria T cells, IgA+ plasma cells, and germinal center B cells (Round and Mazmanian, 2009). The colonization of particular commensal microbes shape the differentiation of these subsets and contribute to regionalization of mucosal immune responses in the intestine. For example, *Segmented filamentous bacteria* which specifically colonizes the distal ileum induces a Th17 response in this region (Farkas et al., 2015; Ivanov et al., 2009). SFB does not colonize or adhere to the colon which is relatively devoid of Th17 cells. *Lactobacillus reuteri* which colonizes the small intestine generates indole derivatives from tryptophan metabolism which activates the aryl-hydrocarbon receptor and drives a downregulation of Thpok in CD4 T cells in the ileum. This decrease of Thpok leads to the reprograming of CD4 IELs to upregulate CD8α and become double positive IELs (Cervantes-Barragan et al., 2017). *Bacteroides fragilis* colonizes the colonic crypts and produces polysaccharide A where it is sensed by DCs and polarizes CD4 T cells into FOXP3+ regulatory T cells (Tregs). These FOXP3 Tregs secrete IL10 and protect mice from inflammatory disease and experimental colitis (Lee et al., 2013; Mazmanian et al., 2005; Round et al., 2011; Round and Mazmanian, 2010). *Helicobacter hepaticus* colonizes the cecum and colon where it induces the differentiation of FOXP3+ RORγt+ cells and suppresses proinflammatory Th17 cells in this region. In genetically susceptible IL10 deficient mice, the *H. hepaticus* specific T cells instead differentiate to colitogenic Th17 cells and drive spontaneous colitis (Xu et al., 2018). Clostridia from cluster IV and XIVa metabolize polysaccharides and ferment short chain fatty acids such as butyrate which induces the differentiation of FOXP3+

Tregs in the colon. (Atarashi et al., 2013; Atarashi et al., 2011; Furusawa et al., 2013; Smith et al., 2013). Butyrate acts as an HDAC inhibitor and leads to the increase acetylation of the CNS1 enhancer of the FOXP3 locus which drives the differentiation of Tregs (Arpaia et al., 2013). In all these examples, the colonization and niche of the bacteria contribute to shaping local immune responses throughout the intestine. Understanding the mechanisms of bacterial colonization will elucidate how certain commensal microbes interact with the host to regulate these immune responses. *Segmented filamentous bacteria* is a model mucosal-associated commensal bacteria which is region-specific and highly immunogenic. Its genome, colonization, and induction of host immune responses will be reviewed in further detail to illustrate detailed mechanisms of region specific host-microbe interactions and its role in homeostasis and disease.

1.6 *Segmented filamentous bacteria* genome and ileal colonization

In the small intestine, *Segmented filamentous bacteria* (SFB) preferentially attach to IECs and PPs of the terminal ileum (Klaasen et al., 1992). The mechanisms of how SFB preferentially colonize the ileum and evade the host immune response to thrive in this unique ecological niche is not yet known. At the moment, SFB is not widely amenable to bacterial culture or genetic manipulation (Schnupf et al., 2015). This has hindered discovering mechanisms of SFB colonization and host interaction. Instead studying this bacteria has relied on metagenomic sequencing and monocolonization of GF mice (Klaasen et al., 1991; Kuwahara et al., 2011). SFB have been identified in mice, rats, chickens, turkeys, pigs, and potentially humans (Snel et al., 1995). These bacteria are anaerobic spore forming filamentous bacteria that are related to Clostridia but are phylogenetically distinct. Their 1.57 Mb genome is much smaller than Clostridial genomes and shows similarities to other host depend bacteria such as Mycoplasma and Borrelia (Sczesnak et al., 2011). SFB's genome reveals extensive auxotrophy and lacks the

metabolic pathways required the biosynthesis of amino acids, cofactors, and nucleotides (Prakash et al., 2011; Sczesnak et al., 2011). Instead SFB is enriched in various amino acid and carbohydrate transporters and proteases suggesting it acquires these nutrients from the environment. SFB contains complete glycolysis and pentose phosphate pathways. It is lacking genes of the electron transport chain and likely produces ATP by substrate level phosphorylation (Pamp et al., 2012). This symbiotic relationship between SFB and the host likely explains why this bacteria evolved to live in close proximity to IECs. SFB's genome encodes flagellin proteins, fibronectin-binding proteins, and numerous secreted adhesins which may facilitate attachment to the extracellular matrix of host IECs (Sczesnak et al., 2011). Flagellin is a ligand for TLR5 which may be a mechanism of host sensing of SFB colonization and induction of immune responses (Kuwahara et al., 2011). However, whether SFB binds to a host receptor to facilitate its ileal colonization is unknown.

SFB colonization peaks in mice at weening around day 28 and then quickly declines around day 42 where it remains stable in the adult mouse. Early colonization of SFB depends on the immune status of the dam which was determined by performing littermate crosses with dams that are scid/scid or scid/+ (Jiang et al., 2001). Pups that born from dams that are scid deficient, which are lacking T, B, and NK cells, reveal earlier colonization of SFB regardless of their genotype. This suggests that early colonization of SFB in the pups is independent of the host immune system but dependent on the maternal immune response. However, the decline of SFB from weaning to adult was dependent on the host immune status. Pups which were scid deficient were unable to control the levels of SFB which remained high. These data suggest that the immune response of the mother and host control SFB colonization dynamics. IgA is one mechanism that could explain these data. IgA is transferred from the breast milk of the mother to

the pups before weaning. High levels of SFB specific IgA were detected in the stomach contents of pups 12 days after birth (Jiang et al., 2001). After weaning, the mice switch from breast milk to solid food and luminal IgA levels decline until the host develops an adaptive immune response. This increase of IgA and CD4 T cells in the host correlate with the sharp decline of SFB from days 28 to 42. Expansion of SFB in adult mice has been seen in various immunodeficient hosts: RAG-/-, AID-/-, Myd88-/-, IgA-/-, and IL17Ra-/- (Donaldson et al., 2018; Kumar et al., 2016; Mao et al., 2018; Suzuki et al., 2004; Wang et al., 2015). The host genetics also play a role in controlling SFB colonization and attachment. SFB is able to colonize C57BL6 (B6) mice higher than BALB/C mice. BALB.B mice, which contain the H-2^b haplotype from B6 mice, exhibit higher levels of SFB colonization similar to the levels of B6 mice (Khan et al., 2019). The lower levels of SFB in BALB/C mice were not due to the adaptive immune response as BALB/C RAG-/- mice did not change SFB colonization (Khan et al., 2019). These data are intriguing because SFB induces antigen specific adaptive immune responses such as Th17 cells, which recognize the 3340 epitope, and the fact SFB is highly IgA coated in a T cell-dependent manner (Bunker et al., 2015; Yang et al., 2014). These data suggest a complex model of SFB colonization whereby the adaptive immune response both limits and supports the colonization of SFB. Future experiments will be needed to understand the mechanism of how the H-2b haplotype supports SFB colonization and why the H-2d haplotype is inhibitory. It is important to note that SFB is not cleared, but rather controlled by the immune system. The presence of this bacteria provides protection to the host against enteric pathogens, but can be harmful in specific genetic contexts (Ivanov et al., 2009; Lee et al., 2011). Therefore regulating the colonization of this bacteria is critical for host immune homeostasis.

1.7 *Segmented filamentous bacteria* induction of host immune responses

SFB colonization induces a broad mucosal immune response of the host triggering changes of the IECs, IELs, LP lymphocytes, and inducing immune activation and germinal centers (GCs) within the PPs, MLNs, and ILFs of the tissue. To colonize the host, SFB forms holdfast segments which is a hook like appendage that enables its attachment to IECs (Jepson et al., 1993). Following attachment there is a rearrangement of the actin cytoskeleton of the IEC at the point of attachment (Jepson et al., 1993). SFB does not puncture the plasma membrane of the epithelial cell but rather causes an invagination of the plasma membrane (Ladinsky et al., 2019). SFB forms endocytic vesicles which bud off the bacteria and are transferred into the host endolysosomal network. These vesicles contain SFB antigens such as the immunodominant Th17 epitope 3340 which is from a cell surface protein of unknown function. This process of endocytosis is clathrin-independent and dynamin-dependent. It is regulated by the host actin cytoskeleton in a CDC42 dependent mechanism (Ladinsky et al., 2019). Mice that are CDC42 deficient in IECs disrupt SFB antigen uptake and have a loss of intestinal Th17 cells (Ladinsky et al., 2019).

In addition to transferring antigens to IECs, SFB colonization induces many other physiologic changes in IECs. SFB induces the upregulation of MHCII expression in IECs (Umesaki et al., 1995). Deleting MHCII in IECs with H2-Ab1flox villin cre mice, led to equivalent levels of Th17 cell induction in the LP indicating the MHCII expression in IECs was not required for this process (Goto et al., 2014b). SFB also induces an upregulation of fucosylation and *Fut2* expression in IECs which is important in maintaining homeostasis of the microbiota and tolerance to *Citrobacter rodentium* (Goto et al., 2014a; Pickard et al., 2014; Umesaki et al., 1995). The adherence of SFB with IECs is required for the induction of Th17 and

IgA+ plasma cells (PC) in the lamina propria (Atarashi et al., 2015). Genetically similar SFB strain found in rats, R-SFB, is unable to adhere to IECs in mice or induce a Th17 response. The adherence of SFB induces IL22 expression by ILC3s in the tissue which feedbacks on the IECs to upregulate STAT3 and serum amyloid 1/2 (SAA) (Sano et al., 2015). SAA induces production of IL6 and IL23 on dendritic cells (DCs) in vitro to polarize naive CD4 T cells into the Th17 effector program (Ivanov et al., 2009). SAA1/2 double knockout mice have a decreased frequency of IL17+ CD4 T cells with no change in the frequency of RORγt+ CD4 T cells in the LP (Sano et al., 2015). This suggests that serum amyloid A is also important for controlling the effector function of Th17 cells in the tissue. In addition, it controls the pathogenicity of the Th17 cells. Classically, IL6 + TGFβ activates RORγt and induces the expression of the IL17 locus. However, SAA can substitute for TGFβ and induce Th17 differentiation and effector function in vitro (Lee et al., 2020). The SAA induced Th17 cells express a more pathogenic signature marked by coexpression of *Tbx21* (Tbet), *IL23r*, and *Gzmb* (Lee et al., 2020). Whereas TGFβ induced Th17 cells expressed a non-pathogenic signature marked by *Ahr*, and Il10 (Lee et al., 2020). The regulation of SAA expression plays a role in disease. There is a potential role of these cytokines in inflammatory diseases, SAA1/2 is upregulated in the ileum of IBD patients, and SAA1/2/3 triple knockout mice are protected from the IL10r neutralization model of colitis and EAE (Lee et al., 2020).

SFB monocolonization in GF mice is sufficient to induce CD8αβ IEL T cell recruitment and cytolytic activity in the ileum (Umesaki et al., 1995; Umesaki et al., 1999). It also drives an increase of IgA+ PC in the lamina propria (Umesaki et al., 1995). Furthermore, SFB leads to an increase of IFNγ, TNFα, IL17, and IL10 gene expression in the ileum (Gaboriau-Routhiau et al., 2009). The CD4 IL17 response is particularly interesting because unlike the other SFB induced

cytokines, IL17 production is nearly absent in GF mice. In fact, mice from different SPF facilities have varying amount of Th17 cells. It was noted that mice from Taconic had Th17 cells whereas those from Jackson labs did not have many Th17 cells (Ivanov et al., 2008). Cohousing Taconic with Jackson (JAX) mice and antibiotic treatments suggested that the Th17 cells in Taconic were driven by a specific microbe that was absent in JAX mice (Ivanov et al., 2008). 16s sequencing identified a candidate microbe, SFB, which was sufficient to restore Th17 cells in GF mice to the level observed in JAX mice (Ivanov et al., 2009). These Th17 cells were shown to be specific to SFB antigens in vitro and in vivo. To identify the specific SFB antigen and corresponding TCR, a whole genome shotgun cloning and expression screen was performed (Yang et al., 2014). The SFB genome was cloned and expressed in a *E.coli* library and used to activate NFAT-GFP hybridoma cell lines expressing TCRs from Th17 cells. The bacterial clones which stimulated these cells lines mapped to two genes of SFB named, SFBNYU_3340 and 4990, both predicted to be unique to SFB, extracellular proteins, and have an unknown function (Yang et al., 2014).

As described above, the 3340 antigen of SFB is endocytosed in IECs, it is then presented on MHCII to naïve CD4 T cells, which are subsequently polarized to the Th17 effector program. In the intestine, DCs, macrophages, B cells, IECs, and ILC3s all express MHCII and are able to present antigens. To identify the antigen-presenting cell required in promoting intestinal Th17 cells, MHCII floxed mice were crossed with different cre drivers to ablate MHCII on these cell types. H2-Ab1floxed CD11c-cre mice revealed a defect in Th17 differentiation following SFB colonization indicating that DCs or intestinal macrophages were the key cell type in presenting SFB antigens (Goto et al., 2014b). In the intestine CD11c is expressed on numerous dendritic cell or macrophage subtypes which both express CD11c and MHCII. Intestinal macrophages are

CD64+ or F4/80+ whereas DCs are negative for both markers. Furthermore, DCs can be further segregated into migratory MHCII^{hi} CD103+, CD11b+ or CD11b-, and resident MHCII^{int}, CD8α+ CD11b+, (Esterházy et al., 2016). To distinguish which subtype were important in Th17 differentiation, each cell type was systemically depleted. Depleting CD103+ CD11b+ DCs with Langerin-DTA mice did not alter the frequency of Th17 cells. CD8α DCs are able to cross present antigens and require the TF BATF3 for their development. BATF3-/- mice are deficient in CD8α DCs and CD103+ DCs (Edelson et al., 2010). Double deficient BATF3-/- and Langerin-DTA mice lacked both CD103+ CD11b- and CD103+ CD11b+ DCs without disturbing the CD103-CD11b+ cells. These mice still generated equivalent levels of Th17 cells indicating that CD103+ DCs are dispensable for Th17 differentiation (Panea et al., 2015). Transient depletion of monocytes with CCR2-DTR mice and CSF1R neutralization both ablated the CD64+ intestinal macrophages and Th17 differentiation. Furthermore transfer of exogenous monocytes, Ly6C+ CCR2+ BM cells, to CCR2-DTR mice recovered the Th17 cell defect (Panea et al., 2015). These data collectively showed that intestinal CD64+ macrophages are the key antigen presenting cell which drives Th17 differentiation to SFB. How these cells acquire the 3340 antigen from IECs and where they prime Th17 cells is still an unanswered question.

T cell priming in the intestine is able to occur in the MLNs, PP, or isolated lymphoid follicles. SFB colonization induces an expansion of GC B cells, an increased number of CD4+ T cells, and an increase of IgA+ class-switched B cells in all these inductive sites (Lecuyer et al., 2014; Sano et al., 2015). Transferred SFB specific CD4+ TCR transgenic cells, are first primed in the MLNs and then subsequently in the PPs (Sano et al., 2015). B cell deficient mice, which lack PPs, do not have a defect in the number of Th17 cells in the LP (Goto et al., 2014b). These data suggest that PPs are dispensable for Th17 priming. However, lymphotoxin-α (Ltα) deficient

mice which lack secondary lymphoid organs, MLNs, PPs, and ILFs, still generate equivalent Th17 cells following SFB colonization indicating that organized GALT is dispensable for Th17 differentiation (Goto et al., 2014b; Lécuyer et al., 2014). These data support a model where SFB likely primes Th17 responses in the MLNs, PPs, and locally in the intestinal LP and that in the absence of secondary lymphoid tissue, local T cell priming can compensate.

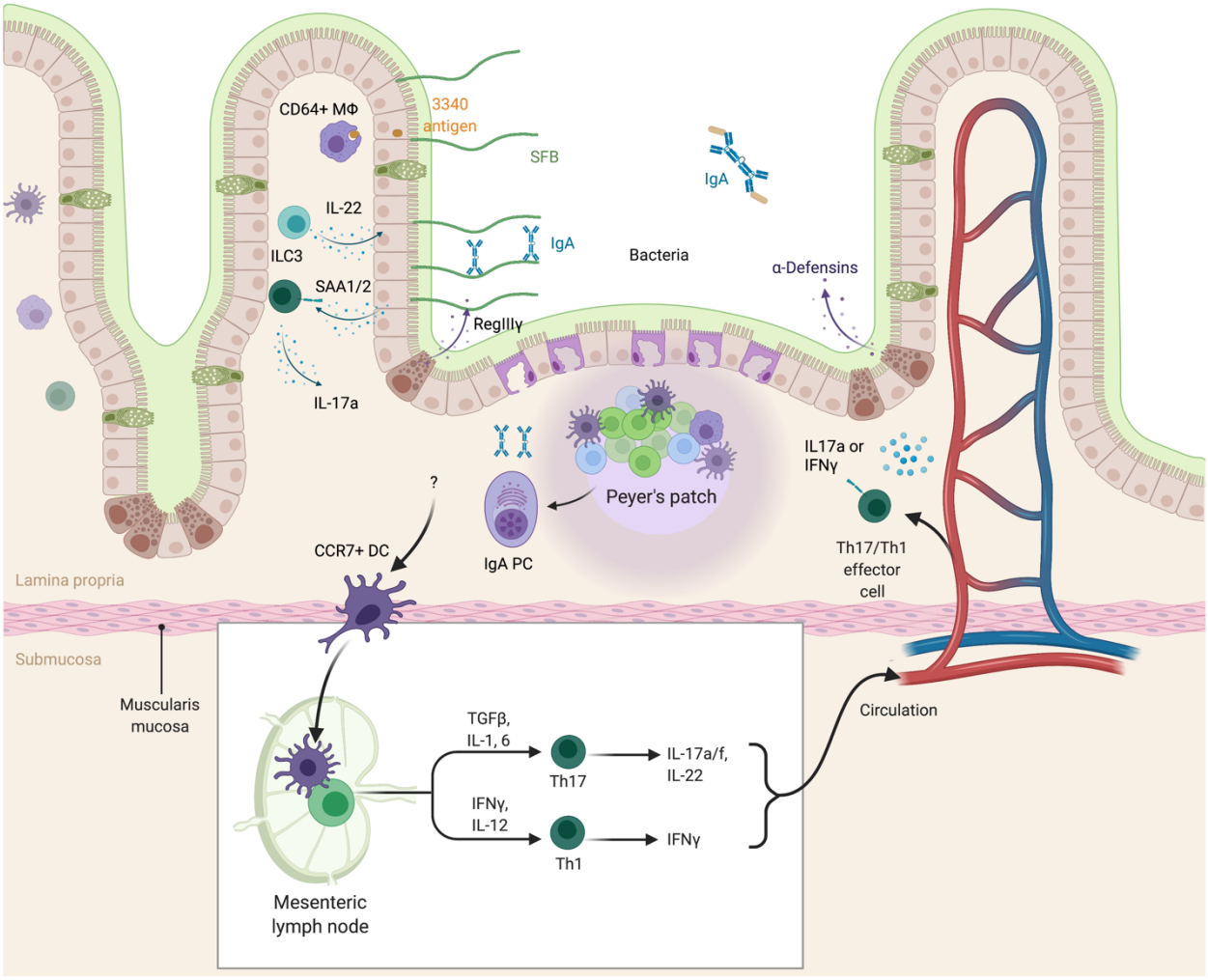

Figure 2. SFB regulation of mucosal immune responses

Following SFB colonization the 3340 antigen is endocytosed by IECs. It is then transferred to CD11c+ CD64+ macrophages where they present the antigen on MHCII to induce the differentiation of Th17 cells. Where these cells prime the Th17 cells is not clear. There are likely other antigen presenting cells involved in Th17 cell differentiation, since migrating CCR7+ DCs play a role. These cells traffic to the MLNs to prime the T cells. Also, SFB colonization induces an IL22, SAA circuit in the tissue. ILC3s are activated following the colonization and secrete IL22 which activates STAT3 in IEC to induce SAA expression. The SAA provides a second hit on RORγt+ CD4 T cells to induce IL17a expression in the tissue. IL17a activates REGIIIγ and other AMP expression in Paneth cells. SFB also induces a GC B cell response in Peyer's patches. These B cells class switch to IgA and traffic back to the tissue to become IgA plasma cells. These plasma cells secrete IgA which is transcytosed into the lumen and binds SFB and other antigens.

