Unveiling the Impact of *Tritrichomonas musculus* Colonization and its Interplay with Host Metabolism and Immunity

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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Basel, 2024

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

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List of Abbreviations

ATMs	Adipose Tissue Macrophages
BSA	Bovine serum albumin
CCR	Chemokine receptor
CD	Cluster of Differentiation
CyTOF	Cytometry by time of flight
DMB	3,3-dimethyl-1-butanol
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraaceticacid
FBS	Fetal Bovine Serum
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GF	Germ-free
GI	Gastrointestinal tract
GLP1	Glucagon like peptide 1
GSIS	Glucose stimulated insulin secretion
GTT	Glucose tolerance test
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High-fat diet
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
IMDM	Iscove's Modified Dulbecco's Medium

Abbreviations

i.p.	Intraperitoneal
ITS	Internal Transcribed Sequences
ITT	Insulin tolerance test
MΦ	Macrophage
MHC	Major Histocompatibility Complex
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide + hydrogen
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NIH	National Institute of Health
Nlrp3	NLR Family Pyrin Domain Containing 3
PBS	Phosphate buffered saline
PCoA	Principal Coordinate Analysis
P/S	Penicillin and Streptomycin
PTT	Pyruvate tolerance test
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
Rag1	Recombination activating gene 1
RIP2	Receptor interaction protein 2
Rorγt	RAR-related orphan receptor gamma
rRNA	Ribosomal Ribonucleic Acid
SPF	Specific pathogen-free
TMA	Trimethylamine
TMAO	Trimethyl-N-amineoxide
TNF	Tumor Necrosis Factor
WGS	Whole-Genome Shotgun
β-cell	Pancreatic beta-cell

Abstract

The murine eukaryotic protozoa, *Tritrichomonas musculus*, is a commensal member of the gut microbiota that was initially considered non-pathogenic. Recent research has delved into the adverse effects of various Tritrichomonad protists upon colonization in their rodent hosts. However, the implications of chronic *T. musculus* colonization on metabolic health and mucosal immunity are not yet fully understood. By utilizing a chow- and high-fat diet (HFD)-fed wild-type mouse model, we aim to study the metabolic and mucosal immunological consequences of *T. musculus* colonization. Alongside monitoring the *in vivo* metabolic phenotype, we assessed gut mucosal immune cells via flow cytometry and conducted caecal microbiota sequencing to evaluate microbial composition.

Our results indicated that *T. musculus* colonization in wild-type C57BL/6N mice substantially impaired the metabolic state and induces a pro-inflammatory shift in mucosal immune cells. This impairment was initiated at an earlier timepoint and further exacerbated by HFD feeding. The engraftment of *T. musculus* influences mucosal immunity, with significant expansions in pro-inflammatory macrophages and $CD4^+$ T-cell responses. Crucially, the Nlrp3 inflammasome and IL-1β play key roles in these phenotypes, as demonstrated through genetic knockout and pharmacological inhibition models. Microbiota composition analysis revealed significant shifts in the abundances of the *Bacteroides*, *Akkermansia*, and *Desulfovibrio* bacterial families in *T. mu*-colonized mice. The increased abundance of choline-processing bacterial families, such as *Desulfovibrio* and *Lachnoclostridium*, upon *T. musculus* colonization suggests a potential involvement of the choline-TMAO pathway in the metabolic phenotype. Administering choline to uncolonized HFD-fed mice significantly impairs glycemic control, leading to hyperinsulinemia and the development of insulin resistance.

Overall, *T. musculus* colonization adversely affects mucosal immune cell homeostasis and glycemic control through IL-1β secretion and choline metabolism, with these effects accentuated by HFD feeding. Our findings provide additional evidence that the integration of *T. musculus* into the gut microbiome induces substantial alterations in bacterial communities, with these changes being dependent on dietary nutrients. This study sheds new light and builds upon previous research regarding the metabolic and immunological implications of *T. musculus* colonization in rodent hosts.

Chapter 1

Background and Statement of Aims

1.1 The Microbiome and Gut Microbiota

The composition of microorganisms varies significantly across the different anatomical regions of the human gastrointestinal tract, mainly due to the differing physiological and chemical environment in each region (Figure 1.1). This variability is due to alterations in parameters such as the rate at which digesta (partially digested food) moves through the intestines, the composition and thickness of the mucus layer lining the intestines, the presence and activity of immune cells, secretions of digestive enzymes by the host, the concentration of bile acids, pH levels, and redox potential (Touhy & Scott, 2015). These dynamic factors collectively influence and shape the microbial communities in distinct areas of the gastrointestinal tract. As a result, the gut microbiota exhibits changes in both the diversity of microbial species and their relative abundance along the length of the gastrointestinal tract. Additionally, the gut microbiota varies across the width of the gut, with microbial populations located near the intestinal wall differing from those residing within the central lumen of the gut. The composition of the gut microbiota is highly diverse and can vary from person to person, but almost universally includes Bacteria, Archaea, Viruses, Fungi and eukaryotic Protozoa.

Numerous studies have sprung up highlighting the importance of the role of bacterial communities within the microbiota in maintaining a homeostatic balance between health and disease pathology (Hou et al. 2022). Sometimes referred to as "the hidden organ," the human microbiota contributes 150-times more genetic information than the entire human genome (Figure 1.2). While "microbiota" refers to the living microorganisms in a particular environment, "microbiome" includes all microbial elements, metabolites, and environmental conditions, making it a broader term. In the gut, the microbiota performs crucial roles like food fermentation and immune response stimulation, containing major phyla such as *Firmicutes* and *Bacteroidetes*. The gut microbiota also contains notable fungal species such as *Candida* and *Saccharomyces* which play significant roles in maintaining gut homeostasis.

Oral microbiota, the second largest microbial community, consists of multiple regions within the buccal cavity, each incorporating various bacterial species, which includes *Proteobacteria* and *Actinobacteria*. Additionally, the lungs, previously thought to be sterile, harbor a great deal of microbiota that can have significant impact on chronic respiratory diseases. Lastly, the microbiota found on the skin varies geographically based on the distribution of sweat glands and follicles and consists of bacterial species such as *Cyanobacteria*.

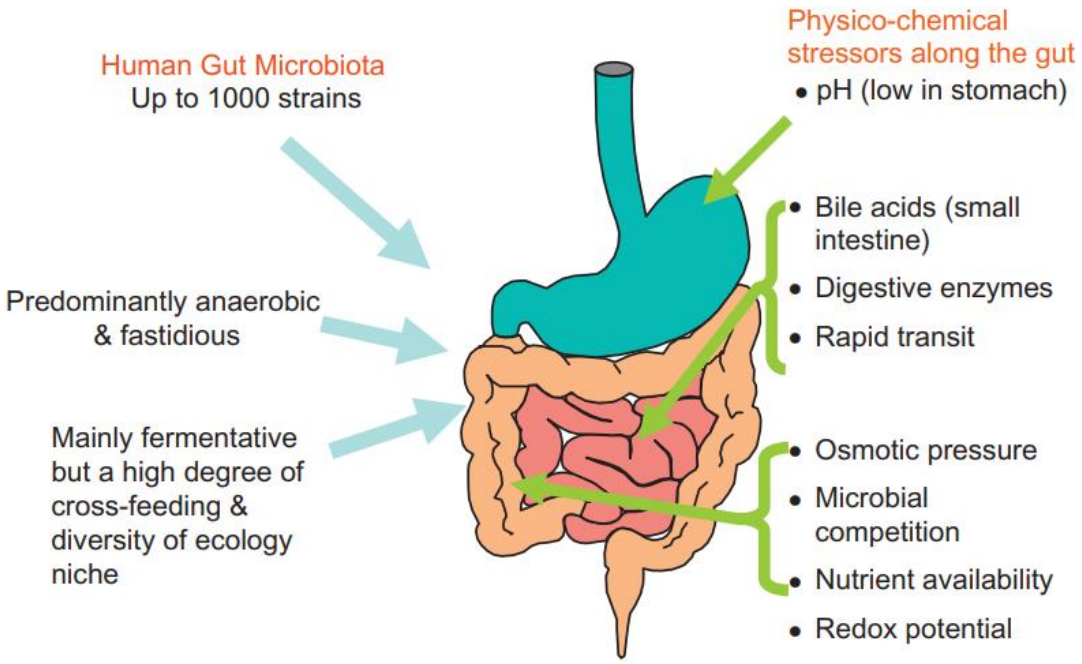


Figure 1.1. Physical and chemical obstacles by the gut microbiota as it travels through the various sections of the gastrointestinal tract. Adapted from Tuohy & Scott, 2015.

1.1.1 Bacteria

Bacteria are single-celled microorganisms that are classified as prokaryotes, lacking a true nucleus and membrane-bound organelles. They are the most abundant microorganisms in the gut, comprising the majority of the gut microbiota. The human gastrointestinal tract harbors trillions of bacteria, representing thousands of different species all of which play a central role in the gut microbiota, and their composition is vital for maintaining various aspects of human health. An online database compiling all the bacterial species identified in humans was published in 2015 and remains one of the most up-to-date comprehensive analyses of the collection of microbial communities

associated with humans (Hugon et al. 2015). In their report, the authors identified 2,172 distinct species found in humans. These species have been categorized into 12 different groups, with the vast majority, about 93.5%, falling into the *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* phyla.

More recently, a study in 2019 identified 1,952 distinct species of bacteria from a metagenome-assembled genome library of 11,850 human gut microbiomes (Almeida et al. 2019). These genome libraries from uncultured microorganisms significantly broaden our understanding of the various species present in the human gut microbiota, increasing the phylogenetic diversity by 281%. The three most common families in this dataset were *Coriobacteriaceae* (making up about 20.6% of the bacteria), *Ruminococcaceae* (comprising 9.9%), and *Peptostreptococcaceae* (representing 7.4%). Among the bacteria genera, *Collinsella* was the most prevalent, accounting for around 17.7%, followed by *Clostridium* at 7.3%, and *Prevotella* at 4.4%. The dominant species in the human gut microbiota are primarily members of the *Bacteroidetes* phylum (Forster et al. 2019). Eight of the top 20 prevalent species belong to the *Bacteroides* genus, such as *Bacteroides vulgatus*, *Bacteroides uniformis*, and *Bacteroides ovatus*. The *Bacteroides* and *Parabacteroides* genera are significantly overrepresented. In contrast, the *Firmicute*s phylum, with over 346 species, has only six distantly related species highly represented across individuals. These findings suggest that specific members of the *Bacteroides* play crucial roles in the maintenance of homeostasis in the human gastrointestinal tract. In contrast, the greater diversity observed in *Firmicutes* suggests a more variable and potentially functionally redundant group.

As alluded to above, the gut microbiota, including the bacterial composition, varies across different regions of the gastrointestinal tract due to distinct environmental conditions. For example, the small intestine contains Proteobacteria like *Enterobacteriaceae*, while the colon is typically inhabited by *Bacteroidetes*, including *Bacteroidaceae*, *Prevotellaceae*, and *Rikenellaceae* (Flint et al. 2012). Additionally, the gut microbiota changes with age, with increased diversity from childhood to adulthood, a relative stability during adulthood, and a decrease in diversity in older age, especially beyond 70 years (Rinninella et al. 2019). Children's microbiota is initially dominated by *Akkermansia muciniphila*, *Bacteroides*, *Veillonella*, *Clostridium coccoides spp*., and

Clostridium botulinum spp. By age 3, it becomes more similar to that of adults, characterized by three major microbial phyla: *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*. In older individuals, dietary and immune system changes can impact the composition of the gut microbiota, often resulting in decreased *Bifidobacterium* and increased *Clostridium* and *Proteobacteria*. Such distinct changes in the microbiota composition can have serious implications in dictating the inflammatory status of the gut and the overall health of an individual.

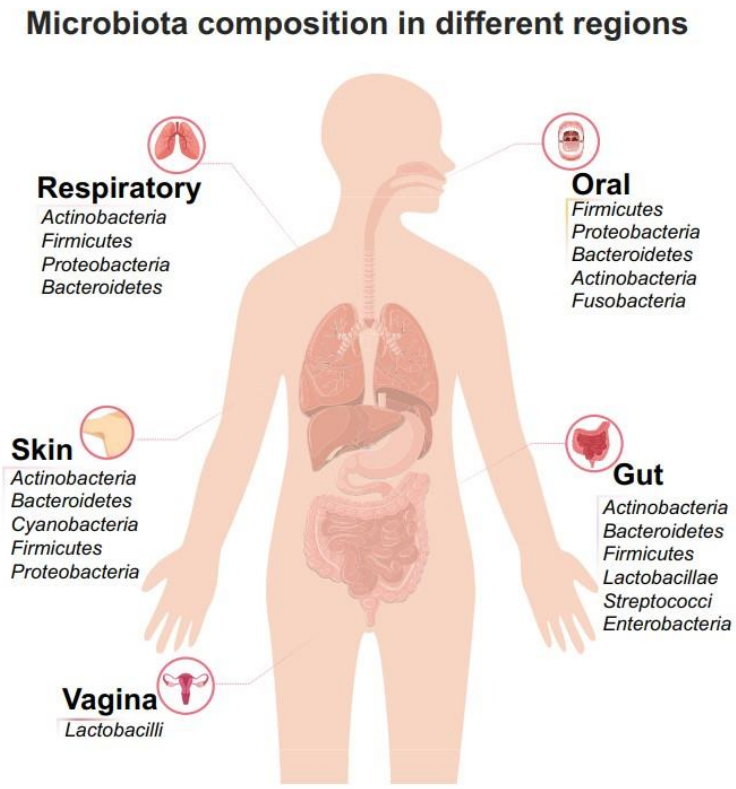


Figure 1.2. *The composition of the human microbiota in various locations of the human body, including the oral cavity, respiratory tract, skin, gut, and vagina. Adapted from Hou et al. 2022.*

1.1.2 Archaea

Archaea are single-celled microorganisms that share some similarities with bacteria but belong to a separate domain of life. In 1990, the domain Archaea was proposed as a distinct group of prokaryotic microorganisms based on their ribosomal RNA gene sequences (Gupta, 1998). While most archaea are commonly associated with extreme environments, a separate class of archaea known as mesophilic archaea, have been discovered in more moderate settings like soil and oceans. Moreover, several recent studies have confirmed the presence of archaea on human skin, in the oral cavity, and

the gastrointestinal tract (Pausan et al. 2019). Although archaea represent a relatively small portion of the human microbiome, they are still a significant member, suggesting that they may form intricate communities with varying compositions and structures across different parts of the human body.

Most microbiome studies have not given much attention to the human archaeome. Several factors contribute to the underrepresentation of archaea in these studies (Moissl-Eichinger et al. 2017). These include problems with "universal primers" that do not match archaeal sequences well, the low abundance of archaeal DNA in the samples under study, inadequate DNA extraction techniques, and incomplete 16S rRNA gene reference databases. Additionally, the lack of clinical interest in archaea is due to the absence of identified members that can cause pathogenic diseases. Members of the archaeome are commonly found along the human digestive tract beginning from the oral cavity all through to the gastrointestinal tract, where they can sometimes outnumber the most abundant bacterial species by up to 14%. However, most studies on archaea in humans have utilized cultivation or qPCR-based detection methods, with only a few focusing on 16S rRNA-based approaches. These studies have revealed that archaea are also present in the human respiratory tract and on the skin.

Archaea display specific patterns in different areas of the body, similar to human-associated bacteria (Koskinen et al. 2017). For instance, the gastrointestinal tract is predominantly inhabited by methanogens, while the skin is colonized by *Thaumarchaeota* and the lungs by *Woesearchaeota*. This information underscores the significant presence of archaea in various human tissues, possibly in all of them. To date, four species of methanogenic archaea have been successfully isolated from human body samples. These include Methanobrevibacter smithii, *Methanosphaera stadtmanae*, *Methanomassiliicoccus luminyensis*, and *Methanobrevibacter oralis*. Most of these isolates were obtained from human feces, while *Methanobrevibacter oralis* was cultured from oral mucosa. Notably, *Methanobrevibacter smithii* is present in the gut of up to 96% of individuals, though its abundance can vary between subjects, comprising 10% of anaerobic microbes in the colon (Dridi et al. 2009). *Methanosphaera stadtmanae* is highly abundant, detected in around 30% of tested individuals, while *Methanomassiliicoccus luminyensis* is less prevalent at 4% in the population studied. Together, *Methanobrevibacter smithii*, *Methanosphaera*

stadtmanae, and *Methanomassiliicoccus luminyensis* are the primary archaea members known to predominantly inhabit the human gut.

1.1.3 Virus

The gut virome, which encompasses both eukaryotic viruses and bacteriophages, is an integral part of the overall gut microbiome. Eukaryotic viruses are capable of infecting human cells while bacteriophages infect the gut bacteria. Long-term studies in this field are lacking, making it challenging to distinguish between viruses that establish persistent infections and are considered part of the normal gut flora and those responsible for acute infections, especially among human eukaryotic viruses (Lecuit & Eloit, 2017). Bacteriophages, are influenced by the presence of their host bacteria and can regulate bacterial populations (Ogilvie & Jones, 2015). The gut phage communities are highly individualized and are primarily composed of DNA phages with a temperate lifestyle. Due to bacteriophages, the viral load in the gut is roughly equivalent to that of the bacterial composition. Studies have shown that the ratio of phages to bacteria is approximately 1:1, and they are found at higher levels within the gut mucosa, with a ratio of around 20:1 (Barr et al. 2013).

The composition of the gut virome can vary due to multiple factors, including exposure to viral species, the makeup of the gut microbiota, and host-related factors such as immune functions. Clinicians have observed that immunodeficient patients often experience diarrhea as a common symptom of opportunistic viral infections (Roos-Weil et al. 2011). For instance, individuals undergoing immunosuppressive therapy following organ transplantation may develop chronic diarrhea caused by viruses like noroviruses and sapoviruses, which can lead to continuous fecal shedding and hospital-based transmission. Most of the dominant phages in the gut appear to have a temperate lifestyle, meaning they integrate into host chromosomes or exist quiescently (Minot et al. 2011). This symbiotic relationship is important for genetic exchange between bacterial hosts, altering host characteristics, and influencing the competitive abilities of bacterial strains in the gut. Indeed, bacteriophages have been shown to be capable of facilitating horizontal gene transfer between bacterial strains and species, potentially carrying genes encoding toxins, virulence factors, or alternative metabolic pathways.

The human gut virome exhibits substantial interpersonal variation but maintains remarkable temporal stability in structure. Over extended periods, most viral genotypes are retained within an individual, with minimal changes in their relative abundances. The high degree of inter-individual variation has been linked to variations in the bacterial component of the gut microbiome carried by individuals, their dietary influences, and the ability of viral populations to undergo rapid evolution (Minot et al. 2013). The sheer diversity of enteric virus species, their ability to infect a wide range of hosts, their capacity for genetic recombination, high transmissibility, and interactions with the host's microbiota and immune functions make it challenging to fully understand how a single viral species contributes to the complexity of the gut virome. However, it's important to note that many of these assumptions have been based on the analysis of a relatively limited number of individuals. Future research should involve larger cohorts and a more detailed examination of the human gut virome over a broader range of temporal and spatial scales to fully understand this viral ecosystem.

1.1.4 Fungi

The human gastrointestinal tract is not only inhabited by bacteria, archaea and viruses but also by a significant population of fungi, comprising about 0.1% of the total gut microbes (Sender et al. 2016). Technological advancements in deep sequencing and microbial cultivation have revealed a diverse fungal community in the human gut, including key fungal species that play essential roles in human health and are linked to the onset and progression of various diseases. Imbalances in the composition of the gut mycobiota have been associated with a range of diseases, including autoimmune, metabolic, neurological disorders, and cancer (Zhang et al. 2022). Opportunistic fungal pathogens can colonize the gut and trigger dysregulated immune responses, impacting disease development. Furthermore, gut fungi, particularly Candida species, can influence the assembly and function of the gut bacterial microbiome, affecting host physiology through various mechanisms, such as competition for nutrients, production of antimicrobial peptides, and physiochemical changes in the gut environment.

The interactions between gut fungi, bacteria, and host immunity are crucial for maintaining human immune homeostasis, which in turn influences overall health and disease. Recent studies have shed light on the composition of the gut mycobiome in

humans, laying the groundwork for understanding the roles of gut fungi in various diseases and potential therapeutic interventions (Nash et al. 2017). However, gut fungi have been somewhat overlooked in the development of microbiota-based therapies, and their full potential in clinical practice remains underutilized. In mice, for example, the analysis of fecal samples from C57BL/6 mice showed that over 97% of fungal sequences were attributed to only 10 fungal species, with *Candida tropicalis* and *Saccharomyces cerevisiae* being the most abundant commensal fungi (Iliev et al. 2012).

In the study by Nash et al. 2017, the fecal mycobiome of a large cohort of healthy individuals (over 100 volunteers) was comprehensively analyzed and described. Participants in the study provided stool samples collected longitudinally, totaling 317 samples. It is the first study to include sequencing data for the Internal Transcribed Spacer (ITS), 18S rRNA gene, 16S rRNA gene, and whole-genome shotgun (WGS) metagenomic data on the same samples, allowing for method validation and correlative analyses. The study's findings revealed that fungal diversity in the gut is lower than bacterial diversity. The most abundant yeast genera in this cohort included *Saccharomyces*, *Malassezia*, and *Candida*. *Candida* species have previously been identified as part of the healthy human mycobiome in various body sites, including the gut, oral cavity, vagina, and skin (Hoffmann et al. 2013). Furthermore, the aforementioned study examined the compositional changes of the gut mycobiome in relation to diet and found *Saccharomyces* and *Candida* fungal species but did not identify *Malassezia* in the gut mycobiota. This discrepancy may be due to differences in study methodologies, specifically the use of different primers for amplifying fungal DNA. Additionally, differences in cohort characteristics, such as diet and geographical location, could also contribute to variations in the gut mycobiome.

Fungi have been implicated in the exacerbation of various human diseases, including inflammatory bowel disease, graft versus host disease, Hirschsprung-associated enterocolitis, colorectal cancer, and the advanced progression of hepatitis B virus infections (Zhang et al. 2022). The presence of fungi as a part of the human microbiome and their potential roles in both health and disease emphasize the importance of delving deeper into the characterization of a healthy human mycobiome. A better understanding of a healthy mycobiome can facilitate research aimed at identifying disease-

contributing fungal species and elucidating the interactions between fungi and bacteria that are critical for maintaining health.

1.1.5 Protozoa

The commensal eukaryotic protozoa and helminth communities have always been an underappreciated member of the gut microbiota. With most research focusing on gut-resident bacteria, the role of intestinal protozoa in the human gut microbiota is a relatively new and emerging field of research (Lukeš et al. 2015). Protozoa are single-celled eukaryotic organisms found in various environments, either as free-living or parasitic/symbiotic microbes. Traditionally, they have been classified into four subgroups based on their morphological features: amoebas, flagellates, coccidians and ciliates (Dubik et al. 2022). However, more recent classifications consider two subkingdoms (Sarcomastigota and Biciliata), with distinct groups falling into each. The evolutionary relationship between protozoa and humans has likely impacted the gut microbiota dynamics.

Understanding the role of commensal protozoa in shaping the gut's immune landscape remains a challenge. Commensal microbes coexist with the host without causing harm and are tolerated by the immune system. However, the dynamic nature of host-microbiota interactions can sometimes blur the line between commensal and parasitic protozoa (Chabé et al. 2017). Additionally, factors like experimental design, protozoa species, and geographical variations in gut microbiota contribute to the lack of consensus regarding the precise role of intestinal protozoa in maintaining mucosal immune balance. Among the protozoa that are known to cause diseases are *Giardia lamblia*, *Entamoeba histolytica*, and *Cryptosporidium spp*. (Thompson & Ash, 2016). These are common enteric protozoa parasites that have been linked to the pathogenesis of acute gastroenteritis and diarrhea on a global scale. They are well-studied in terms of their virulence factors and mechanisms of intestinal invasion. Infections with these protozoa can lead to severe conditions like amoebic liver abscess and amoebic colitis. Despite the health burden posed by parasitic infections, many intestinal protozoa species are non-pathogenic.

Examples of these "commensal-like" protozoa include Blastocystis, Entamoeba, and Trichomonads, which can be found in the stool of healthy individuals or may not cause any symptoms during disease (Parfrey et al. 2014). The murine commensal protist, *Tritrichomonas spp*., has emerged in recent years as a focal point of study on the deleterious impact of otherwise “harmless” eukaryotic protozoa on host immunity. Tritrichomonads are a group of protists that can impact intestinal homeostasis, particularly in animals and humans. They are typically 5 to 10 μm, unicellular and flagellated protozoa that primarily inhabit the gastrointestinal tract (Cao et al. 2020). While Tritrichomonads are usually considered non-pathogenic and routinely monitored in mouse colonies, recent studies have indicated their potential role in exacerbating gut inflammation in experimental mice.

Intriguingly, researchers conducted an analysis in specific pathogen-free (SPF) animal facilities and found multiple species of *Tritrichomonas spp*. colonizing the gastrointestinal tract of their mouse colonies (Figure 1.3). Surprisingly, these Tritrichomonad infections did not cause any noticeable symptoms in the mice, resembling the situation reported in human patients (Chudnovskiy et al. 2016). These apparently innocuous commensal organisms are more commonly found in developing countries and have a moderate presence in closed communities within developed countries. This observation implies that they play a substantial role as a component of our microbiome. Transmission between different hosts, known as zoonotic transmission, has been observed via the fecal-oral route, raising questions about host-specificity and the evolution of pathogenicity during interspecies transmission (Maritz et al. 2014).

Certain members of the Trichomonad family, such as *Tritrichomonas vaginalis*, are known to be pathogenic and can cause diseases. On the other hand, species like intestinal *Pentatrichomonas hominis* have been linked to enteric diseases and are considered opportunistic pathogens (Suzuki et al. 2016). However, the overall classification of Trichomonads as true pathogens is a subject of ongoing debate, with many experts leaning towards classifying *Tritrichomonas spp*. as commensal pathobionts, meaning they normally coexist with their hosts but can become harmful under certain conditions.

1.1.6 Metabolism of Tritrichomonad protists

Tritrichomonads are anaerobic protists that undergo metabolism by producing molecular hydrogen in specialized organelles called “hydrogenosome” (Tachezy et al. 2022). The hydrogenosomes were first identified in the cattle parasite *Tritrichomonas foetus* and later in the human pathogen *Trichomonas vaginalis*. Initially, the granules in these organelles were believed to be mitochondria, but further investigation through electron microscopy and biochemical studies revealed that they were unique structures with no known biochemical markers of mitochondria. Biochemical studies indicated that the microbodies contain enzymes for anaerobic metabolism, including hydrogenase, leading to the conclusion that these organelles produce hydrogen. Further research showed that hydrogenosomes resemble mitochondria in certain aspects, such as the presence of respiratory pathways and adenylate kinase. Molecular phylogenetic studies and investigations into protein import mechanisms provided evidence supporting the common ancestry of hydrogenosomes and mitochondria. Notably, the presence of mitochondrial proteins, the import machinery, and the assembly machinery for iron-sulfur clusters were found in hydrogenosomes (Smutná et al. 2009)

After several decades of research, it was established that hydrogenosomes in trichomonads are modified mitochondria with an α-proteobacterial origin. Enzymes such as pyruvate:ferredoxin oxidoreductase (PFO) and hydrogenase are common in anaerobic prokaryotes but typically absent in aerobic eukaryotes. The core catabolic hydrogenosomal pathway involves PFO and hydrogenase, which oxidize pyruvate and release CO2, acetate, and hydrogen gas as waste products. This pathway contributes to ATP production through substrate-level phosphorylation, a process known as "extended glycolysis." (Muller et al. 2012). One of the important functions of reduced ferredoxin in hydrogenosomes is its role in the reductive activation of anti-trichomonad drugs like metronidazole. The reduced ferredoxin helps convert metronidazole to a highly reactive anion radical, which damages the cell and causes cell death. The metabolic fuel pyruvate is imported from the cytoplasm, but it may also be formed inside the hydrogenosome from malate through the activity of malic enzyme. The enzyme is crucial for reductive activation of metronidazole and plays a role in hydrogenosomal malate oxidation. Another important process is the recycling of NAD+ during the oxidative decarboxylation of malate. A two-subunit remnant of mitochondrial Complex

1 has been found in hydrogenosomes, which may link the reoxidation of NADH with the production of hydrogen. Lastly, the hydrogenosomal proteome contains many proteins involved in various metabolic pathways, suggesting that the metabolic capabilities of hydrogenosomes are more extensive than currently understood (Smutná et al. 2022). Further research is still needed to fully uncover the range of functions these unique organelles possess.

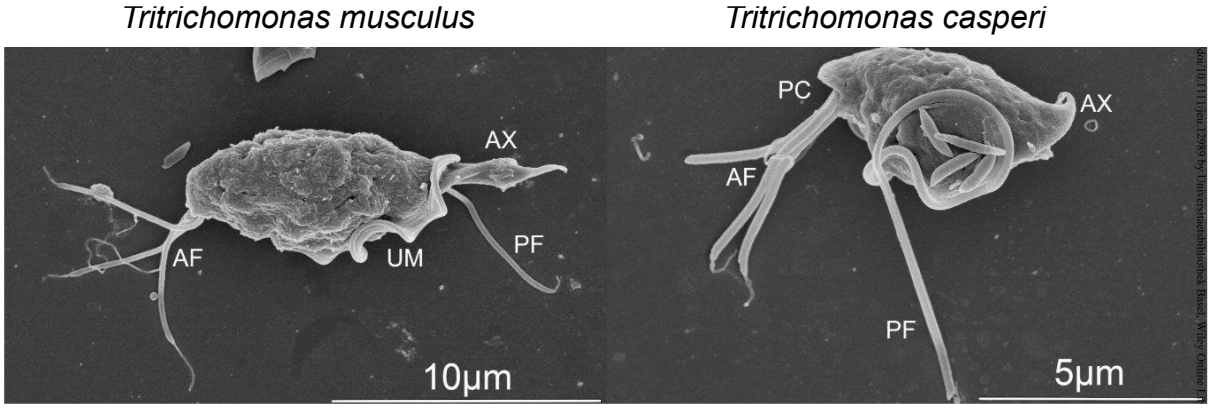


Figure 1.3*. Scanning electron microscopy images of two separate species of murine Tritrichomonad protists. Tritrichomonas musculus has a larger size of approximately 12.4 µm and is considered one of the more common protists that colonizes laboratory mice. Tritrichomonas casperi is a recently discovered species of Tritrichomonad protist and is smaller in size at approximately 6 – 7 µm. Both species are multi-flagellated and are isolated from the caecal content of Mus musculus. AX: axostyle, AF: anterior flagella, PF: posterior flagella, UM: undulating membrane, PC: periflagellar canal. Figure adapted from Tuzlak et al. 2023.*

1.1.7 Closest human homolog *Dientamoeba fragilis*

Dientamoeba fragilis, *D. fragilis*, is a human protozoan parasite that shows a close genetic relationship to the murine Tritrichomonad species. It is considered an intestinal commensal, similar to the most studied murine Tritrichomonad species, *Tritrichomonas musculus, T. mu*. *(also known as Tritrichomonas musculis)*. *D. fragilis* is a single-celled protozoan parasite that has been largely overlooked in the field of medicine as a cause of gastrointestinal (GI) disease (Stark et al. 2016). Despite reports spanning a century linking *D. fragilis* to human GI disorders, including diarrhea, it is often disregarded as a pathogen, and routine testing for it may not be conducted by diagnostic laboratories. The lack of knowledge about its basic biology has left us unaware of its host distribution, life cycle, and other essential aspects of its biology. However, recent advancements in *D. fragilis* research are changing this perspective. Modern diagnostic tests for *D. fragilis* have been developed, leading to an increase in its detection in cases

of GI disease. This has prompted a reevaluation of historical knowledge, especially regarding its potential role as a human pathogen.

The available evidence regarding *D. fragilis* and its association with GI disease is complex and sometimes contradictory. Numerous studies over the years have suggested that treatment to eliminate *D. fragilis* leads to significant improvement in GI symptoms, indicating its potential role as a pathogen. However, recent reports from northern Europe, particularly Denmark, have shown remarkably high rates of *D. fragilis* infection, even among healthy individuals, which is puzzling given the good hygiene and sanitation practices in these developed countries (Petersen et al. 2013). Some studies from Denmark have even proposed that *D. fragilis* might have a protective effect against inflammatory bowel disease (IBD), while others have associated it with a low frequency of defecation in patients with irritable bowel syndrome (IBS). These findings are in contrast to the majority of reports from various research groups that link *D. fragilis* to GI symptoms such as abdominal pain and diarrhea (Start et al. 2010). Despite the discrepancies, many studies still support the notion that *D. fragilis* can be a cause of GI disease, and treatment to eradicate the parasite often leads to symptom resolution. Further investigation and validation of diagnostic methods are needed to clarify the true prevalence and role of *D. fragilis* in causing GI disease, especially in regions with unusually high reported infection rates.

D. fragilis has been investigated in various animal species, but only a few have been confirmed as natural hosts. Early studies reported the presence of *D. fragilis* in primates, sheep, and a wild rat. These investigations relied on microscopic examination of stained fecal smears, but some details on staining techniques were not provided. Recent studies have confirmed the natural occurrence of *D. fragilis* in pigs and gorillas using microscopy and PCR. Gorillas with *D. fragilis* infection displayed symptoms similar to irritable bowel syndrome, which can be resolved with metronidazole treatment (Cacciò et al. 2012). Pigs were also found to carry *D. fragilis*, and the same subtype as found in humans, suggesting a zoonosis transfer of disease. Earlier attempts to establish animal models for *D. fragilis* were unsuccessful, but in 2013, a rodent model was successfully established (Munangsinghe et al. 2013). BALB/c mice were highly susceptible to infection with cultured *D. fragilis* trophozoites, while other mammals, including rats, were not. These studies provide a critical incentive that the

murine Tritrichomonad species may pose similar pathogenesis in their rodent hosts and the notion that these protists are accepted commensal pathobionts may need further reconsideration in the future.

1.1.8 Life cycle of Tritrichomonad protists

Protists can act as commensals, parasites, or pathobionts and interact with resident bacterial communities. These interactions are influenced by the host's immune pathways. As outlined above, protists like *Tritrichomonas musculus* (*T. mu*) and *Tritrichomonas muris* (*T. muris*) can colonize the gastrointestinal tract of rodent hosts, leading to asymptomatic infections and influencing intestinal immune activation. A recent study aimed to investigate the interactions between *T. mu*, gut bacteria, and the host's immune response using 16S rRNA sequence surveys and transcriptomics in healthy mice and B cell-deficient mice (Popovic et al. 2023, bioRxiv).

Using B-cell deficient mouse model, the authors discovered that *T. mu* colonization induces significant diversification in gut bacterial populations within the first 28 days, with B cells, particularly crucial for IgA production, implicated in maintaining microbial homeostasis. Core metabolic processes and virulence factors of *T. mu* are upregulated upon colonization, dramatically altering gut bacterial composition and increasing bacterial richness.

Popovic et al. assembled the metagenome of *T. mu* and used it to map and analyze metatranscriptomic reads upon *T. mu* colonization. They identified 17,212 genes with unique mapping of 20% of the total metatranscriptomic reads. Their analysis revealed dynamic gene expressions in *T. mu,* notably in carbohydrate metabolism. By performing single-cell transcriptomics on *T. musculus* protists, the authors delved into the diverse life cycle stages of *T. mu* within the intestinal tract. Fifteen subpopulations, organized into three superclusters, represent various stages such as trophozoites, amoeboid forms, and potential pseudocysts. Dynamic metabolic activities, encompassing carbohydrate metabolism, cytoskeleton remodeling, and biosynthetic processes, characterize *T. mu* during colonization. Exploring the influence of the bacterial microbiome, the authors compared *T. mu* isolated from conventionalized and

germ-free mice, finding distinct cell state distributions. The bacterial microbiome has a significant impact on the protists' life cycle, influencing cell cycle progression, meiosis, and sexual or parasexual replication.

Using *in situ* fluorescent labelling of specific protozoan mRNAs, the authors validated the presence of distinct subpopulations of *T. mu* in the intestinal lumen of colonized mice. Fluorescent *in situ* hybridization flow cytometry (FiSH-Flow) allowed the authors to detect the expression of specific transcripts in different clusters of *T. mu* cells freshly isolated from the caeca. One particular transcript was uniquely expressed in a supercluster of cells believed to be *T. mu* pseudocysts. Pseudocysts are thought to form during the protists' egress from the gastrointestinal tract. By tracking the expression of this transcript in freshly isolated protists from the caecum and colon, as well as during *in vitro* culturing, the authors revealed that the proportion of cells expressing the transcript increased during culturing which closely represents the fluctuating levels of chitin in culture. Overall, the research shows the dynamic interplay between *T. mu*, gut bacteria, and the host's immune response, providing insights into the complex interactions within the gut microbiome and its influence on host physiology.

1.2 Impact of *Tritrichomonas spp*. on host immunity

The colonization of *Tritrichomonas spp*. in the intestinal tract can have a significant impact on gut physiology and immune homeostasis (Figure 1.4). Studies of *Tritrichomonas spp.* colonization in wild-type and gustducin-deficient mice, which lack the taste-chemosensory G protein subunit, show an increase in goblet cell hyperplasia, epithelial cell proliferation, cell death, and DCLK1-expressing tuft cells in mice (Howitt et al. 2016). This expansion of goblet cells was initiated by IL-13 stimulation of the epithelium through taste receptors on tuft cells. *Tritrichomonas spp.* and enteric helminths can activate tuft cells via the succinate receptor (GPR91) through the release of succinate (Schneider et al. 2018). The interaction between protozoa and host tuft cells triggered the release of the cytokine, IL-25, which stimulate the expansion and activation of ILC2s. These ILC2s, in turn, released cytokines like IL-5 and IL-13, promoting tuft cell and goblet cell differentiation in a closed positive feedback loop. Schneider et al., also discovered the importance of the E3-Ubiquitin ligase, A20, in the

negative regulation of this circuit. The activity of A20 leads to decreased ILC2 proliferation and cytokine production downstream of the IL-25 receptor. In the absence of A20, higher tuft cell counts and significant elongation of the small intestine are observed due to uncoupled ILC2 activation. Dietary metabolites acetone and succinate released by the microbiota also trigger ILC2 proliferation and cytokine production, upstream of A20-mediated inhibition (Nadjsombati et al. 2018). The authors found that *Tritrichomonas rainier*, a relative of *T. musculis* and *T. muris*, triggered a unique pathway for communication between tuft cells and ILC2s, which was dependent on the presence of GPR91 and the release of IL-25. This interaction was crucial for the host's anti-helminth responses, indicating that protozoa colonization can influence the tuft cell-ILC2 crosstalk.

