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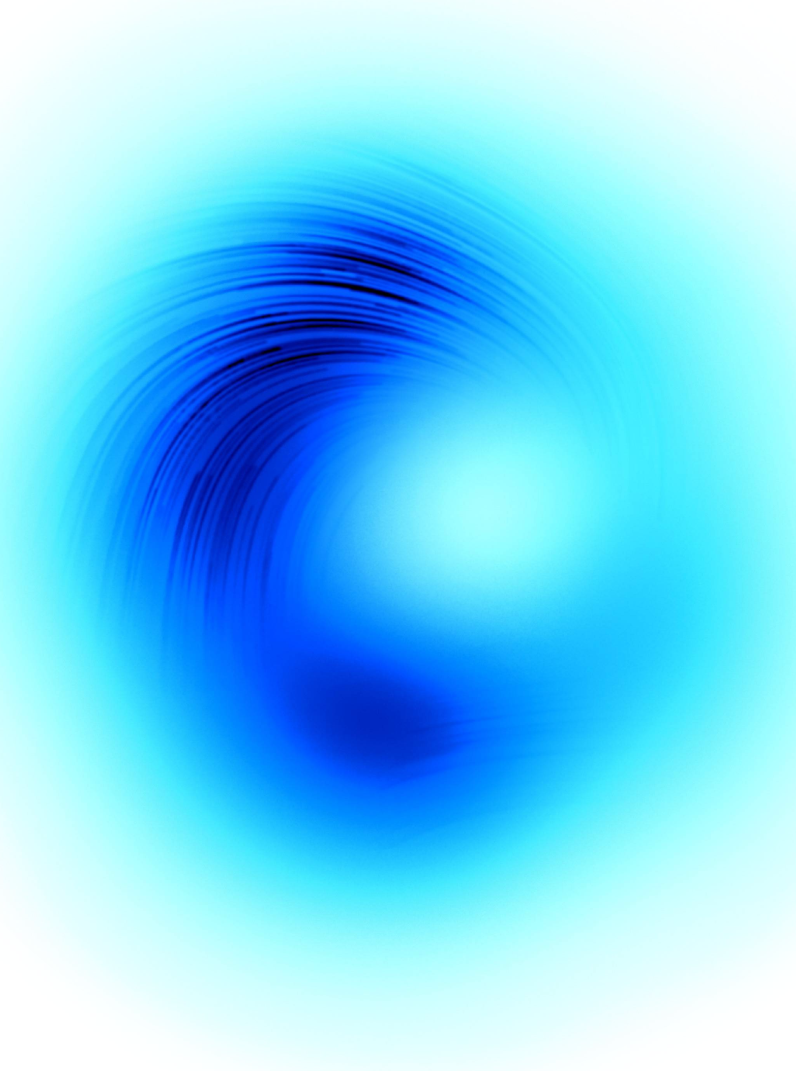

Saeed Katiraei

ROLE OF INTESTINAL MICROBIOTA IN CARDIO-METABOLIC DISEASES

Saeed Katiraei

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1

GENERAL INTRODUCTION

Saeed KATIRAEI

1.1. ROLE OF INTESTINAL MICROBIOTA IN CARDIO-METABOLIC DISEASES

THE obesity epidemic forms one of the major healthcare challenges of the current era and forms a global healthcare challenge [1]. Obesity is strongly associated with a disturbed glucose and lipoprotein metabolism and is also associated with a proinflammatory state [2, 3]. Obesity plays a causal role in a range of comorbidities like type 2 diabetes (T2D) and cardiovascular disease (CVD) [4]. One of the most prominent factors causing obesity is the modern lifestyle characterized by a disbalance between caloric consumption and energy expenditure due to an overall sedentary lifestyle. However, since not all obese individuals develop T2D or CVD, additional factors like genetic background and specific environmental exposures must contribute to the development of obesity and obesity associated diseases.

Within the past decade, our understanding of the intestinal microbiota, the microbes harbouring our gastrointestinal tract (GI-tract), has increased remarkably. It has been shown that intestinal microbiota are associated with different diseases, and that they play a role in our metabolism and immunity [5, 6].

Our intestinal microbiota are the cumulative result of (early and late) environmental exposure, life style and genetics and are strongly implicated in the development of obesity and associated diseases [7, 8, 9, 10]. As such, they may explain at least a portion of the interindividual variability in susceptibility to T2D and CVD. In this thesis, we focus on the role of the intestinal microbiota in cardiometabolic disease. This was conducted by studying the effects of *A. municipia* on plasma lipids, immunity and neointima formation in the hyperlipidemic E3L.CETP mouse model and by studying the effects of cohousing in mice on immunity in the setting of bone marrow transplantation (BMT). Because of the inherent limitations and biases of the DNA sequencing technologies. In addition, we investigated the effects of different DNA sequencing technologies on the interpretation of experiment outcomes.

1.1.1. RISK FACTORS FOR CARDIOMETABOLIC DISEASE

CVD is a leading cause of death responsible for 32% of deaths globally in 2019 and the contribution of CVD to mortality is expected to increase over the next decades [1]. The main underlying cause of CVD is atherosclerosis, which is chronic deterioration of the heart and blood vessels, promoted by multiple risk factors including, dyslipidemia, high blood pressure, disturbed glucose metabolism and inflammation. The co-occurrence of metabolic risk factors that specifically increase the risk of T2D and CVD is termed the Metabolic syndrome (MetS). MetS is defined by the presence of three out of the five following factors: abdominal obesity, hypertriglyceridemia, hypertension, fasting hyperglycemia and low High Density Lipoprotein (HDL)-cholesterol [11]. The prevalence of MetS is currently increasing rapidly in parallel with the obesity epidemic.

Atherosclerosis is initiated by damage to the endothelium, which can be caused by dyslipidaemia, smoking and/or high blood pressure. Atherogenic lipoproteins, predominantly LDL and very low-density lipoprotein (VLDL) particles, accumulate in the vessel wall and after oxidation are taken up by macrophages via endocytosis [12]. Excess uptake of oxidized lipids causes macrophages to differentiate into foam cells [13]. Macrophages

and foam cells secrete pro-inflammatory mediators such as cytokines like TNF-α and IL-6, which activate other immune cells and result in exacerbation of inflammation. Vascular inflammation subsequently induces smooth muscle cell activation. Foam cells, smooth muscle cells, and accumulated lipoproteins together form lesions or atherosclerotic plaques, narrowing the vascular lumen [14].

Since atherosclerosis is driven by dyslipidaemia and local inflammation, it is likely exacerbated by a systemic pro-inflammatory state [15, 14]. Obesity is clearly associated with systemic inflammation. The obesity associated systemic inflammation originates at least partly from white adipose tissue (WAT) [16, 17]. Expansion of WAT leads to infiltration of immune cells like macrophages and T-cells in this tissue. These infiltrated immune cells express pro-inflammatory cytokines such as TNF-α and IL-6, that spill over in the circulation, resulting in systemic inflammation, that subsequently may aggravate atherosclerosis [18].

Another cause for the induction of systemic inflammation in obesity is leakage of bacterial components like lipopolysaccharides (LPS) from the intestine into the circulation [19]. Consumption of a high fat diet has been associated with dysfunction of the mucin producing goblet cells and degradation of the mucin layer in the gastrointestinal tract, which forms a physical barrier between the intestinal content and the system [19]. Consumption of a high fat diet also decreases intestinal tight junction protein content [20]. Tight junctions are intercellular junctions in the intestinal epithelial cell monolayer, which seal neighbouring epithelial cells together and control paracellular permeability of the intestinal cell monolayer [20, 21, 22]. Dysfunction of the mucin layer and disrupted tight junctions results in a so called 'leaky gut' and translocation of bacterial components like LPS into the portal vein blood and circulation [23, 19, 20]. This LPS binds to Toll-like receptors (TLRs) on various immune cells. Binding of LPS to TLRs leads to activation of immune cells and results in an (innate) immune response [24]. Studies in obese and diabetic mice have shown that restoration of the mucin layer thickness by bacterial interventions ameliorates metabolic endotoxemia induced inflammation [25, 26, 27]. Thus, leakage of LPS from the intestinal lumen into the circulation results in systemic inflammation, which may also promote atherosclerosis [25, 28].

1.1.2. MOUSE MODELS TO STUDY ATHEROSCLEROSIS

Wild type mice are relatively resistant to the development of atherosclerosis. That is because in mice, unlike in humans, plasma cholesterol is mainly present in the HDL fraction and not in the pro-atherogenic LDL or VLDL fraction [29]. However, several mouse models have been developed to study the effects of experimental interventions on atherogenesis. The Apolipoprotein E*3Leiden. Cholesteryl Ester Transfer Protein (E3L.CETP) mouse, expressing a mutated human apolipoprotein E (APOE) and the human Cholesteryl Ester Transfer Protein (CETP) gene, is a hyperlipidemic, diet sensitive, atherosclerosis model characterized by a human-like lipid and lipoprotein metabolism [29, 30]. On a cholesterol containing Western type diet, E3L.CETP mice develop atherosclerosis in the aortic root area after ~8-16 weeks. This model is particularly responsive to human drugs that target lipid metabolism [30].

In addition, more rapid models of vascular damage and response have been developed. One such model entails placement of a non-constricting polyethylene cuff around

the femoral artery of E3L.CETP mice on a Western type diet. Vascular remodelling with signs of accelerated atherosclerosis takes place in a relatively short time frame of 2–3 weeks in the cuffed femoral artery [31]. This model of cuff-induced neointima formation resembles both restenosis as it occurs after balloon angioplasty in humans as well as the very early steps of atherosclerotic plaque formation, as the lesions formed after cuff placement contain both smooth cells and macrophages that might become foam cells [32]. This model is particularly responsive to immune modulation [33].

1.1.3. BONE MARROW TRANSPLANTATION, A TOOL TO STUDY GENE FUNCTION IN ATHEROGENESIS

A widely used tool to study the role of immune cells in many immune-associated disorders such as atherosclerosis, is experimental bone marrow transplantation (BMT) in mouse models. With this technique, host hematopoietic cells are depleted by lethal total body irradiation (TBI) and replaced by donor bone marrow cells harbouring genetic alterations in a relevant inflammatory pathway [34]. Both the more natural and cuff-induced models of atherosclerosis development have been used in conjunction with bone marrow transplantation (BMT) to determine the effects of deletion or addition of a gene to the bone marrow compartment on atherosclerosis development in mouse models [35, 36].

Experimental BMT in mice is relatively easy, effective and cost-efficient. However, a drawback is that BMT, and in particular the lethal TBI that is part of the procedure, may induce metabolic disturbances *per se* such as decrease in body weight, reduced adiposity, reduced organ weight, reduced insulin secretion and glucose intolerance [37, 38, 39]. While BMT is a relatively easy and effective method to assess gene function, the BMT procedure has multilevel effects on the organism and affects metabolically important organs and processes that are involved in development and progression of atherosclerosis. Therefore, BMT might affect atherosclerosis development *per se*, which should be taken in consideration in experimental design and interpretation of experimental outcome.

1.2. MICROBIOTA

1.2.1. INTESTINAL MICROBIOTA

Over the past decade, our understanding of the mutual symbiotic relationship between mammals and microorganisms has evolved rapidly. The human microbiome project revealed that the human body is populated by hundreds of different bacterial species, collectively termed microbiota [40], harbouring our skin, body cavities and our internal organs like the gastrointestinal tract (GI tract) [41, 8]. The composition, complexity and abundance of microbiota are shaped by both the local micro-environment and environmental exposure and therefore differ at different body sites [42].

It is becoming clear that disruption of homeostasis of the intestinal microbiota (i.e. microbial dysbiosis) is associated with and is potentially causal in the development of various diseases in humans. Dysbiosis may not only play a role in intestinal diseases such as inflammatory bowel disease and ulcerative colitis, but also in diseases with a metabolic and an immune component such as type-2 diabetes [43, 44, 45].

Multiple factors like age, sex, lifestyle, medication use, host genetics, geography and

diet modulate and shape intestinal microbiota acutely and longitudinally. Diet is one of the most direct modulators and dietary intervention changes the activity and composition of intestinal microbiota already within 24 hours [46]. Individuals consuming a paleolithic diet like the Hadza hunter gatherers were shown to have a distinct GI tract microbiota composition as compared with European lean subjects [47]. It has also been shown that intestinal microbiota of lean and obese human individuals and mice differ. Obese individuals and obese mice have, in general, a less diverse microbiota and it has been shown that certain gut microbial patterns correlate with obesity [48, 7]. More recently, the first evidence for the causality of gastrointestinal microbiota in metabolic disease by direct interaction with the immune system, by either their metabolites or cell components, have been shown in animal studies [49, 50, 51]. For instance transplanting uncultured fecal microbiota of obese people to germ-free mice promoted obesity in these mice, showing the causal role of intestinal microbiota in obesity [52]. Although causality of intestinal microbiota in disease in different mouse studies has been shown, proving the causality of intestinal microbiota with disease in human subjects remains challenging.

1.2.2. BENEFICIAL INTESTINAL BACTERIA

Over the past decade, investigations of beneficial microorganisms that reside in the human gut have attracted much attention, in order to study the utilization of intestinal microbiota as a potential treatment agent for metabolic and intestinal inflammatory diseases. To this end, controversial approaches like Fecal Microbiota Transplantation (FMT) and less controversial approaches like oral administration of single bacterial specie have been used. The aim of FMT is to transplant healthy fecal microbiota to an individual with unhealthy fecal microbiota, while the aim of administration of single bacterial specie is to expose an individual to a beneficial bacterium.

Fecal Microbiota Transplantation is one of the oldest strategies for modulation of intestinal microbiota as it was applied in Traditional Chinese medicine back in the 4th century B.C. and described in western medicine in 1958 [53]. In humans, Fecal Microbiota Transplantation is achieved by blending donor fecal matter with saline solution to a homologous liquid solution. This liquid solution is delivered to the recipient either via a duodenal tube or via colonoscopy [54]. More recently, FMT studies have been conducted, using capsules containing fecal matter for oral administration [55]. FMT in rodents can be achieved by oral gavage of donor fecal material into the recipient or by co-housing. Since rodents are coprophagic, co-housing of a donor and a recipient rodent, results in fecal microbiota exchange [52]. Currently, FMT is applied as an effective therapy for the treatment of recurrent *Clostridium dificile* infection (CDI) in humans. Multiple studies have shown more than 90 percent efficacy in resolving recurrent CDI by FMT [56]. Animal studies have also demonstrated the ability of co-housing to alter the metabolic phenotype of the host [52].

Although FMT is an effective therapy for CDI and showed promising experimental outcomes in the onset of other inflammatory and metabolic diseases, it is a black box approach. FMT transplants several hundred or thousands bacterial species from the donor to the recipient and thus the procedure does not pinpoint which bacterial species are responsible for the beneficial outcome. Oral administration of a single bac-

terial specie rather than a complex bacterial population has also been extensively studied within the past decade. *Akkermansia muciniphila* (*A. muciniphila*) and *Faecalibacterium prausnitzii*(*F. prausnitzii*) are two potential beneficial bacterial species that have been subjected to multiple animal and human studies for their antiglycemic and anti-inflammatory properties [57, 58, 59, 60, 61].

A. muciniphila is a strictly anaerobic, gram-negative commensal bacterium of the human and mouse GI-tract and has been isolated from the human GI-tract. *A. muciniphila* degrades mucin and produces propionate and acetate [62]. Treatment with viable *A. muciniphila* decreased fat mass, restored mucus layer thickness, decreased endotoxemia and improved glycaemia in obese mice [26]. More recently, a clinical trial showed beneficial metabolic effects of *A. muciniphila* administration in overweight/obese insulin-resistant humans [63]. Administration of pasteurised *A. muciniphila* tended to reduce body weight and fat mass. Plasma total cholesterol, insulinemia were reduced and insulin sensitivity was improved compared to the placebo group [63]. Numerous in vivo and in vitro studies have addressed the anti-inflammatory properties of *F. prausnitzii*. *F. prausnitzii* exerts its anti-inflammatory properties via different mechanisms [64]. *F. prausnitzii* produces a 15kDa peptide named microbial anti-inflammatory molecule (MAM), which enhances the anti-inflammatory milieu in the gut by inhibition of pro-inflammatory cytokines such as IL-8, Th1 and Th17 [65, 66]. Furthermore, *F. prausnitzii* exerts its anti-inflammatory effects by inducing secretion of anti-inflammatory IL-10 and enhancing the intestinal barrier function via expression of tight junction proteins claudin-1 and claudin-2 [60, 67]. *A. muciniphila* and *F. prausnitzii* are examples of beneficial bacteria, which potentially can be used as a single bacterial species with therapeutic properties.

1.3. INTESTINAL MICROBIOTA AND THE IMMUNE SYSTEM

1.3.1. PHYSICAL AND IMMUNOLOGICAL BARRIERS OF THE INTESTINE

The gastrointestinal tract organ contains the largest number of microorganisms of all body sites. These microorganisms are comprised of viruses, fungi, parasites and, predominantly, bacteria [68]. The main purpose of intestinal bacteria is degradation of otherwise indigestible compounds and thus supply additional nutrients to the host [69]. Their concentration per gram luminal content increases from the upper intestine towards the colon [70]. To prevent exposure and migration of the intestinal luminal bacteria to the circulation, the lumen is confined by both physical and immunological barriers [71].

The first physical barrier or the extrinsic barrier is the mucus layer consisting of mucin glycoproteins, produced by goblet cells. The mucus layer prevents exposure of bacteria to the epithelium layer which forms the second physical barrier or the intrinsic barrier. The epithelial cells are circumferentially linked together by tight junctions making them impermeable for whole bacteria [72].

In addition to physical barriers, the intestinal lumen is also guarded by immunological barriers. The immune system has a variety of tools like anti-microbial peptides and immune cells belonging both to innate and adaptive immunity which can recognize and respond to microbiota and their components. The first immunological barrier is via secretion of anti-microbial peptides such as defensins and lysozyme by Paneth cells and

immunoglobulin alpha (IgA) by B cells into the mucus layer [73].

On top of secretion of antimicrobial compounds, different immune cells are able to recognize and eliminate microorganisms that have penetrated the intestinal epithelial layer and entered the underlying connective tissue called lamina propria, before they enter the circulation. Neutrophils which can phagocytose these intruders along with monocytes and T-cells are patrolling the lamina propria. Upon infection, inflammatory monocytes and T-cells can be recruited to the lamina propria [74]. It seems likely that the immune system can distinguish between symbiotic microorganism which are beneficial for the host and the pathogenic bacteria which might have a negative effect on the host health [71].

1.3.2. Intestinal microbiota interact direct and indirectly with host immune system

Intestinal microbiota interact directly with antigen presenting immune cells (APC), such as dendritic cells (DCs). Pattern recognition receptors (PRR) like the TLRs recognize a variety of microbial components and allow (innate) immune cells to sense pathogen associated molecular patterns (PAMPs). These receptors are expressed on the intestinal epithelial cells (IEC) and the APCs [74]. Dendritic cells are also able to sense the intestinal lumen directly by protruding dendrites and take up epitopes from the intestinal lumen. The APCs migrate to secondary lymphoid organs like the Peyer's patches or mesenteric lymph nodes (MLNs) for antigen presentation to T cells, which results in T cell differentiation. T cells can differentiate into anti-inflammatory regulatory T cells (TReg) or pro-inflammatory T helper 1 (TH1) and TH17 cells [73].

Intestinal microbiota not only directly interact with the immune system, but also indirectly via the metabolites they produce. Intestinal microbiota convert indigestible carbohydrates to Short Chain Fatty Acids (SCFAs) which have been shown to interact with immune cells. Kim et all. demonstrated SCFA production in the intestine of mice after feeding them with a mixture of dietary fibres pectin and inulin. This was associated by antibody production in B cells [75]. SCFAs also seem to control gene expression to express molecules necessary for plasma B cell differentiation [75]. They also showed that increased SCFA levels not only affected local immunity in the intestine but also systemic immunity, because of increased numbers of IgA+ cells in mesenteric lymph nodes and the spleen.

Thus, the interaction of intestinal bacteria with the host does not only affect local immunity but also affects systemic immunity. Experiments in germ-free mice, born and raised in sterile conditions and therefore lacking intestinal bacteria, showed that the splenic lymphocyte population had decreased CD4+ T cell proportions compared to conventionally raised mice [76]. Mono-colonization of these germ-free mice with *Bacteroides fragilis* restored the splenic CD4+ T cell proportions. Therefore it can be concluded that intestinal microbiota play a crucial role in regulation of the systemic immune system. CD4+ T cells also play a role in atherogenesis as TH1 cells seem to be pro-atherogenic and Treg cells athero-protective [77].

1.4. NEXT GENERATION SEQUENCING

1.4.1. NEXT GENERATION SEQUENCING AS A TOOL TO PROFILE THE INTESTINAL MICROBIOTA AND MICROBIOME

Microbiota are defined as an ecological community of commensal, symbiotic and pathogenic microorganisms whereas the microbiome is defined as the collective genomes of these microorganisms [78, 40]. Studying the microbiome has been made possible with the rapid evolution of Next Generation Sequencing (NGS). Due to NGS technology, it is now possible to sequence billions of nucleotides in multiple samples in limited time and at limited cost. Roche 454 pyrosequencing was one of the first commercially available NGS platforms. Pyrosequencing technology yielded an approximately 100-fold increase in throughput over conventional Sanger sequencing, reducing sequencing time and cost significantly [79]. This technological improvement made the fast rise of the microbiome field possible. Currently, there are multiple NGS platforms available, with different specific properties like for e.g. differences in amplicon sequence length, sequencing time per run and sequencing depth (number of sequence copies per run), from different companies like Oxford Nanopore, Pacific Bioscience and Illumina.

Every gram of stool sample contains billions of bacteria belonging to hundreds of different taxa. In order to distinguish these bacterial taxa the 16s ribosomal RNA gene (16s rRNA) serves as a marker gene [80]. 16s rRNA gene consists approximately of 1500 base pairs with nine hypervariable regions. The hypervariable regions are 70 up to 250 base pairs long and each of them is flanked by highly conserved regions [81]. Due to the hypervariability of these regions, bacterial taxa within a sample can be distinguished by sequencing one or multiple hypervariable regions and aligning the sequences to a reference sequence.

1.4.2. 16S SEQUENCING AND METAGENOMIC SEQUENCING

For studying the bacterial composition of a sample, sequencing one or multiple V-regions of the 16s gene is sufficient to distinguish different bacteria. This strategy is called 16s V-region sequencing and is currently the most commonly applied strategy for studying bacterial communities [82]. However, 16s sequencing provides only information about the bacterial taxa, not about eukaryotes, viruses or their gene content. Furthermore, 16s sequencing has a limited taxonomic resolution (~genus level) because of the relatively short sequenced amplicon and overlap in these sequences between different species.

For studying microbiomes, metagenomic sequencing has a substantially higher taxonomic resolution. With metagenomic sequencing, all the DNA present in the sample is sequenced. When using Illumina technology this will result in short reads. Subsequently, genomes are reassembled from these short reads and aligned against reference databases. Reads from the same genome are merged into a single contiguous sequence or so called contig [83]. After assembly, genes are predicted and functionally annotated using a sequence database such as for example KEGG [83]. Since metagenomic sequences deal with all the DNA present in the sample and genomes are reassembled from the sequence DNA it is also possible to identify eukaryotes, viruses and their gene content. Metagenomic sequencing provides far more information about the microbiota and their genomic potential compared to 16s sequencing. However it is a more compu-

tational and cost expensive strategy.

1.5. OUTLINE OF THE THESIS

As introduced above, atherogenesis and CVD are promoted by multiple risk factors including dyslipidaemia, high blood pressure, disturbed glucose metabolism and inflammation. Intestinal microbiota have been associated with many of these CVD risk factors. In this thesis, we set out to investigate the role of intestinal microbiota in cardiometabolic disease and its underlying risk factors.

We used different strategies for manipulation of intestinal microbiota and studied the effects of modified intestinal microbiota on cardiometabolic risk factors. In chapter 2 and chapter 3, we investigated the role of intestinal microbiota in bone marrow transplantation (BMT) studies, since the microbiota are more than likely severely affected by the drastic BMT procedure. We exploited natural coprophagy while co-housing BMT-treated and control mice, as a tool to cross-transfer fecal microbiota. Chapter 2 describes the effects of autologous BMT on metabolic parameters, since the BMT procedure itself was found to have pleiotropic organ-specific effects. In chapter 3, we show that the autologous BMT procedure itself triggers a pro-inflammatory immune response which is mediated and transferred via the intestinal microbiota from a BMT-treated mouse to its co-housed partner. These data are proof for the causality of intestinal microbiota in the modulation of the systemic immune response.