1.8 The role of SFB in disease

As previously described, SFB induces an inflammatory mucosal immune response without inducing pathology in its host. The immune response tightly regulates the levels of SFB without completely clearing the bacteria suggesting a potential beneficial role of SFB for the host. Previous work has shown that in the context of intestinal infections with *Citrobacter rodentium* (*C. rodentium*) or Rotavirus, SFB protects mice from these pathogens (Ivanov et al., 2009; Shi et al., 2019). JAX colonized mice had increased *C. rodentium* load and colitis compared to JAX + SFB colonized mice (Ivanov et al., 2009). SFB induces IL22 production from ILC3s which feeds back on epithelial cells to induce the expression of AMPs (Basu et al., 2012). The ILC3 response plays an essential role early in the infection whereas later, IL22 from CD4+ T cells is important in controlling the pathogen and SFB colonization increases IL22 production from both cell types. Future work will be needed to understand more precise mechanisms of how SFB specific Th17 cells which are generated in the ileum protect against *C. rodentium* which colonizes the colon. In the context of Rotavirus infection, SFB positive mice were protected against infection. This mechanism of protection was independent of SFB's impact on adaptive or innate lymphocytes since SFB positive RAGγc deficient mice were still protected (Shi et al., 2019). SFB instead increased the turnover and shedding of IECs which impeded Rotavirus entry into host tissue (Shi et al., 2019). These data indicate that SFB provides a benefit to the host against intestinal infections. On the other hand, the presence of SFB increases the susceptibility of mice to numerous autoimmune or inflammatory diseases. SFB monocolonized mice showed an increase susceptibility to experimental autoimmune encephalomyelitis (EAE) compared to GF mice (Lee et al., 2011). SFB monocolonized K/BxN mice had increased susceptibility to autoimmune arthritis compared to GF mice (Wu et al.,

2010). The presence of SFB in the dam increased the susceptibility of the pups to maternal immune activation (MIA) model of autism (Kim et al., 2017). In all these models, the increase of IL17+ CD4+ T cells from the SFB colonization is linked with the increased susceptibility to disease. For the EAE model, the authors found an increase of IL17+ CD4+ T cells in the spinal cord of mice colonized with SFB. For the K/BxN mice and the MIA model, IL17 neutralization impeded the development of arthritis or MIA. These data collectively show that SFB colonization protects the host against enteric pathogens but increases the susceptibility to various autoimmune diseases. Therefore, tight regulation of SFB load in the host may be required by the adaptive immune response to prevent this pathobiont from inducing IL17 dependent inflammatory diseases.

1.9 Summary

The intestinal tract is regionally compartmentalized to coordinate digestion and absorption. The epithelial cells are critical for regulating these metabolic processes along the intestine. Specific transcription factors control spatial patterning of epithelial cells and confer region specific functions. GATA4 is an epithelial transcription factor that is expressed in the duodenum and jejunum and is absent from the ileum and colon. It activates the jejunal transcriptional identity while restricting the ileal program. GATA4 deficient mice reveal a loss of regionalization where the jejunum loses its identity and upregulates ileal specific genes. These mice reveal a defect in lipid metabolism in the jejunum and an upregulation of genes involved in bile acid uptake and handling. These data suggest that GATA4 deficient mice may be a unique genetic model of altering small intestinal regionalization to study its impact on host physiology.

The intestine is also colonized with trillions of microbes termed the microbiota. The diversity and load of these microbes differ along the intestinal tract. A key function of the

microbiota is the development and maintenance of host immune responses. Specific commensal immunomodulatory microbes colonize discrete niches along the intestinal tract where they induce local tissue immune responses. SFB is one particular microbe which attaches to IECs of the ileum. Its colonization triggers the activation of the immune response at the level of the IECs, antigen-presenting cells, innate lymphoid cells, CD4 and CD8 T cells, GC B cells, and IgA PC. These host responses are induced locally in the ileum or draining lymph nodes and are absent from the colon. On the other hand, *Bacteroides fragilis* another commensal microbe which colonizes the colon induces distinct immune responses than SFB. Whereas SFB induces a Th17 response, *B. fragilis*, induces an IL10 Treg response in the colon but not small intestine. These examples suggest that commensal microbes shape regional tissue immune responses.

Intestinal epithelial cells interact with both the microbiota and host immune cells. These cells express pattern-recognition receptors to sense conserved microbial patterns. Goblet cells produce mucus and Paneth cells produce antimicrobial peptides which maintain spatial segregation of the bacteria with the host. They uptake bacterial antigens through M cells of the PPs or through enterocytes. For SFB, endocytosis of a particular antigen 3340 into enterocytes is then transferred to host antigen presenting cells where it drives Th17 differentiation. Epithelial cells express MHCII and stress ligands which are upregulated following infection or damage and signal to activate adaptive immune cells. Since epithelial cells have a region specific transcriptional signature and interact with both the microbiota and host immune cells, we hypothesized that IECs coordinate regional tissue immune responses through the microbiota. Furthermore, GATA4 controls regional immunity through regulating bacterial colonization. The goals of this thesis are first to define how the tissue and microbiota shape regional tissue immune responses. Few studies have explored this concept, or have studied regional immune responses in

GF mice to illustrate how the tissue shapes immunity independently of the microbiota. On the other hand, the regions strongly impacted by the microbiota and what responses are specific to those regions are unclear. Second to test whether GATA4 shapes regional tissue immune responses. If so, which immune responses are GATA4 dependent and are they microbiota dependent or independent. We will define which microbes are involved and how GATA4 controls the colonization of these microbes. Third, we will identify specific mechanisms of how GATA4 in IECs shape regional tissue immune responses and bacterial colonization. Finally, we will show the impact of intestinal regionalization by GATA4 on host response to infection or autoimmune disease. These experiments will reveal how the host establishes regional immune responses in the intestine and what role this has in homeostasis and disease.

2. MATERIALS AND METHODS

This chapter includes experimental procedures from an article that will be submitted for publication.

2.1 Mice

7-12 week old mice were used for experiments and housed in *Helicobacter hepaticus*, Murine norovirus free barrier level or GF. Mice were also kept SFB+ or SFB-. GATA4fl/fl villin-cre SPF mice were previously generated in the CD1 background and obtained from the Matzinger laboratory (Shulzhenko et al., 2011). This line was rederived GF for this study and backcrossed for 10 generations to C57BL/6J background for T cell transfers. C57BL/6J, B6-Tg(Tcra, Tcrb)2Litt/J SFB TCRtg, B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ, B.6129S7-Rag1^{tm1mom}/J were obtained from Jackson laboratory. CD-1 IGS mice were obtained from Charles river laboratory. Mice deficient for IgH J segment locus (Jh) were obtained from the Bendelac laboratory and generated using Cas9 with the protospacers GCTACTGGTACTTCGATGTC and GCCATTCTTACCTGAGGAGA. IgA deficient mice where the Sα (IgA switch region) and C1α (first exon) were deleted were obtained from the Bendelac laboratory and generated using Cas9 with the protospacers AAGCGGCCACAACGTGGAGG and TCAAGTGACCCAGTGATAAT. Jh and IgA deficient mice were rederived GF at Taconic laboratory.

2.2 Isolation of IEC, IEL, and LP cells

The segments of the intestine were excised as follows to isolate cells for flow cytometry: duodenum was taken 12cm from the stomach, jejunum 12cm from the middle, and ileum 12cm from the cecum. Any leftover segments were discarded. The entire colon was taken after the cecum to the rectum. PPs were first removed from the small intestine, the segments were then

opened longitudinally and washed briefly in PBS. The intestine was cut into 2mm pieces and then transferred to 50ml falcons containing 12ml of RPMI 1640 media with 1% dialyzed FBS, 2mM EDTA, and 1.5mM $MgCl_2$ (IEL media). The cells were placed horizontally in an incubator set to 37C and shaking at 250rpm for 20min. The media was strained through 100µm filters, the tissue was collected back into the original 50ml conical, fresh 12mls of IEL media were added, and the tissue was placed back into the shaking incubator for another 20mins. Following the second shake both IEL fractions were combined. 12mls of LP media, RPMI 1640 with 20% FBS and 0.23mg/ml of collagenase VII (Sigma-Aldrich, C2139) were added to the tissue and then placed back in the shaker for 2X 20min incubations. The IEL and LP compartments were then subjected to a percoll density gradient centrifugation step to remove dead cells and debris. Both fractions were pelleted and resuspended in 9ml of 40% percoll (GE Healthcare) and spun for 12min at 3000rpm set to acceleration and deceleration 3. After the spin the debris was removed and the percoll was diluted out. The IEL and LP cells were then counted on a hemocytometer.

2.3 Cytokine stimulation

Up to $2x10^6$ cells/ sample were collected and resuspended in 270µl of complete RPMI1640 (RPMI, 10% FBS and 1% glutamine, penicillin, streptomycin). 250µl of media and cells were added to a 48well tissue culture plate and 250µl of 2X stimulation media was added. The 2X stimulation media was complete RPMI with 1.5µl/ml of ionomycin (stock 1mg/ml), .1µl/ml phorbol 12-myristate 12-acetate (PMA) stock (stock 1mg/ml), and 3µl/ml golgi-stop, BD. Final concentration of ionomycin is 750ng/ml and 50ng/ml of PMA. The cells were incubated for 2 hours at 37C with 5%CO_2. After stimulation the reaction was quenched with 500µl of ice cold FACS buffer (1X PBS with 2% FBS) and the cells were subsequently stained with antibodies for flow cytometry.

2.4 Flow cytometry

Cells were first stained with FC block (CD16/32) to block nonspecific binding for 10min at 4C. The cells were then stained with dead dye to exclude dead cells (Aqua, ThermoFisher or Zombie NIR, Biolegend) for 15min at 4C. Cell surface markers were next stained for 20min at 4C. For intracellular cytokine staining the BD cytofix/cytoperm kit was used and the manufacturers protocol was used. The incubation for intracellular cytokines was 40min at 4C. For intracellular TFs the Foxp3 ebioscience kit was used and the manufacturers protocol was followed. The fixation and intracellular staining steps were both 1hr. The cells were run on the LSRFortessa™ X-20 Flow Cytometer or the Cytek® Aurora.

2.5 DNA isolation

5cm of tissue were taken for mucosal scrapings for DNA isolation from the jejunum and ileum. 5cm proximal to the middle for the jejunum and 5cm from the ileocecal valve for the ileum. The entire colon was used for mucosal scrapings. The tissue was excised, opened longitudinally, scraped with a glass slide, transferred to 2ml screw cap tube with 500µl of 0.1mm glass beads (Bio-spec), and snap frozen on dry ice. 50-100mg of content was taken as close to the middle and possible and from the last 5-7cm of the ileum. 1ml of inhibitex buffer (Qiagen) was added and homogenization was done using the bead ruptor elite – bead mill homogenizer (Omni, 19040E) on speed 6 for 3mins. After homogenization the DNA was extracted using the QIAmp Fast DNA stool mini kit (Qiagen) following the manufacturers protocol with the optional high temp (95C) lysis step. DNA was eluted in 50µl of H_20 or buffer EB (Qiagen) and the concentration was determined using the nanodrop UV spectrophotometer (ThermoFisher).

2.6 RNA isolation

1cm of tissue was excised from the beginning of the duodenum, the middle of the jejunum, the end of the ileum, and the center of the colon and preserved in RNAprotect (Qiagen) overnight at 4C and then transferred to -80C for long term storage. The tissue was transferred to 600µl of Buffer RLT with β-mercaptoethanol (Qiagen) and homogenized for 30seconds with a hand held rotor (tissue-tearor, biospec). RNA was purified using RNAeasy plus mini kit (Qiagen) following the manufacturers protocol with the optional on column DNase digest (Qiagen).

2.7 qPCR

RNA was first reverse transcribed to cDNA using GoScript Reverse Transcriptase kit (Promega) following the manufacturers protocol. 10ng of cDNA or 20ng of DNA from mucosal scrapings and content was used for qPCR. SYBR green TB green qPCR master mix (Takara) was used and the target gene was quantified and normalized to the housekeeping gene using $1000*2^{-(\text{Ct target- Ct housekeeping})}$ formula. For host gene expression the target gene was normalized to GAPDH. For bacterial load target gene was normalized to either host DNA with ASL primers or universal 16s primers. The qPCR was performed on the LightCycler® 480 System (Roche). *ASL* forward 5'-TCTTCGTTAGCTGGCAACTCACCT-3', *ASL* reverse 5'-ATGACCCAGCAGCTAAGCAGA TCA-3'; *16S UNI 340* forward 5'-ACTCCTACGGGAGGCAGCAGT-3', *16S UNI 514* reverse 5'-ATTACCGCG GCTGCTGGC-3'; *SFB 736* forward 5'-GACGCTGAGGCATGAGAGCAT-3', *SFB 844* reverse 5'-GACGGCACGGATTGTTATTCA-3'; *C. rodentium espB* forward 5'-ATGCCGCAGATGAGACAGTTG-3', *C. rodentium espB* reverse 5'-CGTCAGCAGCCTTTTCAGCTA-3'

2.8 Histology

The tissue was collected in the same manner as for RNA, placed in cassettes and fixed in 10% formalin for H&E staining or Carnoy solution (ThermoFisher) for fluorescent *in-situ* hybridization (FISH) staining overnight at room temperature. Cassettes were transferred to 70% ethanol for formalin or 100% ethanol for Carnoy to wash out the fixative. The tissue was embedded in paraffin and slides were cut at 5µm thickness. The H&E staining was performed by the Human Tissue Resource Center at the University of Chicago. For FISH staining, the paraffin was first removed by running the slides through 4X 3min incubations in xylene and 4X 3min incubations in 100% ethanol. The slides were then moved to a polypropylene slide container and filled with 20mls of hybridization solution containing the diluted 16s probe (.9M NaCl, 20mM Tris-HCL pH 7.5, 0.1% SDS with 0.2ng of probe specific for SFB 16s or universal 16s). SFB1008 5'-Alexa488-GCGAGCTTCCCTCATTACAAGG-3' SFB1008 5'-Cy5-GCGAGCTTCCCTCATTACAAGG-3'. EUB338 5'-Alexa546-GCTGCCTCCCGTAGGAGT-3'. EUB338 5'-Cy3-GCTGCCTCCCGTAGGAGT-3'. The slides were incubated overnight at 50C in the dark. The slides were washed 3X with the hybridization buffer, briefly rinsed in H20, and then mounted with Prolong diamond antifade with DAPI (ThermoFisher). The slides were scanned with the CRi Pannoramic SCAN 40x Whole Slide Scanner at the University of Chicago Integrated Light Microscopy core.

2.9 ELISA

To quantify luminal IgA levels, content was collected from the jejunum and ileum and weighed in 2ml bead beating tubes containing 500µl of 0.1mm glass beads. 1ml of 1X cell lysis buffer with protease inhibitors (Cell Signaling Technologies) was added and the content was homogenized on a vortex for 5min. The debris was pelleted at 13000rpm for 10min and the

supernatant was collected for ELISA. For tissue explants, 1cm of tissue was excised and opened longitudinally, washed in PBS, and placed in complete RPMI at 37C for 24hours. The culture supernatant was collected and used for ELISA. The supernatant was diluted 500, 1000, 2000, times in 1X assay diluent A (ThermoFisher) to pick the dilution that appeared in the middle of the standard curve. IgA mouse uncoated ELISA kit (ThermoFisher) was used following the manufacturers protocol and absorbance was read at 450nm. The values of IgA were back calculated to the original sample and normalized relative to the weight of the content.

2.10 Luminal IgA isolation

To isolate luminal polyclonal sIgA from the intestine, content was pooled from the small intestine, large intestine, and cecum from 8-12 week old WT CD1 mice (Charles river). Content from 10 mice was transferred to 50ml falcons with 20ml of TBST buffer with proteinase inhibitor (Roche). Falcons were then vortexed for 5 min on max speed and centrifuged for 10min at 5000 rpm. The supernatant was collected and spun again 2X to further remove bacteria and debris. 1.25ml of Pierce Protein L Magnetic Beads (Thermo Scientific) were added for 20mls of TBST and incubated for 1hr at room temperature while shaking. After 1hr beads selectively bound to IgA through kappa light chain were separated from a supernatant with EasyPrep magnetic stand. Supernatant was discarded and beads washed 3X. IgA was separated from the beads with 1ml of Pierce IgG Elution Buffer pH 2.0 (ThermoFisher) for the content from 10mice. Elution buffer was incubated with the beads for 10min at room temperature on a shaker. 150µl of Tris-HCl 1M pH 8.5 was added to neutralize the solution. IgA concentration was measured using NanoDrop. The isolated IgA was then filtered with 0.22µm sterile syringe filter unit and protease inhibitors were added (ThermoFisher). The IgA was kept up to one week at 4C.

For the IgA gavage, the isolated IgA was further concentrated with Amicon Ultra-4 Centrifugal Filter Units (MilliporeSigma) until 250-350µg/0.1ml final concentration was achieved.

2.11 Bacterial staining with luminal IgA

Content was taken from WT and GATA4$^{\Delta IEC}$ mice and resuspended in 1X PBS with protease inhibitors at a concentration of 0.1mg/µl, vortexed for 5mins, and spun at 8000rpm for 5min. 3 fecal pellets of RAG-/- mice were homogenized as previously described, and pelleted. The bacterial pellet was resuspended in 50µl PBS and combined with 50µl of luminal supernatant containing IgA. The IgA was incubated with the bacteria for 1hr at 4C. The bacteria were then washed, pelleted, and stained with SYTO BC (ThermoFisher) diluted 1:5000 and anti-IgA APC diluted 1:200 for 30mins. Bacteria were gated FSC, SSC, SYTOBC+, and IgA+.

2.12 *Citrobacter rodentium* infections

C. rodentium strains DBS100, DBS120 *pler-lux*, or DBS100 ΔEAE were grown overnight shaking at 37C in LB broth. The cultures were diluted 100X in the morning and grown up to log phase until the OD600nm reached 0.75. 162mls of culture were spun down and resuspended in 3ml of PBS. 200µl of bacteria were used for gavage which gave a dose of 2.5X10^{9} CFU/mouse. DBS100 or DBS100 ΔEAE strains were given to GF mice and DBS120 *pler-lux* was given to SPF mice. The DBS120 strain has a genomic kanamycin resistance cassette inserted through Tn5. To determine CFUs of DBS120, 2 fecal pellets/mouse was resuspended in 1ml of PBS, and plated on MacConkey agar containing 50µg/ml of kanamycin. CFU/mg feces were determined by (#CFU counted*Dilution factor/(vol plated in mls (5µl)))/mg feces. To determine the amount of bacterial translocation, the MLN, liver, and spleen were aseptically dissected, weighed, and homogenized with the tissue-tearor rotor (Biospec) in 500µl of PBS. 200µl of homogenate was plated on MacConkey agar containing 50µg/ml of kanamycin.

2.13 SFB TCRtg adoptive transfer

Naïve SFB TCRtg Vβ8 CD4 T cells were isolated from congenically marked CD45.1 Vβ8+/- female mice using the naïve CD4 T cell isolation kit (Miltenyi). The cells were isolated from LNs and spleen, and labeled with Cell Trace Violet (stock 5mM). CTV was diluted 1:4000 in PBS. 1ml of cells with up to $20x10^6$ cells was combined with 1ml of CTV (1:4000) and the cells were incubated at 37C in the dark for 20 mins. The reaction was quenched by adding complete RPMI spun down and diluted to a concentration of $2x10^5$ cells/.1ml. 100µl was injected retroorbitally into CD45.2 WT and $GATA4^{\Delta IEC}$ mice to give $2x10^5$ cells/mouse. 3 days after transfer the mice were euthanized to determine the priming and activation of cells in the jejunal and ileal draining MLN as described previously (Esterházy et al., 2019). For determining expansion in the tissue, 50,000 cells were injected/mouse and the mice were analyzed 9 days after transfer.

2.14 16s sequencing libraries and analysis

Extracted DNA was amplified, barcoded and sequenced as described previously (Barlow et al., 2020; Bogatyrev and Ismagilov, 2020; Bogatyrev et al., 2020). Briefly, amplification of the variable 4 (V4) region of the 16S rRNA gene was performed in 20 uL duplicate reactions: 8 uL of 2.5X 5Prime Hotstart Mastermix (VWR, Radnor, PA, USA), 1 uL of 20X Evagreen (VWR), 2 uL each of 5 uM forward and reverse primers (519F, barcoded 806R, IDT, CoralVille, IA, USA), variable input volumes of extracted DNA template and nuclease free water. Total DNA input (determined by NanoDrop) was limited to 400ng to prevent inefficient amplification. Amplification reactions were monitored on a CFX96 RT-PCR machine (Bio-Rad Laboratories, Hercules, CA, USA) and samples were removed in late exponential phase to minimize chimera formation and non-specific amplification (1,4,5). Amplification cycling conditions were as

follows: 94 °C for 3 min, up to 50 cycles of 94 °C for 45 s, 54 °C for 60 s, and 72 °C for 90 s. Successfully amplified duplicates were pooled together and quantified with KAPA library quantification kit (Roche, Basel, Switzerland) and then all samples were combined at equimolar concentrations with up to 96 samples per library. AMPureXP beads (Beckman Coulter, Brea, CA, USA) were used to clean up and concentrate libraries before final library quantification with a High Sensitivity D1000 Tapestation Chip (Agilent, Santa Clara, CA, USA). Illumina MiSeq sequencing was performed with a 2x300bp reagent kit by Fulgent Genetics (Temple City, CA, USA). Raw reads were demultiplexed by Fulgent Genetics. Demultiplexed forward and reverse reads were processed with QIIME 2 2020.2 (Bolyen et al., 2019). Loading of sequence data was performed with the demux plugin followed by quality filtering and denoising with the dada2 plugin (Callahan et al., 2016). Dada2 trimming parameters were set to the base pair where the average quality score dropped below thirty. All samples were rarefied to the lowest read depth present in all samples (48,305 reads) to decrease biases from varying sequencing depth between samples (Weiss et al., 2017). The q2-feature-classifier was then used to assign taxonomy to amplicon sequence variants (ASV) with the Silva 132 99% OTUs references (Bokulich et al., 2018; Quast et al., 2013). Resulting read count tables were used for downstream analyses in IPython notebooks.