The protozoa-tuft cell-ILC2 circuit influenced host immune responses, and its activation could lead to systemic dissemination of ILC2s. Indeed, in addition to the expansion of naïve ILC2s in the gut, colonization with *T. mu* can promote the accumulation of eosinophils both locally in the gut and remotely in organs such as the lungs (Burrows et al. 2022). The authors found that colonization of *T.mu* results in increased frequencies of eosinophils in the blood, colon, bone marrow, and lung. This increase is associated with elevated levels of IL-5 and IL-13, which are important cytokines for eosinophil survival and are released by tissue-resident ILC2s. Further investigation revealed that lung ILC2s produce higher levels of IL-5 and IL-13 after colonization with *T.mu*. Lung ILC2s are divided into two populations: natural ILC2s (nILC2s) and induced ILC2s (iILC2s). The colonization of *T.mu* led to a significant increase in iILC2s but not nILC2s in the lungs. The activation and trafficking of iILC2s from the gut to the lung in *T.mu*-colonized mice are dependent on the S1P receptor 1 (S1PR1). Inhibition of S1PR1 with the antagonist FTY720 prevents the accumulation of iILC2s in the lungs and mesenteric lymph nodes (MLN) after colonization with *T.mu*. Moreover, antibiotic-mediated eradication of *T.mu* from the intestine reverses the trafficking of iILC2s to the lung and decreases lung eosinophil numbers. Overall, this emerging field of research demonstrates that colonization with the protozoan commensal *T.mu* can regulate tissue-specific and systemic innate type 2 immune cell distribution in healthy mice. These findings provide valuable insights into how the gut microbiota can influence immune responses at distant sites and may have implications

for understanding diseases associated with type 2 immunity, such as allergic asthma and type 2 diabetes mellitus.

The microbiota plays a significant role in regulating the host's immune system, affecting not only local tissue immunity but also distal immune responses in other parts of the body. Despite the identification of multiple enteric Tritrichomonad species, our knowledge about their phylogenetic relationships and functional heterogeneity is limited, which makes it challenging to understand their impact on the diversity of the gut microbiome and the overall health of the host (Chudnovskiy et al. 2016, Escalante et al. 2016). This highlights a gap in our understanding of how these protozoans interact with the host's microbiome and immune system. However, a recent publication from a group in the National Institutes of Health (NIH), Bethesda, Maryland aimed to bridge this current knowledge gap through structural identification and molecular characterization of two Tritrichomonad species (Tuzlak et al. 2023). The authors focused on characterizing *Tritrichomonas musculus* (*T. musculus*) and *Tritrichomonas casperi* (*T. casperi*). Through Sanger sequencing of the 18S rRNA gene sequence and phylogenetic analysis, *T. musculus* was identified to have 100% sequence similarity to *T. musculis*, originally described by Chudnovskiy et al. in 2016. Moreover, *T. casperi* share 100% sequence similarity to a sequence submitted to GenBank from a group in China in 2017 (GenBank Acc. No.: MF375342).

T. musculus was identified to be the larger protist when compared to *T. casperi*, with a median length of 12.4 µm and 6.8 µm, respectively. *T. muris*, the most common Tritrichomonad protist colonizing laboratory mice, was previously characterized to be of much larger in size: 16-26 µm in length and 10-14 µm in width (Baker et al. 2007). Through light microscopy and scanning electron microscopy, distinct morphological features were observed, including the structure of the undulating membrane, anterior flagella, and posterior flagellum (Tuzlak et al. 2023). Phylogenetic analysis based on 18S rRNA gene sequences confirmed that *T. musculus* and *T. casperi* are distinct species from *T. muris* and other related trichomonads. Their diversification into different species may be a result of adaptations to specific micro-niches within the gastrointestinal tract, driven by factors such as oxygen tensions, bacterial community structure, and metabolites. Additionally, the authors aimed to characterize the varying extent of Tritrichomonad protist colonization across separate NIH facilities.

Parabasalids were screened from mouse stool content across multiple animal facilities at the NIH, and various parabasalid sequence types were detected. *T. casperi* was the dominant sequence type recovered from mice in two different animal facilities and was present in four out of five facilities. *T. musculus* and *T. rainier* were also detected across multiple facilities. The study highlighted the need for further research on these protists' ecological niches, possible micro-niche adaptations, and their interactions with bacterial communities in the gut. The authors suggested that protist-bacteria symbioses, as observed in the termite gut, might also play a role in the gut ecosystem of mice and contribute to sympatric speciation for these protists that naturally colonize rodents (Peterson et al. 2015).

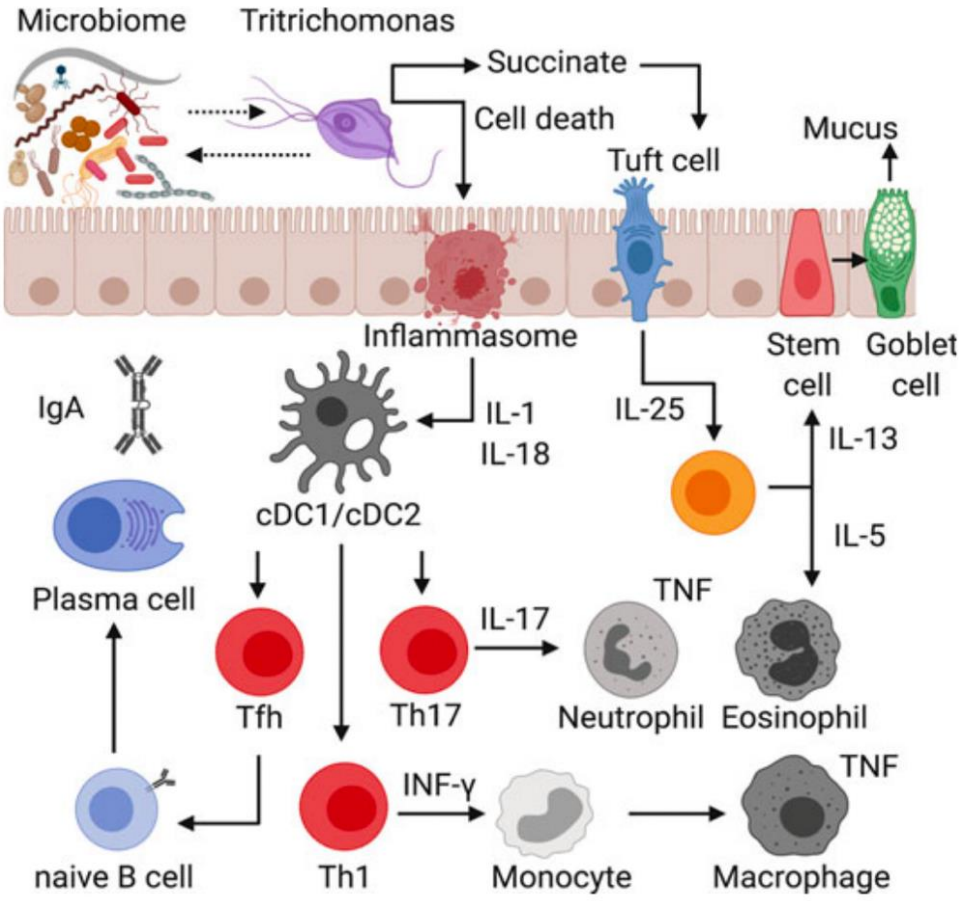


Figure 1.4. Impact of *Tritrichomonas spp*. on mucosal intestinal immunity. Adapted from Cao et al. 2020.

1.2.1 *Tritrichomonas spp*. and colonic immunity

Innate immune cells play a crucial role in recognizing and responding to microbial invaders, initiating the activation of the adaptive immune system. This recognition occurs through innate pattern recognition receptors, leading to the activation of inflammasomes that support the differentiation of $CD4^+$ T helper (Th) cells. For instance, the clearance of the pathogenic intracellular protist *Toxoplasma gondii* involves the activation of the Nlrp1 and Nlrp3 inflammasomes (Gorfu et al. 2014). Indeed, it was initially described that in the case of *T. mu* colonization in the gastrointestinal tract of mice, colonic macrophages and epithelial cells produce elevated levels of IL-1 and IL-18, which require inflammasome activation for their release (Chudnovskiy et al. 2016; Escalante et al. 2016). IL-1 boosts Th17 cell activation, while IL-18 supports Th1 cell differentiation and production of interferon-gamma (IFN-γ).

In brief, Chudnovskiy et al. set out to investigate the role of *T. mu* in the expansion of gut tissue-resident hematopoietic cells using flow cytometry and CyTOF analysis of the colonic lamina propria (LP) at different time points after colonization. Following a one-week colonization period with *T. mu*, the authors observed a significant increase in the populations of $Ly6C^{hi}$ monocytes, $CD103^-CD11b^+$ dendritic cells (DCs), macrophages, and neutrophils. In contrast, migratory $CD103^+$ and $CD103^+CD11b^+$ double-positive DCs were reduced in the colonic mucosa, indicating a possible migration into the draining mesenteric lymph nodes to activate $CD4^+$ T-helper cells (Th1). Moreover, *T. mu* colonization affected innate lymphoid cells (ILCs), with a significant increase in IL-5 and IL-13 producing ILC2s in the colonic mucosa, as previously reported by Howitt et al. 2016. The colonization with *T. mu* also resulted in a notable expansion of interferon-gamma (IFN-γ)-producing $CD4^+$ T helper cells (Th1 cells) as early as one-week post-colonization. Additionally, there were significant increases in the expansion of activated $CD44^{hi}CD4^+$ T cells, proliferating $CD4^+Ki67^+$ T cells, and $CXCR3^+CD4^+$ T cells in the MLN and colonic LP, which may indicate the priming and accumulation of tissue-specific T effector responses two weeks after colonization by *T. mu*. To confirm that the immune modulation observed upon *T. mu* colonization was not due to other factors, the authors inoculated mice with purified *T. mu* cultured in the presence of antibiotics. Importantly, similar induction of colonic Th1 and Th17 immune responses was observed in mice inoculated with purified and cultured *T. mu*, indicating

that *T. mu* is the main driver of the colonic immune-modulatory effects observed in colonized animals.

Another publication that began elucidating the effects of Tritrichomonad protists colonization on gut immunity was the paper by Escalante et al. from 2016. In the process of studying colitis in a T cell transfer model using separate non-littermate mouse lines ($Rip2^{-/-}Rag1^{-/-}$ and $Rag1^{-/-}$), the authors happened upon a transmissible factor between littermates that led to the exacerbation of disease progression and severity. Further investigation revealed that only the $Rip2^{-/-}Rag1^{-/-}$ mouse line was colonized with the protozoa *T. muris*, resulting in an accelerated T cell transfer colitis pathology. To investigate the effect of *T. muris* infection and the observed exacerbation of colitis pathology, the authors isolated *T. muris* from the caecal contents of $Rip2^{-/-}Rag1^{-/-}$ mice and administered the protists orally into Tritrichomonas-free $Rag1^{-/-}$ mice. $CD4^{+}CD45RB^{High}$ T cells were subsequently transferred into the *T. muris*-infected $Rag1^{-/-}$ mice and their non-infected littermate controls. Following a four-week colonization period, the *T. muris*-infected mice exhibited significantly increased weight loss, loss of crypt structure, epithelial damage, and lymphocyte infiltration in the colon compared to the non-infected controls. The infected mice also showed elevated levels of pro-inflammatory cytokines IFN-γ and IL-12/IL-23p40 in the colon, indicating an increased Th1 pro-inflammatory environment. These findings suggest that *T. muris* infection can contribute to the exacerbation of colon pathology in T cell transfer colitis mouse model.

Following this discovery, Escalante et al. aimed to investigate whether *T. muris* could influence immune activation in the gut of immunocompetent mice during natural infection. They established a line of chronically infected C57BL/6 mice by colonizing breeders and compared the immune responses of their offspring with Tritrichomonas-free mice (littermates). Mice colonized with *T. muris* showed increased frequencies of IFN-γ^{+} CD4 T cells in the caecal lamina propria and decreased frequencies of IL-$17A^{+}$ CD4 T cells in the colon lamina propria. While $Ror\gamma t^{-}Foxp3^{+}$ regulatory T cell frequencies remained similar between colonized and uncolonized mice, $Ror\gamma t^{+}Foxp3^{+}$ T reg cell frequencies were decreased. The presence of *T. muris* lead to a shift towards a more pro-inflammatory mucosal environment, indicated by the increase in IL-12/IL-23p40 protein and IFN-γ-producing CD4 T cells. This suggested that *T. muris* might act

as a general stimulator of the mucosal immune system rather than specifically favoring a Th1 response, as reported by Howitt et al. 2016. Interestingly, although Chudnovskiy et al. and Escalante et al. demonstrated increases in Th1 responses in their *Tritrichomonas*-colonized mouse models, there were inconsistencies in other readouts such as the secretion of pro-inflammatory cytokines. This difference in Tritrichomonad-induced pathology may be due to chronic (from weaning) versus acute (oral administration at later age) infections or differences induced by separate protozoan species (*T. musculis* vs *T. muris*).

1.2.2 The NLRP inflammasome

The NOD-, LRR- and pyrin domain-containing protein 3, or simply NLRP3 inflammasome, is a large protein complex first characterized as being the driver for the dominantly inherited auto-inflammatory disease known as cryopyrin-associated periodic syndrome (CAPS) (Hoffman et al. 2001). CAPS consists of a number of autoinflammatory diseases of varying degrees of severity but symptoms typically include fever, joint pains and the development of skin rashes throughout the body. It was soon discovered that the NLRP3, along with other similar NLRP complexes, can lead to the activation of caspase 1 to induce the cleavage of pro-IL-1β and pro-IL-18 into the inflammatory cytokines, IL-1β and IL-18, respectively (Martinon et al. 2002). This discovery provided a molecular basis to the pathogenicity observed in CAPS patients and led to the development of small molecule inhibitors that regulate the activity of the NLRP inflammasomes (Swanson et al. 2019).

In brief, the NLRP3 inflammasome is made up of three main components: NLRP3 (a sensor protein), ASC (an adaptor protein), and caspase 1 (an effector protein). NLRP3 has three distinct parts: a pyrin domain (PYD) at the beginning, a NACHT domain in the middle, and a leucine-rich repeat domain (LRR domain) at the end. The ATPase activity of the NACHT domain is important for NLRP3 function, while the LRR domain is hypothesized to inhibit its activity through autoinhibition. The adaptor protein, ASC has two domains for protein-protein interaction: a PYD at the start and a caspase recruitment domain (CARD) at the end. When the NLRP3 inflammasome is activated, the NLRP3 molecules oligomerizes through interactions between their

NACHT domains. ASC is recruited to the complex through PYD-PYD interactions and forms filamentous structures (Lu et al. 2014). Several filaments converge to form a large macromolecular structure known as an ASC speck. This speck mediates the recruitment of caspase 1 through CARD-CARD interactions, leading to the cleavage and activation of caspase 1. The activation of caspase 1 involves self-cleavage into two parts: p33 (containing CARD and p20) and p10, both of which stay attached to the ASC speck. Recent findings have shown that the NIMA-related kinase 7 protein, NEK7, a kinase usually involved in cell division, is a crucial component of the NLRP3 inflammasome (Schmid-Burgk et al. 2016). NEK7 interacts specifically with NLRP3 and promotes the formation of a complex that is vital for ASC speck formation and caspase 1 activation.

The activity of the NLRP inflammasomes is a tightly-regulated inflammatory process that occurs in two main steps. The first step, called priming, has two key functions. It increases the expression of inflammasome components like NLRP3, caspase 1, and pro-IL-1β through the recognition of specific pathogen or damage-associated molecular patterns, PAMP and DAMP, respectively. This can be triggered by receptors like Toll-like receptors (TLRs) or cytokines such as TNF and IL-1β, leading to increased gene transcription activity (Bauernfeind et al, 2009). Priming also involves the modification of NLRP3 through post-translational modifications (PTMs) that stabilize it in an inactive, but ready-to-respond state. The priming step prepares the cell for inflammasome activation, while the second step involves the full activation and formation of the inflammasome complex. The activation stage is triggered by a variety of PAMPs and DAMPs, which include particles, mineral formations, and ATP, all of which incite several precursory signaling activities. Following activation, intracellular processes such as the efflux of potassium ions (K^+), the flux of calcium ions (Ca^+), the breakdown of lysosomes, the creation of mitochondrial reactive oxygen types (mtROS), the shifting of cardiolipin to the external mitochondrial membrane, and the discharge of oxidized mitochondrial DNA (Ox-mtDNA), culminated in the efflux of chloride ions (Cl^-) from the cell. The result of these processes is the assembly of the NLRP inflammasome, which activates the catalytic activity of caspase 1. Caspase 1 cleaves the precursor proteins, pro-IL-1β and pro-IL-18, into IL-1β and IL-18. These inflammatory cytokines are subsequently released into the extracellular space where they mediate their inflammatory functions. An additional outcome of Nlrp

inflammasome activation involves the fragmentation of gasdermin D (GSDMD) by caspase 1, where it is embedded into the inner membrane, oligomerizes and forms pores, thereby causing a specific form of cell death known as pyroptosis.

As opposed to the other NLRP complexes, NLRP3 is unique because it can be activated by a wide range of stimuli, including infections and sterile inflammation caused by internal damage or environmental irritants (Swanson et al. 2019). What connects these activators is their ability to induce cellular stress, which is sensed by NLRP3. The exact pathways and mechanisms through which NLRP3 senses and responds to cellular stress are not fully understood, but several signals are proposed to be involved, such as changes in ion concentrations (potassium and calcium ions), disruptions in cellular compartments such as lysosomes and mitochondria, metabolic shifts, and alterations in cellular organization. Despite significant amount of research in recent years, there is still no unified model for NLRP3 activation due to the complexity and interrelated nature of the signaling pathways. Different signals might act together or independently to trigger NLRP3 inflammasome activation.

1.2.3 *Tritrichomonas spp.* and the NLRP inflammasome

Recent findings from the group of Arthur Mortha at the University of Toronto, provided evidence that colonization with *T. musculis* in C57BL/6 mice has the capability of increasing the levels of extracellular ATP in the intestinal tract (Chiaranunt et al. 2022). Extracellular ATP has been known to be released by some bacteria during growth and plays a role in the activation of immune cells, such as Th17 cells. Indeed, the authors found an increased accumulation of IFN-γ^+ Th1 and IL-17A$^+$ Th17 cells in the colonic lamina propria of mice following a two-week colonization period with *T. musculis*. Resistance against *Toxoplasma gondii*, a parasitic protozoan of the intestinal epithelium, was shown to require the activation of Nlrp1b and Nlrp3 inflammasomes (Ewald et al. 2014).

Activation of these NLRs happens through flux in intracellular or extracellular ATP levels and leads to the processing of pro-IL-1β and pro-IL-18. As extracellular ATP can come from other sources such as commensal bacterial communities or dying host cells,

the authors revealed that the increased luminal ATP levels following *T. musculis* colonization are derived from the protist themselves, presumably through metabolism in the hydrogenosome. Given the increased luminal ATP levels in wild-type mice colonized with *T. musculis*, Chiaranunt et al. questioned whether the NLRP inflammasomes contribute to the observed inflammatory shift in the colon. By using genetic whole-body knock-out of NLRP1b and NLRP3 mice, Chiaranunt et al. showed that the NLRP1b- and NLRP3-deficient mice did not lead to significant induction of Th1 and Th17 cells in the colonic lamina propria after colonization, unlike their wild-type counterparts. NLRP3-deficient mice displayed a slight induction of certain Th cells, hinting at a partial requirement for NLRP3 in regulating Th cell responses after *T. musculis* colonization. Notably, the lack of Th1 and Th17 response in NLRP1b- and NLRP3-deficient mice was not due to altered colonization efficiency, as both types of mice carried similar numbers of the protist. As opposed to wild-type mice, NLRP1b- and NLRP3-deficient mice did not show increase in intestinal cell death or significant elevation in colonic IL-1β and IL-18 levels when colonized with *T. musculis*. These results suggest that the immune adaptation in the colon of T. musculis-colonized mice requires protozoa-derived ATP and the activation of NLRP1b and NLRP3. The lack of these inflammasomes appears to hinder the typical immune response, indicating their crucial role in the defense mechanism against protozoan colonization.

An additional conclusion of the study is that the introduction of *T. musculis* into the microbiome altered the host's intestinal immune landscape and aided in the mitigation of the severity of Salmonella-induced disease. *T. musculis* was shown to act as a "natural antibiotic" against Salmonella infections, with its derived ATP activating NLRP1b and partly NLRP3 to boost the innate and adaptive immune response against Salmonella infections. Variations in protozoan and Salmonella strains could affect the protection offered, and more research is needed to fully understand the influence of protozoan commensals on host immunity and infectious disease epidemiology.

1.3 Gut Microbiota in Health and Disease

The intestinal microbial balance has a profound connection to human health and diseases. Particularly, the human gastrointestinal (GI) tract hosts around 100 trillion microorganisms, including bacteria, yeasts, and viruses (Ley et al. 2006). This vast microbial community plays a pivotal role in various human biological processes such as nutrient absorption, metabolism, and immunity. These microorganisms help extract energy from food, produce essential molecules like vitamins, and play a protective role by producing substances that ward off harmful pathogens (Turnbaugh et al. 2006). They also contribute significantly to the development of the intestinal lining and the immune system. A "healthy" gut microbiota is characterized by stability, adaptability, and a beneficial relationship with its host. Typically, it possesses diverse species and maintains a stable core of microbes. While every individual has a unique microbial distribution, the composition of the gut microbiota can change based on factors like age, medications, and other environmental conditions (Flint et al. 2012). Given the critical role of the microbiota in health and its potential implications in diseases, research has shifted from just understanding its composition to exploring its functions. Advanced techniques such as high-throughput sequencing are aiding this exploration. The ultimate goal is to harness this knowledge to create microbiome-based diagnostic tools and pave the way for personalized medical treatments.

The multitude of complex microbial communities form complex biological systems that can interact with various bodily functions. Recent research has increasingly focused on how alterations in microbiota composition are linked to the pathogenesis of different diseases, including cardiovascular diseases (CVDs), cancer, respiratory issues, diabetes, inflammatory bowel diseases, brain disorders, kidney, and liver diseases (Lau et al. 2017). The gut microbiota, considered the largest endocrine organ in the body, has an essential influence on the CVD development. Certain bacterial species such as, *Porphyromonas gingivalis*, are involved in the metabolism of specific compounds, leading to the production of trimethylamine-N-oxide (TMAO). TMAO has been linked to cholesterol regulation, early atherosclerosis, and a higher risk of long-term mortality from CVDs (Roncal et al. 2019). Mechanistically, TMAO activates inflammatory pathways that results in increased expressions of IL-6 and TNF-α, contributing to atherosclerotic disease progression. The gut microbiota also metabolizes substances

into short-chain fatty acids (SCFAs), such as acetates, butyrates, or propionates. These SCFAs have been linked to the development of CVDs. For instance, butyrates play a role in maintaining the integrity of the intestinal barrier, and propionates are involved in balancing immune cells crucial for hypertension and related organ damage. These SCFAs, specifically propionates and butyrates, have been shown to protect against hypertensive cardiovascular damage in animal studies (Bartolomaeus et al. 2019).

Additionally, recent research highlights the connection between gut microbiota and the development and progression of colorectal cancer (CRC). Microbiota dysbiosis, characterized by an imbalance in gut microbial communities, can provoke inflammation and immune responses that indirectly lead to cancer formation. Secreted microbial metabolites can cause a breakdown in the epithelial barrier, which triggers tumor-related inflammation and drives CRC pathogenesis (Grivennikov et al. 2012). The relationship between gut microbiota and CRC disease initiation and progression can be seen in the influence on particular signaling pathways, such as E-cadherin/β-catenin, TLR4/MYD88/NF-κB, and SMO/RAS/p38 MAPK. Both commensal and pathogenic bacteria are involved in CRC progression by taking advantage of defects in the tumor surface barrier, invading healthy colonic tissue to induce inflammation, and secreting metabolites that can lead to the transformation of normal colonic epithelial cells into oncogenic cells (Chen et al. 2017). Key bacteria associated with CRC include E*. faecalis, E. coli, B. fragilis, S. bovis, F. nucleatum,* and *H. pylori*. For example, studies focusing on *F. nucleatum* have shown a higher abundance of this bacterium in CRC patients compared to healthy individuals (Kostic et al. 2012).

Despite notable progress in microbiota and disease pathogenesis research, our current comprehension predominantly centers on bacterial components, leaving the roles of fungi, viruses, and other microbes, such as eukaryotic protozoa like Tritrichomonads, largely unexplored. Moreover, while imbalances in microbiota are often observed in illnesses, the direct causative role of microbiota in these diseases has yet to be definitively established. This still remains the forefront of current and future microbiota research.

1.3.1 The Gut Microbiota and Diabetes Mellitus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by an impairment in maintaining normal glycemic control. This is mainly due to a defect in insulin secretion from pancreatic beta-cells as in the case of Type 1 DM or the development of insulin resistance, where tissues do not respond normally to insulin signaling as observed in type 2 DM patients. Type 1 DM is an autoimmune condition where the body's immune system attacks and destroys the insulin-producing cells in the pancreas. As a result, little or no insulin is produced. Type 1 DM often develops in childhood or adolescence but can occur at any age. Type 2 DM stands as the most prevalent form of diabetes, impacting nearly 500 million individuals in 2017 and thereby constituting a substantial global health burden. (Khan et al. 2020). Type 2 DM is characterized by the development of insulin resistance and is often linked to obesity and usually develops in adulthood, although it has been reported to have been increasingly diagnosed in children and adolescents. Lastly, gestational DM occurs during pregnancy and is characterized by high blood sugar levels that develop during gestation. It usually resolves after birth but increases the risk for both mother and child to develop Type 2 DM later in life. The connection between microbial communities and DM has been thoroughly researched, and the link between microbiota dysbiosis and the initiation and progression of DM is firmly recognized in the scientific community.

Type 1 Diabetes Mellitus

A multinational research initiative, focused on unraveling the environmental factors influencing the development of Type 1 DM children, has revealed a substantial involvement of the gut microbiome in the pathogenesis of the disease (Rewers et al. 2018). In Type 1 DM, the gut microbiota plays a significant role in the development of chronic inflammation and the modulation of host’s immune system. Numerous studies have highlighted differences in the microbial composition in the oral and fecal samples of Type 1 DM patients. For example, bacterial species such as *Christensenella* and *Bifidobacteria* were found to be more prevalent in fecal samples of Type 1 DM patients, whereas differences in levels of Streptococcus in oral and fecal samples may contribute to pathology (de Groot et al. 2017). Other studies have provided evidence to suggest that Type 1 DM patients might have fewer bacteria that produce beneficial compounds

such as SCFA butyrate, crucial for maintaining normal gut health and reducing inflammation (Mariño et al. 2017). Elevated levels of specific inflammatory markers, such as IL-6, IL-1β, and TNF-α have been found in Type 1 DM patients, and are correlated with the abundance of bacterial communities including *Ruminococcaceae* and *Ruminococcus*. Increased levels of lipopolysaccharide (LPS) and upregulated TLR2 and TLR4 activity have also been found to be influenced by certain bacterial species and can mediate release of pro-inflammatory cytokines causing damage to pancreatic beta-cells (Acosta-Montaño et al. 2019). Unfortunately, inconsistences in the results obtained between separate studies suggest that further research on the matter is required to draw a solid conclusion. While some results are inconsistent, it has been suggested that Type 1 DM-related microbial factors may be diverse in nature but united in function. Most research in this field has been conducted on animal models, indicating a need for further human studies to validate the pathogenic mechanisms.

Type 2 Diabetes Mellitus

Over the years, a growing body of research has consistently demonstrated the correlation between the composition of gut microbiota and the onset of Type 2 DM, the most prevalent form of diabetes in humans. The abundances of certain bacterial communities such as *Firmicutes*, *Clostridia* and *Bacteroides* have been shown to correlate with the incidence of Type 2 DM (Larsen et al. 2010). For example, the ratio between *Bacteroides* to *Firmicutes* was widely reported to positively correlate with the blood glucose levels. Importantly, the use of anti-diabetic medications can lead to the improvement of bacterial diversity and richness in the gut of Type 2 DM patients. Tight regulation of inflammation, gut barrier permeability and control of glucose metabolism may all be involved in the mechanisms linking gut microbiota composition to Type 2 DM pathophysiology. Higher levels of low-grade inflammatory molecules such as LPS have been observed in the circulation of Type 2 DM patients (Gomes et al. 2017). LPS can promote the recruitment of inflammatory macrophages and the activation of the NF-κB signaling pathway, leading to the secretion of pro-inflammatory cytokines that can impair insulin secretion. Dysbiosis in gut microbiota may also cause abnormal bile acid metabolism affecting glucose metabolism and insulin sensitivity (Shapiro et al. 2018). SCFAs secreted by the gut microbiota can play significant roles in multiple signaling pathways modulating the secretion of insulin and glucagon as well as limiting

host's autoimmune response. Butyrate, a SCFA secreted by certain class of bacteria, has important roles in maintaining intestinal barrier's integrity and the secretion of anti-inflammatory cytokines mediated by increased recruitment of regulatory T-cells (Takahashi et al. 2020). Additionally, the oral microbiota has also been implicated in the development of Type 2 DM (He et al. 2014). Alterations in oral bacterial communities can have significant impact on the gut microbiota, influencing immune response at the gut. Some studies have found differences in oral microbiota between Type 2 DM patients and healthy controls, suggesting that Type 2 DM may increase the risk and severity of conditions like periodontal disease. Overall, the relationship between microbiota and Type 2 DM incidence emphasizes the complexity of the disease and offers potential avenues for treatment development.

Recently, a long-term longitudinal study of a large cohort of Finnish adults provided significant insights in the relationship between gut microbiota composition and the development of Type 2 DM (Ruuskanen et al. 2022). The fecal samples of 5,572 healthy Finnish adults (after exclusion) were harvested in 2002 and again in 2017, after a 15-year follow-up period. Among the 5,572 adults tested, 432 cases of type 2 DM were detected at the end of the follow-up period. Through shotgun metagenomics, the microbiota compositions from the fecal samples collected at baseline and at the conclusion of the study were analyzed. The results showed four species from the *Lachnospiraceae* family to be robustly linked to a higher risk of Type 2 DM in two geographically distinct regions of Finland. The four species identified were *Clostridium citroniae*, *Clostridium bolteae*, *Tyzzerella nexilis* and *Ruminococcus gnavus*. All four species have previously been associated with other metabolic diseases and the development of obesity. For example, *C. citroniae* and other members of this genera, have been associated with the production of the metabolite, TMAO, known to cause the development of insulin resistance (Li et al. 2022). There has been an increasing number of studies aimed to show the association between bacterial-produced TMAO and a host of pathologic conditions such as in CVD and glucose and lipid homeostasis (Fennema et al. 2016). The pathway of the generation and metabolism of TMAO by the gut microbiota involves the released form of the metabolite choline and will be discussed below. *C. bolteae* was found to have increased abundance in patients with Type 2 DM, while its abundance was markedly reduced upon treatment the antidiabetic drug, acarbose. *T. nexilis* and *R. gnavus* are related to the intake and availability of different

polysaccharides in the colon, influencing their niche. The results of this study are unique due to its long follow-up period for the study participants as opposed to a cross-sectional study. The study aimed to help detect early microbial signals related to Type 2 DM development with its results unconfounded by the use of antidiabetic drugs. The study also highlighted the advantages of using high-end techniques such as shotgun sequencing or full-length 16S rRNA gene sequencing over amplicon sequencing for higher taxonomic resolution, a crucial prerequisite in the efforts to delineate further the association between gut microbiota composition and Type 2 DM development.

Gestational Diabetes Mellitus

Lastly, in pregnancies affected by gestational diabetes mellitus (GDM), studies have shown that changes in gut microbiota play a role in mediating insulin resistance and inflammation (Radzicka et al. 2018). The composition of microbial communities of the gut shifts significantly throughout pregnancy. Several correlations were found between specific bacterial communities and metabolism such as the gram-positive gut bacteria, *Collinsella*, and insulin secretion. A crucial factor determining GDM disease progression was may be due to increased gut microbiota diversity but decreased community richness from the first to the third trimesters (Koren et al. 2012). GDM patients were found to have an altered ratio of *Firmicutes* to *Bacteroidetes*, promoting obesity and worsened inflammation (Cortez et al. 2019). Bacterial-derived SCFAs were also found to be lower in GDM pregnancies, possibly leading to increased blood glucose levels (Crusell et al. 2018). Additionally, the gut microbiota composition in the infants of GDM mothers was different, with an increase in pro-inflammatory bacteria (Ponzo et al. 2019). These findings suggest that the gut microbiota may have an essential role in both the development of GDM and its effects on infants.

1.3.2 Interplay between host and microbiota

The intestinal microbiota plays a significant role in influencing our body's physiology. Host-microbe interactions occur through the recognition of microbial-derived ligands by host pattern-recognition receptors. Bacteria produce metabolites, like short-chain fatty acids (SCFAs), which support bacterial metabolism and suppress host inflammatory responses. The presence of luminal bacteria activates gut-resident RORγt-expressing innate immune cells, leading to the release of the cytokine IL-22. IL-22 functions through the modification of epithelial-expressed glycoproteins, providing a potential nutrient resource for microbes, promoting host-microbe commensalism and maintaining intestinal homeostasis (Sanos et al. 2009).

Tritrichomonad protists also produce host-modulatory metabolites. They can generate ATP from anaerobic fermentation of pyruvate via the reduction of ferredoxin and increase the accessibility of succinate from dietary fibers, which serve as nutrients for other microbes. These metabolites activate the host epithelium and immune cells, leading to the release of inflammatory cytokines. Indeed, Schneider et al. (2018) revealed a tripartite interplay between the small intestinal circuit, diet, and microbiota, involving the protist-derived fermentative end-product succinate. The authors discovered that the presence of eukaryotic pathosymbionts, such as *Tritrichomonas spp*., in the gastrointestinal tract of murine models influenced the activation of ILC2s and tuft cell expansion, particularly during weaning and colonization. *Tritrichomonas spp*. was found to degrade dietary fiber and produce succinate, which activate the tuft cell–ILC2 circuit. This signal promotes luminal detection of succinate and leads to adaptive intestinal remodeling. Moreover, the activation of the circuit caused small intestine lengthening and remodeling, associated with the maintenance of systemic energy balance, suggesting a conserved physiological response to sustain host metabolic needs. This circuit alteration contributes to concomitant immunity, a state where further infections are attenuated, and suggests an innate tissue memory or 'training' in the intestinal niche.

In a recent study, the authors demonstrated cross-kingdom microbial communication, showing how a commensal protozoan, *T. musculis*, affected the gut bacterial community composition (Wei et al. 2020). They also found evidence of cooperative support from

certain bacterial species such as *Bacteroidetes*, *Deferribacteres* and *Spirochaetes* for *T. musculis* colonization. The impact of bacterial communities on the colonization capabilities of Tritrichomonad protists depends heavily on the dietary nutrient conditions i.e., a standard chow or high-fat diet. *T. musculis* required fermentable dietary fibers for colonization and stability, and antibiotics allowed successful engraftment under fiber-limited conditions, suggesting a potential cross-kingdom competitive interaction between the protozoan and bacteria. However, when sufficient food fiber was provided, specific bacterial species displayed competitive or cooperative effects on *T. musculis* growth, indicating that the protozoan's growth in the gut might depend on a delicate balance of relationships with other members of the microbial community. These interactions between protozoa and bacteria may play a significant role in forming a functional gut microbiota that influences host health.

1.3.3 Microbiota-secreted metabolites impact on host metabolism

The development of obesity and Type 2 DM are linked to alterations in the gut microbiota, inflammation, and a compromised intestinal barrier (Li et al. 2020). Both conditions often exhibit a mild but consistent inflammatory reaction that is believed to promote insulin resistance. As discussed above, numerous studies have surfaced in mice that have demonstrated the contribution of the gut microbiota to the heightened inflammation observed in obesity. Additionally, components of the gut microbiota have the capacity to interact with dietary factors, affecting insulin sensitivity, gut integrity, as well as glucose and fat processing (Gurung et al. 2020). Usually portrayed as an independent metabolic organ, numerous metabolic activities in the gut microbiota interplay closely with the development of insulin resistance and diabetes. As alluded to earlier, recent research developments have revealed distinct gut microbial compositions in Type 2 DM patients compared to healthy individuals, with a noted decrease in bacteria that produce the metabolite, butyrate, potentially leading to glucose metabolism issues. External factors, like diet, can lead to microbiota dysbiosis, impacting the production of microbial by-products and contributing to the development of insulin resistance. Additionally, gut microbes can modify the metabolic response to food, affecting diabetes risk (Sonnenburg & Bäckhed, 2016). Over the past decade, research has delved into the ways the gut microflora impacts insulin resistance, focusing on microbiota-secreted metabolites such as lipopolysaccharide (LPS) and short-chain

fatty acids (SCFAs). The central role of gut microbiota and its secreted metabolites in inducing markers of diabetes, such as chronic mild inflammation and triggering the immune response has been a primary focus of many studies.

LPS, derived from the cell walls of Gram-negative bacteria, has the capacity to trigger chronic low-grade inflammation by interacting with the Toll-like receptor 4 (TLR4) found on the surface of immune cells. The activation of this signaling pathway mediates the release of pro-inflammatory cytokines such as TNF and type 1 interferons (Lu et al. 2008). The activation of this pathway is widely regarded as a key instigator of the chronic mild inflammation related to obesity development. Saturated fatty acids such as lauric acid and palmitic acid can further exacerbate this inflammation via the TLR4 signaling pathway (Rogero & Calder, 2018). Additionally, another toll-like receptor, TLR2, has also been implicated in driving this inflammation process. Microbiota-derived ligands such as peptidoglycans, diacyl and triacylglycerol metabolite products, are recognized by TLR2. Upon recognition, a signaling cascades occurs leading to the activation of the NF-κB pathway and the expression of type 1 interferon genes (Oliveira-Nascimento et al. 2012). The intestinal epithelium acts as a protective barrier against pathogens and is vital in managing obesity and the control of gut inflammation. This barrier maintains the separation of the gut's microbes' and the host's basic immunity, ensuring both optimal absorption of nutrients and robust defense mechanisms. Higher LPS levels instigated by gut microbiota dysbiosis can lead to the weakening of this barrier, exacerbating inflammation through increased LPS absorption (Bron et al. 2017).

