ENTERIC PARASITE INFECTION-INDUCED ALTERATION OF THE GUT MICROBIOTA REGULATES INTESTINAL GOBLET CELL BIOLOGY AND MUCIN PRODUCTION VIA TLR2 SIGNALLING

ENTERIC PARASITE INFECTION-INDUCED ALTERATION OF THE GUT MICROBIOTA REGULATES INTESTINAL GOBLET CELL BIOLOGY AND MUCIN PRODUCTION VIA TLR2 SIGNALLING

By

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Abstract

In the gastrointestinal (GI) tract, goblet cells are the major source of mucins, the main structural components of the mucus layer, which functions as the front line of innate defense. The GI tract contains trillions of commensal microbes, and these microbes can manipulate mucin production by activating different signalling cascades initiated by pattern recognition receptors (PRRs), including bacterial sensing Toll-like receptors (TLRs). In addition, sterile α motif pointed domain-containing ETS transcription factor (SPDEF) is a transcription factor that modulates goblet cell differentiation and positively regulates mucin production. During helminth infections, due to the co-existence of parasites and microbiota in close proximity of goblet cells in the gut, it is likely that helminth-microbiota interactions play an important role in mucin production. Indeed, goblet cell hyperplasia and increased mucin production are observed in many enteric helminth infections, including *Trichuris muris*, and these processes play key roles in host infection clearance. However, it should be noted that the role of microbiota within this axis is not yet understood. Here, we hypothesize *T. muris*-induced altered microbiota modulates goblet cell differentiation and mucin production via SPDEF-mediated transcriptional regulation and TLR2 signalling.

C57BL/6 mice were gavaged with ~300 *T. muris* eggs. Mice were sacrificed 36 days post-infection. Microbiota from these *T. muris*-infected and non-infected mice were transferred into two groups of germ-free (GF) mice. Microbiota analysis revealed that treatments in both experiments (infection with *T. muris* and microbiota transfer from *T. muris*-infected mice into GF mice) significantly account for the among-sample variations in the composition of the gut microbiota between groups ($p <= 0.001$).

In GF mice, transfer of *T. muris*-infected microbiota significantly increased goblet cell numbers and *TLR2* expression as well as upregulated *Muc2* expression compared to

GF mice with non-infected microbiota. Antibiotic-treated (ABX-treated) *TLR2* knockout (KO) mice after receiving microbiota from *T. muris*-infected mice showed significantly decreased expression of *Muc2* and *Muc5ac* compared to ABX-treated wild-type (WT) mice receiving the same microbiota. To investigate whether SPDEF is a driving factor for *Muc2* production in response to *T. muris* microbiota stimulation, we next transferred *T. muris*-infected microbiota into antibiotic-treated *SPDEF* KO and WT mice. We observed a slight, though not significant, the influence of SPDEF on the stimulation of mucin production by *T. muris* microbiota. These findings reveal important interactions among parasites, resident microbiota, and host in relation to goblet cell response in the gut. In addition, this study provides new information on TLR2-based innate signalling in the regulation of goblet cell biology and mucin production.

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5-HT	5-hydroxytryptamine
ABX	Antibiotic
APCs	Antigen-presenting cells
DMEM	Dulbecco's modified Eagle medium
EC cells	Enterochromaffin cells
EECs	Enteroendocrine cells
ELISA	Enzyme-linked immunosorbent assay
ESPs	Excretory/secretory products
FBS	Fetal bovine serum
FDR	False discovery rate
GF mice	Germ-free mice
GI	Gastrointestinal
Gfi1	Growth factor independence 1
Hes1	Hairy and enhancer of split 1
Igs	Immunoglobulins
Klf4	Krüppel-like transcription factor 4
IL	Interleukin
ILC2s	Type-2 Innate lymphoid cells
LPS	Lipopolysaccharides
LTA	Lipoteichoic acids
Math1	Mouse atonal homolog 1
MEM	Modified Eagle medium
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
Nod protein	Nucleotide-binding oligomerization domain-containing protein

PAMPs	Pathogen-associated molecular patterns
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
p.i.	Post-infection
PTS	Proline, threonine, and/or serine
PRRs	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
RELM-β	Resistin-like molecule β
Sat-1	Sulphate anion transporters 1
s.c.	Subcutaneous
SCID	Severe combined immunodeficiency
SPDEF	Sterile α motif pointed domain-containing ETS transcription factor
STAT6	Transducer and activator of transcription 6
TFFs	Trefoil factors
TJ proteins	Tight junction protein
Th2	T helper 2
TLRs	Toll-like receptors
TSLP	Thymic stromal lymphopoietin
WT mice	Wild-type mice
ZO-1	Zonula occludens protein-1

Declaration of academic achievement

In 2021, I attended the Canadian Digestive Disease Week (CDDW™) conference and was honored to give an oral presentation in Research Topics discussing "Enteric parasite infection-induced alteration of the gut microbiota regulates intestinal goblet cell biology and mucin production via TLR2 signalling". Along with this, the abstract regarding this work was published in the Journal of Canadian Association of Gastroenterology.

During the course of my Master's degree, I was the first author on the review article titled "*Trichuris muris* model: role in understanding intestinal immune response, inflammation and host defense" (Yousefi, Y., Haq, S., Banskota, S., Kwon, Y. H. & Khan, W. I. 10, 925 (2021)) published in *Pathogens*.

Chapter 1: Introduction

1.1. Gastrointestinal (GI) tract and mucus barrier

The GI tract is a dynamic environment comprised of specialized epithelial cells, including absorptive enterocytes and secretory endocrine cells and goblet cells, as well as sentinel immune cells and the residing microbiota. Throughout the GI tract, the epithelial lining is coated by a viscoelastic mucus layer which represents the first line of innate immunity. This defense helps to protect the epithelium from invading luminal pathogens, poisons, and other environmental stimuli[1]. The mucus layer also acts as a simple lubricant, facilitating the movement of feces down the intestinal canal. Along with its physical aspects, recent studies have shown that the intestinal mucus layer modulates a variety of intracellular signalling pathways and intensely interacts with the gut microbiota, which is important for host intestinal function and health status[2,3]. The GI mucus layer is primarily composed of goblet cell-secreted mucins, which are large, high-molecular-weight glycoproteins. These highly glycosylated molecules are the main structural component of the mucus layer, which retain water and contribute to the mucus layer's viscoelastic features[1,2]. In addition to these structural mucins, water, immunoglobulins (Igs), and some secreted bioactive molecules, including trefoil factors (TFFs), resistin-like molecule β (RELM-β), as well as antimicrobial peptides such as defensins, are also found in the mucus layer[1].

Both host innate and adaptive immune responses regulate mucus layer formation and maintain the integrity of the mucus layer[1,4]. There are different types of tight junction (TJ) proteins, including zonula occludens protein-1 (ZO-1), ZO-2, and ZO-3 proteins, as well as members of the membrane-associated guanylate kinase protein family, occludin, claudins, and cingulin that impacts immune homeostasis of GI tract[5].

The small intestine has a single mucus layer, whereas the colon and stomach are covered by a dual layer of mucus[6]. The attached inner layer is normally devoid of commensal microbiota, and gut microbes tend to be limited to the 'loose' outer layer[7]. The physiological function of mucus is associated with its structural properties, and many studies have indicated that the mucus composition is altered in various GI disorders, including ulcerative colitis (UC), colorectal cancers, and parasite infections[8].

Mucins

In general, mucins consist of a protein core that contains tandem repeats rich in threonine, proline, and/or serine (PTS). These PTS domains are extended by large numbers of O-linked oligosaccharide chains. These extensive O-glycosylated chains give mucin a "bottle-brush" appearance, help protect the protein core from proteases, and thus, result in the preservation of mucus integrity[1,2]. Along with their protective role with regard to the epithelial barrier, mucins forms a permeable layer for gases and nutrient exchange[9].

Mucins are categorized into two subfamilies based on their structural and functional features: membrane-bound and secretory mucins. Secreted mucins, such as MUC2, MUC5AC, MUC5B, and MUC6, contribute to the structural properties of the mucus barrier, while cell surface mucins including MUC1, MUC3, MUC4, MUC13, and MUC17 constitute a carbohydrate-rich physical barrier known as glycocalyx, which restricts infections by responding to microorganisms that reach the cell membrane[8]. Currently, 21 human mucin genes have been described, and a large number of human mucin homologues have been detected in rats and mice[10]. Among secretory mucins, several studies confirmed that MUC2 *(*Muc2 in mice) is the prominent gel-forming mucin in both the small intestine and colon[1]. It has also been shown that *Muc2* secretion is associated with goblet cell morphology and has an important function in colonic protection[11,12]. Many common human GI disorders such as

carcinoma, inflammatory bowel disease, and celiac disease are associated with altered or aberrant production of *MUC2*[13–15]. In addition to *MUC2*, *MUC5AC*, secretory mucin normally present in the stomach, can also be upregulated within the intestine in various enteric infections[16].

Goblet cells

Goblet cells are intestinal secretory cells that act as a component of the innate immune system and sustain intestinal hemostasis by mucin synthesis and secretion of other bioactive proteins, including TFF3 and RELM- β[11].

Mucins are produced and secreted by goblet cells through two pathways, constitutive/basal and regulated pathways[1,9]. Continuous or basal secretion occurs under normal physiological conditions to replenish and replace the mucus barrier that is sloughed off by the peristaltic movement of the GI tract. This mode of secretion is dependent on the intracellular transportation of mucin vesicles from the Golgi to the cell surface. In contrast, potent mucin secretagogues, such as hormones, neuropeptides, and cytokines, stimulate the regulated secretion of mucin[1].

It has long been recognized that goblet cells play a critical role in mucosal biology. However, goblet cells' regulatory mechanisms and the impact of innate immune components in the control of goblet cell biology are only beginning to be investigated. As touched on previously, changes in goblet cell numbers and/or mucin secretion activity are related to several mucosal disorders[11]. For example, intestinal helminth infections such as *Trichuris muris* are accompanied by goblet cell hyperplasia and increased mucin production, which aid worm expulsion[17]. The goblet cell response against nematode infection is thought to be mainly under the control of a T helper (Th2) type immune response and is considered a potential effector mechanism in this regard[18,19]. While our understanding of how adaptive immune

factors impact goblet cell biology in helminth infections has significantly expanded over the last decade, the role of innate immune networks play in regulating goblet cell responses to helminth infection remains largely unexplored. It has been shown that multiple genes, including mouse atonal homolog 1 (*Math1*), Krüppel-like transcription factor 4 (*Klf4*), sterile α motif pointed domain-containing ETS transcription factor (SPDEF), and growth factor independence 1 (*Gfi1*) are involved in the proper differentiation and function of goblet cells in a normal mucosal environment[11]. Studying genetic mechanisms that regulate the differentiation of these cells may further enable us to understand the impacts of goblet cell malfunction in different mucosal disorders.

1.2. Intestinal cell fate determinants and goblet cell differentiation

Within the gut, specialized epithelial cell types (enterocytes, enteroendocrine cells, goblet cells, and Paneth cells) are constantly renewed by multipotent stem cells residing at the bottom of intestinal crypts[20]. Asymmetric stem cell division and upward migration are required for the renewal and production of enterocytes, enteroendocrine cells, and goblet cells that occupy the villi while Paneth cells terminally differentiate at the crypt bottoms[4,21]. Studies have shown various signalling pathways, including Notch and Wnt, regulate stem cell proliferation and differentiation via their transcription factor networks **(Figure 1)**[22–24]. In short, the Notch pathway is predominant in enterocyte differentiation, whereas the Wnt signalling is a secretory cell lineage-specific pathway **(Figure 1)**[23–25]. *Hes1* (hairy and enhancer of split 1) expression is transcriptionally elevated by Notch pathways[26]. It has been demonstrated that partially inactivated Notch in *Hes1* depletion commits to secretory lineages[27]. *Hes1*, the Notch target gene, inhibits the expression of *Math1*, a transcriptional factor required for secretory cell lineages. It has been shown that *Math1* null mice lacked goblet cells, enteroendocrine cells, and Paneth cells while absorptive cells appeared normal[26]. One of the epithelial-specific

transcription factors, SPDEF, has been described as a lineage-specific transcript and acts downstream of Math1, which is essential for the proper allocation of intestinal secretory cells[11,28,29]. SPDEF has a role in goblet cell maturation, terminal differentiation, and function in both the intestines and lungs[30,31]. It has also been reported that loss of SPDEF leads to defects in the terminal differentiation of goblet cells[32]. Interestingly, inactivating SPDEF does not affect TFF3 expression, implying goblet cell specification is not SPDEF-dependent[32]. In pulmonary T helper 2 (Th2-derived) inflammation, SPDEF is required for goblet cell hyperplasia and increased *Muc5ac* secretion under the regulatory control of signal transducer and activator of transcription 6 (STAT6)[31,33]. In the intestinal epithelium, *SPDEF* expression promotes goblet cell numbers with the reduction in other epithelial cell numbers, and *in vitro* experiments have indicated it is required for the expression of goblet cell genes, including *MUC2*[30].

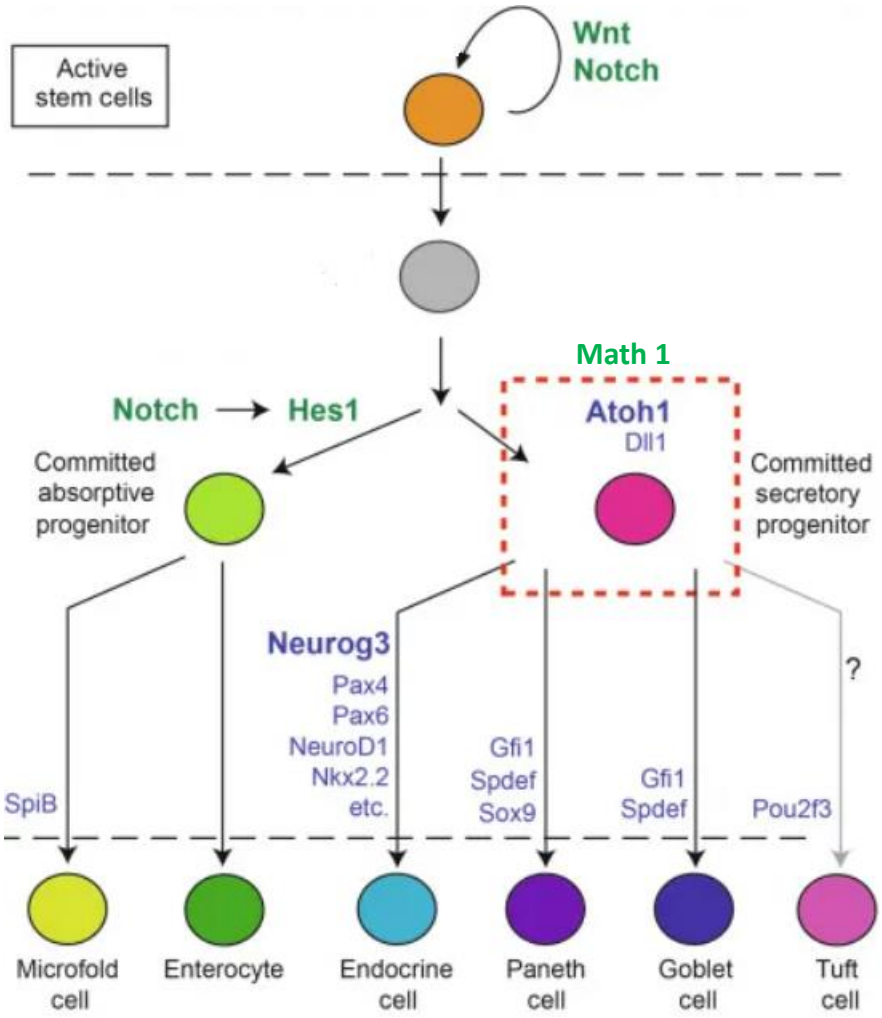


Figure 1: Epithelial cell lineage differentiation in mouse intestine[24].

Stem cell renewal results in enterocyte and secretory cell linage differentiation via Wnt and Notch signalling, and their transcription factor networks, Atoh1 and Hes, respectively [Figure adapted from www. abdominalkey.com].

1.3. Gut microbiota, toll-like receptors (TLRs), and mucins

The gut is colonized by a substantial number (approximately 10^{14} CFU) and a diverse community of microorganisms. In addition to viral and fungal components, the majority of this microbiota is composed of various bacterial species. Obligate anaerobic bacteria predominate in the colon, and the most abundant phyla amongst these anaerobic microbes are *Firmicutes* and *Bacteriodetes*[34]. The intestinal microbial composition differs between various parts of the GI tract, and it is affected by multiple factors such as diet, illness, antibiotic use, emotional stress, and age [35]. The commensal microbiota has a symbiotic relationship with its mammalian hosts. The microbiota contributes to host nutrition, metabolism, normal intestinal physiology, and maturation of the immune system[36].

Epithelial cells generate a physical barrier (mucus layer) protecting underlying cells and tissues from potentially invasive bacteria species and other luminal contents. Mucosal immunity has evolved to distinguish indigenous bacteria from pathogens by pattern recognition receptors (PRRs) that sense conserved pathogen-associated molecular patterns (PAMPs)[1,36]. TLRs are one of the best characterized PRRs and have essential roles in maintaining intestinal homeostasis by differentiating commensals microbes from pathogens. There are 13 types of TLRs expressed on or within innate immune cells in mammals[37,38]. A series of genetic studies have revealed their respective ligands. For example, TLR2 recognizes various bacterial components, including peptidoglycan, lipopeptide, and lipoprotein of Gram-positive bacteria, while lipopolysaccharides (LPS) of Gram-negative bacteria are recognized by TLR4[39].

Within the gut, symbiotic intestinal microbes rely on the ecological advantages mucus provides, including its roles as an endogenous source of nutrients and as a protective niche against the intestine's peristaltic activity in order to grow[1].

On the other hand, microbiota-induced immune responses are also required to maintain intestinal homeostasis[34]. Gut microbes and microbial products can modulate mucin synthesis and secretion via activating signalling pathways and modulating the expression of secretory elements generated by epithelial and lamina propria cells[1,4]. For instance, an investigation of probiotic bacteria confirms that probiotic strains such as *Lactobacillus GG or Bifidobacterium lactis Bb12* can modulate intestinal mucins and exert beneficial influences on host immunity by increasing IgA and IgM production, important factors in mucosal immunity[40–42]. Furthermore, *in vivo* experiments have shown that probiotic bacteria such as *Lactobacillus plantarum* induce *MUC2* and *MUC3* expression and occupy binding sites of enteropathogenic *Escherichia coli,* implying *L. plantarum* protects against invasive pathogens by enhancing mucins secretion and inhibiting the adherence of pathogenic microorganisms[34,43]. It has also been reported that a multispecies probiotic mixture including *Lactobacilli, Bifidobacteria,* and *Streptococci* species acts as a mucin secretagogue, and was particularly adept at increasing *Muc2* expression in comparison to other mucins, including *Muc1* and *Muc3*[44].

Probiotic strains also have the ability to prevent pathogen invasion by promoting qualitative alternations in the mucus layer. Bacterial products such as LPS and flagellin A from Gram-negative bacteria and lipoteichoic acids (LTA) from Gram-positive bacteria are the most common bacterial products that are able to induce the expression of both *Muc2* and *Muc5ac*[45].

Further support for the role of microbiota in the development of mucus barrier is the findings in GF mice. Intriguingly, altered intestinal morphology and function have been consistently observed in germ-free (GF) mice. In these mice, the absence of indigenous microbiota is correlated with reduced goblet cell numbers as well as altered goblet cell morphology and, consequently, affects the mucosa characteristics in GF mice[4,46,47]. The mucus barrier in GF mice is less stable compared to conventional mice as mucus layer thickness, structure, and mucin content are highly affected by intestinal microbiota. It has also been shown

that GF animals have lower mucin expression, such as *Muc1*, *Muc2*, *Muc3*, and *Muc4*, as well as a lower amount of Muc2 protein levels in comparison to conventional animals, implying mucin expression and secretion are dependent on the gut microbiota.animals[48,49].

1.4. Intestinal parasite infection (*Trichuris muris*)

Enteric parasites are one of the most important parasites in terms of their widespread prevalence and have major socio-economic impacts on both developing and developed countries by affecting human and animal wellbeing, productivity, and agriculture. Among the intestinal parasites, intestinal helminth infections are the most prevalent and occur through contact with parasite eggs and larvae. According to recent estimates, about two billion people worldwide are infected with helminths[50].

Trichuris trichiura is a soil-transmitted helminth, and current estimates indicate that roughly 465 million individuals globally are infected with *T. trichiura*[51–53]. The majority of infected individuals are children in developing countries and humans; this infection is associated with several adverse health consequences[54,55]. Chronic *T. trichiura* infection results in iron deficiency anemia, starvation, and growth retardation, particularly in children, whereas high worm burdens may cause ulcerative colitis and rectal prolapse[56,57]. *T. muris* is a murine pathogen and shares extensive homology at genomic, transcriptomic, and morphological levels to *T. trichiura* and, thus, has been extensively used as a laboratory model for *T. trichiura* in mice[58]. In addition, *T. muris* has the unique advantage of producing heterogeneous immunological outcomes in different mouse strains and, therefore, can provide a distinct understanding of host-parasite interactions[59]. Due to this well-defined immunity and biology, it is common to use the *T. muris* model to study inflammatory changes, the function of the epithelial barrier, immunological responses, and the mechanisms of host defence in the context of intestinal inflammation and infection.