Absolute abundance

The total microbial load (bacteria and archaea) of each sample and the absolute abundance of each taxon in individual samples was determined as described previously (Barlow et al., 2020; Bogatyrev and Ismagilov, 2020). Briefly, the Bio-Rad QX200 droplet dPCR system (Bio-Rad Laboratories) was utilized to measure the 16S concentration in each sample with the following reaction components: 1X QX200 EvaGreen Supermix (Bio-Rad), 500 nM forward

primer, and 500 nM reverse primer (Uni 16S 519F, Uni 16S 806R) and thermocycling conditions: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 52 °C for 30 s, and 68 °C for 30 s, followed by a dye stabilization step of 4 °C for 5 min and 90 °C for 5 min. The final concentration of 16S rRNA gene copies in each sample was corrected for dilutions and normalized to the extracted sample total DNA measurement from NanoDrop. Total DNA levels provide a good proxy for tissue mass in biopsy samples. For each sample, the input-DNA-normalized total microbial load from dPCR was multiplied by each amplicon sequence variant's (ASV) relative abundance to determine the absolute abundance of each ASV.

Poisson quality filtering

Two separate quality-filtering steps based on Poisson statistics were used to determine the statistical confidence in the measured values. First, a 95% confidence interval was calculated from the repeated measures of water blanks. Samples with a total microbial load below the upper bound of this confidence interval were removed from further analysis.

Second, the limit of detection (LOD) in terms of relative abundance was determined for each sample. Sequencing can be divided into two separate Poisson sampling steps. First, an aliquot of sample is taken from the extracted sample and input into the library amplification reaction. The LOD of the library amplification step was determined by multiplying the total microbial load from dPCR by the input volume into the library amplification reaction and then finding the relative abundance corresponding to an input of three copies. Poisson statistics tells us that the likelihood of sampling one or more copies with an average input of three copies is 95%. The second Poisson sampling step in sequencing arises from the number of reads generated from the amplified library. The accuracy of the second Poisson sampling step was previously shown to follow a negative exponential curve, $LOD = 7.115 * read\ depth^{-0.115}$, between the

total read depth and relative abundance at which 95% confidence of detection is observed (Barlow et al., 2020). The minimum of the two described LODs (first determined per sample by total load, and second by sequencing depth) was then determined for each sample. For each sample, the abundance of any ASV with a relative abundance below the LOD was set to zero. After filtering, data tables for each taxonomic level were generated.

Statistical analysis and correlations

Group comparisons were analyzed using the non-parametric Kruskal-Wallis rank sums tests with Benjamini–Hochberg multiple hypothesis testing correction using *SciPy.stats Kruskal* function and *statsmodels.stats.multitest multipletests* function with the *fdr_bh* option.

2.15 Intestinal epithelial cells and tissue mRNA sequencing and analysis

Purified cell subset RNA-seq

The SMART-Seq v4 Ultra Low Input RNA Kit (TaKaRa) was used to generated amplified cDNA, using 7500pg of RNA input. The cDNA was generated and purified according to the manufacturer's specifications. cDNA was amplified 12 cycles. The Nextera XT DNA Library Preparation Kit (Illumina) was used to generate the RNA-seq libraries, with an input of 125pg cDNA, according to the manufacturer's specifications. Subsequently, the libraries were multiplexed and sequenced at a depth of 20 million reads per sample (50bp SR) on a HiSeq4000.

Whole tissue RNA-seq data analysis

Whole tissue biopsies stored at -80C were thawed on ice and transferred to Starstedt tubes containing 350uL RLT Plus supplemented with 1% 2-mercaptoethanol and equal quantities of 1.0mm and 0.5mm zirconium oxide beads (Next Advance). Biopsies were bead beat 3 times

for 1min at a setting of 9 on a Bullet Blender 24, with one minute of cooling one ice between each beating. Lysates were processed using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). 500ng of purified RNA was used as input in the TruSeq Stranded mRNA Library Prep kit (Illumina) to generate sample libraries according to manufacturer's specifications. Libraries were multiplexed and sequenced at a depth of 20 million reads per sample (50bp SR) on a HiSeq4000.

Table 1. List of reagents

Reagent or Resource	Source	Identifier
Antibodies		
CD45 Pac Blue (30-F11)	Biolegend	Cat#103126
TCRgd FITC (eBioGL3)	Thermo Fisher	Cat#11-5711-82
CD4 BV785 (GK1.5)	Biolegend	Cat#100453
CD4 BV605 (GK1.5)	Biolegend	Cat#100451
CD8β BUV395 (H35-17.2)	BD	Cat#740278
CD8α PerCp/Cy5.5 (53-6.7)	BD	Cat#551162
NK1.1 PE-CF594 (PK136)	BD	Cat#562864
TCRβ BUV737 (H57-597)	BD	Cat#612821
TCRβ BV711 (H57-597)	BD	Cat#563135
CD3ε BUV737 (145-2C11)	BD	Cat#612771
IFNγ APC (XMG1.2)	BD	Cat#554413
IL10 PEcy7 (JES5-16E3)	Biolegend	Cat#505026
IL17a PE (ebio17B7)	Thermo Fisher	Cat#12-7177-81
CD45.1 Pac Blue (A20)	Biolegend	Cat#110722
CD45.2 BUV395 (104)	BD	Cat#553772
vβ14 TCR FITC (14-2)	BD	Cat#553258
RORγt BV786 (Q31-37)	BD	Cat#564723
FOXP3 eflour450 (FJK-16s)	Thermo Fisher	Cat#48-5773-82
FOXP3 FITC (FJK-16s)	Thermo Fisher	Cat#11-5773-82
FOXP3 PE-cy7 (FJK-16s)	Thermo Fisher	Cat#25-5773-82
Tbet PE (4B10)	Biolegend	Cat#644810
CD44 PE-CY7 (IM7)	Biolegend	Cat#103030
CD62L PE (MEL-14)	Biolegend	Cat#104408
Epcam PerCp/Cy5.5 (G8.8)	Biolegend	Cat#118220
CD19 FITC (1D3/CD19)	Biolegend	Cat#152404

Table 1. List of reagents continued

NK1.1 BV605 (PK136)	Biolegend	Cat#108753
CD11C BV605 (N418)	Biolegend	Cat#117334
TER119 BV605 (TER-119)	Biolegend	Cat#116239
F4/80 BV605 (BM8)	Biolegend	Cat#123133
CD3ε BV605 (145-2C11)	Biolegend	Cat#100351
Ly6g BV605 (1A8)	Biolegend	Cat#127639
B220 PE-cy7 (RA3-6B2)	Biolegend	Cat#103222
IgA PE (mA-6E1)	Thermo Fisher	Cat#12-4204-81
IgA AF647 goat polyclonal	Southern Biotech	Cat#1040-31
Biological Samples		
Fetal Bovine Serum	Biowest	Cat#S01520; Lot#A11504E
Normal Goat Serum	JacksonImmunoResearch	Cat#005-000-121
Chemicals, Peptides, and Recombinant Proteins		
EDTA, 0.5M, pH8.0	Corning	Cat#46-034-CI
1M MgCl2	Thermo Fisher	Cat#AM9530G
Cytiva Percoll™ Centrifugation Media	GE Healthcare	Cat#45-001-747
RNAprotect Tissue Reagent	Qiagen	Cat#76106
2-Mercaptoethanol (BME)	Sigma-Aldrich	Cat#M7154
Phorbol Myristate Acetate	Sigma-Aldrich	Cat#P1585
Ionomycin Calcium Salt from Streptomyces conglobatus	Sigma-Aldrich	Cat#10634
BD GolgiStop Protein Transport Inhibitor	BD	Cat#554724
Ethanol 200 Proof	Decon Labs Inc	Cat#DSP-MD 43
Inhibitex Buffer	Qiagen	Cat#19593
Nuclease-free Water	Ambion	Cat#AM9932

Table 1. List of reagents continued

Carnoy Solution	Ricca Chemical	Cat#R18510004C
10% Formalin Solution	Thermo Fisher	Cat#SF98-4
RPMI 1640 with L-Glutamine	Corning	Cat#MT-10043CV
Collagenase from Clostridium histolyticum	Sigma-Aldrich	Cat#C2139-500MG
1M TRIS-HCL pH 7.5	Thermo Fisher	Cat#15567027
10% SDS solution	Thermo Fisher	Cat#15553027
Sodium chloride	Sigma-Aldrich	Cat#S9888
Critical Commercial Assays		
SytoBC	Thermo Fisher	Cat#S34855
LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit	Thermo Fisher	Cat#L34966
Zombie NIR™ Fixable Viability Kit	Biolegend	Cat#423106
BD Cytofix/Cytoperm Plus Fixation/Permeabilization Solution Kit	BD	Cat#554714
eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set	Thermo Fisher	Cat#00-5523-00
QIAamp Fast DNA Stool Mini Kit	Qiagen	Cat#51604
RNeasy Plus Mini Kit	Qiagen	Cat#74136
RNeasy Micro Kit	Qiagen	Cat#74004
GoScript Reverse Transcriptase Kit	Promega	Cat#A5001
SYBR Advantage qPCR Premix	Clontech	Cat#639676
ProLong™ Diamond Antifade Mountant with DAPI	Thermo Fisher	Cat#P36962
Pierce™ Protein L Magnetic Beads	Thermo Fisher	Cat#88850
Pierce™ IgG Elution Buffer, pH 2.0	Thermo Fisher	Cat#21028
1.0 M Tris HCl pH 8.5	VWR	Cat#76236-402
ALDEFLUOR™ Kit	Stemcell technologies	Cat#01700
IgA mouse ELISA	Thermo Fisher	Cat#88-50450-86

Table 1. List of reagents continued

Oligonucleotides		
GAPDH Forward 5′-AGGTCGGTGTGAACGGATTTG-3′	(Abadie et al., 2020)	N/A
GAPDH Reverse 5′-TGTAGACCATGTAGTTGAGGTCA-3′	(Abadie et al., 2020)	N/A
IFNγ Forward 5′-ATGAACGCTACACACTGCATC-3′	(Abadie et al., 2020)	N/A
IFNγ Reverse 3′-TCTAGGCTTTCAATGACTGTGC-5′	(Abadie et al., 2020)	N/A
ASL Forward 5'-TCTTCGTTAGCTGGCAACTCACCT-3'	(Klose et al., 2014)	N/A
ASL Reverse 5'-ATGACCCAGCAGCTAAGCAGATCA-3'	(Klose et al., 2014)	N/A
Uni 16s 340F 5'-ACTCCTACGGGAGGCAGCAGT-3'	(Sano et al., 2015)	N/A
Uni 16s 514R 5'-ATTACCGCGGCTGCTGGC-3'	(Sano et al., 2015)	N/A
SFB 736F 5'-GACGCTGAGGCATGAGAGCAT-3'	(Sano et al., 2015)	N/A
SFB 844R 5'-GACGGCACGGATTGTTATTCA-3'	(Sano et al., 2015)	N/A
C. rodentium espBF 5'-ATGCCGCAGATGAGACAGTTG-3'	(Sagaidak et al., 2016)	N/A
C. rodentium espBR 5'-CGTCAGCAGCCTTTTCAGCTA-3'	(Sagaidak et al., 2016)	N/A
EUB338 5'-Alexa546-GCTGCCTCCCGTAGGAGT-3'	(Amann et al., 1990)	N/A
EUB338 5'-Cy3-GCTGCCTCCCGTAGGAGT-3'	(Amann et al., 1990)	N/A
SFB1008 5'-Alexa488-GCGAGCTTCCCTCATTACAAGG-3'	(Sano et al., 2015)	N/A

Table 1. List of reagents continued

SFB1008 5'-Cy5-GCGAGCTTCCCTCATTACAAGG-3'	(Sano et al., 2015)	N/A
Other		
Bead ruptor elite bead mill homogenizer	Omni International	Cat#19-040E
Tissue-Tearor	Biospec Products	Cat#985370-XL
Glass Beads	Biospec Products	Cat#11079101
LightCycler® 480 System	Roche	N/A
LSRFortessa™ X-20 Flow Cytometer	BD	N/A
Cytek® Aurora	Cytek	N/A
CRi Pannoramic SCAN 40x Whole Slide Scanner	3DHistech	N/A
EasyEights™ EasySep™ Magnet	Stemcell technologies	Cat#18103

3. RESULTS

This chapter includes results from an article that will be submitted for publication.

3.1 Regionalization of intestinal immunity

The intestinal tract is compartmentalized into distinct regions which perform coordinated digestive and metabolic functions. This segregation establishes various gradients along the intestine such as the decrease of metabolism, digestion, dietary antigens, and oxygen concentration moving proximal to distal (Mowat and Agace, 2014). In contrast, there is an increase of microbial load, microbial diversity, and short chain fatty acids. Dietary and microbial antigens in the small intestine are sampled through PPs, whereas the thick mucus layer in the colon excludes most microbes from interacting with the host tissue. Specific commensal immunomodulatory microbes are found in discrete niches throughout the intestine and induce different immune responses. For example, SFB colonizes the ileum and induces a Th17 response which restricts *C. rodentium* colonization. *B. fragilis* colonizes the colon and induces IL10 which dampens the immune response and helps establish tolerance. Therefore, we sought to further understand how both the region of the intestine and the microbiota contribute to shaping local immune responses. To address this, we profiled B cell and T cell populations throughout the intestine in SPF and GF mice by multicolor spectral flow cytometry. We observed that the number of IgA PCs greatly varied throughout the intestine in both SPF and GF mice (Figure 3A, B). There was a striking reduction of IgA PCs moving from the jejunum to ileum in SPF mice. Interestingly, GF mice still had a propensity of more IgA PCs in the proximal small intestine than the ileum or colon. These data suggest that there is a contribution of both the region and microbiota in regulating the number of IgA+ PCs throughout the intestine. We next profiled GC B cells and Tfhs. We identified GC B cells and Tfh cells only in the ileum and colon of SPF

mice (Figure 3C, D). This shows that the microbiota drives GC responses particularly in the ileum and to a lesser extent in the colon and fit with the observations that isolated lymphoid follicles are typically present in the distal intestine of SPF mice (Agace and McCoy, 2017). Next we analyzed T cell effector responses in the IEL and LP compartments by intracellular cytokine staining. The frequency of IFNγ+ CD8αβ T cells greatly varied by region and was microbiota dependent (Figure 3G). The impact of the microbiota on IFNγ+ CD8αβ T cells was greatest in the ileum. The microbiota appeared to be required for this response as GF mice did not show much variation between regions. This is in contrast to the IFNγ CD4 T cell response. Here, GF mice differed by region and had elevated levels of IFNγ+ CD4 T cells in the duodenum and jejunum (Figure 3G). Interestingly although the colon has the greatest amount of microbes, it was also a region of low IFNγ and IL17 T cell responses in SPF mice. Instead, the microbiota skewed the immune response in the colon to a tolerogenic IL10 CD4 T cell response (Figure 3G). These data show great heterogeneity of the immune response throughout the intestine and identify how both the region and microbiota shape tissue immunity. We observed that the ileum was a region of strong microbiota dependent immune responses characterized by high levels of IFNγ and IL17 T cell responses, GC B cells, and Tfhs. These responses were muted in the proximal intestine and more similar to GF mice. GF mice on the other hand had elevated IgA PCs, CD4 IFNγ+ IELs, and IL10+ CD4 T cells in the proximal small intestine suggesting the duodenum and jejunum induce these immune responses independently of the microbiota. We were intrigued why the small intestine compartmentalized microbiota dependent inflammatory immune responses in the ileum. We sought to further understand the role of regional immunity in homeostasis and disease and the molecular mechanisms driving these processes.

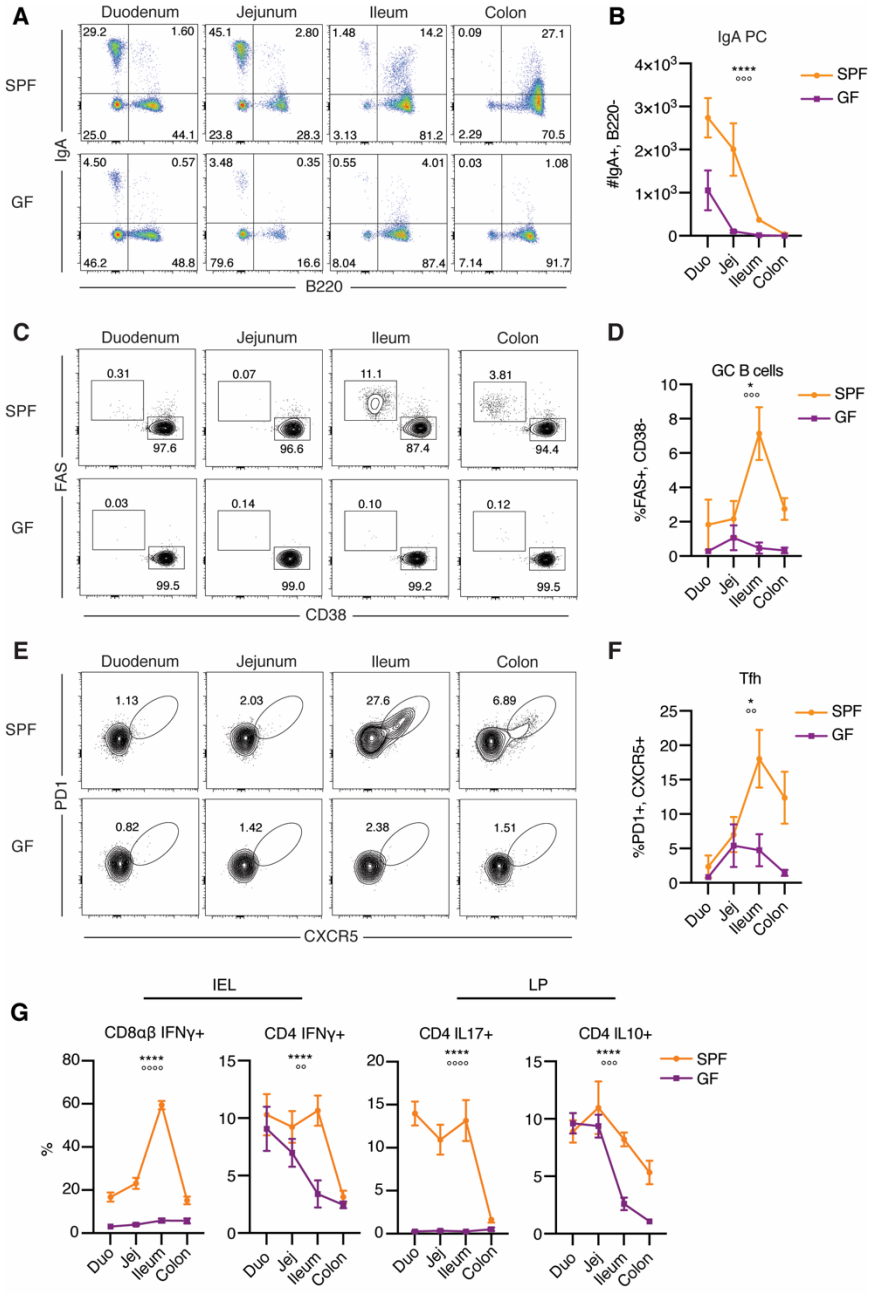

Figure 3. Regionalization of intestinal immunity in SPF and GF mice.

A, B. Flow cytometry of IgA PCs in the LP of the duodenum, jejunum, ileum, and colon of SPF and GF mice. The cells were gated CD45+, lin-, IgA+, B220-. Absolute numbers were calculated by using counting beads. C, D. GC B cells were gated CD19+, FAS+, CD38-. E, F. Tfh were gated TCRαβ+, CD4+, PD1+, CXCR5+. G, Intracellular flow cytometry of PMA/Ionomycin stimulated cells from IEL (left) or LP (right). Frequency of IFNγ+, IL17+, or IL10+ among CD4+, CD8αβ+ T cells. Two-way ANOVA of microbiota and region impact on cells populations or cytokine levels. **** p<0.0001, * p<0.05 effect due to region. °°°° p<0.0001, °°° p<0.001, °° p< 0.01 effect due to microbiota. N= 5-7 mice/group.

3.2 Epithelial regulation of regional immunity and bacterial colonization by GATA4

Summary

The intestine is regionally compartmentalized to provide essential functions for host metabolism. However, the mechanisms involved in these processes and its impact on the mucosal immune response in homeostasis and disease are unclear. Here, we show that intestinal epithelial cells (IECs) through the transcription factor GATA4 control regionalization of tissue immune and metabolic functions in the small intestine. In the absence of GATA4, the regionalization between the jejunum and ileum is lost shifting the balance from host protective immune IgA responses in the proximal intestine towards microbiota dependent inflammatory T cell IFNγ, IL17 responses predominant in the distal small intestine. We find that GATA4 is fundamental to coordinating adherent bacterial colonization and retinol metabolism, which maintains luminal IgA levels and protects proximal IECs from bacterial attachment. This compartmentalization of the proximal small intestine by GATA4 is critical for host response to disease from an enteric pathogen, *Citrobacter rodentium*, and the pathophysiology of celiac disease.

Introduction

The primary purpose of the gastrointestinal tract is to provide efficient digestion and absorption of nutrients necessary to sustain life. Along the intestine, IECs acquire unique genetic programs which drive regional metabolic functions. These metabolic gradients create unique niches which facilitate microbial colonization and support various host immune cells. However, the physiologic impact of intestinal regionalization on mucosal immune homeostasis or disease is largely unexplored, and the mechanisms controlling this remain elusive.