Specific intestinal microbiota have been proposed to play beneficial roles in the modulation of inflammation. Since fecal transplantation is not a selective manipulation strategy and potentially transfers multiple bacterial and eukaryotic strains, viruses and host material, we aimed to introduce a single bacterial strain as a selective manipulation strategy. In chapter 4, we modulated the intestinal microbiota directly by oral administration of *A. muciniphila* and studied its local and systemic immune modulating properties and determined whether *A. muciniphila* administration would prevent neointima formation. Although lipid metabolism and the immune system showed signs of improvement by *A. muciniphila* administration, this was insufficient to ameliorate neo-intima formation. In chapter 5, we focussed on strategies to analyse the bacterial composition of the microbiota. Using two different sequencing platforms, we studied whether full length versus V4-region sequencing of the 16S rRNA gene affected the interpretation of the bacterial composition. Our results do indicate that the sequencing platform and strategy indeed affect the results and interpretation of the bacterial composition of the microbiota before and after an intervention. The implications of the findings described in this thesis and the future perspectives of these findings are discussed in chapter 6.

1.6. ABBREVIATIONS

T2D	Type 2 diabetes
CVD	Cardiovascular disease
MetS	Metabolic syndrome
BMT	Bone marrow transplantation
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
LPS	Lipopolysaccharides
E3L.CETP	APOE*3Leiden.humanCholesteryl Ester Transfer Protein
APOE	Apolipoprotein E
CETP	human Cholesteryl Esther Transfer Protein
IgA	Immunoglobulin alpha
SCFAs	Short Chain Fatty Acids
APC	Antigen presenting cells
DC	Dendritic cell
MLN	Mesenteric lymph node
PRR	Pattern recognition receptors
PAMPs	Pathogen associated molecular patterns
IEC	Intestinal epithelial cells
TReg	Regulatory T cells
TH1	T helper 1
TLR	Toll-like receptor
GI tract	Gastrointestinal tract
A. muciniphila	*Akkermansia muciniphila*
NGS	Next Generation Sequencing
16s rRNA	16s ribosomal RNA gene

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2

BMT DECREASES HFD-INDUCED WEIGHT GAIN ASSOCIATED WITH DECREASED PREADIPOCYTE NUMBER AND INSULIN SECRETION

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Abstract

Experimental bone marrow transplantation (BMT) in mice is commonly used to assess the role of immune cell-specific genes in various pathophysiological settings. The application of BMT in obesity research is hampered by the significant reduction in high-fat diet (HFD)-induced obesity. We set out to characterize metabolic tissues that may be affected by the BMT procedure and impair the HFD-induced response. Male C57BL/6 mice underwent syngeneic BMT using lethal irradiation. After a recovery period of 8 weeks they were fed a low-fat diet (LFD) or HFD for 16 weeks. HFD-induced obesity was reduced in mice after BMT as compared to HFD-fed control mice, characterized by both a reduced fat (-33%; $p<0.01$) and lean (-11%; $p<0.01$) mass, while food intake and energy expenditure were unaffected. As compared to control mice, BMT-treated mice had a reduced mature adipocyte volume (approx. -45%; $p<0.05$) and reduced numbers of preadipocytes (-38%; $p<0.05$) and macrophages (-62%; $p<0.05$) in subcutaneous, gonadal and visceral white adipose tissue. In BMT-treated mice, pancreas weight (-46%; $p<0.01$) was disproportionally decreased. This was associated with reduced plasma insulin (-68%; $p<0.05$) and C-peptide (-37%; $p<0.01$) levels and a delayed glucose clearance in BMT-treated mice on HFD as compared to control mice. In conclusion, the reduction in HFD-induced obesity after BMT in mice is at least partly due to alterations in the adipose tissue cell pool composition as well as to a decreased pancreatic secretion of the anabolic hormone insulin. These effects should be considered when interpreting results of experimental BMT in metabolic studies.

2.1. INTRODUCTION

Overweight and obesity are presently affecting close to 50% of the adult population in many Western countries and are leading to an epidemic of associated metabolic comorbidities such as type 2 diabetes and cardiovascular diseases [1]. Obesity is associated with adipose tissue inflammation, characterized by abnormal production of proinflammatory cytokines and chemokines by adipocytes and infiltrating immune cells, which ultimately leads to chronic systemic inflammation [2]. Most of the immune cells are derived from bone marrow [3]. A widely used tool to study the role of immune cells in many immune-associated disorders is experimental bone marrow transplantation (BMT) in mouse models. With this technique, host hematopoietic cells are depleted by lethal total body irradiation (TBI) and replaced by donor bone marrow cells harbouring genetic alterations in a relevant inflammatory pathway [4].

Experimental BMT in mice is relatively easy, effective and cost-efficient. However, a drawback is that BMT, and in particular the lethal TBI that is part of the procedure, may induce metabolic disturbances per se. Poglio *et al.* [5] showed that mice have a lower body weight seven days post BMT and that irradiation dose dependently and acutely reduced white adipose tissue (WAT) fat pad weight by decreasing both adipocyte volume and number. Another study showed that BMT reduced adiposity in genetically obese ob/ob mice. These mice stopped gaining weight two months after TBI, while the control mice gained weight continuously [6].

We set out to investigate the mechanism underlying the metabolic phenotype of mice fed a low-fat normal diet (hereafter called LFD) or high-fat diet (HFD) after BMT, and focused on body composition as well as glucose and insulin metabolism. We show that BMT treatment indeed significantly decreased diet-induced obesity which involves altered characteristics of white adipocytes and a decreased insulin secretion in response to a HFD.

2.2. MATERIAL & METHODS

2.2.1. ANIMALS

Male C57BL/6 mice were purchased from Charles River (Maastricht, The Netherlands) and housed under standard conditions with free access to water and food. After a period of two weeks acclimatisation, half of the mice underwent syngeneic BMT. In short, mice received 8 Gy X-ray radiation using an Orthovolt and the day thereafter an intravenous injection in the tail with donor bone marrow cells. The donor mice were male C57BL/6 mice from similar age. All mice, both BMT-treated and non BMT-treated control mice, received antibiotics-water (Amfotericine B, Ciprofloxaci, Polymixin B) from 3 days before until 4 weeks after BMT. After 8 weeks recovery on chow diet, mice were fed low-fat normal diet (LFD; 10% energy from lard D12450B) or a HFD (60% energy derived from lard fat D12492, Research Diet Services, Wijk bij Duurstede, The Netherlands) for 16 weeks to induce obesity (n=7-12 per group). The experiment started with n=12 per group. During the study one mouse died from the BMT-treated HFD-group within the recovery period and 4 mice were euthanized during the HFD-feeding due to deteriorated general health condition. This resulted at the end in n=7 mice for the BMT-treated HFD-group and n=12 mice for the other 3 groups. Body weight was measured weekly during

the entire experiment. Lean and fat mass were monitored by MRI-based body composition analysis (Echo MRI, Echo Medical Systems, USA). Twenty-four weeks after BMT, mice were anesthetized by a subcutaneous injection of a mixture of Neurotranq, Midazolam and fentanyl. Mice were bled via the eye and the following organs were dissected: heart, liver, pancreas, thymus, spleen, skeletal muscle quadriceps, and white adipose tissue pads from the gonadal (gWAT, unilateral), subcutaneous (sWAT, unilateral) and visceral (vWAT) region. All experiments were approved by the animal ethics committee of the Leiden University Medical Center (protocol no. 121031).

2.2.2. INTRAVENOUS GLUCOSE TOLERANCE TEST

At 12 weeks LFD or HFD feeding, an intravenous glucose tolerance test (IVGTT) was performed. Prior to the IVGTT, mice were fasted for 6 hours. Blood samples were collected from the tail vein immediately before (t=0 min) and 2, 5, 15, 30, 90 and 120 minutes after intravenous injection with glucose (2 mg/g body weight). Plasma glucose concentrations were quantified using the Glucose Start Reagent Method according to manufacturer's instructions (Instruchemie, Delftzijl, The Netherlands). Plasma insulin and C-peptide levels were measured at 12 weeks LFD or HFD using the Ultra Sensitive Mouse Insulin ELISA Kit and Mouse C-Peptide ELISA Kit, respectively, according to manufacturer's instructions (Crystal Chem, Downers Grove, USA).

2.2.3. ADIPOSE TISSUE CHARACTERIZATION

Adipose tissue from the gWAT (unilateral), sWAT (unilateral) and vWAT region were removed from the mice after 16 weeks of LFD or HFD and kept in PBS. The tissues were minced, digested with 0.5 g/l collagenase (Type 1) in DMEM/F12 with 20 g/l of dialyzed bovine serum albumin for 1 h at 37°C (BSA, fraction V; Sigma, ST Louis, USA), and filtered through a nylon mesh (236 µm pore). Adipocytes were obtained from the surface of the filtrate, and washed two times with PBS. Cell size and volume of mature adipocytes were determined from micrographs (approx. 1,000 cells per WAT sample) using image analysis software that was developed in house in MATLAB (MathWorks, Natick, MA). The adipocyte number per fat pad was calculated from the fat pad mass and adipocyte size.

Stromal vascular cells were isolated from the adipose tissue filtrate and fixed in 0.5% paraformaldehyde as described [7]. The number of stromal vascular cells per fat pad was determined using an automated cell counter (TC10, Biorad, CA, USA). The percentage of preadipocytes within the stromal vascular fraction was measured using flow cytometry. The stromal vascular cells were stained with fluorescently labeled antibodies for CD45, CD31, CD34 and F4/80 (BioLegend, CA, USA). Cells were measured on an LSR II flow cytometer (BD Biosciences, Breda, the Netherlands). Data was analyzed using FlowJo software (FlowJo, Oregon, USA). Preadipocytes were determined by selecting the CD45-CD31-CD34+ cells. Macrophages were determined by selecting the CD45+F4/80+ cells.

2.2.4. HISTOLOGICAL EXAMINATION OF THE PANCREAS

The pancreas was isolated, weighed and fixed in a random orientation in 4% paraformaldehyde and embedded in paraffin. Beta cell area and mass of n=5 mice per group were quantified as described before [8]. For the identification of beta cells, sections were immunostained with rabbit anti-insulin IgG (1:200 dilution; Santa Cruz Biotechnology,

Santa Cruz, CA, USA) for 1 hour followed by HRP- or AP-conjugated secondary antibodies (1:100 dilution) for 1 hour. Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. For determining the beta cell mass, 3-4 insulin-DAB stained sections (200 mm apart) were digitally imaged (Panoramic MIDI, 3DHISTECH, Hungary). The area of the beta cells within the pancreas were determined using an in house developed image analysis program (Stacks 2.1, LUMC, Leiden, The Netherlands), excluding large blood vessels, larger ducts, adipose tissue and lymph nodes. The area of clusters containing beta cells was individually measured and used to determine the average beta cell cluster area. Beta cell mass was determined by the percentage of beta cell area to pancreas area multiplied by the total pancreas weight, as described previously [8].

2.2.5. FAECAL TRIGLYCERIDES AND FREE FATTY ACIDS MEASUREMENT

Faecal samples were weighted and manually pulverized. Faecal powder was dissolved in a mixture of MilliQ water, methanol and hexane. Samples were vortexed, centrifuged at 14,000 g for 1 minute and the hexane supernatant was transferred into a clean tube. Additional hexane was added. After again vortexing and centrifuging at 14,000 g for 1 minute, the hexane fractions were transferred into new clean tubes. The hexane was evaporated using N2. Triton-chloroform was added to the pellets and the samples were incubated 10 minutes at 37 °C. Triglycerides were measured using the enzymatic kit 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA). Free fatty acids were measured using Wako NEFA-C kit from Wako Diagnostics (Instruchemie, Delfzijl, The Netherlands)."

2.2.6. STATISTICS

Data are presented as means ± SD. BMT-treated mice were compared to control mice using unpaired student T-test analysis. Correlation analysis was performed using linear regression analysis. The regression lines of the BMT-treated mice versus control mice were compared to see whether the correlations differed between BMT-treated and control mice. First it was tested whether slopes of the lines differed and then whether intercepts of the lines differed. When the slopes and intercepts were not significantly different, linear regression analyses was performed on pooled data of both groups,. All statistical analyses were performed using GraphPad Prism version 6 (GraphPad software, San Diego, CA, USA).

2.3. RESULTS

2.3.1. AFTER LFD OR HFD, BODY WEIGHT GAIN, BUT NOT ENERGY EXPENDITURE AND FOOD INTAKE, IS DECREASED IN BMT-TREATED MICE

We first determined the effects of LFD or HFD on body weight gain and body composition as well as on food intake and energy expenditure in mice that had undergone a syngeneic BMT versus controls. The BMT procedure caused a reduction in body weight already within a few days after BMT while mice were fed chow diet (Figure 2.1A and B). After the diet switch to LFD or HFD at t=0 weeks, the body weight gain diverged between BMT-treated and control mice (Figure 2.1A and B). This was most apparent for HFD-fed BMT mice. Analysis of body composition by Echo-MRI showed decreased lean mass (Figure 2.1C) and fat mass (Figure 2.1D) both on LFD and HFD in BMT-treated mice as compared to controls. However, the decreased body weight gain was mainly due to decreased fat mass expansion, especially in the HFD-fed BMT-treated mice. Individual indirect calorimetry measurements using metabolic cages revealed that the decrease in body weight gain could neither be explained by decreased food intake nor by increased energy expenditure in LFD- or HFD-fed BMT-treated mice. There was no difference in fat oxidation or carbohydrate oxidation between BMT-treated and control mice (Figure S2.1).

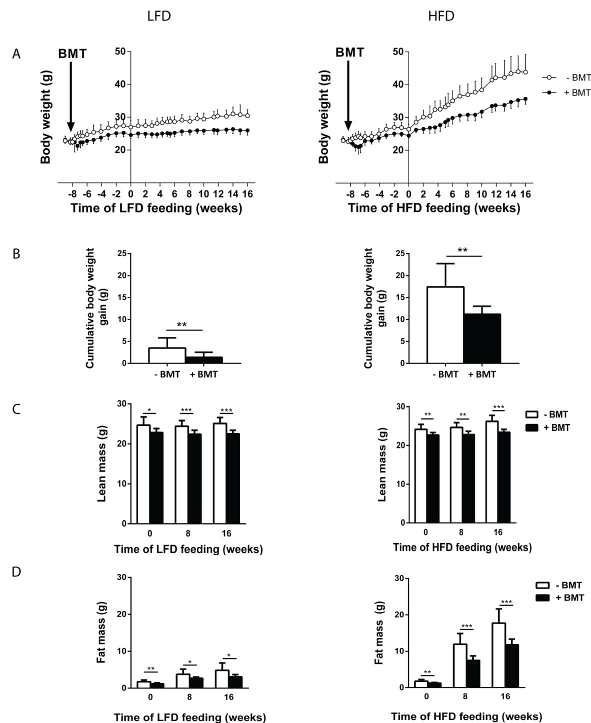

Figure 2.1: BMT decreased diet-induced obesity. (A) Male C57BL/6 mice underwent BMT at time point 0 weeks. From 0 weeks until week 8, mice were fed a chow diet and from time point 8 weeks, mice were fed either a 10% LFD. (B) Body weight gain of BMT-treated mice, over a period of 16 weeks LFD, was reduced compared to control mice. Values are means ± SD; n=7-12; * p<0.05,** p<0.01, *** p<0.001.

2.3.2. LFD- AND HFD-INDUCED INCREASES IN WHITE ADIPOSE TISSUE WEIGHT, ADIPOCYTE SIZE AND MACROPHAGE INFILTRATION ARE DIMINISHED BY BMT TREATMENT

To investigate whether BMT affects adipose tissue after LFD or HFD, sWAT, vWAT and gWAT of BMT-treated and control mice were characterised. On LFD, the weights of all individual fat pads were reduced at the end of the study in BMT-treated mice as compared to control mice (Figure 2.2A). On HFD, the weight of sWAT and vWAT, but not gWAT, was reduced in the BMT-treated group as compared to control mice (Figure 2.2A). The adipocyte volumes in the various fat pads decreased in parallel with decreased fat pad weights (Figure 2.2B). The number of mature adipocytes per fat pad was not affected by BMT in the WAT pads neither on LFD nor on HFD (Figure 2.2C). However, FACS analyses revealed that in BMT-treated mice the absolute number of preadipocytes present in adipose tissue was lower in all fat pads on HFD and in the gWAT and vWAT on LFD, than in the controls (Figure 2.2D). In addition, the absolute numbers of macrophages per fat pad in almost all fat pads in mice on LFD and HFD was lower in BMT-treated mice than in controls (Figure 2.2E). A clear correlation was observed between gWAT fat pad weight and macrophage number per fat pad (Figure 2.2F). Interestingly, at similar fat pad weight, the number of macrophages was lower in BMT-treated mice than in control mice, as demonstrated by the downward shifted correlation line (comparison regression lines: slopes Fslopes=2.91, p=NS, Fintercepts=18.49; p<0.01). Collectively, these findings show that LFD- and HFD-induced WAT expansion is significantly impaired after BMT treatment.

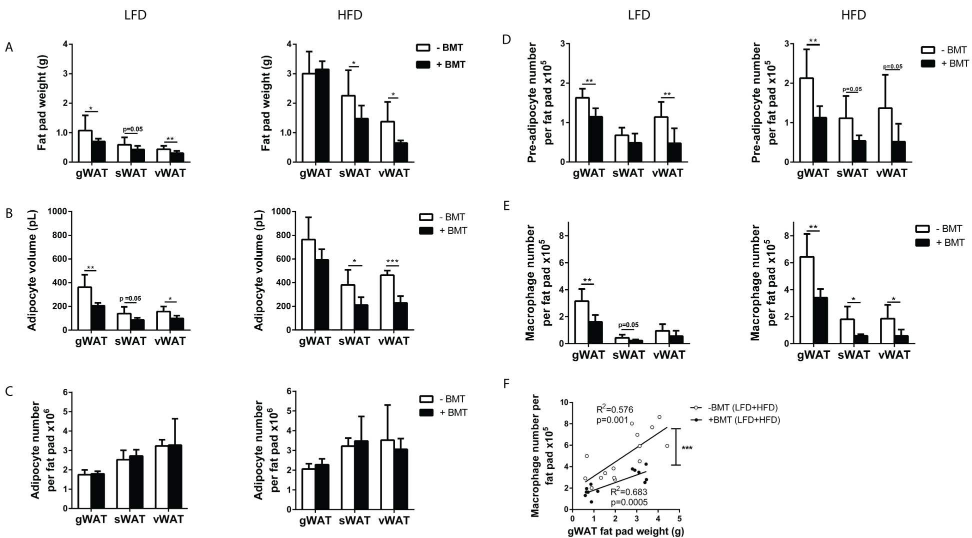

Figure 2.2: BMT has long term effects on white adipose tissue. (A) Weight of sWAT and vWAT pads was decreased in BMT-treated mice as compared to control mice. (B) The volume of mature adipocytes was lower in BMT-treated mice than control mice. (C) The number of mature adipocytes was not affected in the three WAT pads, (D) but the number of preadipocytes in all three WAT pads was lower in BMT-treated mice than control mice. (E) Macrophage number per fad pad was lower in the three fat pads of BMT-treated mice vs. control mice. (F) Linear regression analysis of macrophage number per fat pad and gWAT pad weight showed decreased macrophage numbers in gWAT in BMT-treated mice compared to control mice. Values are means ± SD; n=7-12; * p<0.05, ** p<0.01, *** p<0.001.

2.3.3. THE WEIGHT OF NON-ADIPOSE TISSUE ORGANS IN LFD- AND HFD-FED MICE IS DECREASED AFTER BMT TREATMENT

Further characterisation of organs revealed that in LFD- and HFD-fed mice, weight of liver, thymus and spleen was lower in BMT-treated mice than in controls (Figure 2.3A). Heart weight was lower in LFD-fed but not in HFD-fed BMT-treated mice. BMT treatment did not affect skeletal muscle quadriceps weight. Remarkably, both in LFD- and HFD-fed mice, pancreas weight was reduced by approx. 45% in BMT-treated mice ($p<0.01$) (Figure 2.3A). Liver, thymus, spleen and pancreas weight correlated with body weight (data not shown). However, BMT-treated mice had lower pancreas weights than control mice at similar body weights, as demonstrated by the significantly downward shifted correlation line (comparison regression lines: Fslopes=0.109, p=NS, Fintercepts=56.81; $p<0.01$; Figure 2.3B). This difference in organ weight relative to body weight between the mouse groups was not seen for liver, thymus and spleen. The pancreas was further characterized by histological examination. Surprisingly, total beta cell area or beta cell mass per total pancreas was not affected by BMT treatment (Figure 2.3).

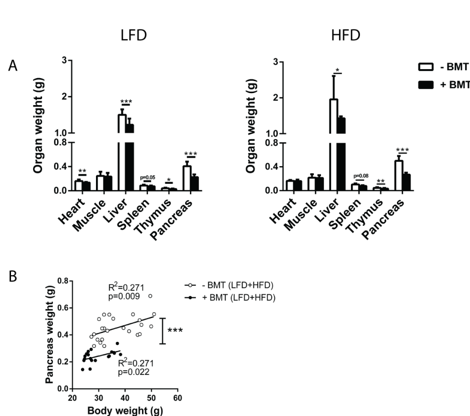

Figure 2.3: BMT decreased weight of different organs. (A) BMT decreased the weight of liver, thymus, spleen and the pancreas both on LFD and HFD. (B) Linear regression analysis of pancreas weight and body weight showed a lower pancreas weight in the BMT-treated mice, independently of body weight. Values are means ± SD; n=7-12; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

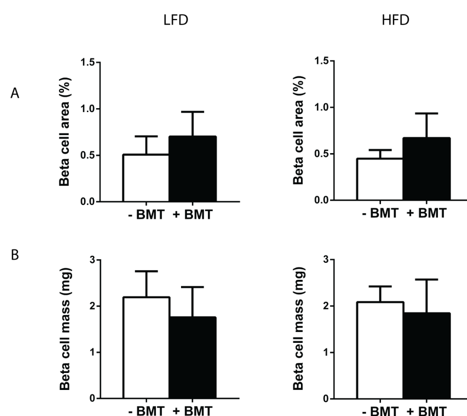

Figure 2.4: BMT did not affect pancreatic beta cell area and beta cell mass. (A) Beta cell area and (B) beta cell mass were not different in the BMT-treated mice. Values are means ± SD; n=5.

2.3.4. INSULIN PRODUCTION AND GLUCOSE TOLERANCE ARE LOWER IN HFD-FED MICE AFTER BMT TREATMENT

As pancreas weight relative to body weight was disproportionally reduced in both LFD- and HFD-fed mice after BMT treatment, we further assessed the effect of BMT treatment on glucose and insulin metabolism. Neither on LFD nor on HFD fasting plasma glucose concentrations were affected by BMT treatment (Figure 2.5A). To investigate whether BMT itself also affected fasting plasma glucose levels independently of body weight, linear regression analysis was performed on fasting plasma glucose levels versus body weight for BMT-treated and control mice separately. BMT-treated mice tended to have a higher fasting plasma glucose concentration with similar body weights (Figure 2.5B: comparison regression lines: Fslopes=0.065, p=NS, Fintercepts=3.72; p=0.06). Interestingly, fasting plasma insulin concentration was only lower in BMT-treated mice than in controls when they were fed a HFD (-68%; p<0.05; Figure 2.5C). These decreased insulin concentrations in the metabolically-challenged BMT-treated mice may be the result of a lower insulin production and, therefore, fasting plasma C-peptide levels were measured. Indeed, BMT reduced fasting plasma C-peptide levels compared to controls only in HFD-fed mice (-37%; p<0.01; Figure 2.5D). The pancreas weight positively correlated with C-peptide levels (R2=0.244; p<0.01; Figure 2.5E), indicating that the reduced pancreas volume in BMT mice was linked to lower insulin secretion. In line with reduced insulin secretion, HFD-fed BMT-treated mice were more glucose intolerant during an IVGTT than control mice, as reflected by an increased glucose clearance half-life (Figure 2.5F).