Recent evidence indicates that along with the gut microbiota sharing its environment with *T. muris*, *T. muris* also develops bidirectional interactions with these microbes. For example, the gut microbiota facilitates parasite survival in the colon by inhibiting effective immune response against parasites (Th2 immune response). TLRs are triggered by the microbial products, resulting in a Th1 response and a suppression of Th2 responses. Also newly revealed is that *T. muris* is able to acquire particular intestinal bacterial subsets from the mouse host's gut environment that help its survival. On the other hand, parasites influence the physical habitat for the gut microbiota by changing antimicrobial peptides secretion and nutritional availability and, thus, cause outgrowth of specific species within the microbiota [60,61].

These interactions are also immunologically relevant since they involve the host immune system and therefore have impacts on host defense mechanisms. In this thesis, we focused on the role of *T. muris*-microbiota interactions in immune responses against acute *T. muris* infection[60,62].

Life cycle of *T. muris*

The *T. muris* life cycle starts with the ingestion of infective eggs released in the feces of infected hosts. Upon ingestion, eggs move toward the gut and end up in the cecum after they've been ingested. About 90 minutes after ingestion, the eggs hatch, followed by L1 larval development in the cecum and colon of infected mice. The L1 larvae moult three times, becoming L2 (9–11 days post-infection (p.i.), L3 (17 days p.i.), and L4 (18 days p.i). (22 days p.i.). Interestingly, larval molting activity durations vary in different host strains. The adult form of *T. muris* is discovered in the cecum and proximal colon after larval development (by day 32 p.i.)[17,63,64].

Two different immune response phenotypes dictate either resistance or susceptibility to *T. muris* infection. These “resistant” and “susceptible” phenotypes vary with *T. muris* mouse

strain[17] and, thus, successful completion of the *T. muris* life cycle is dependent also predicated on the host strain. For example, more than 70% of mouse strains such as BALB/k, BALB/c, and C57BL/6 (high dosage of infection) are resistant to *T. muris* infection and eliminate the worms through a Th2-dependent response by day 32 post-infection. In contrast, the mouse strains such as AKR, B10Br, C57BL/6 (low dose of infection) develop a Th1 immune response and, unable to clear the worms, become susceptible to chronic *T. muris* infection[17,63].

Immune responses against *T. muris* infection

Innate immune response

Innate immune components, including innate immune cells and PRRs, as well as the mucus barrier, are involved in generating immunity against *T. muris* infection. Innate immune cells such as dendritic cells, macrophages, granulocytes, natural killer cells, and type-2 innate lymphoid cells (ILC2s) act as antigen-presenting cells (APCs) and participate in the generation of immune responses against *T. muris* by helminth recognition and Th2 cytokine (IL-4, IL-5, IL-9, and IL-13) production[17,65–69].

Furthermore, studies have shown that epithelial cells play an important role in host *T. muris* defense, notably in the early stages of infection. A lack of NF-B signalling in intestinal epithelial cells prevents mice from generating an efficient immune response and expulsion of the worm, indicating that epithelial cells need to be stimulated prior to the participation of adaptive immune cells in the expulsion[70].

Adaptive immune response

Among the different types of CD4+ T-cells, the Th2 type of immune response is mainly linked with protective immunity from intestinal helminth infections, like *T. muris*. The main cytokines of the Th2 immune response, interleukin-4 (IL-4), IL-5, IL-9, and IL-13, are

essential in providing immunity against *T. muris* infections[17,71,72]. Mice lacking either IL-4 or IL-13 secretion were shown to be susceptible to *T. muris* infection[71,72]. In fact, the activation of IL-13 and IL-4 results in goblet cell hyperplasia and mucin production, respectively, which are the effective immune responses to *T. muris* infection and lead to worm expulsion[64,73]. In addition, *T. muris* ejection is suppressed by IL-9 neutralizing antibodies, indicating that IL-9 plays an essential role in generating effective immune responses to *T. muris* infection[74]. During *T. muris* infection, IL-5 regulates eosinophil recruitment in the colonic mucosa. However, the lack of IL-5 and decreased eosinophils did not affect *T. muris* survival and removal[75,76].

In addition, B cells regulate immune responses against *T. muris* infection by acting as APCs, secreting cytokines, and producing *T. muris* specific antibodies[77]. B cells are necessary to shift a mixed Th1/Th2 response observed in some mouse strains, such as C57BL/6 mice, toward a Th2 response but are not required to elicit an efficient immune response to *T. muris* infection in mouse strains with dominant Th2 responses (BALB/c animals)[77]. Accordingly, anti-IL-12 antibody treatment reduced the formation of the susceptibility-related Th1 responses, which resulted in parasite expulsion in B cell-deficient mice[78].

On the other hand, in susceptible mouse strains like AKR, the development of a Th1 response results in chronic infection. Th1 response is characterized by increased levels of IFN-ɣ, IL-12, and IL-18 cytokines[17,63]. IFN-ɣ depletion promotes resistant-associated responses in normally susceptible mice[79].

1.5. *T. muris* effects on the epithelial layer

T. muris effects on epithelial cells

Intestinal epithelial cells secrete various important cytokines such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) that are crucial in the development and activation of the Th2 immune response during *T. muris* infection[80].

Intestinal epithelial cells release IL-25, which enhances the Th2 immune response to gastrointestinal helminth infections by stimulating ILC2s or multipotent progenitor (MPP) cells. *IL-25*-deficient mice generate a susceptible-associated response that can be switched to Th2 immunity through MPPtype2 cell transfer[81,82].

As a result of *T. muris*-induced cellular necrosis, intestinal epithelial cells produce and secrete IL-33, which has been demonstrated to stimulate the Th2 immunity in response to helminth infections[80,83]. Resistant mice exhibit greater amounts of IL-33 on day 3 p.i. compared to susceptible mice. Additionally, administration of recombinant IL-33 treatment to susceptible mice in the early stage of *T. muris* infection can change susceptible-related immune response and thus induce *T. muris* expulsion while late IL-33 treatment does not lead to worm clearance, showing IL-33 initiated Th2 response after *T. muris* infection. It has also been shown that the treatment of *T. muris*-infected severe combined immunodeficiency (SCID) mice with IL-33 fail to produce resistant-associated responses, suggesting that IL-33's ability to elicit Th2 responses is reliant on the presence of T cells[84].

Along with IL-25 and IL-33, TSLP is another member of intestinal epithelial cell cytokines that stimulates Th2 cell differentiation by interacting with a variety of immune cells, including DCs, basophils, monocytes, granulocytes, T cells, and B cells[85]. During *T. muris* infection, TSLP level is significantly higher in resistant mice in comparison with susceptible mice[86]. It has also been shown that *TSLP* knockout (KO) mice show a susceptible phenotype[82].

T. muris effects on enteroendocrine cells (EECs)

EECs, via their chemosensory function, detect helminths and other luminal entities and interact with the immune system to orchestrate an immune response[87]. Enterochromaffin (EC) cells, an important subgroup of EEC in the mucosal layer of the intestine, are the main producers of serotonin [5-hydroxytryptamine (5-HT)]. Infection with *T. muris* promotes EC cell hyperplasia[88] and the production of 5-HT in resistant mice strains[89]. Our lab has demonstrated that during *T. muris* infection, the increased synthesis of 5-HT in the EC cells is controlled by the gut microbiota via a TLR2 dependent mechanism[90]. It has also been shown that chronic *T. muris* infection can alter host microbial composition, decreasing the variety and number of Bacteroidetes, notably *Prevotella* and *Parabacteroides*[91] and, therefore, *T. muris* may also indirectly influence EC cells biology and 5-HT production by affecting the microbial composition of the host.

T. muris effects on goblet cells and mucins

T. muris infections, whether acute or chronic, alter the intestinal mucus barrier's components. *T. muris* exposure causes upregulation of transmembrane mucin secretion, including *Muc4*, *Muc13*, and *Muc17*, which can potentially lead to the increased thickness of the glycocalyx. Moreover, in acute infection, the Th2 cytokines stimulate the expression of secretory mucins into the mucus layer via GABA-α3[92]. This increase in mucin expression and glycocalyx thickness during acute infection enables the host to clear *T. muris* through physically trapping worms within the mucus, impairing worm motility and inhibiting parasite feeding capacity[45].

In the acute *T. muris* infection model, resistant mice develop goblet cell hyperplasia, which occurs via activation of the transcription factors, Math1 and SPDEF[92]. These factors promote the differentiation of multipotent stem cells to the secretory cell phenotype[18,92,93].

Conversely, the transcription factor, Hes1, which promotes absorptive enterocyte differentiation, had increased expression levels in susceptible mice harboring chronic *T. muris* infection[92,94].

Goblet cell hyperplasia is believed to be greatly regulated by Th2 cytokines[19,95], and this expansion of the goblet cell populations in mice with acute *T. muris* infection is accompanied by an upregulation in the secretion of mucins. In opposition, during chronic infection, the decreased goblet cell numbers lead to a depleted mucus barrier despite hypersecretion of transmembrane mucins[92].

Muc2, the principal mucin generated by intestinal goblet cells, has been demonstrated to be important in eliminating the *T. muris* parasite, according to Hasnain and colleagues. *T. muris* eradication was delayed in Muc2-deficient mice, despite an adequate Th2-mediated immune response, confirming Muc2's unique involvement in host immunity. In addition to Muc2, Muc5ac, a mucin normally found in lungs and stomach, was upregulated in colonic tissue during infection, shortly before worm clearance in resistant mice[18]. Mice lacking *Muc5ac* were incapable of eliminating *T. muris* and harboured long-term, chronic infections, showing the vital role of *Muc5ac* in *T. muris* expulsion. Interestingly, Interestingly, mice lacking *Muc5ac* exhibited a susceptible phenotype, despite anti- IFN-ɣ administration producing robust Th2 responses, did not shift into a Th2 dominated response. In addition, human MUC5AC has negative effects on the viability of *T. muris* worms[16]. Despite significant improvements in the identification of both mucin genes and their contributing functions, the precise regulatory networks that mediate mucin production in response to intestinal parasitic infection remain to be identified.

As well as quantitative changes in mucus layer components, mucin quality is also altered by *T. muris* infection. Mice with chronic *T. muris* infection have mucins with low charge, whereas mice with acute *T. muris* infection have mucins with high charge. Mucins with

a higher charge may alter the physicochemical features of the mucus barrier, resulting in worm rejection[92]. Furthermore, an altered glycosylation pattern is present in the mucins during chronic *T. muris* infection; a shift from Sulphomucins (highly charged) to sialomucins (low charged) occurs within the cecum of these mice. *T. muris* acute infections, on the other hand, are linked with high levels of sulphomucin, which is controlled by interleukin IL-13. Notably, sulphate glycan-containing Muc2 is less vulnerable to proteolytic degradation by *T. muris* excretory-secretory products (ESPs) compared to sialomucins[96]. Even with the dominant Th2 immune responses in mice with gene deletion of sulphate anion transporters 1 (Sat-1), *T. muris* susceptible immune responses have been observed to *T. muris* infection due to decreased mucin sulphation[96]. These findings show that changes in mucin glycosylation induce structural changes in the mucus barrier, which can protect the underlying epithelium from parasite degradation and thereby contribute to the host defense.

1.6. Helminth-microbiota-innate immune signalling interactions and goblet cells

Probiotics are beneficial in treating different diseases by influencing the gut microbiota and modifying immune responses in the host[97]. Treatment of *T. muris* infection with live *Lactobacillus rhamnosus* expedited worm expulsion and promoted goblet cell hyperplasia in resistant as well as susceptible mice (AKR) via the IL-10 pathway, which itself has direct effects on goblet cell hyperplasia. Thus, by altering this pathway, live *L. rhamnosus* can play an important role in the treatment of *T. muris* infection by regulation of goblet cell biology and defense response. It should be noted that treatment with γ-irradiation-killed *L. rhamnosus* did not contribute to goblet cell hyperplasia and innate immune response, suggesting that the therapeutic effects of live *L. rhamnosus* in *T. muris* infection are due to factors other than bacterial structure, such as some sort of metabolic products from the bacteria or interactions between the host and the live bacteria[98]. Other parasitic models have illustrated a role of TLR2

activation in the immune response against infection; epithelial cells (HT-29 cells) had increased expression of *TLR2* and *MUC2* in response to human intestinal trematode antigens, *Gymnophalloides seo*[99].

Contributing factors and mechanisms to intestinal goblet cell differentiation and development remain incompletely understood. Due to the gut microbiota's known role in maintaining intestinal homeostasis, it is not a far-reaching hypothesis that microbial recognition receptors are heavily involved in intestinal development; however, more studies are needed in this regard to elucidate the role of microbial receptors in goblet cell biology. Previously, we demonstrated nucleotide-binding oligomerization domain-containing (Nod) receptors (Nod1 and Nod2) have an important role in modulating goblet cell response and mucin production in the context of *T. muris* infection[100]. It has also been shown TLR4 regulates goblet cell differentiation, and loss of *TLR4* promotes goblet cell differentiation and inhibits necrotizing enterocolitis induction in mice independently of microbiota, as no changes in microbial communities were identified between WT and *TLR4*-deficient mice[101]. Recently studies from our laboratory demonstrated that the expression of *TLR1* and *TLR4* were unchanged following antibiotic therapy in naïve mice while *TLR2* expression was reduced, indicating that depletion of microbial content with antibiotics largely affects *TLR2* expression[90]. Taken together, this evidence leads us to postulate that there is an interaction between TLR2 and gut microbiota which can regulate goblet cell biology and mucin production.

Chapter 2: Hypothesis and aims

Based on the literature presented, this thesis explores the role of the altered gut microbiota induced by *T. muris* infection in the regulation of goblet cell biology. Due to the co-existence of both *T. muris* and the gut microbiota in close proximity to intestinal goblet cells during parasitic infection, and as *T. muris* can change the host's microbial composition, we hypothesize that the *T. muris*-altered gut microbiota promotes goblet cell function via activating TLR2. We have studied the role of *T. muris* infection-induced altered gut microbiota in goblet cell biology and the underlying mechanisms involved in the process by using WT mice, GF mice, *TLR2*-deficient, and *SPDEF*-deficient mice, and the human adenocarcinoma cell line, HT-29.

Aim 1

To determine the effect of *T. muris* infection-induced altered gut microbiota on the regulation of intestinal goblet cell response and mucin production.

Rationale: According to our previous findings, GF mice, as well as broad-spectrum antibiotics (ABX)-treated mice, had significantly lower numbers of periodic acid-Schiff (PAS)-stained goblet cells and reduced *Muc2* expression, implying a role for the gut microbiota in intestinal goblet cell biology[100]. Furthermore, *T. muris* infection establishment is dependent on the presence of intestinal microbiota, and it has been shown chronic *T. muris* infections result in changes in fecal bacterial species[60,102]. Taken together, these studies suggest that *T. muris* interacts with the gut microbiota, and these *T. muris*-microbiota interactions can have an impact on host immune system response. Hence, we set out to investigate the effects of the host's altered gut microbiota after *T. muris* infection on the regulation of goblet cell responses by utilizing C57BL/6 and GF mice.

Aim 2

To investigate whether *T. muris*-mediated altered gut microbiota modulates mucin production through TLR2 signalling.

Rationale: NLRs and TLRs are key components of the innate immune system. Our previous study indicated that treatment with Nod1 and Nod2 agonists increased mucin production in GF mice while separate treatment of either Nod1 agonist or Nod 2 agonist had no significant effects. It has also been shown that *Nod1* and *Nod2* double-KO (*Nod* DKO) mice with *T. muris* infection had fewer goblet cells and less *Muc2* expression compared to *T. muris* infected WT mice. These findings, along with delayed worm expulsion in *Nod* DKO mice, imply that innate immune signalling via Nod proteins contributes to goblet cell response in *T. muris* infection[100].

TLRs are expressed in most intestinal epithelial cell lineages, including goblet cells[103–105]. Recent studies from our lab revealed a reduction in goblet cell numbers of *TLR2* KO mice after *T. muris* infection, while that of infected *TLR4* KO and WT mice had no significant difference (unpublished data). We also previously observed *TLR2* expression was reduced by antibiotic treatment,[90] thus, it is very likely that altered microbiota by *T. muris* infection can influence goblet cell response and mucin production via TLR2 signalling in relation to innate defense to this infection.

Aim 3

To elucidate whether transcription factor (SPDEF) plays a role in goblet cell response by helminth-induced altered gut microbiota.

Rationale: SPDEF, a putative transcriptional factor, plays a critical role in controlling the terminal differentiation of goblet cells[29]. Recently, SPDEF has been investigated as a possible regulator of mucin production in infectious diseases such as *Brachyspira hyodysenteriae* and

Citrobacter rodentium, which are bacterial pathogens that cause mucus layer changes[73,106]. However, the role of SPDEF in goblet cell biology and mucin production in parasitic infection remains unexplored. Therefore, in this aim, we investigate whether the transcription program (with a focus on SPDEF) can mediate the regulation of goblet cell function by the *T. muris*-microbiota axis.

Chapter 3: Material and Methods

Animal experiments

Animals

In all experiments, a combination of both male and female mice, aged 6-8 weeks, were used. GF mice on a C57BL/6 background were obtained from the Farncombe Axenic Gnotobiotic Unit (AGU) at McMaster University. *TLR2* KO (C57BL/6 background) and WT mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and Taconic Biosciences (Rensselaer, NY), respectively. *SPDEF* KO mice (BALB/c background) were obtained from Cincinnati Children's Hospital (kindly given by Dr. Jeffrey Whitsett). All mice used in the experiments were acclimatized for at least 7 days prior to the start of any experiments. Mice were housed in sterilized, filter-topped cages under SPF conditions, fed autoclaved food at a temperature of 21-22°C, and with a 12:12 h light-dark cycle in the Central Animal Facility (CAF) of McMaster University. All experimental animal procedures were approved by the Animal Research Ethics Board (AREB)-McMaster University and conducted under the Canadian guidelines for animal research.

T. muris infection

To create a batch of *T. muris* eggs, a number (20-30) of immunodeficient mice were infected by oral gavage with approximately 300 *T. muris* eggs. Mice were sacrificed after 32–35 days, and the cecum of each mouse was removed and placed in a 10 cm petri dish containing Dulbecco's Modified Eagle Medium (DMEM), 500 U/ml penicillin, and 500 g/ml streptomycin. Next, the cecum was gently shaken in phosphate buffered saline (PBS) in order to remove feces, and then it was returned to the petri dish. Afterward, worms were carefully removed using smooth curved forceps and placed in a well of a 6-well plate with 5 ml of DMEM, 500 U/ml penicillin, and 500 g/ml streptomycin and incubated at 37°C for 4 hours.

After 4 hours, the worms were extracted using smooth curved forceps, and the contents of the well were transferred to a 15 ml falcon tube. The tube was centrifuged at 3000 rpm for 5 minutes, and the supernatant was collected as 4-hour antigens before resuspending the egg-containing pellet in autoclaved distilled water. Worms were put back in a new well containing 5mL DMEM, 500 U/ml penicillin, and 500 g/ml streptomycin and were incubated at 37°C overnight. After 24 hours, worms were disposed, and well contents were again transferred to a falcon tube and spun at 3000 rpm for 5 minutes. *T. muris* eggs after 4 hours and overnight incubation were combined and filtered over a through the 70µm mesh filter to eliminate any remaining worms before transferring to a foil-wrapped covered 75 cm2 flask[107,108].

C57BL/6 mice were infected by oral gavage with 300 *T. muris* eggs per 200 µl autoclaved distilled water (high dose of infection). On day 36 p.i. (to exclude the possibility of worm transmission), mice were sacrificed, and the cecum mouse was removed, and the worm burden was measured by counting the number of worms in the cecum according to published method[108].

Antibiotics

In two separate experiments, mice (*TLR2* KO, *SPDEF* KO, and WT mice) received antibiotics, consisting of neomycin (0.5 g/L), ampicillin (0.5 g/L), vancomycin (0.5 g/L), and metronidazole (0.5 g/L) in sterile drinking water for 10 days. During this time, body weight, water, and food intake were monitored and recorded daily. When required, subcutaneous (s.c.) saline injections were administered to mice to prevent dehydration.

TLR2 agonist treatment

Mice were orally sensitized with 100 µg (per mouse) of PAM3CSK (TLR2/1 agonist; InvivoGen, San Diego, CA), reconstituted in PBS. The Control group was given the same volume of PBS (vehicle).

16S rRNA analysis

Amplification of the V3 region of the 16S rRNA gene was used to determine the bacterial diversity profile as previously described[109,110]. The amplicons were sequenced on an Illumina MiSeq Illumina (Farncombe Institute) and compared against the SILVA taxonomy database, and PhyloSeq[111] were used for data analysis. It was essential to reduce data to the minimum library size in order to account for uneven sequencing depth, under-sampling, and other problems with the data. Sample depths range from 15,219 to 202,411 with a mean of 84,102 and all sequences with mean abundance less than 10 were removed.

Beta diversity based on the Bray-Curtis index was employed to exhibit the impact of *T. muris* infection and transplantation of *T. muris* microbiota on the microbial composition. Using permutational multivariate analysis of variance (PERMANOVA), statistically significant differences were examined, and principal coordinates analysis (PCoA) plots were used to visualize dissimilarities.

Statistical comparisons of a single variable data were conducted using the Student's t-test and one-way analysis of variance (ANOVA). Statistical significance was set at a false discovery rate (FDR) of <0.05.

Gut microbiota transfer

Cecums were collected from *T. muris*-infected, and non-infected mice, and cecal contents were diluted 1:1 in PBS. Thereafter, cecum contents were divided into aliquots and snap-frozen in liquid nitrogen and then stored at −80°C for subsequent bacterial composition analysis and experimental microbiota transplantation.

For adoptive microbial transfer, each mouse received 200 µl of diluted cecal content from either non-infected or *T. muris*-infected donors for 3 consecutive days. Cecal content was gavaged from one mouse per day from respective group.

Animal tissue sample preparation

Colonic tissues were obtained according to our lab procedure (**Figure 2**).