Expression of regionally defined transcription factors (TF) endow IECs with spatial metabolic properties (Thompson et al., 2018). GATA4 is a TF highly expressed in IECs of the duodenum and jejunum and absent from the ileum and colon (Battle et al., 2008; Bosse et al., 2006). It is important in defining the functionality between the proximal and distal small intestine by inducing the expression of jejunal lipid transporters and metabolic enzymes (CD36, NPC1l1, FABP1, CYP27a1) and repressing ileal genes involved in the enterohepatic circulation of bile acids (FABP6, SLC10a2) (Aronson et al., 2014; Battle et al., 2008; Thompson et al., 2017). Deleting GATA4 in IECs results in altered intestinal regionalization, where the jejunum loses its identity and upregulates ileal specific genes (Battle et al., 2008). These results suggest a more profound role of GATA4 on the tissue than was previously described. Deleting GATA4 in the epithelium led to an upregulation of 47% of ileal genes and a decrease in 53% of jejunal genes in the jejunum by total tissue microarray. This would suggest that GATA4 in the epithelium regulates the spatial identity of other cells in the tissue. However, these cellular interactions and the mechanisms how GATA4 controls region specific tissue responses have not been dissected.

The intestine is a reservoir of trillions of microbes and the largest number and diversity of immune cells in the body. These microbes colonize discrete niches and induce local tissue immune responses such as Th17 in the ileum from *Segmented filamentous bacteria*, RORγt+ FOXP3+ Tregs in the colon from *Helicobacter hepaticus*, or IL10+ Tregs in the colon from *Bacteroides fragilis* (Round and Mazmanian, 2010; Sano et al., 2015; Xu et al., 2018). However, the impact of intestinal metabolic regionalization in skewing T cell differentiation and supporting immune cell subsets is less well understood. Regional tissue factors such as the production of serum amyloid A, which is restricted to the ileum of the small intestine and induced by the

microbiota, drive pathogenic Th17 responses (Lee et al., 2020). Furthermore, short chain fatty acids, such as butyrate, generated by commensal *Clostridia* drive Treg differentiation in the colon (Furusawa et al., 2013; Smith et al., 2013). Therefore, region specific metabolic factors and the microbiota contribute to immune homeostasis. Since IECs control regional metabolic gradients in the intestine and express spatial pattern-recognition receptors to sense and respond to commensal microbes, it stands to reason that IECs are a central hub in coordinating regional immune responses in the intestine (Price et al., 2018). However, the molecular mechanisms how IECs accomplish this task are unknown.

Revealing host mechanisms behind regional immune homeostasis is important in understanding site specific intestinal autoimmune and inflammatory diseases. Celiac disease is a gluten dependent enteropathy which manifests as villus atrophy and nutrient malabsorption in the duodenum (Green and Jabri, 2003). Inflammatory bowel diseases such as Crohn's disease and Ulcerative colitis can lead to areas of severe inflammation adjacent to uninvolved normal tissue (Xavier and Podolsky, 2007). In addition, pathogenic bacteria such as Salmonella or Listeria evolved to infect the host through M cells of the Peyer's Patches or enterocytes of the small intestine to cause gastroenteritis (Barbuddhe and Chakraborty, 2009; Jones et al., 1994), whereas adherent-invasive *Escherichia coli* or *Clostridium difficile* colonize the large intestine and drive colitis (Abt et al., 2016; Croxen and Finlay, 2010). Understanding the mechanisms driving regional immune responses in homeostasis and disease will be critical for designing therapies for site specific intestinal diseases.

Here we show that IECs through GATA4 control regional tissue immune responses between the proximal and distal small intestine in a microbiota dependent manner. We observe that the jejunum is largely metabolic dependent, devoid of adherent microbes, and has low

inflammatory T cell responses. This is contrast to the ileum which has a decreased lipid metabolic capacity, and contains adherent microbes which promote inflammatory T cell responses. We reveal that GATA4 is the molecular switch controlling metabolic and immune functions between these regions. In GATA4 deficient mice, adherent commensal and pathogenic bacteria change their regionalization and acquire the capacity to colonize the proximal small intestine to shape local immune responses. We observe that compartmentalization of the intestine by GATA4 is critical for host response to pathogenic infections and is associated with the pathogenesis of celiac disease.

RESULTS

The microbiota regulates regionalization of intestinal immunity

Since commensal microbes and metabolic factors of the tissue each shape local T cell responses, we sought to further understand the impact of the anatomical region and the microbiota in controlling T cell effector gradients along the intestine (Honda and Littman, 2016). Therefore, we profiled cytokine responses by flow cytometry throughout the intestine in both the intraepithelial lymphocyte (IEL) compartment and the underlying lamina propria (LP) in SPF and GF mice. We observed substantial heterogeneity in T cell phenotypes which were either microbiota dependent or independent within a particular region (Figure 4A). Strikingly, we observed the ileum as a region of strong microbiota dependent immunity characterized by high levels of IFNγ+, IL17+, and IL10+ CD4 and CD8αβ T cells which were ablated in the absence of microbes (Figure 4A). Although the colon contains magnitudes more microbes and a greater diversity of species than the small intestine, the inflammatory IFNγ and IL17 cytokine responses were markedly reduced in this region instead skewing the T cell phenotype towards a tolerogenic microbiota induced IL10 Treg response (Figure 4A). These data reveal that there is a gradient of

microbiota induced T cell responses along the axis of the small intestine which peak in the ileum. We were intrigued why the microbiota contributes to an inflammatory T cell response in this region and sought to understand the importance of immune compartmentalization for host intestinal homeostasis.

GATA4 controls regionalization of intestinal immunity

The intestinal epithelial cells (IEC) form a barrier between the microbiota and host tissue and are important for integrating signals bidirectionally between these cells (Haber et al., 2017). We hypothesized that part of this cross-talk involves the coordination of regional immune responses from the microbiota. Expression of the TF GATA4 is regionally defined to the duodenum and jejunum of the proximal small intestine and is absent from the ileum and colon (Figure 5A) (Bosse et al., 2006). In mice where GATA4 is specifically deleted in IECs, GATA4fl/fl villin-cre+ ($GATA4^{\Delta IEC}$), the jejunum loses its identity and gains expression of ileal restricted genes (Battle et al., 2008; Thompson et al., 2017). Therefore, we aimed to use $GATA4^{\Delta IEC}$ mice as a genetic model to alter regionalization in the small intestine and test whether IECs shape regional tissue immunity. We first sorted EPCAM+ CD45- IECs from the jejunum and ileum of WT (GATA4fl/fl villin-cre-) and $GATA4^{\Delta IEC}$ mice and performed RNAseq to determine the impact of GATA4 deficiency in IEC regional gene expression. WT jejunal and ileal epithelial cells segregated mainly on PC1, which accounts for 68% of the variance, and the jejunal GATA4 deficient IECs clustered closely with WT ileum indicating that GATA4 strongly controls regionalization of IEC gene expression (Figure 5B, C). Next we tested the impact of GATA4 deficiency on regional tissue immunity by performing total tissue RNA sequencing. We compiled a list of immune genes from the Immport and Iris database and observed segregation of differentially expressed immune genes between the jejunum and ileum

of WT mice indicating distinct immune responses between these segments of the small intestine (Figure 4B). Shockingly, this regionalization was lost in GATA4$^{\Delta IEC}$ mice and the jejunal tissue clustered with the WT ileum demonstrating that IECs through GATA4 control regionalization of tissue immunity (Figure 4B). We observed 625 immune genes which revealed region specific expression in WT mice that were GATA4 regulated (Figure 4C). 238 of these DE genes were contained within previously published IFNγ and IL17 regulated datasets suggesting that these two immune pathways are region specific and controlled by GATA4. We next analyzed T cell cytokine responses from the jejunum and ileum of WT and GATA4$^{\Delta IEC}$ mice. All WT mice analyzed contained an increased frequency of IFNγ+ CD8αβ IEL T cells in the ileum (66%) than the jejunum (13.2%). However in GATA4$^{\Delta IEC}$ mice, this regionalization was lost and the frequency of IFNγ+ CD8αβ T cells in the jejunum (66%) resembled that of the ileum (61.8%) (Figure 4D). Interestingly, we observed that GATA4 not only normalized the immune responses between the jejunum and ileum, but GATA4 deficiency led to an augmented Th17 response in the jejunum surpassing the level seen in the ileum (Figure 4E). These results reveal that intestinal epithelial cells through GATA4 shape regionalization of tissue immunity and suggest GATA4$^{\Delta IEC}$ mice are a unique model to decipher the mechanisms behind these responses.

GATA4 controls regionalization of intestinal immunity in a microbiota dependent manner

Since we observed that the microbiota shaped regional T cell responses in WT mice (Figure 4A), we asked how the microbiota controlled regionalization of immune responses in a GATA4 dependent manner. The GATA4$^{\Delta IEC}$ mice were rederived germ free and we performed whole tissue RNAseq on the jejunum of SPF vs GF WT and GATA4$^{\Delta IEC}$ mice to determine which region specific immune genes were microbiota dependent or independent. We observed that the upregulated immune genes such as the IFNγ and IL17 signaling pathways in the jejunum

of SPF GATA4$^{\Delta IEC}$ mice were dependent on the microbiota (Figure 4F, 5D). Therefore, the increased IFNγ+ CD8αβ and Th17 cells in the jejunum of SPF GATA4$^{\Delta IEC}$ mice were ablated in GF GATA4$^{\Delta IEC}$ mice (Figure 4G,H). On the other hand, the microbiota independent pathways were largely genes involved in cholesterol and lipid metabolism (Figure 5D). Analysis of a published CHIPseq dataset revealed that there was a preference of GATA4 binding to microbiota independent epithelial genes over the microbiota dependent genes (Figure 5E) (Thompson et al., 2017). This suggests that GATA4 positively regulates the expression of lipid metabolic genes in the absence of the microbiota, but the microbiota is required to drive the upregulated immune signature. There were some microbiota independent region specific immune genes that were directly regulated by GATA4: *IL33*, *IL15*, GM-CSF (*CSF2*), and type I IFN receptor (*IFNAR2*) (Figure 4F). These data collectively show that epithelial cells through GATA4 coordinate the regionalization of intestinal immunity through the microbiota.

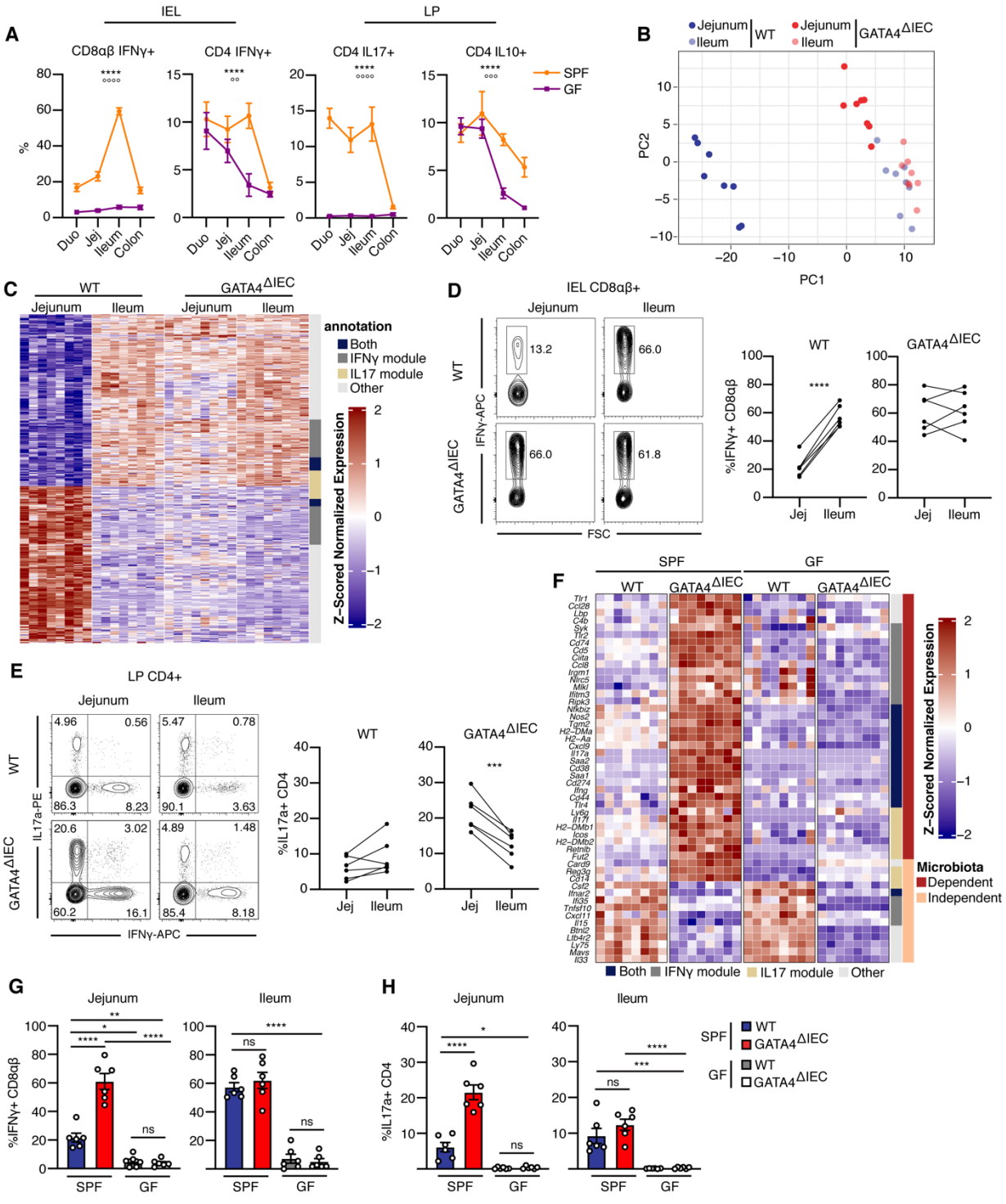

Figure 4. Intestinal epithelial cells control regionalization of tissue immunity through GATA4 and the microbiota.

A. Same as in Figure 3G. Intracellular flow cytometry cytokine straining of phorbol myristate acetate (PMA) and ionomycin stimulated cells taken from the IEL and LP of each intestinal segment of SPF and GF mice.

Figure 4. Intestinal epithelial cells control regionalization of tissue immunity through GATA4 and the microbiota continued.

Two-way ANOVA of microbiota and region impact on cytokine levels **** $p<0.0001$ effect due to region , ^{oooo} $p<0.0001$, ^{ooo} $p<0.001$, ^{oo} $p< 0.01$ effect due to microbiota. N= 5-7 mice/group. B. Principal coordinate analysis of region specific immune genes taken from the Immport and Iris database of tissue RNAseq from WT and GATA4$^{\Delta IEC}$ mice. N= 8 mice/group. C. Heatmap of DE region specific immune genes regulated by GATA4. 625 total genes, 145 present in the IFNγ module, 54 IL17, 39 both, and 387 other. N= 8 mice/group. D. Frequency of IFNγ+ among CD8αβ T cells in the IEL of the jejunum and ileum of WT and GATA4$^{\Delta IEC}$ mice. **** $p<0.0001$ paired t-test. N= 6 mice/group E. Frequency of IL17a+ among CD4 T cells in the LP of the jejunum and ileum of WT and GATA4$^{\Delta IEC}$ mice. *** $p<0.001$ paired t-test. N= 6 mice/group. F. Heatmap of 50 selected microbiota dependent and independent regional specific immune genes in the jejunum of SPF and GF WT and GATA4$^{\Delta IEC}$ mice. IL17 and IFNγ regulated gene modules were generated using IL17 and IFNγ signaling pathways from KEGG and KO studies compiled from MsigDB. G, H. Frequency of IFNγ+ or IL17a+ T cells in the jejunum and ileum of SPF and GF WT and GATA4$^{\Delta IEC}$ mice. ANOVA with Tukey multiple comparison test **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$. N= 6 mice/group

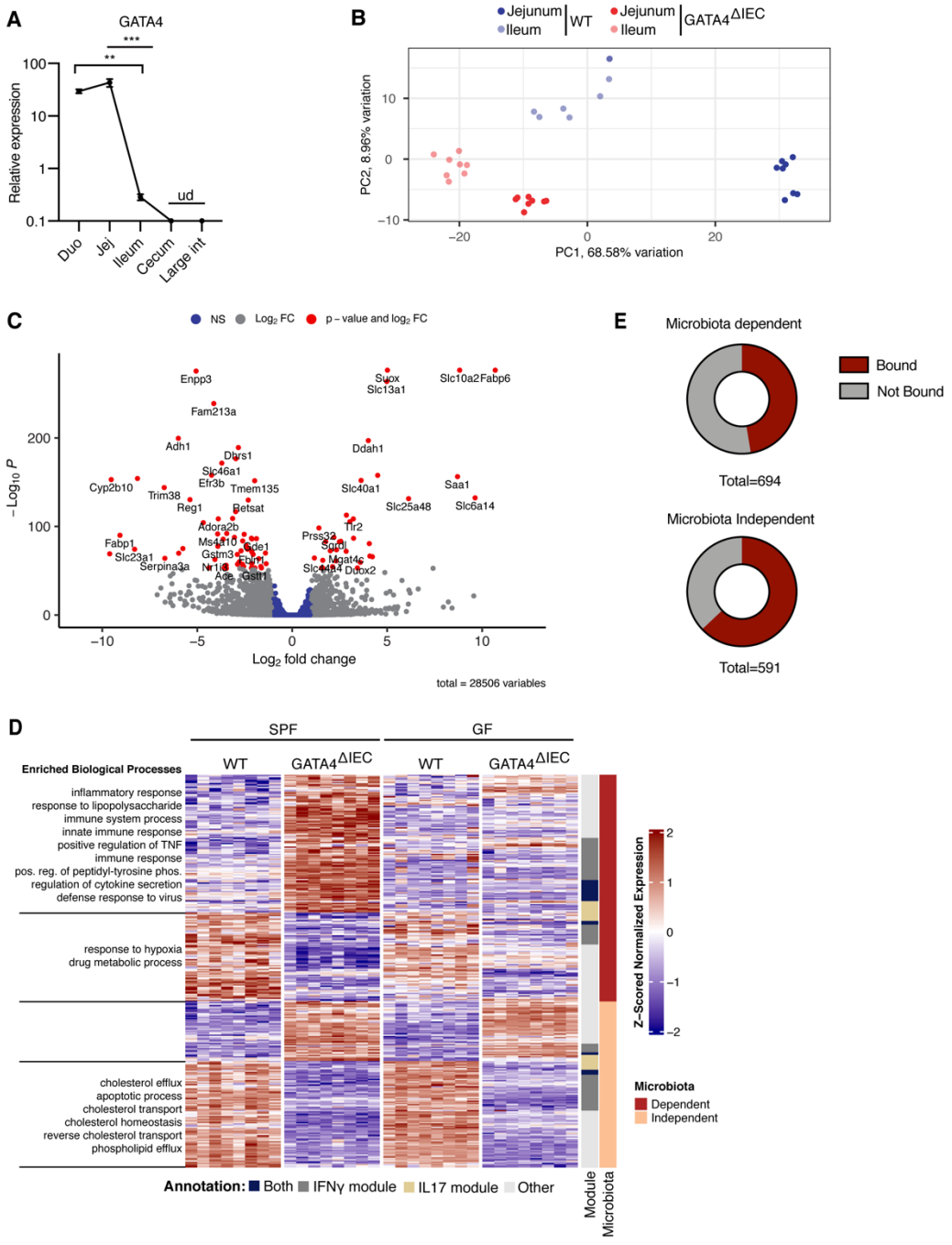

Figure 5. Supplemental GATA4 controls regionalization of small intestinal gene expression

A. qPCR of GATA4 expression relative to GAPDH in the various regions of the intestine. B. PCA of DE genes from sorted Epcam+ CD45- IECs of WT and GATA4$^{\Delta IEC}$ mice jejunum and ileum. C. Volcano plot of region specific GATA4 regulated genes. D. Microbiota dependent or independent region specific GATA4 regulated genes. E. Frequency of GATA4 bound or unbound microbiota dependent or independent genes as determined by CHIPseq. 63% of microbiota independent genes were GATA4 bound compared to 47% of microbiota dependent. ****, p<0.0001, Fisher exact test.

GATA4 controls the colonization of *Segmented filamentous bacteria* to shape tissue immunity

We next sought to determine which microbes were responsible for the upregulated immune phenotype of the GATA4 deficient mice. We performed 16s rRNA sequencing from jejunal and ileal luminal content and mucosal scrapings taken from WT and GATA4 deficient mice. *Segmented filamentous bacteria* (SFB) was the only taxa in our mouse colony which showed regional specificity between the jejunum and ileum preferentially colonizing the ileal mucosa of WT mice. We observed that GATA4 strongly regulated the colonization of SFB in the jejunal mucosa (Figure 6A, 7A). In WT mice, the jejunum is largely devoid of SFB adherence (Figure 6B). However, in the absence of GATA4, SFB's niche expanded and the bacteria gained the capacity to colonize and adhere to the IECs of the jejunum (Figure 6B). The load of SFB expanded up to 1000 fold in the jejunum of GATA4$^{\Delta IEC}$ mice comparable to the amount in the ileum of WT mice (Figure 6C). We did not observe any changes of the ileal microbiota (Figure 7A). These data indicate that epithelial cells control the regionalization of SFB colonization, through GATA4.