The family of SCFAs, which include acetic acid, propionic, and butyric acids, play an essential role in gut health homeostasis and disproportionate levels of SCFAs have been consistently linked to the development of Type 1 and 2 DM. Research indicates that SCFAs can strengthen the gut barrier, with butyrate enhancing the expression of tight junction proteins, supporting mucosal barrier function (Peng et al. 2009). Similarly acetic acid, a metabolic by-product of bacterial carbohydrate fermentation, also reinforces the gut barrier by reducing mucosal permeability (Suzuki et al. 2008). Bacterial-derived SCFAs help regulate glucose homeostasis by triggering the release of GLP-1 and peptide YY (PYY) from L-cells and regulating signaling pathways related to insulin resistance, inflammation, and oxidative stress (Kim et al. 2018). Dietary fiber

is known to stimulate the production of SCFAs by gut microbes. However, in T2D patients, the microbes that produce SCFAs are often less abundant as compared to healthy controls (Zhao et al. 2018). Zhao et al. succeeded in showing that increased fiber intake positively impacts glucose control by transplanting the gut microbiome from humans, pre- and post-dietary fiber interventions, into germ-free mice. Their results showed increased abundance and diversity of SCFAs-producing bacteria, due to fiber intake, were linked to better glycemic control in participants. A genome-wide genotyping study synthesized data from 952 individuals with normal blood glucose levels to investigate the genetic aspects of SCFAs and the development of diabetes (Sanna et al. 2019). The study employed a Mendelian randomization analysis and found a significant causal relationship between certain SCFAs and diabetes: butyrate was linked to improved insulin resistance, while poor absorption of propionic acid was associated with a higher risk of Type 2 DM. This underscores the influential role of the gut microbiome on metabolic health.

In a landmark study more than a decade ago, individuals with insulin resistance underwent fecal microbiota transplants (FMT) from insulin-sensitive donors. The trial yielded significant enhancements in insulin sensitivity, linked to an upsurge in butyrate-producing bacteria. (Vrieze et al. 2012). Conversely, adverse effects may occur after FMT, with reports suggesting that the leakage of transplanted bacteria into the bloodstream can trigger flares of inflammatory bowel disease (Quera et al. 2014). These reports underscore the necessity for in-depth exploration of the role of the gut microbiota in disease pathogenesis. One primary butyrate-producing bacterium identified from human fecal samples is Faecalibacterium prausnitzii (*F. prausnitzii*). Large scale genomic studies across various populations have found that Type 2 DM patients tend to have reduced levels of *F. prausnitzii* and *Roseburia* when compared to healthy individuals (Jumpertz et al. 2011). Further research highlights the potential of *F. prausnitzii* in alleviating inflammatory symptoms and insulin resistance. *Roseburia spp*., another butyrate-producing bacterium, plays an essential role in gut health and immune defense, especially in balancing T cell activities through butyric acid production (Tamanai-Shacoori et al. 2017). The beneficial effects of butyrate include strengthening the gut barrier by promoting mucin synthesis, which reduces gut permeability and bacterial translocation through the intestinal epithelial barrier.

Additionally, butyrate aids in minimizing inflammation and oxidative stress in the colonic epithelium.

Another contribution of metabolic by-products from the microbiota are secondary bile acids, converted from liver bile acids via gut microbiota enzymes (Fiorucci & Distrutti, 2015). In a rat study, the administration of secondary bile acids resulted in significant alterations in bacterial phylum levels, particularly an elevation in the Firmicutes/Bacteroidetes ratio (Islam et al., 2011). These bile acids play a crucial role in regulating insulin sensitivity by activating the Farnesoid X receptor (FXR) and the Takeda G protein-coupled receptor 5 (TGR5). A subsequent investigation employing whole-body FXR knockout mice demonstrated a reduction in insulin resistance, indicating that FXR deficiency enhances the secretion of fibroblast growth factors. This improvement contributes to enhanced glucose tolerance and reduced insulin resistance. (Prawitt et al. 2011).

Branched-chain amino acids (BCAA) have been identified as potential risk markers for Type 2 DM development, with several studies reporting decreased BCAA levels in diabetic patients (Huang et al. 2013). Significant associations between BCAA levels and diabetes risk were uncovered in a large cohort study, emphasizing the potential role of amino acid metabolism in diabetes' early phases. Studies in rats suggest that BCAA supplementation in high-fat diet-induced obesity setting can lead to insulin resistance, a finding confirmed in humans as well (Asghari et al. 2018). The BCAA synthesis pathway is believed to be connected to specific gut bacteria such as *Prevotella copri* and *Bacteroides vulgatus* (Pedersen et al. 2016). Follow-up experiments further revealed that germ-free mice receiving *P. copri* transplants exhibited heightened BCAA levels and increased insulin resistance. The proposed mechanism behind BCAA-induced insulin resistance involves elevated free fatty acid oxidation and the activation of phosphatidylinositol 3-kinase (PI3K), though the precise mechanism remains under investigation.

1.3.4 Tritrichomonad-derived metabolites and host metabolism

As alluded to above, Tritrichomonad protists have the capacity to increase the availability of succinate from dietary fibers through fermentative pyruvate oxidation in their hydrogenosomes (Nadjsombati et al. 2018). While land vertebrates mainly produce lactic acid in anaerobic conditions, helminths, protists, and bacteria have a broader range of anaerobic metabolites such as hydrogen, ethanol, acetate, and others. Several bacterial species, along with specific protists and helminths, use fumarate during fermentation, resulting in succinate production (Muller et al. 2012). Schneider et al. 2018 have shown that succinate, derived from *Tritrichomonas spp*., has the capability of activating the tuft cell-ILC2 circuit through signaling via the succinate receptor, GPR91, on tuft cells.

In a recent study in 2022, the authors investigated an abnormality discovered in their in-house bred specific-pathogen-free (SPF) genetic knock-out mouse model (Kou et al. 2022). The authors found that their herpesvirus entry mediator knock-out ($HVEM^{-/-}$) mice exhibited increased fasting blood glucose levels, impaired glucose tolerance, and heightened gluconeogenesis compared to wild-type (WT) mice from Vital River Laboratory Animal Technology (VRL). On examination of the fecal samples, single-celled protozoa were identified in the $HVEM^{-/-}$ mice but not in VRL WT mice. The protozoa were molecularly identified using DNA sequencing at the 18S and ITS rDNA locus as *T. musculis*, a protist previously identified by the same group (Wei et al. 2020). To discern if the glucose imbalance in the $HVEM^{-/-}$ mice was due to *T. musculis* colonization, the protozoa were eradicated using metronidazole, which subsequently improved the glucose imbalance in these mice. The authors provided further evidence of *T. musculis'* role in glucose regulation by isolating the protists from the $HVEM^{-/-}$ mice and administered them orally to *T. musculis*-free WT mice. This led to a dramatic shift in the gut microbiota composition and an increase in gluconeogenesis in the recipient mice. Collectively, the data indicates that the commensal mouse protozoan *T. musculis* can enhance gluconeogenesis.

The authors delved deeper into the mechanism by which *T. musculis* might influence blood glucose balance in their mice. They theorized that metabolites produced by *T. musculis*, particularly succinate, could be instrumental in this process. When *T.*

musculis-free wild-type mice were colonized with *T. musculis*, elevated levels of succinate were observed in both the cecum and blood serum. However, providing succinate in the drinking water did not lead to increased gluconeogenesis. Moreover, the authors utilized mice deficient in the succinate receptor, Sucnr1, ($Sucnr1^{-/-}$), and demonstrated standard gluconeogenesis levels similar to wild-type mice. Consequently, these findings refute the idea that *T. musculis*-derived succinate plays a role in enhancing host gluconeogenesis.

In an effort to identify other metabolites produced by *T. musculis*, the authors conducted an untargeted metabolomics study on the supernatant from an *in vitro* culture of *T. musculis*. The primary observation was a significant reduction of choline precursors in the media, most notably phosphatidylcholines (PCs) and lyso-PCs. Concurrently, there was an evident rise in the levels of free choline in the culture supernatant. This indicates that *T. musculis* potentially breaks down choline precursors, leading to the release of free choline. When *T. musculis* was administered into wild-type mice, there was a significant increase in the levels of free choline in their cecal contents. The authors also observed increased levels of choline-derived metabolites in the serum of colonized mice. Collectively, these findings suggest a significant role for *T. musculis* in promoting the generation of free choline.

It is important to note that while free choline is less abundant in the human diet and body compared to phosphatidylcholines (PC), bacterial hydrolysis of PC is one of the main sources of free choline (Cohn et al. 2010). Free choline within the host can be converted to various substances, including the neurotransmitter acetylcholine, certain membrane lipids, and the methyl donor betaine. In the intestines, free choline can be transformed into TMA by choline-utilizing bacteria, which is then processed in the liver to TMAO (Fennema et al. 2016). The authors decided to explore the potential role of the gut microbiota in the metabolism of choline, given that certain anaerobic bacteria, such as *Desulfovibrio* with the choline utilization gene cluster, can convert free choline to trimethylamine (TMA). Indeed, the authors found that by introducing *Desulfovibrio vulgaris* into WT mice, with and without the supplementation of choline, there was notable increase in gluconeogenesis and an elevated level of serum TMAO. However, when the mice diet was shifted to a choline-deficient diet, this trend vanished, as did the enhanced gluconeogenesis. Additionally, the authors utilized several inhibitors of

choline downstream metabolism, the small molecular inhibitor, 3,3-dimethyl-1-butanol (DMB) and the phytochemical 3,3'-diindolylmethane (DIM), to show that the treatments were effective in improving the elevated gluconeogenesis exhibited by their *T. musculis*-colonized HVEM$^{-/-}$ mice.

Their findings indicate that for effective gluconeogenesis modulation, dietary free choline and choline from organisms like *T. musculus* might need further processing by choline-metabolizing commensal bacteria. The net metabolic effect of free choline likely hinges on the interplay between microbial and host metabolic processes. While it is known that choline can influence various hormones impacting glucose homeostasis, the exact molecular mechanisms remain elusive (Zeisel & da Costa 2009). TMAO levels in the bloodstream have been proposed as indicators for cardiovascular and cerebrovascular conditions (Haghikia et al. 2018). However, many variables, such as intestinal microbiota composition and genetics, can affect TMAO levels. A recent study has also provided the link between TMAO levels and the enhancement of gluconeogenesis through the activation of an endoplasmic reticulum stress response and the production of FoxO1, a major driver in metabolic diseases (Chen et al. 2019). The study by Kou et al. echoes these findings and emphasizes the gut microbiota's role in phospholipid metabolism, which significantly affects the host's metabolic balance. This was the first study of its kind to show the impact of Tritrichomonad protist colonization on host glycemic control. The basis of this project aims to corroborate their findings and expand upon the knowledge on how *Tritrichomonas musculus* modulates host's glucose homeostasis and gut immunity especially in the context of HFD-induced type 2 DM.

1.4 A Statement of Aims

Given our previous findings that gut inflammation is associated with metabolic disease and impaired glucose control, we decided to explore whether long-term colonization with *Tritrichomonas musculus (*also referred to as *Tritrichomonas musculis)* affects gut immunity and, consequently, glucose homeostasis (Rohm et al. 2022). Understanding this relationship is crucial as it could shed light on how external factors such as microbial infections or environmental pollutants, in addition to diet, might influence

gut immunity and contribute to the development of an impaired glycemic control. We hypothesize that chronic colonization with *Tritrichomonas musculus* in wild-type C57BL/6N mice in the context of HFD-induced obesity leads to a persistent inflammatory environment in the gut, resulting in dysfunctional glucose regulation. Additionally, we aim to show that the immunological shift in *Tritrichomonas*-colonized mice and the resulting impaired metabolism is partly due to the intimate interplay between commensal protozoa, bacteria and host immunity, modulated by a diet-induced shift in gut microbiota composition. The specific objectives of this dissertation are outlined as follows:

A. Investigate the metabolic outcomes following chronic *Tritrichomonas musculus* colonization in wild-type C57BL/6N mice. As of writing, there is no published reports of the glycemic control and insulin sensitivity parameters following Tritrichomonad colonization in wild-type mice. We aim to expand this knowledge further through the use of both a normal chow-fed and a Type 2 DM diet-induced obesity C57BL/6N model. Different metabolic tests such as the standard glucose and insulin tolerance tests will be employed on our study model throughout the colonization period. These findings would broaden our knowledge on the impact *Tritrichomonas musculus* has on the maintenance of glucose homeostasis and insulin secretion.

B. Assessment of the immunological landscape of the colon, small intestine as well as the epididymal adipose tissue, an insulin-sensitive tissue, by flow cytometry. There is an extensive interplay between intestinal immune cells such as macrophages and T-cells and the development of Type 2 DM. We hypothesize that colonization with *Tritrichomonas musculus* mediates an inflammatory shift in intestinal immune cells which contributes to the low-grade chronic inflammation observed in the gastrointestinal tract of diabetic patients. Our findings will corroborate the results of multiple recent publications on the inflammation effectuated by *Tritrichomonas musculus* colonization.

C. Through the use of Nlrp3 inflammasome whole-body knock-out ($Nlrp3^{-/-}$) mice in addition to an *in vivo* pharmacological inhibition of IL-1β in wild-type mice, we aim to show that the Nlrp3 inflammasome and the pro-inflammatory cytokine IL-1β contributes significantly to the inflammation and metabolic outcomes mediated by *Tritrichomonas musculus* colonization. These results will corroborate previous findings that colonization with Tritrichomonad protists is capable of activating the Nlrp inflammasomes, mediating a pro-inflammatory shift. These models would allow us to provide a mechanistic understanding on the Tritrichomonad-driven inflammation.

D. Characterize the composition of the shift in gut microbiota upon colonization with *Tritrichomonas musculus.* in wild-type C57BL/6N mice on both a normal chow and HFD conditions. There are considerable interactions between the eukaryotic protozoa and bacterial kingdoms in the gastrointestinal tract that can influence the overall health of the host and play critical roles in various metabolic and physiological processes. Moreover, the impact of HFD feeding itself on the microbial community is highly significant with notable shifts in certain bacteria taxa linked with the metabolic syndrome. By delineating the microbial shifts under these conditions upon *Tritrichomonas musculus* colonization, we aim to further broaden the knowledge concerning commensal protozoa contribution to microbiome diversity and the physiology of the host.

E. We hypothesize that metabolites secreted from the metabolism of Tritrichomonad protists play a considerable role in mediating the inflammation and metabolic outcomes of the host. It has been previously shown that *T. musculus* can generate free choline and succinate. Furthermore, these secreted metabolites can also interplay with the metabolism of other bacterial communities leading to elevated levels of other microbial-derived metabolites such as SCFAs. Through the supplementation of free choline and the use of small molecule pharmacological inhibitors, we aim to provide a mechanistic understanding of how *Tritrichomonas musculus* engenders the multifaceted effects on the host.

Chapter 2

Materials and Methods

2.1 Mouse models

Male C57BL/6N (B6) mice were purchased from Charles River Laboratories, bred in-house and maintained in specific-pathogen-free conditions within our facility at 22°C on a 12h light/12h dark cycle. Nlrp3$^{-/-}$ mice, backcrossed to a C57BL/6N background, were bred and maintained in-house. Mice are housed in individually ventilated cages that are changed every week and have access to food pellets and water, *ad libitum*. For the germ-free (GF) C57BL/6N mice experiment, GF mice were bred and maintained in flexible-film isolators at the Clean Mouse Facility, University of Bern, Switzerland. Unless stated otherwise, all the mice in this study were used in experiments beginning from 5 to 8 weeks of age and weight-matched into groups. The mouse diets used in this study are the standard chow diet from Granovit (4.5% fat, 18.5% protein, 35% starch, 4.5% fibers) and the coconut-based high-fat diet (HFD) from Research Diets (#D12331, 58 kcal% fat, 16.4% protein, 25.5% maltodextrin 10, 0.5% fibers, sucrose).

Study approval

All animal procedures were approved by the local Animal Care and Use Committee and performed in accordance with Swiss Federal Regulations.

2.2 Isolation of *Tritrichomonas musculus* and colonization of C57BL/6N mice

The following protocol was adapted from the published protocol by O'Leary et al. 2021. The caecal content of *Tritrichomonas*-colonized mice was harvested and resuspended in 50 mL cold PBS. 10 µl of the suspension was pipetted onto a glass slide and checked under a regular brightfield microscope at 20x magnification for the presence of flagellated Tritrichomonad protists. The caecal content suspension is filtered through a 100 µm cell strainer into a new 50 mL falcon tube. The filtrate is centrifuged at 1000 rpm for 10 minutes, pellet resuspended in 50 mL cold PBS and centrifuged once more. Motile protists

were counted using a haemocytometer (a typical colonized mouse caecum contains approximately 60 million protists) and diluted to a final concentration of 50 million protist/mL PBS. To mimic natural vertical transmission of Tritrichomonad protists, two litter-mate C57BL/6N breeding pairs were either orally administered with 20 million protists or left alone. All of the offspring of the *Tritrichomonas*-colonized breeding pair will be colonized with *Tritrichomonas musculus* as the pups exhibit coprophagic behaviour and ingest the protists in their parents' faeces. Offspring from these breeding pairs were used for all subsequent C57BL/6N mice experiments.

2.3 Tritrichomonad colonization of Nlrp3$^{-/-}$ mice

Our Nlrp3$^{-/-}$ C57BL/6N breeding mice were kept Tritrichomonad-free throughout the study. These mice were instead colonized with Tritrichomonad protists starting from age 5 to 7 weeks. In brief, Tritrichomonad protists were harvested and isolated from a donor colonized mouse as outlined above. Following the washing steps, approximately 10 million protists were orally gavaged into experimental Nlrp3$^{-/-}$ groups. The protists were given one week to adjust and colonize the gastrointestinal tract of the mice to ensure complete colonization. Subsequently, the food provided to these mice were swapped from a standard chow to a HFD, *ad libitum*, following this one-week period.

2.4 *In vitro* culture of *Tritrichomonas musculus*

Tritrichomonad protists were isolated from the caeca of colonized mice as outlined previously. Phosphate-buffered saline (PBS) containing 1% penicillin/streptomycin (P/S) and 50 µg/mL vancomycin was used for the washing steps. The culture of Tritrichomonad protists was adapted from the protocol described in Chudnovskiy et al. 2018, with the following modifications. Caecal extract was generated by diluting 1 g of caecal content isolated from *Tritrichomonas*-free C57BL/6N mice with 25mL of BD Trichosel Broth (#298323 Becton Dickinson). The suspension was stirred at 4ºC for 6 hours, centrifuged at 3500 rpm for 10 minutes and the supernatant filtered through a 100 µm strainer. Prior to autoclaving, the caecal extract was titrated to pH 7 with NaOH. Following this, the extract was supplemented with 10% heat-inactivated horse serum (#26050088, Gibco), 1% P/S (#10378016, Gibco), 50 µg/mL vancomycin (#V2002, Sigma-Aldrich), 10 µg/mL

ciprofloxacin (#17850, Sigma-Aldrich), 20 µg/mL gentamicin (#G1272, Sigma-Aldrich) and 0.5 µg/mL amphotericin B (#A2942, Sigma-Aldrich). Protists were inoculated with culture media at 2 million protists per mL media and cultured under anaerobic conditions in BD GasPak EZ Gas Generating Container Systems (#260674, Becton Dickinson) at 37ºC for 72 hours, with shaking. After incubation, the media suspension was diluted up to 50 mL in antibiotic-supplemented PBS, centrifuged at 1000 rpm for 10 minutes and resuspended in PBS for inoculation in GF mice.

2.5 Filtration of Tritrichomonad protists for germ-free mice experiments

The colonization of GF mice with bacterial communities isolated from the caeca of uncolonized and *Tritrichomonas*-colonized mice was achieved by first isolating the caecal contents of mice into cold PBS as described before. Following the initial centrifugation of the suspension at 1000 rpm for 10 minutes, the supernatant is retained and centrifuged once more. To filter out any remaining Tritrichomonad protists from the caecal suspension, two separate methods were implemented: 1) Filtration using 25 mm diameter sterile syringe filters with 5.0 µm pore size hydrophilic PVDF membrane (#SLSV025LS, Millipore) and 2) Filtration using 47 mm diameter, mixed cellulose esters (MCE) membrane filters with 5.0 µm pore size (SMWP04700, Millipore). For Method 1, the caecal suspension was filtered through the syringe filter twice and centrifuged at 5000 g for 5 mins. The bacterial pellet was resuspended in a reduced sterile PBS solution prior to administration into GF mice via oral gavage. For Method 2, a vacuum filtration chamber (Millipore) was used to filter out any remaining protists. In brief, the caecal suspension was subjected to vacuum filtration through the 5.0 µm MCE membrane twice and centrifuged at 5000 g for 5 mins. The colonization of GF mice with bacterial colonies isolated from the donor mice was only performed using the syringe filter filtration method.

2.6 Isolation of immune cells for flow cytometry

Intestinal tissue

Intestinal lamina propria lymphocytes were extracted from the colon of mice following the isolation procedure developed by Baranska et al. 2018. Firstly, the colon samples were measured, and the fat was removed. The tissue was then cut open lengthwise and further divided into 1 cm pieces before being washed with a cold solution of DPBS or HBSS (without Mg/Ca). To eliminate the epithelial layer, the tissue pieces and biopsies were washed twice with HBSS/2mM EDTA while gently shaking for 20 minutes at 37°C. Following this step, they were washed twice with HBSS and transferred into a specialized MACS C-tube (#130-096-334, Miltenyi Biotec) containing 3 mL of Complete IMDM Medium (1x IMDM, 10% FBS, 1% P/S). To initiate enzymatic digestion, 3 mL of 2x Collagenase VIII solution (#C2139, Sigma-Aldrich) was added to the tube, which consisted of Complete IMDM supplemented with 2 mg/mL Collagenase VIII (#C2139, Sigma-Aldrich) and 25 µg/mL DNase I (#11,284,932,001, Roche). The tube was then shaken at 37 °C for approximately 25 and 30 minutes for small intestine and colon tissue, respectively. The digested tissue was homogenized using the gentleMACS Octo Dissociator (#130-096-427, Militenyi Biotec; Program: ms_intestine-01). The digestion process was halted with the addition of EDTA. Subsequently, leukocytes were enriched using a 40%/70% percoll gradient (#GE17-0891-01, GE Healthcare) and centrifuged at 600 g for 20 minutes at 22 °C with brake and acceleration set to 1. The lymphocyte ring that formed at the inter-phase was collected, washed with FACS Buffer (1x DPBS, 0.5% BSA, 5 mM EDTA) through a centrifugation step at 1500 rpm for 5 minutes at 22 °C. Finally, the cells were resuspended in 1 mL of FACS Buffer, filtered using a 35 µM strainer FACS tube (#352235, Corning) and transferred to deep-well plates for antibody staining.

Adipose Tissue

Epididymal adipose tissue samples were minced into small pieces using scissors and then subjected to digestion. The digestion process involved shaking the tissue in a digestive solution containing Collagenase IV (#LS004186, Worthington), which consisted of 1x HBSS, 10 mM HEPES, 1.5 mg/mL Collagenase IV, and 8.25 µg/mL DNase I. The shaking was carried out on a thermomixer for 20 to 30 minutes at 37°C at 400 rpm. To halt the digestion, FACS buffer was added, and the resulting mixture was filtered through cotton

gauze to remove any large undigested debris. To eliminate erythrocytes, Red Cell Lysis Buffer (154 mM NH4Cl, 10mM KHCO3, 0.1 mM EDTA) was added to the solution. Subsequently, the cells were washed, filtered through FACS tubes and transferred to deep-well plates for antibody staining.

2.7 Antibody staining for flow cytometry

To minimize non-specific binding, the Fc receptor was blocked with CD16/32, which was added to the cells along with the monoclonal antibodies (mAbs) for approximately 30 minutes to 1 hour on ice in the dark. The specific mAbs used in this study are listed in (Table 2.2). The gating strategy for intestinal macrophages, intestinal dendritic cells and adipose tissue macrophages can be referred to in (Figures 2.1, 2.2 and 2.3), similar to the strategy described in previous studies for skin macrophages. For the intracellular staining to asses innate lymphoid cells (ILCs) populations (Figure 2.4), the Anti-Mouse/Rat Foxp3 Staining Set (#77-5775-40, eBioscience) was used. In brief, cells were stained with surface antibodies before being fixed with fixation buffer for 40 minutes in the dark. Cells were then washed with permeabilization buffer and stained with intracellular antibodies for 30 to 60 minutes at 4°C. At the end of the staining, cells were washed and resuspended in FACS buffer for flow cytometry analysis. Flow cytometry data were acquired using a BD LSRIIFortessa (BD) and analyzed using FlowJo software version 10.8.2 (BD).

2.8 *In vivo* metabolic assessments

To evaluate the metabolic characteristics of experimental mice, intraperitoneal glucose tolerance tests (ipGTT) were conducted at a monthly basis for up to 3 and 6 months in HFD-fed and standard chow-fed mice, respectively. Intraperitoneal insulin tolerance tests (ipITT) were conducted at the end of the experiment prior to sacrifice. Intraperitoneal pyruvate tolerance tests (ipPTT) were performed on the choline-supplemented mice experiments following a 1-month HFD feeding. Mice were fasted for 6 hours for the ipGTT and 3 hours for the ipITT and ipPTT. Following the fasting period, glucose (2 g/kg body weight), insulin (1-2 U/kg body weight, Actrapid HM Penfill, Novo Nordisk) or pyruvate (2 g/kg body weight) was injected intraperitoneally and blood glucose levels were monitored from the tail vein at 15, 30, 60, 90, and 120 minutes using a glucometer

(Freestyle, Abbot). For the ipGTT experiments, blood was collected from the tail vein at the basal, 15- and 30-minutes time points for insulin measurements.

2.9 Isolation of pancreatic islets and glucose-stimulated insulin secretion (GSIS)

Mouse islets were isolated following a previously established method by Dalmas et al. 2017. The isolation process involved collagenase digestion using 1.5 mg/mL Collagenase IV, followed by filtration and manual selection of the islets. In brief, pancreatic mouse islets were isolated using a method where collagenase IV-containing digestion solution was injected into the pancreas through the common bile duct. The pancreas was then perfused and digested at 37 °C for 30 minutes. After digestion, the process was halted using a solution containing 1x HBSS, 1M HEPES, and 0.5% BSA, and the mixture was filtered. Islets were carefully selected by hand under a stereoscopic microscope and subsequently cultured in RPMI-1640 medium, containing 11.1 mM glucose, 10% FBS, 1% P/S. Approximately 100 islets were collected per sample for further analysis. These isolated islets were then cultured in RPMI-1640 medium with 11 mmol/L glucose and 10% FCS while floating freely overnight. On the following day, the islets were washed and pre-incubated in Krebs-Ringer-bicarbonate buffer (KRB) with 2.8mM glucose. After 1.5 hours incubation, the buffer was replaced with KRB containing either low glucose (2.8 mM, basal) or high glucose (16.7 mM, stimulated). The supernatant was collected after 1 hour to measure basal and glucose-stimulated insulin release. To determine the insulin content of the islets, insulin content was extracted using 0.18 mol/L HCl in 70% ethanol and incubated for at least 1 hour at -20ºC. This extraction process allowed the measurement of insulin content within the islets. Subsequently, secreted and content insulin levels were measured using the Mouse/Rat Insulin Kit (#K152BZC, Meso Scale Discovery). The stimulatory index, indicating the ratio of stimulated insulin secretion at 16.7mM glucose to basal insulin secretion at 2.8mM glucose, was calculated.

2.10 *In vivo* anti-IL-1β antibody treatment

Following two months of HFD feeding, the mice were administered IL-1β antibody via intraperitoneal injections at a dose of 10 mg/kg (01BSUR; similar specificity to canakinumab and used by other research groups (5, from Novartis) or an isotype control (anti-cyclosporin A, from Novartis) once every week, for the first two weeks. Starting from week 3, the dosage was reduced to 5 mg/kg, administered once weekly for the following two weeks prior to sacrifice.

2.11 *Ex vivo* colonic explant culture

The *ex vivo* colonic explant culture was performed as described by Udden et al. 2017, with the following modifications. In brief, whole colon tissue was flushed with cold PBS, excised into approximately 1 cm long sections and weighed. The tissue explants were cultured in IMDM containing 10% FBS and 1% P/S for 2 hours at 37ºC. Following the initial incubation, the explants were rinsed with PBS, fresh media added to the tissue (1 mL per 100 mg tissue) and incubated at 37ºC, 5% CO_2 for 16 to 20 hours. Culture medium was then collected and stored at -20ºC for further cytokine secretion analysis.

2.12 Protein expression analysis

The quantification of plasma insulin, IL-1β, TNF-α, and IL-6 from collected mouse sera and explant culture supernatant was achieved by electrochemiluminescence using the MESO SECTOR S 600 system with kits from Meso Scale Diagnostics (MSD, Rockville, MD, USA). The specific kits used were Mouse/Rat Insulin Kit (#K152BZC), V-PLEX Plus Proinflammatory Panel 1 Mouse Kit (#K15048G), following the manufacturer's instructions. Granulocyte-macrophage colony-stimulating factor (GM- CSF), IL-33, IL-5 and IL-13 were quantified by electrochemiluminescence (MESO SECTOR S 600) using kits from Meso Scale Discovery (USA).

2.13 Measurement of Choline, TMAO and Betaine

Choline, Trimethylamine-N-Oxide (TMAO) and Betaine were measured from the blood sera of chow- and HFD-fed control and *Tritrichomonas*-colonized mice using a LC-HRMS method as outlined in Mueller et al. 2015. The samples were analyzed at the Institute of Clinical Chemistry, University Hospital and University of Zurich, Zurich, Switzerland.

2.14 Caecal DNA extraction, sequencing and microbiota analysis

To isolate genomic DNA from stool samples, the contents from experimental mice caeca were first frozen in 2 mL tubes and stored at -80 °C until the extraction process. The QIAamp PowerFecal Pro DNA kit (#51804 Qiagen) was utilized to extract genomic DNA from feces, following the instructions provided by the vendor. The homogenization step of stool particles was achieved with the use of the TissueLyser II system (#85300, Qiagen). Disruption of samples was performed twice at 25 Hz for 5 minutes. DNA was eluted in 50 µL volumes and their concentrations measured using the Nanodrop2000 system (Thermo Fisher Scientific). Amplicon sequencing was performed on DNA extracts by Novogene. The amplification step was carried out using primers targeting the V4 hypervariable regions of the 16S rRNA gene. PCR products with proper size were selected by 2% agarose gel electrophoresis and pooled in equimolar amounts, end-repaired, A-tailed and ligated with Illumina adapters. Libraries were sequenced on a paired-end Illumina platform to generate 250pb paired-end raw reads. Libraries were quantified on a Qubit Fluorometer (ThermoFisher) and real-time PCR. Quality control, filtering and trimming, dereplication, chimera removal and taxonomic assignment were carried out using the dada2 (Callahan et al. 2016). pipeline on R. Statistical analysis of the fecal microbiome was performed using the phyloseq (McMurdie et al. 2013), vegan (Oksanen et al. 2017) and DESeq2 (Love et al. 2014) packages, and graphical visualizations were made on ggplot2 (Wickham et al. 2016).

2.15 Sequencing of the Tritrichomonad protist genome

Following the *in vitro* culture of Tritrichomonad protists (refer to section 2.4), the DNA of the enriched protists was extracted using the subsequent approach. The QIAamp PowerFecal Pro DNA kit (#51804 Qiagen) was utilized to isolate genomic DNA from the protists following the instructions provided by the vendor. The homogenization step of the protists was achieved with the use of the TissueLyser II system (#85300, Qiagen). Disruption of protists was performed twice at 25 Hz for 5 minutes. DNA was eluted in 50 µL volumes and their concentrations measured using the Nanodrop2000 system (Thermo Fisher Scientific). DNA integrity was verified on a 0.8% agarose gel and sent to Novogene Co., Ltd. (United Kingdom) sequencing service provider to perform whole genome sequencing. Library preparation was achieved using an Illumina 2x150bp paired-end strategy yielding 72.22 million raw reads. Reads were then trimmed and cleaned with fastp (version 0.22.0 (PMID: 30423086)), resulting in 71.76 million reads passing filter (99.36%). Genome assembly was performed with SPAdes (PMID: 22506599) and produced 16546 contigs.

2.16 Phylogenetic Analyses

The 18S rRNA gene and ITS-1 sequence were identified in our Tritrichomonas-assembled contigs using a local BLASTN approach (REF). Briefly, a local BLASTN database was built with the 16546 assembled contigs. The 18S rRNA gene reference sequence (ON927247) from *Tritrichomonas rainier* and the ITS-1 sequence from *Tritrichomonas musculus* (KX000922) were used to blast against our local contigs database. The 18S rRNA gene was identified in a 5470 bp contig (NODE_5658) and the ITS-1 sequence was identified in a 1072 bp contig (NODE_15403). 18S rRNA gene and ITS-1 sequences from other closely related Tritrichomonad species were retrieved from NCBI and included for the alignments using the MUSCLE alignment tool (Edgar, 2004). The alignment for both 18S rRNA gene and ITS-1 sequences were edited manually to remove overhangs before tree construction in MEGA 11 (Tamura K., Stecher G., and Kumar S., 2021). Both consensus tree was inferred using the UPGMA method (Sneath P. H. A. & Sokal R. R., 1973) based on 1000 bootstrap replicates (Felsenstein J., 1985). Trees were annotated with corresponding aligned sequences with iTOL, an online tool for phylogenetic tree display and annotation (PMID: 33885785).

2.17 Gene expression analysis

RNA was isolated from the distal colon, distal small intestine, epididymal adipose tissue and liver samples using either the NucleoSpin RNA kit (#740955250, Macherey-Nagel) or the RNeasy Plus Universal Mini kit (#73404, Qiagen) in accordance with the respective manufacturer's instructions. Reverse transcription was carried out using the GoScript™ Reverse Transcription Mix (#A2801, Promega). For qPCR analysis, the GoTaq qPCR Master Mix (#A6002, Promega) was used on a ViiA7 Real-Time PCR System (Thermo Fisher Scientific). The primer sequences (Microsynth, Balgach, Switzerland) can be found in (Table 2.1). The data was calculated using the 2^{-ddCt} method and normalized to the housekeeping gene GAPDH.

2.18 Quantification and statistical analysis

The results are displayed as mean ± standard deviation of the mean (SD) or mean ± standard error of the mean (SEM), as indicated, and the number of experiments and mice used are specified in the figure legends. Statistical analysis was performed using the Prism8 software (GraphPad Software, San Diego, CA) with the unpaired Mann-Whitney U test and the student's two-tailed distribution t-test. *P-values* of 0.05 or under were considered statistically significant.