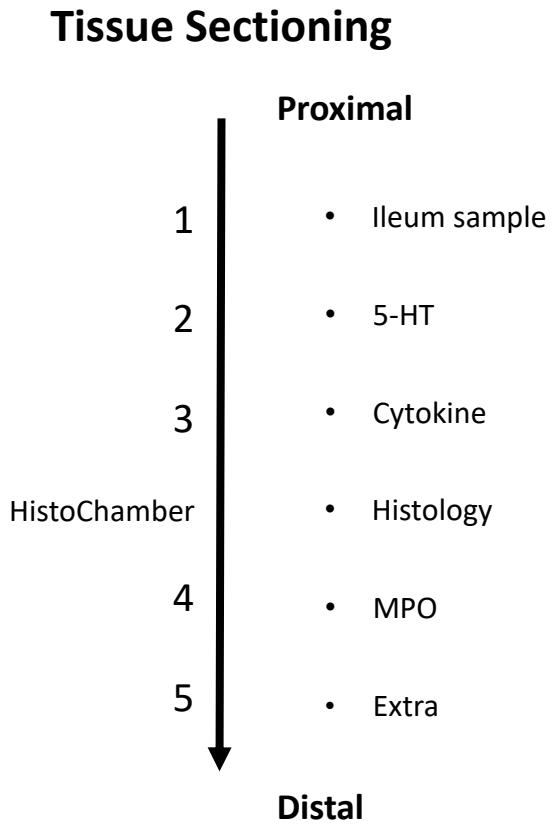


Figure 2: Mouse colonic tissue collection guideline for gene and protein expression analysis.

Tissues were sliced into 1.5 cm sections and kept at -80°C.

Histological analysis (goblet cell staining)

Colon tissues (almost 3mm in length) were placed in histology cassettes and fixed in 10% neutral buffered formalin for 24-48 hours. Before being processed and embedded in paraffin wax, the fixed samples were stored in 70% ethanol. Intestinal goblet cells were detected by staining paraffin-embedded intestinal slices with a periodic acid Schiff (PAS) stain. Per mouse, PAS^+ goblet cells were quantified per 10 villus-crypt units in four distinct regions within the colon.

Immunohistochemistry

Colonic *Muc2* expression levels were evaluated using immunohistochemistry, as previously described[100]. This process was carried out on formalin-fixed, paraffin-embedded sections that were cut to a thickness of 5 μm, heated at 60°C for 30 minutes, and then cleaned with CitriSolv (Fisher Scientific, ON, Canada). After rehydration in a decreasing graded ethanol series, slides were exposed to heat-induced epitope retrieval (10 mM sodium citrate buffer–0.05% Tween 20, pH 6.0), followed by overnight incubation with a polyclonal Muc2 antibody (1:50 dilution; sc-15334; Santa Cruz Biotech). Following the 24-hour incubation, slides were rinsed in PBS–0.5% Tween 20 and incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:1,000) (Molecular Probes/Invitrogen). Nikon Eclipse 80i microscope and NIS-Elements Basic Research imaging software were used to capture images. The number of $Muc2^+$ cells was represented as the number of positive cells per 10 crypts.

***In vitro* experiments**

Cell culture

We utilized the human colonic epithelial cell line (HT-29) to investigate the role of a TLR2 agonist, Pam3CSK4, and *T. muris* ESPs in *MUC2* secretion. HT-29 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). These adherent cells

were cultured in a T75 cm^2 tissue culture flask in Dulbecco's modified Eagle medium–nutrient mixture F-12 (DMEM F-12) (Gibco BRL Life Technologies, Burlington, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin, and streptomycin, 1% modified Eagle medium (MEM) and 1% HEPES buffer (pH 7.5) (all purchased from Invitrogen Life Technologies, Burlington, Canada). Briefly, cells were seeded at 5×10^5 cells per well in a 12-well plate and incubated for 24 h at 37°C (with 5% CO_2) in the complete growth medium. The medium was replaced with serum-free media prior to treatment. For these experiments, Pam3CSK4 (TLR2/1 agonist; InvivoGen, San Diego, CA) was used at the following concentrations: 2, 5, and 20 mg/ml. Similarly, *T. muris* ESPs were used at concentrations of 10, 20, 50, and 100 mg/ml. Following the treatment, a Trypan Blue exclusion assay using Trypan Blue (Thermo Fischer Scientific, Burlington, Canada) was performed to test the viability of drug-treated cells, where blue staining indicates cell death. Cells remained viable upon stimulation with the drug of interest after 24 hours.

RNA extraction

Total RNA was isolated from tissue or cultured cells by using TRIzol™ reagent (catalog no. 15596026, Thermo Fischer Scientific, Burlington, Canada). This reagent protects RNA integrity throughout sample homogenization and cell breakdown by highly efficient inhibition of RNase activity.

Briefly, samples were homogenized in TRIzol™ (1 mL for every 10 mg of tissue or 1×10^6 cells). For 5 minutes, the homogenized mixture was incubated at room temperature to enable particles to settle. The sample was vortexed for 10–15 seconds after adding 100 µg chloroform (Lot no. l1305, APC, Montreal, Canada) per 1 ml of initial TRIzol™ volume, then incubated at room temperature for 5 minutes to facilitate phase separation. Next, samples were centrifuged at 12,000g for 15 min at 4 °C, and centrifuge separated the samples into the lower

phenol-chloroform layer and the upper colorless, aqueous layer containing extracted RNA. Cold isopropanol (Lot no. 93501, Georgetown, Canada) (250 µl per 1 ml of TRIzol^{TM}) was added to the top layer to precipitate RNA. After 10 minutes of incubation at room temperature, the samples were centrifuged at 12,000 g for 10 min at 4°C to remove any debris. The supernatant was removed, and the RNA pellet was washed twice with 75% ethanol. Finally, the pellet was air-dried and dissolved in RNase-free water.

RNA quantification and purity assessment

The RNA yield and purity were determined and assessed using a NanoDrop 2000 (Thermo Fischer Scientific, Burlington, Canada), a UV-Vis spectrophotometer. The wavelength of 260 nm is used to measure nucleic acid concentration, and RNA purity is assessed by using the 260/280 ratio. The 260/230 and 260/280 values for a "pure" nucleic acid are usually in the range of 1.8-2.2. A different ratio from the accepted target may represent contamination by proteins, urea, or phenols that may occur during extraction processes.

Complementary DNA (cDNA) synthesis

cDNA was synthesized according to the manufacturer's manual of iScriptTM cDNA synthesis kit (catalog no. 4368814, Bio-Rad, Mississauga, Canada), containing RNase H+ reverse transcriptase enzyme, which inhibits the breakdown of RNA during the production of cDNA.

Quantitative polymerase chain reaction (qPCR)

Quantifying the expression of the target gene relative to reference genes (expressed in all cells) was performed using qPCR. Each PCR reaction mixture, in a volume of 20 µl, consisted of 10 µl of 2 × SsoFast Evagreen SYBR Green PCR Master Mix (catalog no. 1725201, Bio-Rad, Mississauga, Canada), 7 µl of nuclear-free water, 1 µl of cDNA, and 1 µl

of each forward and reverse primers at a concentration of 10 µmol/L. qPCR was carried out in duplicates and by using a CFX96 qPCR system (Bio-Rad). Expression of target genes was normalized to the housekeeping genes coding for human and mouse 18S rRNA. The relative abundance of mRNA was calculated using $2^{-\Delta\Delta CT}$ methods (mean ± SEM), and the melting curve was used to determine the specificity and quality of amplification. Human and mouse primers are listed in (**Table 1).**

Table 1:qPCR human primer sequences.

	Forward (5'-3')	**Reverse (5'-3')**
18S (human)	TCCACAGGAGGCCTACACGCC	TTTCCGCCGCCCATCGATGTT
MUC2 (human)	ACTCTCCACACCCAGCATCATC	GTGTCTCCGTATGTGCCGTTGT

Table 2: qPCR mouse primer sequences.

	Forward (5'-3')	**Reverse (5'-3')**
18S (mouse)	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Muc2 (mouse)	CTGACCAAGAGCGAACACAA	CATGACTGGAAGCAACTGGA
Muc5ac (mouse)	GTGATGCACCCATGATCTATTTTG	ACTCGGAGCTATAACAGGTCATGTC
TLR2 (mouse)	AAGAGGAAGCCCAAGAAAGC	CGATGGAATCGATGATGTTG
TLR4 (mouse)	ACCTGGCTGGTTTACACGTC	CTGCCAGAGACATTGCAGAA
Occludin (mouse)	ATGTCCGGCCGATGCTCTCTC	CTTTGGCTGCTCTTGGGTCTGTAT
Zo-1 (mouse)	ACCCGAAACTGATGCTGTGGATAGA	AAATGGCCGGGCAGAACTTGTGTA
mBD-1 (mouse)	GGTGTTGGCATTCTCACAAG	ACAAGCCATCGCTCGTCCTTTATG
mBD-3 (mouse)	AGTTGTTTGAGGAAAGGAGGCA	AAAGGAACTCCACAACTGCCA

Enzyme-linked immunosorbent assay (ELISA) and protein assay

First, colonic tissues (15-25 µg) were homogenized in 1ml lysis buffer containing protease inhibitor cocktail (Lot no. 118M4154V, Oakville, Canada) (10 ml of lysis buffer supplemented with 50 µg of protease inhibitor cocktail). Tissue protein concentration was measured by using DC Protein Assay Kit (Catalog no. 5000111; Bio-Rad Laboratories) according to the manufacturer's instructions and compared to a standard curve of known concentration. Supernatants were collected and stored at -80℃.

Mouse cytokines were detected using commercially available ELISA kits, as for IFN-ɣ (Lot no. P133111, R&D System, Minneapolis, USA), IL-4 (Lot no. P247172, R&D System, Minneapolis, USA), IL-13 (Lot no. P293959, R&D System, Minneapolis, USA), and IL-17 (Lot no. P296099, R&D System, Minneapolis, USA). Assays were performed according to the manufacturer's instructions. Results from ELISA were normalized to the total tissue protein concentration.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 8.4.2 (GraphPad Software, La Jolla, CA), and results are shown as mean ± SEM. For comparison of groups, unpaired Student's t-test or one-way ANOVA with Dunnett's and Tukey's was used. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ were considered statistically significant in all tests.

Chapter 4: Results

4.1. *T. muris* infection changes the composition of cecal microbiota.

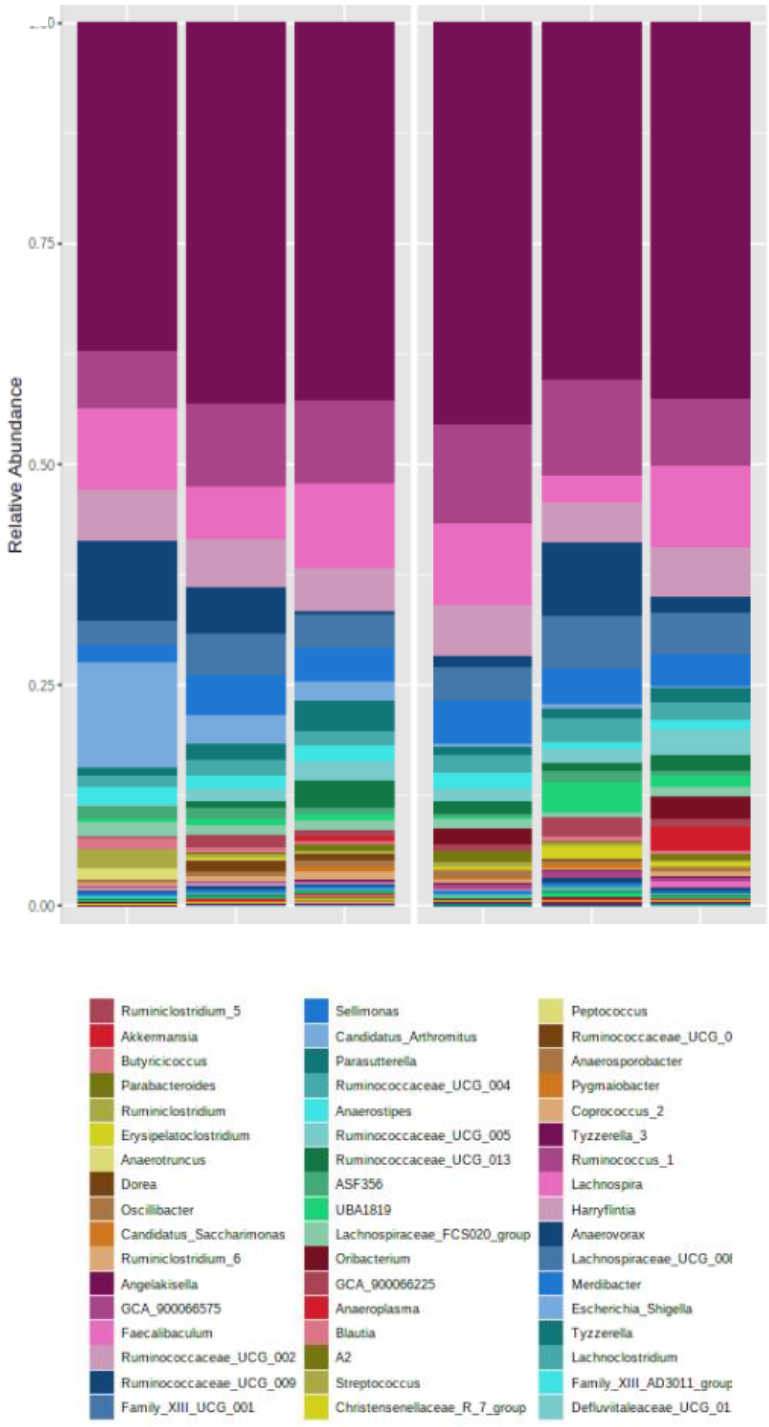
We examined the microbiota composition of *T. muris*-infected mice to that of uninfected mice to investigate whether *T. muris* infection alters the host microbial composition. Our findings revealed that *T. muris*-infected mice had a different gut microbial composition compared to WT littermates on day 36 p.i., suggesting *T. muris*, either directly or indirectly, can impact the microbial make-up of the cecum (**Figure 3**).

Three mice infected with *T. muris* were sacrificed on day 21 p.i. and intestinal worm burden was measured in order to validate *T. muris* infectivity in this experiment. As expected in acute *T.muris*-infected mice, worms were not completely expelled by day 21 post-infection p.i. (**Figure 4a**). Consistent with previous studies, *T. muris*-infected mice had significantly higher levels of IL-13 on day 36 p.i. compared to non-infected mice, thus establishing that these mice were acutely infected (**Figure 4b**).

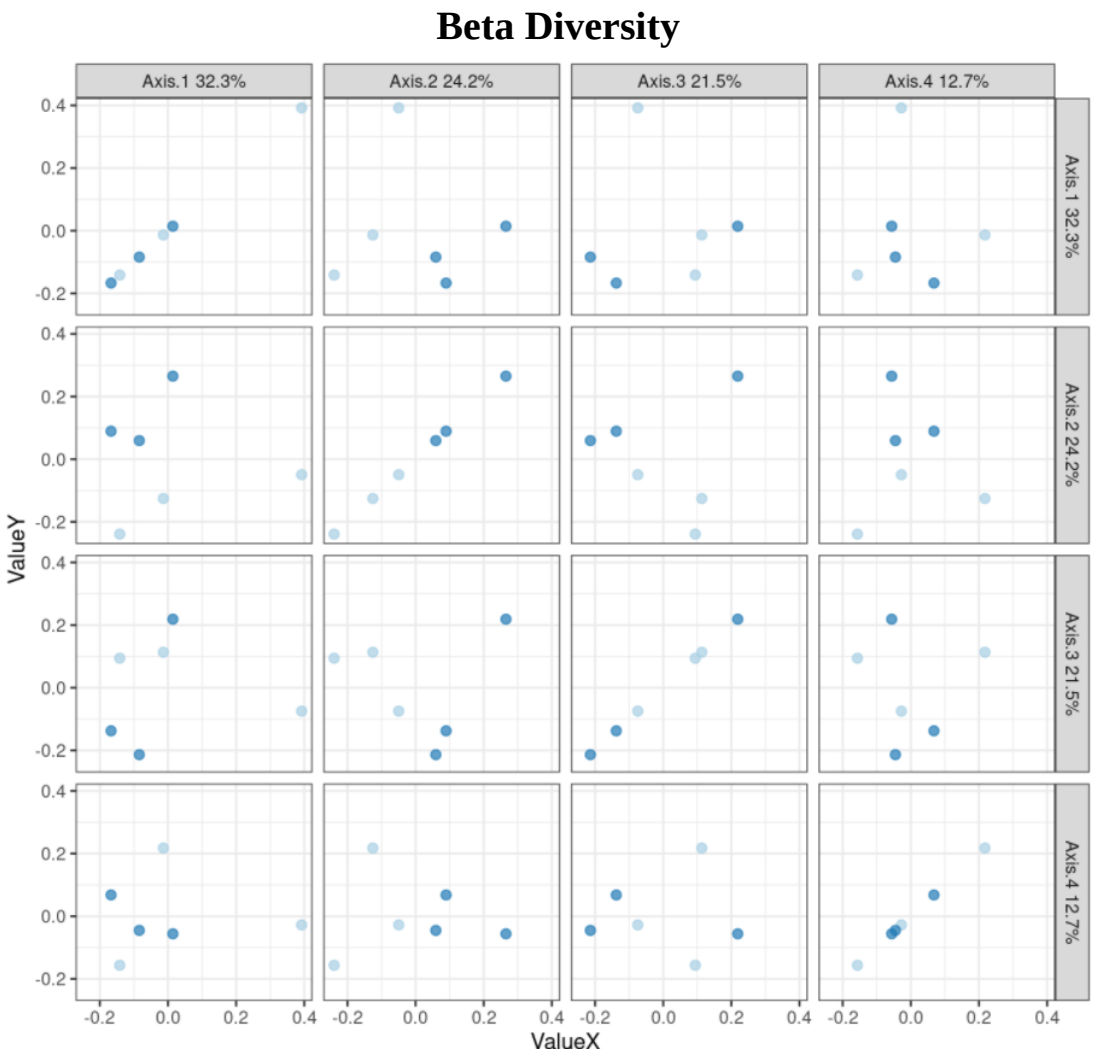
Figure 3: Impact of *T. muris* infection on microbial communities.

(a) Relative abundance of the most abundant genera identified in cecal microbiota samples from *T. muris*-infected (day 36 p.i.) and non-infected mice assessed by 16S rRNA analysis. (b, c) Beta diversity of the cecal microbiota in *T. muris*-infected (day 36 p.i.) and non-infected mice based on the Bray-Curtis index. Mice acutely infected with *T. muris* are clustered separately from mice without *T. muris* infection.

a)


Relative Abundance
0.75
0.50
0.25
0.00
Ruminiclostridium_5
Akkermansia
Butyricicoccus
Parabacteroides
Ruminiclostridium
Erysipelatoclostridium
Anaerotruncus
Dorea
Oscillibacter
Candidatus_Saccharimonas
Ruminiclostridium_6
Angelakisella
GCA_900066575
Faecalibaculum
Ruminococcaceae_UCG_002
Ruminococcaceae_UCG_009
Family_XIII_UCG_001
Sellimonas
Candidatus_Arthromitus
Parasutterella
Ruminococcaceae_UCG_004
Anaerostipes
Ruminococcaceae_UCG_005
Ruminococcaceae_UCG_013
ASF356
UBA1819
Lachnospiraceae_FCS020_group
Oribacterium
GCA_900066225
Anaeroplasma
Blautia
A2
Streptococcus
Christensenellaceae_R_7_group
Peptococcus
Ruminococcaceae_UCG_0
Anaerosporobacter
Pygmaiobacter
Coprococcus_2
Tyzzerella_3
Ruminococcus_1
Lachnospira
Harryflintia
Anaerovorax
Lachnospiraceae_UCG_00
Merdibacter
Escherichia_Shigella
Tyzzerella
Lachnoclostridium
Family_XIII_AD3011_group
Defluviitaleaceae_UCG_01

b)



- Non-infected mice
- *T. muris*-infected mice

c)

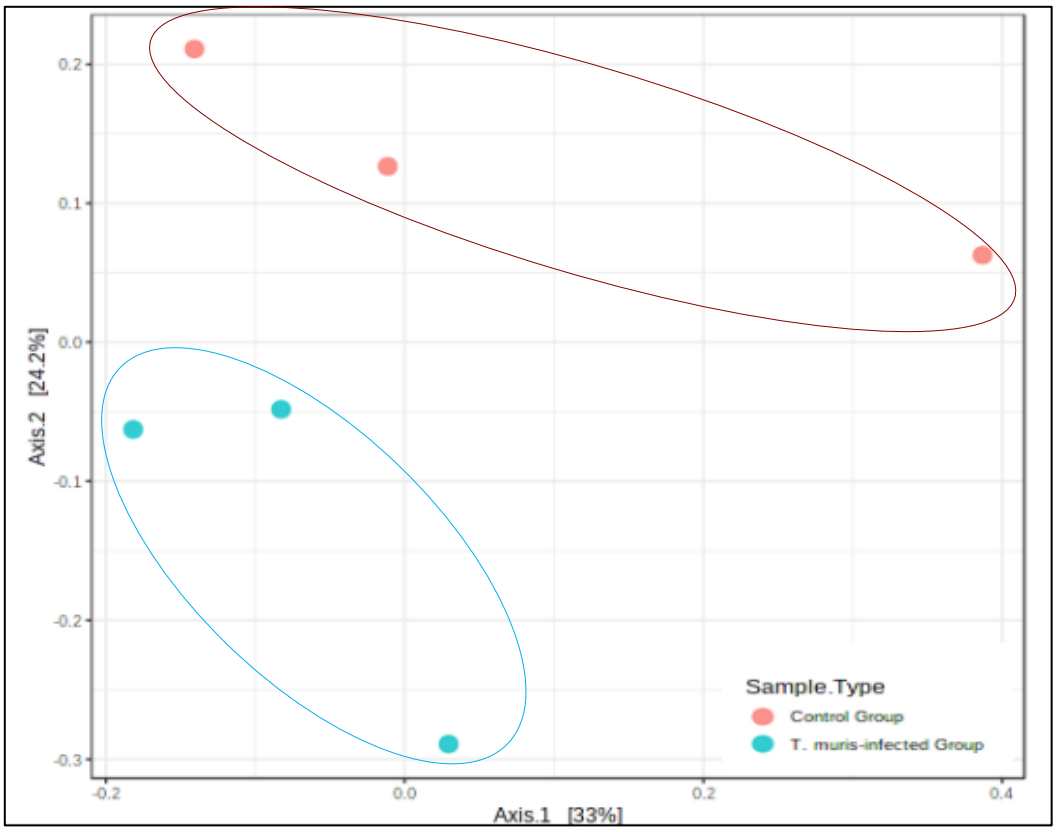
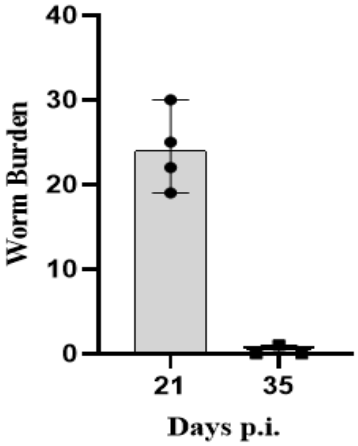

0.2
0.1
0.0
-0.1
-0.2
-0.3
Axis.2 [24.2%]
-0.2
0.0
0.2
0.4
Axis.1 [33%]
Sample.Type
Control Group
T. muris-infected Group

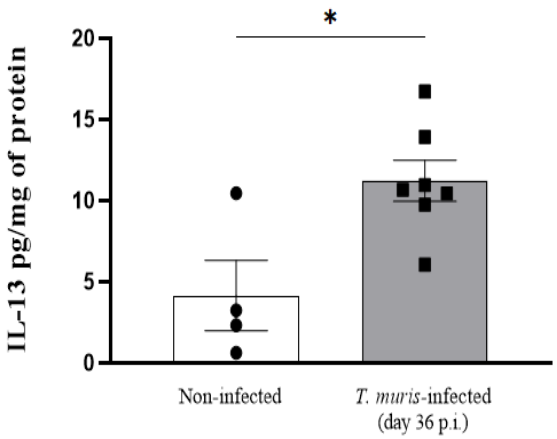
Figure 4: Confirmation of *T. muris* infection in infected group by measuring worm burden (day 21 and 36 p.i.) and IL-13 levels (day 36 p.i.).