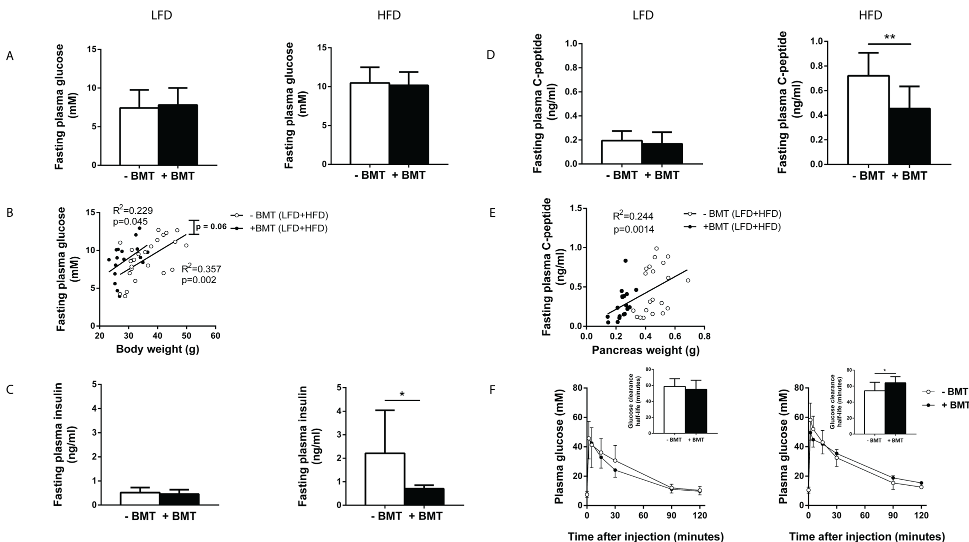

Figure 2.5: BMT mice had lower plasma insulin levels and glucose tolerance on a HFD. (A) Fasting plasma glucose of BMT-treated and control mice did not differ on LFD or HFD. (B) BMT-treated mice tended to have higher fasting plasma glucose concentrations for similar body weights. (C) BMT-treated mice had lowered fasting plasma insulin levels only on HFD. (D) BMT-treated mice had lowered plasma C-peptide concentrations on HFD. (E) There is a linear relation between pancreas weight and fasting plasma C-peptide concentrations. (F) IVGTT revealed that BMT-treated mice have a higher plasma glucose clearance half-life, only on HFD. Values are means ± SD; n=7-12; * p<0.05, ** p<0.01.

2.4. DISCUSSION

The current study shows that BMT induces long-term metabolic abnormalities in mice, with decreased body weight gain upon LFD and, especially, upon HFD feeding. This reduced body weight gain was mainly due to reduced fat mass gain, and to a lesser extent due to reduced lean mass gain. BMT-treated mice displayed decreased adipocyte hypertrophy both on LFD and HFD, whereas adipocyte hyperplasia was not affected 24 weeks after BMT. In addition to reduced adipose tissue, BMT led to a decreased size of other (metabolic) organs such as liver, thymus, spleen and pancreas, which, except for the pancreas, was in line with the reduction in body weight. The pancreas weight disproportionally decreased after BMT and likely explains the decreased plasma insulin levels and insulin secretion on a HFD after BMT.

We observed smaller adipocytes in BMT-treated mice, which could not be explained by a reduced food intake or increased energy expenditure as determined by indirect calorimetry. The smaller adipocytes might be explained by alterations in preadipocyte function. Mature adipocytes are terminally differentiated cells which originate from preadipocytes present in adipose tissue [9, 10, 11]. Recently, Nylander *et al.* [12] showed that a single sub-lethal irradiation dose of 6 Gy attenuated the adipogenic potential of preadipocytes via alterations in intracellular and epigenetic pathways. A reduced preadipocyte differentiation capacity may explain the reduced mature adipocyte volumes in our BMT-treated mice.

Our study revealed that also the number of preadipocytes per fat pad was reduced in the BMT-treated mice, suggesting that the preadipocyte pool used for recruitment of mature adipocytes was decreased by BMT. As both LFD and HFD stimulate energy storage and therefore likely induce preadipocyte recruitment, a decreased preadipocyte pool may also have contributed to the decreased adipose tissue growth seen in BMT-treated mice. The mechanism underlying the lower preadipocyte numbers in BMT-treated mice remains to be determined. It is possible that the TBI procedure has depleted preadipocytes or the mesenchymal stem cells in adipose tissue that are believed to be the precursor cells of the preadipocytes.

Notably, several studies have suggested that progenitor cells derived from bone marrow can contribute to the development of new adipocytes both in mice [13, 14] and humans [15]. This population of bone marrow cells may have been affected by the BMT procedure and thus also contribute to the reduced adipogenesis. Infiltration of bone marrow-derived macrophages into WAT tissue is strongly associated with fat pad expansion [16]. Interestingly, the macrophage content in WAT fat pads after BMT was disproportionally reduced. Thus, BMT not only affects the response of adipocyte precursor cells to LFD and HFD, but also the immune cell composition in the adipose tissue. Since immune cells play a major role in adipose tissue biology [16, 17], our data imply that BMT may also affect WAT function via modulation of the immune status in this tissue.

The weight of organs is in general strongly associated with body weight. In accordance, obesity induces changes in body composition and increases in organ weights [16]. BMT more severely reduced pancreas weight than expected based on the reduction in body weight both in mice on LFD as well as HFD. This was accompanied by a decreased insulin secretion and decreased plasma insulin levels in BMT mice as compared to control mice on HFD. The fact that we only observed this difference in insulin lev-

els on a HFD is explained by a HFD-induced increase in plasma insulin in control mice which was not seen control mice on LFD. BMT-treated mice were slightly more glucose intolerant than control mice on a HFD, despite a lower body weight. Pancreatic beta cell mass, however, was not affected by BMT treatment. Our data together suggest that BMT disproportionally affects the weight of the pancreas, resulting in reduced insulin responses to a HFD, and modest negative effects on glucose metabolism.

It was previously suggested that BMT leads to β-cell regeneration after acute streptozotocin (STZ)-induced injury in mice by triggering recruitment of immature bone marrow-derived cells to the injured pancreas. This recruitment, subsequently, stimulates stem/progenitor cells located in the recipient pancreas, resulting in islet regeneration. It was therefore suggested that BMT can be used as a novel approach to treat T2D [18]. Our results point to the opposite direction and suggest that BMT may lead to metabolic abnormalities, including reduced insulin secretion and glucose intolerance, which is specifically manifest on HFD. Interestingly, reduced pancreas volume was also observed in survivors of childhood acute lymphoblastic leukaemia who were treated with BMT [19]. In line with our study, several clinical studies suggests that BMT survivors have an increased risk of developing insulin resistance and T2D development [16, 10, 20].

In addition to producing the anabolic hormone insulin, the pancreas plays a major role in energy metabolism by secreting digestive enzymes such as pancreatic lipases into the gut where they serve to digest dietary lipids. As BMT reduced the pancreas volume, it is possible that the exocrine function of the pancreas was affected. Reduced absorption of lipids may thus in part explain the reduced body fat gain in BMT-treated mice. To elucidate whether there were differences in pancreatic lipid digestion, we measured faecal triglyceride and free fatty acids content. For faecal triglycerides we did not find differences between the BMT-treated and control mice, whereas for faecal free fatty acids there was a slight decrease in BMT-treated mice on HFD (Figure S2.2). These results indicate that pancreatic lipase production was not affected significantly by BMT. Whether BMT affects the function of other digestive pancreatic enzymes (such as pancreatic proteases) remains to be elucidated.

Taken together, we conclude that the BMT procedure has multilevel effects on the organism and affects metabolically important organs. These multi-level effects are associated with metabolic abnormalities that involve both WAT and pancreas dysfunction in response to a HFD. While BMT is a relatively easy and effective strategy for assessing the role of specific immune-related genes in pathophysiological studies in mice, the metabolic effects of BMT cannot simply be neglected and should be seriously considered in study design, goal and interpretation of the data. Our study may also have clinical implications, as BMT is conducted in humans to treat diseases such as leukaemia. Weight loss and insulin resistance should be closely monitored as potential side effects of this therapy.

2.5. ACKNOWLEDGEMENTS

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2.6. SUPPORTING INFORMATION

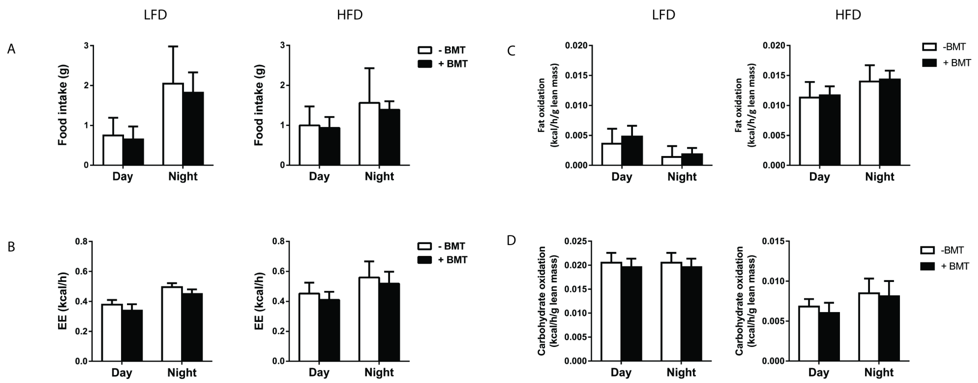

Figure S2.1: BMT did not affect food intake, energy expenditure, fat and carbohydrate oxidation. Indirect calorimetry data using metabolic cages (Phenomaster, TSE Systems, Bad Homburg, Germany) showed (A) Food, (B) energy expenditure, (C) fat oxidation and (D) carbohydrate oxidation of BMT-treated mice did not differed compared to control mice.

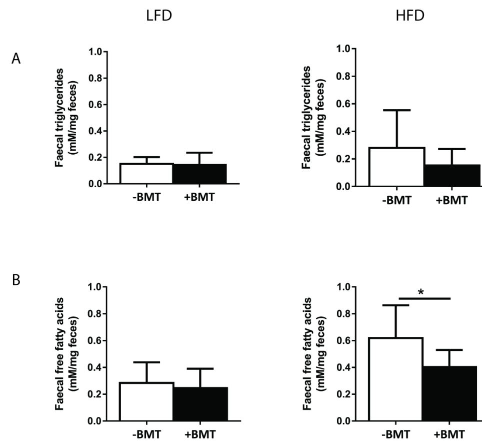

Figure S2.2: BMT did not affect faecal triglycerides content. (A) Faecal triglycerides concentration did not differed between BMT-treated and control mice. (B) There was slightly less free fatty acids in feces of HFD fed BMT-treated mice compared to the control group.

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3

Bone marrow transplantation induces changes in the gut microbiota that chronically increase the cytokine response pattern of splenocytes

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ABSTRACT

Bone marrow transplantation (BMT) involves conditioning regimens which acutely induce side effects, including systemic inflammation, intestinal damage and shifts in the gut microbial composition, some of which may persist chronically. As the gut microbiota affect systemic immune responses, we aimed to investigate whether, post-BMT, the peripheral immune system is modulated as a direct consequence of alterations in the gut microbiota. We show that 24 weeks post-BMT, splenocytes but not peritoneal macrophages display increased cytokine response patterns upon ex-vivo stimulation with various pathogens as compared to untreated controls. The pattern of BMT-induced cytokine responses was transferred to splenocytes, and not to peritoneal macrophages, of healthy controls via cohousing and transferred to germfree mice via transplantation of cecum content. Thus, BMT induces changes in gut microbiota that in their turn increase cytokine responsiveness of splenocytes. Thus, BMT establishes a dominant microbiota that attenuates normalization of the immune-response.

3.1. INTRODUCTION

The intestine is not only the site where food digestion takes place, but it is also home to more than 10^{14} commensal microorganisms that are collectively called the gut microbiota. The microbiota continuously interact with and provide benefits to the host, as they are involved in processes such as fermentation of indigestible fibres, metabolism of xenobiotics and regulation of the immune system. Studies in germfree mice that lack gut microbiota have provided insight into the role of gut microbiota in regulating the immune system. For example, germfree mice have a dysfunctional mucosal immune system with less and smaller Peyer's patches and mesenteric lymph nodes [1, 2]. These mice also have reduced numbers of intra-epithelial lymphocytes with a compromised immune function [3]. The gut microbiota are thus important for shaping a functional mucosal immune system.

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In addition to affecting the mucosal immune system, recent studies have demonstrated that the gut microbiota are important in the development and function of the immune system beyond the intestine. For example, the gut microbiota play a role in the pathogenesis of autoimmune disorders such as rheumatoid arthritis and type-1 diabetes [2, 4, 5]. Clarke *et al.* [6] reported that bacterial components translocate from the gut into the circulation under basal conditions and serve as mediators that systemically prime neutrophils in the bone marrow. However, the effects of the gut microbiota on other key lymphoid tissues such as the spleen have not been fully characterized. Germfree mice have reduced numbers of CD4+ cells and smaller germinal centers within the spleen as well as lower systemic antibody levels [4, 7]. Moreover, oral administration of dietary fibres or short-chain fatty acids (SCFA) to mice increases antibody responses by B cells in the spleen [8], which indicates that metabolites from the gut microbiota affect spleen function.

Bone marrow transplantation (BMT) is applied as therapy for patients with specific cancers of the bone marrow or blood, such as multiple myeloma or leukemia. To minimize residual disease, to create space for the transplant, and to achieve immune-ablation, BMT is preceded by conditioning regimens like total body irradiation (TBI) or chemotherapeutic agents. A drawback of these conditioning regimens is that they induce acute and chronic side effects. TBI not only ablates bone marrow cells, but may also cause acute damage to the gastrointestinal tract. This damage promotes the leakage of bacterial components from the gut into the systemic circulation. Bacterial components act as toll-like receptor ligands, activate immune cells and thus cause systemic inflammation [9]. This phenomenon may certainly explain some of the acute side effects of the BMT treatment.

However, BMT treatment not only resets the host immune system but also affects host microbiota [10]. The long term composition and activity of the intestinal microbiota is the result of intricate interactions between bacteria, host and environment. It is more than likely that BMT treatment thus results in permanently altered microbiota that may contribute to changes in the intra- and extra-intestinal immune system of the host [11].

In the current study, we investigated in mice the effect of BMT on the response patterns of splenocytes and peritoneal macrophages to various pathogenic stimuli as markers of the extra-intestinal immune system. Splenocytes are a mixed population of immune cells and thus represent responses from both the adaptive and innate immune sys-

tem, whereas peritoneal macrophages represent the innate immune system. The potential role of microbiota in the response patterns of splenocytes and peritoneal macrophages was investigated by co-housing control mice with BMT-treated mice and by transfer of cecum content to germfree mice. Our data show that the BMT-induced increase in the cytokine response pattern of splenocytes to pathogenic stimuli can be transferred via the gut microbiota.

3.2. MATERIAL & METHODS

3.2.1. BONE MARROW TRANSPLANTATION AND CO-HOUSING EXPERIMENT WITH MICE

Six week old male C57Bl/6J mice were purchased from Charles River (Maastricht, The Netherlands) and housed under standard conditions with free access to water and food. After a period of two weeks acclimatisation, half of the mice underwent syngeneic BMT. Already before the BMT procedure and during the whole experiment, the BMT-treated mice were co-housed with BMT-treated mice or healthy control mice. This resulted in four different experimental mouse groups: 1) healthy control mice co-housed with healthy control mice, 2) BMT-treated mice co-housed with BMT-treated mice, 3) BMT-treated mice co-housed with healthy controls, 4) healthy controls co-housed with BMT-treated mice. For the BMT procedure, mice received 8 Gy X-ray radiation using an Orthovolt and the day thereafter they received an intravenous injection with donor bone marrow cells in the tail vein. The donor mice were male C57Bl/6J mice of similar age. All mice, both BMT-treated and control mice, received water containing antibiotics (Amphotericin B, Ciprofloxacin, Polymyxin B) from 3 days before until 4 weeks after BMT and were exposed to the same dietary regimen. After 8 weeks recovery on chow diet, mice were fed a low-fat diet (10% energy derived from lard fat; D12450B, Research Diet Services, Wijk bij Duurstede, The Netherlands). Body weight was measured weekly during the entire experiment. Fresh fecal samples from individual mice were obtained 1 week before and 24 weeks after BMT by colon massaging. Each fecal sample was separately stored in a cryovial and snap frozen in liquid nitrogen immediately after collection and subsequently stored at -80°C until time of genomic DNA isolation. Mice were euthanized after anesthetization by a subcutaneous injection of a mixture of Neurotranq, Midazolam and Fentanyl. The spleen, peritoneal macrophages and cecum content were collected. This study was part of a larger study of which recently the metabolic characterization was described 14. All experiments were approved by the animal ethics committee of the Leiden University Medical Center, Netherlands (protocol no. 121031), and conducted in accordance with the European directive 2010/63/UE.

3.2.2. EXPERIMENTS WITH GERMFREE MICE

Eight to twelve weeks old male germfree Swiss/NIH mice were obtained from Taconic Farms and kept in sterile flexible plastic isolators (Standard Safety Equipment) with free access to sterile water and food. Conventional Swiss/NIH mice were obtained from the local Animal Facility at Universidade Federal de Minas Gerais, Brasil. The cecum content of the mice from the four different experimental groups (1- healthy control mice co-housed with healthy control mice, 2- BMT-treated mice co-housed with BMT-treated

mice, 3- healthy controls co-housed with BMT-treated mice, 4- BMT-treated mice co-housed with healthy controls. All mice in the four groups received water containing antibiotics (Amphotericin B, Ciprofloxacin, Polymyxin B) from 3 days before until 4 weeks after BMT and were exposed to the same dietary regimen) were diluted in PBS (10% w/v) and administered by intragastric gavage (200 µL per mice) to the germfree mice. After two weeks of microbiota reconstitution, the colonized mice were euthanized to collect spleen and peritoneal macrophages. Spleen and peritoneal macrophages were also obtained from germfree mice and conventional mice of similar age.

3.2.3. *Ex-vivo* STIMULATIONS OF SPLENOCYTES

Spleen cells were isolated by gently passing spleens through a sterile 70 µm filter chamber. After washing with sterile PBS and centrifugation at 4°C (1,700 rpm for 10 min), cells were counted using a Z1 Coulter Particle Counter (Beckman Coulter, Woerden, The Netherlands), and subsequently cultured in 24-wells plates (Costar, Corning, the Netherlands) at 5×10^6 cells/well in RPMI-1640 containing 1 mM pyruvate, 2 mM L-glutamine, and 50 mg/L gentamicin, in the presence of 10% fetal calf serum (FCS). Different stimuli were added in a final volume of 1 mL. Splenocytes were stimulated with LPS 10 ng/mL, Polyhydroxyalkanoates (PHA) 10 µg/mL, Polyinosinic:polycytidylic acid (poly(I:C)) 50 µg/mL, heat killed *Candida Conidia* $1\text{x}10^6$/mL, heat killed *Salmonella Typhimurium* $1\text{x}10^7$/mL or heat killed *Staphylococcus Aureus* $1\text{x}10^7$/mL. Supernatants were collected after 48 h for IL-10 and TNF-α determination and after 120 h for IL-22 determination. Supernatants were stored at -80°C until concentrations of cytokines were measured.

3.2.4. *Ex-vivo* STIMULATIONS OF PERITONEAL MACROPHAGES

Peritoneal macrophages were isolated from mice by injecting 10 mL of ice-cold sterile PBS (pH 7.4) into the peritoneal cavity, as previously described [12]. After centrifugation and washing, cells were resuspended in RPMI-1640 culture medium containing 1 mM pyruvate, 2 mM L-glutamine, and 50 mg/L gentamicin. Cells were counted and cultured in 96-well round-bottom microtiter plates (Costar, Corning, The Netherlands) at 1×10^5 cells/well, in a final volume of 200 µL. The stimuli were the same as for the splenocytes except that instead of PHA, Pam3Cys was used at 10 µg/mL. Supernatants were collected after 24 h incubation and stored at -80°C for measurement of TNF-α and IL-6.

3.2.5. CYTOKINE MEASUREMENTS

Cytokine concentrations were measured in supernatants of the *ex-vivo* stimulation experiments. TNF-α concentrations were determined by a specific radioimmunoassay as previously described [13]. IL-6 was measured using a commercially available ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA). Similarly, IL-22 and IL-10 concentrations were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA). All according to the instructions of the manufacturer.

3.2.6. 16S rRNA GENE SEQUENCING AND DATA ANALYSIS

From the fecal samples, genomic DNA was extracted using phenol: chloroform: isoamylalcohol (25: 24: 1) (Invitrogen), precipitated with isopropanol, and washed with 70% ethanol. The DNA samples were sent to the Broad Institute of MIT and Harvard (Cam-

bridge, USA) for 16S rRNA gene sequencing. Microbial 16S rRNA gene was amplified targeting the hyper-variable region V4 using forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The cycling conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 60 sec, extension at 72°C for 5 min, and a final extension at 72°C for 10 min. Sequencing was performed using the Illumina MiSeq platform generating paired-end reads of 175 bp in length in each direction. Overlapping paired-end reads were subsequently aligned. Details of this protocol have been described previously [14].

Raw sequence data quality was assessed using FastQC, version: 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads quality was checked with Sickle, version: 1.33 (https://github.com/najoshi/sicklei) and low quality reads were removed. For visualising the taxonomic composition of the fecal microbiota and further beta diversity analysis, QIIME, version: 1.9.1 was used [15]. In brief, closed reference OTU picking with 97% sequence similarity against GreenGenes 13.8 reference database was done. Jackknifed beta-diversity of unweighted UniFrac distances with 10 jackknife replicates was measured at rarefaction depth of 20000 reads/sample.

3.2.7. METABOLITES MEASUREMENT BY 1H-NMR

Weighed cecum content samples (28.4 ± 8.9 mg) were mixed with 5 volumes of milliQ water and prepared and measured using 1H-NMR spectroscopy as described previously [16]. The identification of metabolites was performed using the databases from Bruker (Bruker Biospin Ltd.) and Chenomx (Chenomx NMR suite 8.2) and assignments were verified by 2D 1H-NMR experiments of selected samples. The quantification of metabolites was performed with the Chenomx software and quantities were corrected for sample weight [17].

3.2.8. STATISTICS

Data are presented as means ± SEM. Experimental groups were compared using Mann-Whitney U test. All statistical analyses were performed using GraphPad Prism version 6 (GraphPad software, San Diego, CA, USA).

3.3. RESULTS

3.3.1. THE CYTOKINE RESPONSE PATTERN OF STIMULATED SPLENOCYTES WAS INCREASED AFTER BMT AND TRANSFERRED TO HEALTHY CONTROLS VIA CO-HOUSING

To study the effect of BMT on the response patterns of splenocytes and peritoneal macrophages to various pathogenic stimuli and the potential role of microbiota herein, C57BL/6 mice underwent syngeneic BMT or sham procedure and were co-housed in three combinations to arrive at the following 4 groups: 1) healthy control mice co-housed with healthy control mice, 2) BMT-treated mice co-housed with other BMT-treated mice, 3) BMT-treated mice co-housed with healthy control mice, and 4) healthy controls co-housed with BMT-treated mice. Group 1 served as control for the other three groups. All mice, both BMT-treated and control mice, received water containing antibiotics and were exposed to the same dietary regimen. After recovery from the BMT procedure, mice were fed a low-fat diet for 16 weeks to determine the chronic effects of microbiota exchange via co-housing on the responses of splenocytes and peritoneal macrophages. At the end of the study, peritoneal macrophages were isolated and the spleen was harvested and cells were immediately used for *ex vivo* stimulation assays.

To assess the general health condition of the mice after BMT, we monitored body weight during the study. As reported previously [18, 19, 20], the BMT procedure reduced body weight already within a few days (Figure S3.1). The BMT-induced decrease in body weight was not restored by co-housing the mice with healthy controls and *vice versa* the body weight of the healthy co-housed controls was not affected by co-housing with BMT-treated mice.

Splenocytes of BMT-treated mice showed higher IL-10, IL-22 and TNF-α cytokine release as compared to healthy controls, when stimulated with various pathogenic stimuli such as LPS, Polyinosinic:polycytidylic acid (Poly(I:C)), *Candida Conidia* or *Salmonella Typhimurium* (outer light blue lines versus inner grey lines, Figure 3.1a). Similar IL-10, IL-22 and TNF-α cytokine response patterns were seen in the BMT-treated mice that were co-housed with healthy control mice (outer dark blue lines versus inner grey lines, Figure 3.1b), suggesting that co-housing with healthy controls did not rescue the hyperresponsive phenotype of the splenocytes induced by BMT. Strikingly, the healthy controls that were co-housed with the BMT-treated mice also showed a similar IL-10, IL-22 and TNF-α response pattern upon pathogenic stimulation (outer red lines versus inner grey lines Figure 3.1c), indicating that the BMT-induced responsiveness of the splenocytes was transferred to healthy controls via co-housing.