SFB has a unique ability to adhere to epithelial cells and its colonization triggers the upregulation of various adaptive immune pathways: IL17 and IFNγ T cell responses, recruitment of CD8αβ IELs, and production of IgA+ plasma cells to name a few (Atarashi et al., 2015; Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Umesaki et al., 1999). We asked whether the GATA4 deficient IECs enabled the adherence of SFB and whether this permitted the jejunal colonization of the bacteria. Therefore, we monocolonized GF WT and GATA4$^{\Delta IEC}$ mice with SFB taken from rats (rat SFB) which is genetically similar to mouse SFB but does not adhere to epithelial cells in mice (Prakash et al., 2011). Rat SFB was unable to expand in the jejunum of

GATA4 deficient mice revealing an intriguing relationship between GATA4 deficient IECs and SFB adherence in proximal small intestinal colonization (Figure 6D).

We reasoned that the upregulated inflammatory immunity in the jejunum of GATA4$^{\Delta IEC}$ mice could be explained by either the expansion of SFB or nonspecific immune activation towards any particular microbes. Therefore, to distinguish between these two possibilities we performed microbial transfers where we first transferred an irrelevant minimal microbial consortium, Altered Schaedlers Flora (ASF). ASF was unable to stimulate an inflammatory immune response in the GATA4$^{\Delta IEC}$ mice suggesting that specific microbes are involved (Figure 7B, C). Next, we tested whether a microbe intrinsic to the GATA4 microbiota was involved by transferring the jejunal microbiota from WT or GATA4$^{\Delta IEC}$ mice into GF WT or GATA4$^{\Delta IEC}$ hosts and observed the host genotype determined the immune outcome irrespective of the input microbial community (Figure 7B, C). These results suggest a common microbe is responsible and since SFB is found in both WT and GATA4$^{\Delta IEC}$ mice, we shifted our focus to the role of this microbe in the dysregulated immune response of GATA4 deficient mice. We transferred the microbiota from JAX mice, which lack SFB, or JAX + SFB to GF WT and GATA4$^{\Delta IEC}$ hosts. JAX colonized mice showed a low level of IFNγ+ CD8αβ T cells and Th17 cells in the jejunum of both WT and GATA4$^{\Delta IEC}$ mice (Figure 6E, F). However, the addition of SFB to JAX microbiota was sufficient to lead to an increased IFNγ and IL17 response in GATA4$^{\Delta IEC}$ mice to the levels observed in SPF mice (Figure 6E, F). In addition to JAX microbiota, we also transferred jejunal microbiota from a WT donor within our colony with low to undetectable levels of SFB and observed no difference in the T cell responses between WT and GATA4$^{\Delta IEC}$ mice yielding further support to SFB as the causative microbe in the dysregulated immune response of GATA4 deficient mice (Figure 7B, C). The jejunal colonization and adherence of

SFB in monoassociated ex GF GATA4$^{\Delta IEC}$ mice was sufficient to induce an IFNγ and IL17 response but nonadherent Rat SFB was unable to amount an appreciable response in the proximal intestine (Figure 6G, H, 7B, C).

SFB colonization leads to an antigen specific Th17 cell response against the 3340 epitope of SFB (Yang et al., 2014). To determine whether the increased Th17 cell response observed in the jejunum was a consequence of altered T cell priming we transferred congenically marked CD45.1+, 7B8+ CD4 T cells, specific to the 3340 epitope of SFB, to WT and GATA4$^{\Delta IEC}$ mice. Three days after transfer, we observed the specificity of T cell priming in the ileal draining mesenteric lymph node (MLN) in WT mice was lost in GATA4$^{\Delta IEC}$ mice (Figure 6I). There was an expansion in the number of transferred 7B8+ T cells in the jejunal draining MLN to the level seen in WT ileum. These cells strongly upregulated RORγt to a level higher than what was observed in the WT mice (Figure 6J). Nine days after transfer, we observed an expansion of 7B8+ T cells in the jejunum of GATA4$^{\Delta IEC}$ mice coupled with a downregulation of the TCR indicative of increased T cell activation (Figure 6K). These results collectively show that IECs through GATA4 regulate the regional colonization of SFB which in turn impact host T cell priming and tissue immunity.

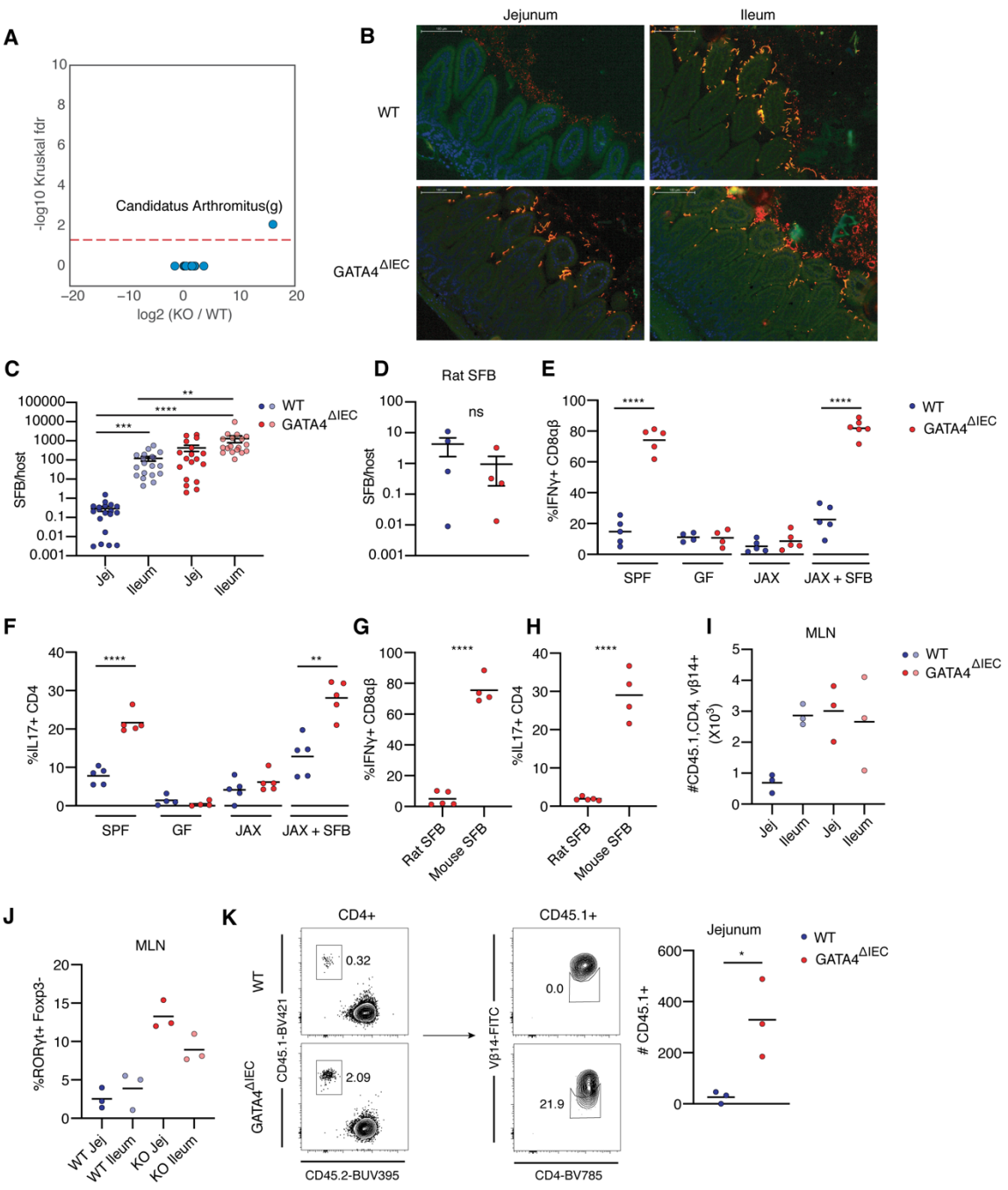

Figure 6. GATA4 controls the colonization of *Segmented filamentous bacteria* to shape tissue immunity.

A. 16s rRNA sequencing of mucosal scrapings from the jejunum of WT and GATA4$^{\Delta IEC}$ mice. B. Representative FISH staining of jejunal and ileal tissue of WT and GATA4$^{\Delta IEC}$ mice. Alexa 488 is SFB and Alexa 546 is universal 16s probe. The overlay represents SFB and is orange. C. qPCR of SFB levels relative to the amount of host DNA in mucosal scrapings of jejunum and ileum from WT and GATA4$^{\Delta IEC}$ mice.

Figure 6. GATA4 controls the colonization of *Segmented filamentous bacteria* to shape tissue immunity continued.

Kruskal-Wallis with Dunn multiple comparison test **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$. N= 18-19 mice/group. D. qPCR of rat SFB levels in the jejunum of WT and GATA4$^{\Delta IEC}$ mice. E, F. Frequency of IFNγ+ or IL17a+ CD8αβ or CD4 T cells in the jejunum of SPF, GF, JAX microbiota transfer, and JAX + SFB transfer WT and GATA4$^{\Delta IEC}$ mice. G, H. Frequency of IFNγ+ or IL17a+ CD8αβ or CD4 T cells in the jejunum of rat or mouse SFB monocolonized WT and GATA4$^{\Delta IEC}$ mice. N= 4-6 mice/group. **** $p<0.0001$, ** $p<0.01$ t-test. I. Absolute number of transferred CD45.1, CD4+, Vβ14+, 7B8 T cells in the jejunal and ileal draining MLN in WT and GATA4$^{\Delta IEC}$ mice. J. Frequency of RORγt+ FOXP3- cells as described in I. K. 7B8 SFB T cell transfer 9 days later in the jejunal LP to determine the absolute number and downregulation of the TCR. * t-test $p<0.05$

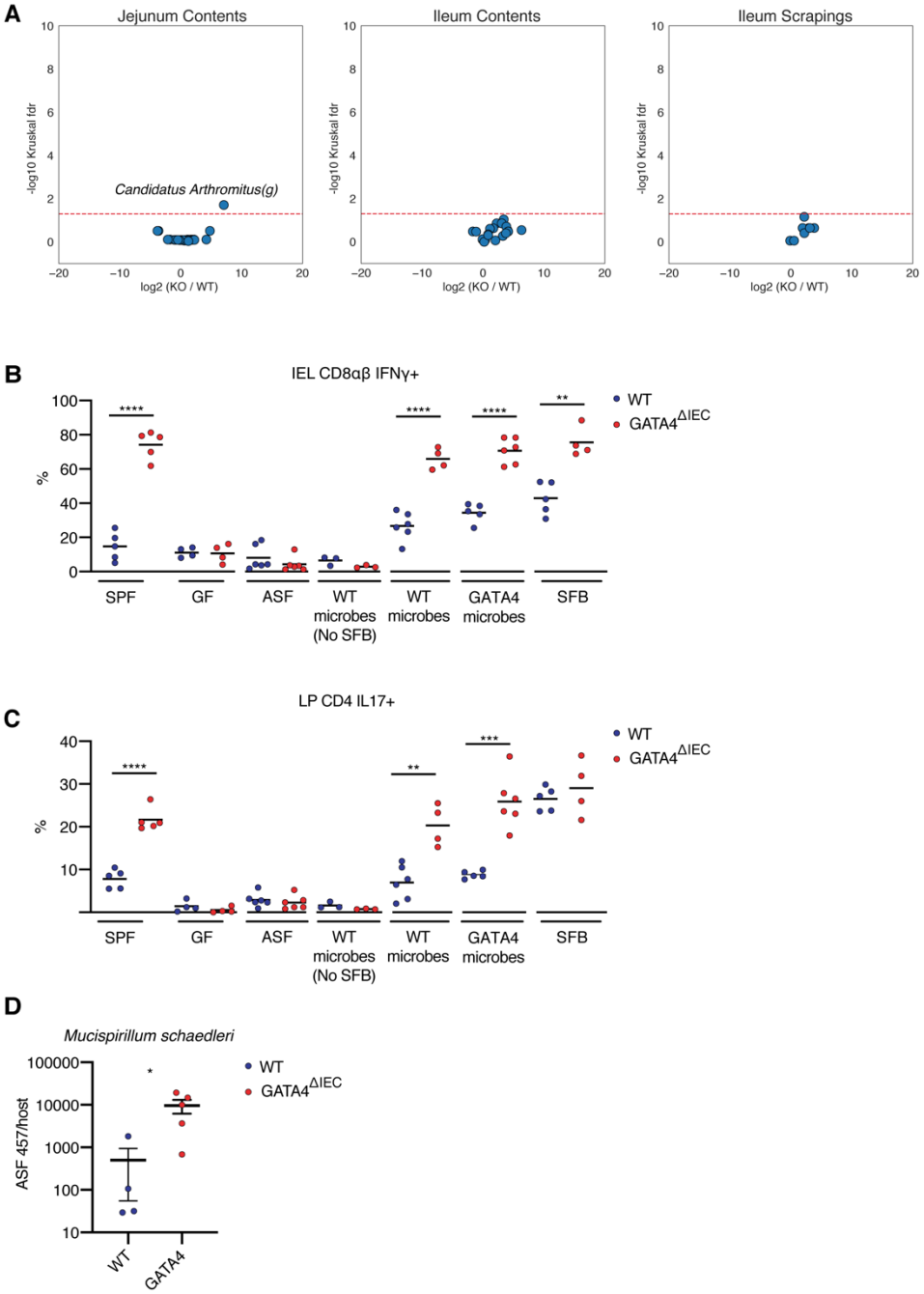

Figure 7. Supplemental GATA4 controls regionalization of mucosal associated bacteria

A. Volcano plot of taxa identified by 16s sequencing from jejunal, and ileal content and ileal scrapings. *Candidatus arthomitus* or SFB was the only significant taxa as determined by Kruskal-Wallis test expanding in the jejunum of GATA4 deficient mice. B. Frequency of IFNγ+ CD8αβ cells in the jejunum of ex GF WT and GATA4$^{\Delta IEC}$ mice receiving the indicated bacteria on the x axis. C. Same as described in B. except frequency of IL17+ CD4 T cells. D. qPCR of *Mucispirillum schaedleri* load relative to host (ASL) DNA in the jejunum following ASF transfer. **** p<0.0001 , *** p<0.001, ** p<0.01, * p<0.05. t-test B, C. Mann-Whitney, D.

GATA4 regulates the colonization of other adherent microbes

A particular feature of SFB is its ability to adhere to IECs and we were intrigued of the possibility that GATA4 regulated the colonization of other adherent or mucosal associated microbes. We observed an expansion of another mucosal associated commensal microbe, *Mucispirillum ASF 457* in the jejunum of GATA4$^{\Delta IEC}$ mice (Figure 7D). These data provided further evidence that GATA4 regulates the colonization of adherent or mucosal associated bacteria. Therefore, we infected WT or GATA4$^{\Delta IEC}$ mice with an adherent pathogen, *Citrobacter rodentium* which regularly colonizes the colon of WT mice (Figure 8A). In the absence of GATA4, the niche for *C. rodentium* was altered. The pathogen acquired the ability to colonize the small intestine, the jejunum and ileum, of GATA4$^{\Delta IEC}$ mice to levels comparable to WT colon. We tested whether *C. rodentium's* ability to adhere to epithelial cells allowed for it to expand in the small intestine of GATA4$^{\Delta IEC}$ mice. We infected GF WT or GATA4$^{\Delta IEC}$ mice with WT or adhesion deficient ΔEAE *C. rodentium*, which lacks intimin the gene required for adherence to intestinal epithelial cells. We observed that both strains colonized the same level in the colon of the GF WT and GATA4$^{\Delta IEC}$ mice indicating no colonization defect in the mutant strain of these GF hosts (Figure 8B). However, only the WT *C. rodentium* was able to expand in the jejunum and ileum of GATA4$^{\Delta IEC}$ mice and the GATA4 deficient mice infected with ΔEAE reverted to the same levels as WT mice infected with WT *C. rodentium* (Figure 8B). Importantly, this suggests that adherence is required for small intestinal colonization in GATA4 deficient hosts and further shows that GATA4 controls the colonization of both commensal and pathogenic microbes.

Loss of small intestinal regionalization increases the host's susceptibility to pathogenic infections and colitis

Since *C. rodentium*'s colonization was altered in GATA4 deficient hosts, we pursued the impact of this altered regionalization on host pathology to an infectious pathogen. We observed severe colitis in the colon and villous atrophy of the ileum in GATA4$^{\Delta IEC}$ mice ten days after infection (Figure 8C). Of note, destruction of small intestinal tissue does not occur in WT mice since this pathogen regularly colonizes the colon. *C. rodentium* infection leads to an increased Th17 response in the colon of WT animals. We asked how the infection alters regionalization of T cell responses in the ileum and colon of GATA4$^{\Delta IEC}$ mice since these regions were the most severely affected. Importantly, in uninfected mice there are low to undetectable levels of Th17 cells in the colon of these mice (Figure 8D left). However, after infection we observed a striking expansion of IL17+ IFNγ-, or IL17- IFNγ+ single positive and IL17+ and IFNγ+ double positive, CD4 T cells in GATA4$^{\Delta IEC}$ mice (Figure 8D right). In addition, these inflammatory T cell responses in GATA4$^{\Delta IEC}$ mice were also observed in the ileum to the same extent as the colon, whereas WT mice showed a moderate Th17 expansion restricted to the colon (Figure 8D right). With the intestinal barrier compromised, there was translocation of *C. rodentium* to systemic sites: mesenteric lymph nodes, liver, and spleen in GATA4$^{\Delta IEC}$ mice. These mice had an increased magnitude and number of positive cultures of systemic pathogen (Figure 8E). The amount of systemic bacteria overwhelmed the host and led to sepsis and high levels of mortality (80%) of GATA4$^{\Delta IEC}$ mice (Figure 8F). These data show that by altering the colonization of *C. rodentium* by GATA4 deficiency it leads to increased pathology of the small and large intestine, loss of regional inflammatory T cell responses, bacterial translocation, and increased mortality of the host.

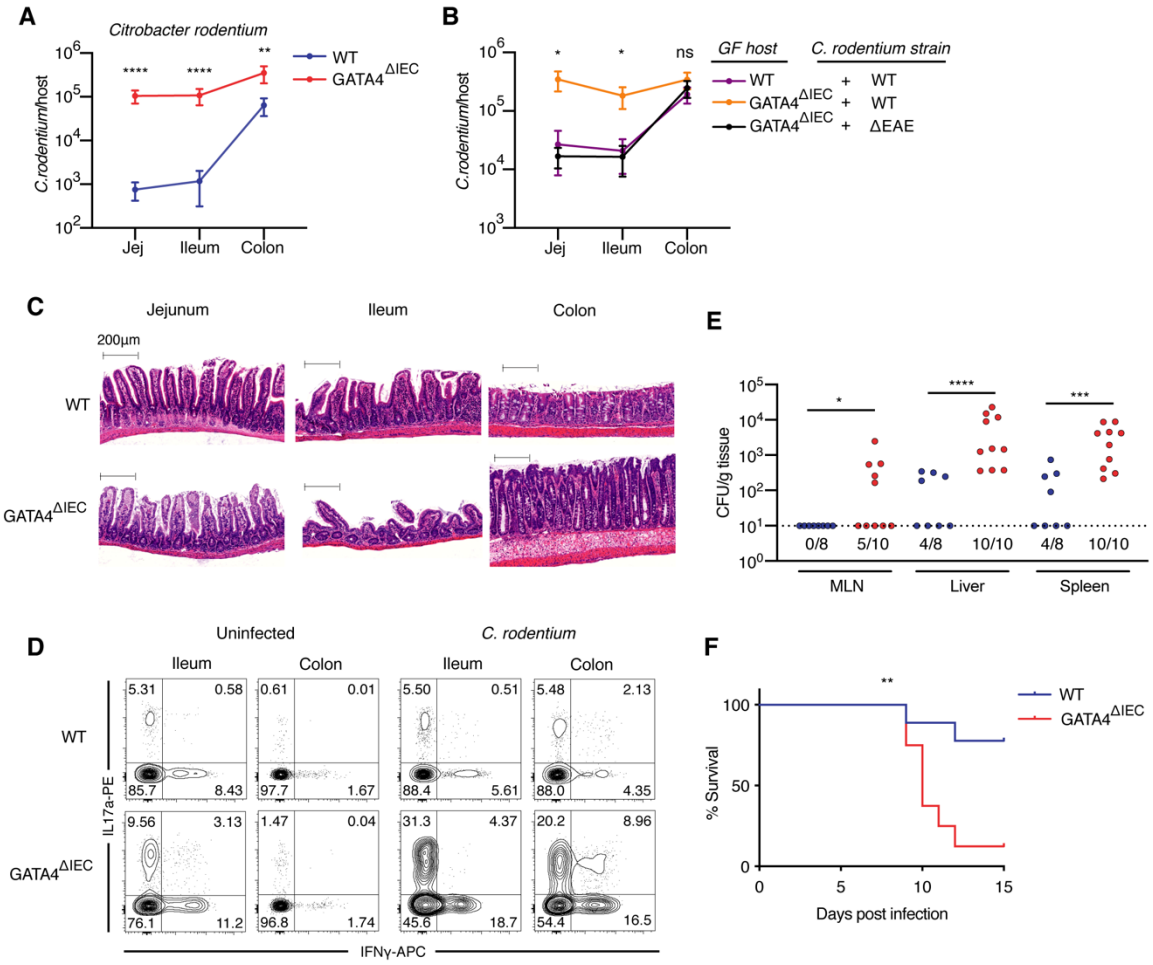

Figure 8. Small intestinal regionalization impacts host susceptibility to enteropathogenic infection by *Citrobacter rodentium*.