Cecal (a) worm burden and colonic (b) IL-13 levels in C57BL/6 mice with or without *T. muris* infection. Data are represented as mean ± SEM; **P*< 0.05; n=3-7 mice/group

a)


Worm Burden
40
30
20
10
0
21
35
Days p.i.

b)


IL-13 pg/mg of protein
20
15
10
5
0
*
Non-infected
T. muris-infected
(day 36 p.i.)

4.2. *T. muris*-induced altered microbiota modulates goblet cell response (*Muc2* production) and stimulates *TLR2* expression.

To explore the role of *T. muris*-induced altered microbiota in modulating goblet cell responses, cecal content from *T. muris*-infected (collected on day 36 p.i. to exclude any possibility of transferring worms) and non-infected C57BL/6 mice were transferred, as previously described[112], into two separate groups of GF mice.

There was a significant increase in PAS-stained goblet cell numbers in GF mice given *T. muris*-infected microbiota, as opposed to GF mice receiving microbiota from non-infected mice (**Figure 5a, c**). We also observed significant upregulation of $Muc2^+$ cells in GF mice with *T. muris*-altered microbiota compared to those received non-infected microbiota, as assessed by immunohistochemical staining (**Figure 5b, d**). In addition, mucin expression, notably *Muc2*, increased in GF mice after being exposed to the *T. muris*-altered microbiota. However, this increase was not statistically significant (**Figure 6a)**. A similar trend was also observed in measures of both occludin and *ZO-1* expression (**Figure 6b**). Expression levels of mouse β-defensin 1 *(mBD-1)* and *mBD-3* were unaltered by both non-infected microbiota or microbiota from *T. muris*-infected mice (**Figure 7a, b**).

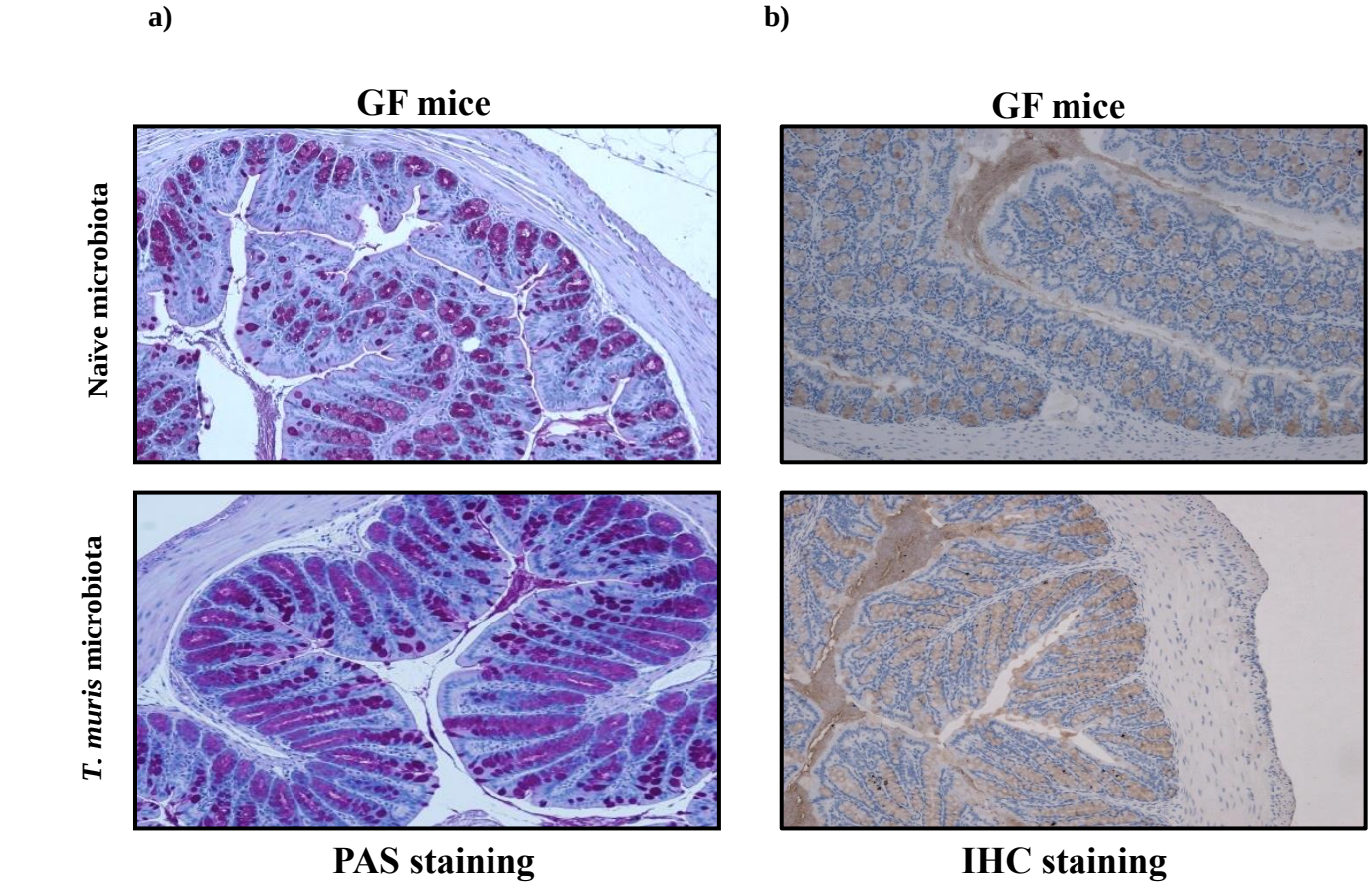
Further, levels of the Th2 cytokines, IL-4 and IL-13, were significantly increased in GF mice colonized with *T. muris*-induced microbiota, as compared to mice colonized with non-infected microbiota (**Figure 8a, b**). In addition to Th2 cytokines, IFN-ɣ and IL-17 levels were measured to investigate whether *T. muris*-induced microbiota can influence goblet cell response through additional adaptive immune responses like Th1 and Th17. Our findings demonstrated that the production of the Th1 cytokine, IFN-ɣ, and the Th17 cytokine, IL-17, did not differ significantly between groups which may indicate there is no association between microbiota altered by *T. muris* infection and the production of both Th1 and Th17 cytokines in order to regulate goblet cell response (**Figure 9a, b**).

TLR2 expression was significantly higher in GF mice receiving *T. muris*-infected microbiota compared to those colonized with microbiota from the non-infected group (**Figure 10a**). In contrast, we did not observe a significant difference in *TLR4* expression between both GF mice groups (**Figure 10b**). TLR2 seems to be involved in the interaction between *T. muris*-altered microbiota and goblet cells, according to these results.

It should be mentioned that, in contrast to conventional mice, GF mice have diminished Muc2 protein levels within the colon, and notably, these levels increase following colonization with microbiota from conventional mice[49]. Therefore, the observed increase in goblet cell number and *Muc2* expression in GF mice colonized with *T. muris*-altered microbiota compared to GF mice colonized with non-infected microbiota support the idea that *T. muris*-induced changes within the gut microbiota stimulate goblet cell responses and mucin secretion, and TLR2 signalling may play a role in this process.

Figure 5: Number of intestinal goblet cells and Muc2$^+$ cells in GF mice after microbiota transplantation from *T. muris*-infected mice compared to GF mice receiving microbiota from non-infected mice.

Representative images of (a) PAS- and (b) Muc2-stained colonic cross-sections in GF mice colonized with *T. muris*-infected or non-infected microbiota. (c) Number of PAS-stained goblet cells, and (d) Number of Muc2$^+$ cells, assessed by IHC, in GF mice received *T. muris*-infected or non-infected microbiota. Data are represented as mean ± SEM; *P< 0.05; n=3-7 mice/group.


a)
b)
GF mice
GF mice
Naïve microbiota
T. muris microbiota
PAS staining
IHC staining

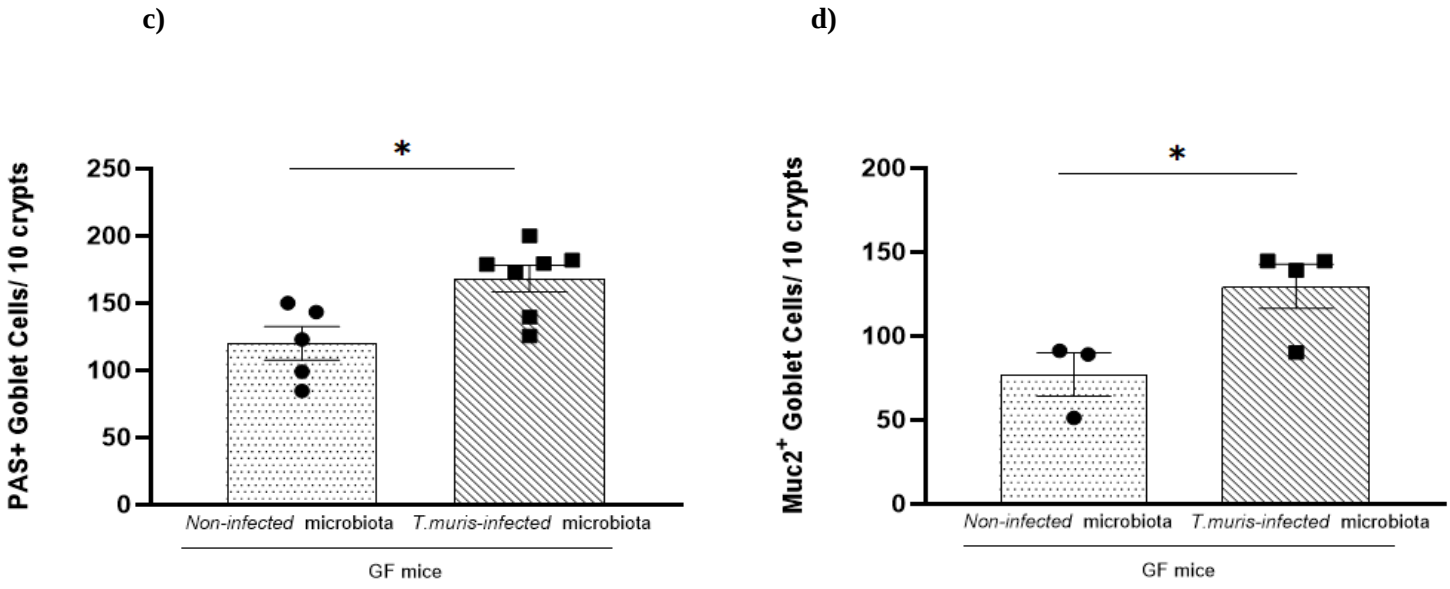
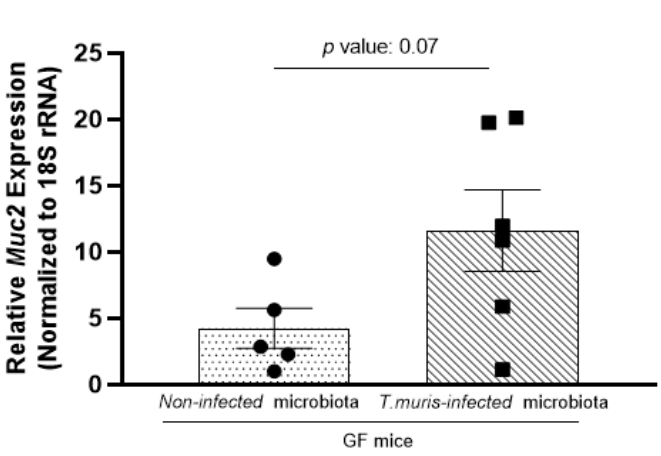
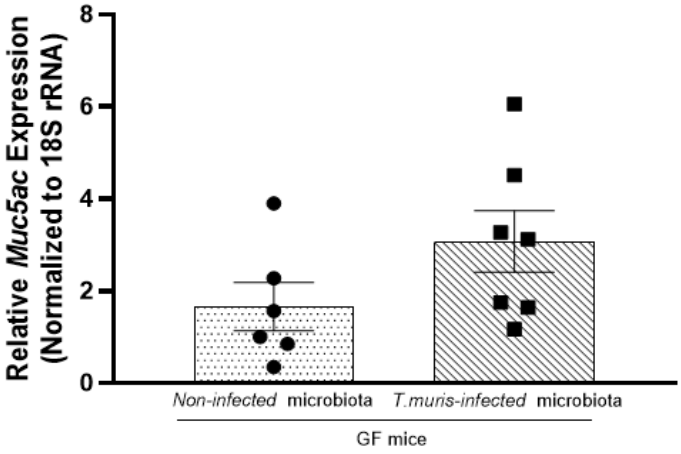

c)
d)
PAS+ Goblet Cells/ 10 crypts
Muc2+ Goblet Cells/ 10 crypts
*
*
250
200
150
100
50
0
200
150
100
50
0
Non-infected microbiota
T.muris-infected microbiota
Non-infected microbiota
T.muris-infected microbiota
GF mice
GF mice

Figure 6: Relative mRNA expression of the mucins, *Muc2* and *Muc5ac*, and the tight junction proteins, occludin and *ZO-1*, in GF mice colonized with *T. muris*-infected microbiota compared to GF mice receiving non-infected microbiota.

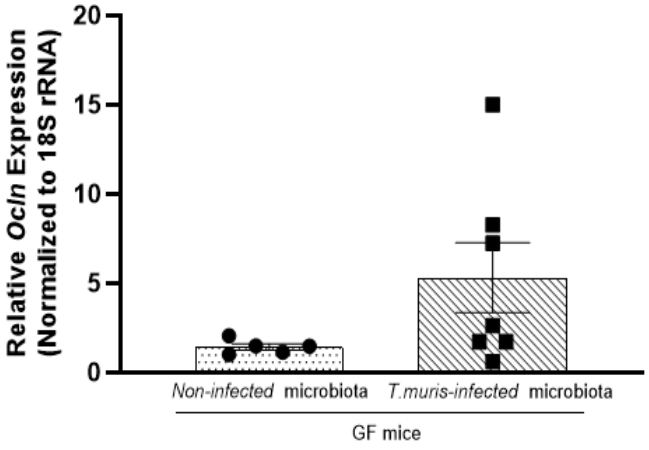
(a) *Muc2*, *Muc5ac*, (b) *Ocln*, and *ZO-1* expression normalized to mouse 18S in colonic tissue of GF mice given *T. muris*-infected microbiota compared to GF mice receiving non-infected microbiota using qPCR. Data are represented as mean ± SEM; n=5-7 mice/group.

a)


p value: 0.07
Relative Muc2 Expression (Normalized to 18S rRNA)
25
20
15
10
5
0
Non-infected microbiota
T.muris-infected microbiota
GF mice


Relative Muc5ac Expression (Normalized to 18S rRNA)
8
6
4
2
0
Non-infected microbiota
T.muris-infected microbiota
GF mice

b)


Relative Ocln Expression (Normalized to 18S rRNA)
20
15
10
5
0
Non-infected microbiota
T.muris-infected microbiota
GF mice

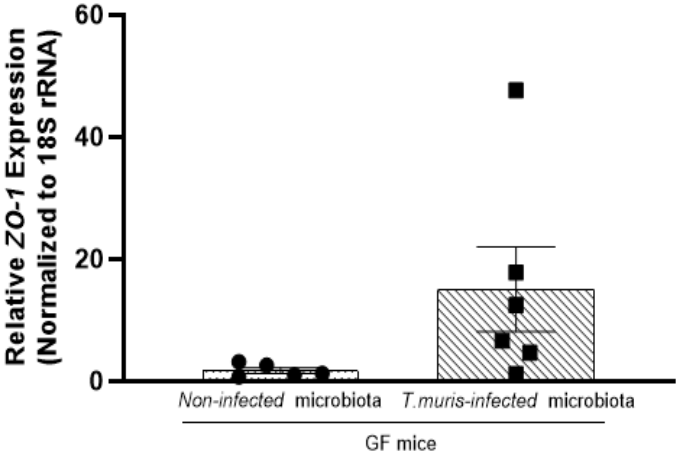
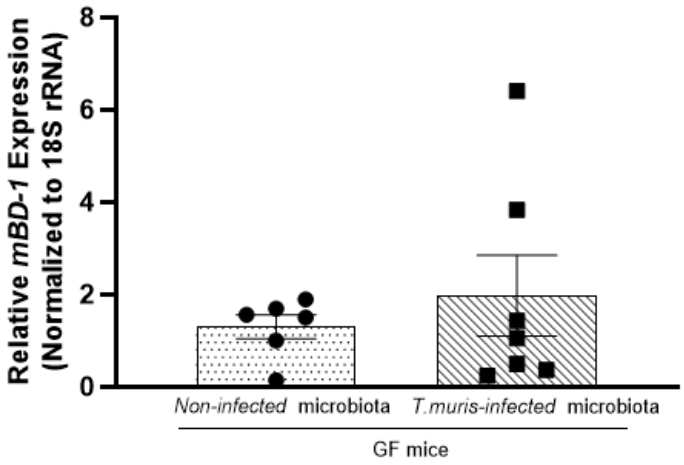

Relative ZO-1 Expression (Normalized to 18S rRNA)
60
40
20
0
Non-infected microbiota
T.muris-infected microbiota
GF mice

Figure 7: Relative mRNA expression levels of *mBD-1* and *mBD-3* in GF mice with *T. muris*-infected microbiota compared to GF mice gavaged with microbiota from non-infected mice.