2.19 Flow cytometry gating strategies

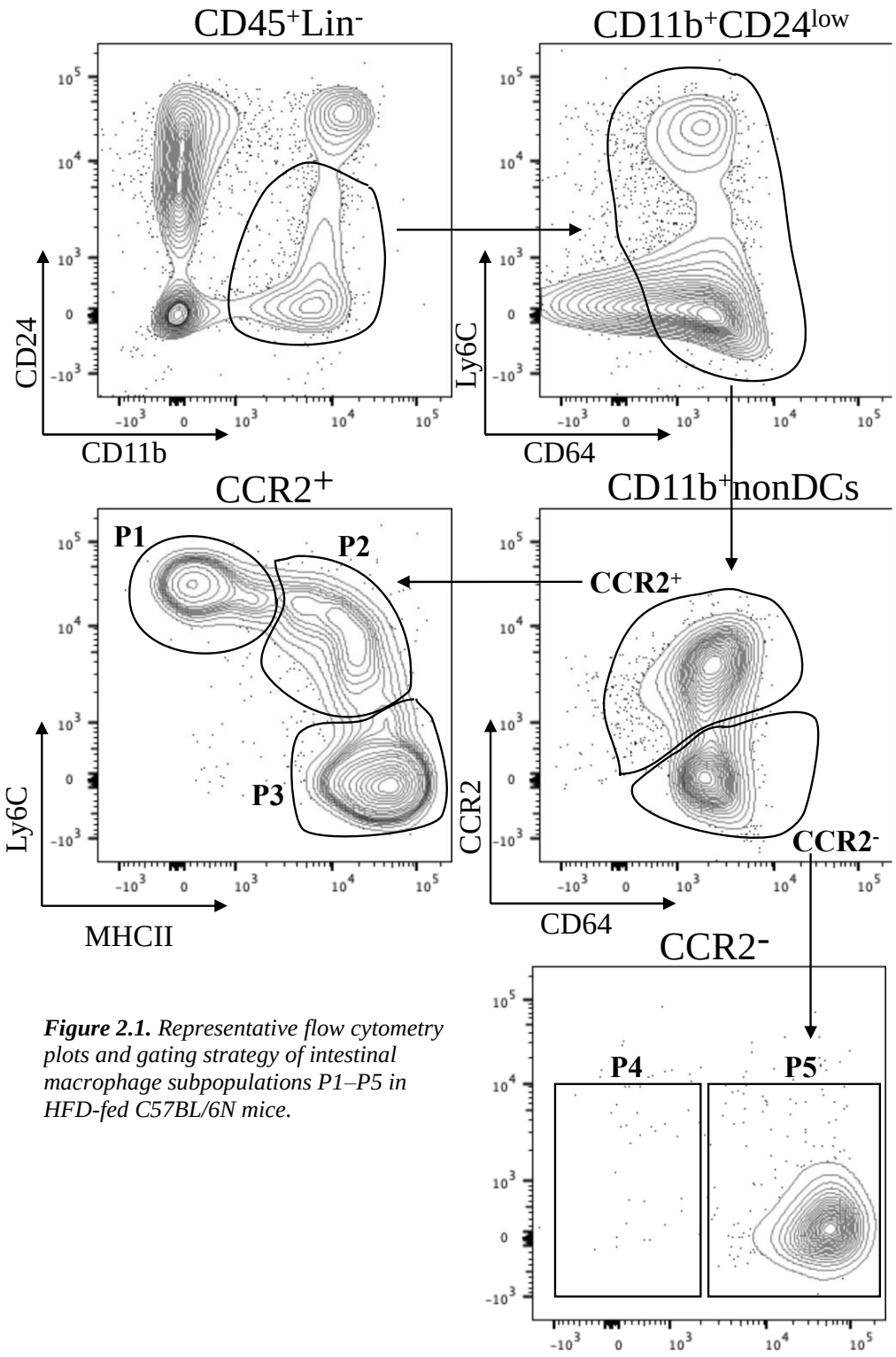


Figure 2.1. *Representative flow cytometry plots and gating strategy of intestinal macrophage subpopulations P1–P5 in HFD-fed C57BL/6N mice.*

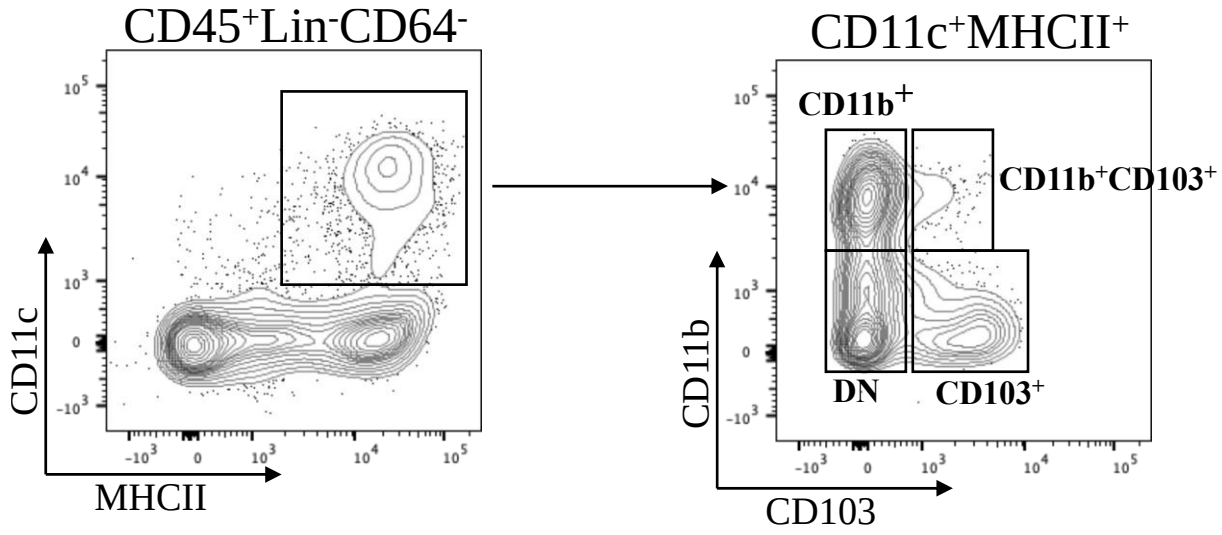


Figure 2.2. *Representative flow cytometry plots and gating strategy of intestinal dendritic cell populations, CD11b+ and CD103+ in HFD-fed C57BL/6N mice.*

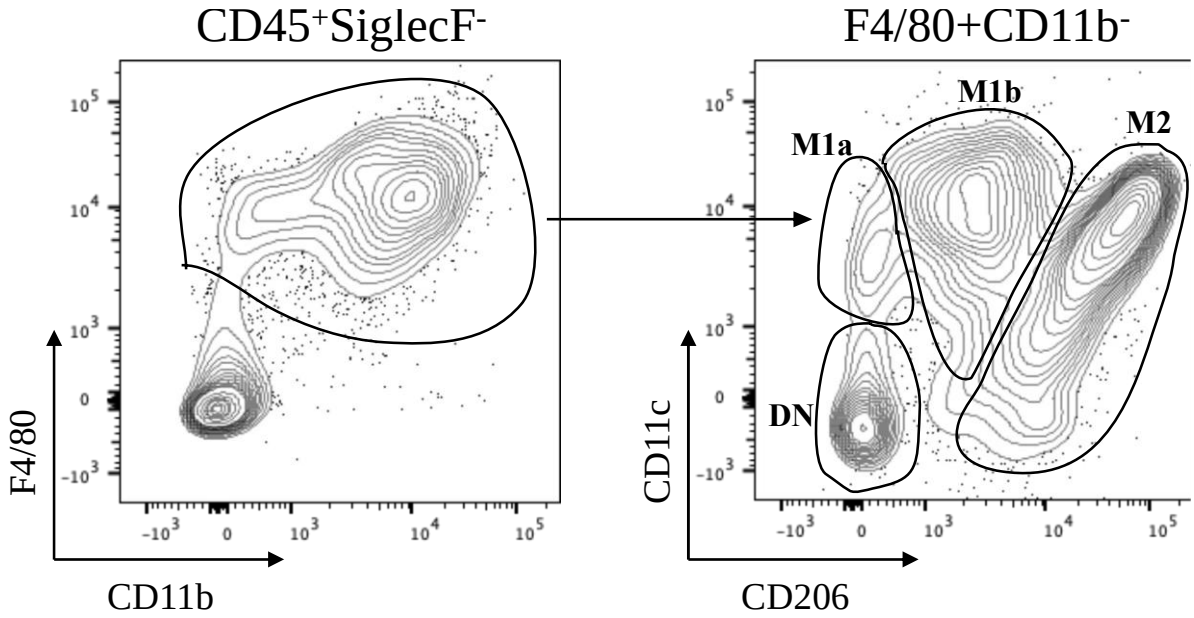


Figure 2.3. *Representative flow cytometry plots and gating strategy of adipose tissue inflammatory (M1) and resident (M2) macrophages, in HFD-fed C57BL/6N mice.*

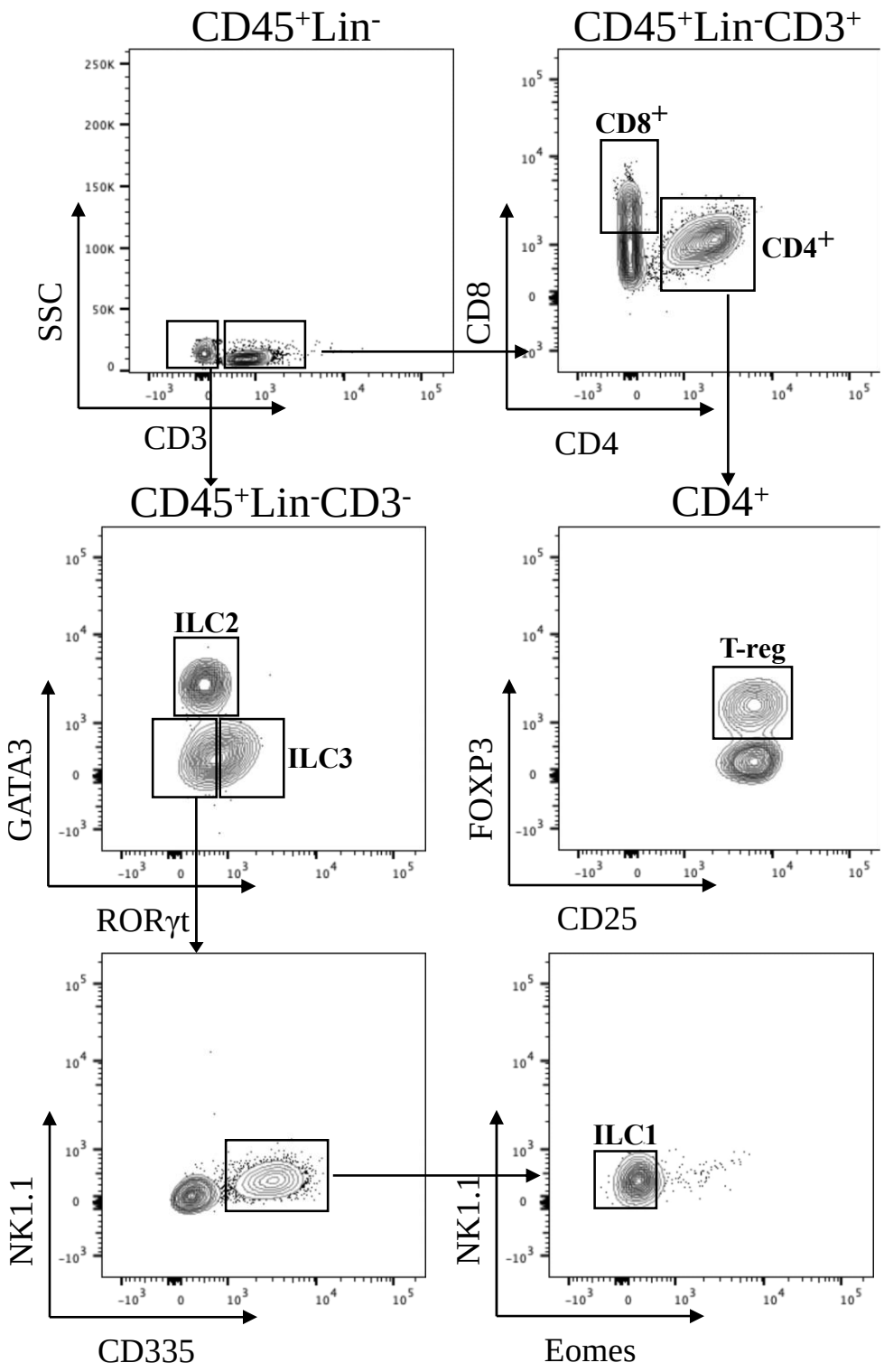


Figure 2.4. *Representative flow cytometry plots and gating strategy of intestinal innate lymphoid cells (ILCs) and T-cell populations in HFD-fed C57BL/6N mice.*

2.20 Materials and Reagents Tables

Table 2.1: Primer sequences used for quantitative RT-PCR

Gene	Forward Primer	Reverse Primer
Housekeeping gene		
GAPDH	5' AGGTCGGTGTGAACGGATTTG	5' TGTAGACCATGTAGTTGAGGTCA
Inflammatory markers		
IL-1β	5′ GCAACTGTTCCTGAACTCAACT	5′ ATCTTTTGGGGTCCGTCAACT
IL-6	5′ GGATACCACTCCCAACAGACCT	5′ GCCATTGCACAACTCTTTTCTC
TNF-α	5′ ACTGAACTTCGGGGTGATCG	5′ TGAGGGTCTGGGCCATAGAA
IFNα	5' CCTGCTGGCTGTGAGGA	5' GGAAGACAGGGCTCTCCAG
IFNg	5' CAGCAAGGCGAAAAAGGA	5' GGTGGACCACTCGGATGA
IL-10	5′ AGGCGCTGTCATCGATTTCTC	5′ GCCTTGTAGACACCTTGGTCTT
MCP1	5′ AGGTCCCTGTCATGCTTC	5′ TCTGGACCCATTCCTTCT
Gluconeogenesis markers		
Pck1	5′ AAGCAAGACAGTCATCATCACCCAA	5′ GGCGAGTCTGTCAGTTCAATACCAA
G6Pase	5′ TGGTAGCCCTGTCTTTCTTTG	5′ TTCCAGCATTCACACTTTCCT
Foxo1	5' ATGGTGAAGAGCGTGCCCTAC	5' CTTTCCAGTTCCTTCATTCTGCAC
CREB1	5' TGAACGAAAGCAGTGACGGAG	5' TAATGTGGCAATCTGTGGCTGGGC
PGC-1α	5' CTCCCTTGTATGTGAGATCACGTT	5' TGCGGTATTCATCCCTCTTGA
Cebpa	5' ATAGACATCAGCGCCTACATCGA	5' GTCGGCTGTGCTGGAAGAG
Choline Transporters and Kinases		
ChKa	5' GCTGCAGTATACTAGATCTCCAGTTGT	5' ATCAGCTTCCGCCTTTCA
ChKb	5' GCAGAGGTTCAGAAGGGTGA	5' CCCCAGAAAAAGTGAGATGC
Slc44aa1	5' TTTGCCCAAGCTACCAG	5' GAGCACAGCGATGGAAGAA
Slc44aa2	5' CCTGGTGCTTGGCTATGG	5' CAAGGTCCAGGGAGA
Slc44aa3	5' GGTCATTTTGGGATTGCTGT	5' ACTGAGGTCGTTGGTGTAGTCA
Slc44aa4	5' ACTCTGTCCCCGTTTCCTTC	5' AAGTTGATGTTGGGGAGTGG
Slc44aa5	5' ATCCAAGTGGCCATCATCC	5' GATTAACGCACTGGGAAGGT
Bacteria genes		
Eubacteria 16S	5' ACTCCTACGGGAGGCAGCAGT	5' ATTACCGCGGCTGCTGGC
A. muciniphila	5' CAGCACGTGAAGGTGGGGAC	5' CCTTGCGGTTGGCTTCAGAT
Bacteroides	5' GTTTAATTCGATGATACGCGAG	5' TTAASCCGACACCTCACGG

Table 2.2: Materials and Reagents

Materials or Reagents	Source	Identifier
Antibodies		
Anti-mouse CD16/CD32	Biolegend	Cat#101310; RRID: AB_2103871
Anti-mouse CD3 (145-2C11) APC-Cy7	Biolegend	Cat#100330; RRID: AB_1877170
Anti-mouse CD19 (6D5) APC-Cy7	Biolegend	Cat#115530; RRID: AB_830707
Anti-mouse NK1.1 (PK136) APC-Cy7	Biolegend	Cat#108723; RRID: AB_830870
Anti-mouse Ly6G (1A8) APC-Cy7	Biolegend	Cat#127624; RRID: AB_10640819
Anti-mouse CD45 (30-F11) PerCP-Cy5.5	Biolegend	Cat#103131; RRID: AB_893344
Anti-mouse CD24 (M1/69) PE-Cy7	Biolegend	Cat#101821; RRID: AB_756047
Anti-mouse CD11b (M1/70) BV421	Biolegend	Cat#101236; RRID: AB_11203704
Anti-mouse CD64 (X54-5/7.1) APC	Biolegend	Cat#139306; RRID: AB_11219391
Anti-mouse Ly6C (HK 1.4) FITC	Biolegend	Cat#128005; RRID: AB_1186134
Anti-mouse I-A/I-E (M5/114.15.2) BV785	Biolegend	Cat#107645; RRID: AB_2565977
Anti-mouse CCR2 (475301) PE	R&D Systems	Cat#FAB5538P-25/100; RRID: AB_10718414
Anti-mouse CD11c (N418) BV650	Biolegend	Cat#117339; RRID: AB_2562414
Anti-mouse CD103 (2E7) PE-Dazzle594	Biolegend	Cat#121430; RRID: AB_2566493
Anti-mouse CD11c (N418) PE-Cy7	Biolegend	Cat#117318; RRID: AB_493568
Anti-mouse CD206 (C068C2) A647	Biolegend	Cat#141712; RRID: AB_10900420
Anti-mouse Siglec F (E50-2440) BV510	BD Biosciences	Cat#740158; RRID: AB_2739911
Anti-mouse F4/80 (BM8) PE	Biolegend	Cat#123110; RRID: AB_893486
Anti-mouse CD3 (17A2) BV711	Biolegend	Cat#100241; RRID: AB_2563945
Anti-mouse CD8a (53-6.7) A700	Biolegend	Cat#100729;RRID: AB_493702
Anti-mouse CD25 (PC61) BV605	Biolegend	Cat#102035; RRID: AB_11126977
Anti-mouse CD4 (GK1.5) PE-Dazzle 594	Biolegend	Cat#100456; RRID: AB_2565845
Anti-mouse CD335, NKp46 (29A1.4) BV421	Biolegend	Cat#137611; RRID: AB_10915472
Anti-mouse NK-1.1 (PK136) PE/Cy7	Biolegend	Cat#108714; RRID: AB_10762291
Anti-mouse Foxp3 (FJK-16s) APC	Thermo Fisher Scientific	Cat#17-5773-82; RRID: AB_469457
Anti-mouse Rorγt (Q31-378) BV786	BD Biosciences	Cat#564723; RRID: AB_2738916

Anti-mouse GATA3 (TWAJ) A488	Thermo Fisher Scientific	Cat#52-9966-43; RRID:AB_2574493
Anti-mouse EOMES (Dan11mag) PE	Thermo Fisher Scientific	Cat#12-4875-82; RRID: AB_1603275

Materials or Reagents	Source	Identifier
Materials, Chemicals and Recombinant Protein		
60% lard-based HFD	Research Diets	Cat# D12492i
Collagenase IV	Worthington	Cat# LS004189
Collagenase VIII	Sigma-Aldrich	Cat# C2139
DNase I	Roche	Cat# 11284932001
Percoll	GE Healthcare	Cat# GE17-0891-01
Anti-IL-1β (01BSUR)	Novartis	MTA
Fix Buffer I	BD Biosciences	Cat# 557870
Perm Buffer III	BD Biosciences	Cat# 558050
DAPI	Biolegend	Cat# 422801
Zombie Aqua Fixable Viability Kit	Biolegend	Cat# 423102
BD Trichosel broth, modified (for Trichomonas species) – K tube	Becton Dickinson	Cat# 298323
Gibco Horse Serum, heat inactivated, New Zealand origin	Gibco	Cat# 26050088
3,3'-Dimethyl-1-butanol	Sigma-Aldrich	Cat# 183105
3,3'-Diindolylmethane	Sigma-Aldrich	Cat# D9568
Choline chloride	Sigma-Aldrich	Cat# C7527
5.0 µm Millex-SV Filter Unit	Millipore	Cat# SLSV025LS
5.0 µm MF-Millipore Membrane Filter	Millipore	Cat# SMWP04700
Antibiotics		
Penicillin-Streptomycin-Glutamine (100X)	Gibco	Cat# 10378016
Vancomycin hydrochloride from *Streptomyces orientalis*	Sigma-Aldrich	Cat# V2002
Ciprofloxacin	Sigma-Aldrich	Cat# 17850
Gentamicin solution	Sigma-Aldrich	Cat# G1272
Amphotericin B solution	Sigma-Aldrich	Cat# A2942
Critical Commercial Assays		
Mouse/Rat insulin kit	MesoScale Diagnostics	Cat# K152BZC
V-Plex custom mouse cytokine proinflammatory panel 1 mouse TNF-α, mouse IL-6 and mouse IL-1β	MesoScale Diagnostics	Cat# K15048

eBioscience Foxp3 staining Kit	Thermo Fisher Scientific	Cat# 00-5523-00
NucleoSpin RNA kit	Macherey Nagel	Cat# 740955
RNeasy Plus Universal Mini kit	Qiagen	Cat# 73404
GoScript™	Promega	Cat# A5003
GoTaq qPCR Master Mix	Promega	Cat# A4472919
QIAamp PowerFecal Pro DNA kit	Qiagen	Cat# 51804
Experimental Mouse Models		
Mouse, C57BL/6NCrl	Charles River Laboratories	RRID:IMSR_CRL:027
Mouse, B6-Nlrp3<tm1Tsc>/N	Prof M. Donath, University of Basel	N/A
Germ-Free Mouse, C57BL/6NCrl	Clean Mouse Facility, University of Bern	N/A
Software and Algorithms		
Prism 8	GraphPad Software, LLc.	https://www.graphpad.com
BD FACS Diva (version 8.0.1)	Becton Dickinson & Company (BD)	https://www.bdbiosciences.com/en-us/instruments/research-instruments/research-software/flow-cytometry-acquisition/facsdiva-software
Flow jo (version 9.9 or higher)	Becton Dickinson & Company (BD)	https://flowjo.com
R version 3.6	The R Foundation	https://www.r-project.org

Chapter 3

Experimental findings and Results

3.1 Identification of Tritrichomonad species

At the outset of my PhD project, I focused on investigating the molecular mechanisms responsible for air pollution-induced Type 2 DM in wildtype mice. However, we encountered difficulties in precipitating the diabetic phenotype as we had managed to show previously (Bosch et al. 2023). Specifically, we observed significant levels of gut inflammation in our in-house bred wild-type mice, even in the control group that had not been exposed to air pollution particles intragastrically. This presented a significant challenge because our research was built on the foundation that the gut exposed to air pollution particles would trigger a chronic inflammatory milieu within the colon and small intestine, leading to the onset of early Type 2 DM. The presence of background gut inflammation in our control mice could mask any inflammation caused by the air pollution particles, potentially skewing the results relating to insulin secretion and glucose homeostasis.

Upon reviewing the health reports for our animal facilities, we discovered that our breeding and experimental mice were infected with Tritrichomonad protists (Refer to section 1.1.5). These protozoa are typically considered commensal, non-pathogenic and are commonly found amongst the gut microbial community of rodents. Given our earlier findings linking gut inflammation to metabolic diseases and impaired glycemic control, we decided to investigate whether the colonization of *Tritrichomonas musculus* in wild-type C57BL/6N mice has a significant impact on gut immunity and, consequently, on glucose homeostasis (Rohm et al. 2022). This investigation is crucial because it sheds light on how factors beyond diet, such as infections or environmental pollutants, can influence gut immunity and susceptibility to metabolic diseases. We hypothesized that chronic colonization of *T. musculus* in wild-type C57BL/6N mice would induce a pro-inflammatory shift in gut immunity, ultimately leading to impaired glycemic control.

Results

Observation of the caecal content diluted in PBS of uncolonized and Tritrichomonas-colonized mice, when viewed under a light microscope at 40x magnification, revealed the presence of tear-dropped shaped, flagellated eukaryotic-like protists in a constant tumbling motion in the colonized-mice (Fig. 3.1a). Quantitative PCR (qPCR) analysis of the caecal content of chow- and HFD-fed C57BL/6N mice using primers against the 28s rRNA of *Tritrichomonas musculus*, confirmed the presence of these protists in the colonized-mice (Fig. 3.1b). It is noteworthy to emphasize that the relative abundance of Tritrichomonad protists experiences a substantial reduction when mice are subjected to a high-fat diet. This observation underscores the dietary and nutrient-dependent assimilation of Tritrichomonad protists into the gut microbial community, aligning with previous findings (Wei et al. 2020). Genomic DNA sequencing of the Internal Transcribed Spacer (ITS) and 18S rRNA regions confirmed that the protists were a Tritrichomonad species, which was phylogenetically indistinguishable from *T. musculus* (*T. mu*) with a high degree of homology (Fig. 3.1c).

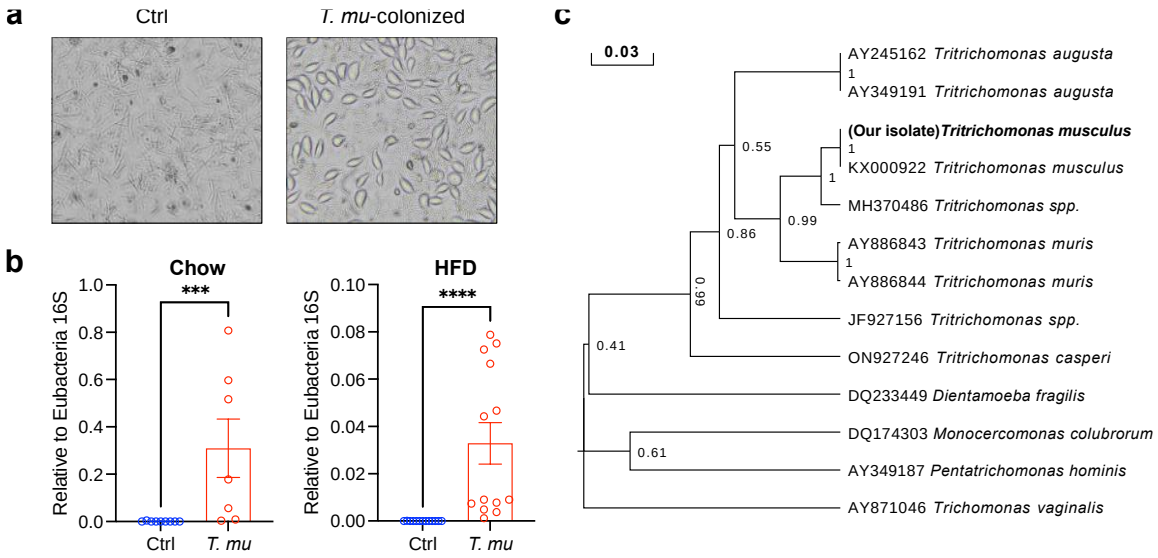


Figure 3.1. Identification of Tritrichomonas musculus in laboratory mice. ***a)*** *Caecal content of uncolonized (Ctrl) and T. mu-colonized mice when viewed under a normal light microscope at 40x magnification.* ***b)*** *Relative abundance of T. mu in caecal content of chow- and HFD-fed C57BL/6N mice (T. mu 28S rRNA relative to Eubacteria 16S rRNA) quantified by qPCR.* ***c)*** *The phylogenetic characterization of Tritrichomonad commensals was established using the conserved ITS gene, inferred by the UPGMA method supported by 1000 bootstrap replications. ITS regions were first aligned by MUSCLE using default settings and trimmed to discard non-homologous overhanging fragments. Evolutionary distances were computed using the Maximum Composite Likelihood method. This analysis involved 13 ITS nucleotide sequences, and the reference sequences downloaded from the NCBI database are labelled with the accession numbers. A total of 284 positions were included in the final dataset. Evolutionary analyses were conducted in MEGA11.*

3.2 Chronic *T. musculus* colonization in chow-fed C57BL/6N mice leads to a pro-inflammatory shift in gut immunity, and is associated with the development of insulin resistance and impaired glycemia

In C57BL/6N mice that were fed a standard chow diet, colonization with *T. mu* led to several noteworthy phenotypic effects during a 6-month follow-up period (at an age of 27-30 weeks). This included a noticeable trend towards impaired glucose tolerance, accompanied by significantly elevated levels of insulin in the bloodstream as a sign of insulin resistance, starting from 5-month post-colonization (Fig. 3.2a and 3.2b). It is important to note that the colonization of *T. mu* did not affect the food intake, body weight and the overall health of the mice. This reaffirms the notion that these protists are a commensal member of the murine gut microbiota and do not cause any significant harm to their hosts upon colonization. Additionally, an insulin tolerance test (ipITT), used to measure the sensitivity of tissues to an intraperitoneally injection of insulin, revealed a significant decrease in insulin sensitivity (Fig. 3.2c). All of these metabolic parameters are indicative of the onset of Type 2 DM in the *T. mu*-colonized mice. Furthermore, in mice colonized by *T. mu*, we observed a significant decrease in glucose-stimulated insulin secretion (GSIS) at higher glucose levels and a decreased stimulation index, indicating an impaired β-cell function (Fig. 3.2d).

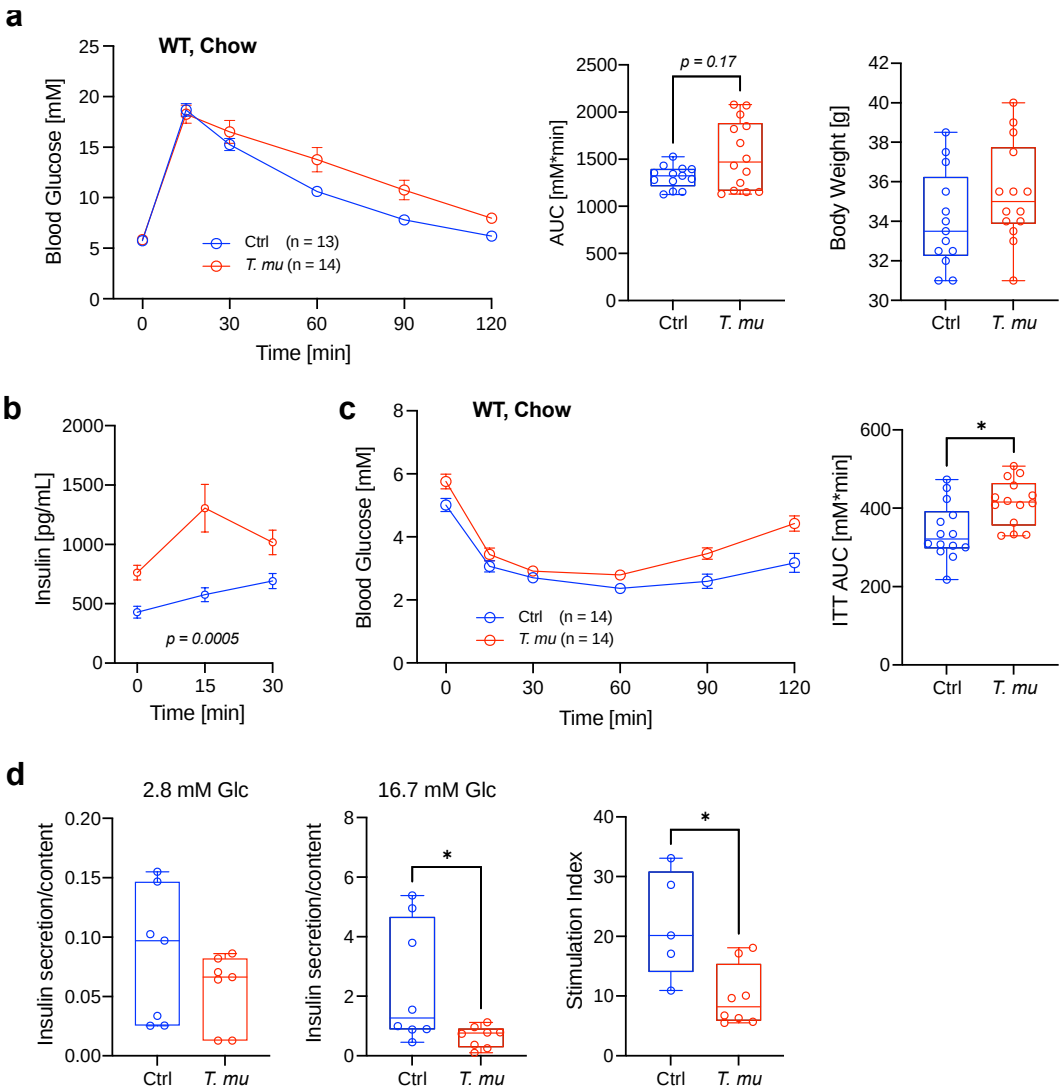


Figure 3.2. Chronic colonization of Tritrichomonas musculus in wildtype C57BL/6N mice fed on standard chow diet leads to an impaired glycemic control, elevated insulin secretion and the development of insulin resistance. ***a)*** *Intraperitoneal glucose tolerance test (ipGTT), area under the curve (AUC) and body weight of chow-fed C57BL/6N mice (Ctrl n = 13, T. mu n = 14; age = 30 weeks).* ***b)*** *Circulating insulin levels measured during the first 30 minutes of the ipGTT.* ***c)*** *Intraperitoneal insulin tolerance test (ipITT), area under the curve (AUC) (Ctrl n = 14, T. mu n = 14; age = 30 weeks).* ***d)*** *Basal and glucose-stimulated insulin secretion (GSIS) in ex vivo cultured pancreatic islets. Data is representative of one experiment with each data point representing one mouse. Statistical data are expressed as mean ± SEM. Data are representative of one* ***(d)*** *or two* ***(a, b, c)*** *independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

We aimed to characterize the immune cell populations of the gut mucosa and conducted flow cytometric analysis to assess macrophage, T-cell, and dendritic cell populations in the intestines of both *T. mu*-colonized and uncolonized wild-type mice (Fig 3.3). We observed a decrease in the anti-inflammatory $CCR2^-$ macrophage population in the small intestine. Further subdividing the pro-inflammatory $CCR2^+$ macrophage population into the P1 to P3 subpopulations revealed significant increases in the $Ly6C^{high}MHCII^{low}$ P1

and P2 population (Fig. 3.3a). These subpopulations represent the newly recruited infiltrating monocytes into the intestinal lamina propria (Rohm et al. 2022). In the colon of the *T. mu*-colonized mice, there is a similar trend towards an increased P2 macrophage subpopulation (Supplementary Fig. 5.2). The most notable changes in the colonic immune cells in the chow-fed colonized mice are the reduction in $CD103^+$ single-positive and double-positive dendritic cells (DCs) indicating the potential of migration towards the draining lymph nodes and the activation of the adaptive immune response (Fig. 3.3b). This was supported by an increased presence of colonic $CD4^+$ and T-regulatory cells (T_{reg}) upon *T. musculus* infection (Fig. 3.3c).

In summation, in wild-type C57BL/6N fed a standard chow diet, colonization with *T. musculus* instigated an inflammatory milieu within the lamina propria of the gut, which was associated with insulin resistance, as evidenced by hyperinsulinemia and impaired insulin tolerance.

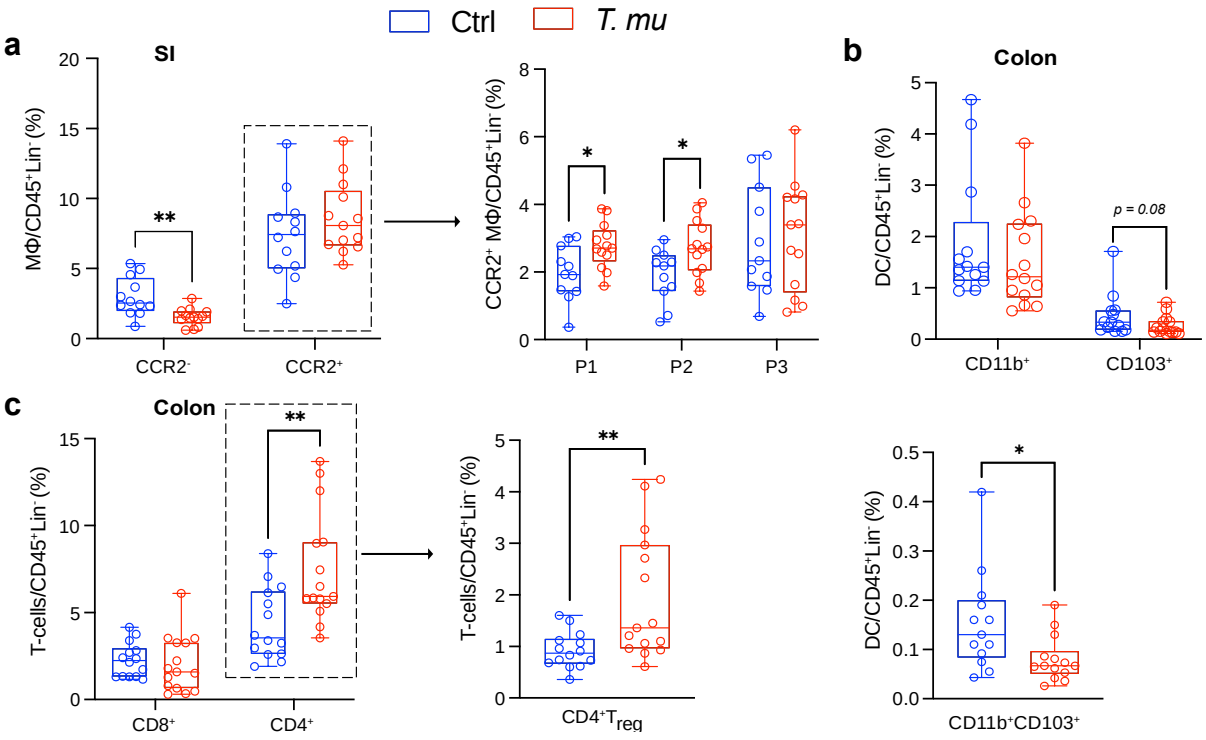


Figure 3.3. Colonization of Tritrichomonas musculus in chow-fed wildtype C57BL/6N mice led to increased pro-inflammatory response in colon and distal small intestine. ***a)*** *Inflammatory $CCR2^+$ macrophages in distal small intestinal tissue and the further subclass distributions of $Ly6C^{high}MHCII^{low}$ P1-P3 macrophage subpopulations.* ***b)*** *Frequencies of colonic dendritic cells (DC) ($CD64^-MHCII^+CD11c^+$) based on the expressions of CD11b and CD103.* ***c)*** *Frequencies of colonic $CD3^+$ T-cell populations. T-regulatory (T_{reg}) cells = $CD4^+CD25^+FoxP3^+$. Data are representative of two* ***(a, b, c)*** *independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

3.3 High-fat diet (HFD) exacerbates the gut inflammation and glucose intolerance induced by *Tritrichomonas musculus*

Following the observations of the metabolic and immunological effects of *T. mu*-colonization on C57BL/6N mice fed on a standard chow diet, we decided to substitute the diet to a 58 kcal% high-fat diet (HFD) in order to precipitate the metabolic phenotype at an earlier timepoint. HFD-fed mice colonized with *T. mu* developed severe glucose intolerance and hyperinsulinemia in just one month, and these effects persisted for up to three months (Fig. 3.4a and b). As with the chow-fed model, there was no significant differences in body weights. However, the impaired insulin sensitivity was lost when the mice were put on the HFD (Fig. 3.4c). This could be attributed to the possibility that the impact of *T. mu*-colonization on insulin sensitivity is obscured by the pre-existing detrimental effects of the HFD. The *ex vivo* GSIS experiments on isolated pancreatic β-cells revealed a significant defect in insulin secretion, primarily due to increased insulin release at basal glucose levels (Fig 3.4d).

Flow cytometry analysis of the intestinal lamina propria revealed a significantly elevated pro-inflammatory shift of immune cells in *T. mu*-colonized mice on HFD (Fig. 3.5). An increased innate immune response was observed in the small intestine of the *T. mu*-colonized mice, characterized by an increase in the inflammatory $CCR2^+$ intestinal macrophages (Fig 3.5a). Additionally, akin to the inflammatory shift observed in chow-fed *T. mu*-colonized mice, *T. musculus* colonization led to increased frequencies of the inflammatory P1 and P2 macrophage subpopulations in the HFD-fed mice. Furthermore, colonization of *T. musculus* led to significantly increased frequencies in the $CD4^+$ T-cell and T-regulatory cell (T_{reg}) response in colonic and distal small intestinal tissue (Fig 3.5b and c). Analysis of the innate lymphoid cells (ILCs) of the gut showed decreased levels of $GATA3^+$ ILC2 cells in the colon but not the small intestine (Fig 3.5d). Infiltration of inflammatory adipose tissue macrophages is a hallmark of chronic inflammation in metabolic syndrome. Interestingly, the colonization of *T. musculus* in mice on a HFD resulted in an increased pro-inflammatory M1 subpopulation and a diminished anti-inflammatory M2 subpopulation of adipose tissue macrophages, signifying the onset of metabolic disease (Fig. 3.5e, Appari et al. 2018).