A. Colonization of *C. rodentium* load relative to host in each segment of the intestine in WT and GATA4$^{\Delta IEC}$ mice. **** p<0.0001, ** p<0.01 Mann-Whitney test. N= 13 mice/group. B. Colonization of WT and ΔEAE load relative to host in each segment of the intestine in either GF WT or GATA4$^{\Delta IEC}$ mice. * p<0.05 Kruskal-Wallis with Dunn multiple comparison test GF WT + WT *C. rodentium* vs GF GATA4$^{\Delta IEC}$ + WT *C. rodentium*. N= 7-9 mice/group. C. Representative IHC of each intestinal region 10 days after infection. D. Representative FACS plot of IL17a by IFNγ in CD4+ T cells from the LP after PMA ionomycin stimulation in uninfected or infected mice 10 days after infection. E. Bacterial translocation of *C. rodentium* 10 days after infection to MLN, liver, and spleen. N= 8-10 mice/group. **** p<0.0001, *** p<0.001, * p<0.05 Mann-Whitney test. F. Percent survival of WT and GATA4$^{\Delta IEC}$ mice 15 days post *C. rodentium* infection. ** p<0.01 Mantel-Cox test. N= 8-9 mice/ group.

GATA4 regulates luminal IgA levels to control regionalization of adherent bacterial colonization

We next sought to understand how epithelial cells through GATA4 restrict adherent bacterial colonization and inflammatory immunity in the proximal intestine. A previous report identified a decrease of GATA4 regulated lipid metabolic genes in the jejunum of B cell deficient mice (Shulzhenko et al., 2011). Comparison of GATA4$^{\Delta IEC}$ mice to B cell deficient mice revealed that 60% of the B cell gene signature was retained in GATA4$^{\Delta IEC}$ mice (Shulzhenko et al., 2011). We reasoned that this correlation of gene expression changes could be explained by a B cell deficiency in GATA4$^{\Delta IEC}$ mice. Therefore, we tested whether B cells control regionalization of adherent bacterial colonization and tissue immune responses as observed in GATA4$^{\Delta IEC}$ mice. To determine the role of B cells on adherent bacterial colonization, we monocolonized GF Jh-/- mice with SFB. The specificity of SFB's ileal colonization in littermate Jh+/- mice was lost in Jh-/- mice and SFB colonized the jejunum to the same level as the control ileum (Figure 9A, C). Next, we tested whether B cells controlled the regionalization of *C. rodentium*. We observed an expansion of *C. rodentium* in the small intestine similar to GATA4$^{\Delta IEC}$ mice suggesting that B cells are required in controlling the colonization of adherent bacteria (Figure 10A). In addition, we observed that B cells were required in controlling the regionalization of IFNγ T cells responses. In SFB monocolonized B cell deficient mice, we observed an increased frequency of IFNγ+ CD8αβ IELs in the jejunum of Jh-/- mice to the same level as the control ileum (Figure 10B). Since a key effector function of B cells is the production of luminal secretory IgA which targets the microbiota, we asked whether IgA controlled the regionalization of SFB colonization. We observed jejunal colonization and adherence of SFB in IgA-/- mice to the same level as the control ileum (Figure 9B, D). Unlike

Jh-/- mice, IgA was not required in controlling the regionalization of *C. rodentium*, or the host immune response (Figure 10E, F). IgA controlled the colonization of *C. rodentium* in the colon in an adhesion dependent manner but was not sufficient to allow the pathogen to expand to the small intestine (Figure 10D). These data show that B cells and IgA control the niche of SFB regionalization and exclude this microbe from adhering to proximal IECs. In addition, B cells are responsible for suppressing activation of IFNγ+ T cell responses in the proximal intestine in an IgA independent manner.

How IECs control regionalization of B cell responses in the intestine is not well understood. Therefore, we asked whether GATA4 regulated the number of IgA+ plasma cells in the lamina propria. Flow cytometry staining of tissue taken from the jejunum and ileum revealed that the jejunum contained a 3 fold higher absolute number of IgA+ B220- plasma cells than the ileum (Figure 9E). This contributed to a greater capacity of jejunal tissue to produce IgA in tissue explants than the ileum (Figure 9F). Interestingly, we observed that GATA4 regulated both the number of IgA+ B220- plasma cells and the production of IgA in the jejunum (Figure 9E, F). Therefore the regionalization of IgA responses between the jejunum and ileum as observed in WT mice is lost in the GATA4$^{\Delta IEC}$ mice. This blunted IgA response contributed to a substantial decrease of luminal secretory IgA in jejunal and ileal contents of GATA4$^{\Delta IEC}$ mice as measured by ELISA (Figure 9G, H). We also noted a decrease in luminal IgA in the jejunum and ileum of GF GATA4$^{\Delta IEC}$ mice suggesting that this decrease is independent of the microbiota (Figure 10G, H). Intrigued as to whether this luminal IgA deficiency impacted the ability of GATA4$^{\Delta IEC}$ mice to coat the microbiota, we stained IgA uncoated microbiota taken from RAG-/- mice with free IgA taken from content supernatant of WT and GATA4$^{\Delta IEC}$ mice. We observed a decreased frequency and level of IgA coating by MFI of microbes from RAG-/- host stained by

GATA4$^{\Delta IEC}$ mice as compared to littermate controls (Figure 9I). The inability of GATA4$^{\Delta IEC}$ mice to coat the microbiota may be a reason why specific IgA dependent adherent microbes, such as SFB, are able to gain a competitive advantage and expand in an otherwise IgA protected region such as the jejunum.

Therefore to test the requirement of IgA and GATA4 in controlling SFB colonization, we supplemented IgA orally in GATA4$^{\Delta IEC}$ mice. We isolated polyclonal secretory IgA from luminal contents of adult WT mice with protein L magnetic beads (Figure 10I). Our isolated IgA was functional and showed a strong capacity to coat bacteria from RAG-/- mice (Figure 10I). We pretreated GF WT and GATA4$^{\Delta IEC}$ mice with IgA by gavage and then colonized the mice with SFB, and gavaged IgA three consecutive times every two hours (Figure 9L). Twenty four hours later we assessed the regionalization of SFB colonization by qPCR in mucosal scrapings. We observed that IgA treatment restored SFB colonization to WT levels in both the jejunum and ileum of GATA4$^{\Delta IEC}$ mice (Figure 9M, N). Therefore, we discovered that IECs through GATA4 control regionalization of IgA plasma cells and luminal IgA which block the proximal intestine from colonization of adherent bacteria such as SFB. Furthermore, B cell deficient but not IgA deficient mice were sufficient to explain the loss of regional immunity seen in GATA4$^{\Delta IEC}$ mice suggesting that B cells may regulate other pathways that impact regional immunity in addition to luminal IgA production.

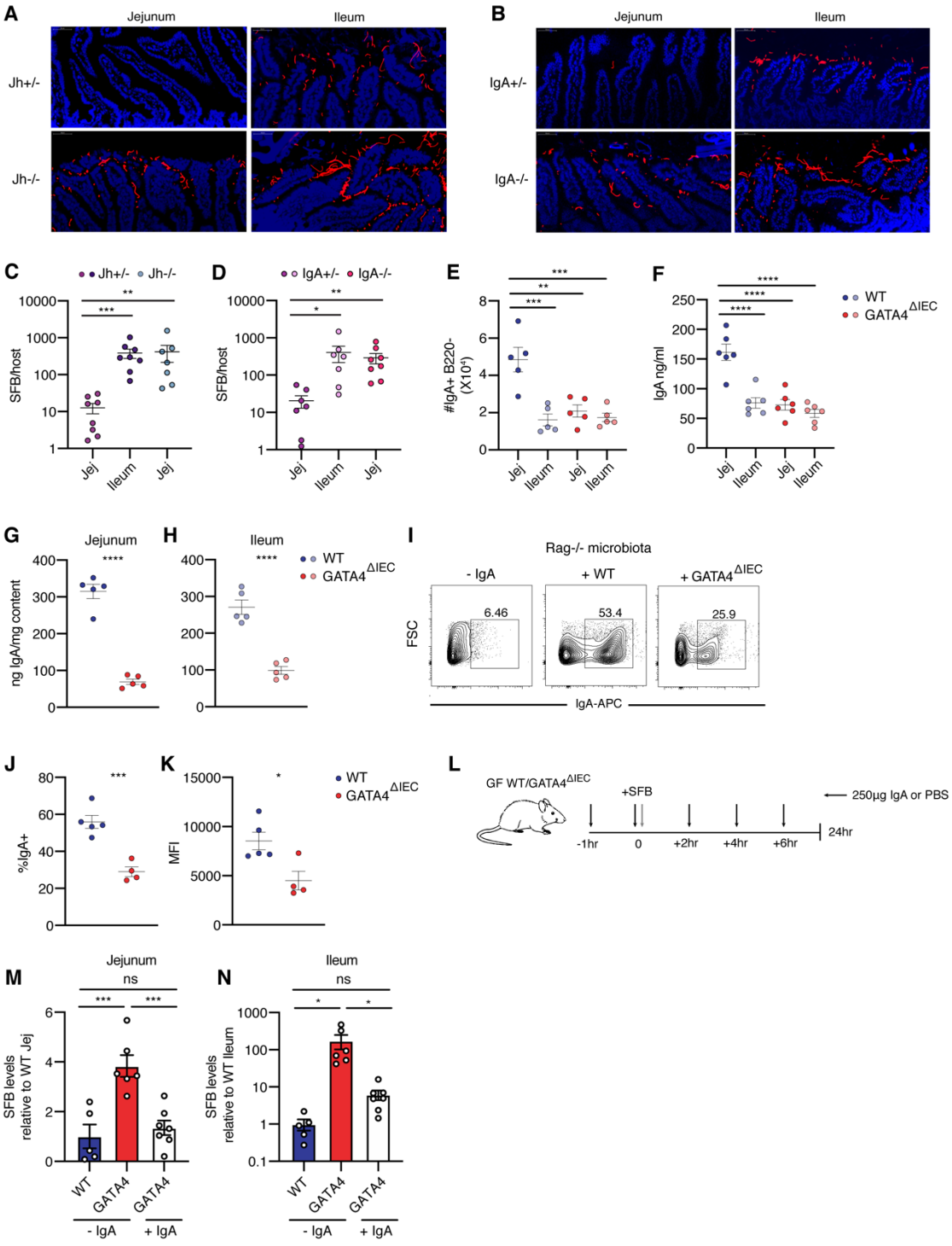

Figure 9. GATA4 regulates regionalization of IgA to limit bacterial adhesion to proximal intestinal epithelial cells.

A. FISH staining of SFB from jejunal and ileal tissue of monocolonized B cell deficient (Jh-/-) and littermate control (Jh+/-). Red is SFB and stained with Cy5 conjugated SFB specific 16s probes. Blue is DAPI counterstain.

Figure 9. GATA4 regulates regionalization of IgA to limit bacterial adhesion to proximal intestinal epithelial cells continued.

B. Same as in A. except in IgA deficient (IgA-/-) and littermate control mice (IgA+/-). C. qPCR of SFB load relative to host DNA in the jejunum and ileum of Jh+/-, and jejunum of Jh-/-. *** $p<0.001$, ** $p<0.01$ Kruskal-Wallis with Dunn multiple comparison test. N= 7-8 mice/group. D. Same as in C. except in IgA deficient mice. ** $p<0.01$, * $p<0.05$ Kruskal-Wallis with Dunn multiple comparison. N= 7-8 mice/group. E. Absolute number of CD45+, lin-, IgA+, B220- plasma cells by FACS in 10cm of tissue taken from the jejunum and ileum of WT and $GATA4^{\Delta IEC}$ mice. *** $p<0.001$, ** $p<0.01$ ANOVA with Tukey multiple comparison test. N= 5 mice/group. F. Amount of IgA (ng/ml) by ELISA in culture supernatant of tissue explants from the jejunum and ileum of WT and $GATA4^{\Delta IEC}$ mice 24 hours later. **** $p<0.0001$ ANOVA with Tukey multiple comparison test. N= 6 mice/group. G, H. Amount of IgA by ELISA in jejunal or ileal content of WT and $GATA4^{\Delta IEC}$. **** $p<0.0001$ t-test. I. Representative FACS plot showing frequency of IgA coating among SYTOBC+ RAG-/- microbes and stained with supernatant from WT and $GATA4^{\Delta IEC}$ content. J. Frequency of IgA+ microbes as described in I. *** $p<0.001$, t-test. N= 4-5 mice/group. K. MFI of IgA+ as described in I. * $p<0.05$, t-test. N= 4-5 mice/group. L. Experimental scheme of IgA gavage and SFB colonization experiment in GF WT and $GATA4^{\Delta IEC}$ mice. M. qPCR of SFB colonization in jejunal mucosal scrapings expressed relative to WT jejunum. *** $p<0.001$, ANOVA with Tukey multiple comparison test. N= 5-7 mice/group. N. qPCR of SFB colonization in ileal mucosal scrapings expressed relative to WT ileum. * $p<0.05$, ANOVA with Tukey multiple comparison test. N= 5-7 mice/group.

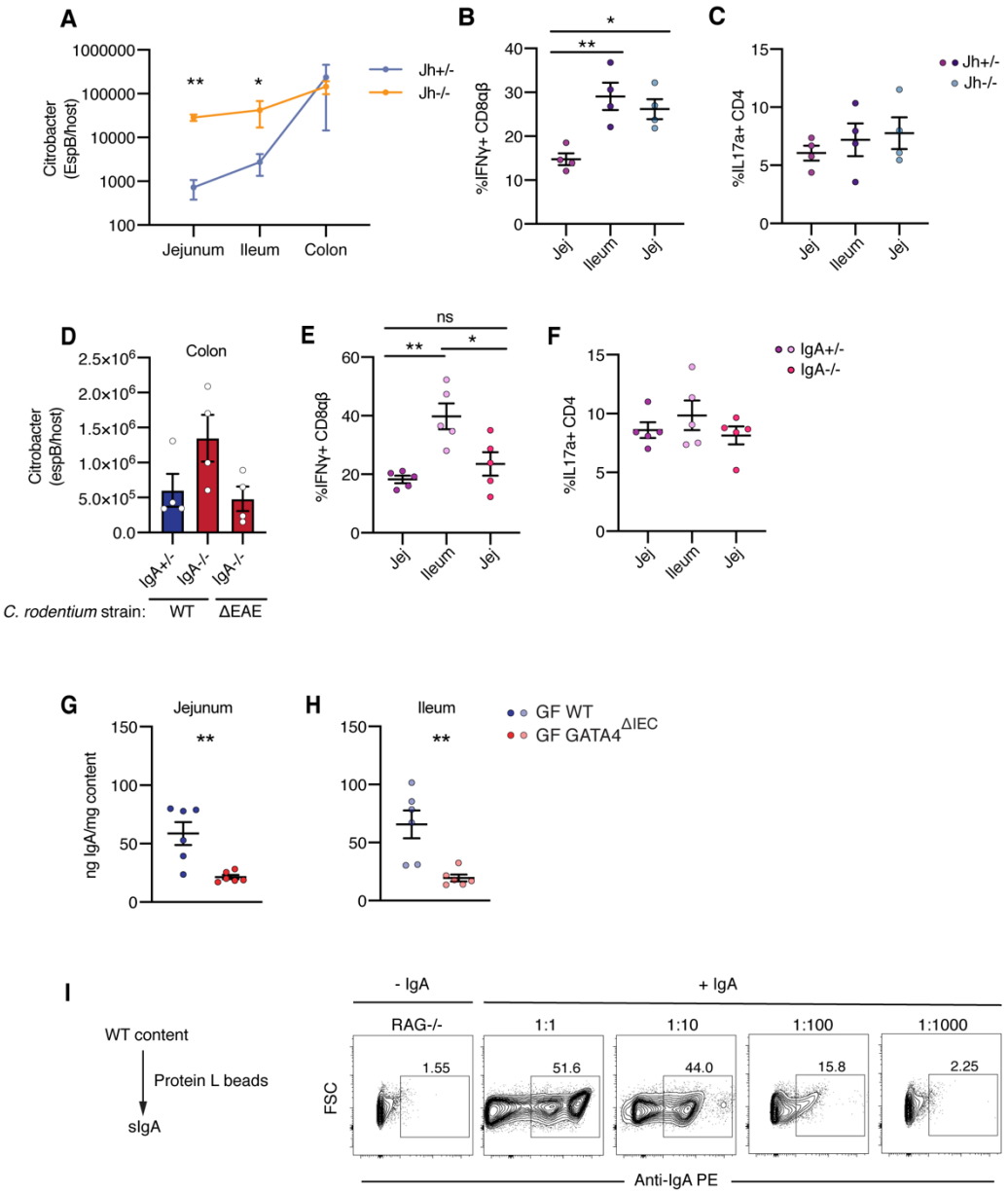

Figure 10. Supplemental B cells and IgA regulation of *C. rodentium* colonization and regional immunity.

A. *C. rodentium* load relative to host DNA in the jejunum, ileum, and colon of Jh+/- and Jh-/-. ** p<0.01, * p<0.05. Mann-whitney. B. Frequency of IFNγ+ CD8αβ T cells in the jejunum and ileum of Jh+/- and jejunum of Jh-/- mice ** p<0.01, * p<0.05 ANOVA with Tukey multiple comparison test. C. Same as in B except IL17+ CD4 T cells. D. WT or ΔEAE *C. rodentium* load relative to host DNA in the colon of IgA+/- and IgA-/- mice. E, F. same experiment as B, C except in IgA-/- mice. G, H Luminal IgA levels in the jejunum and ileum of GF WT and GATA4$^{\Delta IEC}$ mice. ** p<0.01 t-test. I. Isolation of sIgA from intestinal contents of WT mice and staining feces from RAG-/- mice at the indicated concentrations followed by anti-IgA PE staining. Bacteria were pregated SYTO BC+.

Epithelial cells control regionalization of retinol metabolism by GATA4 to maintain luminal IgA levels

Observing that IECs regulate the regionalization of IgA PCs and luminal IgA led us to discover further GATA4 dependent mechanisms which allow IECs to control IgA levels in the proximal intestine. We sorted Epcam+ CD45- IECs from the jejunum and ileum of WT and GATA4$^{\Delta IEC}$ mice and performed RNA sequencing. We looked for pathways which were differentially expressed in the jejunum and ileum and regulated by GATA4. A top hit revealed retinol metabolism as a significant region specific GATA4 controlled pathway (Figure 11A). Retinoic acid has been previously shown to have a multitude of immunomodulatory effects such as regulating Foxp3 CD4 T cell differentiation, T cell effector functions, upregulation of intestinal homing receptors, and modulating B cell responses and IgA class switching (DePaolo et al., 2011; Hall et al., 2011; Mora et al., 2006; Sun et al., 2007). How IECs control retinol metabolism and coordinate with DCs to regulate tissue RA levels is not well understood. We discovered that GATA4 is an essential epithelial TF which maintains the regionalization and expression of numerous genes in the retinol pathway. In the absence of GATA4, the regionalization of genes involved in retinol metabolism between the jejunum and ileum is lost (Figure 11B). Using a published CHIPseq dataset from the jejunum of GATA4 biotinylated mice revealed that GATA4 bound to the promoters of 23/35 (66%) differentially expressed genes in the retinol pathway (Figure 11B) (Thompson et al., 2017). To determine whether this decrease of gene expression contributed to a functional change in the enzymes involved in retinoic acid production, we performed an ALDH fluorometric assay on epithelial cells taken from the jejunum and ileum of WT and GATA4$^{\Delta IEC}$ mice. We identified that GATA4 deficient jejunal IECs have less ALDH enzyme activity and resemble the levels of the WT ileum (Figure 11C, D).

Therefore, we asked whether the epithelial deficiency in RA metabolism seen in GATA4 deficient mice regulated luminal IgA levels. We treated GATA4$^{\Delta IEC}$ mice with RA and observed a partial restoration of luminal IgA levels two weeks later (Figure 11E). We did not see a change in the number of IgA+ B220- plasma cells in the tissue of GATA4$^{\Delta IEC}$ mice following RA treatment (Figure 11F). Since RA treatment did not change the number of IgA PCs in GATA4$^{\Delta IEC}$ mice, we were curious what other pathway RA was modulating in the tissue to impact the luminal IgA levels. We performed RNAseq on the tissue of the mice following RA treatment and looked for which GATA4 regulated genes were restored by RA. Most of the RA regulated genes did not have an obvious role in regulating luminal IgA levels except for *Pigr*, the polymeric immunoglobulin receptor, which is responsible for the transcytosis of IgA in epithelial cells (Figure 11G). The expression of *Pigr* was restored in the GATA4$^{\Delta IEC}$ mice following RA treatment and showed a significant correlation with luminal IgA levels (Figure 11H, I). Therefore, we identified that GATA4 controls regionalization of retinol metabolism in epithelial cells to maintain luminal IgA levels in the intestine.