Expression of (a) *mBD-1* and (b) *mBD-3* normalized to mouse 18S in colonic tissue of GF mice given *T. muris*-infected microbiota compared to GF mice receiving non-infected microbiota using qPCR. Data is represented as mean ± SEM; n=5-7 mice/group.

a)


Relative *mBD-1* Expression
(Normalized to 18S rRNA)
8
6
4
2
0
Non-infected microbiota
T.muris-infected microbiota
GF mice

b)

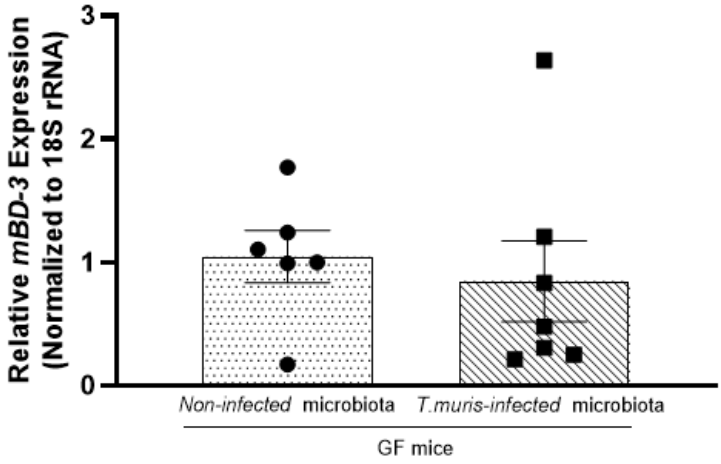
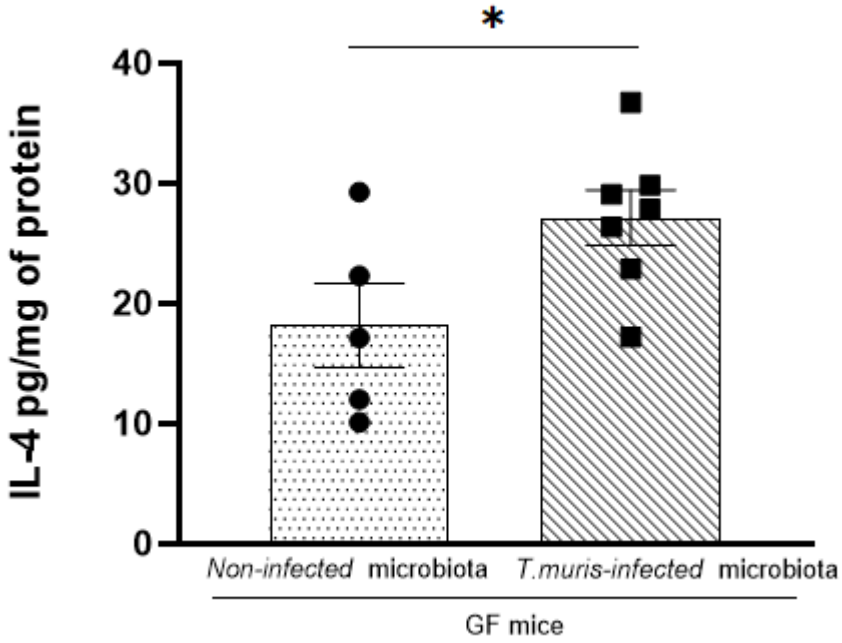

Relative *mBD-3* Expression
(Normalized to 18S rRNA)
3
2
1
0
Non-infected microbiota
T.muris-infected microbiota
GF mice

Figure 8: Levels of the Th2 cytokines, IL-4 and IL-13, in colonic tissue of GF mice with *T. muris*-infected microbiota compared to GF mice with microbiota from non-infected mice.

(a) IL-4 (b) and IL-13 levels in colonic tissue of GF mice given *T. muris*-infected microbiota compared to GF mice receiving non-infected microbiota. Data are represented as mean ± SEM; **P*< 0.05; n=5-7 mice/group.

a)


*
40
30
20
10
0
IL-4 pg/mg of protein
Non-infected microbiota
T.muris-infected microbiota
GF mice

b)

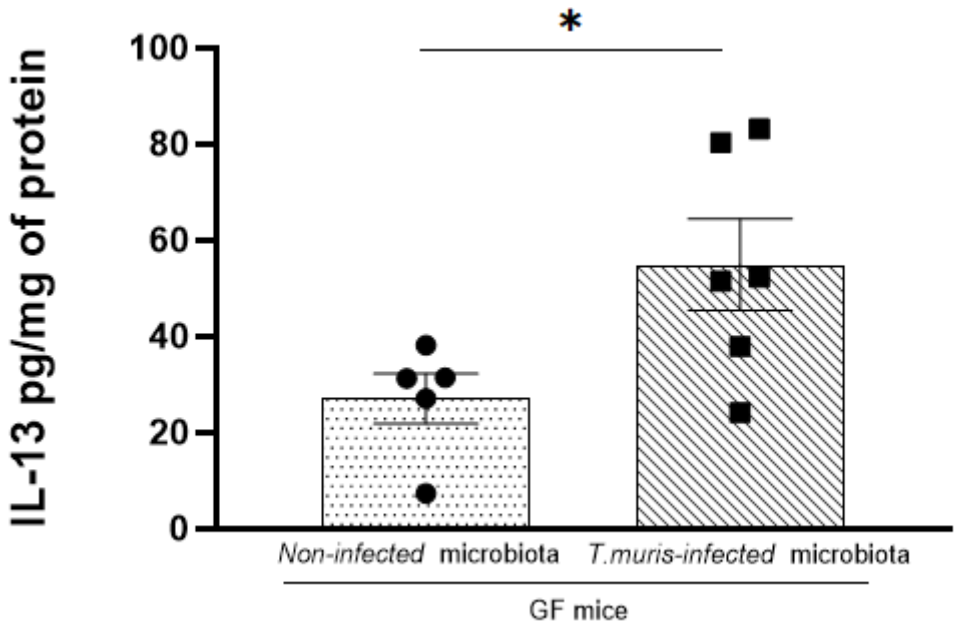
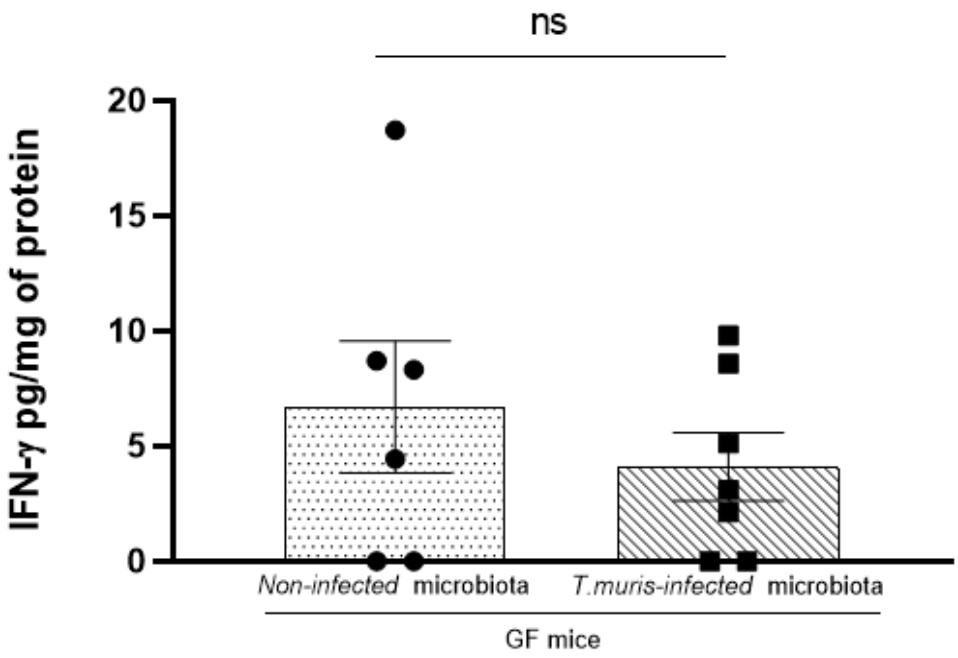

*
100
80
60
40
20
0
IL-13 pg/mg of protein
Non-infected microbiota
T.muris-infected microbiota
GF mice

Figure 9: Levels of IFN-ɣ and IL-17 after transplantation of *T. muris*-induced microbiota/non-infected microbiota into recipient GF mice.

(a) IFN-ɣ (b) and IL-17 levels in colonic tissues of GF mice given *T. muris*-infected microbiota compared to GF mice receiving non-infected microbiota. Data are represented as mean ± SEM; n=5-7 mice/group.

a)


ns
IFN-γ pg/mg of protein
20
15
10
5
0
Non-infected microbiota
T.muris-infected microbiota
GF mice

b)

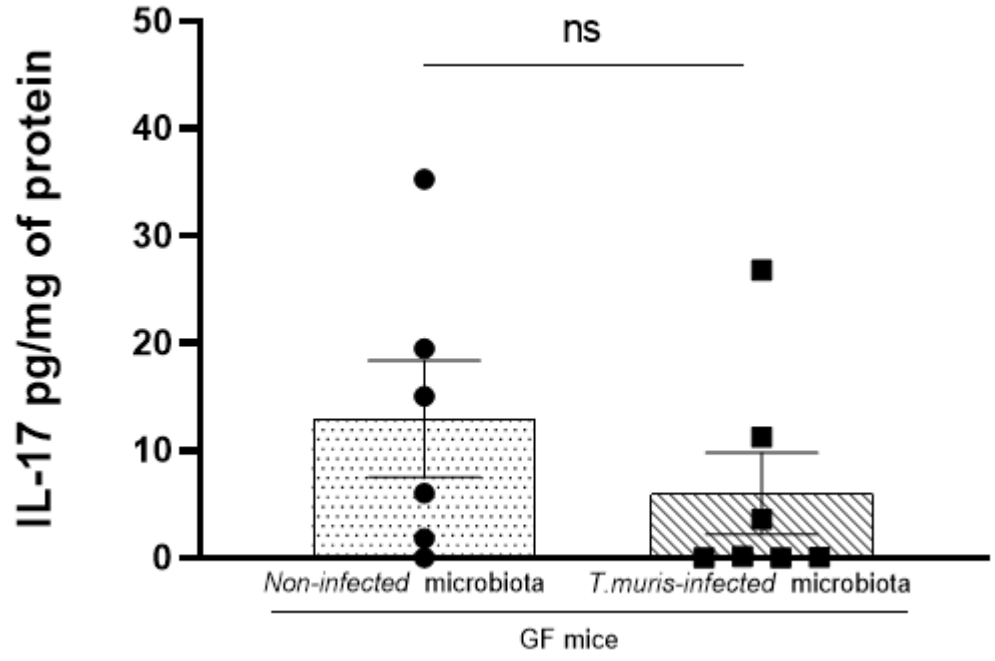
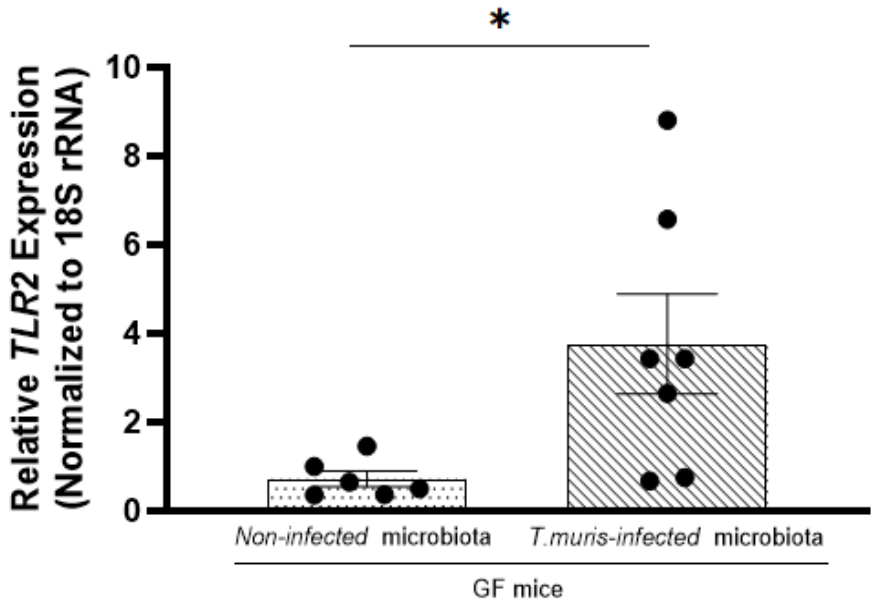

ns
IL-17 pg/mg of protein
50
40
30
20
10
0
Non-infected microbiota
T.muris-infected microbiota
GF mice

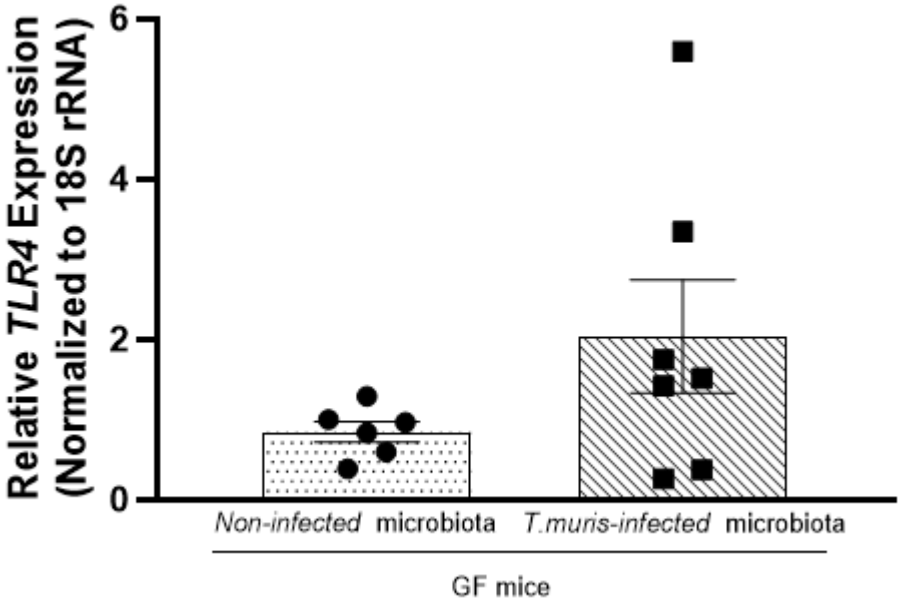
Figure 10: Relative mRNA expression levels of *TLR2* and *TLR4* in GF mice with *T. muris*-infected microbiota compared to GF mice gavaged with microbiota from non-infected mice.

(a) *TLR2* and (b) *TLR4* expression normalized to mouse 18S in colonic tissue of GF mice given *T. muris*-infected microbiota compared to GF mice receiving non-infected microbiota using qPCR. Data are represented as mean ± SEM; **P*<0.05; n=5-7 mice/group.

a)


*
Relative TLR2 Expression
(Normalized to 18S rRNA)
10
8
6
4
2
0
Non-infected microbiota
T.muris-infected microbiota
GF mice

b)


Relative TLR4 Expression
(Normalized to 18S rRNA)
6
4
2
0
Non-infected microbiota
T.muris-infected microbiota
GF mice

4.3. Treatments in both experiments, infection in WT mice and transplantation of *T. muris*-altered microbiota into GF mice, were shown to be responsible for 15% of the among-sample difference and to be statistically significant (p <= 0.001) in both experiments.

We analyzed the microbial composition of GF mice receiving either *T. muris*-infected cecal microbiota or non-infected cecal microbiota. Analysis of 16S rRNA sequencing indicated that there is a significant effect of treatment on microbial composition in this experiment (p ~= 0.006), and thus GF mice colonized with *T. muris*-altered microbiota were divided into different clusters compared to counterpart mice with naïve microbiota, as indicated by PCoA **(Figure 11)**.

To assess the efficacy of microbiota transplantation from non-infected and *T. muris*-infected mice into GF mice groups, we used Bray-Curtis beta diversity to assess microbial composition in recipient GF mice after microbiota transplantation compared to donor groups **(Figure 12)**. GF mice colonized with microbiota either from *T. muris*-infected mice or non-infected mice develop microbiota profiles similar to those of their respective donors.

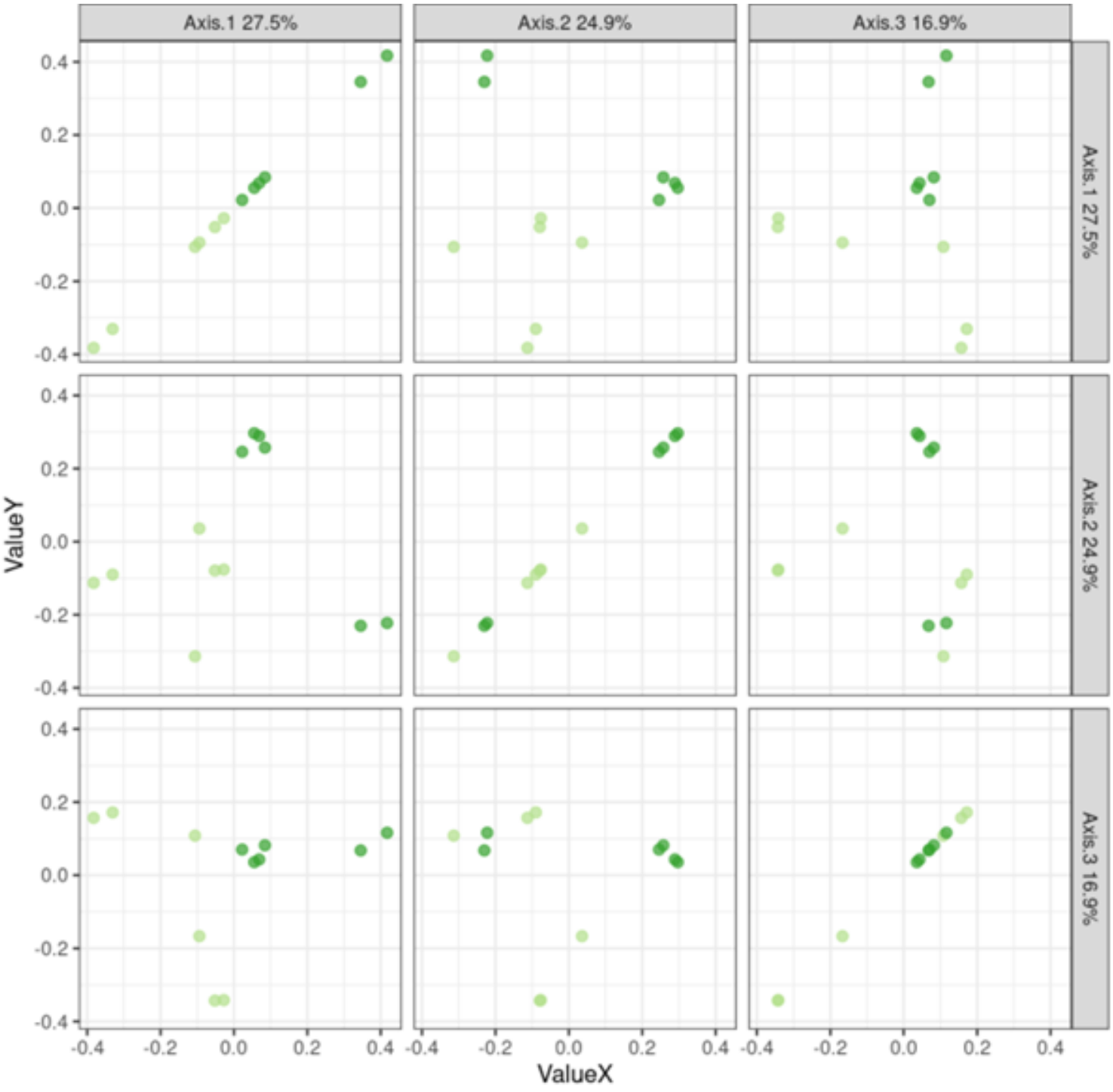
When both experiments, *T. muris* infection in WT mice and transplantation of *T. muris*-altered microbiota into GF mice, are analyzed together, experiment accounts for 16% of the among-sample variation, treatment accounts for 15%, and both are statistically significant (p <= 0.001 in both cases).

In order to distinguish those taxa that are significantly altered by *T. muris*-infection and thus may contribute to observed changes in goblet cell function and mucin production, bacterial taxa that were significantly different in both donor conventional mice and recipient GF under non-infected and infected conditions were identified. Of the top 100 most abundant amplicon sequence variants (ASVs) that are significantly different between *T. muris* microbiota (*T. muris*-infected mice and GF mice colonized with *T. muris*-altered microbiota) and naive microbiota (non-infected mice and GF mice colonized with non-infected microbiota), seven ASVs, from *Lachnospiraceae* and

Muribaculaceae families, were significantly decreased in the relative abundance of *T. muris*-infected mice, and those GF mice colonized with *T. muris* microbiota, compared to non-infected mice and GF mice colonized by non-infected microbiota (**Figure 13**). Two ASVs in *Rikenellaceae* and *Ruminococcaceae* families were increased in GF mice receiving *T. muris*-altered microbiota compared to GF mice with non-infected microbiota.

Figure 11: Analysis of microbial compositions in GF mice after transfer of gut microbiota from *T. muris*-infected and non-infected mice.

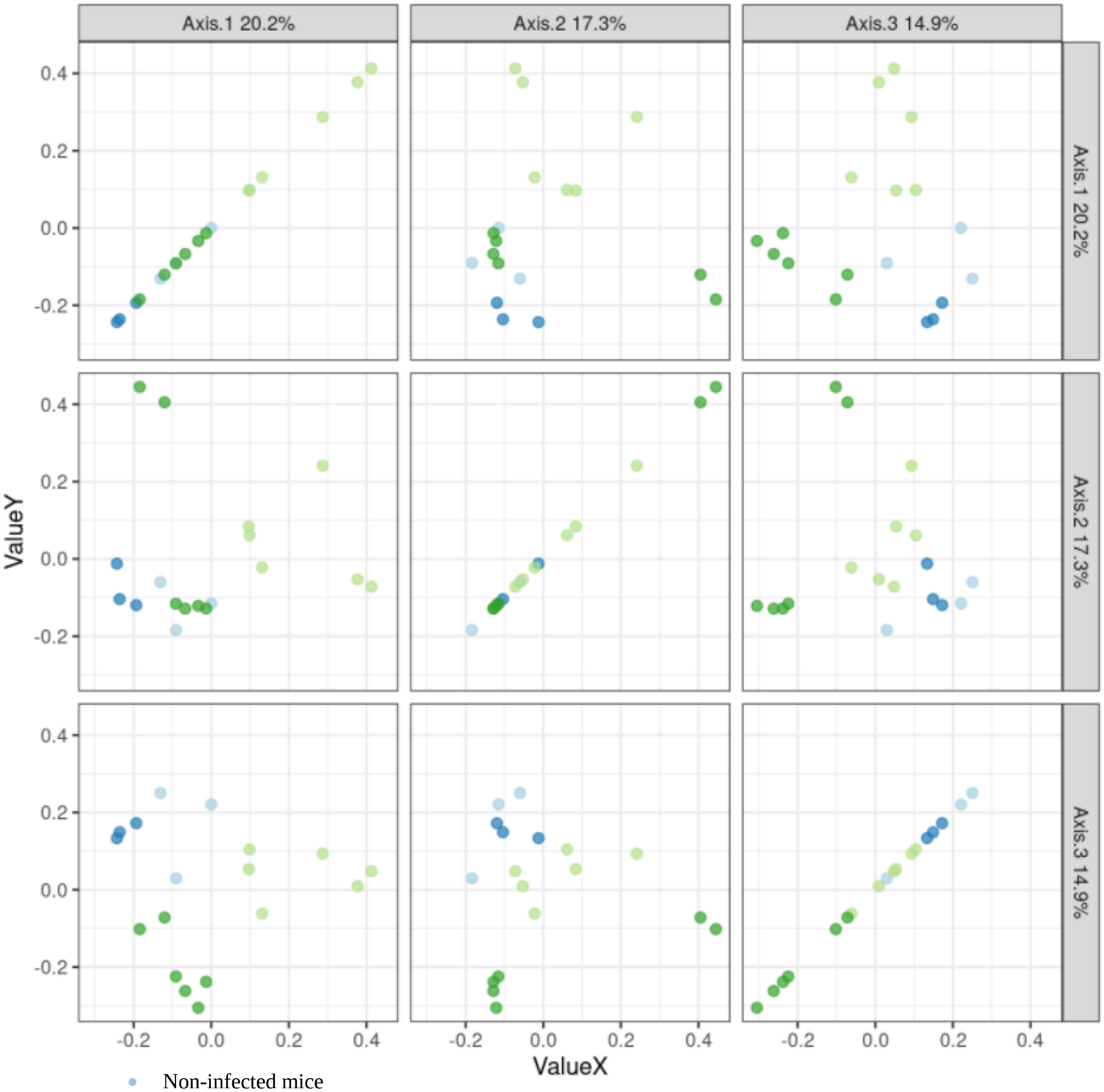
Beta diversity based on the Bray-Curtis index showed distinct microbiota for GF mice after receiving *T. muris* microbiota (dark green) compared to GF mice with non-infected microbiota (light green).