In sum, wild-type C57BL/6N mice colonized with *T. musculus* when fed on a HFD developed severe glucose intolerance, hyperinsulinemia and a dysfunctional β-cell function. Colonization led to elevated innate and adaptive immune responses in the intestinal lamina propria as evidenced by the increased inflammatory macrophage and $CD4^+$ T-cell response, respectively.

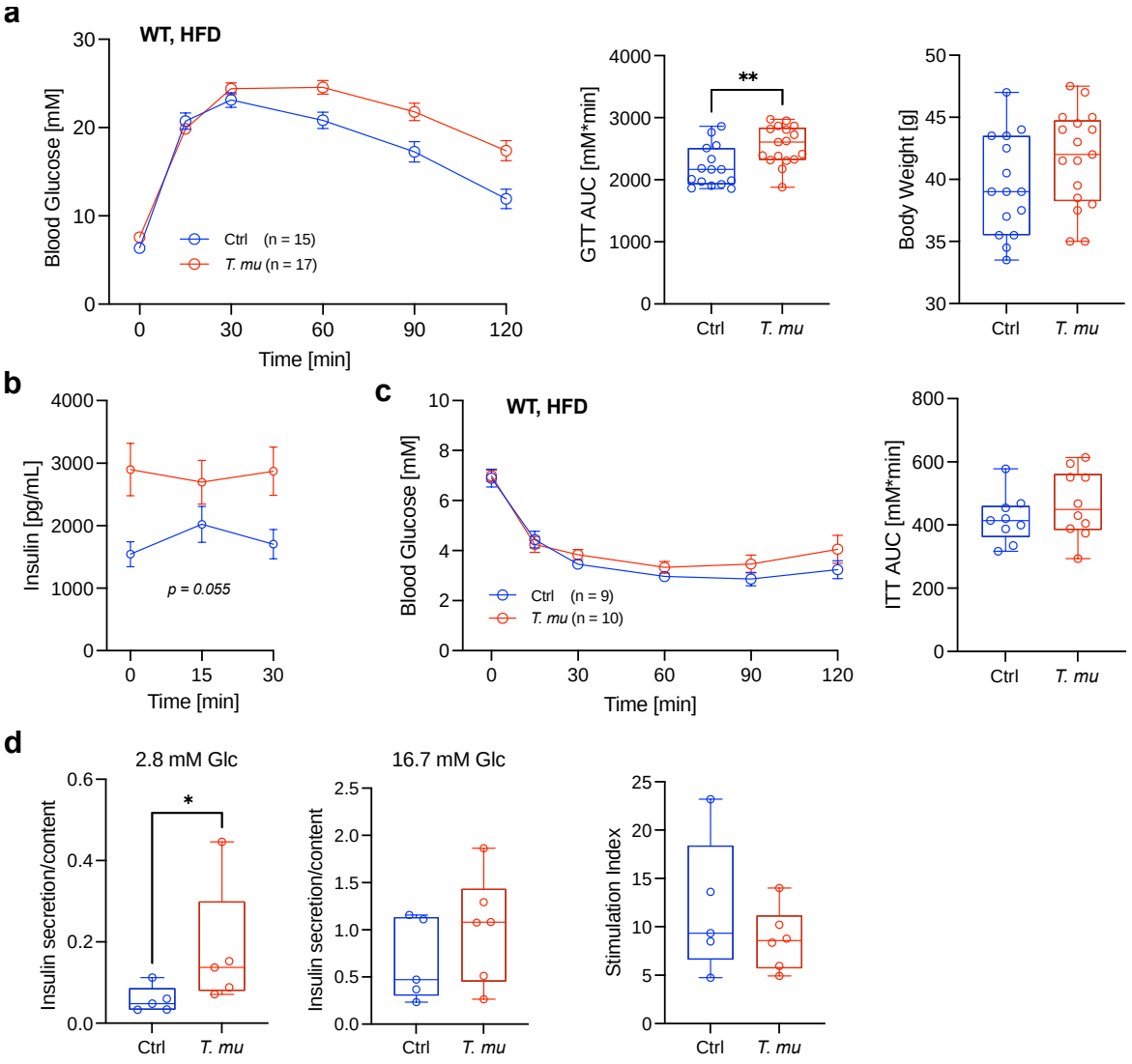


Figure 3.4. Chronic colonization of Tritrichomonas musculus in wildtype C57BL/6N mice fed on a 58 kcal% high-fat diet for 3 months led to a significantly impaired glycemic control, elevated insulin secretion and a dysfunctional β-cell function. ***a)*** *Intraperitoneal glucose tolerance test (ipGTT), area under the curve (AUC) and body weight of HFD-fed C57BL/6N mice (Ctrl n = 15, T. mu n = 17; age = 20-21 weeks).* ***b)*** *Circulating insulin levels measured during the first 30 minutes of the ipGTT.* ***c)*** *Intraperitoneal insulin tolerance test (ipITT), area under the curve (AUC) (Ctrl n = 9, T. mu n = 10; age = 21-23 weeks).* ***d)*** *Basal and glucose-stimulated insulin secretion (GSIS) in ex vivo cultured pancreatic islets. Data is representative of two experiments with each data point representing one mouse. Statistical data are expressed as mean ± SEM. Data are representative of four* ***(a)****, four* ***(b)****, two* ***(c)*** *and two* ***(d)*** *independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

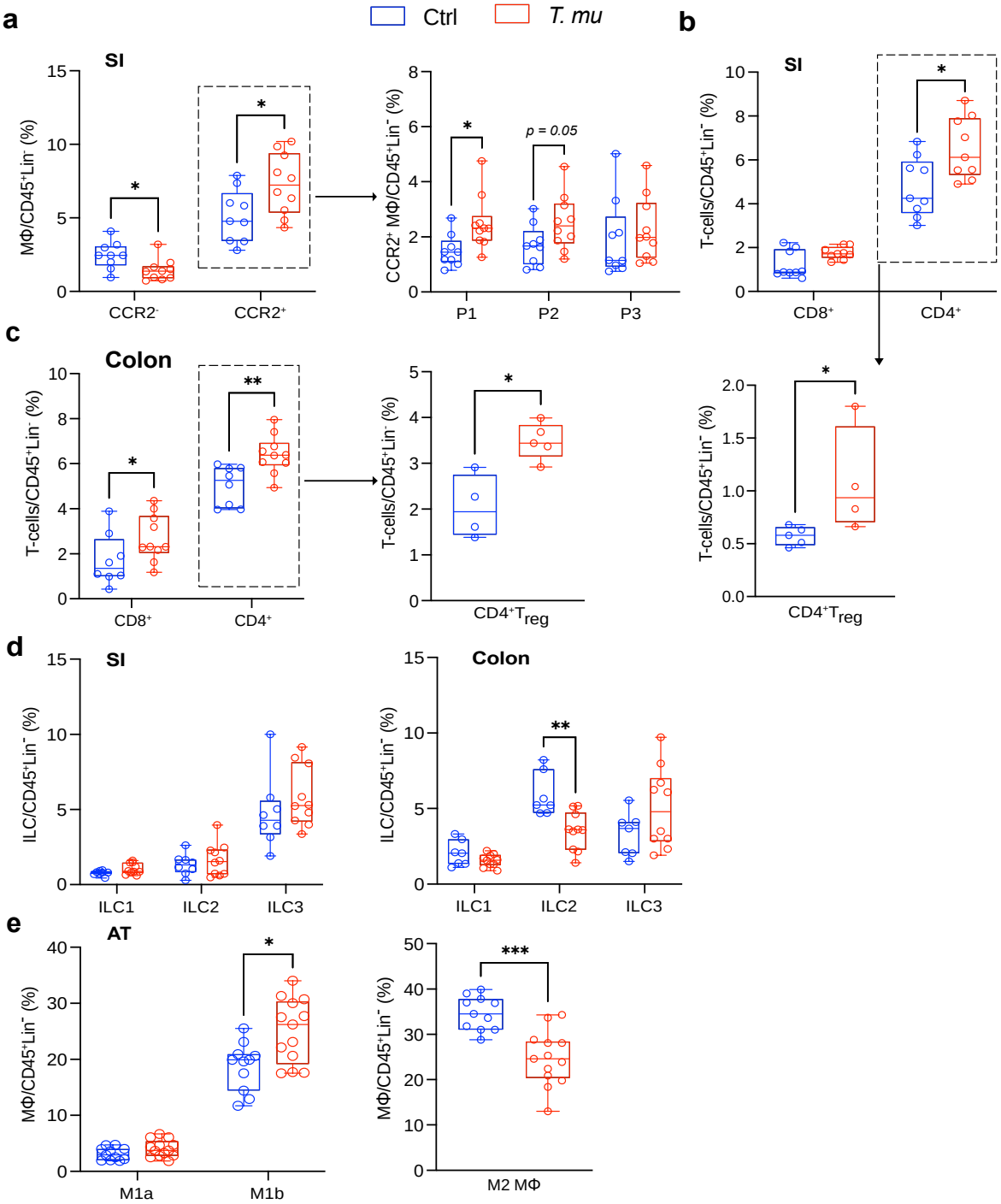


Figure 3.5. Colonization of Tritrichomonas musculus in HFD-fed wildtype C57BL/6N mice led to significant elevated pro-inflammatory response in colon, distal small intestine and epididymal adipose tissue. ***a****) Inflammatory CCR2$^+$ macrophages in distal small intestinal tissue and the further subclass distributions of Ly6C^{high}MHCII^{low} P1-P3 macrophage subpopulations.* ***b****) Frequencies of small intestinal CD3$^+$ T-cell populations. T-regulatory (T_{reg}) cells = CD4$^+$CD25$^+$FoxP3$^+$.* ***c****) Frequencies of colonic CD3$^+$ T-cell populations.* ***d****) Frequencies of distal small intestinal and colonic innate lymphoid cells (ILCs). ILC1 = CD335$^+$Eomes$^-$, ILC2 = GATA3$^+$, ILC3 = RORγt$^+$.* ***e****) Frequencies of epididymal adipose tissue macrophages and the pro-inflammatory (M1a, M1b) and anti-inflammatory (M2) subpopulations. Data are representative of two independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

3.4 Colonization with *Tritrichomonas musculus* instigates the activation of the Nlrp3 inflammasome, leading to IL-1β and pro-inflammatory cytokine secretion

Next, we sought to confirm the increased inflammation observed in *T. mu*-colonized C57BL/6N mice via molecular biology techniques. Using an *ex vivo* culture of colonic tissue, we aim to provide a more accurate representation of the *in vivo* host defense response against *T. musculus* colonization. The technique enables the various cell populations in the intestine to remain metabolically active in culture for at least 48 hours. In line with what was observed previously by Chiaranunt et al. 2022 and others, there was an increased secretion of IL-1β but not IL-6 and TNF-α in the colon tissue from chow-fed *T. mu*-colonized mice (Fig. 3.6a). To determine whether *T. musculus* colonization mediates a local or systemic inflammation, we measured the levels of pro-inflammatory cytokines in the blood sera of both chow- and HFD-fed C57BL/6N mice. Intriguingly, the level of IL-1β was decreased in the circulation of *T. mu*-colonized chow-fed mice, while no difference was detected in the HFD-fed mice (Fig. 3.6b). The levels of IL-5, a pro-inflammatory cytokine secreted by both hematopoietic and non-hemopoietic cells, was significantly increased in the circulation of chow-fed colonized mice. The opposite was observed when the colonized mice were put on HFD. A similar divergent pattern was observed for the levels of IL-33, a pro-inflammatory cytokine secreted by epithelial and endothelial cells. We noted a decrease in levels following T. musculus colonization in mice on a standard chow diet, while an increase was observed in mice fed a high-fat diet (Fig 3.6b). These findings suggest that when C57BL/6N mice are colonized with the commensal protozoan *T. musculus*, it triggers a localized activation of the innate immune system in the gut with varying systemic responses depending on the diet of the mice.

The increased inflammatory response in the intestinal tissue of HFD-fed *T. mu*-colonized mice was confirmed via qRT-PCR using primers against selected Nlrp3-related markers, inflammatory genes and macrophage markers (Fig. 3.7). Across both colonic and small intestinal tissue, we observed a higher expression of the macrophage-related markers, CD68 and Ly6C in *T. mu*-colonized mice. Moreover, as expected, the Nlrp3 gene was expressed higher in the *T. mu*-colonized mice, while IL-1β and IL-18 had significantly higher expression in the small intestine. Lastly, despite observing no increase in the secretion of TNF-α in the *ex vivo* colon explants, there is a significantly higher expression of this gene in the colon and small intestine of HFD-fed *T. mu*-colonized mice.

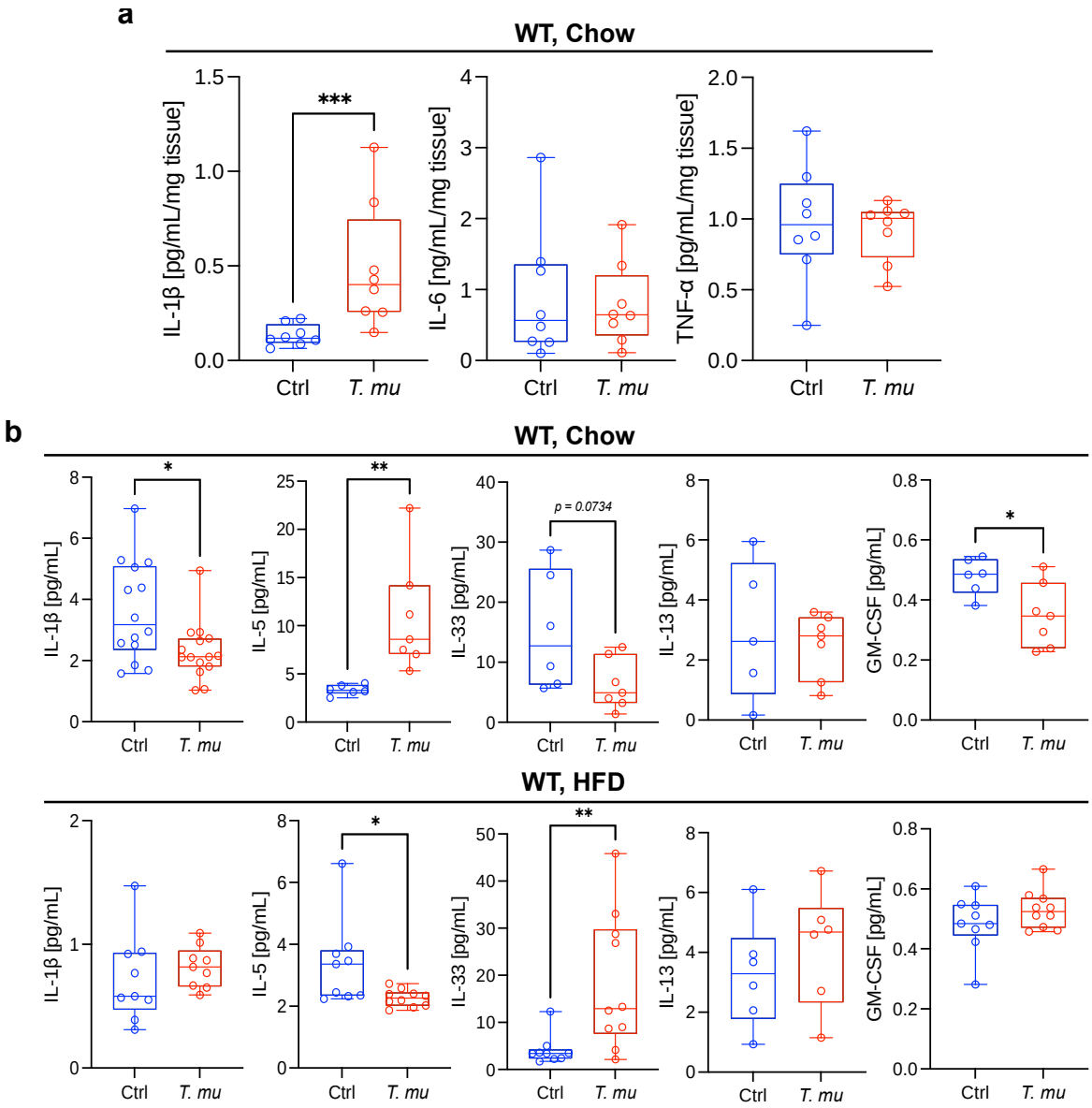


Figure 3.6. Colonization with T. musculus mediates an increased local, but not systemic, secretion of IL-1β in the colon. ***a)*** *Ex vivo colon explant culture of chow-fed uncolonized or T. mu-colonized C57BL/6N mice. Supernatants were collected after an overnight culture and the secretion of IL-1β, IL-6 and TNF-α measured by ELISA.* ***b)*** *Measurement of circulating cytokines in the blood sera collected from both chow- and HFD-fed uncolonized and T. mu-colonized C57BL/6N mice. Data are representative of one (**a**) and two (**b**) independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

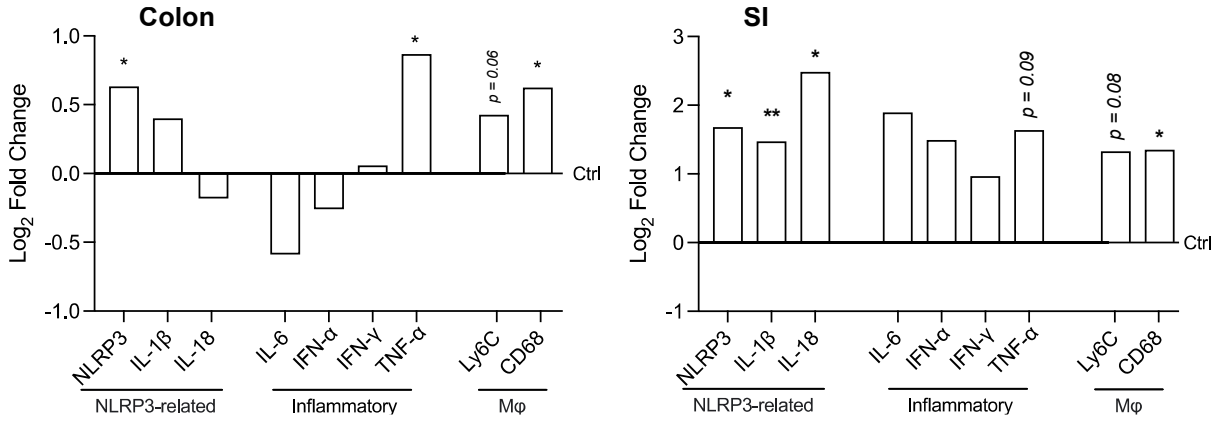


Figure 3.7. qRT-PCR analysis on colon and small intestinal tissue of uncolonized and T. mu-colonized HFD-fed C57BL/6N mice*. The expression of Nlrp3-related, pro-inflammatory and macrophage-related genes were analyzed. Data represents the relative log₂ Fold Change of the T. mu-colonized samples relative to the uncolonized (Ctrl) samples. The -ddCt method was used for the analysis. Data are representative of two independent experiments (n = 11). Student's two-tailed distribution t-test was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

3.5 The Nlrp3 inflammasome is a requisite for the impaired metabolic phenotype and pro-inflammatory shift instigated by *Tritrichomonas musculus* colonization

The elevated levels of IL-1β released from the colon explants and the observed changes in mucosal immune cell populations in *T. mu*-colonized mice indicate a substantial involvement of inflammatory processes in the development of glucose intolerance and hyperinsulinemia. To determine the importance of the Nlrp3 inflammasome in eliciting the impaired metabolic phenotype observed in HFD-fed colonized C57BL/6N mice, we colonized a whole-body Nlrp3 knock-out ($Nlrp3^{-/-}$) mouse model with *Tritrichomonas musculus* (Fig. 3.8a and b). Following a 3-month HFD feeding period, the *T. mu*-colonized $Nlrp3^{-/-}$ mice did not develop glucose intolerance and hyperinsulinemia (Fig. 3.8c and d). Interestingly despite no difference in glycemic control, there was an almost significant trend towards an impaired insulin sensitivity in *T. mu*-colonized $Nlrp3^{-/-}$ mice (Fig. 3.8e). Insulin release remained unchanged at basal glucose levels in cultured pancreatic β-cells from *T. mu*-colonized $Nlrp3^{-/-}$ mice, in contrast to the previously observed increase in insulin release in *T. mu*-colonized wild-type mice on HFD (Fig. 3.4d).

Results

The assessment of the mucosal immune cells of *T. mu*-colonized Nlrp3$^{-/-}$ mice revealed several interesting findings (Fig. 3.9). In the small intestine of colonized mice, there was no longer an increase in the P1 and P2 inflammatory macrophage subpopulation upon colonization (Fig. 3.9a). Conversely, there was a significant expansion in the frequencies of the P3 subpopulation, characterized as Ly6C^{low}MHCII^{high} resident macrophages. However, we observed an increase in the frequency of inflammatory P2 macrophage subpopulation in the colon of the *T. mu*-colonized mice, suggesting that the Nlrp3 inflammasome is not the only driver of the inflammation (Fig. 3.9d). Moreover, there was no increase in the CD4^{+} T-cell response in the small intestine and colon of the *T. mu*-colonized mice (Fig. 3.9b and e). Similar to what was found in the colon of wild-type *T. mu*-colonized mice (Fig. 3.5d), there is a notable decrease in the levels of colonic ILC2 population upon colonization in the Nlrp3$^{-/-}$ mice. In summary, the activation of the Nlrp3 inflammasome plays an important role in *T. mu*-induced gut inflammation and glucose intolerance. This is evident from the fact that Nlrp3$^{-/-}$ mice were protected from the inflammatory and metabolic impairments instigated by *T. musculus* colonization.

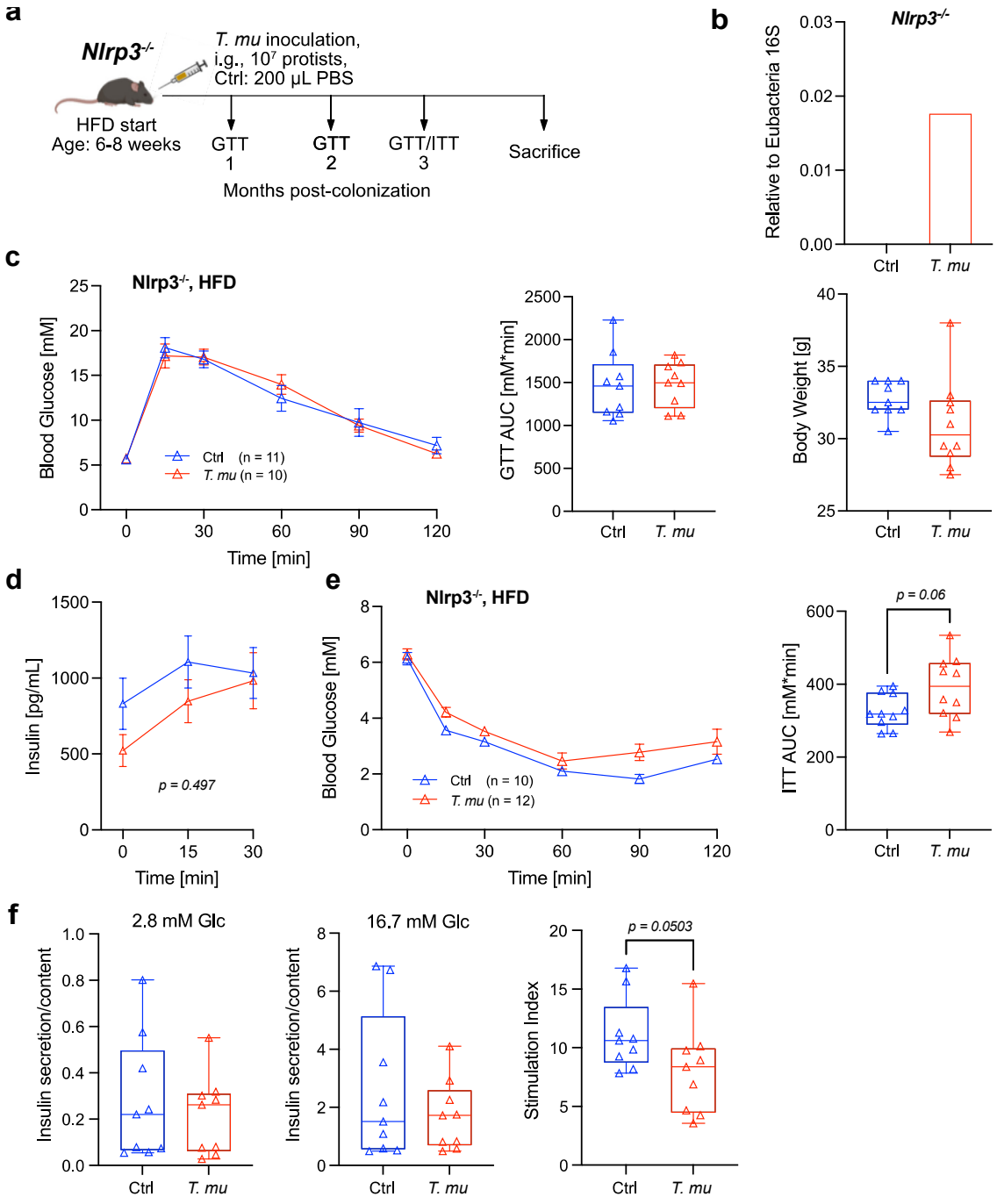


Figure 3.8. The Nlrp3 inflammasome is required to induce the impaired metabolic phenotype in HFD-fed T. musculus-colonized mice. ***a)*** *Schematic illustration of the whole-body Nlrp3$^{-/-}$ model. The mice were inoculated with approx. 10^{7} T. mu protists. Following 1 week colonization period, the mice were fed on HFD for 3 months prior to sacrifice.* ***b)*** *Relative abundance of T. mu in caecal content of the HFD-fed whole-body Nlrp3$^{-/-}$ C57BL/6N mice (T. mu 28S rRNA relative to Eubacteria 16S rRNA) quantified by qPCR.* ***c)*** *Intraperitoneal glucose tolerance test (ipGTT), area under the curve (AUC) and body weight of HFD-fed whole-body Nlrp3$^{-/-}$ C57BL/6N mice (Ctrl n = 11, T. mu n = 10; age = 20-21 weeks).* ***d)*** *Circulating insulin levels measured during the first 30 minutes of the ipGTT.* ***e)*** *Intraperitoneal insulin tolerance test (ipITT), area under the curve (AUC) (Ctrl n = 10, T. mu n = 12; age = 21-22 weeks).* ***f)*** *Basal and glucose-stimulated insulin secretion (GSIS) in ex vivo cultured pancreatic islets. Data is representative of two experiments with each data point representing one mouse. Statistical data are expressed as mean ± SEM. Data are representative of three independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

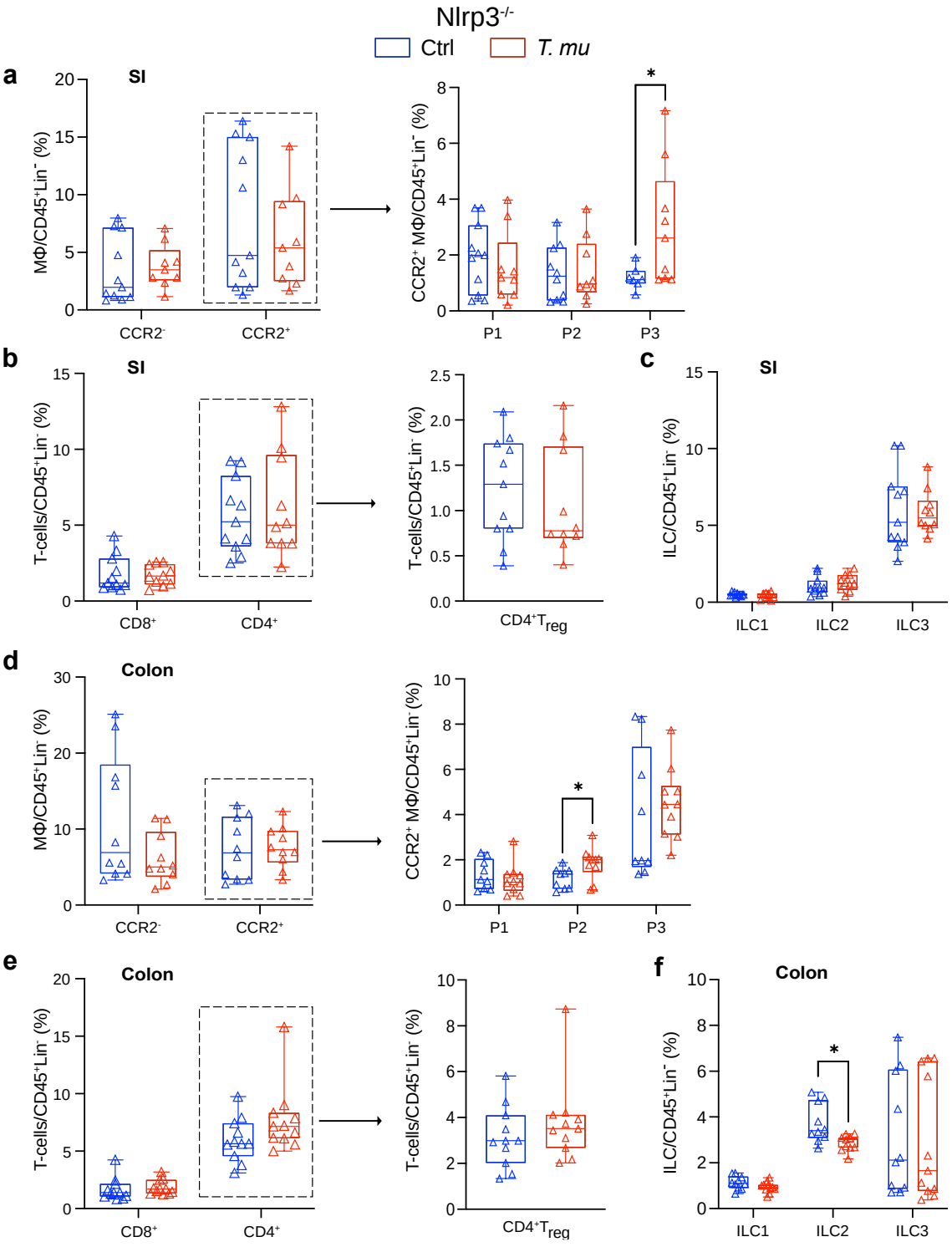


Figure 3.9. The pro-inflammatory shift in mucosal immune cells instigated by Tritrichomonas musculus colonization is dependent on the Nlrp3 inflammasome. ***a and d)*** *Inflammatory CCR2$^+$ macrophages in distal small intestinal tissue* ***(a)*** *and colonic* ***(d)*** *and the further subclass distributions of Ly6C^{high}MHCII^{low} P1-P3 macrophage subpopulations.* ***b and e)*** *Frequencies of small intestinal* ***(b)*** *and colonic* ***(e)*** *CD3$^+$ T-cell populations. T-regulatory (T_{reg}) cells = CD4$^+$CD25$^+$FoxP3$^+$.* ***c and f)*** *Frequencies of distal small intestinal* ***(c)*** *and colonic* ***(f)*** *innate lymphoid cells (ILCs). ILC1 = CD335$^+$Eomes$^-$, ILC2 = GATA3$^+$, ILC3 = RORγt$^+$. Data are representative of three independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

3.6 Pharmacological inhibition of IL-1β reverses the gut inflammation and glucose tolerance induced by *T. musculus* colonization

Previous reports have indicated the importance of macrophage-derived IL-1β in inducing the secretion of insulin and impacting glucose homeostasis (Dror et al. 2017). To investigate whether the involvement of the Nlrp3 inflammasome in *T. mu*-induced glucose intolerance is driven by the release of IL-1β, we intraperitoneally administered a monoclonal antibody against IL-1β to *T. mu*-colonized wild-type C57BL/6N mice on HFD. As our results showed a greater disruption in glucose homeostasis and hyperinsulinemia after 3 months of HFD-feeding in *T. mu*-colonized mice, we began administering anti-IL-1β antibody after 2 months of HFD-feeding for 4 weeks (Fig. 3.10a). The depletion of IL-1β from the antibody treatment was confirmed by measuring IL-1β levels in the circulation of treated mice (Fig. 3.10b). Inhibition of IL-1β in HFD-fed *T. mu*-colonized mice did not result in the amelioration of glucose intolerance as compared to the vehicle-treated controls (Fig. 3.10c). Conversely, the antibody treatment led to a rescue in hyperinsulinemia as shown by the significant reduction in secreted insulin levels (Fig. 3.10d). The decreased insulin secretion did not correspond with an improvement in the insulin sensitivity of anti-IL-1β treated *T. mu*-colonized mice (Fig. 3.10e). The *in vivo* inhibition of IL-1β managed to result in a rescue of pancreatic β-cell dysfunction, as observed in the HFD-fed *T. mu*-colonized mice (Fig. 3.10f & Fig. 3.4d).

The examination of immune cell populations following pharmacological inhibition of IL-1β did not yield significant alterations in mucosal immunity, with a few exceptions (Fig. 3.11). A key aspect of *T. mu*-mediated inflammatory shift of mucosal immune cells is the elevated pro-inflammatory $CCR2^+$ P2 macrophage subpopulations of the small intestine (Fig. 3.3a and Fig. 3.5a). The treatment with anti-IL-1β resulted in a significant reduction in this pro-inflammatory P2 macrophage population (Fig. 3.11a). Another consistent mucosal immunological shift from *T. musculus* colonization is the expansion of the adaptive immune response as measured by increased $CD4^+$ T-cells and T_{reg} cells (Fig. 3.3c and Fig. 3.5b and c). A similar shift towards an increased T-cell response was still detected in the small intestine and colon from the IL-1β treatment in *T. mu*-colonized mice (Fig. 3.11b and e). These results suggest that the secretion of IL-1β stems primarily from the pro-inflammatory macrophage population of the lamina propria and the inhibition of this cytokine can provoke a “rescue” of the inflammatory shift precipitated by *T. musculus*.