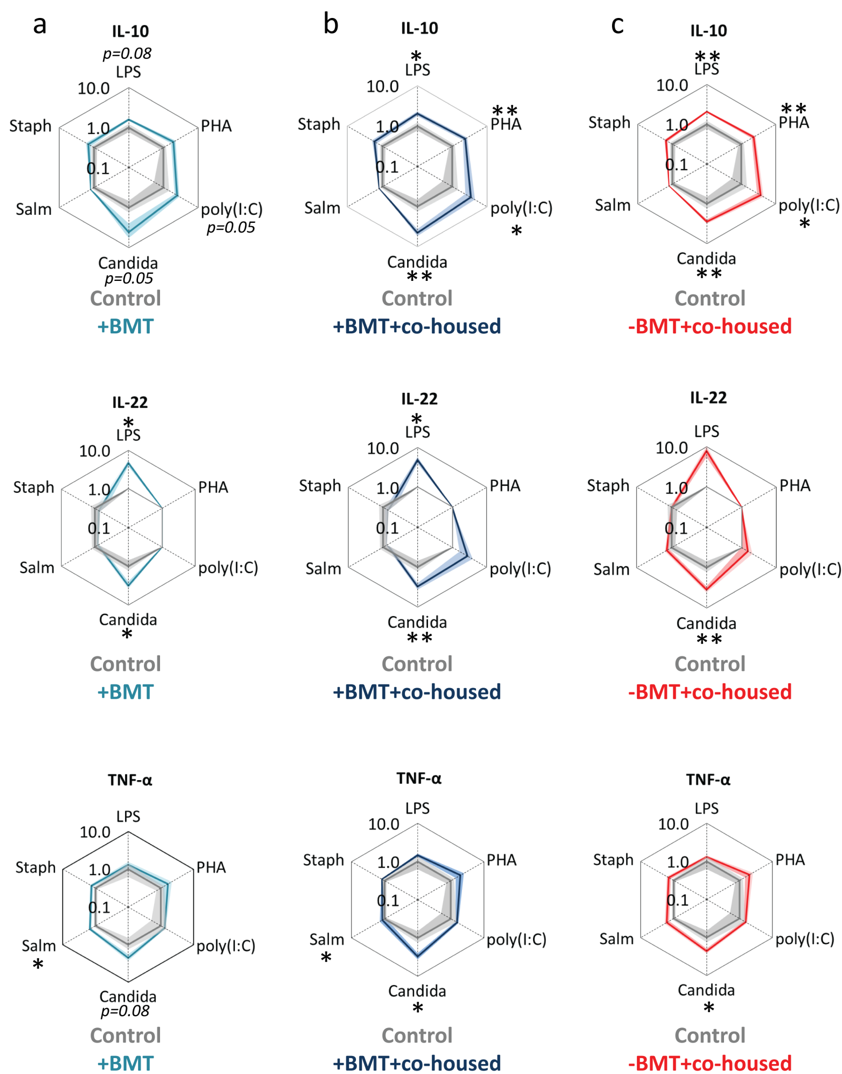

Figure 3.1: The effect of co-housing after BMT on cytokine secretion of stimulated splenocytes. Spiderplots show IL-10, IL-22 and TNF-α release in response to various pathogenic stimuli from splenocytes derived from (a) BMT-treated mice co-housed with BMT mice (outer light blue lines), (b) BMT-treated mice co-housed with healthy control mice (outer dark blue lines), and (c) control mice co-housed with BMT-treated mice (outer red lines). Cytokine concentrations in response to the stimuli are normalized to the cytokine concentrations of healthy control mice co-housed with control mice (inner grey lines), averaged per group and plotted on a log scale. Every corner of the spiderplot hexagon represents the response to one stimulus. The data lines and shades represent means and SEM, respectively; Groups were compared using Mann-Whitney U test; n=5-6 per group; $^*p<0.05$; $^{**}p<0.01$. Candida, *Candida Conidia*; LPS, lipopolysaccharide; PHA, Polyhydroxyalkanoates; poly(I:C), Polyinosinic:polycytidylic acid; Salm, *Salmonella Typhimurium*; Staph, *Staphylococcus Aureus*.

3.3.2. THE CYTOKINE RESPONSE PATTERN OF STIMULATED PERITONEAL MACROPHAGES WAS NOT AFFECTED AFTER BMT

Peritoneal macrophages of BMT-treated mice did not show increased cytokine release upon *ex vivo* stimulation with various stimuli, except for a tendency towards increased IL-6 release upon stimulation with *Salmonella Typhimurium* stimulation (outer light blue lines versus inner grey lines, Figure 3.2a). Peritoneal macrophages of BMT-treated mice that were co-housed with healthy control mice released more IL-6 after stimulation, which was significant for Pam3Cys, poly(I:C), *Salmonella Typhimurium* and *Staphylococcus Aureus* (outer dark blue lines versus inner grey lines, Figure 3.2b). Although the cytokine response pattern of splenocytes upon different pathogenic stimuli was similar between the two groups of BMT-treated mice (Figure 3.1a and b), the IL-6 cytokine response pattern of macrophages from co-housed BMT-treated mice was different (Figure 3.2b). For TNF-α secretion, no differences were observed between the different groups (Figure 3.2a-c). These data indicate that the cytokine response pattern of peritoneal macrophages upon stimulation was not transferred from BMT-treated mice to control mice via co-housing.

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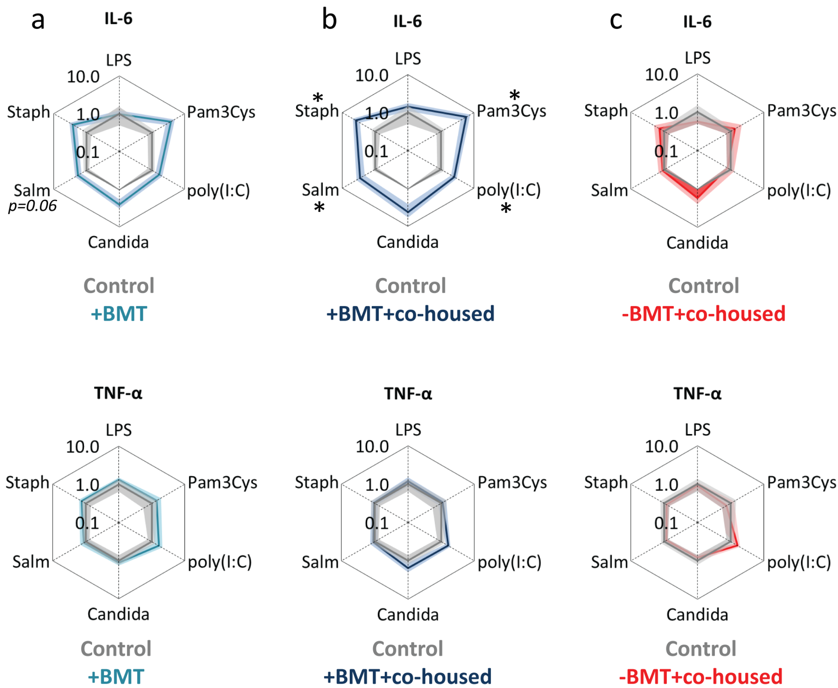

Figure 3.2: The effect of co-housing after BMT on cytokine secretion of stimulated peritoneal macrophages. Spiderplots show IL-6 and TNF-α release in response to various pathogenic stimuli from peritoneal macrophages derived from (a) BMT-treated mice co-housed with BMT mice (outer light blue lines), (b) BMT-treated mice co-housed with healthy control mice (outer dark blue lines), and (c) control co-housed mice co-housed with BMT-treated mice (outer red lines). Cytokine concentrations in response to the stimuli are normalized to the cytokine concentrations of healthy control mice co-housed with control mice (inner grey lines), averaged per group and plotted on a log scale. See legend to Figure 3.1 for more information. Groups were compared using Mann-Whitney U test; n=6; *$p<0.05$.

3.3.3. THE CYTOKINE RESPONSE PATTERN AFTER BMT WAS TRANSFERRED TO SPLENOCYTES OF GERMFREE MICE AFTER TRANSPLANTATION OF CECUM CONTENT

To determine whether intestinal microbiota were causally involved in the altered cytokine response pattern by splenocytes after BMT, germfree mice were inoculated with the cecum content of BMT-treated mice co-housed with or without control mice or with the cecum content of co-housed control mice. Splenocytes derived from germfree mice that were colonized with cecum content of BMT-treated mice as compared to those colonized with cecum content of control mice, secreted more cytokines after stimulation with various pathogenic stimuli, which reached significance for LPS (IL-22 and TNF-α), PHA (IL-10 and TNF-α), poly(I:C) (IL-10, IL-22 and TNF-α) and *Candida Conidia* (IL10 and TNF-α) (outer light blue lines versus inner grey lines, Figure 3.3a). A partly overlapping cytokine response pattern was observed in germfree mice inoculated with cecum content of BMT-treated mice co-housed with BMT mice and in germfree mice inoculated with cecum content of BMT-treated mice co-housed with control mice (outer light and dark blue lines versus inner grey lines, Figure 3.3a and b), with similar (trends in) responses of IL-10, IL-22 and TNF-α secretion to PHA stimulation. Remarkably, splenocytes derived from germfree mice colonized with cecum content of control mice co-housed with BMT-treated mice showed a cytokine response pattern very similar to splenocytes from germfree mice that were colonized with cecum content of BMT-treated mice (outer red lines and light blue lines versus inner grey lines, Figure 3.3a and c). The IL-10 response to PHA, poly (I:C) and *Candida Conidia* were identical, as was the TNF-α response to LPS and *Candida Conidia*. These data are in line with the results derived from the co-housing experiment and indicate that the increased responsiveness of splenocytes after BMT can be largely transferred by cecum content transplantation.

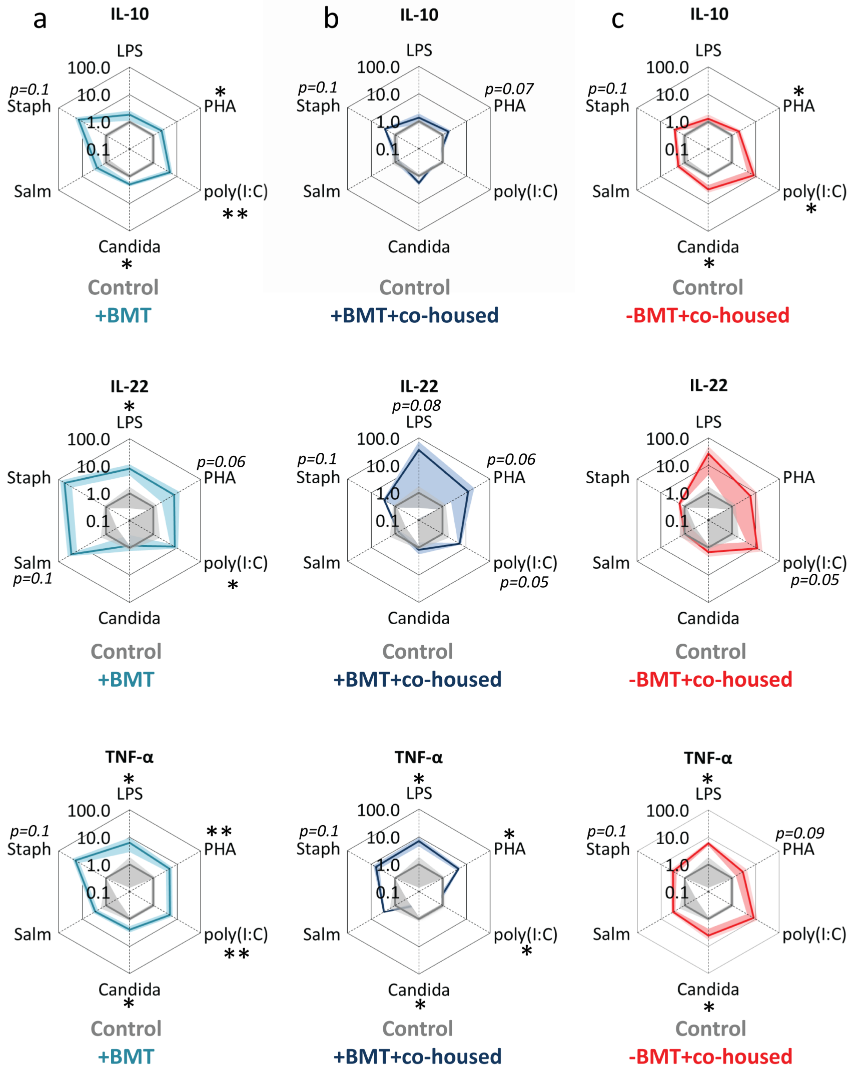

Figure 3.3: The effect of cecum content transfer after BMT on cytokine secretion of stimulated splenocytes. Spiderplots show IL-10, IL-22 and TNF-α release in response to various pathogenic stimuli from splenocytes derived from (a) germfree mice inoculated with cecum content samples of BMT-treated mice co-housed with BMT-mice (outer light blue lines), (b) germfree mice inoculated with cecum content samples of BMT-treated mice co-housed with control mice (outer dark blue lines), and (c) germfree mice inoculated with cecum content samples of control mice co-housed with BMT mice (outer red lines). Cytokine concentrations in response to stimuli are normalized to the cytokine concentrations of splenocytes from germfree mice inoculated with cecum content samples of control mice co-housed with control mice (inner grey lines). See legend to Figure 3.1 for more information. Groups were compared using Mann-Whitney U test; n=3-5; *p<0.05.

Peritoneal macrophages of germfree mice inoculated with cecum content of BMT-treated mice and co-housed BMT-treated mice did not show increased IL-6 and TNF-α secretion upon stimulation (outer light blue and dark blue lines versus inner grey lines, Figure 3.4a-c). Peritoneal macrophages of germfree mice inoculated with cecum content of co-housed controls only showed a minor increase in IL-6 secretion upon *Staphylococcus Aureus* incubation (outer red lines versus inner grey lines, Figure 3.4c). These data indicate that gut microbiota specifically stimulate cytokine secretion of splenocytes but not of peritoneal macrophages after BMT.

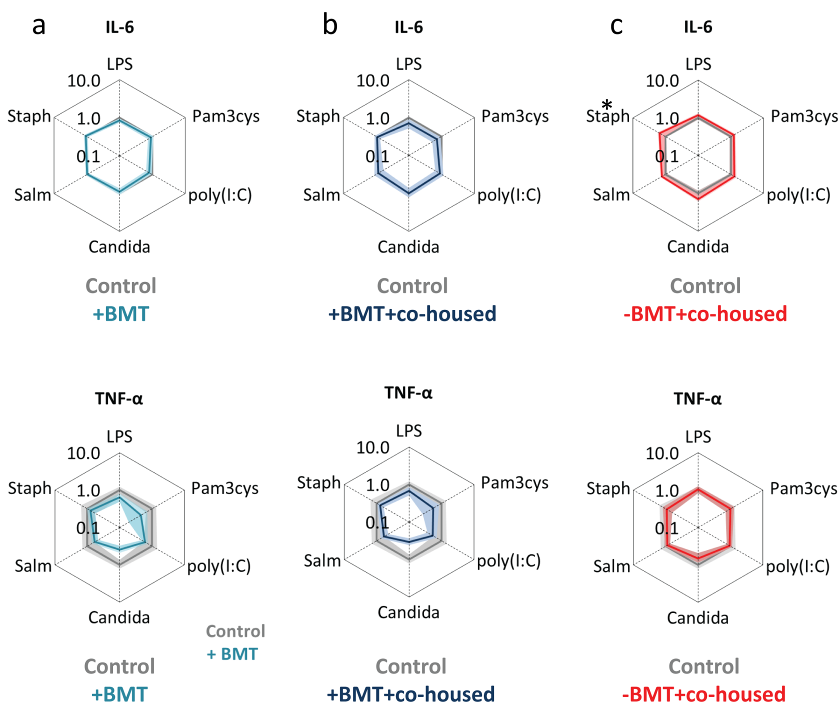

Figure 3.4: The effect of cecum content transfer after BMT on cytokine secretion of stimulated peritoneal macrophages. Spiderplots show IL-6 and TNF-α release in response to various pathogenic stimuli from peritoneal macrophages derived from (a) germfree mice inoculated with cecum content samples of BMT-treated mice co-housed with BMT-mice (outer light blue lines), (b) germfree mice inoculated with cecum content samples of BMT-treated mice co-housed with control mice (outer dark blue lines), and (C) germfree mice inoculated with cecum content samples of control mice co-housed with BMT mice (outer red lines). Cytokine concentrations in response to stimuli are normalized to the cytokine concentrations of peritoneal macrophages from germfree mice inoculated with cecum content samples of control mice co-housed with control mice (inner grey lines). See legend to Figure 3.1 for more information. Groups were compared using Mann-Whitney U test; n=4-5.

3.3.4. SPLENOCYTES OF GERMFREE MICE SHOWED LOWER CYTOKINE SECRETION COMPARED TO CONVENTIONAL MICE

To further investigate the role of gut microbiota in the responsiveness of splenocytes and macrophages, we compared cytokine response pattern of *ex-vivo* stimulated splenocytes and peritoneal macrophages derived from conventionally versus germfree raised mice. Splenocytes from conventional mice secreted more IL-10, IL-22 and TNF-α as compared to untreated germfree mice upon stimulation with LPS, Polyhydroxyalkanoates (PHA) and poly(I:C), and more IL-10 and IL-22 upon stimulation with *Salmonella Typhimurium* and *Staphylococcus Aureus* (outer pink lines versus inner purple lines, Figure 3.5a-c). These data indicate that the presence of gut microbiota increases the cytokine response pattern of splenocytes upon pathogenic stimulation. In contrast to splenocytes, there was no increase of cytokine secretion by *ex-vivo* stimulated peritoneal macrophages between conventional mice and untreated germfree mice (outer pink lines versus inner purple lines, Figure 3.5d-e). These data confirm that cytokine secretion of stimulated peritoneal macrophages is not affected by the presence of gut microbiota.

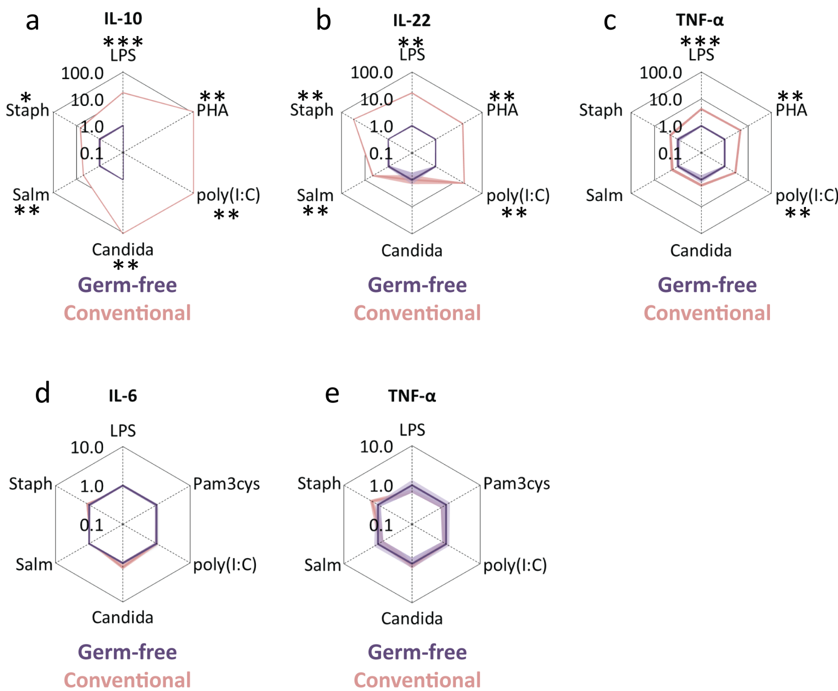

Figure 3.5: Cytokine secretion of stimulated splenocytes and peritoneal macrophages from germfree versus conventionally housed mice. Spiderplots show (a) IL-10, (b) IL-22 and (c) TNF-α release from splenocytes of germfree mice (inner purple lines) upon stimulation with different stimuli compared to splenocytes of conventional mice (outer pink lines). Spiderplots show (d) IL-6 and (e) TNF-α release of peritoneal macrophages of germfree mice (inner purple lines) upon stimulation, compared to conventional mice (outer pink lines). Cytokine concentrations in response to the stimuli are normalized to the cytokine concentrations of splenocytes of germfree mice (inner grey lines), averaged per group and plotted on a log scale. See legend to Figure 3.1 for more information. Values are means ± SEM; Groups were compared using Mann-Whitney U test; n=4-9; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

3.3.5. 16S SEQUENCING DID NOT SHOW CONSISTENT LONGITUDINAL EFFECTS OF BMT ON INTESTINAL MICROBIOTA COMPOSITION

To investigate potential changes in gut microbiota composition induced by BMT that may explain the increased cytokine response pattern of the splenocytes, we determined the intestinal microbial composition by 16S sequencing. We sequenced fecal samples 24 weeks post-BMT, which was just prior to the *ex-vivo* splenocyte stimulations and collection of the cecum contents. Bacterial taxa were not significantly different between BMT-treated and control mice, either with or without co-housing, (Figure 3.6a). There was a large inter-individual variation between the individual samples from the different groups as revealed by the PCoA plots of unweighted UniFrac distance (Figure 3.6b). Individual samples from the different treatment groups clustered together in three subclusters, but no clustering was observed based on treatment. The splenocyte responsiveness could thus not be linked to obvious BMT-induced differences in intestinal microbial composition as determined by 16S sequencing.

3.3.6. CO-HOUSING WITH BMT-TREATED MICE AFFECTED CECUM METABOLITE PROFILES OF HEALTHY CONTROL MICE

To investigate whether BMT induced alterations in gut microbial function that may explain the cytokine response pattern of the splenocytes, we measured 61 metabolites in cecum content samples by 1H-NMR. Metabolite concentrations were quantitatively measured and plotted as z scores in a heat map after unsupervised clustering of all mice (Figure 3.6c). The metabolite profile patterns showed considerable heterogeneity between the four experimental groups (Figure 3.6c). However, the mice from the control group are largely clustered together and are overall different from the other groups. These data indicate that BMT-treated and healthy control mice modulate each other's metabolites via co-housing. However, none of the metabolites were clearly linked to the cytokine responses pattern of splenocytes of BMT-treated mice.

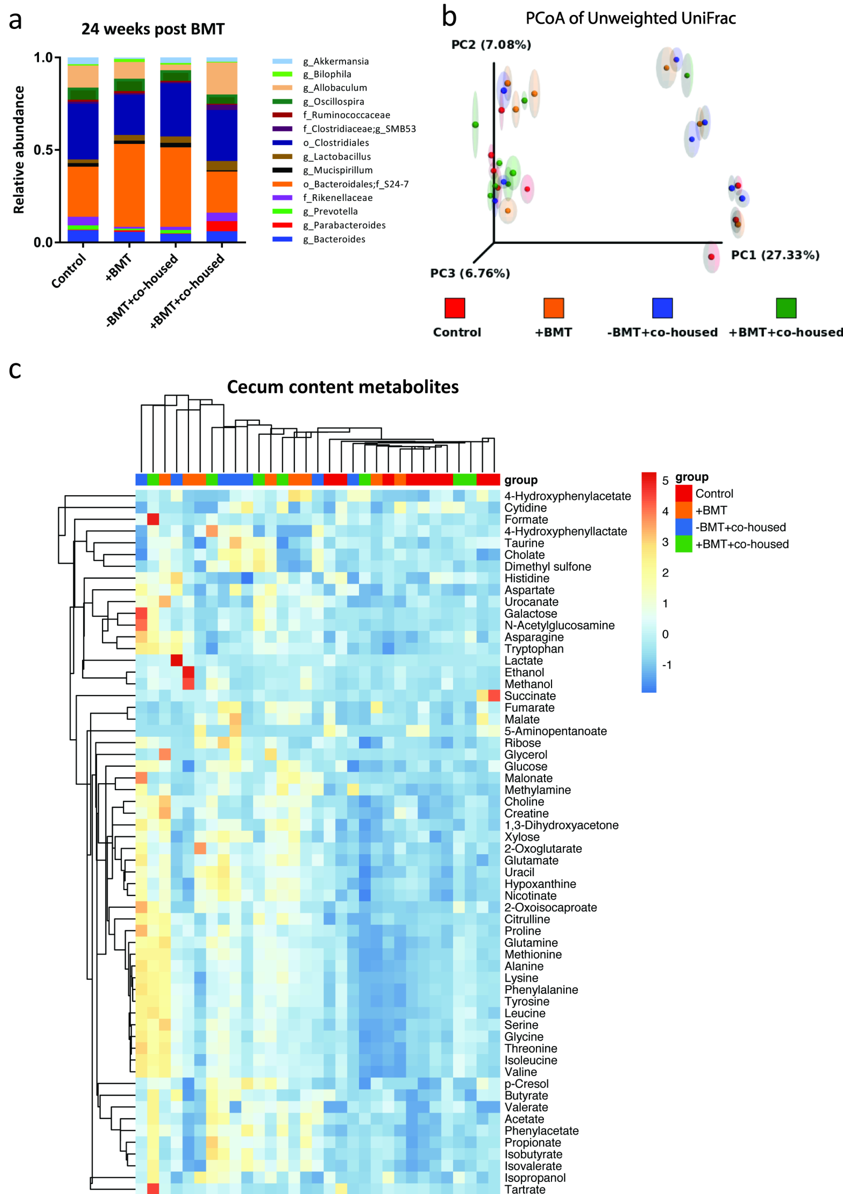

Figure 3.6: The effect of BMT on fecal microbiota composition and metabolites in the cecum. (a) Relative abundance of fecal microbiota in each experimental group as determined by 16S rRNA gene sequencing 24 weeks after BMT. (b) Unweighted UniFrac-based principal coordinate analysis (PCoA) of bacterial communities in fecal samples 24 weeks after BMT. Each dot represents one mouse, each colour represents one experimental mouse group; n=7-8 per group. (c) Heatmap of metabolites in cecum content measured by 1H-NMR; n=7-9 per group.