- GF mice + non-infected microbiota
- GF mice + *T. muris*-infected microbiota

Figure 12: Bray-Curtis dissimilarity of GF mice colonized with microbiota from *T. muris*-infected mice or with microbiota from non-infected mice compared to donors.

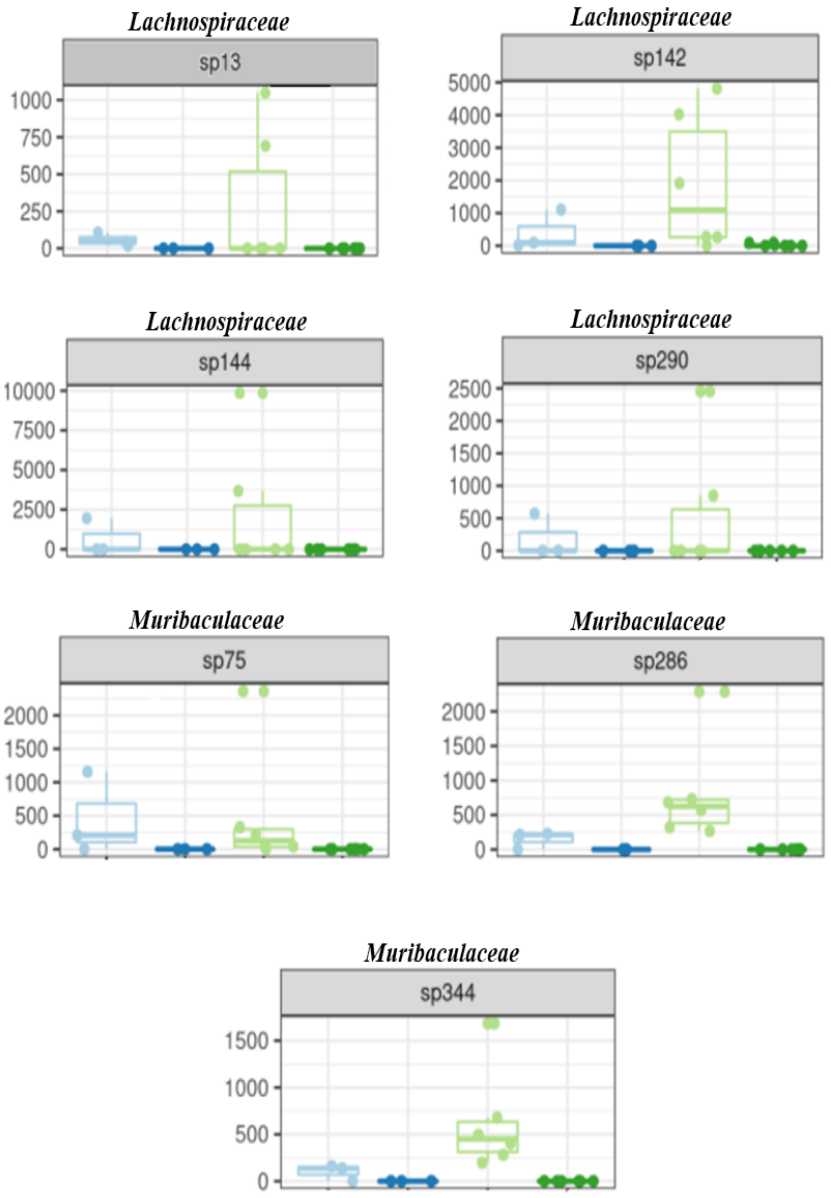
Bray-Curtis dissimilarity revealed that colonization of GF mice with microbiota from either *T. muris*-infected or non-infected mice results in the development of microbiota profiles similar to those of their donors.



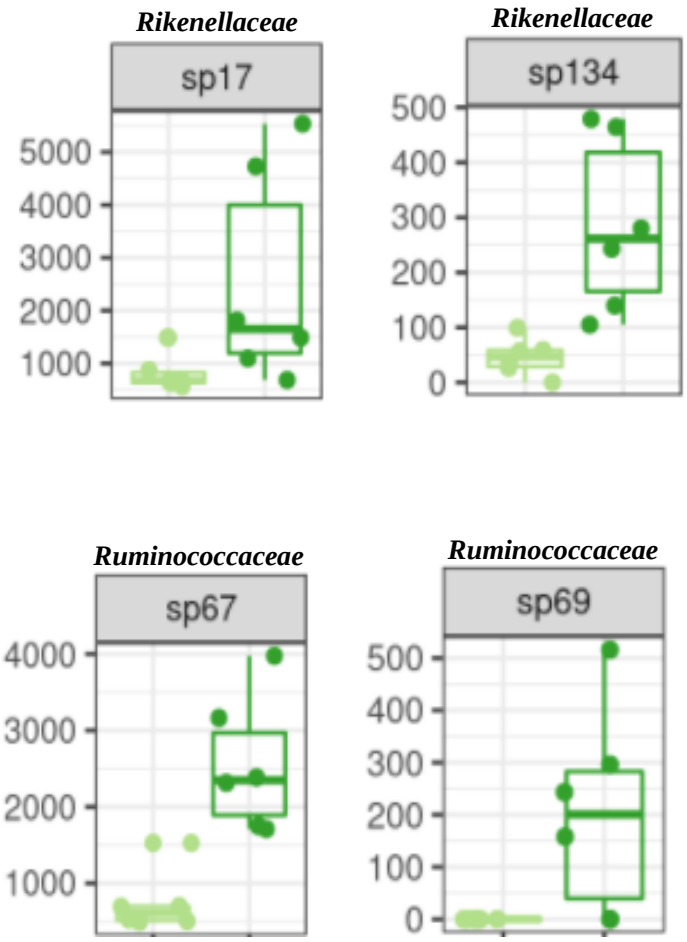
- Non-infected mice
- *T. muris*-infected mice
- GF mice + non-infected microbiota
- GF mice + *T. muris*-infected microbiota

Figure 13: Box-whisker plots representative of statistically significant ASVs present in the top 100 most abundant ASVs.

ASVs differed significantly between the groups of mice with either *T. muris* microbiota (*T. muris*-infected mice and GF mice colonized with *T. muris*-altered microbiota) and mice with naive microbiota (non-infected mice and GF mice colonized with non-infected microbiota). The ASVs shown are those of statistically different that were present in the top 100 most abundant ASVs. Box-whisker plots of relative abundance for visualization purposes for each ASV are presented.



- Non-infected mice
- *T. muris*-infected mice
- GF mice + non-infected microbiota
- GF mice + *T. muris*-infected microbiota



- GF mice + non-infected microbiota
- GF mice + *T. muris*-infected microbiota

4.4. TLR2 agonist and *T. muris* ESPs directly stimulate the human colonic epithelial cell line, HT-29, to produce *MUC2*.

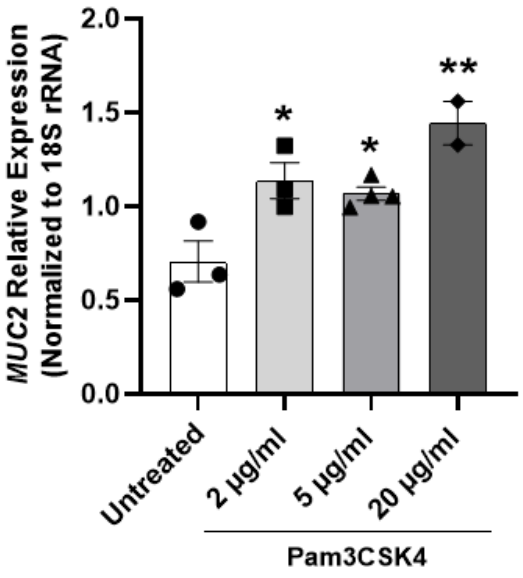
Given the observed elevated *TLR2* expression in GF mice after receiving *T. muris*-altered microbiota **(Figure 10a)**, as well as the known close proximity between *T. muris*, the gut microbiota, and epithelial cells, it is plausible that the interactions between *T. muris* and the gut microbiota can have effects on goblet cell responses and mucin production via TLR2 activation. Herein, HT-29 cells (human epithelial cell line) were treated with the TLR2 agonist, Pam3CSK4, at concentrations of 2, 5, and 20 µg/ml and *T. muris* ESPs at concentrations of 10, 20, 50, and 100 µg/ml for 24 hours to investigate the role of TLR2 and *T. muris* ESPs in the regulation of *MUC2* expression. In addition, co-treatment was performed by incubating HT-29 cells with both Pam3CSK4 and ESPs (both at concentrations of 20 g/ml) over a 24-hour period.

It was found that the stimulation of HT-29 cells with Pam3CSK4 and *T. muris* ESPs for 24 hours increased *MUC2* expression compared to control (untreated) groups **(Figure 14a, b)**. Results of Pam3CSK4 and *T. muris* ESPs co-treatment showed that Pam3CSK4-stimulated HT-29 cells expressed a higher level of *MUC2* expression compared to ESPs-treated and co-treated cells with the same concentrations **(Figure 14c)**. These findings imply a modulatory role of TLR2 and *T. muris* ESPs in *MUC2* expression in human intestinal epithelial cells.

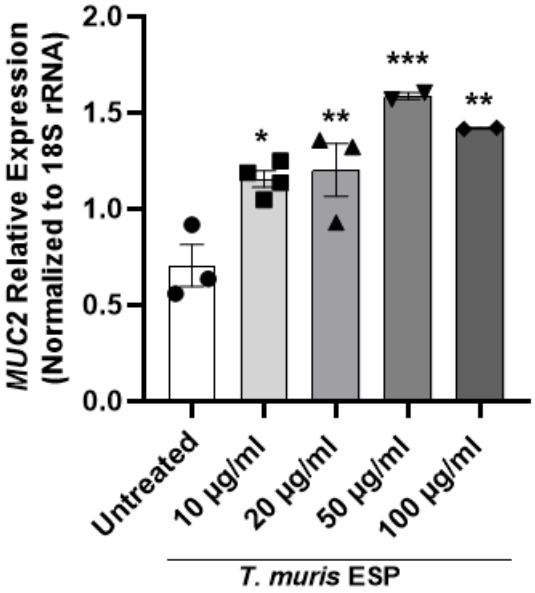
Figure 14: TLR2 agonist (Pam3CSK4) and *T. muris* ESPs impacts on the expression of *MUC2* in HT-29 cells.

mRNA expression of *MUC2* upon 24 h of (a) Pam3CSK4 (2, 5 and 20 µg/ml) and (b) ESPs (10, 20, 50 and 100 µg/ml) treatments. (c) *MUC2* levels after Pam3CSK4 and ESPs (at the concentration of 20 µg/ml) co-treatment for 24 hours. Data are representative of three independent experiments (mean ± SEM); **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (*p values* are representative differences in groups relative to untreated group).

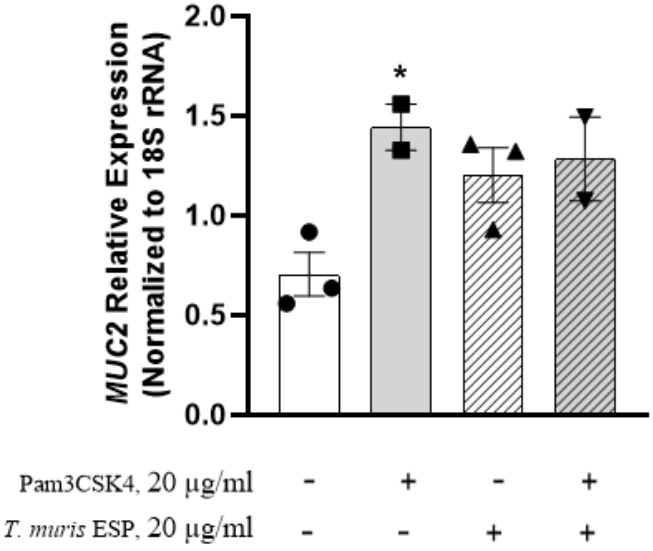
a)


MUC2 Relative Expression
(Normalized to 18S rRNA)
2.0
1.5
1.0
0.5
0.0
*
*
**
Untreated
2 µg/ml
5 µg/ml
20 µg/ml
Pam3CSK4

b)


MUC2 Relative Expression
(Normalized to 18S rRNA)
2.0
1.5
1.0
0.5
0.0
*
**
***
**
Untreated
10 µg/ml
20 µg/ml
50 µg/ml
100 µg/ml
T. muris ESP

c)


MUC2 Relative Expression
(Normalized to 18S rRNA)
2.0
1.5
1.0
0.5
0.0
*
Pam3CSK4, 20 µg/ml - + - +
T. muris ESP, 20 µg/ml - - + +

4.5. *Muc2* expression was significantly increased in mice treated with the TLR2 agonist, Pam3CSK4.

Based on *in vivo* observations that *TLR2* and *Muc2* expression were increased in mice given *T. muris*-altered microbiota compared to those gavaged with non-infected microbiota **(Figure 6a and 10a)** and *in vitro* findings indicating that human intestinal cells (HT-29) exposed to a TLR2 agonist increases *MUC2* expression **(Figure 14a)**, we investigated whether treatment with the aforementioned TLR2 agonist enhances *Muc2* expression in C57BL/6 mice.

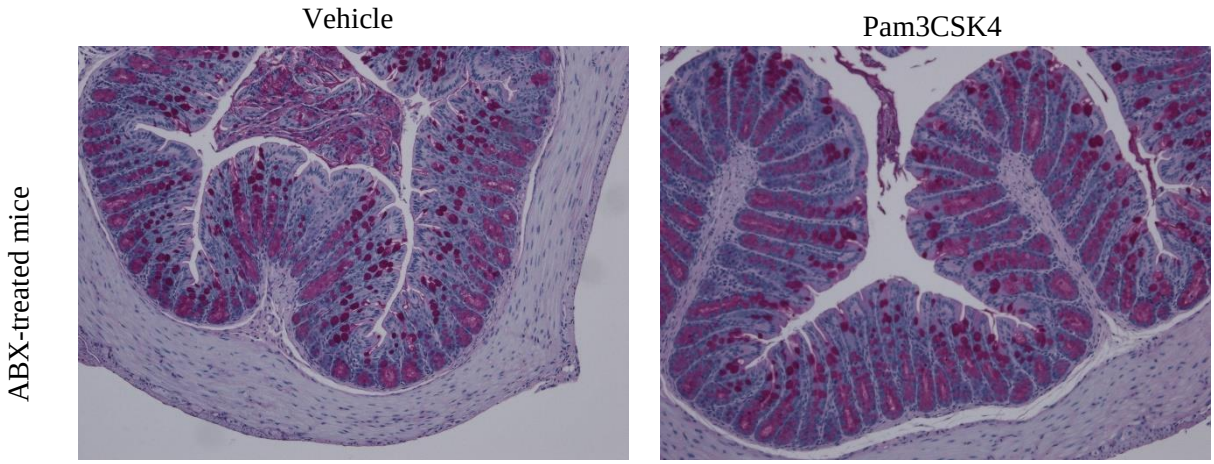
For ten days, two groups of C57BL/6 mice were given a cocktail of broad-spectrum antibiotics (neomycin, vancomycin, metronidazole, and ampicillin) in sterile drinking water for microbiota decimation. Weight, water, and food consumption were monitored during this time. One group of ABX-treated mice was gavaged with Pam3CSK4 (one dose of 100 µg, as previously described[113], per mouse), while the control group received vehicle (PBS) treatment, and both groups were sacrificed 48 hours after treatment. A significant increase in the number of goblet cells **(Figure 15)** was accompanied by a substantial upregulation of *Muc2* expression in TLR2 agonist-treated mice compared to controls **(Figure 16a)**. *Muc5ac* expression, on the other hand, did not increase in mice treated with Pam3CSK4 **(Figure 16b)**.

No significant changes between groups were observed in measures of Th1, Th2, or Th17 cytokines, although TLR2 agonist-treated mice did show a minor upregulation in IL-13 and a minor diminishment in IL-17 **(Figure 17)**. These findings and decreased Th2 cytokines, including IL-13 **(Figure 20a)** in *TLR2* KO mice, suggest that there can be a link between IL-13 and TLR2 signalling. Therefore, further studies are needed to investigate the adaptive immune response that is implicated in Pam3CSK4-mediated stimulation of *Muc2* expression.

Figure 15: PAS-stained micrographs of colonic tissue in ABX-treated mice following TLR2 agonist treatment compared to ABX-treated mice left untreated.

Representative images of (a) PAS-stained intestinal sections and (b) numbers of PAS-stained goblet cells in ABX-treated mice with and without TLR2 agonist treatment. Data are represented as mean ± SEM; *P< 0.05; n=4-5 mice/group.

a)


Vehicle
Pam3CSK4
ABX-treated mice

b)

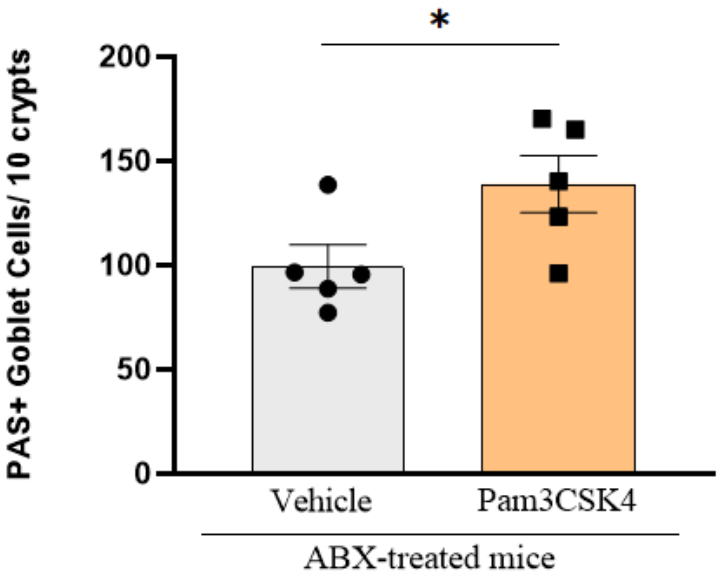
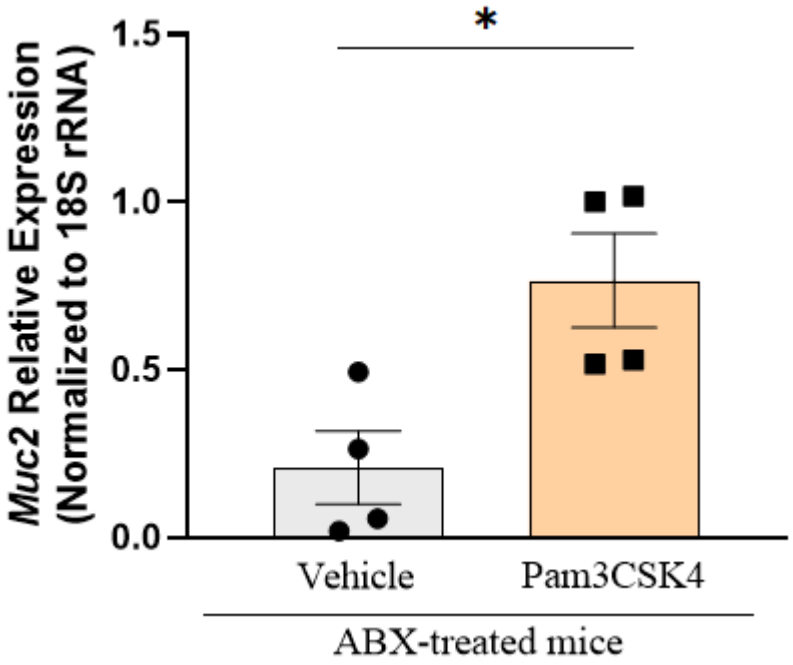

*
PAS+ Goblet Cells/ 10 crypts
200
150
100
50
0
Vehicle
Pam3CSK4
ABX-treated mice

Figure 16: Relative mRNA levels of *Muc2* and *Muc5ac* expression in ABX-treated mice with and without TLR2 agonist treatment.