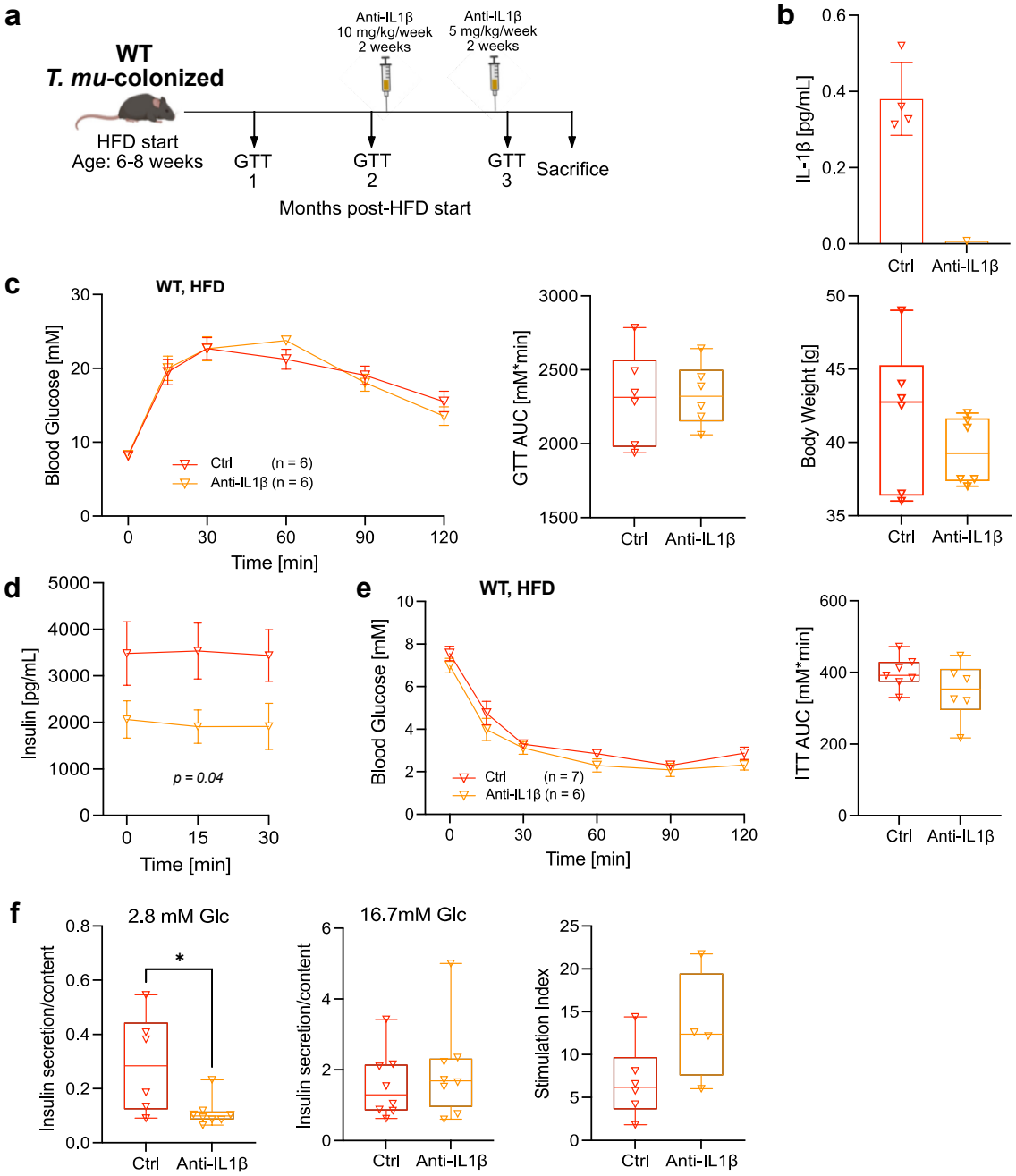


Figure 3.10. Pharmacological inhibition of IL1β in HFD-fed T. mu-colonized wild-type mice rescued the mice from hyperinsulinemia. ***a)*** *Schematic illustration of the in vivo administration of anti-IL1β model. T. mu-colonized C57BL/6N mice were put on HFD for 2 months prior to administration of 10 mg/kg/week anti-IL1β for 2 weeks followed by 5 mg/kg/week anti-IL1β for the final 2 weeks.* ***b)*** *IL-1β levels in the blood sera of control and antibody-treated mice.* ***c)*** *Intraperitoneal glucose tolerance test (ipGTT), area under the curve (AUC) and body weight of HFD-fed T. mu-colonized C57BL/6N mice (Ctrl n = 6, Anti-IL1β n = 6; age = 21-22 weeks).* ***d)*** *Circulating insulin levels measured during the first 30 minutes of the ipGTT.* ***e)*** *Intraperitoneal insulin tolerance test (ipITT), area under the curve (AUC) (Ctrl n = 7, Anti-IL1β n = 16; age = 22 weeks).* ***f)*** *Basal and glucose-stimulated insulin secretion (GSIS) in ex vivo cultured pancreatic islets. Data is representative of one experiment with each data point representing one mouse. Statistical data are expressed as mean ± SEM. Data are representative of three independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

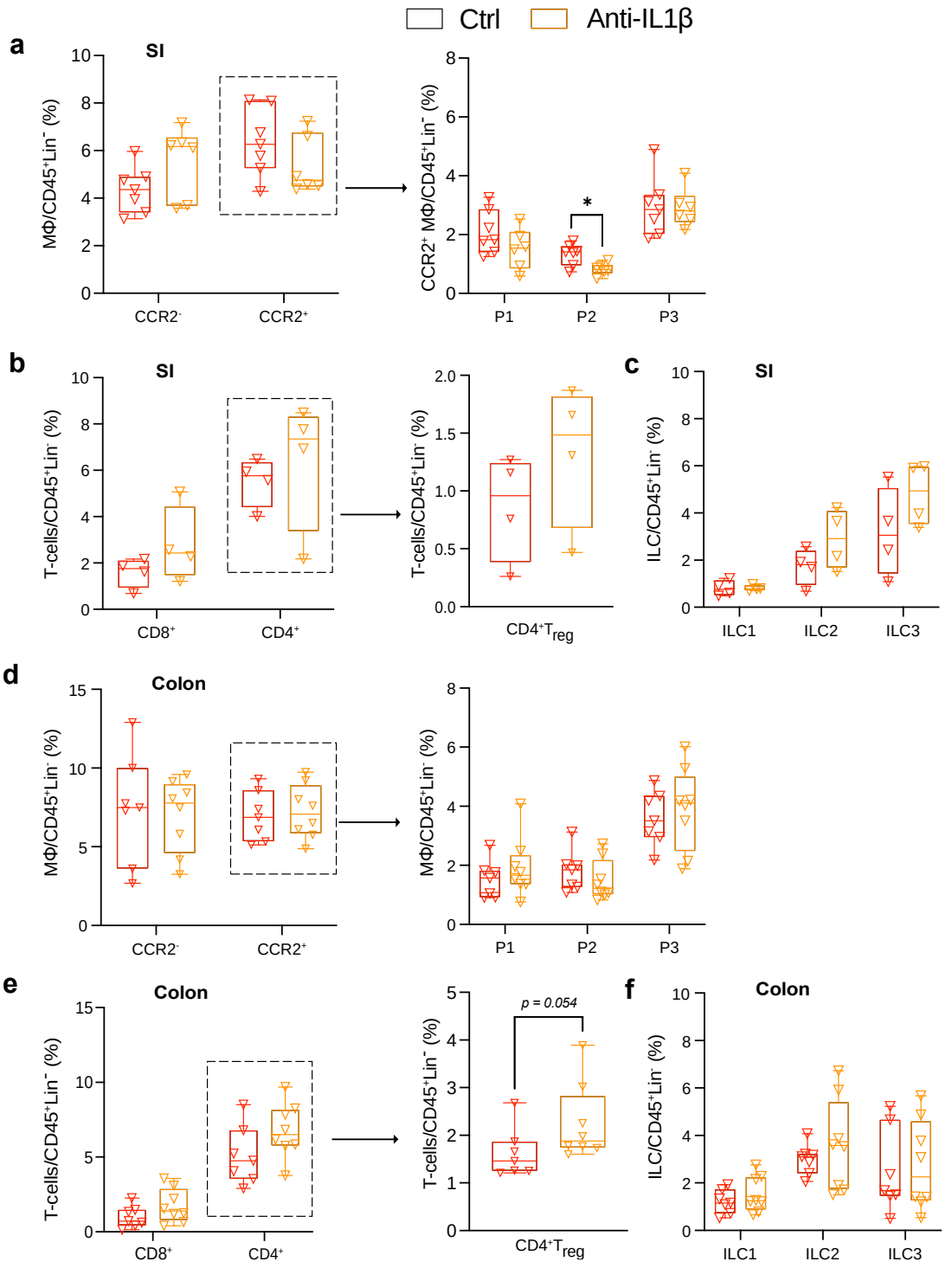


Figure 3.11. Pharmacological inhibition of IL1β reversed the gut inflammation elicited by Tritrichomonas musculus colonization. ***a and d)*** *Inflammatory CCR2$^+$ macrophages in distal small intestinal tissue* ***(a)*** *and colonic* ***(d)*** *and the further subclass distributions of Ly6C^{high}MHCII^{low} P1-P3 macrophage subpopulations.* ***b and e)*** *Frequencies of small intestinal* ***(b)*** *and colonic* ***(e)*** *CD3$^+$ T-cell populations. T-regulatory (T_{reg}) cells = CD4$^+$CD25$^+$FoxP3$^+$.* ***c and f)*** *Frequencies of distal small intestinal* ***(c)*** *and colonic* ***(f)*** *innate lymphoid cells (ILCs). ILC1 = CD335$^+$Eomes$^-$, ILC2 = GATA3$^+$, ILC3 = RORγt$^+$. Data are representative of three independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

3.7 Colonization of *Tritrichomonas musculus* significantly alters the composition of the gut microbiota and is dependent on dietary nutrients

As alluded to in the introduction, a healthy or dysbiotic composition of gut microbiota plays an essential role in regulating the immune system, affecting the immunity of tissues locally as well as distally throughout the body (Refer to Section 1.3). Despite the identification of several enteric Tritrichomonad species, our understanding of their genetic relationships and functional diversity remains limited. This lack of knowledge makes it challenging to comprehend their impact on the diversity of the microbiome and the overall health of the host. We aimed to bridge this knowledge gap by performing 16S ribosomal gene sequencing to analyze the gut microbiota composition in the caeca of *T. mu*-colonized wild-type C57BL/6N mice on both chow and HFD (Fig. 3.12). We observed distinct microbial community shifts upon *T. musculus* colonization in a dietary nutrient-dependent manner as shown by the Principal Coordinate Analysis (PCoA) plot, which summarizes 3243 Amplicon Sequence Variants (ASVs) into two principal components. The samples clustered together according to diet and *T. musculus* colonization with PC1 separating the chow- and HFD-fed samples (45.8% variance) and PC2 separating based on the colonization status (20.3% variance) (Fig. 3.12a). The alpha diversity indices at the family level provided insights into the richness and evenness of bacterial communities between uncolonized and *T. mu*-colonized mice and were primarily influenced by dietary factors (Fig. 3.12b). We observed a trend towards an increased bacterial diversity upon *T. mu*-colonization in the chow-fed mice, with a significant increase at the Inverse Simpson index. However, *T. mu*-colonization did not significantly impact bacterial richness in HFD-fed mice. This data suggests that the nutrient composition of the diet is paramount to the symbiotic relationship between *T. musculus* and various members of the gut microbial community.

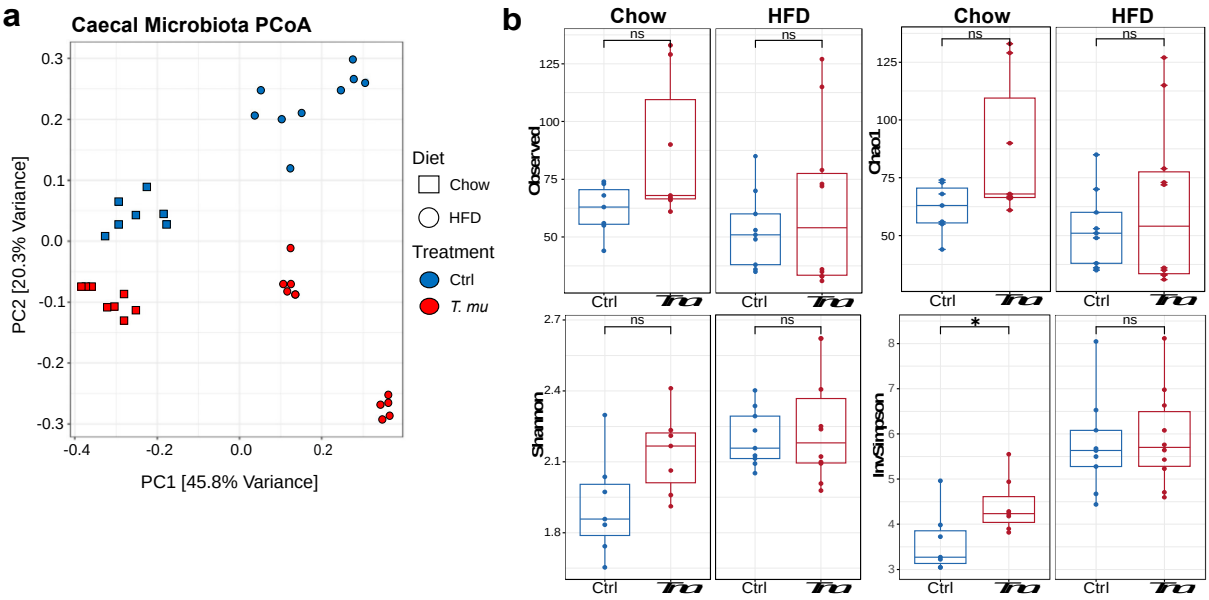


Figure 3.12. The composition of the gut microbiota is significantly altered in the caeca of Tritrichomonas musculus-colonized mice. ***a)*** *Principal coordinate analysis (PCoA) plot based on the Amplicon Sequence Variant (ASV) cluster abundances from the sequencing of caecal bacterial 16S rRNA.* ***b)*** *The alpha-diversity at the family level of commensal bacteria composition, as assessed by the Observed, Chao1, Shannon and inverse Simpson indices. Data are representative of two independent chow- and HFD-fed experiments. Each datapoint in the PcoA* ***(a)*** *plot represents an individual experimental mouse. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

At the family level, there were considerable shifts in a number of key microbial communities from the diet and *T. mu*-colonization (Fig. 3.13a). Most notably, *T. mu* colonization led to a significant depletion in the *Akkermansiaceae* family across both chow and HFD diet. The abundances of the *Bacteroidaceae* and *Desulfovibrionaceae* families were both significantly expanded upon *T. musculus* colonization only in the HFD-fed mice (Fig. 3.13b). As alluded to in the introduction, both the *Akkermansia* and *Bacteroides* family of commensal bacteria play crucial roles in the development of Type 2 DM, with decreased and increased levels of *Akkermansia* and *Bacteroides*, respectively, directly correlating with Type 2 DM incidence. The qPCR analysis using primers against *Akkermansia muciniphila* in the caecal content of HFD-fed showed similar decreases in the abundance of *A. muciniphila* upon *T. mu*-colonization (Fig. 3.13c).

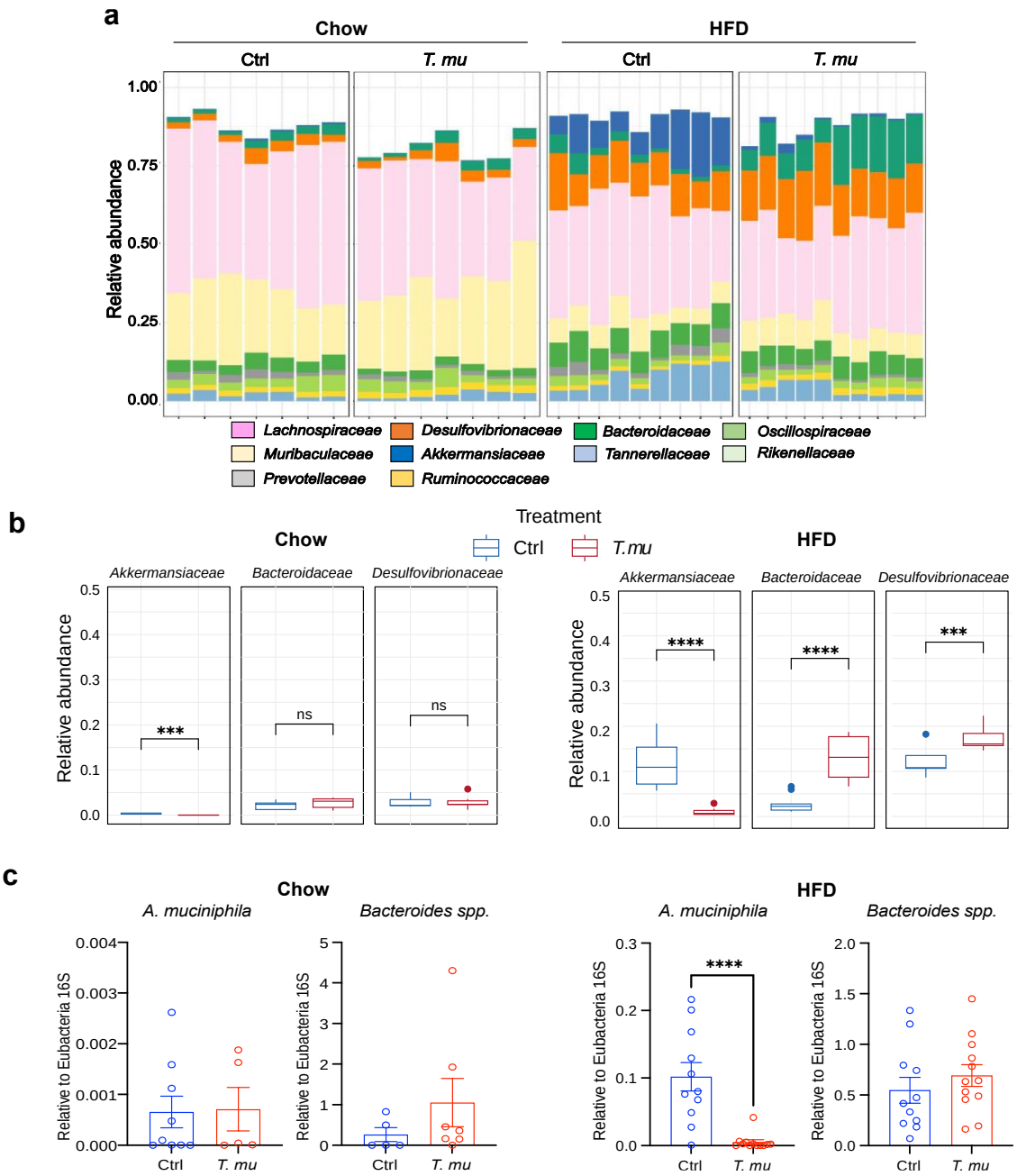


Figure 3.13. Tritrichomonas musculus colonization instigate substantial shifts in the abundances of key commensal bacterial community. ***a)*** *Relative abundances of the top 10 most abundant bacterial families that are perturbed by diet and T. mu colonization.* ***b)*** *Boxplots representing the relative abundances of the three most significantly altered bacterial family members.* ***c)*** *qPCR analysis of Akkermansia muciniphila and Bacteroides spp. from the caecal content of chow- and HFD-fed uncolonized and T. mu-colonized mice. Data are representative of two independent chow- and HFD-fed experiments* ***(a, b, c)****. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

3.8 Choline secreted by *Tritrichomonas musculus'* metabolism is a key driver of the impaired metabolic phenotype upon protozoan colonization

Our findings from the microbiota sequencing analysis showed significant expansion in the relative abundances of several key choline-utilizing bacteria in the caeca of HFD-fed *T. mu*-colonized mice (Fig. 3.13b, Supplementary Fig. 5.8). *T. musculus* colonization precipitated the growth of the *Proteobacteria*, *Desulfovibrio*, as well as the *Firmicutes* bacteria*, Lachnoclostridium*, all of which are capable of producing the choline-derivative, trimethylamine (TMA) (Fennema et al. 2016). As referenced earlier in the introduction (Refer to Section 1.3.4), a recent study by Kou et al. found choline secreted by *T. musculis* infection to be a chief instigator in enhancing hepatic gluconeogenesis. The authors found that *T. musculis*-derived choline requires further processing by choline-utilizing bacteria into TMA and subsequently trimethyl N-amine oxide (TMAO) in the liver to facilitate the observed metabolic phenotype. Thus, we sought to delve deeper into the metabolic and immunological phenotype brought about by administering free choline in the drinking water of HFD-fed uncolonized C57BL/6N mice.

We first measured the levels of choline and TMAO in the blood sera of chow- and HFD-fed uncolonized and *T. mu*-colonized C57BL/6N mice. We observed significantly elevated levels of choline in the *T. mu*-colonized mice across both diets, while TMAO was only increased in the chow-fed colonized mice (Fig. 3.14a). Subsequently, we assessed the expression of choline-related genes in intestinal samples from HFD-fed uncolonized and *T. mu*-colonized mice by qRT-PCR (Fig. 3.14b). The results of these findings indicated significantly greater expressions of the choline kinase genes, *ChKa* and *ChKb*, both of which play key roles in the biosynthesis of phosphatidylcholine in the small intestinal tissue but not colon tissue. Moreover, we found significantly higher expressions of the *Slc4aa1*, *Slc4aa2* and *Slc4aa3* genes, which encode the choline transporter proteins CTL1, CTL2 and CTL3, respectively. These results indicate that *T. musculus* colonization mediates the secretion of choline, which facilitates its uptake into mucosal tissue in the small intestine.

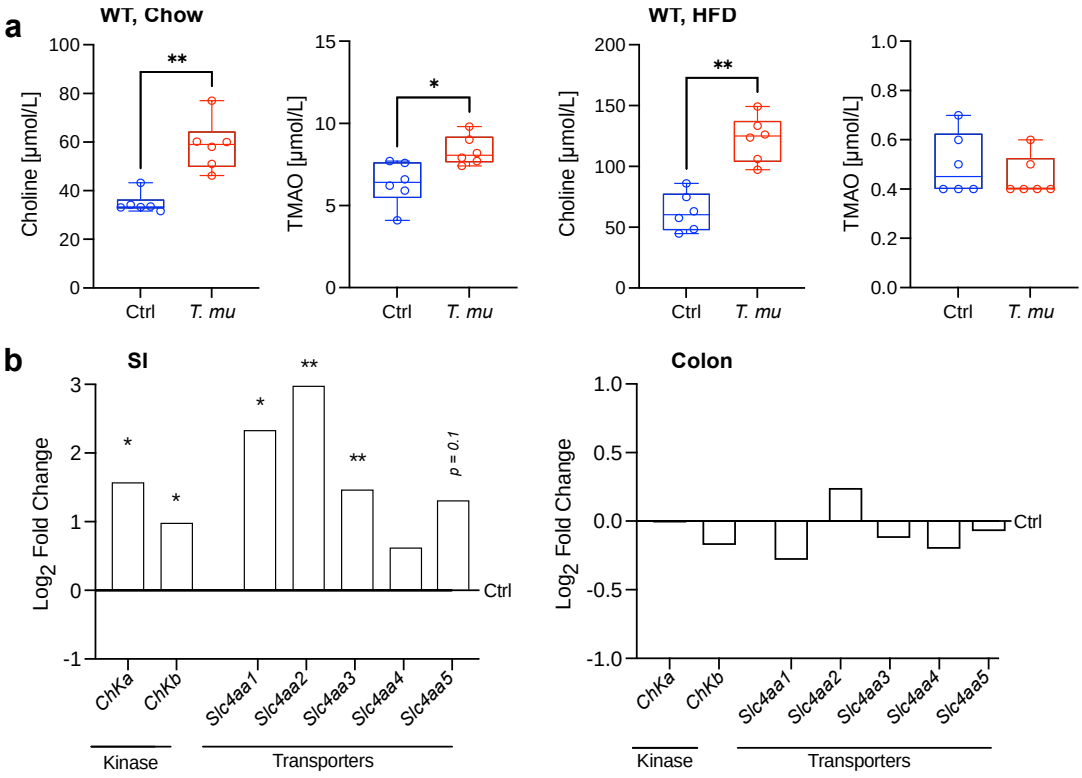


Figure 3.14. Tritrichomonas musculus colonization led to increased choline secretion and expression of choline-related genes in wild-type C57BL/6N mice. ***a)*** *The concentration of Choline and its derivative, Trimethylamine N-Oxide (TMAO) in the sera of chow- and HFD-fed uncolonized and T. mu-colonized mice.* ***b)*** *qRT-PCR analysis of choline kinase and transporter genes in the distal small intestine and colon of uncolonized vs T. mu-colonized mice. Data represents the relative log_2 Fold Change of the T. mu-colonized samples relative to the uncolonized (Ctrl) samples. The -ddCt method was used for the analysis. Data are representative of two independent experiments (n = 16). Student's two-tailed distribution t-test was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

Next, we administered 0.1% (w/v) free choline in the drinking water of HFD-fed uncolonized mice and assessed their metabolic and gut immunological phenotype after 1 month (Fig. 3.15, Supplementary Fig. 5.11). We noted significantly impaired glucose tolerance and hyperinsulinemia in the choline-treated mice (Fig. 3.15a and b). Additionally, similar to the results of the chow-fed *T. mu*-colonized mice (Fig. 3.2c), choline treatment precipitated an impaired insulin sensitivity, a hallmark of the development of Type 2 DM (Fig. 3.15c). As insulin resistance and hepatic gluconeogenesis are intimately linked in the pathology of Type 2 DM, we performed an intraperitoneal pyruvate tolerance test (ipPTT) by administering a pyruvate bolus in the mice and measuring glucose output, as pyruvate serves as a central precursor for hepatic gluconeogenesis. Similar to the findings of Kou et al., we found significantly enhanced hepatic gluconeogenesis in the HFD-fed choline-treated uncolonized mice (Fig. 3.15d). In summation, our results suggest that administering free choline to HFD-fed wild-type

mice leads to the onset of hyperinsulinemia and hepatic insulin resistance, manifested by increased liver gluconeogenesis and elevated circulating blood glucose levels. Persistent insulin resistance and heightened hepatic gluconeogenesis contribute to the development of chronic hyperglycemia, a hallmark of Type 2 DM.

An assessment of the mucosal immune cells by flow cytometry found mostly no significant changes in the innate and adaptive immune responses in the choline-treated mice (Supplementary Fig. 5.11). The only observable difference we found was a decrease in the $CD4^+$ T-cells and T_{reg} cells upon choline treatment. This is in contrast to the increased in the frequencies of these cells in the HFD-fed *T. mu*-colonized mice (Fig. 3.5c). These results indicate that the primary impact of choline administration in HFD-fed mice is the impaired metabolic phenotype. This lends credence to the hypothesis that *T. musculus* colonization in HFD-fed wild-type mice impacts glucose metabolism and mucosal immune cells through different mechanisms, possibly through the extensive interaction with the commensal gut microbiota.

3.9 *In vivo* administration of 3,3-dimethyl-1-butanol to HFD-fed *T. mu*-colonized mice did not ameliorate the impaired metabolic and immunological phenotype.

Free choline can be metabolized by choline-utilizing bacteria, such as *Desulfovibrio* or *Lachnoclostridium*, into trimethyalamine (TMA) or betaine through the action of choline TMA lyase or choline dehydrogenase, respectively (Fig. 3.16). The small molecular choline analog, 3,3-dimethyl-1-butanol (DMB) is capable of inhibiting the downstream metabolism of choline into its derivatives (Bollenbach et al. 2020). Previously, Kou et al. utilized an *in vivo* administration of DMB to instigate a "rescue" of the impaired hepatic gluconeogenesis in their *Tritrichomonas*-colonized $HVEM^{-/-}$ mouse strain (Refer to section 1.3.4). Based on the similarity in the metabolic phenotype and microbial shift between our findings and that of Kou et al.'s, as well as the increased choline secretion in our *T. mu*-colonized mice, we sought to inhibit the bacterial conversion of choline into its derivates to discern the importance of this pathway in our experimental mouse model.

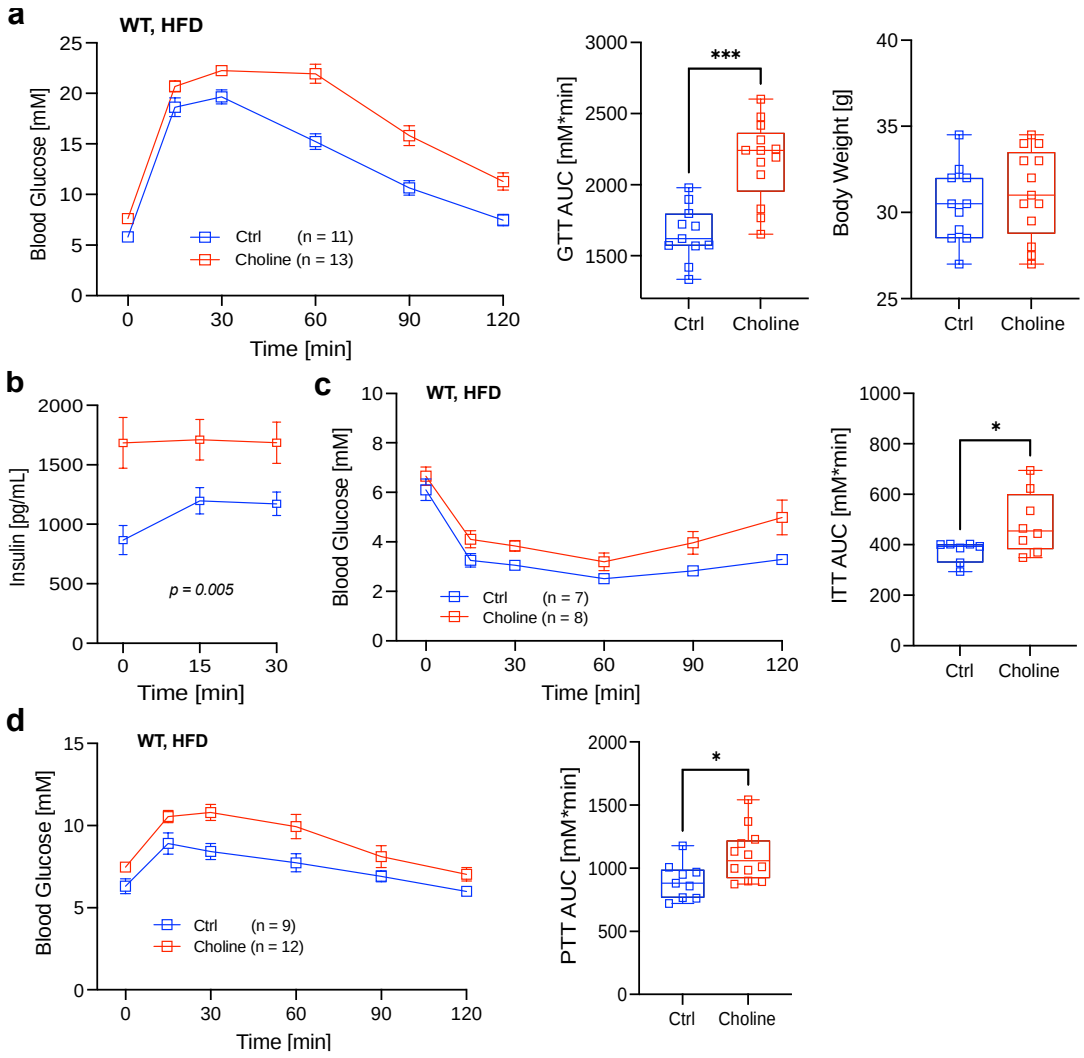


Figure 3.15. Administration of 0.1% (w/v) choline in the drinking water significantly impairs the metabolic phenotype of HFD-fed uncolonized C57BL/6N mice. ***a)*** *Intraperitoneal glucose tolerance test (ipGTT), area under the curve (AUC) and body weight of HFD-fed C57BL/6N mice (Ctrl n = 11, Choline n = 13; age = 10-12 weeks).* ***b)*** *Circulating insulin levels measured during the first 30 minutes of the ipGTT.* ***c)*** *Intraperitoneal insulin tolerance test (ipITT), area under the curve (AUC) (Ctrl n = 7, Choline n = 8; age = 11-13 weeks).* ***d)*** *Intraperitoneal pyruvate tolerance test (ipPTT) and the area under the curve (AUC) of HFD-fed C57BL/6N mice (Ctrl n = 9, Choline n = 12; age = 11-13 weeks). Statistical data are expressed as mean ± SEM. Data are representative of three* ***(a, b)*** *and two* ***(c, d)*** *independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

We administered 1% (w/v) DMB in the drinking water of HFD-fed *T. mu*-colonized mice after approximately 3 weeks of HFD feeding (Fig. 3.17). Following the treatment with DMB for one week, we found no significant differences in the glucose excursion of the DMB-treated mice as compared to the control *T. mu*-colonized mice (Fig. 3.17a). DMB treatment led to a significant rise in insulin secretion, mirroring the observations made in our models involving *T. mu* colonization and choline treatment in mice (Fig. 3.17b). One plausible explanation may be the elevation in circulating choline levels resulting from

DMB treatment, contributing to hyperinsulinemia. An alternative explanation could be attributed to the role of DMB in mitigating pancreatic tissue injury caused by the detrimental effects of high-fat diet consumption, thereby enhancing β-cell functions (Yang & Zhang, 2022). DMB treatment similarly did not lead to the amelioration of pro-inflammatory small intestinal macrophages and ILC populations (Fig. 3.17c and e). Notably, we noticed a reduction in the frequency of small intestinal $CD4^+$ T_{reg} cells upon DMB treatment (Fig. 3.17d), contrasting with the elevated cell population observed after *T. mu*-colonization in HFD-fed C57BL/6N mice (Fig. 3.5b). Collectively, the administration of DMB in *T. mu*-colonized mice did not lead to an improvement of glycemic control, but instead mediated an increased insulin secretion and attenuated the $CD4^+$ T-cell response associated from Tritrichomonad colonization.

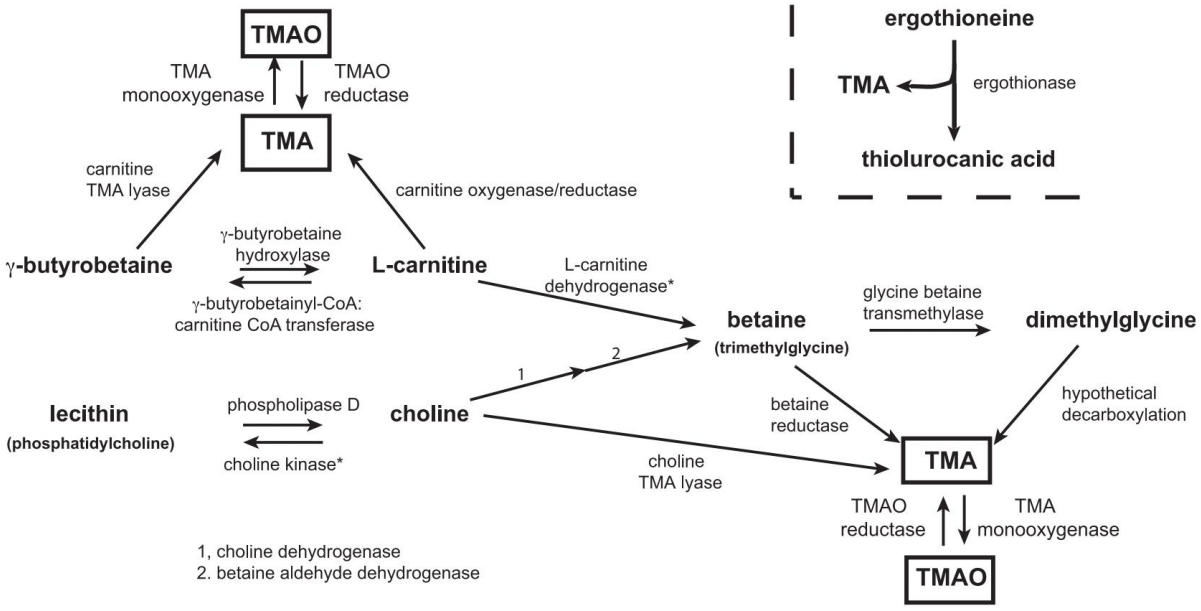


Figure 3.16. Metabolic pathways for the production and metabolism of TMA by the human microbiota. *TMA can be generated from trimethylamine oxide (TMAO) through the action of TMAO reductase. Choline, found in free form or released from lecithin, can contribute to TMA production directly by choline-TMA lyase or indirectly by conversion to betaine. L-carnitine, present in red meat or derived from g-butyrobetaine, can also contribute to TMA formation directly via carnitine reductase/oxidase or indirectly by conversion to betaine or g-butyrobetaine. Betaine itself can contribute directly to TMA formation through betaine reductase or indirectly through conversion to dimethylglycine. TMA can be produced from ergothioneine by ergothionase, and TMA can be further oxidized to TMAO via TMA monooxygenase. Figure adapted from Fennema et al. 2016.*

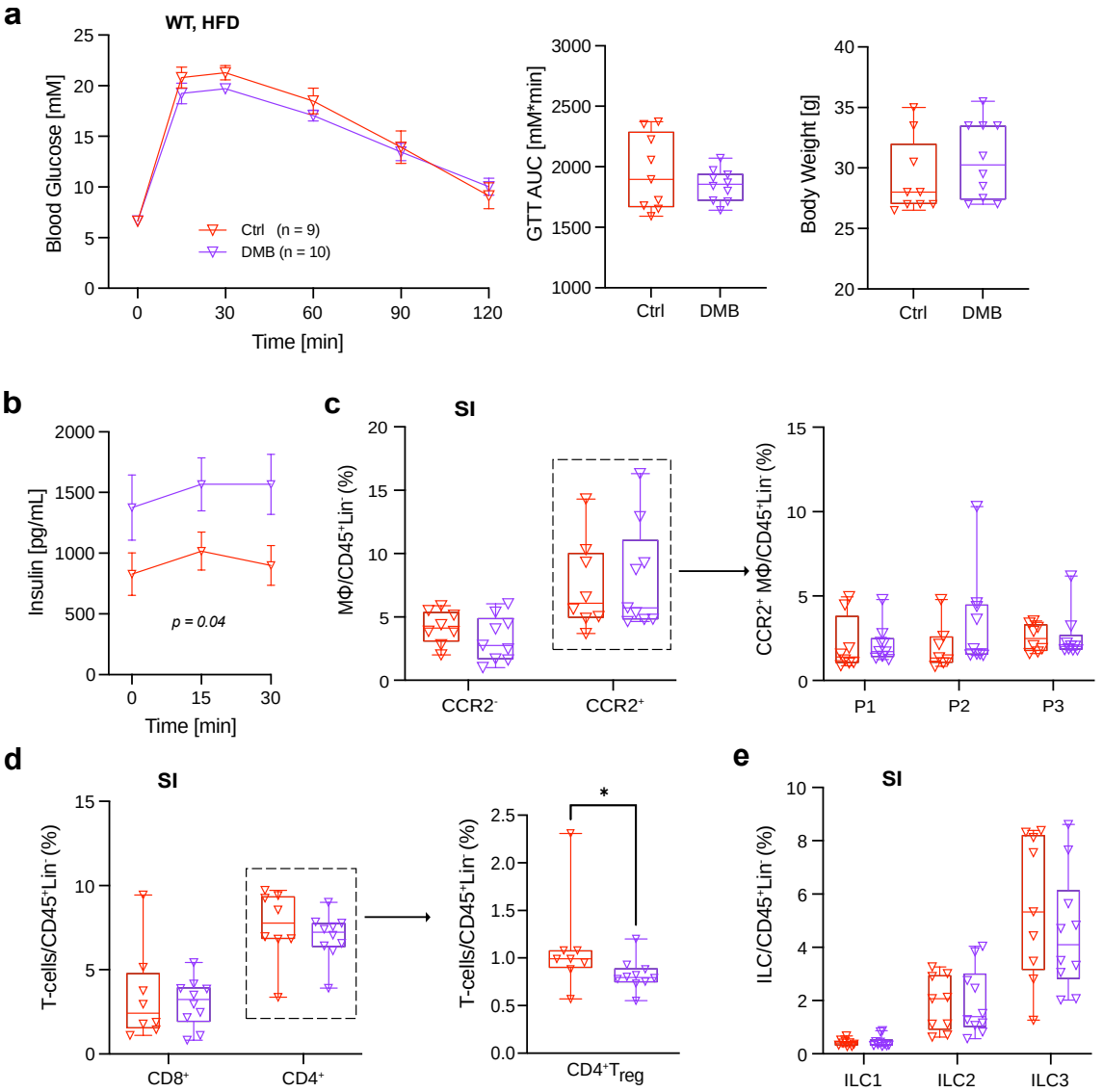


Figure 3.17. Administration of 1% (w/v) 3,3-dimethyl-1-butanol in the drinking water failed to rescue the metabolic phenotype of HFD-fed T. mu-C57BL/6N mice. ***a)*** *Intraperitoneal glucose tolerance test (ipGTT), area under the curve (AUC) and body weight of HFD-fed T.mu-colonized C57BL/6N mice (Ctrl n = 9, DMB n = 10; age = 11-13 weeks).* ***b)*** *Circulating insulin levels measured during the first 30 minutes of the ipGTT.* ***c)*** *Inflammatory CCR2$^+$ macrophages in distal small intestinal tissue and the further subclass distributions of Ly6C^{high}MHCII^{low} P1-P3 macrophage subpopulations.* ***d)*** *Frequencies of small intestinal CD3$^+$ T-cell populations. T-regulatory (T_{reg}) cells = CD4$^+$CD25$^+$FoxP3$^+$.* ***e)*** *Frequencies of distal small intestinal innate lymphoid cells (ILCs). ILC1 = CD335$^+$Eomes$^-$, ILC2 = GATA3$^+$, ILC3 = RORγt$^+$. Statistical data are expressed as mean ± SEM. Data are representative of three independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

Chapter 4

Discussion, Outlook and Limitations of this study

4.1 *Tritrichomonas musculus* colonization leads to significant impairment in glycemic control, hyperinsulinemia and instigates a pro-inflammatory shift in mucosal cells

In this study, we unveiled a newfound parasitic relationship between the intestinal commensal protozoan *Tritrichomonas musculus* and its rodent host. Our research shows that *T. mu* can integrate into the host's ecosystem, leading to considerable alterations in the gut microbial communities, modulation of the host immunity and ultimately, impacting glucose metabolism (Figure 4.1). The findings of this study further substantiate the conclusions drawn from an increasing body of recent publications, all highlighting the detrimental effects of Tritrichomonad protist colonization on its host (Refer to section 1.2). Screening for Tritrichomonad protist is not a common practice across animal facilities, so the current prevalence of Tritrichomonad protists in laboratory mice remains uncertain. In a recent study by Tuzlak et al. (2023), Tritrichomonad protists were found to be widespread in various NIH facilities, suggesting a potentially high prevalence of these protists. For research on the adverse effects of Tritrichomonad protists to be impactful in the current scientific landscape, it is imperative to critically reassess the notion that these protists can colonize their host without inflicting any harm or pathological damage.