3.4. DISCUSSION

Our results demonstrate that BMT induced chronically increased cytokine responses of splenocytes but not peritoneal macrophages upon *ex-vivo* stimulation with various pathogenic stimuli. For splenocytes, and not for peritoneal macrophages, the BMT-induced cytokine response patterns were at least partly mediated via the gut microbiota, as the increased response pattern was largely transferred to splenocytes of healthy control mice via co-housing and via cecum content transplantation to germfree mice. In line with a role for microbiota, splenocytes, but not peritoneal macrophages from germfree mice showed lower stimulated cytokine response patterns as compared to conventional mice. The BMT-induced increased splenocyte response pattern could not be linked to obvious BMT-induced differences in gut microbiota composition or metabolites in cecum. Nevertheless, our data clearly show that gut microbiota are an important determinant of the cytokine response pattern of splenocytes, but not of peritoneal macrophages.

The differential role of microbiota in the cytokine responses pattern of splenocytes but not peritoneal macrophages may be associated with physical access of (components of) the intestinal content to splenocytes. In contrast to the peritoneal cavity, the spleen is an integral part of the blood circulation. In addition to accessibility, the immune cell composition and function of the spleen differs from peritoneal macrophages. The spleen contains multiple populations of leukocytes from both the innate and adaptive immune system, whereas peritoneal macrophages belong to the innate immune system. The increased secretion of IL-10 and IL-22 by splenocytes upon BMT suggests an elevated adaptive immune response, as these cytokines are mainly secreted by T cells. However, it remains to be investigated whether the microbiota affect specific subsets of immune cells from the adaptive or innate immune system in the spleen.

The higher response pattern of splenocytes after BMT did not coincide with detectable changes in gut microbial composition as determined by 16S sequencing. It is possible that the resolution of the 16S sequencing technique to distinguish bacterial taxa was not sufficient to show such alterations. Metagenomic sequencing in this respect might have revealed differences at the species level. However, the intra-individual variation in microbial composition between the mice at baseline before BMT-treatment was extremely large. This more than likely hampered detection of significant changes in microbial composition induced by the BMT.

To identify mechanisms underlying the higher response pattern of splenocytes induced by gut microbiota, we further focused on alterations in the function of the gut microbiota induced by BMT. We considered that multiple species of bacteria can exert the same function and produce the same metabolites. For instance, multiple bacterial species can produce SCFA which can cross the intestinal border and have extra-intestinal immune modulatory effects in several tissues including the spleen [8, 21]. By performing 1H-NMR on the cecum content samples that were used to inoculate the germfree mice and subsequent unsupervised clustering on the mice, we observed significant heterogeneity in the experimental groups. However, the mice from the control group largely clustered together and are overall different from the other groups. These data indicate that both the BMT and co-housing procedure affected metabolite levels in the gut. Although we can conclude that BMT and co-housing led to alterations in microbial function in our study, the underlying mechanisms linking the gut microbiota to splenocyte

cytokine response pattern remain to be elucidated.

In addition to bacterial metabolites, bacterial components such as LPS and peptidoglycans can modulate the immune system. BMT conditioning results in intestinal damage with the likely consequence of LPS leaking into the system. Our data show that the response pattern of splenocytes of BMT-treated mice was transferrable to splenocytes of healthy controls which have no damaged intestine. This makes LPS leakage an unlikely cause for the hyper-responsiveness of the splenocytes in the healthy controls cohoused with BMT mice. On the other hand, bacterial peptidoglycans have been reported to translocate from the gut into the circulation also under basal conditions [6]. It remains to be investigated whether peptidoglycans can explain the hyper-responsiveness of splenocytes after BMT. Although we conclude that the microbiota are at least partly responsible for the long term transfer of splenocyte cytokine response patterns via cohousing and cecum transplantation, we cannot exclude that other factors are involved. Recently, Liu *et al.* [21] identified microRNAs (MIR) in feces that are produced by epithelial cells in the intestine and affect microbial function by regulating bacterial gene transcription. It is thus possible that BMT-induced alterations in epithelial cell MIR secretion play a role in the altered responsiveness of splenocytes after BMT.

Our study may have clinical implications. Here, we focussed on the role of microbiota in syngeneic BMT. However, in humans allogenic BMT is common practice and is associated with graft versus host disease (GVHD). In GVHD, donor immune cells (mainly the T cells) are activated by recipient cells and cause severe inflammation and damage to skin, liver, hematopoietic system and gut. The initiation of GVHD depends not only on activation of the donor T cells, but also on activation of the recipient's antigen presenting cells and the interaction between these two. As the current study shows, also gut changed microbiota influence the recipient's immune response and could affect GVHD activation and outcome. Further research is needed to monitor gut microbiota dysbiosis and the possibility of restoring the gut microbiome to a healthy state in BMT patients to reduce the risk of GVHD development.

In conclusion, gut microbiota increase the cytokine responses pattern of splenocytes after BMT. This phenotype can be transferred to splenocytes of healthy controls by cohousing or to germfree mice via transfer of cecum content, indicating that they are independent of BMT-induced intestinal damage and microbial leakage. Whether the BMT-induced higher cytokine response pattern of splenocytes is due to changes in microbial composition or activity, or other transferable factors, remains to be further investigated.

3.5. ACKNOWLEDGEMENTS

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3.6. SUPPORTING INFORMATION

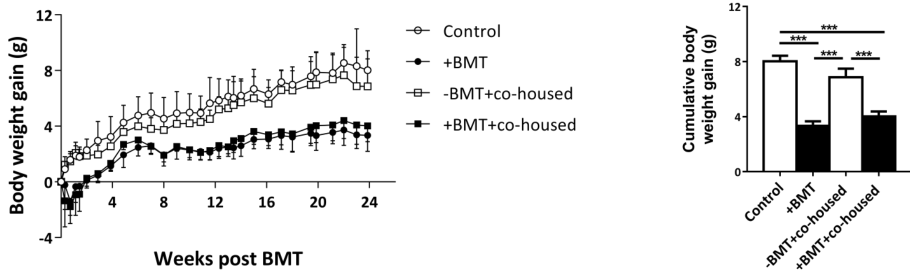

Figure S3.1: Co-housing BMT treated mice with healthy control mice did not affect body weight. Mice underwent BMT at time point 0 weeks. From 0 weeks until week 8, mice were fed a chow diet. From time point 8 weeks on, mice were fed a 10% LFD. Body weight gain was reduced by the BMT-treatment over the period of 16 weeks LFD, but not affected by co-housing control mice with BMT-treated mice. Values are means ± SEM; Groups were compared using Mann-Whitney U test; n=7-12; ***p<0.001.

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4

Akkermansia muciniphila EXERTS LIPID-LOWERING AND IMMUNOMODULATORY EFFECTS WITHOUT AFFECTING NEOINTIMA FORMATION IN HYPERLIPIDEMIC APOE*3-LEIDEN.CETP MICE

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Abstract

Scope: *Akkermansia muciniphila (A. muciniphila) is an intestinal commensal with anti-inflammatory properties both in the intestine and other organs. We aimed to investigate the effects of oral administration of A. muciniphila on lipid metabolism, immunity and cuff-induced neointima formation in hyperlipidemic APOE*3-Leiden (E3L).CETP mice.*

Methods and results: *Hyperlipidemic male E3L.CETP mice were daily treated with* $2x10^8$ *CFU A. muciniphila by oral gavage for 4 weeks and the effects were determined on plasma lipid levels, immune parameters and cuff-induced neointima formation and composition. A. muciniphila administration lowered body weight as well as plasma total cholesterol and triglycerides levels. A. muciniphila influenced the immune cell composition in mesenteric lymph nodes, as evident from an increased total B cell population, while reducing the total T cell and neutrophil populations. Importantly, A. muciniphila reduced the expression of the activation markers MHCII on dendritic cells and CD86 on B cells. A. muciniphila also increased whole blood ex vivo lipopolysaccharide-stimulated IL-10 release. Finally, although treatment with A. muciniphila improves lipid metabolism and immunity it did not affect neointima formation or composition.*

Conclusions: *Four weeks of treatment with A. muciniphila exerted lipid-lowering and immunomodulatory effects, which were insufficient to inhibit neointima formation in hyperlipidemic E3L.CETP mice.*

4

4.1. INTRODUCTION

Our understanding of the role and importance of the intestinal microbiome in health and disease has increased remarkably over the last decades. It is becoming clear that disruption of homeostasis of the intestinal microbiota (i.e. microbial dysbiosis) is involved in the development of various diseases in humans. Dysbiosis does not only play a role in intestinal complications such as inflammatory bowel diseases and ulcerative colitis, but also in rheumatoid arthritis and type-2 diabetes [1, 2, 3]. The α-diversity (i.e. the species richness) within the gut microbiota is reduced in these patients, implying that particular bacterial species have disappeared during or preceding the development of these diseases [4, 5].

Akkermansia muciniphila (*A. muciniphila*) is one of the specific bacterial species of which the abundance is inversely correlated with diseases, such as inflammatory bowel diseases, autism, atopy and metabolic syndrome [6, 7, 8, 9]. *A. muciniphila* is a commensal member of human and rodent intestinal microbiota and has been identified as a beneficial bacterium [10, 11]. Indeed, daily exposure to *A. muciniphila* protected mice against high-fat diet-induced obesity and improved their metabolic parameters such as obesity, fat mass, glucose tolerance and hypercholesterolemia [12, 13]. In addition, oral supplementation of *A. muciniphila* reduced experimental alcoholic or immune related liver disease in mice [14, 15].

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Although the mechanisms underlying the beneficial effects of *A. muciniphila* have not been completely resolved, it has been proposed that they involve effects on gut barrier integrity and inflammation. *A. muciniphila* is thought to counteract metabolic endotoxemia and to improve gut barrier integrity as a consequence of the increased mucin production that is caused by *A. muciniphila*'s mucin degrading capacity [12, 13, 16, 17]. Oral administration of *A. muciniphila* to high-fat diet-fed mice upregulated colonic expression of the intestinal antimicrobial peptide RegIIIγ and attenuated inflammation in visceral adipose tissue by inducing T-regulatory cells [12, 16]. Moreover, administration of *A. muciniphila* had anti-inflammatory effects on splenocytes and macrophages as indicated by an increased ratio of IL-10/TNF-α secretion by these cells [18]. Taken together, these studies suggest that *A. muciniphila* has beneficial immunomodulating properties both in the intestine and in other tissues.

Atherosclerosis is a chronic, low-grade inflammatory disease of the vessel wall [19]. One of the major triggers for vessel wall inflammation is hyperlipidemia. Studies in experimental models support the beneficial effects of inhibiting inflammation to delay atherosclerosis progression [20, 21, 22]. Recently, a clinical study of Ridker *et al.* [23] showed anti-inflammatory therapy by canakinumab lowered the rate of recurrent cardiovascular events, without reducing plasma lipid levels. Therefore, reduction of inflammation as strategy to reduce cardiovascular risk is a viable approach. Given its beneficial immunomodulatory effects, *A. muciniphila* might be promising in attenuating atherosclerosis. Indeed, in a recent study, *A. muciniphila* reduced systemic inflammation and atherosclerotic lesion development in *apoe*$^{-/-}$ mice, presumably by ameliorating endotoxemia [24].

In the present study, we extended these findings and investigated the effect of oral administration of *A. muciniphila* on lipid metabolism, immunity and cuff-induced neointima formation in hypercholesterolemic APOE*3-Leiden.cholesteryl ester transfer pro-

tein (E3L.CETP) mice, an established model for human-like lipoprotein metabolism [25, 26]. In this double transgenic mouse model, both a mutated human *APOE*3* gene and the human *CETP* gene are expressed which leads to diet-induced hyperlipidemia and atherosclerosis development [27]. After placement of a non-constricting polyethylene cuff around the femoral artery, vascular remodelling with signs of accelerated atherosclerosis takes place in the femoral artery in approximately 2 weeks [25]. This model of neointima formation resembles both restenosis, as it occurs in patients after balloon angioplasty, as well as accelerated atherosclerotic lesion formation, as the lesions formed after cuff placement contain mainly both smooth muscle cells and foam cells [28].

We aimed to investigated the effects of oral administration of *A. muciniphila* on lipid metabolism, immunity and cuff-induced neointima formation in hyperlipidemic APOE*3-Leiden (E3L).CETP mice. We determined the effect of 4 weeks oral *A. muciniphila* administration in E3L.CETP mice on 1) plasma lipid levels, 2) the immune response by measuring portal vein lipopolysaccharide (LPS) levels, mesenteric lymph node (mLN) immune cell composition and *ex vivo* responses of circulating leukocytes to LPS, and 3) neointima formation and composition. We found that 4 weeks of treatment with *A. muciniphila* beneficially affects hyperlipidemia and had broad immunomodulatory effects in E3L.CETP mice, but that these effects did not result in reduced neointima formation.

4.2. MATERIAL & METHODS

4.2.1. ANIMALS

Male E3L.CETP mice [29] between 9-13 weeks old were fed an atherogenic Western-type diet containing 1% cholesterol and 0.05% cholate (4021.37 AB Diets, Woerden, The Netherlands) for 7 weeks. All mice received water and food ad libitum. Body weight and food intake per cage were measured weekly. After 3 weeks of run-in, mice were divided in 2 groups based on age, body weight and plasma lipid levels, and thereafter orally gavaged daily for 4 weeks with 100 µL of either 1) anaerobic PBS or 2) 2x10^8 CFU viable *A. muciniphila* in anaerobic PBS; both containing 2.5% glycerol.

Tail blood samples were obtained in week 0, 2 and 4 after 4 hours fasting for plasma lipid determination. Fresh fecal samples were obtained in week 0 and 4. Neointima formation was induced by placement of a non-constricted polyethylene cuff (Portex, UK) around the femoral artery of both legs. Mice were anesthetized with a mixture of midazolam/medetomidine/fentanyl. After the surgery, the anesthesia of the mice was antagonized with a mixture of atipamezol/fluminasenil. Buprenorphine was given directly after surgery to relieve pain. The cuffs were placed 2 weeks after starting the *A. muciniphila* treatment and 2 weeks thereafter the mice were killed. After 4 weeks of treatment with *A. muciniphila* or vehicle, mice were anesthetised by a mixture of midazolam/fentanyl/dexdomiter/NaCl mix and portal vein samples were obtained in a laminar flow cabinet in pyrogenic-free tubes. Mice were killed by bleeding via the portal vein and thereafter mesenteric lymph nodes and cecum were dissected. The femoral arteries were harvested, fixed overnight in 4% formaldehyde in PBS, paraffin-embedded and sectioned. Animal experiments were performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament and all exper-

iments were approved by the animal ethics committee of the Leiden University Medical Center (protocol no. 14143).

4.2.2. CULTURE AND PASTEURIZATION OF AKKERMANSIA MUCINIPHILA

Viable A. muciniphila was prepared by the Laboratory of Microbiology, Wageningen University as described previously [13]. In brief, A. muciniphila MucT (ATTC BAA-835) was cultured anaerobically in a basal mucin-based medium as previously described [10]. Cultures were washed and concentrated in anaerobic PBS with 25% (vol/vol) glycerol under strict anaerobic conditions. Cultures were then immediately frozen and stored at -80 °C. A representative glycerol stock was thawed under anaerobic conditions to determine the CFU/ml by plate counting using mucin media containing 1% agarose (agar noble; Difco). Before administration by oral gavage, glycerol stocks were thawed under anaerobic conditions and diluted with anaerobic PBS to an end concentration of 2×10^8 CFU/100 µl and 2.5% glycerol.

4.2.3. *A. muciniphila* QPCR

To quantify *A. muciniphila* in feces or cecum, quantitative PCR was performed. The primers to detect *A. muciniphila* were based on the 16S rRNA gene sequences: forward CAGCACGTGAAGGTGGGGAC, reverse CCTTGCGGTTGGCTTCAGAT as described previously [12]. Detection was achieved with CFX96 real-time PCR system and software (BioRad, Hercules, CA, USA) using EvaGreen (Biotium Inc., Hayward, CA, USA) according to the manufacturer's instructions. Each assay was performed in triplicate in the same run. The cycle threshold of each sample was then compared with a standard curve (performed in triplicate) made by diluting genomic DNA of *A. muciniphila* (5-fold serial dilution).

4.2.4. DETERMINATION OF PLASMA TRIGLYCERIDES AND CHOLESTEROL

After 4 h fasting, tail blood was collected. Commercially available kits were used to determine plasma total cholesterol (236691 Roche Molecular Biochemicals, Indianapolis, IN, USA) and triglycerides (1488872, Roche Molecular Biochemicals, Indianapolis, IN, USA).

4.2.5. PORTAL VEIN SERUM LPS MEASUREMENTS

LPS concentrations were measured in portal vein serum samples using an Endosafe-Multi-Cartridge System (Charles River Laboratories, MA, USA) based on the Limulus Amaebocyte Lysate (LAL) kinetic chromogenic methodology, as described before [12]. Each sample was diluted when necessary with endotoxin-free LAL reagent water (Charles River Laboratories, MA, USA) and treated in duplicate. Two spikes for each sample were included in the determination in order to validate the recovery of LPS. All samples were validated for the recovery and the coefficient variation. The lower limit of detection was 0.005 EU/mL.

4.2.6. Determination of immune composition of mesenteric lymph nodes by flow cytometry

Single cell suspensions were prepared from mouse mesenteric lymph nodes (mLNs) for analysis by flow cytometry. Following 20 minute digestion with collagenase II/ DNase in 12 well plates, digested mLNs were dispersed through 100μm cell strainers. Single cell-suspensions were plated in 96-well v-bottom plates at concentrations of $5x10^5$ or $1x10^6$ cells per well. Cells were washed, incubated with aqua live-dead staining (20 minutes, RT) and stained for respective antibody panels for 20 minutes (-4°C). Cells were stained with antibodies against: CD1d, CD3, CD4, CD5, CD11b, CD23, CD44, CTLA-4, FoxP3 (eBioscience, San Diego, CA, USA) CD11c, CD19, CD21, CD25, CD86, MHC-II, GR-1 (BD Biosciences, San Diego, CA, USA), CD24, CD64, GITR, CD103 (BioLegend, San Diego, CA, USA) and F4/80 (SanBio, Uden, The Netherlands). Additionally, FC block (CD16/CD32) was added to the antibody mix to prevent non-specific binding. For intracellular staining, antibody incubation was preceded by 1 h fixation with eBioscience Fix/Perm buffer and antibody staining mixes were created with Bioscience permeabilization buffer. Cells were acquired with a FACS Canto-II and analysis was performed with FlowJo (Tree Star, San Carlos, CA, USA) software. For gating strategies see Figure S4.1.

4.2.7. Whole blood stimulation with LPS

Portal vein blood samples (16 μL blood in 400 μL RPMI) were stimulated in triplo overnight with 10 ng/mL LPS or vehicle and supernatants were collected and stored at -80°C. IL-10 and TNF-α were subsequently measured by ELISA (BD Biosciences, San Diego, CA, USA).

4.2.8. Neointima formation assessment

Serial cross sections of 5 μm thick were made throughout the entire length of the cuffed femoral artery for histological analysis. Weigert's elastin staining was used to visualize elastic laminae. Smooth muscle cells were visualized with smooth muscle cell α-actin staining (Boehringer Mannheim, Germany), Mac-3 (Accurate Chemical, Westbury, NY, USA) macrophage staining was used to detect monocytes/macrophages and Sirius red stain (VWR International) was used to detect collagen. Six equally spaced cross sections were used in all mice to quantify intimal lesions. Using image analysis software (Leica, Wetzlar, Germany), total cross-sectional medial area was measured between the external and internal elastic lamina; total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina [25]. For macrophage, smooth muscles cell and collagen composition absolute immuno-positive area and neointimal area were measured and used to calculate the percentage of immune-positive area within the neointima area.

4.2.9. Statistics

Data are presented as mean ± SD. Statistical analyses were performed using Student T-test analysis. Portal vein serum LPS levels were log normalized before applying Student T-test analysis. (GraphPad Software Inc., La Jolla, Ca, USA). $P<0.05$ was considered statistically significant.

4.3. RESULTS

4.3.1. *A. muciniphila* DECREASED PLASMA LIPIDS

To study the effect of administration of *A. muciniphila* on metabolic and immune parameters as well as on neointima formation, hyperlipidemic male E3L.CETP mice were orally gavaged with $2x10^8$ CFU *A. muciniphila* daily for 4 weeks. As controls, E3L.CETP mice were treated with equivalent volume of vehicle. *A. muciniphila* abundance, body weight and food intake were monitored, as well as fasted plasma lipid levels. Daily gavage with *A. muciniphila* led to approx. 100,000-fold higher CFU levels in feces and approx. 1,000-fold higher CFU in cecum at the end of the study (Figure S4.2A). A single gavage with *A. muciniphila* led to a similar increase in CFU levels (Figure S4.2B), indicating that *A. muciniphila* colonized the gastrointestinal tract of the mice. *A. muciniphila* administration resulted in slightly decreased body weight (Figure 4.1A), without affecting food intake (data not shown) as compared to control treatment. *A. muciniphila* markedly reduced fasting plasma total cholesterol (TC) (Figure 4.1B) and triglycerides (TG) (Figure 4.1C) levels after 2 and 4 weeks of treatment as compared to vehicle. Taken together, exposure to *A. muciniphila* decreased body weight as well as plasma TC and TG levels.

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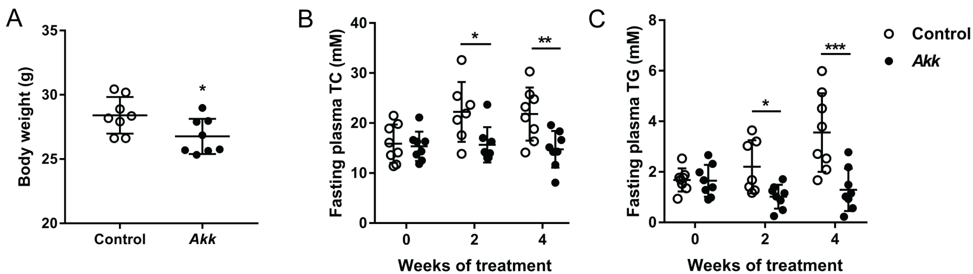

Figure 4.1: *A. muciniphila* decreased body weight and plasma lipid levels. (A) Body weight (after 4 weeks) as well as fasting plasma (B) total cholesterol (TC) and (C) triglyceride (TG) levels were determined at the indicated times after daily oral administration of hyperlipidemic E3L.CETP mice with *A. muciniphila* (Akk) or vehicle (control). Data are means ± SD; n=8 per group. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

4.3.2. *A. muciniphila* TENDED TO LOWER PORTAL LPS LEVELS

To assess whether *A. muciniphila* counteracted endotoxemia, LPS levels were measured in portal vein serum. *A. muciniphila* administration tended to decrease portal vein serum LPS levels by approx. 60% (p=0.06) as compared to control treatment (Figure S4.3).

4.3.3. *A. muciniphila* MODULATED THE COMPOSITION AND REDUCED THE ACTIVATION STATUS OF IMMUNE CELLS IN MESENTERIC LYMPH NODES

We next assessed the effect of *A. muciniphila* on the immune system *in vivo*. The mesenteric lymph node (mLN) is the "first pass" organ for nutrients and microbial substances entering the lymph fluid from the intestinal lamina propria [30]. As such, it serves as a key site for tolerance induction to food particles and commensals, but at the same time it acts as a firewall to prevent systemic spread of microorganisms [30, 31, 32]. Therefore, we studied the effect of *A. muciniphila* on immune cells populations locally in the mLNs, using flow cytometry.