(a) *Muc2* and *Muc5ac* expression normalized to mouse 18S in colonic tissue of ABX-treated mice with and without TLR2 agonist treatment using qPCR. Data are represented as mean ± SEM; *$P < 0.05$; n=4-5 mice/group.

a)


*
1.5
1.0
0.5
0.0
Muc2 Relative Expression
(Normalized to 18S rRNA)
Vehicle
Pam3CSK4
ABX-treated mice

b)

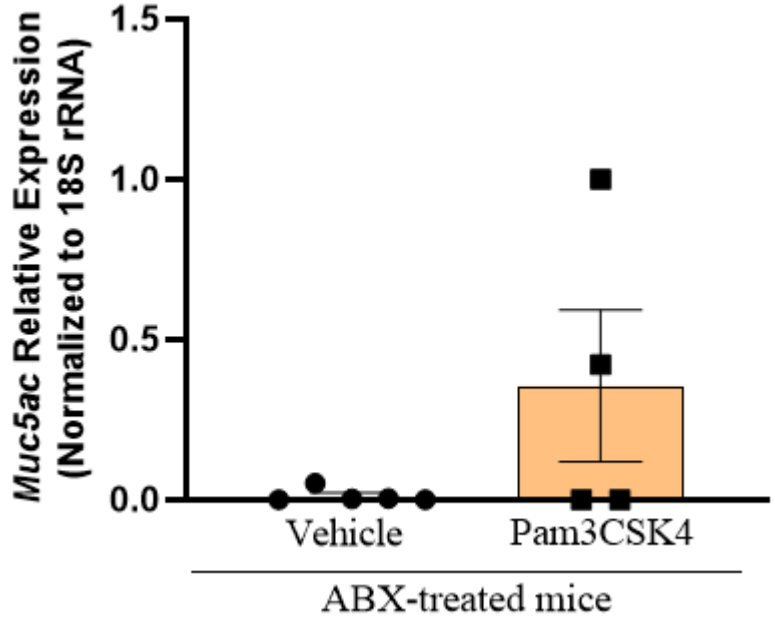
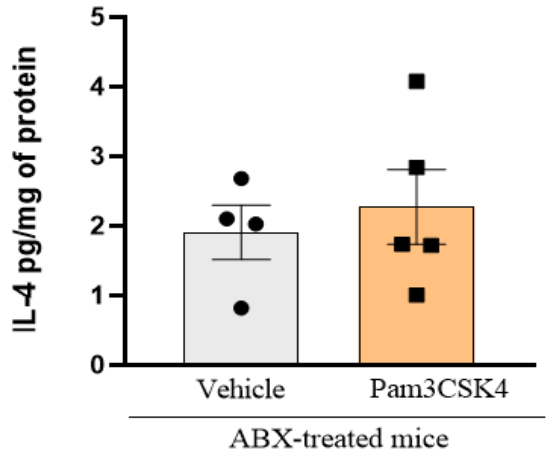

1.5
1.0
0.5
0.0
Muc5ac Relative Expression
(Normalized to 18S rRNA)
Vehicle
Pam3CSK4
ABX-treated mice

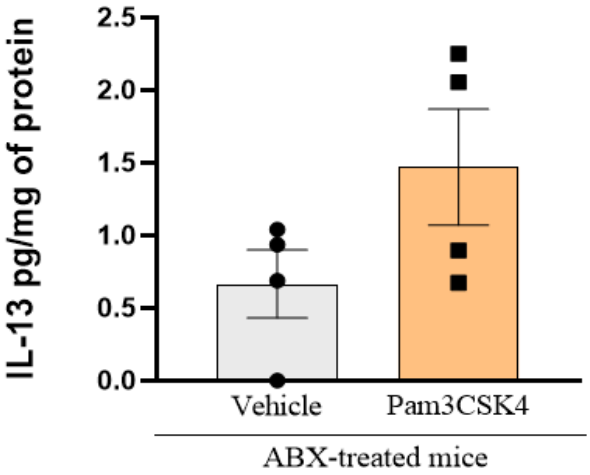
Figure 17: Effects of the administration of a TLR2 agonist on the production of IL-4, IL-13, IFN-ɣ, and IL-17.

(a) IL-4, (b) IL-13, (c) IFN-ɣ and (d) IL-17 levels in colonic tissues of ABX-treated mice with and without TLR2 agonist treatment. Data are represented as mean ± SEM; n=4-5 mice/ group.

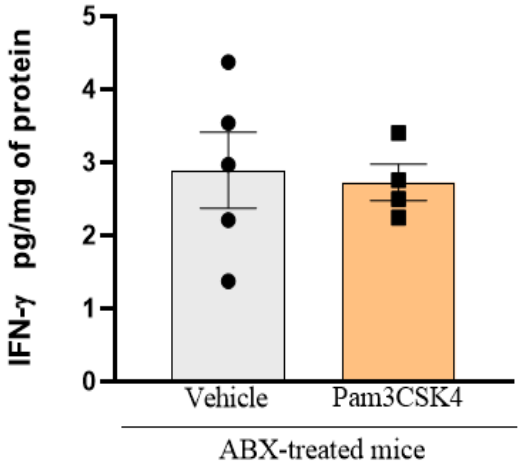
a)


IL-4 pg/mg of protein
5
4
3
2
1
0
Vehicle
Pam3CSK4
ABX-treated mice

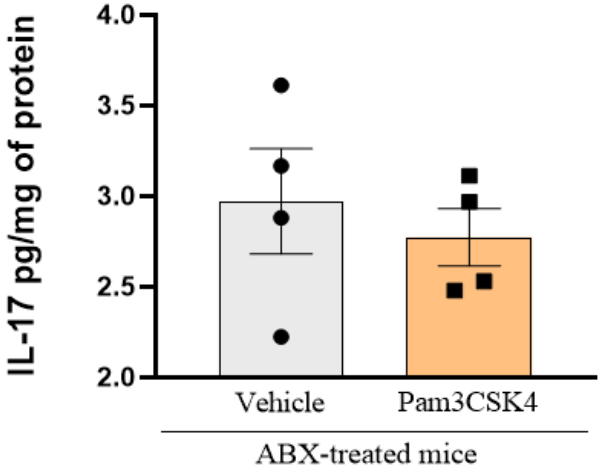
b)


IL-13 pg/mg of protein
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
Pam3CSK4
ABX-treated mice

c)


IFN-γ pg/mg of protein
5
4
3
2
1
0
Vehicle
Pam3CSK4
ABX-treated mice

d)


IL-17 pg/mg of protein
4.0
3.5
3.0
2.5
2.0
Vehicle
Pam3CSK4
ABX-treated mice

4.6. Interactions between *T. muris*-altered microbiota and TLR2 regulate goblet cell and mucin production.

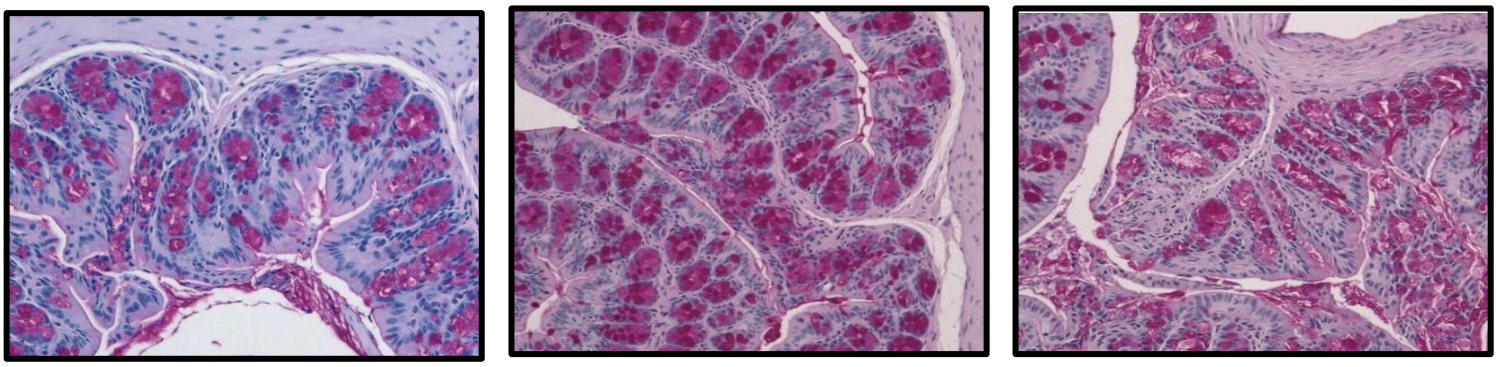
To determine if *T. muris*-altered microbiota regulates goblet cell responses through TLR2, we treated one group of *TLR2* KO and two groups of WT mice with broad-spectrum antibiotics (neomycin, vancomycin, metronidazole, and ampicillin) in sterile drinking water over ten days for microbiota decimation. During this time, body weight, water, and food intake were recorded.

Following antibiotic treatment, the *TLR2* KO group and one group of WT mice received microbiota from *T. muris*-infected mice, whereas the second group of WT mice served as the controls and were sacrificed without microbial transfer. After receiving microbiota from *T. muris*-infected mice, ABX-treated WT mice showed significantly higher numbers of PAS-stained goblet cells (**Figure 18**) compared to the ABX-treated *TLR2* KO mice administered the same microbiota and the control group which received no microbial transplantation. We also observed a significantly lower expression of *Muc2* in ABX-treated *TLR2* KO mice with *T. muris* microbiota compared to both ABX-treated WT groups (**Figure 19a**). It should be noted that no significant differences in *Muc5ac* expression between groups were detected (**Figure 19b**). Th2 cytokines levels (IL-4 and IL-13) were higher in the colonic tissue of ABX-treated WT mice colonized with *T. muris* microbiota compared to *TLR2* KO mice with the same microbiota and the control group (**Figure 20**).

Figure 18: PAS-stained micrographs of colonic tissue in ABX-treated *TLR2*-deficient mice gavaged with *T. muris* microbiota compared to ABX-treated WT littermates colonized with same microbiota and the control group.

(a) Representative images of PAS-stained colonic sections, and (b) numbers of PAS-stained goblet cells per 10 crypts in the colon of ABX-treated WT and *TLR2* KO mice with *T. muris* microbiota as well as ABX-treated WT mice which served as controls. Data are represented as mean ± SEM; **P*<0.05 and ***P* < 0.01; n=4-5 mice/group.

a)



ABX-treated WT mice | **ABX-treated WT mice** | **ABX-treated *TLR2* KO mice**

***T. muris* microbiota**

b)

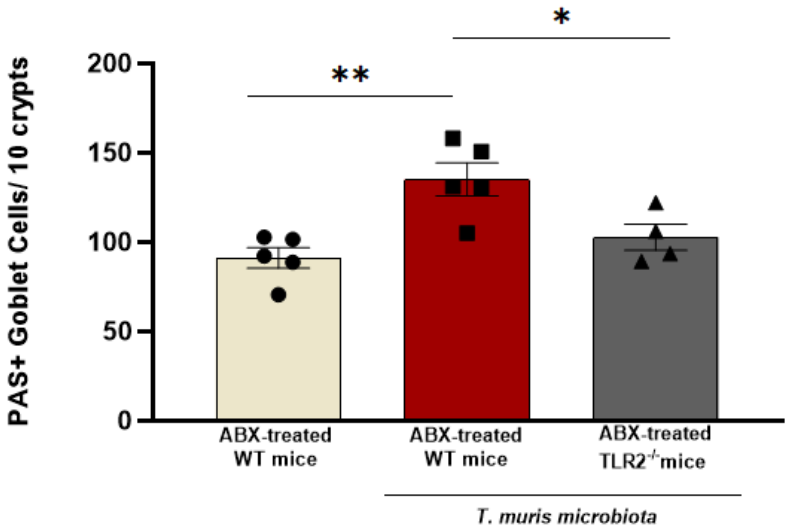
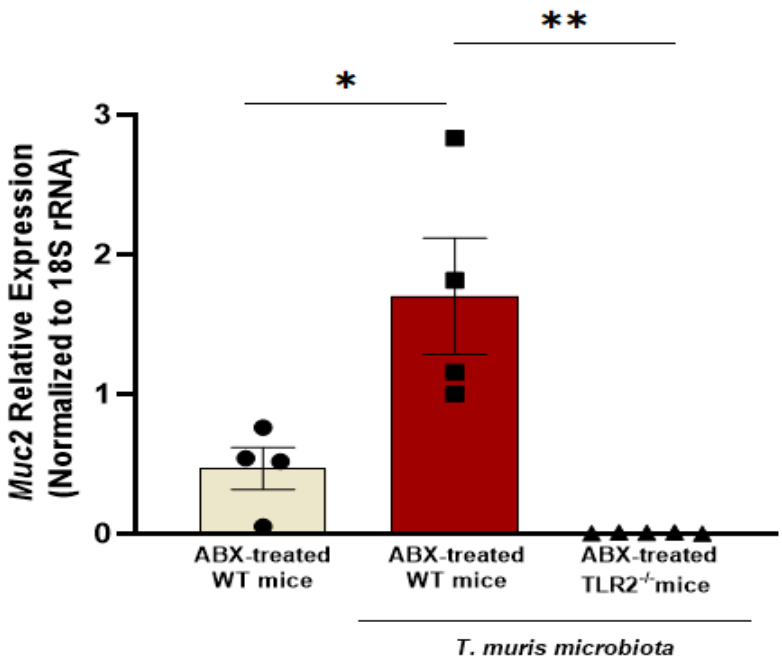


Figure 19: Relative mRNA levels of *Muc2* and *Muc5ac* expression in ABX-treated *TLR2*-deficient mice gavaged with *T. muris*-altered microbiota compared to ABX-treated WT littermates colonized with same microbiota and the control group.

(a) *Muc2* and (b) *Muc5ac* expression in intestinal tissue of ABX-treated WT and *TLR2* KO mice with *T. muris* microbiota as well as ABX-treated WT mice which served as controls using qPCR. Data are represented as mean ± SEM; **P*<0.05 and ***P* < 0.01; n=4-5 mice/ group.

a)


**
*
Muc2 Relative Expression
(Normalized to 18S rRNA)
3
2
1
0
ABX-treated
WT mice
ABX-treated
WT mice
ABX-treated
TLR2-/- mice
T. muris microbiota

b)

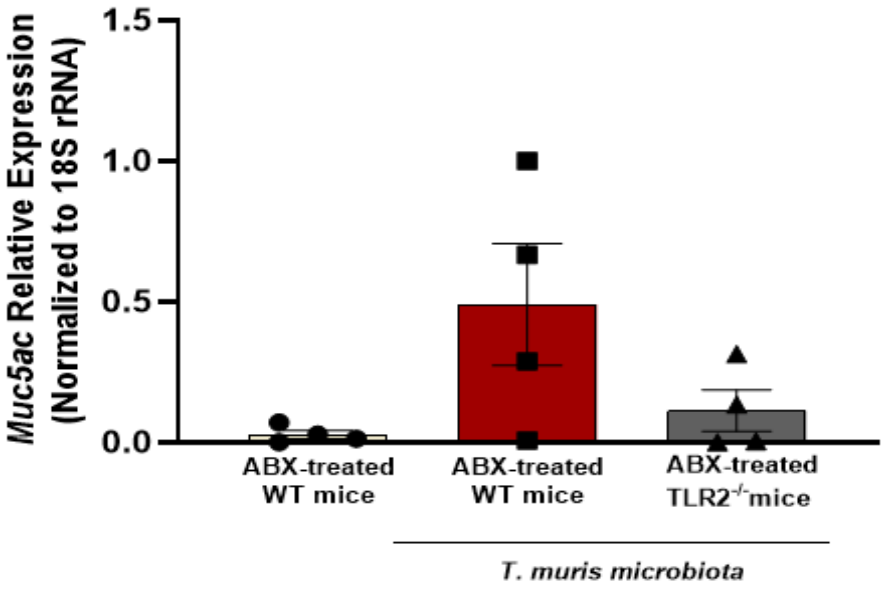
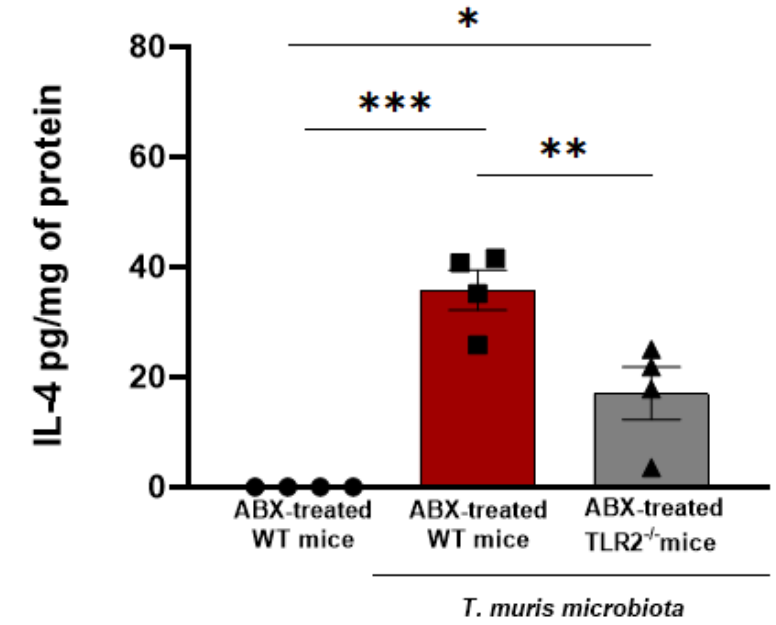

Muc5ac Relative Expression
(Normalized to 18S rRNA)
1.5
1.0
0.5
0.0
ABX-treated
WT mice
ABX-treated
WT mice
ABX-treated
TLR2-/- mice
T. muris microbiota

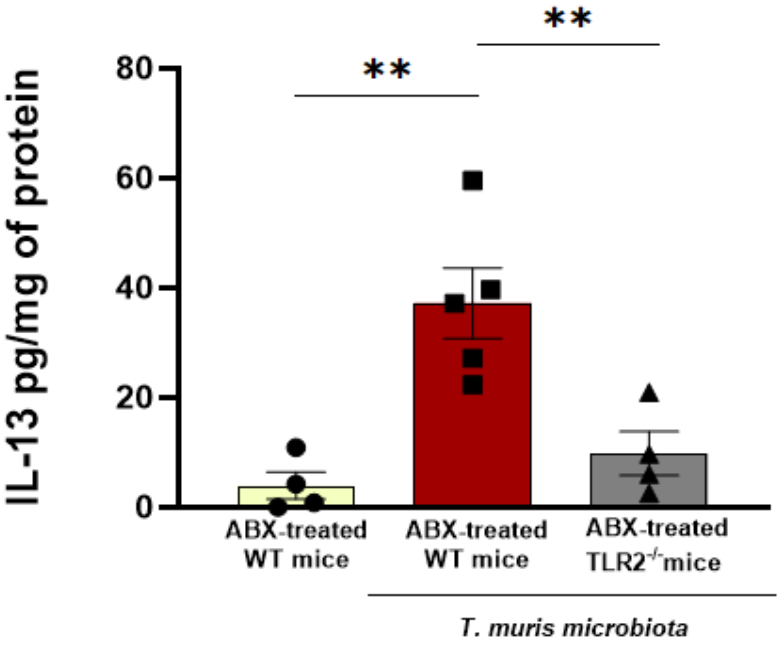
Figure 20: Levels of the Th2 cytokines, IL-4 and IL-13, in ABX-treated *TLR2*-deficient mice gavaged with *T. muris*-altered microbiota compared to ABX-treated WT littermates colonized with similar microbiota and the control group.

(a) IL-4 and (b) IL-13 levels in intestinal tissues of ABX-treated WT and *TLR2* KO mice with *T. muris* microbiota as well as ABX-treated WT mice which served as controls. Data are represented as mean ± SEM; **P*<0.05 and ***P* < 0.01, and ****P* < 0.001; n=4-5 mice/ group.

a)


*
***
**
80
60
40
20
0
IL-4 pg/mg of protein
ABX-treated WT mice
ABX-treated WT mice
ABX-treated TLR2-/- mice
T. muris microbiota

b)


**
**
80
60
40
20
0
IL-13 pg/mg of protein
ABX-treated WT mice
ABX-treated WT mice
ABX-treated TLR2-/- mice
T. muris microbiota

4.7. Absence of the transcription factor, SPDEF, reduced goblet cells numbers after *T. muris*-altered microbiota transplantation in ABX treated mice.

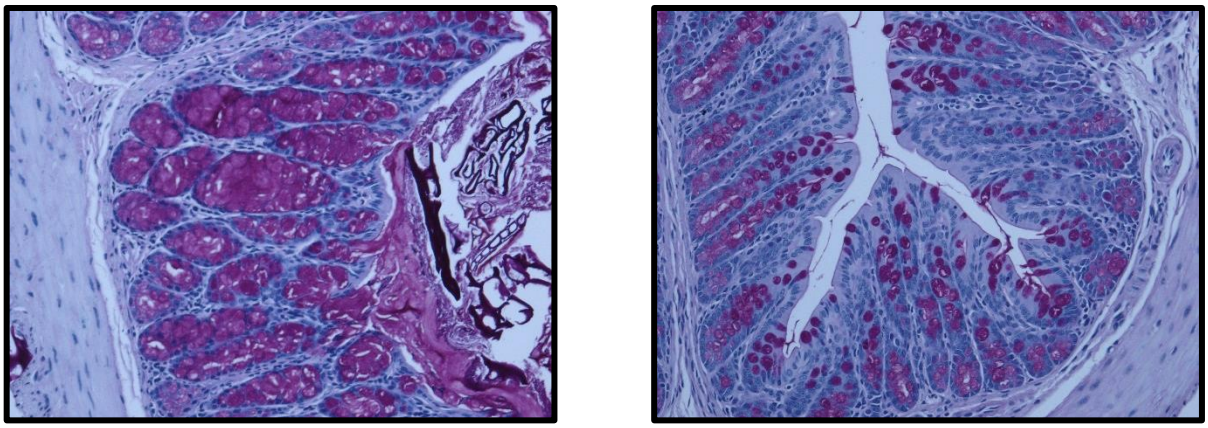
To investigate the transcriptional mechanism by which *T. muris*-altered microbiota modulates goblet cell responses, we focused on the transcription factor, SPDEF, which has been previously identified as a crucial component in goblet cell maturation and response regulation[30,31].