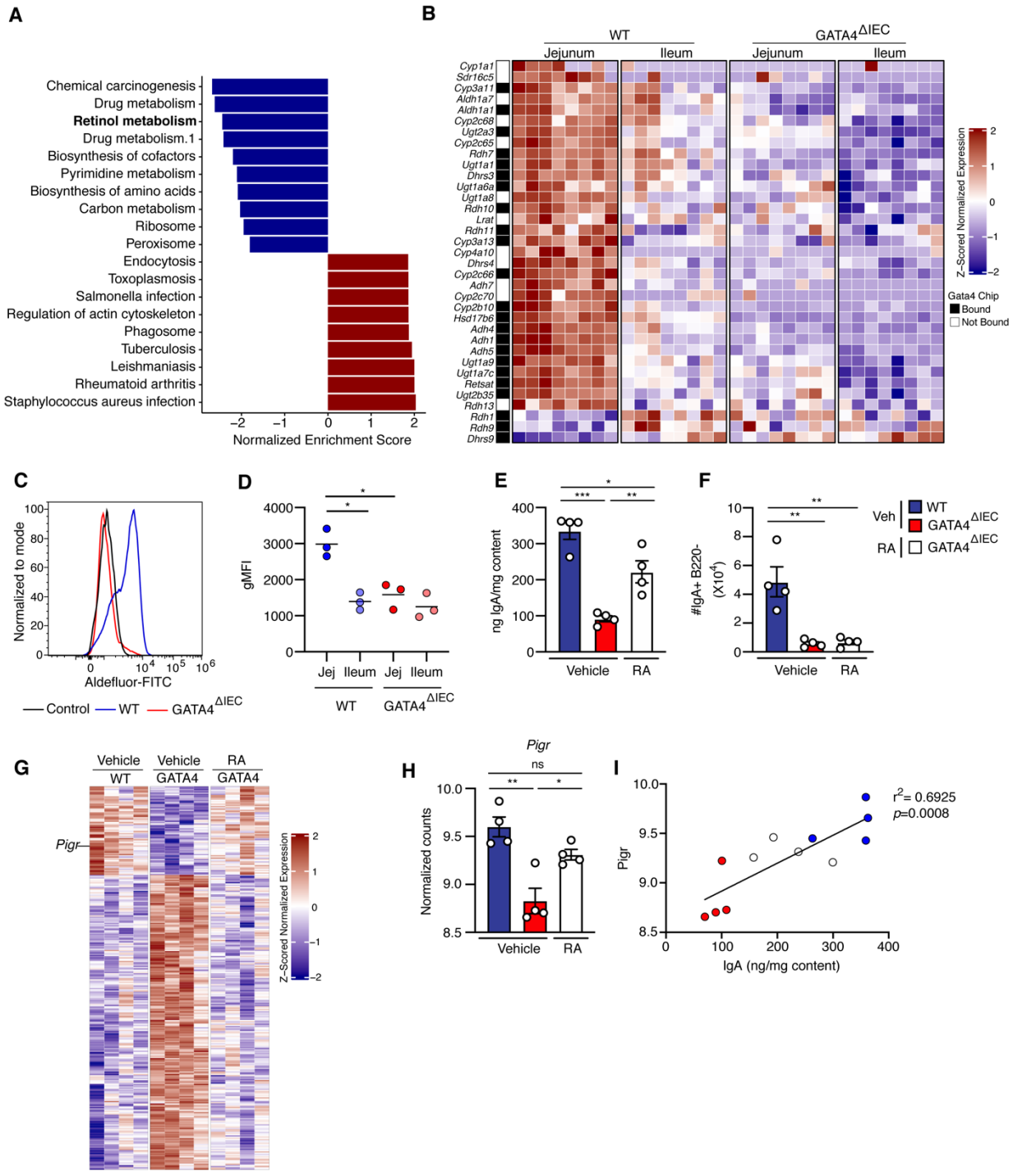

Figure 11. GATA4 regulates retinol metabolism in IECs to maintain luminal IgA levels.

A. Pathway enrichment analysis of GATA4 regulated region specific genes from sorted Epcam+, CD45- IECs of the jejunum and ileum of WT and GATA4$^{\Delta IEC}$ mice. B. Heatmap of DE genes in KEGG retinol pathway of the jejunum and ileum of WT and GATA4$^{\Delta IEC}$ mice. Genes are annotated as GATA4 bound or unbound by CHIPseq analysis.

Figure 11. GATA4 regulates retinol metabolism in IECs to maintain luminal IgA levels continued.

C. Histogram of ALDEFLUOR staining in jejunal epithelial cells of WT and GATA4$^{\Delta IEC}$ mice and WT cells treated with ALDH inhibitor. D. gMFI from jejunal and ileal epithelial cells of WT and GATA4$^{\Delta IEC}$ mice from C. E. Total IgA ELISA from Jejunal content of WT and GATA4$^{\Delta IEC}$ vehicle and GATA4$^{\Delta IEC}$ RA treated mice after 14 days. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ ANOVA with Tukey multiple comparison test. N= 4 mice/group. F. Absolute number of CD45+, lin-, IgA+, B220- plasma cells by FACS in the jejunum after 14 days of RA treatment. ** $p<0.01$, ANOVA with Tukey multiple comparison test. N= 4 mice/group. G. Heatmap of genes restored after RA treatment from RNAseq of jejunal tissue. H. Normalized counts of PIGR expression from G. ** $p<0.01$, * $p<0.05$ ANOVA with Tukey multiple comparison test. N= 4 mice/group. I. Correlation of PIGR expression and luminal IgA levels of WT and GATA4$^{\Delta IEC}$ vehicle and GATA4$^{\Delta IEC}$ RA treated mice. P= 0.0008, $r^2 = .6925$, Pearson correlation.

GATA4 is associated with an IL17 gene signature in celiac disease

We next sought to determine the role of GATA4 in the pathophysiological consequences of intestinal inflammatory diseases. RNAseq of duodenal biopsies from a cohort of celiac patients revealed a significant decrease of GATA4 expression in active celiac patients which was restored on a gluten free diet (Figure 12A). In addition, we observed undetectable levels of GATA4 protein in apical epithelial cells of active celiac patients by IHC (Figure 12B). We were intrigued by previous reports which identified an increase of IL17 in duodenal biopsies of active celiac disease patients (Monteleone et al., 2010). Furthermore, tetramer staining of isolated CD4 T cells revealed that the IFNγ+ producing T cells were gluten specific whereas the IL17 producing T cells were not (Bodd et al., 2010). We observed an increased IL17 gene signature in active celiac patients and a significant negative correlation between GATA4 levels and IL17 inducible genes in active celiac patients but not in controls (Figure 12C, D). Furthermore, we observed a positive correlation of the retinol gene set enrichment with GATA4 expression in active celiac patients but not in controls (Figure 12E). Therefore in a human disease context, loss of GATA4 expression correlated with an increase of IL17 signaling and a decrease in retinol metabolism similar to GATA4 deficient mice. Since the development of Th17 cells are dependent on adherent microbes in the intestine, we asked whether there was an association between GATA4 levels and particular mucosal associated bacteria in duodenal biopsies of celiac patients. Of all the taxa detected, we observed a significant association with the presence of Actinobacillus in active celiac patients and lower GATA4 expression, higher IL17 signaling, and decreased retinol enrichment (Figure 12F). Furthermore, these celiac patients who had detectable Actinobacillus had increased tissue destruction and villus atrophy through APOA4/Ki67 expression (Figure 12F). Interestingly, these patients did not have increased IFNγ signaling

indicating the presence of this bacteria is associated with a specific IL17 response in celiac disease (Figure 12F). These data suggest an intriguing association between GATA4 levels, atypical IL17 immunity, and mucosal associated Actinobacillus in celiac disease.

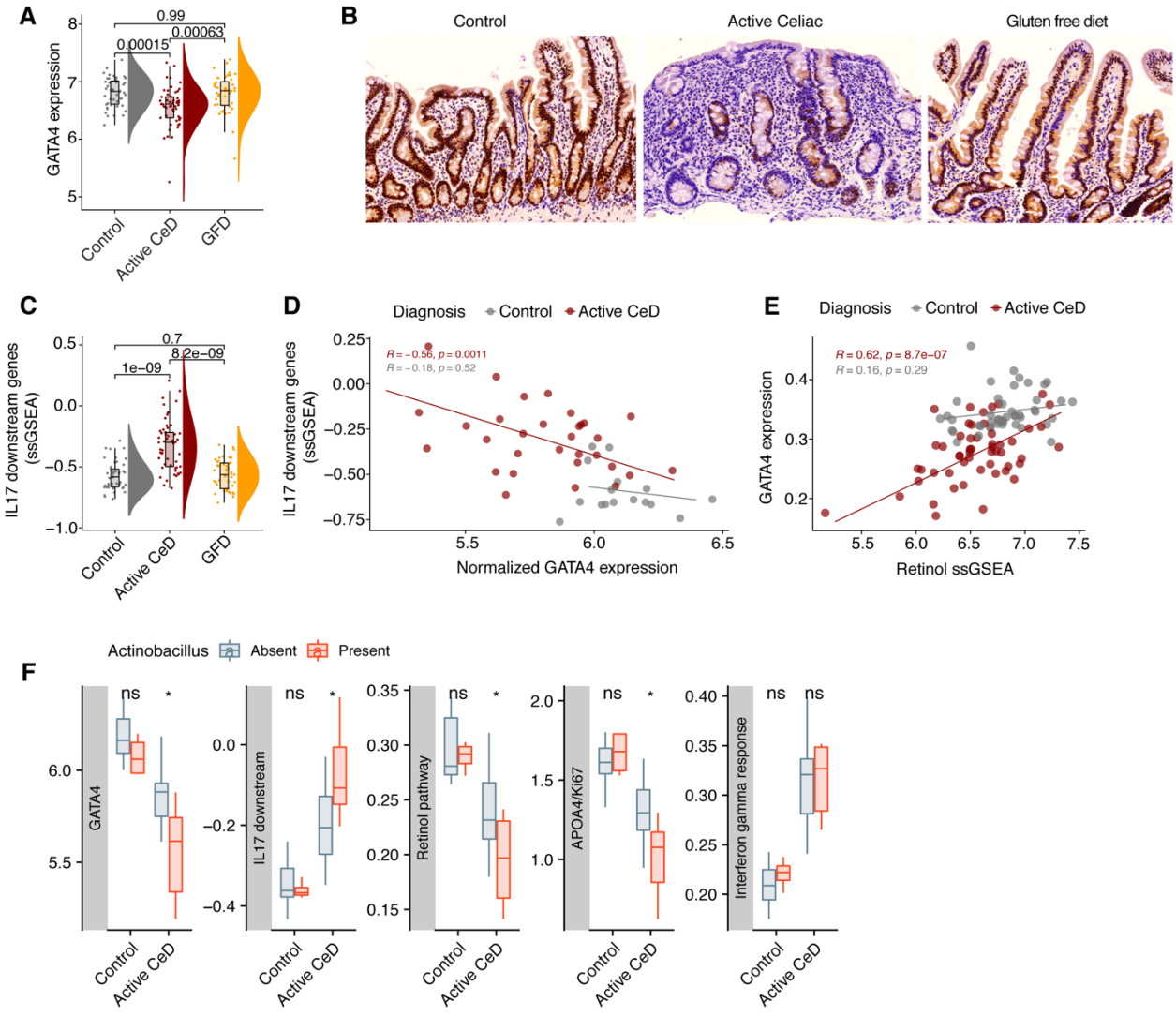

Figure 12. GATA4 and IL17 association in celiac patients

A. GATA4 expression in duodenal biopsies of control, active celiac disease, or gluten free diet. B. IHC of GATA4 in duodenal biopsies of the previous groups. C. IL17 downstream signaling genes GSEA. D. Pearson correlation of IL17 GSEA and GATA4 expression between control (grey) or active CeD (red). E. Pearson correlation of Retinol GSEA and GATA4 expression between control (grey) or active CeD (red). F. Presence or absence of detectable Actinobacillus levels in duodenal biopsies of control or active CeD and GATA4 expression or pathway enrichment scores.

Discussion

In this report, we identify mechanisms controlling regionalization between the proximal and distal small intestine and reveal the importance of this segregation in homeostasis and disease. The proximal IECs regulate retinol metabolic processes and induce protective immune responses, such as IgA+ PCs and luminal IgA, to limit bacterial adhesion and allow the host to focus on digestion and absorption of nutrients. On the other hand, the ileum focuses on bile acid reabsorption and produces less IgA, which allows for adherent microbes such as SFB to induce inflammatory IFNγ and IL17 T cell responses required for controlling pathogenic infections. The IEC TF GATA4 is the molecular mechanism which links all these processes and an essential regulator of small intestinal regionalization.

Regionalization of intestinal immunity

Profiling effector T cell responses throughout the intestine in SPF and GF mice revealed that both the region and microbiota greatly shape local mucosal immune responses. The role of specific commensal bacteria driving immune responses within particular regions have been reviewed elsewhere (Honda and Littman, 2016). However, less attention has been given to the role of the tissue in shaping local immune responses independent of the microbiota. We observed that IL15, IL33, and IL18 were all microbiota independent cytokines that were preferentially expressed in the jejunum compared to the ileum. We observed GATA4 binding sites in these genes and they were all downregulated in GATA4$^{\Delta IEC}$ mice suggesting that GATA4 may directly regulate the expression of all these cytokines in the jejunum. Other reports have suggested that the immune response in the proximal intestine is driven by dietary antigens or that MLNs draining the proximal intestine preferentially support tolerogenic responses (Esterházy et al., 2019; Kim et al., 2016). Our data fit this narrative that the immune responses in the proximal

intestine are largely tolerogenic, protective, and microbiota independent. What was unclear in the literature was the mechanisms regulating these tolerogenic responses in the proximal intestine. Our data indicate that IECs coordinate the induction of tolerogenic or protective immunity through the TF GATA4. In the absence of GATA4, the jejunum becomes more ileal like and inflammatory microbiota dependent immune mechanisms, IL17, IFNγ, TNFα, are all induced at the expense of tolerogenic responses. This heightened inflammatory state likely increases intestinal permeability and renders GATA4 deficient hosts more susceptible to translocation of pathogens to extraintestinal organs and sepsis.

Retinoic acid and luminal IgA

The data presented here demonstrate that GATA4 deficiency dramatically alters regionalization of the intestine. 2964 region specific genes were differentially expressed in the jejunum of GATA4 deficient mice. The most downregulated GATA4 dependent pathways were involved in lipid metabolism such as cholesterol and retinol metabolism. Retinoic acid has been previously shown to synergize with TGFβ to induce IgA class switch, upregulate homing receptors on B cells, and maintain the number of IgA+ PC in the lamina propria (Mora et al., 2006; Seo et al., 2013). IECs express all the genes involved in converting vitamin A to retinoic acid but how compartmentalization of vitamin A metabolism by IECs contributes to maintaining luminal IgA responses is not known. Here we show that GATA4 controls the regionalization of retinol metabolism IECs. Treating GATA4$^{\Delta IEC}$ mice with retinoic acid did not influence IgA class switching in the PPs or the number IgA+ PCs in the lamina propria, but instead regulated expression of *Pigr* on epithelial cells. This reveals an interesting novel mechanism where production of RA by proximal IECs induces a feedback loop on these cells to upregulate the receptor required in transporting IgA. Once, in the lumen sIgA acts to enforce the mucosal

barrier by coating commensal and pathogenic bacteria (Bunker et al., 2017; Palm et al., 2014). Previous work has demonstrated opposing roles of IgA in regulating colonization of mucosal associated microbes such as *Bacteroides fragilis* and SFB (Donaldson et al., 2018). In addition, increased frequency of IgA+ bacterial coating has been observed in the small intestine than the colon suggesting possible region specific functions of IgA (Bunker et al., 2015). Our data show that GATA4 is a key mechanism in regulating regionalization of IgA+ PC and luminal IgA in the small intestine. Both GATA4 deficiency and adhesion of SFB was required in driving the upregulation of IFNγ and IL17 T cell responses in the jejunum. IgA deficiency was required in controlling the regionalization of SFB but not sufficient to explain the upregulation of IFNγ and IL17. In contrast, B cells were required in controlling both SFB regionalization and the IFNγ T cell response in the tissue. Future experiments will need to assess the how B cells restrain CD8αβ+ IFNγ producing T cells in the proximal intestine in an IgA independent mechanism. These experiments, while out of the scope of this paper, may yield insights into other mechanisms whereby B cells suppress inflammatory T cell responses in the proximal intestine.

GATA4 in the context of infections and disease

Understanding how compartmentalization of the intestine shapes the mucosal immune response is critical for understanding site specific intestinal diseases. Here we show the impact of small intestinal regionalization in the host response to a colonic specific pathogen, *Citrobacter rodentium*, and human celiac disease. We unexpectedly observed that defects in the proximal intestine can have an impact on distant regions. We observed this phenomenon in the case of *C. rodentium* infection of GATA4$^{\Delta IEC}$ mice. GATA4 is expressed in the duodenum and jejunum and absent from the ileum and colon. Most phenotypes reported with *C. rodentium* susceptibility are in the colon, where the pathogen preferentially colonizes (Collins et al., 2014). However, we

observed in GATA4$^{\Delta IEC}$ mice, *C. rodentium* acquired the capacity to colonize the small intestine and cause villus atrophy and an upregulated Th17 response in the ileum. In addition, we also observed very severe colitis in the colon of GATA4$^{\Delta IEC}$ mice. These data show that GATA4 regulation of small intestinal regionalization impacts host susceptibility in both the small and large intestine to *C. rodentium*. These data are intriguing in the context of host pathogen interactions and inflammatory bowel disease. Traditionally in studying intestinal diseases, inflamed tissue is sampled and adjacent uninflamed regions are used as controls. However, it is possible that defects in specific regions of the intestine may increase host susceptibility in other distal sites. This may require rethinking intestinal physiology as a whole in order to understand complex local inflammatory diseases.

In addition to understanding the role of compartmentalization to host pathogen interactions, we asked whether the mechanisms observed in GATA4$^{\Delta IEC}$ mice applied to human autoimmune diseases. We were intrigued by the upregulation of IL17 expression previously reported in duodenal tissue of active celiac patients and the data that IL17+ CD4 T cells were not gluten specific unlike the IFNγ+ CD4 T cells (Bodd et al., 2010; Monteleone et al., 2010). The role of Th17 cells in contributing to tissue destruction or repair mechanisms in celiac patients is unknown. Single cell sequencing from CD4 T cells isolated from duodenal biopsies will be needed to determine whether these cells represent the pathogenic or homeostatic Th17 cells that have been previously described (Aschenbrenner et al., 2018; Lee et al., 2012). We identified an inverse correlation with GATA4 levels and IL17 downstream genes, suggesting a potential role for GATA4 in regulating Th17 cells in active celiac patients. Since Th17 cells are induced by gut bacteria in mice and humans, we asked whether there were increased mucosal associated duodenal bacteria which had an association with GATA4 and IL17 levels (Britton et al., 2019;

Ivanov et al., 2009; Weaver et al., 2006). We observed an association of the presence of Actinobacillus in GATA4 low active celiac patients that had increased IL17 gene signatures. Future studies will be needed to try to cultivate these bacteria from celiac biopsies and test whether they induce an IL17 response.

4. DISCUSSION

4.1 Regionalization of intestinal immunity by GATA4

The results presented illustrate the importance of both the microbiota and the intestinal region in controlling immune responses throughout the intestine. Furthermore, we reveal a novel mechanism which describes that IECs control regional immune responses through the TF GATA4 and the microbiota. Finally, we show the impact of loss of intestinal regionalization in GATA4 deficient mice to the susceptibility of an enteric pathogen and human celiac disease. When surveying the immune responses throughout the intestine in SPF and GF mice, we observed that many responses in the proximal small intestine were microbiota independent such as the IL10+ CD4 T cells and the IFNγ+ CD4 T cells. Instead, the distal small intestine contained more microbiota dependent inflammatory responses such as higher IFNγ, IL17, GC B cells, and Tfhs. We also observed an increased number of IgA+ plasma cells in the duodenum and jejunum, which strikingly dropped off in the ileum. The microbiota contributed to the numbers of the IgA+ plasma cells in the proximal intestine however there were still much more IgA+ plasma cells in the duodenum and jejunum of GF mice than the ileum. These data confirms previous reports that GF mice contain IgA plasma cells but that the microbiota contributes to the overall number and level of luminal IgA (Macpherson et al., 2000). These data suggest an important point not emphasized in the literature, the location of the intestine regulate the amount of IgA+ plasma cells. Therefore there must be certain tissue specific metabolites or cytokines which regulate IgA cell maintenance or homing. We identified that this regionalization of IgA+ plasma cells in the jejunum and ileum is regulated by IECs and the TF GATA4. Since GATA4 directly regulated genes involved in the retinol metabolic pathway we pursued whether supplementing RA in GATA4 deficient mice would restore the regional IgA plasma cell defect. We observed

that RA treatment partially restored luminal IgA without impacting the number of IgA plasma cells. This revealed that RA was not sufficient to regulate IgA cell numbers and that GATA4 controls a different mechanism that regulates IgA plasma cells in the tissue. Its known that tissue metabolites play a key role of shaping effector lymphocyte functions. Fatty acid oxidation is critical for the formation of long lived memory CD8 T cells following an infection (O'Sullivan et al., 2014; Pearce et al., 2009). In contrast, glycolysis is critical for the ability to T cells to clonally expand following activation (Klein Geltink et al., 2020). Activated B cells require glucose and glycolytic pathways to undergo germinal center reactions (Buck et al., 2017). However the metabolic requirements of intestinal IgA PCs are unclear. The GATA4 deficient mice may represent a useful model for dissecting the mechanisms of tissue metabolites on IgA plasma cell maintenance.