A. muciniphila had no effect on the percentage of dendritic cells (DCs) infiltrating the mLNs (Figure 4.2A), but increased the total B cell population (Figure 4.2C) and markedly decreased the total neutrophil population (Figure 4.2B) and the total T cell population (Figure 4.2D). Subsequently, we studied the composition of various subpopulations of DCs, B cells and T cells in the mLNs. *A. muciniphila* had no effect on the percentages of various DC subpopulations (Figure 4.3A-D). However, *A. muciniphila* tended to decrease the antigen-presenting molecule MHCII on CD11b-CD103+ (tissue resident) DCs ((p=0.07; Figure 4.3E) and reduced the expression of this marker on the CD11b+CD103- DCs subpopulation (Figure 4.3G). These data indicate reduced activity of these DC subpopulations.

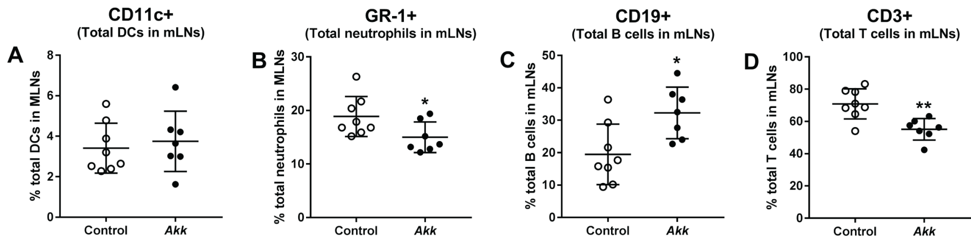

Figure 4.2: *A. muciniphila* modulated immune cell populations in mesenteric lymph nodes. Mesenteric lymph nodes (MLNs) were isolated from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control), and the percentage of total (A) dendritic cells, (B) neutrophils, (C) B-cell and (D) T-cell populations were studied using flow cytometry. Data are means ± SD; n=7-8 per group. * *p<0.05* and ** *p<0.01*.

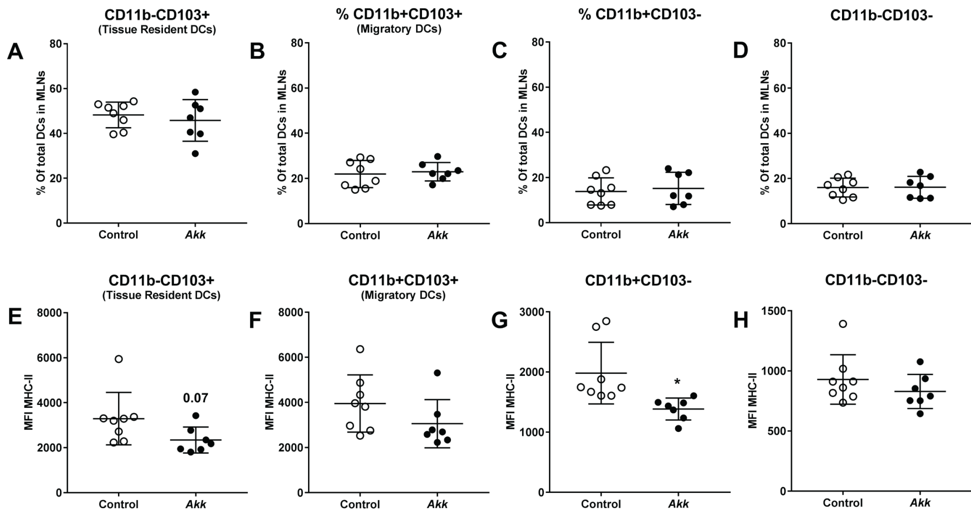

Figure 4.3: *A. muciniphila* did not affect percentage of dendritic cell subpopulations but decreased expression of MHC-II on DCs in mLNs. Mesenteric lymph nodes (MLNs) were isolated from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control), and subsets of dendritic cells (DCs) were studied using flow cytometry. (A-D) The percentage as well as (E-H) the mean fluorescence intensity (MFI) of MHC-II were determined of (A, E) CD11b-CD103+ (tissue resident DCs), (B, F) CD11b+CD103+ (migratory DCs), (C, G) CD11b+CD103- and (D, H) CD11b-CD103- DCs. Data are means ± SD; n=7-8 per group. * *p<0.05*.

A. muciniphila did not affect the percentage of follicular B cells (Figure 4.4A) or mucosal B cells (Figure 4.4B) within the total B cell population, indicating that the increase in total B cell population could not be ascribed to changes in these specific B cell subpopulations. *A. muciniphila*, however, reduced the expression of the T cell co-stimulatory molecule CD86 on both the follicular (Figure 4.4C) and mucosal (Figure 4.4D) B cell population. *A. muciniphila* did not affect regulatory T cells in the mLNs (Figure S4.4). Altogether, *A. muciniphila* influences the immune cell composition and may reduce the activation status of the immune cells, thereby exerting immunomodulatory properties on mLNs *in vivo*.

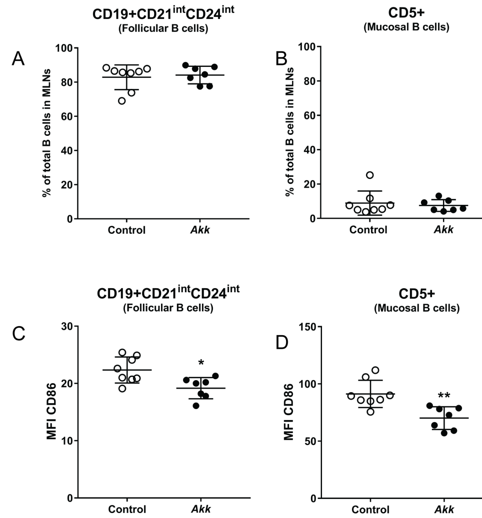

Figure 4.4: *A. muciniphila* did not affect abundance of follicular and mucosal B cells in mLNs, but decreased their CD86 expression. Mesenteric lymph nodes (MLNs) were isolated from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control), and subsets of B cells were studied using flow cytometry. (A-B) The percentage as well as (C-D) the mean fluorescence intensity (MFI) of MHC-II were determined of (A, C) $CD19+CD21^{int}CD24^{int}$ (follicular B cells) and (B, D) CD5+ (mucosal B cells). Data are means ± SD; n=7-8 per group. * $p<0.05$ and ** $p<0.01$.

4.3.4. *A. muciniphila* INCREASED THE ANTI-INFLAMMATORY IL-10 RESPONSE AFTER WHOLE BLOOD STIMULATION

As *A. muciniphila* modulated the immune system locally in the mLNs, we further studied the effect of *A. muciniphila* on *ex vivo* LPS-stimulated whole blood as a measure for systemic inflammation. Although the overall IL-10 levels were very low, *A. muciniphila* tended to increase unstimulated whole blood IL-10 levels (p=0.09; Figure 4.5A) and increased the LPS-stimulated IL-10 response (Figure 4.5B) as compared to whole blood of control mice. Treatment with *A. muciniphila* did not influence the unstimulated or LPS-stimulated TNF-α response (Figure 4.5C-D). These findings indicate that *A. muciniphila* increased the whole blood-(un)stimulated IL-10 response, and thus modulates systemic cytokine secretion.

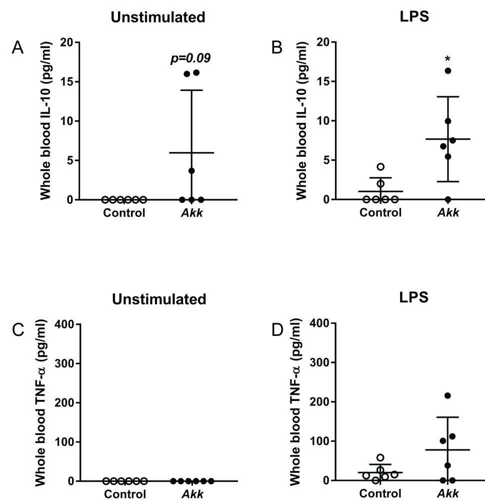

Figure 4.5: *A. muciniphila* increased IL-10 secretion in ex vivo LPS-stimulated blood. Whole blood was collected from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control) and, subsequently, stimulated in vitro (A, C) without or (B, D) with 10 pg/ml LPS for 24 h. (A-B) IL-10 and (C-D) TNF-α secretion was determined by ELISA. Data are means ± SD; n=6 per group. * $p<0.05$.

4.3.5. *A. muciniphila* did not prevent neointima formation

We next assessed whether exposure to *A. muciniphila* prevented neointima formation, a common feature of restenosis and atherosclerosis. Four weeks daily treatment with *A. muciniphila* did not reduce neointimal area as compared to vehicle (Figure 4.6A-B). In addition, *A. muciniphila* did not modulate neointimal content of macrophages (Figure 4.6C), vascular smooth muscle cells (Figure 4.6D) and collagen (Figure 4.6E). These data show that, despite its lipid-lowering and immunomodulatory properties, *A. muciniphila* did not reduce neointima formation and vascular lesion composition within this period of time.

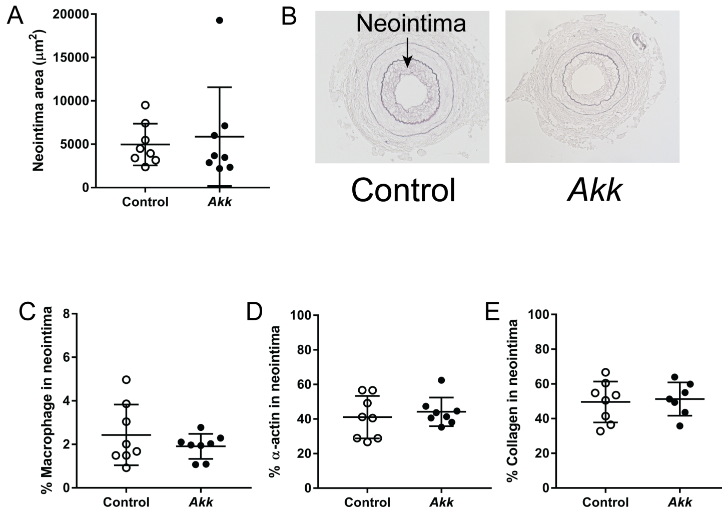

Figure 4.6: *A. muciniphila* did not affect neointima formation and composition. (A) The neointimal area in the cuffed femoral artery was determined in E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control) and (B) representative pictures are shown. In addition, neointimal content of (C) macrophages, (D) vascular smooth muscle cells and (E) collagen was determined. Data are means ± SD; n=7-8 per group.

4.4. DISCUSSION

We investigated the effect of oral administration of *A. muciniphila* on plasma lipid levels, immunity and cuff-induced neointima formation in hyperlipidemic E3L.CETP mice, a mouse model for accelerated atherosclerosis driven both by lipids and inflammation [25, 26]. Four weeks of treatment with *A. muciniphila* rapidly reduced hyperlipidemia and exerted immunomodulatory effects. However, within this time-frame these effects were not accompanied by a reduction in neointima formation or alterations in the composition of the neointima.

We and others have shown extensively that the E3L.CETP mouse model responds in a human-like fashion to many of the currently prescribed lipid-lowering drugs such as atorvastatin and fenofibrate [26, 29, 33]. Since *A. muciniphila* administration markedly decreased plasma TC and TG, this seems to be a promising lipid-lowering approach for human interventions that deserves further exploration. Future studies are required to clarify the mechanism of lipid-lowering by *A. muciniphila* administration. The main trigger for cuff-induced neointima formation in E3L.CETP mice is the local injury induced by the cuff, which evolves into a lesion in a two week time span. Hypercholesterolemia is required for this process. Apparently, the reduction in plasma TC and TG levels after *A. muciniphila* treatment was insufficient to affect the pathological process of neo-intima formation in the used time-frame.

A. muciniphila has been shown to have systemic and organ-specific immunomodulatory properties [34, 35, 36], but the underlying mechanisms have not been entirely resolved. Our data clearly showed that *A. muciniphila* administration modulates the immune cell composition of the mLNs. Importantly, *A. muciniphila* also reduced the expression of the antigen peptide complex MHCII on DCs as well as the expression of the co-stimulatory signal CD86 on B cells. Both MHC-II and CD86 are involved in T-cell mediated immune responses [37, 38]. In line with this, *A. muciniphila* reduced the percentage of total T cells in the mLNs upon administration. Combined, these observations may suggest that *A. muciniphila* reduces pro-inflammatory T cell-mediated immune responses, at least in the mLNs.

Intriguingly, the putative immunomodulatory effects of *A. muciniphila* were not restricted to the mLNs. *A. muciniphila* administration also increased IL-10 secretion from circulating immune cells in whole blood upon ex-vivo LPS stimulation, although overall IL-10 levels remained very low. *A. muciniphila* thus seems to have the capacity to exert complex modulatory effects *in vivo*, which is in line with a previous study in which daily oral gavage with *A. muciniphila* diminished Western-type diet-induced serum levels of pro-inflammatory monocyte chemoattractant protein-1 (MCP-1) and interleukin-1β (IL-1β) [24]. These studies substantiate the findings that intestinal microbiota regulate peripheral immunity, which is an interesting area of further investigation.

The cuff-induced neointima formation model was shown to be susceptible to immunomodulation in previous studies. For instance, IL-10 deficiency in hyperlipidemic E3L mice increased cuff-induced neointima formation, whereas a decrease was observed after IL-10 overexpression [39]. Moreover, treatment of hypercholesterolemic E3L mice with abatacept, a protein that prevents CD28–CD80/86 co-stimulatory T-cell activation, profoundly inhibited neointima formation [40]. Although we found that *A. muciniphila* modulated both the peripheral IL-10 response and the T cell activation in the mLNs,

these effects were apparently insufficient to exert an anti-atherogenic response in this mouse model, within the period of investigation.

A. muciniphila administration tended to reduce the portal vein LPS levels by approx. 60% in our study. The lack of a significant effect may be explained by the low LPS levels observed after the Western-type diet in our study, which were comparable to previously reported LPS levels in mice fed a chow diet [12]. Previously, Li *et al.* [24] showed that *A. muciniphila* administration reduced atherosclerotic lesion development in *apoe*$^{-/-}$ mice. In their study, 8 weeks of treatment with 2x10^9 CFU *A. muciniphila* inhibited atherosclerosis presumably via ameliorating endotoxemia induced by their Western-type diet. These data thus imply that an increased dose of *A. muciniphila*, the diet and/or the duration of treatment might explain the discrepant effects of *A. muciniphila* on atherosclerosis development between the studies.

Recently, it was observed that pasteurized cells of *A. muciniphila* were as active as live *A. muciniphila* cells in protecting mice from diet-induced obesity [13]. The outer membrane protein of *A. muciniphila*, Amuc_1100*, also stimulated TLR2 signaling *in vitro*, similar to viable *A. muciniphila* [13, 41]. Although our experiments clearly indicate that administration of viable *A. muciniphila* colonized the mice very well and exerted lipid-lowering and immunomodulatory effects, it remains to be investigated whether *A. muciniphila* needs to be viable to retain its efficacy.

In conclusion, administration of *A. muciniphila* lowered hyperlipidemia in hypercholesterolemic E3L.CETP mice and had immunomodulatory properties. As both hyperlipidemia and immune responses are involved in the pathogenesis of atherosclerosis these observations suggest that *A. muciniphila* has anti-atherogenic potential. However, *A. muciniphila* was unable to ameliorate atherosclerosis in our cuff-induced neointima formation model, suggesting that the anti-atherogenic effects of *A. muciniphila* were not sufficiently strong in this mouse model.

4.5. ACKNOWLEDGEMENTS

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4.6. Supporting Information

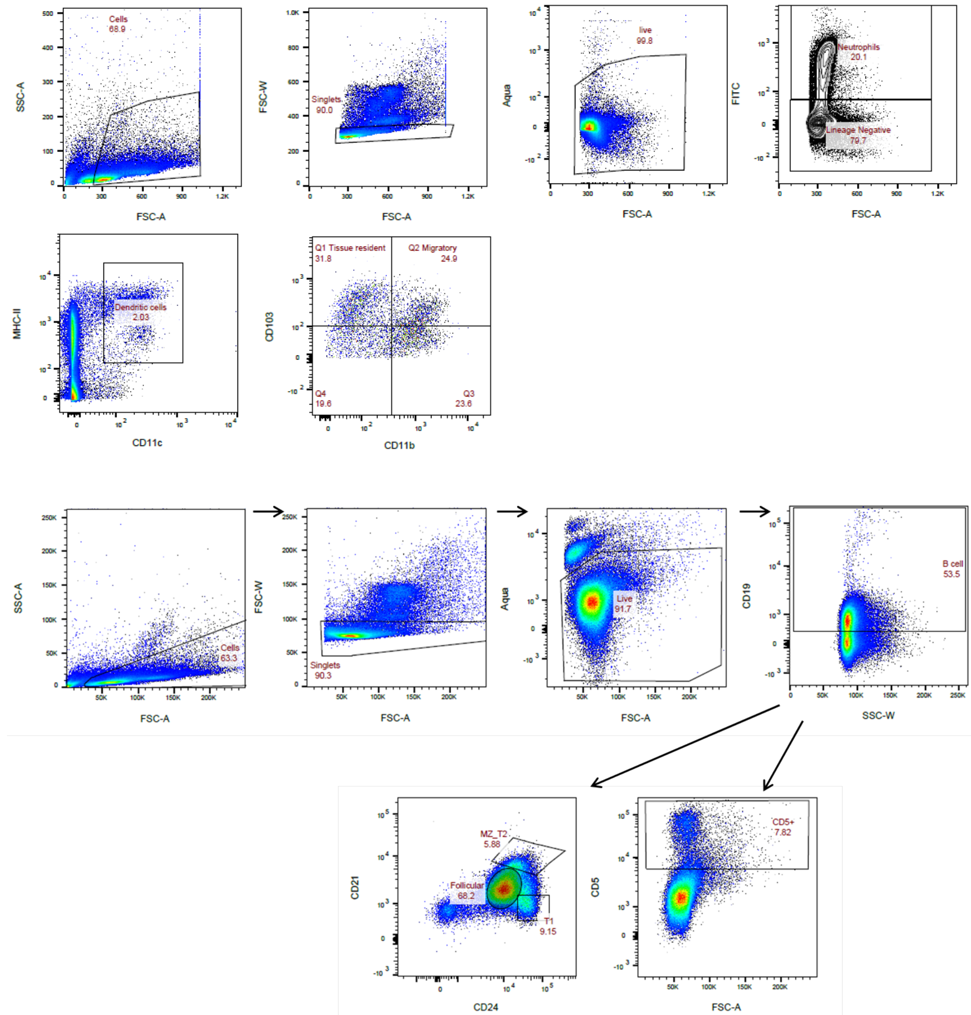
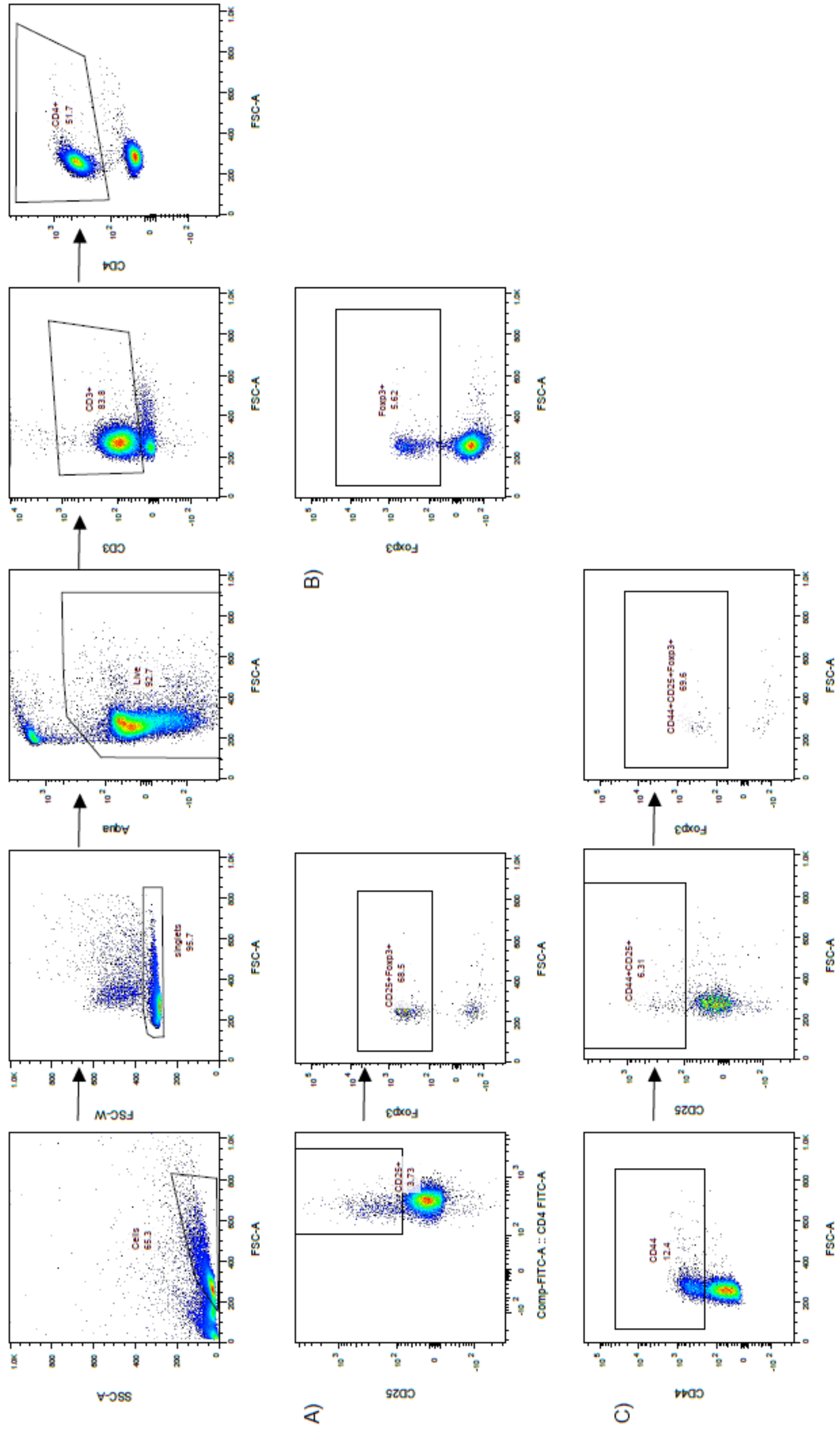


Figure S4.1: Gating strategies for the various cell populations. The gating strategies that were used for analysing (A) dendritic cells (DCs) and neutrophils, (B) B cells, and (C) T cells are shown.



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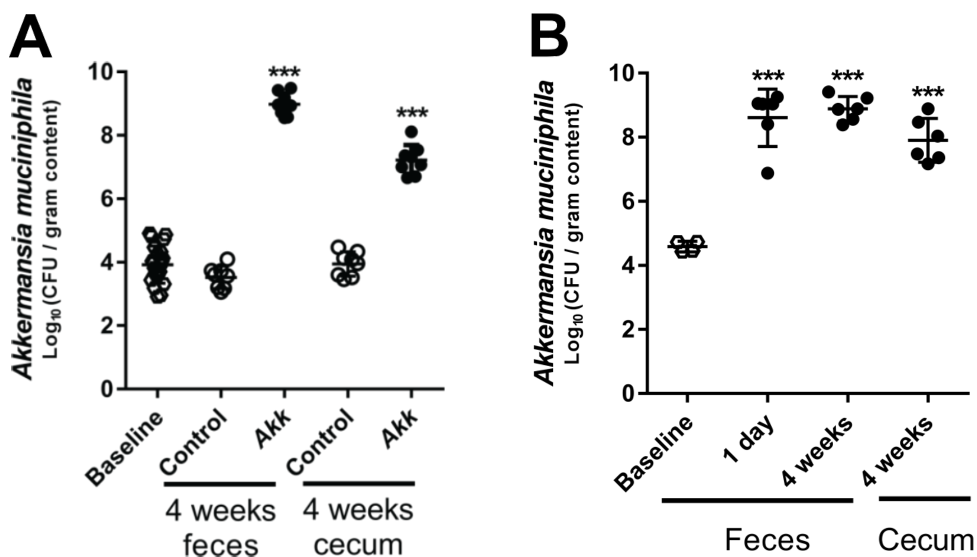

Figure S4.2: *A. muciniphila* colonized E3L.CETP mice efficiently. (A) The concentration of *A. muciniphila* was determined by qPCR in feces and cecum of E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control); n=8 per group. (B) The concentration of *A. muciniphila* was determined by qPCR in feces and cecum of E3L.CETP mice in time , after oral administration of one single dose *A. muciniphila* (Akk); n=5. Data are means ± SD. *** *p<0.001*.