SPDEF KO and WT mice were given broad-spectrum antibiotics (neomycin, vancomycin, metronidazole, and ampicillin) in sterile drinking water for ten days prior to receiving *T. muris*-altered microbiota. ABX-treated WT mice colonized with microbiota from *T. muris*-infected mice showed significantly higher numbers of PAS-stained goblet cells compared to *SPDEF* KO mice with the same microbiota (**Figure 21**). The induction of *Muc2* and *Muc5ac* expression after transferring *T. muris* microbiota (which was observed in GF mice after receiving *T. muris* microbiota) was inhibited in ABX-treated *SPDEF* KO mice **(Figure 22)**. Furthermore, a trend of increased Th2 cytokine levels (IL-4 and IL-13) was observed in ABX-treated WT mice compared to ABX-treated *SPDEF* KO mice. **(Figure 23)**.

Figure 21: PAS-stained micrographs of colonic tissue in ABX-treated WT mice after microbiota transplantation from *T. muris*-infected mice compared to ABX-treated *SPDEF* KO mice after receiving the same microbiota.

(a) Representative images of PAS-stained intestinal sections and (b) numbers of PAS-stained goblet cells in ABX-treated WT and *SPDEF* KO mice after receiving microbiota from *T. muris*-infected mice. Data are represented as mean ± SEM; $^{**}P < 0.01$; n=4-6 mice/group.

a)



ABX-treated WT mice **ABX-treated *SPDEF* KO mice**

***T. muris* microbiota**

b)

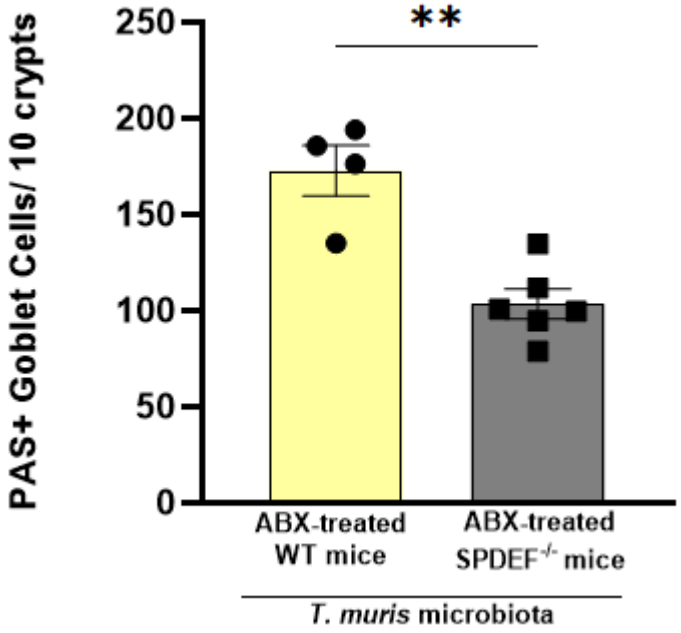
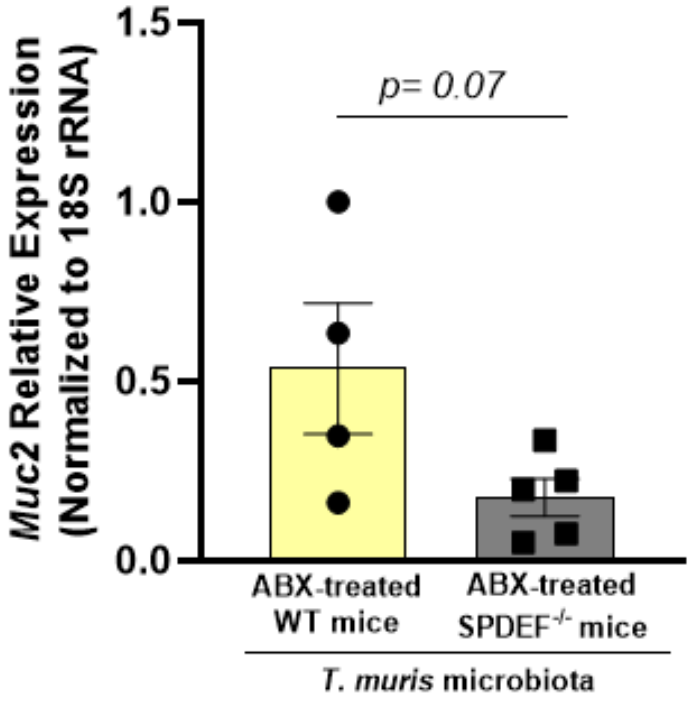


Figure 22: Relative mRNA levels of *Muc2* and *Muc5ac* expression in ABX-treated *SPDEF* KO and WT mice gavaged with *T. muris*-altered microbiota.

(a) *Muc2* and (b) *Muc5ac* gene expression in colonic tissue sections of ABX-treated WT and *SPDEF* KO mice after receiving microbiota from *T. muris*-infected mice, assessed by qPCR. Data are represented as mean ± SEM; n=4-6 mice/group.

a)


Muc2 Relative Expression
(Normalized to 18S rRNA)
1.5
1.0
0.5
0.0
p= 0.07
ABX-treated
WT mice
ABX-treated
SPDEF-/- mice
T. muris microbiota

b)

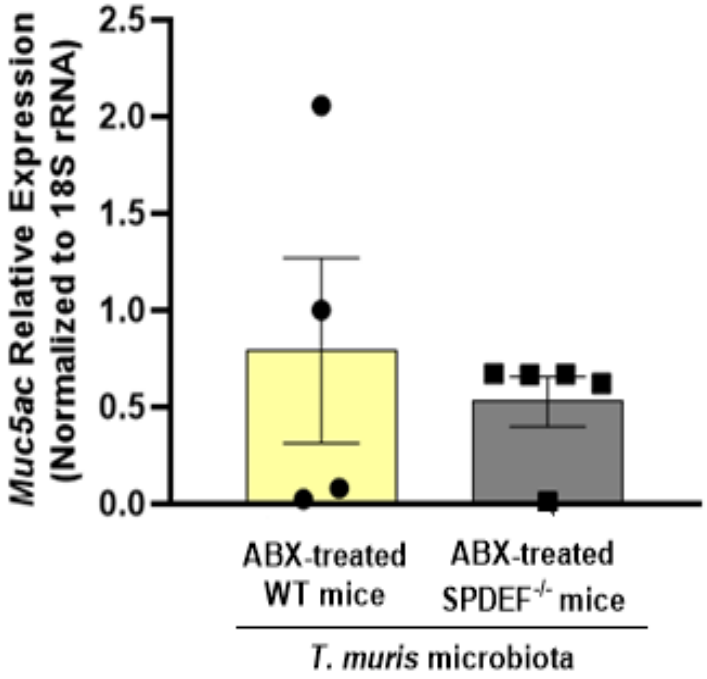
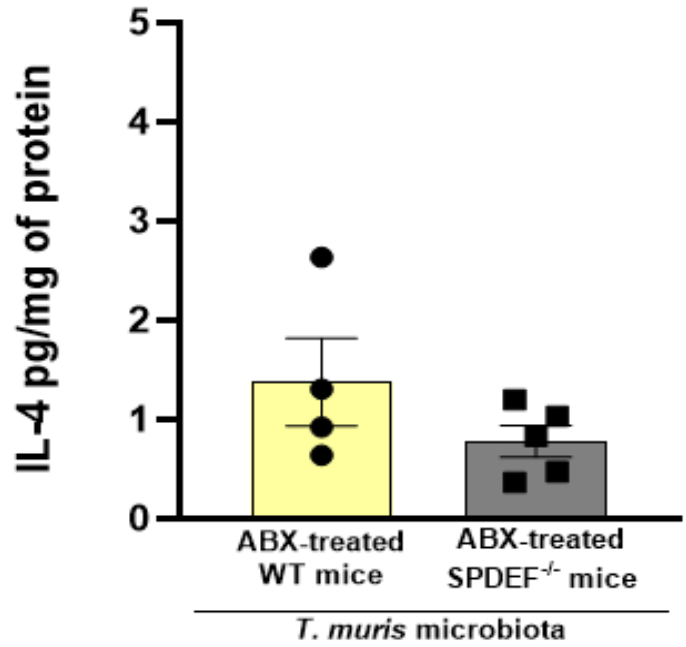

Muc5ac Relative Expression
(Normalized to 18S rRNA)
2.5
2.0
1.5
1.0
0.5
0.0
ABX-treated
WT mice
ABX-treated
SPDEF-/- mice
T. muris microbiota

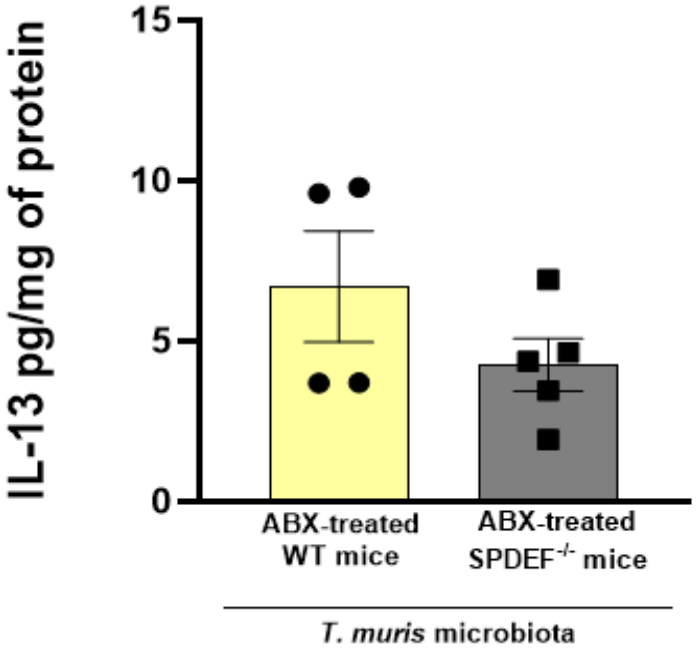
Figure 23: Levels of the Th2 cytokines, IL-4 and IL-13, in ABX-treated *SPDEF* KO and ABX-treated WT mice after receiving *T. muris* microbiota.

(a) IL-4 and (b) IL-13 levels in colonic tissues of ABX-treated WT and *SPDEF* KO mice after receiving microbiota from *T. muris*-infected mice. Data are represented as mean ± SEM; n=4-6 mice/ group.

a)


IL-4 pg/mg of protein
5
4
3
2
1
0
ABX-treated WT mice
ABX-treated SPDEF-/- mice
T. muris microbiota

b)


IL-13 pg/mg of protein
15
10
5
0
ABX-treated WT mice
ABX-treated SPDEF-/- mice
T. muris microbiota

Chapter 5: Discussion

T. muris infection has well-documented impacts on host immunological responses, including goblet cell hyperplasia and mucin production[82]. Due to the gut microbiota's close contact with *T. muris* within the GI tract, studies are now questioning the effects of *T. muris* infection on the host microbial composition and the impacts of *T. muris*-mediated changes in the microbiota on the host immune responses.

Across several studies, controversial findings have been uncovered regarding the role of infection with *Trichuris* species in altering the mammalian host's gut microbiota. For example, it has been reported that *T. trichiura*[114] infection, as well as an acute model of *T. muris*[115] infection, had no effects on the intestinal microbial composition of their respective hosts. In contrast, other studies on *T. trichiura*[116], *T. muris* (chronic infection)[91,117], and *Trichuris suis* in pigs[118,119] found infection considerably changed hosts' microbial communities. It should be noted, however, that variations in diet, genetic background, age, gender, and different housing conditions may have contributed to the conflicting results between these studies. In the present work, 16S rRNA sequencing and analysis revealed differing cecal microbial compositions in WT mice with acute *T. muris* infection compared to non-infected littermates.

Consistent with our findings that *T. muris* infection alters microbial communities, we found that several ASVs were significantly different in *T. muris*-infected mice and GF mice with *T. muris*-altered microbiota compared to non-infected mice and GF mice received non-infected microbiota, respectively. We identified two significant negative associations between bacterial taxa and *T. muris* infection: the family *Lachnospiraceae* within the order Clostridiales and the family *Muribaculaceae* from the order Bacteroidales.

Decreased *Lachnospiraceae* abundance was present in the microbial composition of the initial *T. muris*-infected mice as well as GF mice following *T. muris*-altered microbiota

transplantation. In parallel with our findings, Li et al. discovered a negative correlation between *Blautia*, a member of the *Lachnospiraceae* family, and *T. suis* infection[118]. Moreover, human studies conducted in Liberia and Indonesia found that the family *Lachnospiraceae* were significantly decreased in three parasitic infections, including *Ascaris lumbricoides, Necator americanus,* and *T. trichiura*[120]. Commensal A4 bacteria, a member of *Lachnospiraceae* family, has been demonstrated to hinder lamina propria Th2 cell proliferation[121], which may explain the lower abundance of *Lachnospiraceae* family members in *T. muris*-infected mice and GF mice following *T. muris*-altered microbiota transplantation, since it has been well-documented that mice with acute *T. muris* infection have a strong Th2 response, and our results suggested that Th2 cytokines production increased in GF mice with *T. muris*-altered microbiota. Further, our findings showed that three distinct ASVs assigned to *Lachnospiraceae NK4A136* were lower in *T. muris*-infected mice as well as GF mice gavaged with *T. muris*-altered microbiota compared to their respective controls. Intriguingly, the results of a recent study on *Trichuris*-associated microbial species also revealed a consistent negative link between *Trichuris* infection and *Lachnospiraceae NK4A136* across both human and mouse samples[122]. However, more work needs to be done to strengthen further the negative association of this microbial family with intestinal parasites and how these microbial changes either help or hinder host defense.

Within our study, *Muribaculaceae* abundance, known as the main gut microbiota in healthy individuals, was also negatively correlated with *T. muris* infection and *T. muris*-altered microbiota transfer; this family was found to be greater in non-infected mice as well as GF mice receiving non-infected microbiota compared to *T. muris*-infected mice and GF mice with *T. muris*-altered microbiota. Currently, no known negative association between *Muribaculaceae* and intestinal parasitic infection has been described in the literature. However, the Bacteroidetes phylum of which *Muribaculaceae* is a member, has been shown to have diminished abundance in the presence of *T. muris*[91,123].

In contrast, the families, *Ruminococcaceae* and *Rikenellaceae* (*Alistipes* genus), were found in greater abundance in GF mice with *T. muris*-altered microbiota compared to GF mice receiving microbiota from non-infected mice, but not in original *T. muris*-infected/non-infected mice. In parallel, an investigation performed in 2018 on patients infected with *Strongyloides stercoralis*, human intestinal nematode, consistently found that the population of *Ruminococcaceae* was higher in infected individuals than in those individuals that were parasite-free[124]. Interestingly, mice treated with genetically modified bacteria that secrete a protein associated with the flatworm genus, *Schistosome,* had a shift in microbial composition and an increased *Ruminococcaceae* population[125]. The presence of an increased population of *Alistipes* (within the *Rikenellaceae* family) in GF mice colonized with *T. muris*-infected microbiota was consistent with an earlier study that demonstrated an increased abundance of *Alistipes* in *T. muris*-infected mice on day 20 p.i.[117].

The effects of *T. muris*-induced altered microbial composition on host immune responses and, specifically, on the outcome of *T. muris* infection are not fully understood. Some studies, however, have highlighted the function of several bacterial taxa in modifying immunological responses during *T. muris* infection. For instance, it has been reported that increased relative abundance of *Lactobacillaceae* coincides with decreased IFN-ɣ response in both the lamina propria and mesenteric lymph nodes of chronic *T. muris*-infected mice, which promotes *T. muris* survival. Moreover, alterations in microbial communities following chronic *T. muris* infection are associated with changes in Treg populations and intestinal metabolites, which influence the health of the host[91,117].

Intriguingly, it has been demonstrated that the microbial changes associated with *T. muris* infection play a role in other intestinal pathologies such as colitis. Using *Nod2* KO mice that develop spontaneous colitis, it was observed that *T. muris* infection induces Th2 immunity that leads to the expansion of bacterial symbionts, *Clostridiales*, which competitively inhibits the colitogenic

species, *Bacteroides vulgatus*, and, ultimately, decreases the severity of colitis[126]. In contrast, the transfer of a *Heligmosomoides polygyrus*-altered microbiota enhances *Citrobacter* infection and colitis by stimulating the development of regulatory T cells that dampen protective responses[127]. These findings have shown that helminth-induced alterations in the intestinal microbiome can influence host immune responses regardless of live helminth infection.

Several studies have already established that in resistant mice, the Th2 immune response is dominant during acute *T. muris* infection, resulting in increased mucin production and consequent worm expulsion[17]. Although it is already well recognized that the gut microbiota stimulates goblet cells to produce mucins[2], there is no data on the contribution of *T. muris*-induced altered microbiota in the development of the acute immune response and mucin production. Here, by transferring cecal microbiota (collected on day 36 p.i. to preclude the possibility of worm transmission) from *T. muris*-infected and non-infected mice into GF mice, we demonstrated that microbiota from previously infected mice is able to induce mucin production upon transfer. These findings indicate that *T. muris*-induced changes within the microbiota play an important role in the formation of an effective immune response against *T. muris* infection.

Our findings indicated that GF mice with *T. muris*-altered microbiota had increased goblet cell number and mucin secretion, which are typically related to Th2 immunity. Consequently, we found that the production of colonic Th2 cytokines (IL-4 and IL-13) was higher in GF mice receiving *T. muris*-altered microbiota in comparison with those receiving non-infected microbiota. Increased Th2 cytokines were also observed in WT but not in *TLR2* KO mice when colonized with the same *T. muris*-altered microbiota. These findings demonstrated that the absence of TLR2 signalling results in reduced Th2 cytokines production. A study using a mouse cheek model found that pre-administration of TLR2 agonists increased the scratching behavior induced by IL-13 in atopic dermatitis, demonstrating that TLR2 may modulate IL-13 signalling[128]. Further studies are

needed to elucidate the adaptive immune response through which *T. muris*-induced microbiota and TLR2 signalling regulate goblet cell function.

The role of several PRRs in the regulation of goblet cell responses has been identified. For example, TLR2 and TLR4 regulate goblet cell responses in *Gymnophalloides seoi* infection[99] and necrotizing enterocolitis[101], respectively. In a previous study, we found that NOD proteins play a key role in the induction of goblet cell hyperplasia and mucin production in the context of *T. muris* infection[100]. In addition to NOD receptors, additional innate receptors, including TLRs, can regulate goblet cell responses following *T. muris* infection. Here, we observed an increase in mucin (*Muc2*) production upon TLR2 agonist treatment in both human cell line (HT-29) and WT mice. Furthermore, *TLR2*-deficient mice colonized with *T. muris*-altered microbiota exhibited fewer goblet cells and lower *Muc2* expression than WT mice with the same microbiota. Thus, TLR2 stimulation induces goblet cell hyperplasia and *Muc2* expression, while TLR2 deficiency inhibits *T. muris* microbiota-induced goblet cell responses and *Muc2* production, indicating a role for TLR2 in regulating host defensive response to *T. muris.*

Interestingly, both IL-4 and IL-13 showed a reduced expression in *TLR2* KO mice after receiving *T. muris*-altered microbiota compared to WT mice receiving the same microbiota, implying that TLR2 signalling activates Th2 immune responses through which it modulates goblet cell responses. In our previous study, we also showed that TLR2 signalling modulates ECs function by activating Th2 cytokines during *T. muris* infection[90]. Goblet cell hyperplasia is a specific characteristic of the Th2 response in the gut and is induced by IL-13[129]. However, the understanding of the interactions between TLR2 signalling and IL-13 remains in its infancy.

Our results showed that *T. muris* infection induces alterations in host microbial composition and this *T. muris*-induced changed microbiota has an immunoregulatory role in goblet cell responses via TLR2. These findings imply that TLR2 signalling, along with altered microbial composition,

acts as an essential arm of the innate immune response against *T. muris* infection and has a role in the regulation of goblet cell secretions.

According to recent studies, the transcription factor, SPDEF, is necessary for generating a Th2 inflammatory response in the lungs and modulating goblet cell maturation and differentiation in both the lung and the intestines[6]. It has also been shown that intestinal *MUC2* production is stimulated by SPDEF[30]. In addition, increased *SPDEF* expression was reported in acute *T. muris* infection, which is associated with goblet cell hyperplasia and increased mucin secretion[92]. Based on this information, we next examined whether SPDEF has a role in the intracellular pathway through which *T. muris* microbiota affects goblet cell response and mucin production by utilizing *SPDEF* KO mice. We observed diminished *Muc2* production (not significant) in *SPDEF* KO mice after colonization of *T. muris*-altered microbiota compared to WT littermates given the same microbiota, suggesting a role for SPDEF in the regulation of *T. muris* microbiota-mediated mucin production.

Altogether, this study gives us further knowledge on the role of *T. muris*-induced altered microbiota in the intestinal goblet cell response as well as mucin production and suggests an important involvement of TLR2 signalling in the regulation of contributing pathways. Studies on the immunomodulatory effects of helminth-modified microbiota open up the opportunity of treating infectious and inflammatory diseases that may in the future benefit patients, including those suffering from inflammatory bowel disease.

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