We first faced challenges in our initial goal of studying the molecular underpinnings of air pollution-induced diabetes. Notably, we observed gut inflammation in our mouse colonies at the Department of Biomedicine in Basel, including in control mice that had not been exposed to air pollution particles previously. This led us to discover that our mice were colonized with *Tritrichomonas musculus*, a well-accepted commensal member of the gut microbiome. We report here that the colonization of wild-type C57BL/6N mice with *T. musculus* significantly impairs the metabolic state and mediates the pro-inflammatory shift of mucosal immune cells. This deleterious effect is accentuated when the mice were fed a 58% kcal HFD.

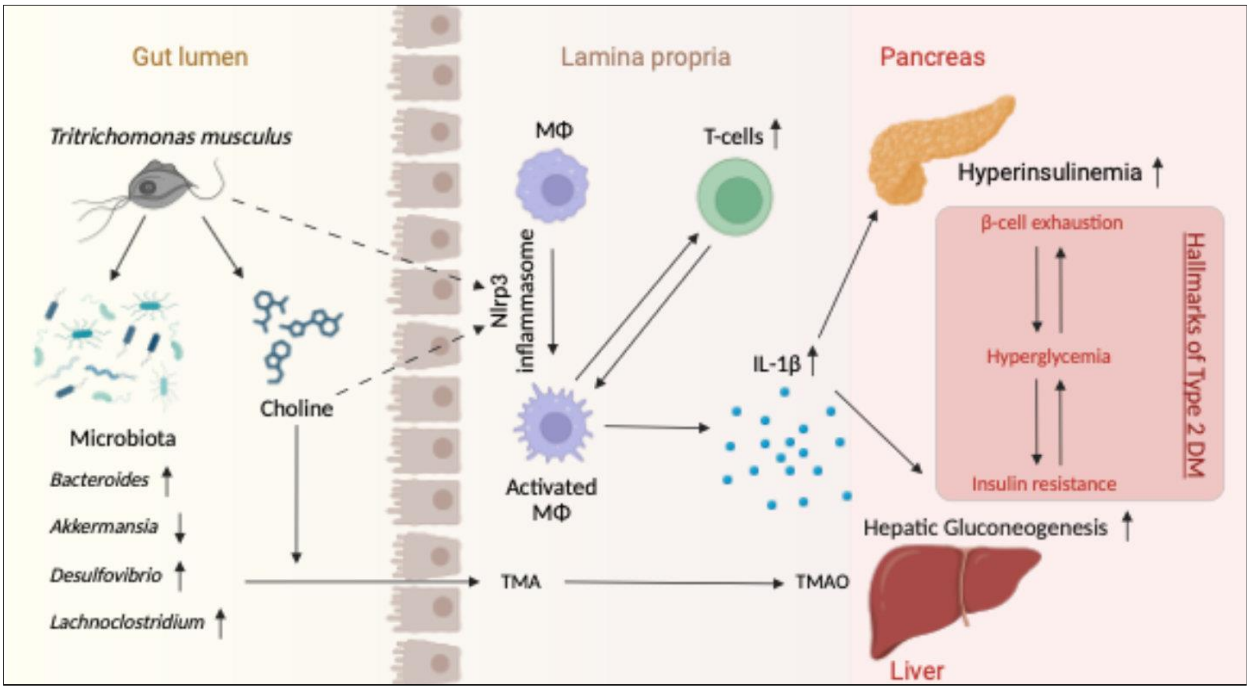


Figure 4.1. Schematic illustration of the Impact of Tritrichomonas musculus Colonization on Gut Inflammation and Glucose Homeostasis*. Colonization of T. musculus in wild-type C57BL/6N instigates significant changes in the gut microbial community, mucosal immunology as well as increasing the levels of free choline through the protists' metabolism. The modulation of mucosal immunology leads to a chronic pro-inflammatory state and elevated levels of secreted inflammatory cytokines such as IL-1β. All of which have considerable impact on the onset of insulin resistance, β-cell exhaustion, hyperglycemia – Hallmarks of Type 2 DM. Figure was created with BioRender.com.*

The engraftment of *T. musculus* into the gut microbiota has a notable impact on other commensal bacteria communities and is also influenced by dietary nutrients. We observed significant expansions in the pro-inflammatory macrophage populations and an increased $CD4^+$ T-cell response in the intestinal lamina propria of *T. mu*-colonized mice. Moreover, we observed a noteworthy elevation in the infiltration of pro-inflammatory adipose tissue macrophages, a characteristic inflammation associated with the onset of Type 2 DM, in the HFD-fed *T. mu*-colonized mice. We showed the importance of the Nlrp3 inflammasome and the actions of the pro-inflammatory cytokine, IL-1β, in instigating the metabolic and immunological phenotype brought about by *T. musculus* colonization. By utilizing a whole-body genetic knockout of Nlrp3 mouse model and the pharmacological inhibition of IL-1β, we showed that the *T. mu*-colonized mice are protected from impaired glucose tolerance and hyperinsulinemia. Additionally, we observed an amelioration of the pro-inflammatory shift in mucosal immunity in these mouse models. The contribution of the Nlrp3 inflammasome in the modulation of mucosal immune cells by Tritrichomonad colonization was first elucidated by Chudnovskiy et al., explored further by Chiaranunt et al. (Refer to Section 1.2.3) and expanded in this report.

Discussion

By conducting 16S ribosomal gene sequencing to examine the composition of the gut microbiota in the ceca of wild-type C57BL/6N mice colonized with *T. musculus*, we showed significant increase and decrease in the populations of *Bacteroides* and *Akkermansia* families, respectively. An increase in the abundance of the *Bacteroides* enterotype has been identified as a significant risk factor for Type 2 DM (Wang et al. 2020). Conversely, members of the *Akkermansia* family such as *Akkermansia muciniphila* is a promising member of the gut microbiota for the treatment of Type 2 DM due to its capabilities of reducing insulin resistance and increasing insulin sensitivity (Li et al. 2023). Additionally, we found notable expansions in bacterial families involved in choline-processing such as *Desulfovibrio* and *Lachnoclostridium*, in the caeca of *T. mu*-colonized mice. Kou et al. had previously implicated the involvement of *Desulfovibrio vulgaris*, in mediating the processing of choline into trimethylamine which are subsequently processed into trimethylamine-N-oxide in the liver, contributing to increased hepatic gluconeogenesis. We corroborated their observations through our gut microbiota sequencing, revealing a substantial expansion of the *Desulfovibrio* family—a group of choline-processing bacteria—in mice colonized by HFD-fed *T. mu*-colonized mice.

Moreover, our results also showed an increase in the levels of circulating choline in the sera of *T. mu*-colonized mice, thus indicating the generation of free choline from the metabolism of these protists. By administering choline in the drinking water of HFD-fed uncolonized C57BL/6N mice, we found that choline led to glucose intolerance, hyperinsulinemia, insulin resistance and an increased hepatic gluconeogenesis. Choline and its downstream metabolite, trimethylamine-N-oxide, have been shown to be positively associated with the onset of Type 2 DM (Dibaba et al. 2020 & Qi et al. 2023). We attempted to solidify the importance of the choline-TMAO pathway to be a crucial mechanism in instigating the impaired metabolism by inhibiting the processing of choline with the choline analog, DMB. Treatment with DMB in *T. mu*-colonized mice did not result in a significant improvement in glycemic control; however, an increase in insulin secretion was observed. This could be attributed to elevated circulating choline levels resulting from the inhibition of choline metabolism. Our results from choline treatment already emphasize the crucial role of choline in shaping a hyperinsulinemic phenotype. Moreover, DMB treatment mitigated the notable expansion of $CD4^+$ T_{reg} cells, although it did not affect mucosal innate immunity.

Discussion

It is still a matter of debate within the scientific community on whether colonization with Tritrichomonad protists leads to a symbiotic relationship with other bacterial species as well as the host. The colonization by Tritrichomonad protists holds the potential to confer crucial benefits to the host. Firstly, protist colonization can trigger the activation of the epithelium, promoting increased mucin release and reinforcing the integrity of the mucus barrier (Howitt et al. 2017). Secondly, heightened levels of activated innate immune cells and T-cells can enhance the responsiveness of the local gut immunity against enteric pathogens. Indeed, Chudnovskiy et al. had previously shown in 2016 that *T. mu*-colonization can protect against *Salmonella typhimurium* mucosal infection. Thirdly, *Tritrichomonas spp*. have the ability to prompt the migration of locally activated immune suppressive cells from the intestine to the brain, offering protection against autoimmunity. While these effects present a clear advantage for animals colonized with *Tritrichomonas spp*., detrimental consequences may arise when *Tritrichomonas spp*. colonization supports uncontrolled Th cell activation in the absence of sufficient immune suppression (Cao et al. 2020). In a recent unpublished finding, the authors found that *T. mu*-colonization can intensify the severity of asthma by fostering lung eosinophilia through the activation and dissemination of ILC2s (Burrows et al. 2022, bioRxiv).

As alluded to above (Refer to Section 1.3), gut microbiota dysbiosis is associated with a host of intestinal disorders. Our findings indicate an increase in inflammatory mucosal immune cells upon *T. musculus* colonization, particularly exacerbated by HFD-feeding. Despite this shift toward pro-inflammatory mucosal cells, *T. mu*-colonization did not adversely affect the overall well-being of the mice. In inflammatory bowel diseases like Crohn's disease and ulcerative colitis, patients exhibit a decrease in the Firmicutes bacterial family and a simultaneous increase in the Bacteroidetes family (Hansen et al. 2010). As these are commensal members of the gut microbiota, colonization by these bacterial families under homeostatic conditions does not lead to disease pathogenesis. Our results suggest a primed immunological state of the intestinal mucosa and an increase in insulin secretion in *T. mu*-colonized mice. However, in a challenged *T. mu*-colonized model, such as chronic HFD feeding, the balance shifts toward a deleterious effect of colonization. Therefore, future studies on Tritrichomonad colonization should carefully consider the rodent model used to accurately delineate the metabolic and immunological impact of colonization.

In summation, our findings revealed that *Tritrichomonas musculus* colonization in C57BL/6N laboratory mice disrupts mucosal immune cell homeostasis and impairs glucose metabolism. These effects are driven by the pro-inflammatory environment instigated by Il-1β, as well as the secretion and processing of choline. Additionally, engraftment of *T. musculus* into the gut microbiome significantly shifts the relative abundances of key bacterial families involved in Type 2 DM pathogenesis and choline metabolism. Importantly, these adverse effects of *T. mu* colonization are initiated at an earlier timepoint and exacerbated by HFD feeding.

4.2 Study Limitations

In highlighting the crucial role of commensal members in the gut microbiota, it becomes imperative to uphold a consistent microbiome in mice for subsequent experiments. The reproducibility of experiments in mouse models is shaped by a complex interplay of genetic and environmental factors. Notably, the diverse composition of microbiota among mice from various commercial vendors, along with the gut microbiota shift occurring through generations of in-house breeding across different animal facilities, significantly contributes to variability in experimental reproducibility in mouse models (Mandal et al. 2020). Therefore, the diverse composition of gut microbiota across animal facilities globally may pose a challenge in precisely reproducing the results obtained in this study. Indeed, a recent report revealed a substantial alteration in the gut microbiota profile of C57BL/6N mice obtained from vendors, indicating a significant shift over time while housed in their respective facilities, despite exhibiting low bacterial diversity at the time of purchase (Long et al. 2021). A notable instance is the absence in the expansion of the ILC2 stimulatory circuit, previously observed in *Tritrichomonas*-colonized wild-type mice by other researchers, was not replicated in our *T. mu*-colonized chow-fed mouse model (Howitt et al. 2017 & Schneider et al. 2018). On the other hand, this distinction might arise from diverse Tritrichomonad species, considering the ongoing sequencing of a growing number of these protists as they are progressively identified in various animal facilities.

Discussion

We offered some insights into the mechanism behind *T. mu*-driven impaired glycemic control and heightened mucosal inflammation by pinpointing choline as the primary driver of these phenotypes. However, our study did not clearly establish the connection between the increased circulating choline resulting from *T. musculus* colonization and the impaired metabolic and immunological phenotype. To delve deeper into the mechanistic aspects of Tritrichomonad protists, it is imperative to establish a well-defined method for culturing these anaerobes for further investigations. This would facilitate more comprehensive *in vitro* experiments involving these protists, such as the co-culture with immune cells to directly investigate the activation of the Nlrp3 inflammasome. Furthermore, while implicated to be correlated with Type 2 DM, our study did not demonstrate the involvement of the downstream choline metabolite, TMAO, in the *T. mu*-driven metabolic impairment (Dambrova et al. 2016). Among the various metabolites secreted by *T. mu*, succinate has been identified as a trigger for the tuft cell-ILC2 inflammatory circuit, driving intestinal remodeling (Refer to Section 1.2; Figure 1.4). Kou et al. (2022) previously explored the role of succinate in hepatic gluconeogenesis, finding that supplementation in their mouse model did not result in the anticipated impaired metabolic phenotype. These findings highlight the potential adverse implications of other *T. mu*-derived metabolites in mediating intestinal inflammatory responses and impaired glycemic control.

Our key observations in this report reveal that *Tritrichomonas musculus* colonization has a heightened impact on host metabolism, particularly when mice are subjected to a diet-induced obesity diet (HFD). Notably in initial publications on *Tritrichomonas spp*., the authors initially encountered issues with their mouse models, discovering later that their mice were colonized with this protist. This underscores the importance of screening for the presence of *Tritrichomonas spp*. in mouse models investigating metabolic syndrome and gut immunity, highlighting the potential impact on study outcomes.

4.3 Outlook for further studies

Despite our attempts to provide a clear mechanistic link between *T. mu*-colonization, the pro-inflammatory mucosal immune cell shifts and the impaired glycemic control, additional experiments can still be performed to elucidate this relationship clearly. Firstly, it is still unclear whether free choline secreted by *T. musculus* has an impact on the pro-inflammatory shift directly. It has previously been shown that choline uptake is increased in LPS-primed macrophages through the induction of the choline transporter, CTL1 (Slc4aa1 gene) (Sanchez-Lopez et al. 2019). The authors further showed that the uptake of choline into the macrophages is a key driver in the activation of the Nlrp3 inflammasome and IL-1β secretion. Thus, we hypothesize that the increased choline levels detected in *T. mu*-colonized mice triggers an elevated choline uptake in intestinal macrophages, expediting the pro-inflammatory milieu in the gut mucosa. We first showed in our study that the expression of choline-related kinase and transporter genes are significantly upregulated in the small intestine of *T. mu*-colonized mice, lending credence to our hypothesis. Nevertheless, our analysis of the immune cells in the colonic and small intestinal lamina propria did not yield evidence of a pro-inflammatory shift in the innate and adaptive immune cells (Supplementary Fig. 5.11). Our analysis of mucosal immune cells primarily examines surface and intracellular markers of different innate and adaptive immune cells. However, we did not assess the activation state based on secretion markers in our study. It is conceivable that choline treatment could induce a transition toward a more activated immune cell state, leading to the release of pro-inflammatory cytokines, similar to observations in mouse experiments of rheumatoid arthritis (Guma et al. 2015). Future experiments should be focused on elucidating the direct interaction of choline secreted by *T. musculus* or choline-derived metabolites on mucosal immune cells.

Notwithstanding the increasing number of recent reports exploring the impact of various enteric Tritrichomonad species, our comprehension of their phylogenetic relationships and functional diversity remains constrained. This knowledge gap has a substantial impact on our comprehension of the protist's role in host physiology. Given that different Tritrichomonad species can elicit varied effects on metabolism and immunity, bridging this gap is crucial for a more comprehensive understanding. Despite the fact that *T. musculus* and other species are commensal members of the gut microbiota, the integration of *T. musculus* into the microbiome results in significant shifts in bacterial populations, as

demonstrated by our research and others'. These changes in microbial communities can exert a notable impact on host physiology and health (Sekirov et al. 2010). In order to illuminate the role of *T. musculus* in host metabolism and mucosal immunity, independent of the gut microbiota's influence, we conducted experiments using germ-free mice. As of writing, there is scarce to no reports on the colonization of Tritrichomonad species in germ-free mice.

We attempted to monocolonize germ-free mice with cultured *T. musculus* in collaboration with Prof. S. Ganal-Vonarburg (University of Bern). In an effort to deplete the bacterial population from our isolates, we cultured *T. musculus* in horse serum-supplemented Trichosel media broth, along with a cocktail of antibiotics. We then intragastrically gavaged the protist isolate into HFD-fed germ-free mice and assessed their metabolic readouts by ipGTT and mucosal immune cells by flow cytometry. Regrettably, our attempts to achieve mono-colonization of *T. musculus* in our germ-free mice were unsuccessful, as we detected several bacterial species in the caeca of these mice (Supplementary Fig. 5.13). We faced a similar challenge, as discussed with other researchers who tried to colonize germ-free mice with Tritrichomonad protists. Consequently, we are currently unable to advance our experiments with germ-free mice until a more effective method for achieving monocolonization with Tritrichomonad protists has been developed. Conducting experiments with germ-free mice would be imperative to discern whether the observed metabolic and immunological phenotype in *T. musculus*-colonized mice originates from the protists themselves or from the shift in gut microbial composition upon colonization.

Finally, our goal is to evaluate whether human-related Tritrichomonad species, such as *Dientamoeba fragilis*, are overrepresented in the gut microbiota of human stool samples from type 2 diabetic patients compared to non-diabetic controls. This investigation aims to uncover any potential modulations by human Tritrichomonad species in the host's metabolism. As alluded to in the introduction (Refer to Section 1.3.1), a comprehensive, long-term longitudinal study involving a substantial cohort of Finnish adults yielded significant insights into the correlation between gut microbiota composition and the development of Type 2 diabetes mellitus (Ruuskanen et al. 2022). The authors identified strong species-level taxonomic features that serve as predictors for incident Type 2 DM. These observations not only augment but also extend existing predominantly cross-

sectional evidence. They reinforce the connections between dietary habits, metabolic diseases, and Type 2 DM, emphasizing the influential role of the gut microbiome in modulating these relationships. We have requested for permission to the Finnish Institute for Health and Welfare Biobank in order to gain access to the human microbiota dataset. We propose that the human homologs of Tritrichomonad protists or bacterial communities, which exhibit a shift upon *T. mu*-colonization in our findings, experience an increased abundance in individuals diagnosed with Type 2 DM during the follow-up study. The analysis of this dataset will be done in collaboration with Prof. P. Vonaesch (University of Lausanne). As of writing, we are in the process of obtaining the dataset from the Biobank and will initiate the analysis promptly upon its availability to us.

4.4 Conclusion

In this project, we uncovered an anomaly in our initial mouse models, prompting an investigation into the causes of abnormal glucose tolerance and mucosal inflammation. Our findings revealed the presence of unicellular, flagellated, motile eukaryotic-like organisms, approximately 10 μm in size, colonizing the gastrointestinal tract of our in-house bred C57BL/6N mice. Confirmation through ITS and 18S rRNA genomic DNA sequencing identified these organisms as a commensal Tritrichomonad species, phylogenetically identical to *Tritrichomonas musculus*. By meticulously colonizing *Tritrichomonas*-free wild-type C57BL/6N breeding mice with *T. musculus*, we established a model to study the metabolic and mucosal immunological impact of chronic *T. musculus* colonization. Experimental mice were subjected to either a standard chow or a 58% kcal HFD, with monthly monitoring of their metabolic status.

Our findings unveiled that *T. mu*-colonized mice developed severe glucose intolerance, hyperinsulinemia, and insulin resistance, with these effects exacerbated and accelerated by HFD feeding. Flow cytometric analysis of colonic and small intestinal lamina propria immune cells indicated a significant pro-inflammatory shift in intestinal macrophages and an expansion of the $CD4^+$ T-cell response due to *T. mu*-colonization. To delve deeper into the mechanistic action, we analyzed IL-1β secretion in colonic explants and measured the expression of Nlrp3-related genes in intestinal tissue. *T. mu*-colonized mice exhibited a significant increase in IL-1β secretion and upregulated Nlrp3-related genes. Utilizing

whole-body Nlrp3 inflammasome knockout mice and an *in vivo* pharmacological inhibition of IL-1β models, we demonstrated their crucial role in initiating the observed metabolic impairment and pro-inflammatory shift caused by *T. mu*-colonization.

Additionally, 16S rRNA gene sequencing of the caecal gut microbiota composition in uncolonized and *T. mu*-colonized mice displayed significant shifts in bacterial community abundances, notably the depletion of *Akkermansiaceae* and an increase in *Desulfovibrionaceae* bacterial families. Building on previous reports on the choline-utilizing activity of *Desulfovibrio* bacteria and the increased circulating choline and its derivative, TMAO, levels in *T. mu*-colonized mice, we investigated the metabolic consequences of administering choline to HFD-fed uncolonized mice. The results demonstrated that providing 0.1% (w/v) choline in the drinking water of HFD-fed mice induced hyperglycemia, hyperinsulinemia, increased hepatic gluconeogenesis, and hyperinsulinemia — all hallmarks in the development of Type 2 DM.

The results outlined in this thesis provide new insights and expands upon previous studies to delineate the consequences of *T. musculus* colonization in experimental mouse models. It underscores the importance of challenging and reevaluating the acceptance of Tritrichomonad protist colonization as non-harmful commensal members of the gut microbiota. Finally, our findings offer evidence suggesting that the potential inhibition of the metabolism of the human homolog of Tritrichomonad protists could serve as a prospective therapeutic intervention for ameliorating Type 2 DM symptoms.

Chapter 5

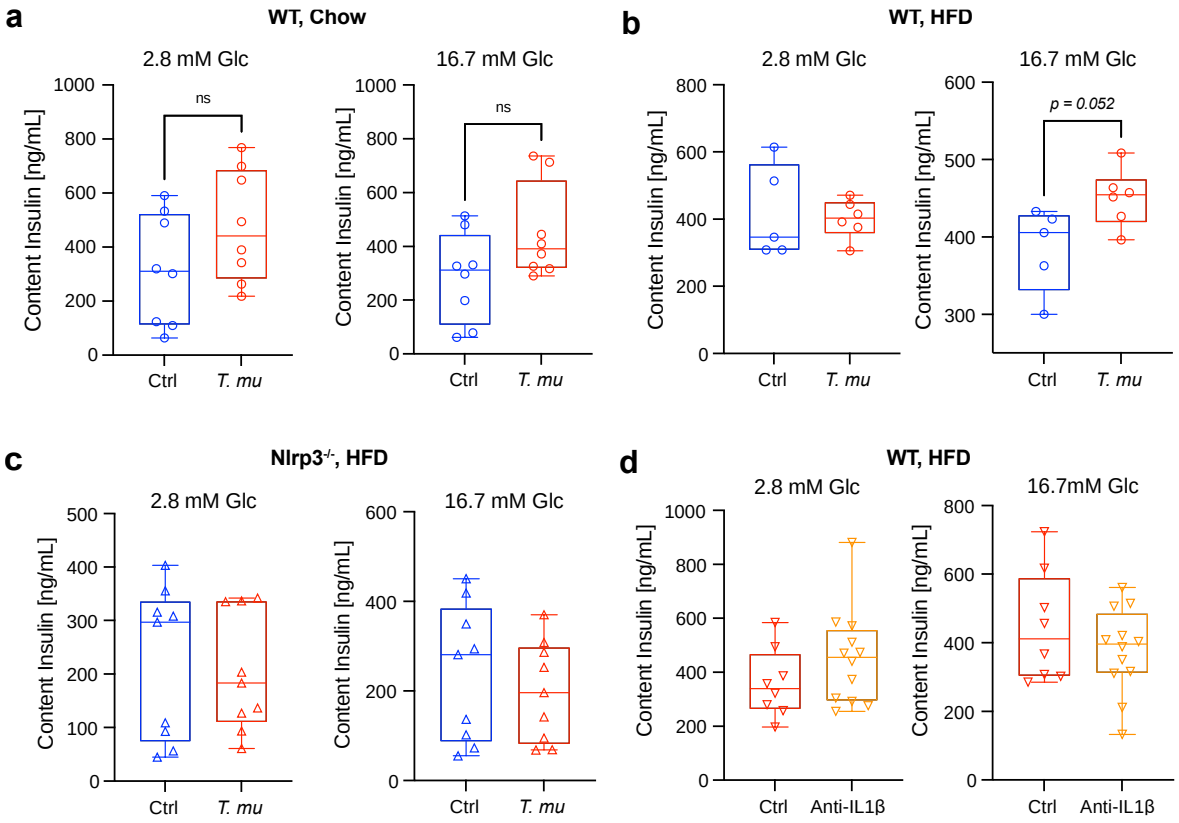
Appendix

5.1 List of Tables

5.2 List of Figures

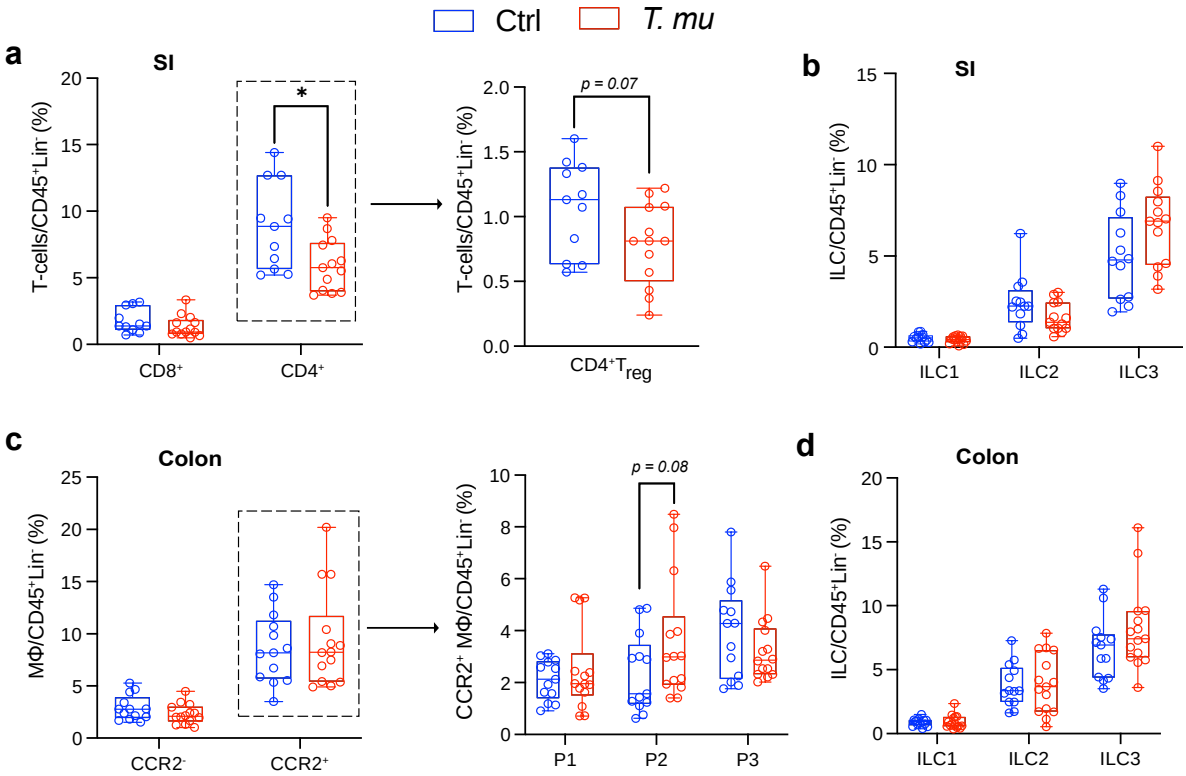
Appendix

5.3 Supplementary Figures

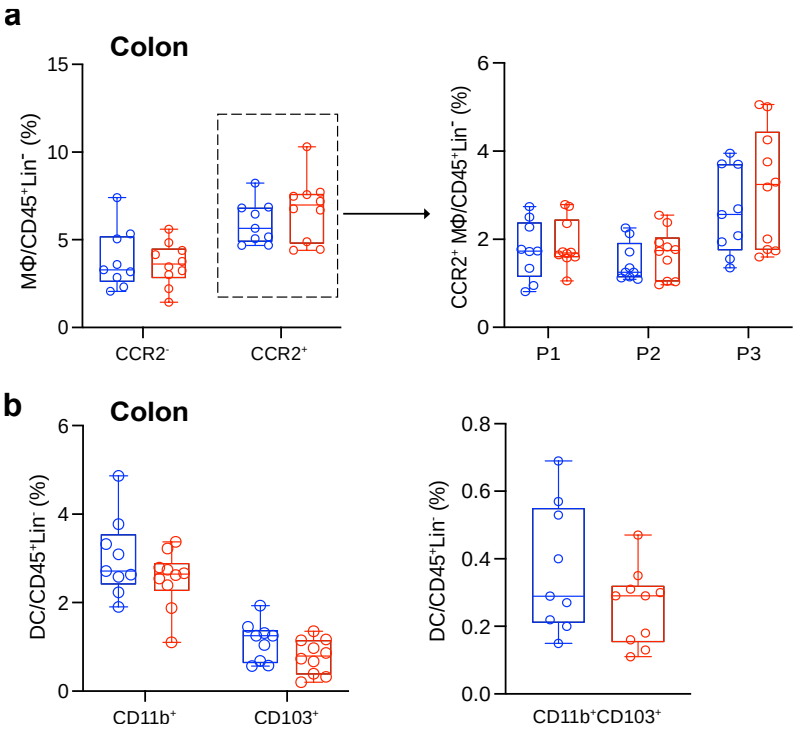


Supplementary Figure 5.1. Total content of ex vivo pancreatic islet culture for Glucose Stimulated Insulin Secretion experiments. *Total insulin content of chow-fed (**a**), HFD-fed (**b**), Nlrp3-/- HFD-fed (**c**) and anti-IL1β HFD-fed (**d**) experiments.*

Appendix



Supplementary Figure 5.2. Further mucosal immune cells characterization in chow-fed wildtype C57BL/6N mice the in distal small intestine and colon. ***a)*** *Frequencies of small intestinal $CD3^+$ T-cell populations. T-regulatory (T_{reg}) cells = $CD4^+CD25^+FoxP3^+$* ***b and d)*** *Frequencies of distal small intestinal (**b**) and colonic (**d**) innate lymphoid cells (ILCs). ILC1 = $CD335^+Eomes^-$, ILC2 = $GATA3^+$, ILC3 = $ROR\gamma t^+$.* ***c)*** *Inflammatory $CCR2^+$ macrophages in distal small intestinal tissue and the further subclass distributions of $Ly6C^{high}MHCII^{low}$ P1-P3 macrophage subpopulations. Data are representative of two (**a, b, c**) independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*



Supplementary Figure 5.3. Further mucosal immune cells characterization in HFD-fed wildtype C57BL/6N mice the in the colon. ***a)*** *Inflammatory $CCR2^+$ macrophages in distal small intestinal tissue and the further subclass distributions of $Ly6C^{high}MHCII^{low}$ P1-P3 macrophage subpopulations.* ***b)*** *Frequencies of colonic dendritic cells (DC) ($CD64^-MHCII^+CD11c^+$) based on the expressions of CD11b and CD103. Data are representative of two (**a and b**) independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

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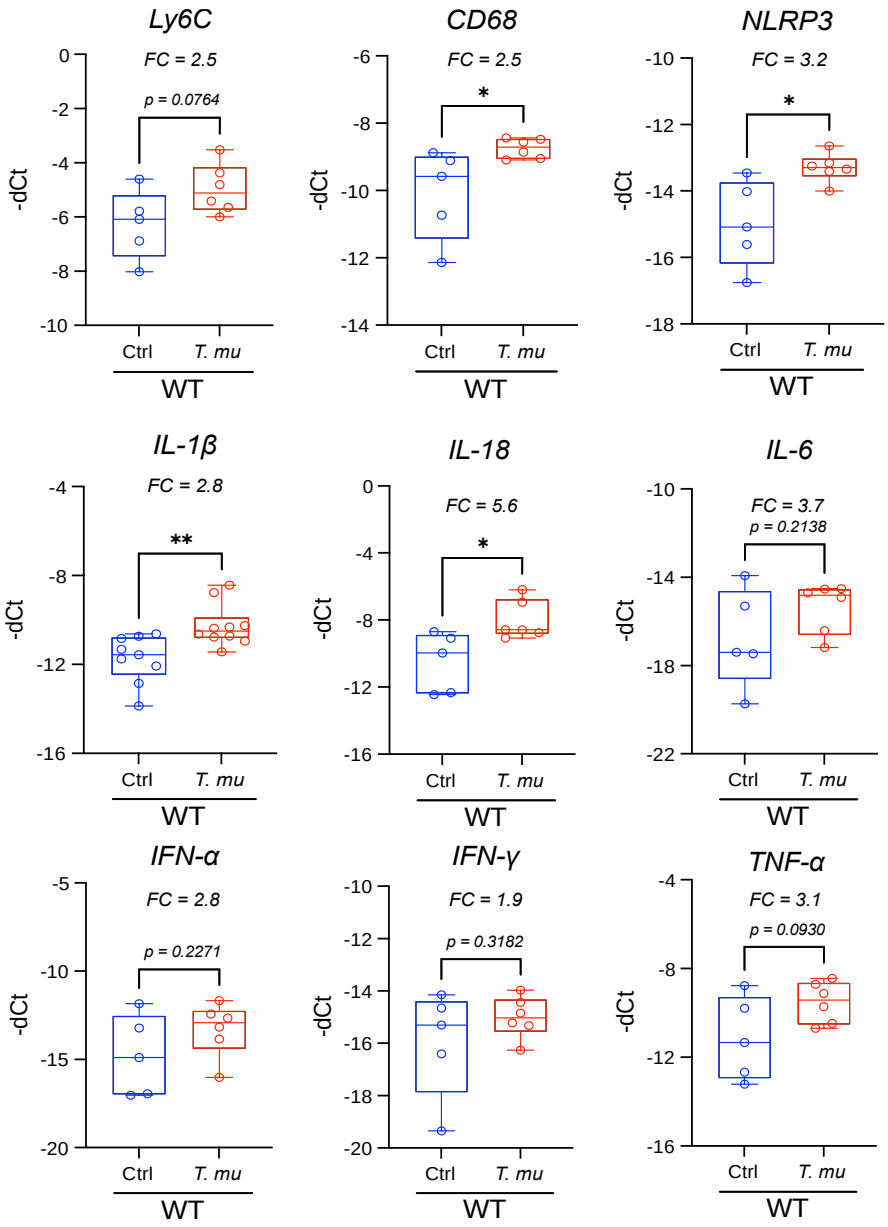

Supplementary Figure 5.4. -dCt values of qRT-PCR of HFD-fed inflammatory genes in the small intestine. *Data are representative of two independent experiments. Refer to Figure 3.7 (right figure) for Log_2Fold-Change. The -ddCt method was used for the analysis. Student's two-tailed distribution t-test was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