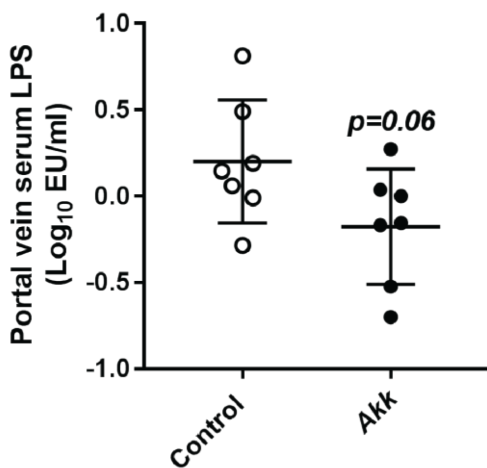

Figure S4.3: *A. muciniphila* tended to lower portal vein serum LPS levels. Portal vein blood was collected from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control). LPS levels were determined in portal vein serum. Data are means ± SD; n=7 per group.

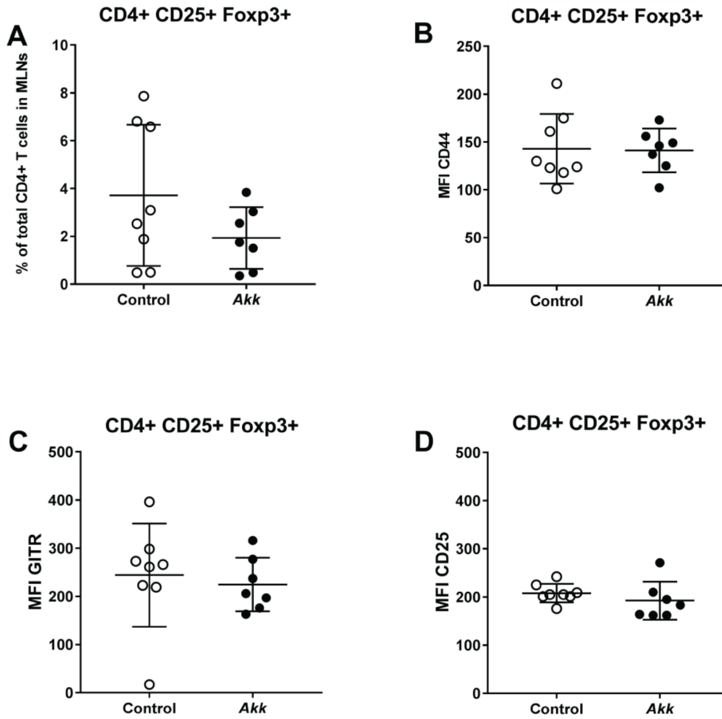

Figure S4.4: ***A. muciniphila* did not influence regulatory T cells.** Mesenteric lymph nodes (mLNs) were isolated from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control), and (A) CD4+CD25+Foxp3+ (regulatory T cells) were studied using flow cytometry. In addition, the mean fluorescence intensity (MFI) of (B) CD44, (C) GITR, and (D) CD25 were determined on these regulatory T cells. Data are means ± SD; n=7-8 per group.

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Evaluation of full-length versus V4-region 16S rRNA sequencing for phylogenetic analysis of mouse intestinal microbiota after a dietary intervention

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ABSTRACT

The composition of microbial communities is commonly determined by sequence analyses of one of the variable (V) regions in the bacterial 16S rRNA gene. We aimed to assess whether sequencing the full-length versus the V4 region of the 16S rRNA gene affected the results and interpretation of an experiment. To test this, mice were fed a diet without and with the prebiotic inulin and from cecum samples, two primary data sets were generated: 1) a 16S rRNA full-length data set generated by the PacBio platform; 2) a 16S rRNA V4 region data set generated by the Illumina MiSeq platform. A third derived dataset was generated by in silico extracting the 16S rRNA V4 region data from the 16S rRNA full-length PacBio data set. Analyses of the primary and derived 16S rRNA V4 region data indicated similar bacterial abundances, and α- and β-diversity. However, comparison of the 16S rRNA full-length data with the primary and derived 16S rRNA V4 region data, revealed differences in relative bacterial abundances, and α- and β-diversity. We conclude that the sequence length of 16S rRNA gene and not the sequence analysis platform affected the results and may lead to different interpretations of the effect of an intervention that affects the microbiota.

5.1. INTRODUCTION

The composition of gut microbiota have been associated with a variety of pathophysiological conditions, including obesity, low grade inflammation and overt disease [1, 2, 3]. We [4, 5, 6] and others [7, 8] have exploited possibilities to beneficially affect microbiota using probiotics or dietary compounds that affect the composition and/or activity of the gut bacteria. To determine the success of intervention, the composition of gut microbiota are commonly determined by massive parallel sequencing of one of the variable (V) regions of the bacterial 16S rRNA gene [9]. Sequence analysis of the V-region of 16S rRNA has proven to be a powerful tool to describe the composition of bacterial communities [10, 11]. However, the resolution of the taxonomic description of the communities is limited by the uniqueness of the V-region sequences and available reference databases [12]. Numerous different bacterial species have almost identical V-region sequences which makes distinguishing between these bacteria based on a single V-region impossible. The currently available 16S reference databases that are used for taxonomic classification of 16S sequencing data are still quite limited and do not contain a reference sequence for all experimentally obtained 16S sequences [13, 14]. Therefore some 16S V-region sequences can only be assigned up to the family and/or genus level or cannot be assigned at all.

Massive parallel sequencing of 16S rRNA V-regions has been made possible by the development of next generation sequencing technology (NGS). A typical NGS run on for example an Illumina MiSeq will provide several million 250 bp paired-end reads per flow cell. The advantage of high throughput is countered by the relatively short reads that are produced by NGS. Although many of the limitations of short read sequencing can be addressed using computational approaches, it is extremely challenging, if not impossible, to assemble longer sequences composed of highly homologous parts. Examples of this are repeat sequences in the human genome, but also the genomes of the various bacteria that constitute the microbiota. A number of so-called third generation sequencing technologies have been developed to overcome these limitations by sequencing very long amplicons. One such approach is developed by Pacific BioSciences (PacBio) and is termed single-molecule real-time (SMRT) sequencing [15].

We aimed to assess whether sequencing the full-length 16S rRNA gene using SMRT sequencing affected the results and interpretation of a dietary intervention compared to sequencing only the V4 region of this gene. This study included two experimental conditions; a Western-type diet (WTD) and a WTD complemented with the fibre inulin. Inulin is a fructose polymer that can only be degraded by intestinal microbiota and therefore strongly favours the expansion of specific intestinal microbiota [16, 17, 18, 19]. To compare the effects of the dietary intervention measured on either the PacBio or Illumina MiSeq platform, we performed taxonomic analysis and diversity analysis on primary and derived data sets.

5.2. MATERIALS & METHODS

5.2.1. CECUM SAMPLES

Cecum content was collected for microbial analysis. The cecum samples used in study were obtained in the context of a larger study of which the results were published recently [5].

5.2.2. DNA ISOLATION

From cecum samples, genomic DNA was extracted using phenol: chloroform: isoamylalcohol (25: 24: 1) (Invitrogen), precipitated with isopropanol, and washed with 70% ethanol.

5.2.3. PACBIO SEQUENCING

16S rRNA full-length amplification was performed using degenerate primers containing 5' M13 universal tail sequences (Table S5.1). The 16S locus was amplified using LA Taq polymerase (Takara) with 400 µM dNTPs, 50ng DNA template, and 400 nM of each primer in 1x LA buffer + magnesium with 30 cycles of PCR (20 sec 94°C, 30 sec 48°C, 2 min 68°C). PCR reactions were size selected using 0.65x AMPure XP beads (Beckman Coulter). Amplicons were barcoded in a second PCR reaction containing universal tail oligos complementary to the M13 universal tail sequences (Table S5.1). Barcodes were added using Herculase II Taq polymerase (Agilent) with 250 µM dNTPs, 2ul of purified PCR product, and 400 nM of each primer in a 1x reaction buffer with 5 cycles of PCR (20 sec 95°C, 20 sec 58°C, 2 min 72°C). The barcoded amplicons were size selected using 0.65x AMPure XP beads (Beckman Coulter). 500 ng of barcoded amplicons were prepared for sequencing using the amplicon template preparation protocol, 2015 release (Pacific Biosciences) including DNA damage repair and SMRTbell adapter ligation. Libraries were sequenced on the Pacific Biosciences RSII using MagBead loading with 6 hours of movie time and P6-C4 chemistry.

5.2.4. IN SILICO ISOLATION OF V4 REGIONS FROM FULL-LENGTH 16S RRNA PACBIO SEQUENCING DATA

V4 regions from full-length 16S rRNA PacBio data set were in silico isolated by the V-ripper script [20] using forward primer (5'-GTGCCAGCMGCCGCGGTAA-3') and the reverse primer (5'-GGACTACHVGGGTWTCTAAT-3'). Subsequently, isolated sequences with length between 100-300 bp were retained.

5.2.5. ILLUMINA SEQUENCING

Genomic DNA was sent to the Broad Institute of MIT and Harvard (Cambridge, USA). Microbial 16S rRNA was amplified targeting the hyper-variable V4 region using forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The cycling conditions consisted of an initial denaturation of 94 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 45 sec, annealing at 50 °C for 60 sec, extension at 72 °C for 5 min, and a final extension at 72 °C for 10 min. Sequencing was performed using the Illumina MiSeq platform generating paired-end reads of 175 bp in length in each direction. Overlapping paired-end reads were subse-

quently aligned. Details of this protocol have previously been described [21].

5.2.6. SEQUENCING DATA ANALYSIS

All three data sets were analysed using the operational taxonomic unit (OTU) approach. This was done by using the QIIME pipeline [22]. We used SILVA 132 QIIME release as reference OTU taxonomy database. Prior to OTU picking, each data set was quality filtered by sickle version 1.33 and low quality reads were discarded. Open reference OTU picking strategy with 97% sequence similarity and minimum OTU size of two reads was used. The α-diversity metric based on observed OTUs was calculated continuously from 50 reads/sample up to 3300 reads/sample with increasing steps of 50 reads, with 10x rarefaction. Unweighted UniFrac distances, with 10 jack-knifed replicates was measured at rarefaction depth of 3000 reads per sample, based on the unfiltered OTU table and relative bacterial abundance was determined. Prior to relative abundance visualisation, rare taxa that were present at less than 0.1% were filtered. Sequence data is submitted to SRA database and is accessible with BioProject accession number PRJNA786882.

5.3. RESULTS

5.3.1. SEQUENCING DEPTH

Cecum content from mice fed a WTD without or with 10% inulin for 11 weeks was collected (n=2 per group) and genomic DNA was extracted. The full-length 16S rRNA gene was amplified for PacBio sequencing, and the V4 region of the bacterial 16S rRNA gene was PCR amplified for Illumina short read sequencing. To determine platform bias in the data sets obtained from the PacBio and Illumina platforms, a 16S rRNA V4 region data set was generated in silico from the full-length 16S rRNA PacBio data set (V4 PacBio). Table S5.2 shows that the read count obtained by PacBio and Illumina sequencing are in range of a typical run for the platforms, and the reads have the correct mean read length for the full-length 16S rRNA (approx. 1500 bp) and V4 region (approx. 250 bp). Interestingly, V4 PacBio read count for individual samples are approximately 50% of the read count for the full-length 16S rRNA PacBio data set they were derived from (Table S5.2). The V-ripper script in combination with the used primer sequences, apparently does not recognize 50% of the full-length 16S rRNA sequences.

5.3.2. SEQUENCING DATA ANALYSIS

For operational taxonomic unit (OTU) picking, open reference OTU picking strategy with 97% sequence similarity and minimum OTU size of two reads was used. The minimum OTU size of at least two sequences/OTU ensured that singletons are excluded from the data. Table 5.1 shows the number of OTUs for individual samples and the number of sequences that these OTUs contained. In the 16S rRNA full-length PacBio data set, proportionally more reads were discarded in the OTU picking step compared to both 16S rRNA V4 data sets. These discarded reads, were singletons and sequences that failed to align with the reference database. Furthermore, sequencing full-length 16S rRNA resulted in a higher percentage of unassigned taxa (2.9-8.4% of total reads) compared to both V4 data sets (0.05-0.6% of total reads; Table 5.1). These were reads without any reference sequence available in the reference database. The number of unassigned reads in the full-length 16S rRNA data set was in particular higher for samples of inulin fed mice compared to samples of control mice.

Table 5.1: Statistics of data sets.

Group	Data set	Sample	Read Count	OTU Count	Reads assigning taxonomic labels (coverage %) Class	Order	Family	Genus	Unassigned
Control	FL	C1	15261	1821	14781 (96.9%)	14781 (96.9%)	14769 (96.8%)	13455 (88.2%)	480 (3.1%)
	FL	C2	7022	1198	6819 (97.1%)	6819 (97.1%)	6810 (97.0%)	5919 (84.3%)	202 (2.9%)
	V4 PacBio	C1	9195	435	9181 (99.8%)	9181 (99.8%)	9178 (99.8%)	8071 (87.8%)	14 (0.2%)
	V4 PacBio	C2	3979	366	3955 (99.4%)	3955 (99.4%)	3951 (99.3%)	3386 (85.1%)	23 (0.6%)
	V4 Illumina	C1	105426	1424	105323 (99.9%)	105323 (99.9%)	105174 (99.8%)	87984 (83.5%)	103 (0.1%)
	V4 Illumina	C2	139693	1572	139583 (99.9%)	139583 (99.9%)	138285 (99.0%)	114513 (82.0%)	110 (0.1%)
Inulin	FL	In1	13481	2199	12986 (96.3%)	12986 (96.3%)	12968 (96.2%)	10098 (74.9%)	492 (3.6%)
	FL	In2	5350	1056	4900 (91.6%)	4900 (91.6%)	4780 (89.3%)	3131 (58.5%)	449 (8.4%)
	V4 PacBio	In1	9609	618	9595 (99.9%)	9595 (99.9%)	9568 (99.6%)	6919 (72.0%)	14 (0.1%)
	V4 PacBio	In2	3309	353	3290 (99.4%)	3290 (99.4%)	3181 (96.1%)	2060 (62.3%)	19 (0.6%)
	V4 Illumina	In1	144897	2036	144761 (99.9%)	144761 (99.9%)	144228 (99.5%)	85122 (58.7%)	135 (0.1%)
	V4 Illumina	In2	198271	2036	198171 (99.9%)	198171 (99.9%)	195756 (98.7%)	96617 (48.7%)	99 (0.05%)

5.3.3. FULL-LENGTH 16S RRNA RESULTS INTO HIGHER α-DIVERSITY

The OTU richness was assessed by plotting α-diversity versus sequencing depth. The α-diversity expressed as number of unique observed OTUs was calculated continuously from 50 reads/sample up to 3300 reads/sample with increasing steps of 50 reads, with 10x rarefaction. Already at a sequencing depth of 300 reads/sample, α-diversity of 16S rRNA full-length PacBio samples was increased compared to both V4 PacBio and V4 Illumina data sets for control and inulin fed samples (Figure 5.1), while α-diversity of the V4 PacBio and V4 Illumina data sets were comparable. These data show that sequencing the full-length 16S rRNA resulted in a higher number of unique OTUs, already at a relatively low sequencing depth.

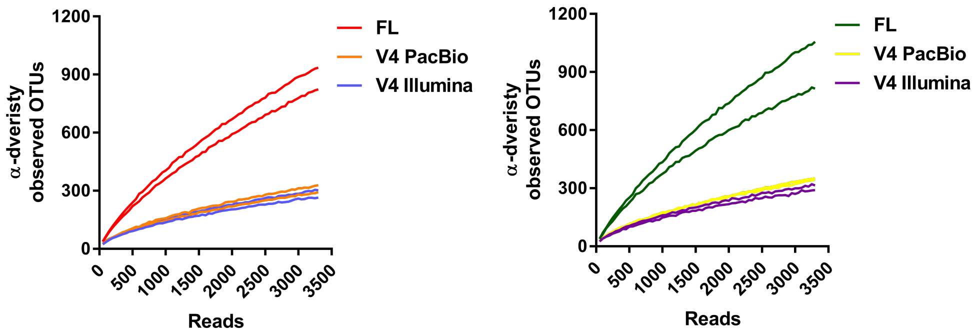

Figure 5.1: α-diversity. α-diversity metric observed species was calculated continually both for control and inulin-fed mice (n=2) with 10x rarefaction from 50 reads/sample up to 3300 reads/sample with steps of 50 reads. Each line represents one individual sample. FL, 16S rRNA full-length PacBio.

5.3.4. USING FULL-LENGTH 16S RRNA REVEALS A DIFFERENT BACTERIAL PHYLOGENY AS COMPARED WITH V4 REGION

The between sample diversity, or β-diversity, was determined by calculating unweighted UniFrac distances. This is a validated and widely used quantitative distance metric for studying microbial community clustering that takes the phylogeny of communities into account [23, 24]. Principal coordinate analysis was performed and the variation explained by the first two principal coordinates is plotted in Figure 5.2. Principal coordinate (PC)1, which explains 34.8 % of the data, clearly separates the full-length 16S rRNA PacBio data from the V4 amplicon data. The unweighted UniFrac distance for the V4 PacBio data set was comparable with the UniFrac distance of Illumina V4 regions, indicating limited sequencing platform bias in determining β-diversity. In order to assess the robustness of the UniFrac distance 10x jack-knifing at 3000 reads/sample was performed for all samples. The jack-knifing variance, indicated by the ellipsoids around the data points, was smaller for the full-length 16S rRNA sequenced samples compared to both V4 data sets (Figure 5.2). This indicates that a longer amplicon length provided a more robust UniFrac distance assignment.

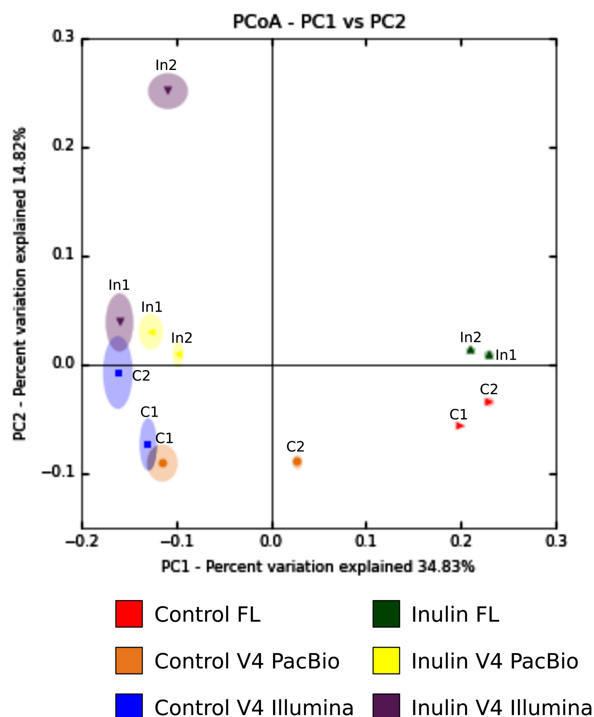

Figure 5.2: β-diversity, unweighted UniFrac distances. Unweighted UniFrac distances for individual samples were calculated both for control and inulin-fed mice (n=2) using PacBio and Illumina MiSeq platform. Identical sample names in the graphs indicate individual mouse samples studied using different approaches. 10x Jack-knifing at 3000 reads/sample was performed. C1 and C2 are individual samples from the control group. In1 and In2 are individual samples from the inulin group. FL, 16S rRNA full-length PacBio.

5.3.5. USING FULL-LENGTH 16S RRNA GENE RESULTS IN A DIFFERENT BACTERIAL COMPOSITION AND RELATIVE ABUNDANCE

In addition to diversity analyses, we aimed to study if sequence length affected the taxonomic analysis outcome. We hypothesized that a longer amplicon length increased the resolution of the analysis by detecting additional taxa which would not be observed by sequencing the V4 region only. Therefore, we compared the full-length 16S rRNA PacBio samples with the V4 PacBio and V4 Illumina data sets. In this way we could exclude platform bias and detect the effects of amplicon length on taxonomic analysis after a dietary intervention.

Genus level is considered as the maximum resolution of 16S sequencing. Therefore, we compared relative abundance of bacterial taxa in the three data sets at genus level. Sequencing the full-length 16S rRNA gene showed a different relative abundance at genus levels compared to both V4 data sets, both for samples of control and inulin-fed mice (Figure 5.3). Bacterial relative abundances of V4 PacBio and V4 Illumina data sets were comparable for control samples. For inulin-fed mice, sample In2 showed variation in relative abundance for several taxa between the V4 PacBio and V4 Illumina data set (Figure 5.3). Interestingly relative abundance of the genus Faecalibaculum that blooms with inulin intervention was higher in the full-length 16S rRNA data set compared to both V4 data sets. Relative abundance of the uncultured genus of Muribaculacea family that increases with inulin intervention was lower in the full-length 16S rRNA data set compared to both V4 data sets (Figure 5.3). Relative abundance of the Bacteroides genus that decreases with inulin intervention was higher in full-length 16S rRNA data set compared to both V4 data sets (Figure 5.3). Remarkably, the genus Lactobacillus was detected in the V4 PacBio and V4 Illumina data sets for both dietary conditions, but was completely absent inform the full-length 16S rRNA PacBio data set for both dietary conditions. After inulin intervention, other taxa like GCA-900066575, Lachnospiraceae-UCG006, Lachnospiraceae uncultured genus, Oscillibacter and Ruminiclostridium 9 were detected in both V4 data sets, and were also almost or completely absent in the full-length 16S rRNA PacBio data set. Taken together, this taxonomic analysis shows that sequencing the full-length 16S rRNA gene results in a different bacterial composition and relative abundance of bacterial species both for control and inulin-fed mice compared to determining the sequence of the V4 region only.

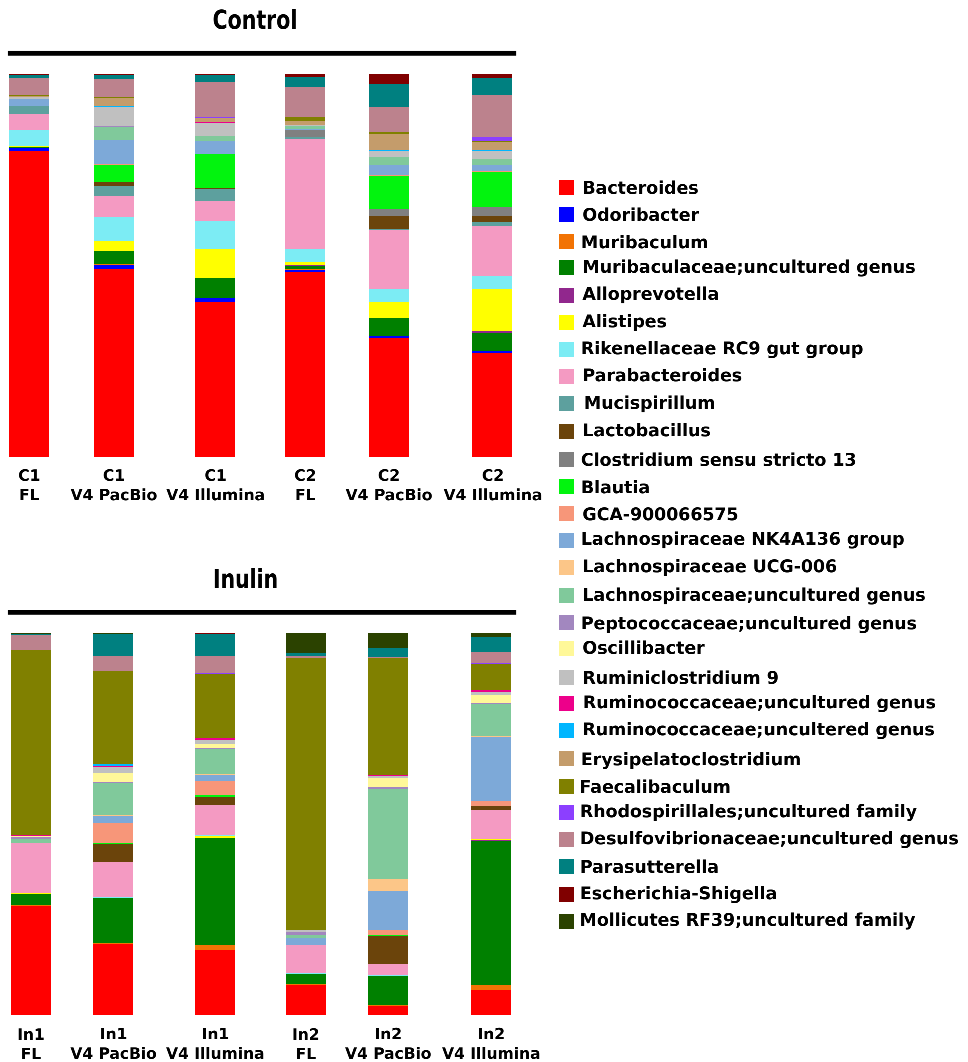

Figure 5.3: Comparison of microbial composition. Relative bacterial abundance in the cecum content of control and inulin fed-mice (n=2) visualised at genus level. Taxa abundant less than 0.1% of total population are filtered out. C1 and C2 are individual samples from the control group. In1 and In2 are individual samples from the inulin group. FL, 16S rRNA full-length PacBio.