Another key differentially expressed pathway in the GATA4 deficient mice is cholesterol metabolism. NPC1L1 is the key transporter for cholesterol which is expressed on the apical brush border of enterocytes in the jejunum (Huang et al., 2020). GATA4 strongly regulates the expression of this gene and it is repressed in GATA4$^{\Delta IEC}$ mice. In addition CD36, and SCARB1 are other receptors for cholesterol and fatty acid uptake and are also repressed in in GATA4$^{\Delta IEC}$ mice. These genes are regulated by sterol regulatory element binding protein 1, SREBF1, an essential transcription factor for controlling cholesterol metabolism which is also decreased in epithelial cells of GATA4$^{\Delta IEC}$ mice (Eberlé et al., 2004). There is rationale in the literature for a potential role of cholesterol metabolism in regulating B cells and IgA plasma cells. For example, B cell and IgA deficient mice reveal a decrease of lipid metabolic genes and in particular genes in cholesterol metabolism (Shulzhenko et al., 2011). In addition, GC B cells express SREBP2 which senses 25-hydroxycholesterol and regulates their class switch to IgA+ PCs following

infection or immunization (Trindade et al., 2021). Furthermore, cholesterol metabolism is linked to IgA nephropathy, which is a disorder characterized by high levels of serum IgA that complexes in the glomeruli and can lead to renal failure (Wyatt and Julian, 2013). Cholesterol lowering drugs, ezetimibe and statins are common treatments for IgA nephropathy patients. Therefore, it is plausible that GATA4 controls the levels of cholesterol metabolites in the jejunum which shape the regionalization of IgA plasma cells. Treatment of WT mice with the NPC1L1 inhibitor ezetimibe would lower intestinal cholesterol levels and test whether cholesterol impact the numbers of IgA plasma cells. Furthermore, sorting IgA+ B220- PCs from the jejunum and ileum of WT and GATA4$^{\Delta IEC}$ mice may reveal clues as to which pathways are differentially expressed in IgA+ cells from the GATA4$^{\Delta IEC}$ mice.

The B cell deficient mice recapitulated many of the defects observed in GATA4$^{\Delta IEC}$ mice, such as the increased SFB and *C. rodentium* colonization in the jejunum, and increased IFNγ+ CD8αβ IEL T cells. However, Jh-/- mice did not have higher levels of IL17+ CD4 T cells in the jejunum that were observed in the GATA4$^{\Delta IEC}$ mice. Therefore, B cell deficiency is not sufficient to explain the upregulated IL17 response. This suggests that GATA4 must regulate another mechanism in addition to B cells to restrain IL17 induction in the proximal intestine. In WT mice SAA is regularly expressed in the ileum and provides a second hit to RORγt CD4 T cells in the tissue to regulate effector function of Th17 cells (Lee et al., 2020). Interestingly, SAA1 was also upregulated in the jejunum of GF GATA4$^{\Delta IEC}$ mice. This suggests that GATA4 can regulate *SAA1* expression in the absence of SFB. The increased levels of SAA1 in the jejunum, a region where it is not regularly expressed, may further augment the IL17 expression of Th17 cells in GATA4$^{\Delta IEC}$ mice. To test whether the increase of SAA1 drives the IL17 signature, SAA1-/- mice can be crossed to GATA4$^{\Delta IEC}$ mice to see whether the IL17 levels are restored in the absence of

SAA1. It is also important to note that cholesterol metabolism controls the effector function of Th17 cells. RORγt is a nuclear hormone transcription factor. Oxysterols generated from cholesterol catabolism bind RORγt and activate the transcription of its target genes (Hu et al., 2015; Santori et al., 2015). Cyp51 deficient mice are unable to produce the sterols which are endogenous ligands for RORγt have smaller lymph nodes and a decrease of IL17+ CD4 T cells (Santori et al., 2015). Although GATA4$^{\Delta IEC}$ mice are deficient in cholesterol uptake, some genes in the sterol biosynthetic pathway are upregulated to compensate for this decrease. Metabolomics will need to be done to determine the levels of the various sterols and RORγt ligands in the jejunum of the GATA4$^{\Delta IEC}$ mice.

4.2 B cells and IgA regulation of adherent bacterial colonization

Our data show that B cells and IgA control the regionalization of adherent bacterial colonization. In the absence of B cells and IgA, SFB was able to expand and colonize the jejunum to the same level as the WT ileum. This shows that IgA restricts the niche of SFB to the ileum and protects proximal IECs from bacterial adhesion. Luminal IgA has been previously shown to bind commensal or pathogenic bacteria (Bunker et al., 2015; Palm et al., 2014). However, until now it has not been reported to control the regionalization of bacterial colonization. IgA-/- mice have a decrease of *Bacteroides fragilis* in the colon and an expansion of SFB in the ileum (Donaldson et al., 2018). This suggests that IgA has opposing roles in different regions of the intestine. In the colon, *B. fragilis* induces a specific IgA response dependent on its surface capsular polysaccharides which binds to *B. fragilis* in the lumen (Donaldson et al., 2018). This adhesion enables *B. fragilis* to stick to the mucus within colonic crypts. However, IgA restricts SFB colonization in the ileum. IgA can be generated from T cell dependent and T cell independent mechanisms (Fagarasan et al., 2010). In TCRβδ-/- mice, the

commensal microbes are coated in the same frequency as WT mice (Bunker et al., 2015). However, SFB and Mucispirillum have decreased IgA coating in the absence of T cells showing that these bacteria are coated by T cell dependent mechanisms (Bunker et al., 2015). There are still many unanswered questions concerning the role of IgA with SFB. For instance, how does IgA block the colonization of SFB? Along the same line why does IgA in the jejunum restrict its colonization while the IgA in the ileum enables SFB to colonize but still controls its overall load? Finally, what role does natural IgA have versus SFB specific T cell dependent IgA have in regulating SFB? Future studies will be needed to decipher these questions. The function of IgA in the proximal versus distal small intestine is intriguing. We observed that the microbiota had less impact on driving IgA+ PC in the proximal compared to the distal small intestine. We also observed more Tfh and GC B cells in the ileum than the jejunum which were microbiota dependent. An increased frequency of IgA+ cells were CD19+ in the ileum than the jejunum. Also, the IgA+ PCs expressed CD138 in the ileum but were CD138 negative in the jejunum. These data suggest that the IgA+ PCs may be functionally different in the ileum compared to the jejunum. In addition, the microbiota reactive IgA+ cells may be generated preferentially in the ileum. The specificity of IgA was assessed previously using PCs sorted from the entire small intestine (Bunker et al., 2017). It may be necessary to further define the specificity of IgA plasma cells in each region of the small intestine to be able to elucidate the regional functions of IgA. We showed that gavaging polyclonal luminal sIgA isolated from intestinal contents of WT mice to GATA4$^{\Delta IEC}$ mice was sufficient to block the colonization of SFB. This IgA came from SFB positive donor mice. Therefore it likely contains a mix of SFB specific and non-specific IgA. To determine whether IgA from SFB negative mice also regulates SFB colonization JAX mice could be used as donors. It is possible that IgA of a particular specificity against SFB blocks its

colonization. However it is also possible that polyreactive IgA regardless of the specificity sterically hinders the binding of SFB to enterocytes. Future experiments transferring IgA from JAX mice and repertoire analysis of IgA PCs in the jejunum and ileum will be needed to understand how IgA controls SFB colonization.

We next asked whether B cells and IgA controlled the regionalization of *C. rodentium*. We observed that B cells controlled the regionalization of *C. rodentium* whereas IgA controlled the colonization of *C. rodentium* only in the colon. This reveals distinct mechanisms of how B cells or IgA control *C. rodentium* infection. It was previously reported that B cell deficient (μMT-/-) mice were unable to clear *C. rodentium* and have more severe colitis (Maaser et al., 2004). This defect was not due to IgA, PIGR, J-chain, secreted IgM, or IgG3. Another report analyzed *C. rodentium* bacterial coating in the lumen. They observed that the bacteria was highly IgG coated but not IgA bound (Kamada et al., 2015). However there are contradicting reports of the role of IgA in *C. rodentium* infection. *C. rodentium* induces an increase of IgA PCs and luminal IgA in the colon (Atarashi et al., 2015). This IgA from infected mice is specific to intimin and TIR, the genes used by *C. rodentium* to adhere to IECs (Ghaem-Maghami et al., 2001). We observed that IgA controlled the colonization of *C. rodentium* in the colon in an adhesion dependent manner. WT *C. rodentium* expanded in IgA-/- mice but the adhesion deficient ΔEAE reverted to WT levels. However IgA deficiency was not sufficient to alter the niche of *C. rodentium*. The fact that IgG coats *C. rodentium* in the lumen of the colon is intriguing because there is no known mechanism for how IgG can be transported into the intestine of adult mice. The neonatal FC receptor (FcRn) is required for transporting maternal IgG into breastmilk and transports luminal IgG across the intestinal epithelium of neonates where it can bind to the microbiota (Koch et al., 2016). However in adult mice, FcRn expression

is lost in the epithelial cells and there is no detectable levels of IgG in the intestinal lumen (Roopenian and Akilesh, 2007). It is possible that IgG leaks through the damaged epithelium and binds to *C. rodentium* and is not actively transported.

It will be of future interest to understand how GATA4 deficiency potentially alters the IgA repertoire or controls other B cell functions. Understanding these mechanisms may reveal how B cells control the regionalization of *C. rodentium* or why SFB expands in GATA4$^{\Delta IEC}$ mice. Although we observed the total level of IgA reduced in the jejunum and ileum of GATA4$^{\Delta IEC}$ mice, it is possible that the specificity to SFB is also impaired. Repertoire analysis and total mRNA sequencing from IgA PCs will define whether there is a change in IgA repertoire in GATA4$^{\Delta IEC}$ mice and whether the IgA PC are functionally different.

4.3 Role of retinoic acid in controlling regional immune responses and bacterial colonization in the intestine

The proximal small intestine is critical for the uptake of vitamin A and the production of retinoic acid which elicits numerous immunological functions on the host. Mice fed a vitamin A deficient diet show a substantial decrease of B cells in the PPs, IgA+ PC in the LP, and CD4+ T cells in the LP (Iwata et al., 2004; Mora et al., 2006). These observations indicate an important requirement of vitamin A in establishing mucosal intestinal immunity. Dietary vitamin A, carotenoids or retinyl esters, are first absorbed by intestinal epithelial cells in the proximal small intestine. Inside the enterocyte, alcohol dehydrogenase enzymes (ADH) convert retinol to retinal and retinal dehydrogenases (RALDH) convert retinal to retinoic acid (RA) (Erkelens and Mebius, 2017). All-trans retinoic acid is the natural ligand for the nuclear hormone receptor RARα and RARβ which induce the transcription of RAR dependent genes. Numerous cell types in the intestine express various isoforms of RALDH and have the capacity to produce retinoic

acid. Small intestinal CD103+ migratory DCs express RALDH2 and make retinoic acid with synergizes with TGFβ to induce Treg differentiation (Coombes et al., 2007; Sun et al., 2007). DC's of proximal gut draining lymph nodes express higher levels of RALDH2 than those at distal sites and are important for establishing oral tolerance to dietary antigens (Esterházy et al., 2019). Therefore, retinoic acid regulates the reciprocal Treg/Th17 balance by promoting Treg and blocking Th17 differentiation (Mucida et al., 2007). Retinoic acid production by PP DCs imprints gut homing receptors α4β7 and CCR9 on T cells and B cells (Iwata et al., 2004; Mora et al., 2006). In addition, RA synergizes with TGFβ or IL6 to induce IgA class switch in B cells (Mora et al., 2006; Seo et al., 2013). Finally RA signaling in CD4 T cells is essential for driving effector responses during infection, vaccination, and inflammatory disease (DePaolo et al., 2011; Hall et al., 2011). Intestinal epithelial cells express RALDH1 instead of RALDH2 and also contain the capacity to produce retinoic acid. Deletion of RARα in IECs leads to an underdeveloped immune system and decreased lymphoid follicles (Jijon et al., 2018). In addition it leads to an alteration of antimicrobial peptide expression and changes of the microbiome (Jijon et al., 2018). Finally, RARα deficient mice are more susceptible to infection by *C. rodentium* (Jijon et al., 2018). RARβ an isoform of RARα also has important functions in establishing intestinal immunity in IECs. RARβ in IECs regulates the expression of SAA, T cell homing, and generation of IgA+ PCs (Gattu et al., 2019).

We observed that GATA4 controls regionalization of the retinol metabolic pathway in jejunal and ileal epithelial cells. In GATA4$^{\Delta IEC}$ mice, there was a decreased ALDH activity in jejunal epithelial cells which indicates a decreased capacity to make retinoic acid. We observed a decrease in expression of numerous ADH enzymes in GATA4$^{\Delta IEC}$ jejunal epithelial cells, *ADH1/4/5*. This indicates a decreased ability of IECs to produce retinal. We also observed a

significant decrease in expression in *ALDH1a1* which produces retinoic acid. In GATA4$^{\Delta IEC}$ mice there appeared to be a global decrease in many of the enzymes required in converting retinoic acid. Interestingly, 66% of the retinol metabolic genes were directly bound by GATA4 from CHIPseq analysis. Previous work has indicated that RARα or RARβ controls the expression of these genes so it was surprising to find another TF which regulates these genes in IECs. We did not observe a decreased expression of RARα/β TFs in GATA4$^{\Delta IEC}$ mice. Future experiments will need to dissect the role of GATA4 and RARs in regulating retinol metabolism in IECs and whether these TFs interact. We hypothesized that the decrease in retinoic acid contributed to a decrease in luminal IgA in GATA4$^{\Delta IEC}$ mice. Therefore we treated GATA4$^{\Delta IEC}$ mice with RA and observed a partial restoration in luminal IgA. These results suggest other mechanisms in addition to RA regulate luminal IgA levels in GATA4$^{\Delta IEC}$ mice. RA was not sufficient to restore all the IgA defects observed such as the decrease in IgA plasma cells. To identify how RA treatment restored luminal IgA in GATA4$^{\Delta IEC}$ mice we sequenced the tissue and looked at which genes were restored to WT levels in the RA treated group. We identified two potential mechanisms of how RA regulated luminal IgA. First, RA treatment restored the expression of PIGR in GATA4$^{\Delta IEC}$ mice to WT levels. PIGR is the polymeric immunoglobulin receptor which transcytosis IgA across the epithelium to the lumen. The expression of PIGR correlated with the amount of luminal IgA in the various treatment groups. Second we observed that RA treatment increased the expression of *Igha* in the tissue suggesting that it boosted the production of IgA in PCs. We observed that IgA+ PCs sorted from GATA4$^{\Delta IEC}$ mice and cultured for 24 hours secreted less IgA than WT PCs. This indicates that the IgA PCs in GATA4$^{\Delta IEC}$ mice are functionally impaired in the ability to secrete IgA and that RA restores this defect. The restored expression in PIGR is interesting because the mechanisms of how RA can

regulate gene expression in IECs are unclear. The regulation of PIGR expression by RA can be direct or indirect. It would be of future interest to culture epithelial organoids from WT and GATA4$^{\Delta IEC}$ mice to determine whether RA directly regulates PIGR expression and IgA transport in an epithelial intrinsic manner. The alternate explanation is that RA directly regulates IgA production in IgA PCs and the increased IgA in the tissue signals to IECs to upregulate PIGR and IgA transport. We don't expect the GATA4$^{\Delta IEC}$ mice to be deficient in RA production globally. It is possible the duodenum is still able to produce equivalent levels. Future experiments will need to determine the impact of GATA4 deficiency in the duodenum. The previous experiments which showed the requirement of RA in maintenance of LP CD4 T cells and IgA+ PCs fed mice a vitamin A deficient diet from birth (Iwata et al., 2004; Mora et al., 2006). In this system, we would not be able to determine the role of RA in controlling regional immune responses with the mucosal immune response ablated. Shortened treatments of vitamin A deficient diet may reveal whether this is required for establishing regional IgA and T cells responses in the intestine. Although both IECs and DCs can produce RA, the contribution of both cells in shaping tissue RA levels and specific immune responses will need to be assessed separately. Floxed mice of key enzymes in the retinol metabolic pathway will need to be developed to ablate these genes in IECs or DCs. It will be of interest to determine the role of ALDH1a1 in IECs or ALDH1a2 in DCs in shaping specific intestinal immune responses. Finally, it will be of importance to determine whether retinoic acid regulates adherent bacterial colonization. Mice where RARα is selectively deleted in the epithelium are more susceptible to *C. rodentium* and had an increased pathogen load in the colon (Jijon et al., 2018). The authors did not report a change in pathogen regionalization or any impact of the pathogen in the small intestine. Future experiments will need to determine whether RAR signaling controls

regionalization of adherent bacterial colonization. The regionalization of SFB and *C. rodentium* colonization should be studied in mice fed a vitamin A deficient diet or when RAR signaling is inhibited with the small molecule LE540. These data will provide a more complete understanding of how RA production by IECs or DCs regulate intestinal T and B cell responses to control bacterial colonization and regional immunity.

4.4 Conclusion and model

The results presented here demonstrate that GATA4 is a critical regulator of regional metabolic and immune system processes between the proximal and distal small intestine. In the absence of GATA4, we observed an upregulation of microbiota dependent IFNγ and IL17 producing T cells in the proximal intestine. These inflammatory T cell responses were driven by a change in regionalization of SFB. GATA4 regulation of retinoic acid production in jejunal IECs was critical for maintaining luminal IgA levels and preventing SFB from adhering to the proximal intestine. We propose that this compartmentalization by GATA4 allows the host to focus on lipid metabolic processes while restricting adherent bacterial colonization and inflammatory immunity in the proximal intestine. The consequences of altered intestinal regionalization by GATA4 deficiency is an increased host susceptibility to *C. rodentium* induced colitis and mortality, and an upregulated IL17 signature in active celiac patients.

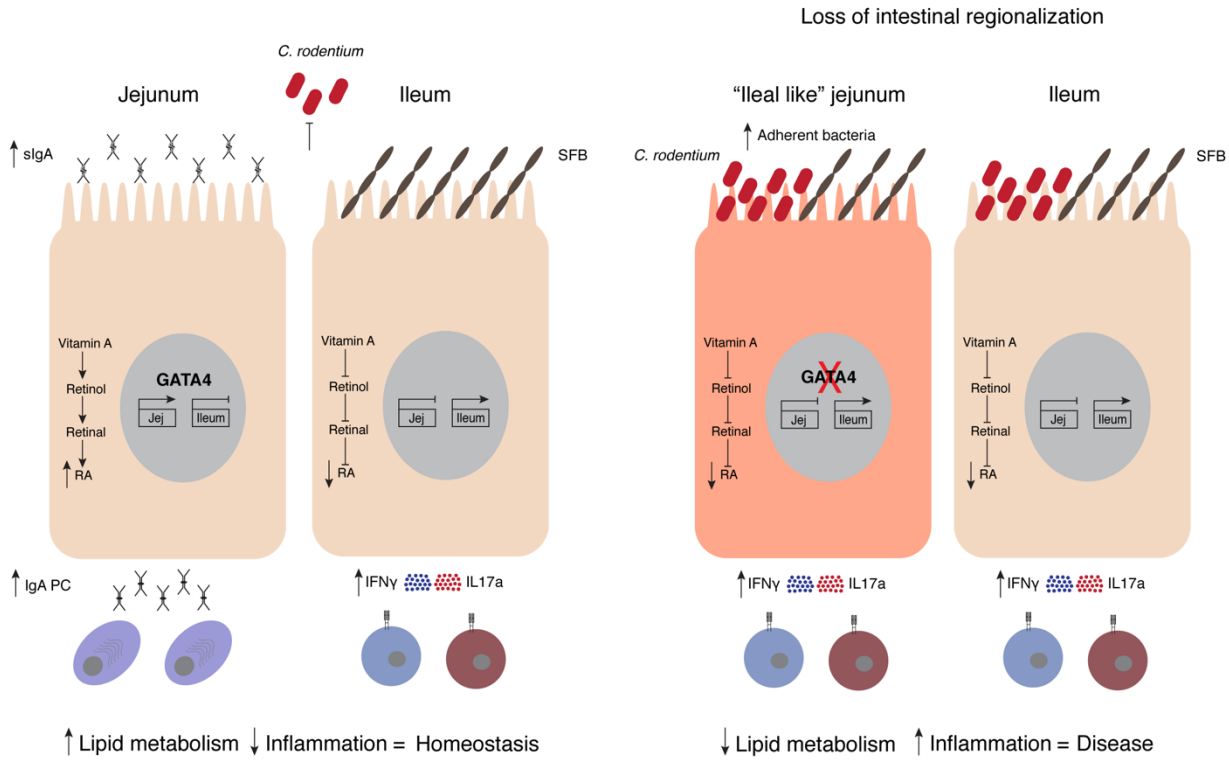

Figure 13. Model of GATA4 regulation of intestinal regionalization

The TF GATA4 promotes the expression of jejunal genes and restricts the expression of ileal genes. This induces the expression of enzymes involved in the production of retinoic acid in the jejunum. Retinoic acid modulates the levels of luminal IgA to restrict the colonization of SFB. This compartmentalization of GATA4 induces high levels of IgA PCs in the jejunum and restricts inflammatory immune responses and SFB to the ileum. In the absence of GATA4, the jejunal defining genes are inhibited and the production of retinoic acid drops. The levels of IgA PCs decrease and there is less luminal IgA. The decrease of IgA allows for SFB to change its regionalization and colonize the jejunum and induce IFNγ and IL17 T cell responses. This loss of intestinal regionalization increases the colonization of *C. rodentium* in the jejunum and ileum and increases the susceptibility to disease.

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