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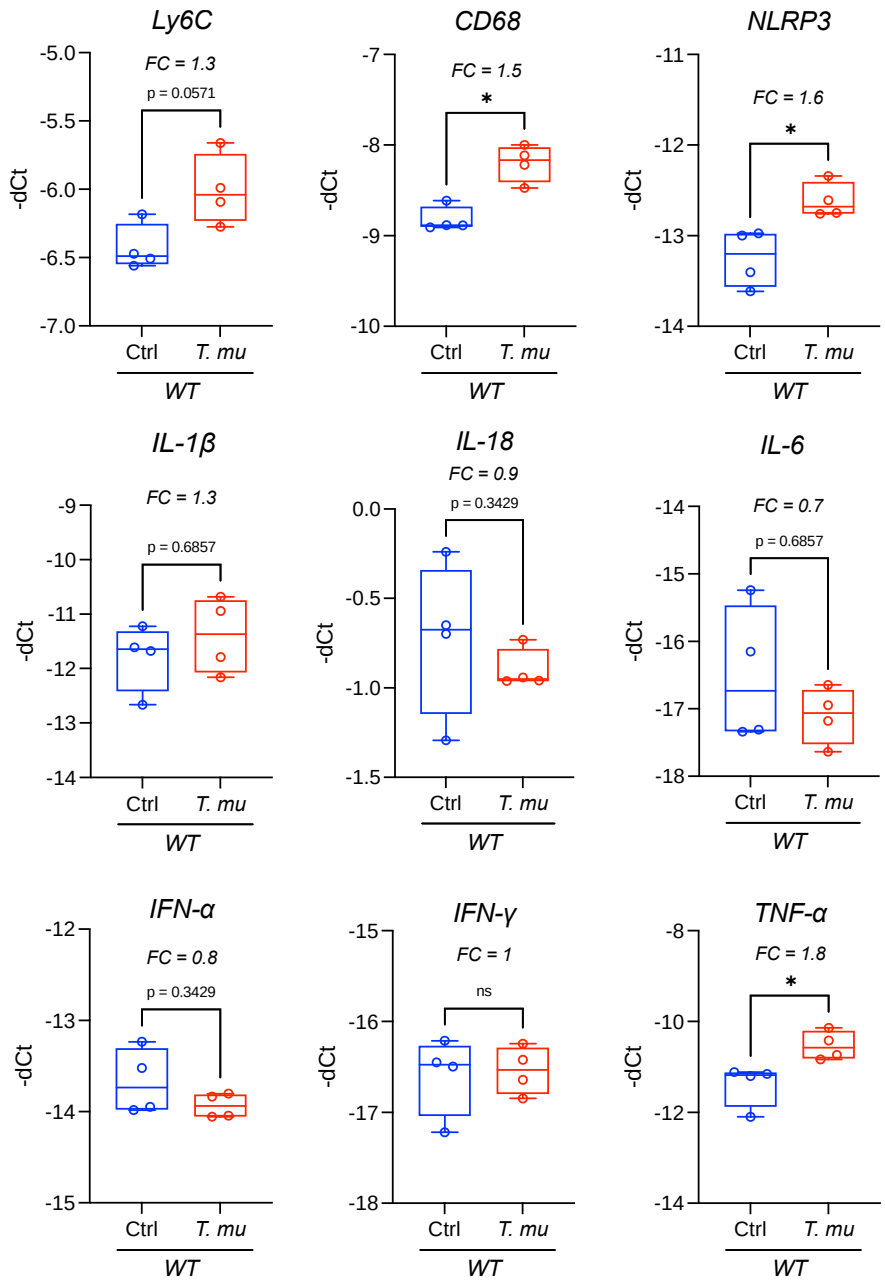
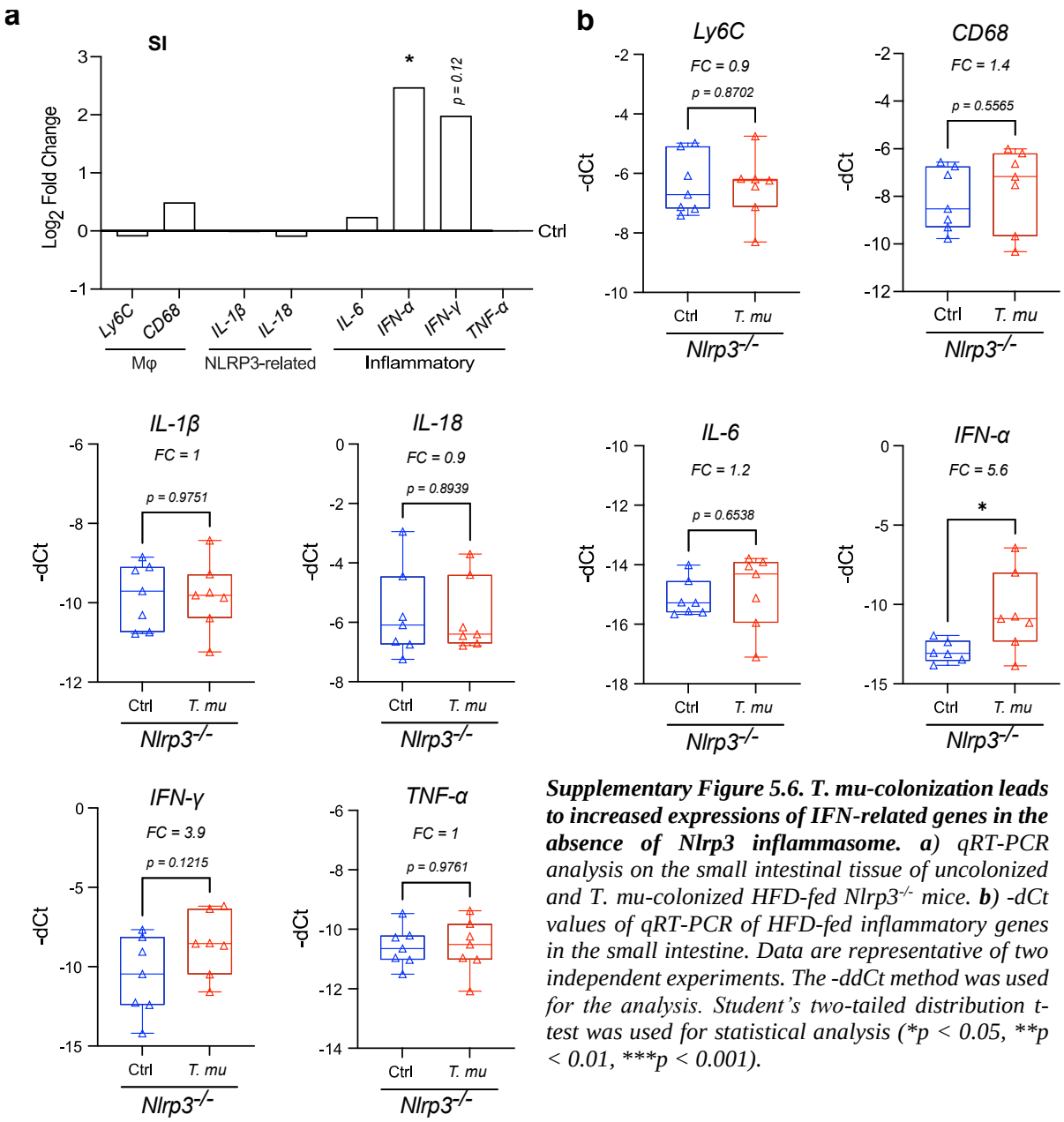
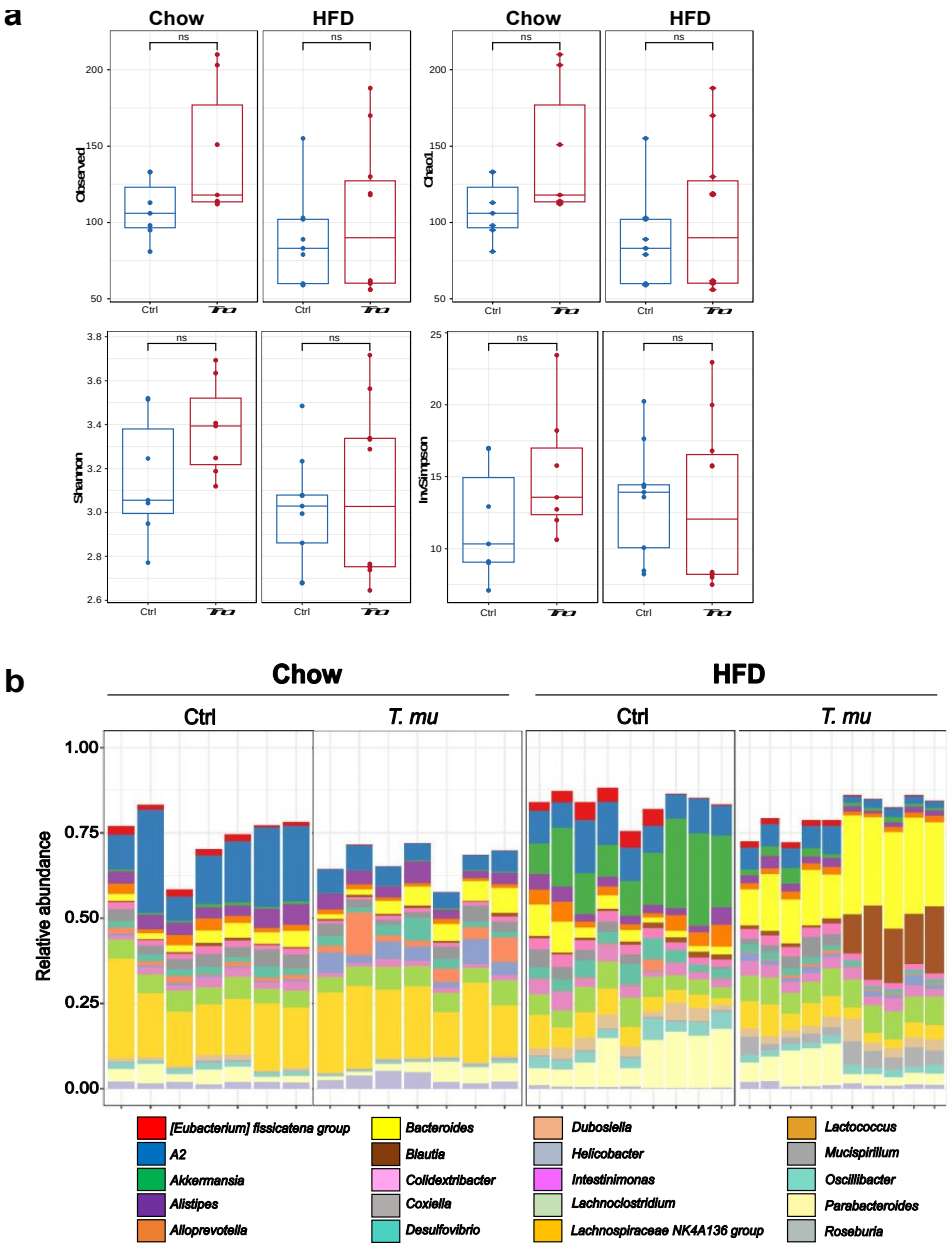


Supplementary Figure 5.5. -dCt values of qRT-PCR of HFD-fed inflammatory genes in the colon. *Data are representative of two independent experiments. Refer to Figure 3.7 (left figure) for Log_2Fold-Change. The -ddCt method was used for the analysis. Student's two-tailed distribution t-test was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*



Supplementary Figure 5.6. T. mu-colonization leads to increased expressions of IFN-related genes in the absence of Nlrp3 inflammasome. ***a)*** *qRT-PCR analysis on the small intestinal tissue of uncolonized and T. mu-colonized HFD-fed Nlrp3$^{-/-}$ mice.* ***b)*** *-dCt values of qRT-PCR of HFD-fed inflammatory genes in the small intestine. Data are representative of two independent experiments. The -ddCt method was used for the analysis. Student's two-tailed distribution t-test was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*



Supplementary Figure 5.7. Caecal gut microbiota analysis at the Genera level in T. mu-colonized C57BL/6N mice. a*) The alpha-diversity at the genera level of commensal bacteria composition, as assessed by the Observed, Chao1, Shannon and inverse Simpson indices.* ***b****) Relative abundances of the top 10 most abundant bacterial genera that are perturbed by diet and T. mu colonization. Data are representative of two independent chow- and HFD-fed experiments (****a and b****). Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

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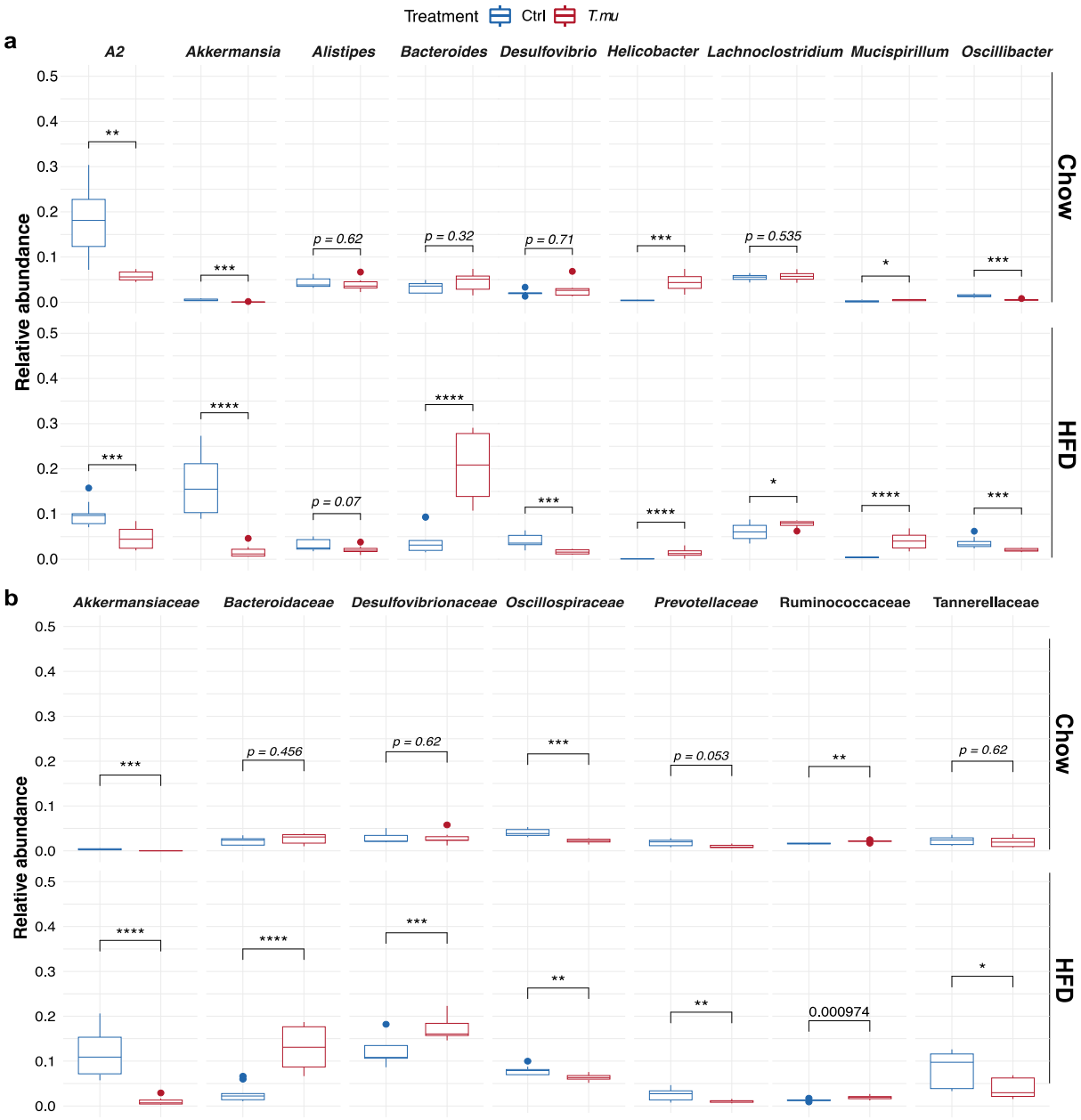

Supplementary Figure 5.8. Boxplots showing the relative abundances of the top most abundance bacterial Genera (a) and Families (b) from T. mu-colonization. *Data are representative of two independent chow- and HFD-fed experiments (**a and b**). Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

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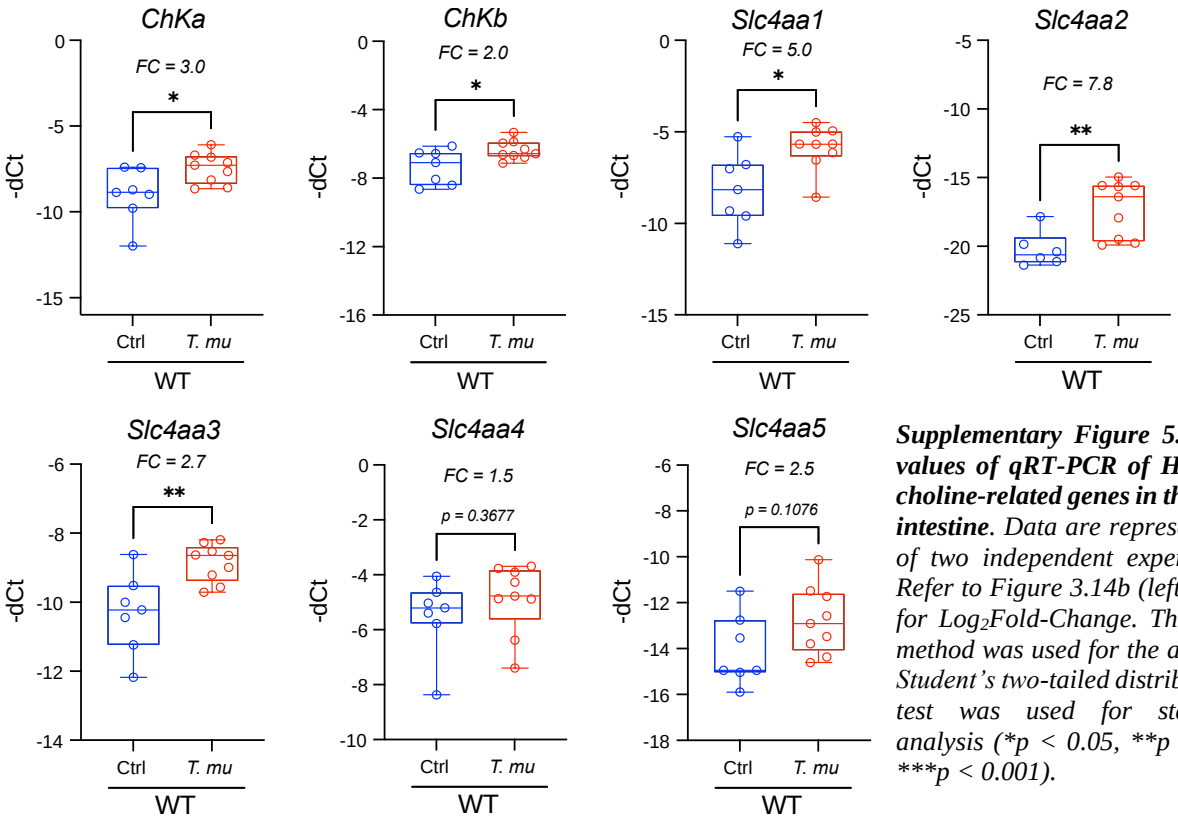
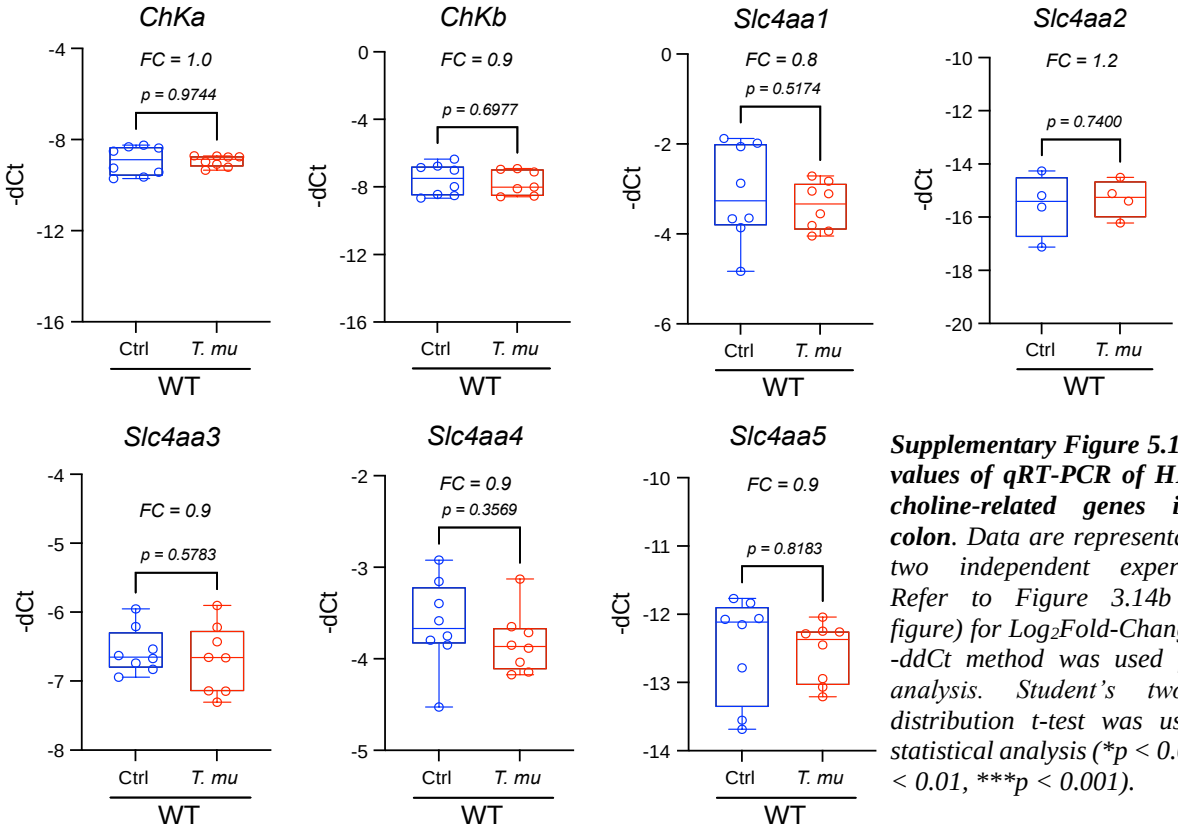
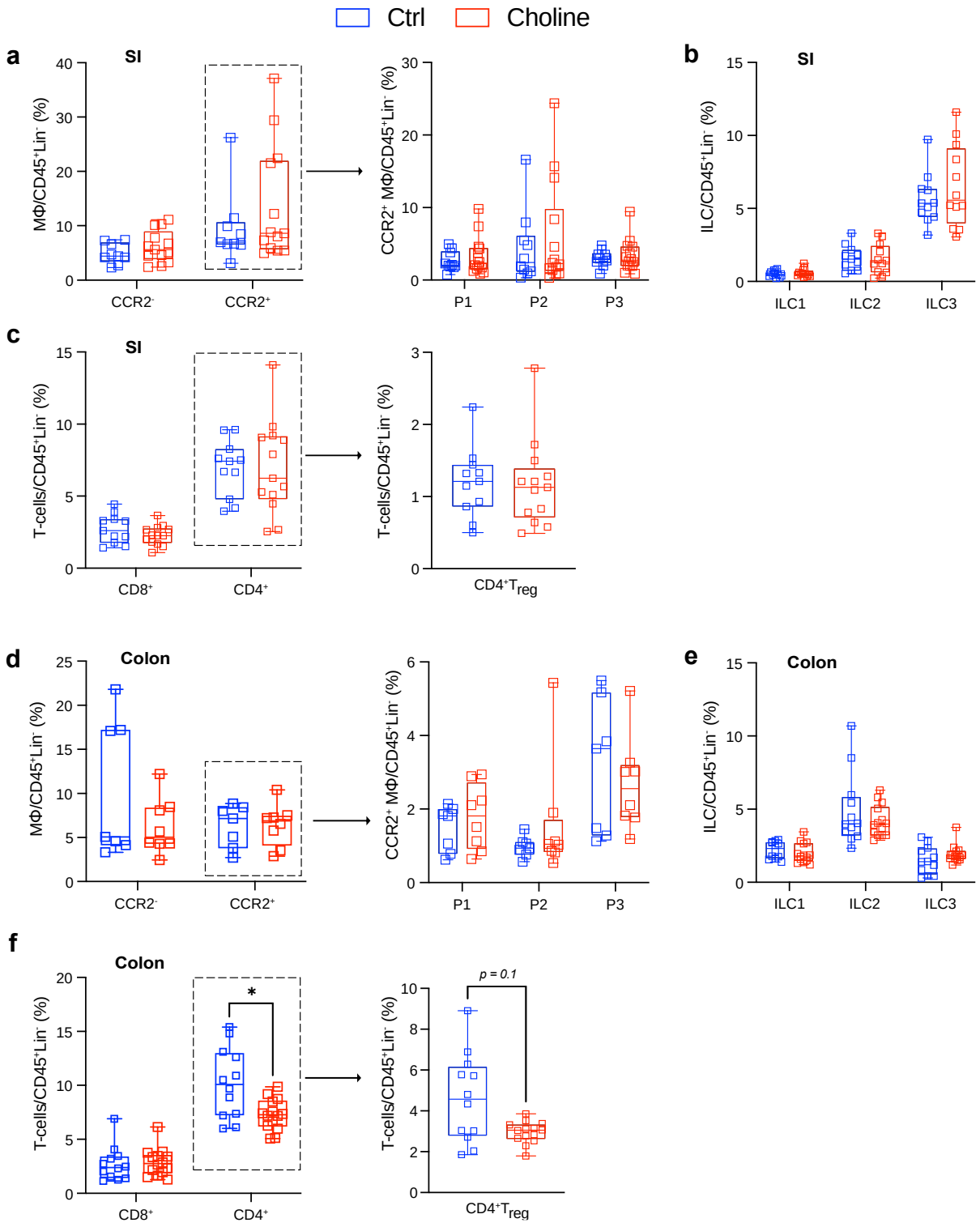


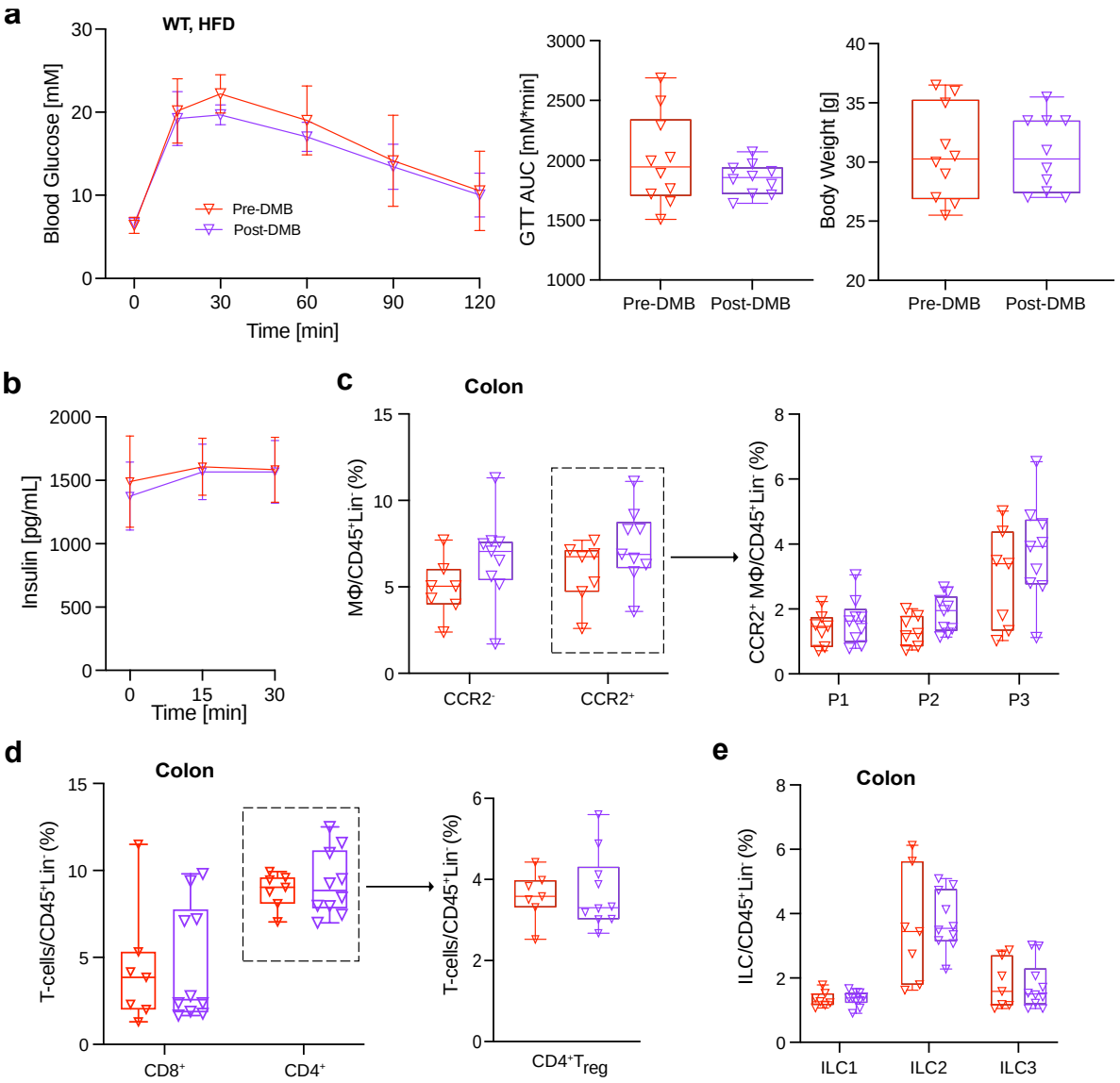
Supplementary Figure 5.9. -dCt values of qRT-PCR of HFD-fed choline-related genes in the small intestine. *Data are representative of two independent experiments. Refer to Figure 3.14b (left figure) for Log_2Fold-Change. The -ddCt method was used for the analysis. Student's two-tailed distribution t-test was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*



Supplementary Figure 5.10. -dCt values of qRT-PCR of HFD-fed choline-related genes in the colon. *Data are representative of two independent experiments. Refer to Figure 3.14b (right figure) for Log_2Fold-Change. The -ddCt method was used for the analysis. Student's two-tailed distribution t-test was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*



Supplementary Figure 5.11. In vivo administration of 0.1% (w/v) choline in drinking water of HFD-fed uncolonized C57BL/6N mice did not lead to a significant shift in pro-inflammatory mucosal immune cells. a and d) *Inflammatory CCR2$^+$ macrophages in distal small intestinal tissue* ***(a)*** *and colonic* ***(d)*** *and the further subclass distributions of Ly6C^{high}MHCII^{low} P1-P3 macrophage subpopulations.* ***b and e)*** *Frequencies of distal small intestinal* ***(b)*** *and colonic* ***(e)*** *innate lymphoid cells (ILCs). ILC1 = CD335$^+$Eomes$^-$, ILC2 = GATA3$^+$, ILC3 = RORγt$^+$.* ***c and f)*** *Frequencies of small intestinal* ***(b)*** *and colonic* ***(e)*** *CD3$^+$ T-cell populations. T-regulatory (T_{reg}) cells = CD4$^+$CD25$^+$FoxP3. Data are representative of three independent experiments. Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*



Supplementary Figure 5.12. Metabolic readouts comparison between pre- and post-DMB treatment and colonic mucosal immune cell phenotype in HFD-fed T. mu-colonized C57BL/6N mice. ***a)*** *Intraperitoneal glucose tolerance test (ipGTT), area under the curve (AUC) and body weight of HFD-fed T.mu-colonized C57BL/6N mice (pre-and post-DMB n = 10; age = 11-13 weeks).* ***b)*** *Circulating insulin levels measured during the first 30 minutes of the ipGTT.* ***c)*** *Inflammatory CCR2$^+$ macrophages in colonic tissue and the further subclass distributions of Ly6C^{high}MHCII^{low} P1-P3 macrophage subpopulations.* ***d)*** *Frequencies of colonic CD3$^+$ T-cell populations. T-regulatory (T_{reg}) cells = CD4$^+$CD25$^+$FoxP3$^+$.* ***e)*** *Frequencies of colonic innate lymphoid cells (ILCs). ILC1 = CD335$^+$Eomes$^-$, ILC2 = GATA3$^+$, ILC3 = RORγt$^+$. Statistical data are expressed as mean ± SEM. Data are representative of three independent experiments. Student's two-tailed distribution t-test (**a & b**) and Unpaired Mann-Whitney U test (**c, d & e**) with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

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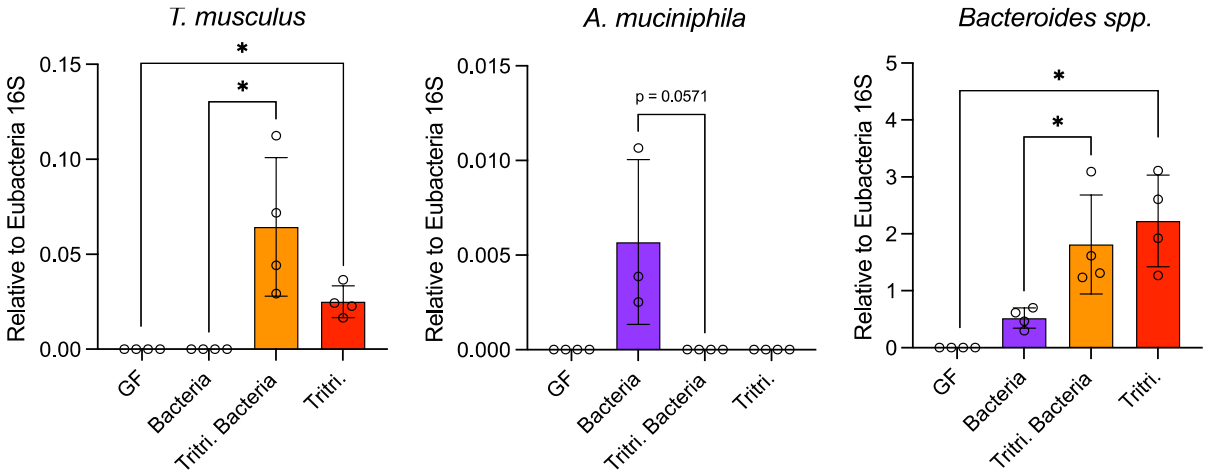

Supplementary Figure 5.13. qPCR analysis of T. musculus, A. muciniphila and Bacteroides spp. in the caecal content of germ-free mice. *The bacteria group represents GF mice colonized with the caeca bacterial communities isolated from uncolonized HFD-fed C57BL/6N mice. The Tritri. Bacteria group represents GF mice colonized with the caecal bacteria communities of T. mu-colonized HFD-fed C57BL/6N mice, following syringe filtration through 5.0 μm filters. The Tritri. group represents GF mice colonized with T. musculus isolated from the caeca content of HFD-fed T. mu-colonized mice, following in vitro culture of the protists. Refer to section 2.5 of Chapter 2 for more detailed explanation of the methodology. Statistical data are expressed as mean ± SEM. Data are representative of one independent experiment (n = 4/group). Unpaired Mann-Whitney U test with two-tailed distribution was used for statistical analysis (*p < 0.05, **p < 0.01, ***p < 0.001).*

Chapter 6

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Chapter 7

Acknowledgements

Embarking on a journey across continents to pursue higher education, distanced from the familiar faces of friends and family, marked the most challenging chapter of my life. The decision to pursue a doctoral degree materialized during my first semester of my first year at the University of Melbourne, Australia. Drawn to the remarkable accomplishments of renowned scientists, I delved into non-fiction scientific writings, fascinated by the historical dimensions of their achievements. The allure of understanding Nobel Laureates' journeys, from their humble beginnings to overcoming hardships, ignited a deep-seated passion to tread a similar path. Choosing Basel for my dream pursuit meant bidding farewell to my partner, friends, and family, a transition that never ceased to challenge me. Although I never fully adapted to life here, the promise of fulfilling my aspirations and returning triumphant to my loved ones fueled my resolve.

I express my gratitude to Dr. Claudia Cavelti-Weder for granting me the opportunity to pursue a PhD in the Translational Diabetes laboratory at the University of Basel. Despite the initial project's setbacks, the shift provided a much-needed boost of motivation and enthusiasm. With that said, I would like to extend my deepest gratification to past and present members of the group. Special shoutouts to Lena Keller and Zihan Ding, who has always been a dear friend of mine throughout my time here. Moreover, I would also like to thank members of Prof. Dr. Marc Donath's Diabetes Research group for their unwavering support and advice throughout the project. Furthermore, I express my gratitude to the members of my PhD Advisory Committee, Prof. Dr. Christoph Hess and Prof. Bart Deplancke, for their constant guidance and valuable insights provided during our annual meetings.

As I continued working on this project, we realized early on that we would require external expertise on the subject matter as we were inexperienced and not optimally equipped for some experiments. I would like to thank Prof. Christoph Schneider from the University of Zurich who was the first to advise us on this project and for his continuous support throughout. This project had given me the opportunity to collaborate with the foremost

experts in this field. The guidance and invaluable assistance regarding experiments provided by both Prof. Pascale Voenaesch (University of Lausanne) and Prof. Stephanie Ganal-Vonarburg (University of Bern) had helped me tremendously and for which I would always be appreciative of. Addtionally, I would like to thank Dr. Jeanne Tamarelle and Dr. Julian Garneau from Pascale's group in Lausanne for helping me out with the microbiota and sequencing analyses performed for this project.

For more than four years, I have been separated from home, navigating the challenges of immense time zone differences that have isolated me from familiar faces and voices. To my friends and family, thank you for all your support through all these years. A heartfelt appreciation to my Mom for consistently reaching out to check on me and make sure I was doing well. Lastly, to the love of my life, my high-school sweetheart, Si Ying Chan, thank you for always being there for me. I eagerly anticipate the moment when I can embrace you in my arms once more, and we can embark on the journey of spending the rest of our lives together, never to be parted again. The time has come for me to reunite with my loved ones and commence the next chapter of my life.

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Presentations and Posters

Poster: Seminar: Swiss Society of Endocrinology and Diabetes (SGED) 16-17 November 2023 Inselspital, University Hospital Bern, Switzerland – "Chronic colonization by the common mouse protozoa *Tritrichomonas spp.* instigates a persistent inflammatory gut microenvironment, impacting glycemic control and insulin secretion".

Poster: Seminar: Swiss Society of Endocrinology and Diabetes (SGED) 17-18 November 2022 Inselspital, University Hospital Bern, Switzerland – "Chronic colonization by the common mouse protozoa *Tritrichomonas spp.* instigates a persistent inflammatory gut microenvironment, impacting glycemic control and insulin secretion".

Poster: Seminar: Progress and New Approaches in Biomedical Research (DBM PhD Retreat) 22-24 September 2022 – "Chronic colonization by the common mouse protozoa common mouse protozoa *Tritrichomonas spp.* instigates a persistent inflammatory gut microenvironment, impacting glycemic control and insulin secretion".

Poster: Seminar: Cell symposium: Translational Immunometabolism 26-28 June 2022 University Hospital Basel, Switzerland – "Colonization by the common mouse and protozoa *Tritrichomonas spp.* modulates gut innate immunity, impacting glucose tolerance and insulin secretion".

Oral: Seminar: Swiss Society of Endocrinology and Diabetes (SGED) 11-12 November 2021 Inselspital, University Hospital Bern, Switzerland – "Colonization by the common mouse protozoa *Tritrichomonas spp.* modulates gut innate immunity and impacts glucose tolerance".

Poster: Seminar: Progress and New Approaches in Biomedical Research (DBM PhD Retreat) 8-10 October 2020 – “Air pollution mediates the development of type 2 diabetes via oral exposure by disrupting innate mucosal immunity”.

Poster: Seminar: Swiss Society of Endocrinology and Diabetes (SGED) 12-13 November 2020 Inselspital, University Hospital Bern, Switzerland – “Air pollution mediates the development of type 2 diabetes via oral exposure by disrupting innate mucosal immunity”.

Poster: Seminar: 23rd Annual Lorne Proteomics Symposium 1-4 February 2018 – “Charting the tumor suppressive signaling networks of CHK kinase in colorectal cancer cells with a novel tyrosine-specific phosphoproteomics approach”.