5.4. DISCUSSION

We hypothesized that sequencing the full-length 16S rRNA gene would provide a higher resolution in terms of diversity and taxonomic analyses compared to sequencing a single short amplicon of the 16S rRNA marker gene such as the V4 region.

Our results show that in the in silico extracted V4 PacBio data set, individual samples have approximately 50% of the read count of the full-length 16S rRNA PacBio data set. This reduction in read count after in silico isolation of the V4 sequences from the full-length 16S rRNA data set might be caused by variability in the primer sequences. It is known that primer choice for sequencing hypervariable regions of 16S rRNA influences sequencing outcome, due to the fact that primers do not cover the 16S rRNA V4 flanking region for all bacteria [25, 26, 27]. These data could indicate that a proportion of the taxa that are identified by full-length 16s rRNA gene sequencing are not detected by sequencing the V4 region only. Alternatively, although the circular consensus sequencing approach of PacBio has a very low error rate, this could also explain a proportion of the V-regions that could not be extracted using the V-ripper script. However, since PacBio sequencing errors are random, this would have no consequences on the distribution and phylogenetic assignment of the extracted sequences.

In addition to primer choice, other factors including the DNA extraction method and choice of the 16S V-region may affect experimental outcome and introduce biases to the diversity and taxonomic analysis. DNA extraction method: Mackenzie *et al.* studied the effects of different DNA extraction methods, including commercially available DNA isolation kits and the phenol: chloroform: isoamylalcohol method [28]. Different DNA isolation methods resulted in different DNA yield, DNA quality and relative abundance of taxon-assigned OTUs. Other studies addressing microbial DNA extraction methods report similar issues [29, 30]. These results emphasize that it is important, if at all possible, to be consistent in the use of a DNA extraction method. Choice of 16S V-region: Sequencing the V4 region in combination with Illumina MiSeq platform has been widely used for taxonomic and diversity analysis [11, 31]. More recently, a combination of two regions like the V2-V3 or V3-V4 region have been used for this purpose [32]. Burkin *et al.* compared V2-V3 with V3-V4 regions in water samples and reported that V2-V3 sequencing has higher resolution for lower-rank taxa [32]. Abellan-Schneyder *et al.* conducted an extensive study including six different combinations of the V-regions on human gut and mock samples [33]. They recommended sequencing of V3-V4 regions for human gut samples, but also mentioned that primer choice has significant influence on the resulting microbial composition [33]. Since there seems no consensus on which V-regions provides the best results, investigators should consider the choice for their desired V-region carefully based on the experimental design and sample type. The cecum samples used in study were obtained in the context of a larger study of which the results were published as mentioned in the Materials and Methods section [5]. In order to maintain comparability with previously obtained data we have used the V4 region in this current study.

Diversity analyses and taxonomic analysis are based on OTUs. An OTU is described as a cluster of sequences with a minimum amount of sequence identity; in the case of genus level the threshold for sequence identity is set on 97% similarity [9]. Since OTU picking is based on sequence identity, sequence length can thus affect the number and

composition of OTUs in a given data set. α-diversity metric observed OTUs confirmed that indeed for both dietary conditions, the full-length 16S rRNA PacBio data set resulted in an increased number of unique OTUs compared to both V4 data sets. This finding shows that sequencing the full-length 16S rRNA provides more detail about within sample diversity compared to V4 sequencing. Interestingly, already at a depth of 300 reads/sample the full-length 16S rRNA PacBio data set resulted in an increased number of unique OTUs compared to both V4 data sets.

In addition, our results showed that β-diversity is affected by the sequence length. β-diversity analysis performed by calculating the unweighted UniFrac distances shows that full-length 16S rRNA and V4 sequencing of individual samples did resulted in separation in the PCoA plot. The unweighted UniFrac distance is a qualitative distance metric which takes the phylogeny of the sample into account [24]. The PCoA plot of unweighted UniFrac distance is based on the number of shared and unshared branches of the phylogenetic tree of the samples and is therefore a measure of heterogeneity of the bacterial population [24, 23]. Since 16S rRNA full-length PacBio and V4 Illumina sequenced samples are separated, we can conclude that these samples had different phylogenetic trees which reflected different bacterial compositions. As samples of the V4 PacBio data set and the V4 Illumina clustered together, we can conclude that the difference in phylogenetic trees and thus bacterial composition is not due to platform bias (PacBio vs Illumina), but caused by the difference in sequence length. Furthermore jack-knifing variance, which determines how often the cluster results are recovered using random subsets of the data, was smaller for the full-length 16S rRNA PacBio samples compared to both V4 data sets and shows that sequencing full-length 16S rRNA resulted in increased robustness of the data [24]. It has previously been shown that the PacBio platform can be used for studying microbiota communities [34, 35]. Based on our findings and the fact that β-diversity metric UniFrac can distinguish bacterial communities at a depth of 50 reads/sample [23], we suggest that PacBio platform can be used to study intestinal microbial communities at a lower sequencing depth. This allows multiplexing multiple samples on a single molecule real-time (SMRT) cell in order to reduce resources and sequencing costs.

In addition to diversity analysis, interpretation of experimental outcome requires insight into the bacterial composition of a sample to understand e.g. which bacterial species are able to convert a dietary compound. Taxonomic analysis of the three data sets showed that sequencing full-length 16S rRNA resulted in a different bacterial composition as relative abundances of taxa were increased or decreased with 16S rRNA full-length PacBio after inulin intervention compared to both V4 data sets. Interestingly, the genus Lactobacillus was completely absent in the full-length 16S rRNA PacBio data set, while being detected in both V4 data sets. This difference in taxa detection is of major importance for interpretation of biological data. It should be mentioned that in our previous article, that exclusively relied on 16S rRNA V4 region sequencing by Illumina, we reported that the genus Allobaculum bloomed after inulin intervention [5]. However, here we report that Faecalibaculum bloomed after inulin intervention. Faecalibaculum is closely related to Allobaculum with 86.9% sequence similarity and was recently isolated from laboratory mice [36]. Microbial data of our initial article was analysed using the Greengenes 13.8 reference database and for the current work we used the SILVA 132

reference database which likely explains this discrepancy in annotation.

Sequencing the full-length 16S rRNA gene resulted in detection of a higher percentage of unassigned reads compared to sequencing the V4 regions only. Interestingly, in our study the percentage of unassigned reads was higher in samples of inulin-fed mice. This finding might suggest that at least part of the bacterial taxa blooming on inulin are in this unassigned fraction of the data. Since we cannot assign these reads, we cannot fully utilize the advantage of full-length 16S rRNA gene sequencing compared to V4 sequencing.

5.5. CONCLUSIONS

Taken together, we conclude that sequencing the full-length 16S rRNA gene provides a different view regarding bacterial relative abundance, in sample diversity and in in-between sample diversity, as compared to V4 sequencing regardless of sequence analysis platform. This clearly has implications for interpretation of biological data after a dietary intervention.

5.6. ACKNOWLEDGMENTS

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5.7. SUPPORTING INFORMATION

Table S5.1: Primer sequences used for PacBio sequencing.

Primer name	Primer sequence
M13-F-GM3F	TGTAAAACGACGGCCAGTAGAGTTTGATCMTGGC
M13-F-GM3F-YM	TGTAAAACGACGGCCAGTAGAGTTTGATYMTGGC
M13-R-GM4R	CAGGAAACAGCTATGACCTACCTTGTTACGACTT
Universal Tail Forward	CCATC|16-nt-barcode|TGTAAAACGACGGCCAGT
Universal Tail Reverse	GGTAG|16-nt-barcode|CAGGAAACAGCTATGACC

Table S5.2: Statistics of data sets

Group	Data set	Sample	Read Count	Joined reads	Mean read length (bp)	Total number of bases (bp)
Control	FL	C1	19389	-	1514	29354586
	FL	C2	9404	-	1494	14052243
	V4 PacBio	C1	9288	-	253	2347424
	V4 PacBio	C2	4061	-	251	1019710
	V4 Illumina	C1	240682	109650	253	27738445
	V4 Illumina	C2	304190	142428	253	36025327
Inulin	FL	In1	20986	-	1518	31863815
	FL	In2	7782	-	1505	11714148
	V4 PacBio	In1	9799	-	253	2475902
	V4 PacBio	In2	3386	-	251	850296
	V4 Illumina	In1	330802	151169	253	38200080
	V4 Illumina	In2	430798	202756	253	51215941

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6

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Saeed KATIRAEI

6.1. The role of intestinal microbiota in lipid metabolism and cardiovascular disease risk factors

Obesity is associated with a variety of comorbidities and predisposes an individual to type 2 diabetes and cardiovascular disease (CVD). The globally increasing prevalence of obesity challenges the medical scientific community to find new targets and strategies for the treatment of obesity and associated cardiometabolic diseases. Because of their role in host metabolism and immunity, intestinal microbiota form a new and interesting target in treating obesity and associated comorbidities such as the prevention and treatment of CVD.

6.1.1. *A. muciniphila*: a candidate species for lowering circulating triglycerides and total cholesterol?

In Chapter 4 we showed that administration of *Akkermansia muciniphila* (*A. muciniphila*) decreased plasma TG and TC levels in the hyperlipidemic E3L.CETP mouse model remarkably. The E3L.CETP mouse model is characterized by a human-like lipoprotein metabolism and its circulating cholesterol is, unlike in wild-type (WT) mice, mainly present in the pro-atherogenic LDL and VLDL fraction. Since high circulating TG and TC levels are main risk factors for atherosclerosis, lowering these plasma lipids by *A. muciniphila* administration should help in prevention of atherosclerosis progression. Additional evidence for an anti-atherogenic potential of *A. muciniphila* has been described by Li *et al.* They have shown that administration of *A. muciniphila* decreased atherosclerotic lesion area in apolipoprotein E-deficient (apo$E^{-/-}$) mice by restoring gut barrier function and preventing LPS translocation, and thus preventing systemic inflammation [1]. Whether the beneficial effects of *A. muciniphila* administration are translatable to humans has recently been assessed by Depommier *et al.* [2]. They reported that administration of *A. muciniphila* to overweight and obese humans modestly improved plasma lipid levels. However, although *A. muciniphila* administration significantly decreased TC, it did not decrease LDL-C. The question thus remains whether this reduction in TC was truly anti-atherogenic, since it may well have occurred in the HDL-C fraction, which is not causally associated with CVD. In addition, *A. muciniphila* administration did not affect plasma TG nor inflammatory parameters. However, it should also be noted that the overweight and obese humans in this study were not overtly hyperlipidemic. In conclusion, whether *A. muciniphila* administration has anti-atherogenic potential in humans should be addressed in more appropriate study populations.

A major challenge in developing a novel treatment for hyperlipidemia to reduce CVD is the fact that effective and safe pharmacological treatments are already available. Statins are the most commonly used class of drugs to reduce LDL-C. Statins are relatively cheap and have demonstrated efficacy. Since these treatments cannot ethically be withheld from patients at risk, novel treatments have to be tested on top of statin therapy in prolonged and large outcome trials. The cost of these type of large scale trials is prohibitive. It seems likely that more convincing and clear evidence from both pre-clinical as well as (small scale) clinical trial needs to be generated before such trials for a microbial species or component could be started.

6.1.2. Potential mechanism for lowering circulating triglycerides and cholesterol by intestinal microbiota

As previously described, administration of well-studied commensal bacteria like members of *Bifidobacterium* and *Lactobacillus* genera reduce circulating TG and TC levels [3, 4]. The exact mechanism of TG and TC reduction by these commensals has not been revealed yet. Most *Bifidobacterium* and *Lactobacillus* species that are known to be able lowering TC levels contain bile salt hydrolase (BSH) genes in their genomes and are thus able to deconjugate bile salts [5, 4]. However, the relationship between BSH and lowering TC is still not completely understood [4]. Several hypotheses have been proposed to explain the cholesterol-lowering effect of these intestinal bacteria [4], including the deconjugation of bile salts that promote the consumption of blood cholesterol to synthesize bile lost in feces [6, 5] and cholesterol assimilation in bacterial cells [7]. A study conducted by Wang *et al.* compared the *in vivo* cholesterol reducing effects of two BSH containg *Lactobacillus casei* strains with a *Lactobacillus casei* strain not containing the BSH gene, in high cholesterol diet fed mice. The BSH containing strains showed lowered serum TC levels compared to the control group, downregulated farnesoid X receptor (FXR) expression and upregulated expression of cholesterol liver X receptor (LXR) and low density lipoprotein receptor (LDLR) genes in the liver [4]. These findings indicate that BSH loweres cholesterol levels in vivo by reducing cholesterol absorption and increasing cholesterol catabolism [4].

Interestingly, the genome of *A. muciniphila* has recently been sequenced and it seems not to harbour a bile salt hydrolase gene. Therefore, *A. muciniphla* probably lowers plasma TC by another mechanism of action [8]. *A. muciniphila* mainly produces SCFAs acetate and propionate upon mucin degradation [9, 10]. A previous study by Lukovac *et al.* explored the transcriptional response of mouse intestinal organoids to *A. muciniphila* by incubating the organoids with *A. muciniphila* culture supernatants [11]. This study revealed that *A. muciniphila* metabolites affect various transcription factors and genes involved in fatty acid, cholesterol and bile acid metabolism. Some of these effects may be attributable to the short chain fatty acids (SCFAs) produced by *A. muciniphila*, as a previous study in rats showed that SCFAs down regulate cholesterol synthesis in both liver and intestinal tissue [12]. Alternatively, acetate can be converted to butyrate by buterogenic bacteria and affect host lipid metabolim by another mode of action [13]. Li *et al.* recently suggested that butyrate increases brown adipose tissue activity by neural activation, leading to increased oxidation of intracellular fatty acids resulting in a compensatory influx of TG-derived fatty acids and lowering of plasma TG [14]. These findings show that bacterial species have multiple mechanisms that could explain the reduction in TG and TC after administration.

6.1.3. Boosting bacterial lipid lowering potential by genetic modification

In order to increase the lipid lowering ability of intestinal bacteria in humans, it is an interesting thought to enhance these particular properties by genetic manipulation. Genetically modified bacteria with enhanced lipid lowering ability can serve as a future treatment to reduce cardiometabolic risk factors by lowering host peripheral lipid levels. Well studied commensal bacteria like members of *Bifidobacterium* and *Lactobacil-*

lus genera might be appropriate candidates for a genetically engineered enhanced lipid lowering capability. For instance, bile salt hydrolase overexpressing strains with potential cholesterol lowering ability can be constructed by creating a genetic construct with the bile salt hydrolase gene downstream of a strong overexpression promoter and transforming this construct into the desired strains. With use of *in vitro* tests one can assess whether the overexpression of this gene results in increased bile salt deconjugation and *in vivo* experiments can provide the evidence for its potential supra-physiological cholesterol lowering properties.

The idea of a genetically modified microorganism treatment might not be as far-fetched as it sounds, since similar approaches have been exploited in different contexts. Póhlmann *et al.* engineered the probiotic yeast strain *Saccharomyces boulardii* (*S. boulardii*) for secretion of biologically active viral IL-10 homologs for treatment of inflammatory bowel disease. The *in situ* data of this particular study were promising but *in vivo* data about the secretion of IL-10 are lacking [15]. Another group showed that an engineered mutant of *S. boulardii* could survive the mouse gastrointestinal tract and they could detect viable *S. boulardii* cells in the mouse Peyer's patches (PP) [16]. In particular, the idea of cytokine expression by micoorganisms in peripheral immune active sites like the PP might be interesting to modulate the local or systemic immune response to bacterial triggers from the intestine.

Another example showing the therapeutic potential of genetically engineered microorganisms is a study of Chen *et al.* They designed and constructed a N-acylphosphatidylethanolamines (NAPEs) overexpressing *E. coli* Nissle 1917 strain and administered it for 8 weeks to obese mice, via drinking water. NAPEs are precursors to the N-acylethanolamide (NAE) family of lipids, which are synthesized in the small intestine in response to feeding and they reduce food intake and obesity. Mice that received engineered bacteria had lower food intake, adiposity, insulin resistance and hepatosteatosis compared to control mice [17]. This particular study showed the therapeutic potential of such a strategy in the treatment of obesity related comorbidities. Although design and creation of such a genetically modified therapeutic bacterial strains nowadays is not a technological breakthrough anymore, the public acceptance of such a treatment is still very challenging.

6.2. Strategies for manipulating intestinal microbiota

6.2.1. Controlled and uncontrolled strategies for modulation of intestinal microbiota composition

Intestinal microbiota interact with host immunity and metabolism as shown in Chapter 3 and Chapter 4 of this thesis. There are different strategies for modulation of intestinal microbial communities such as Fecal Microbiota Transplantation (FMT) or oral administration of defined bacterial cultures.

With FMT procedure, the recipient is (re)populated with the fecal microbiota of a donor, sometimes after ablation of the endogenous microbiota [18]. This seems to be an effective strategy for modulation of intestinal microbiota in humans and mice. Currently, FMT is used as a treatment for patients suffering from intestinal diseases like Clostridium difficile-associated diarrhoea (CDAD) and inflammatory bowel disease (IBD) for

restoration of the intestinal microbiota [19, 20]. Despite its effectiveness, FMT is a poorly controlled tool for modulation of the intestinal microbiota. Although the FMT procedure is relatively easy to perform, there is wide inter-institutional variability in methodology, for example in preparation of FMT and or in processing and mode of delivery of the fecal matter [21]. In most cases, prior to FMT, the recipient is treated with antibiotics in order to reduce the native, dysbiotic population [21, 22]. The total fecal composition of the donor is transplanted to the receiver, and the receiver is repopulated with a wide range of bacterial, fungal and viral species of the donor. As a consequence, it is impossible to pinpoint which donor species was responsible for the beneficial (clinical) outcome.

Another uncontrolled strategy for modulation of intestinal microbiota composition is oral administration of prebiotic compounds. Prebiotics are indigestible dietary fibres, which are degraded by intestinal microbiota [23]. A prebiotic compound, such as e.g. inulin, can be degraded by several different species like members of the genera *Bifidobacterium* and *Allobaculum* which as a consequence increase their abundance [24]. To our knowledge, there are no prebiotics known that modulate the abundance of only one specific species. Upon fibre degradation, the bacteria secrete metabolites in the lumen, as side or waste products of their metabolism. These metabolites can serve as precursors for metabolism of the host and/or other bacteria and in this way there is a cross feeding between different species. By using labelled [^{13}C] lactate, Belenguer *et al.* showed that lactate produced by *Bifidobacterium adolescentis* L2-32 in culture is used by *Eubacterium hallii* L2-7 for butyrate production and confirmed the cross feeding between these two human intestinal commensal species [25]. In this way, administration of one single prebiotic compound can up and down regulate the abundance of several additional species, genera or even bacterial families.

Modulation of the intestinal microbial composition can be somewhat more directly and targeted by administration of a single bacterial culture, compared to FMT or oral administration of prebiotic compounds. In this way, a defined strain with a defined concentration can be administered to the host and its effects on host physiological parameters can be described, as applied in Chapter 4 of this thesis. This modulation strategy has been well described in humans and mice for aerobic, facultative aerobic and anaerobic bacteria [26, 27, 28]. However, because of the intricate interplay and cross feeding between different bacterial taxa and species, administration of one single strain can also up or down regulate the abundance of it cross feeding partners or competitors. It seems reasonable to conclude, that even a controlled strategy for manipulation of intestinal microbiota is still only partly controlled. In Chapter 4, we cannot formally exclude the possibility that some of the observed effects are not mediated by *A. muciniphila* but mediated by other bacteria that were affected by administration of *A. muciniphila*.

6.2.2. MODULATION OF INTESTINAL MICROBIOTA BY ELIMINATION OF SINGLE BACTERIAL SPECIES; A FUTURE TOOL

We (Chapter 4) and others have shown that the potentially beneficial effects of bacterial species on host lipid metabolism and immunity can be studied by introducing this bacterial species into the intestinal microbiota population of the host by oral administration. However, it might also be interesting to study the absence of potentially detrimental bacterial species by eliminating these from the intestinal microbiota population

of the host. Elimination of bacterial species in a complex bacterial population can be achieved by bacteriophages. Bacteriophages are viruses which infect bacterial cells and not eukaryotic cells [29, 30]. Like other viruses, they inject their viral DNA into the host cell and use the host cell machinery for the replication and assembly for new bacteriophages until the host cell is eliminated by eruption. This idea has inspired many scientists and even the industry. One of the commercial pioneers in this field is the in France based start-up company Eligo Biosciences. By using CRISPR-Cas technology, Eligo Biosciences aims to design bacteriophage based antimicrobials. By using RNA-guided nucleases (RGNs) targeting specific DNA sequences or polymorphisms, including antibiotic resistance and virulence determinants, a specific bacterium can be targeted and eliminated from the community without affecting other bacterial members of that community [31, 32, 33]. In this way, the effects of presence or absence of a single species within a complex community can be studied and assessed for its contribution or causality in host (patho)physiology. However, as mentioned in the previous paragraph, eliminating one species from a complex bacterial population might have further downstream effects on the whole bacterial population because of the intricate interplay and cross feeding between different bacterial taxa and species in the population.

6.3. Local and systemic immune modulation by intestinal microbiota

6.3.1. Intestinal microbiota modulate the host immune response locally at the intestinal immune active sites

In Chapter 4 we showed that oral administration of *A. muciniphila* modulated the immune cell composition locally in the mesenteric lymph nodes (mLNs) by changing the abundance of different immune cell populations and their activation markers. There is a very fine bidirectional interaction between the host GI immune system and its microbiota. The immune system is constantly surveying the luminal environment and secretes anti-microbial compounds into the mucus layer to control bacterial outgrowth and control overexposure of the epithelial cells to bacteria [34]. Dendritic cells (DCs) survey the lumen and lamina propria constantly and capture bacteria that have crossed the epithelial layer. These antigen loaded DCs migrate to the mLNs for antigen presentation. This antigen presentation leads to the differentiation of commensal specific regulatory cells (Treg) Th17 cells and IgA producing B cells [35]. In Chapter 4, oral administration of *A. muciniphila* decreased total T cell population in the mLNs. Simultaneously, the total B cell population was increased and the expression of the T cell co-stimulatory molecule CD86 on both the follicular and mucosal B cell populations was reduced. *A. muciniphila* also tended to decrease the antigen-presenting molecule MHCII on different DCs subpopulation, indicating reduced activity of these DC subpopulations. Furthermore, the total amount of neutrophils in the mLNs was also decreased upon oral administration of *A. muciniphila*. These findings clearly indicate that *A. muciniphila* has immune modulating properties.

The immune modulation by *A. muciniphila* could be explained by the SCFA producing capability of *A. muciniphila* [11]. SCFAs modulate the host's immune system in different manners. SCFAs increase the expression of antimicrobial peptides secreted

into the lumen, modulate the production of cytokines and chemokines and regulate the differentiation, recruitment and activation of immune cells including neutrophils, macrophages and DCs [36]. A recent study showed that oral administration of butyrate reduced neutrophil associated inflammation in a DSS induced colitis mouse mode [37]. These findings are in line with the hypothesis that the decreased total number of neutrophils upon oral administration of *A. muciniphila* is due to its SCFA production. Thus, oral administration of *A. muciniphila* seems to excert a local anti-inflammatory response in the mesenteric lymph nodes, which may be both mediated via presentation of *A. muciniphila* antigens by the DCs in the mLNs and SCFA production and secretion of *A. muciniphila* in the lumen.

6.3.2. INTESTINAL MICROBIOTA MODULATE HOST PERIPHERAL IMMUNITY

Several human commensals, e.g. members of *Bifidobacterium* are known to affect the circulating immune cells and signaling molecules. Bernini *et al.* showed that daily intake of *B. lactis* HN109 over a period of 45 days decreased the pro-inflammatory cytokines TNF-α and IL-6 in blood samples of obese subjects [26]. Martin *et al.* showed that daily oral gavage for a period of 10 days with *Bifidobacterium animalis ssp. lactis* CNCM-I2494 increased splenic secretion of anti-inflammatory cytokines IL-4, IL-5 and IL-10 in a low-grade inflammation mouse model [38]. In Chapter 4, we showed that administration of *A. muciniphila* affected the host IL-10 response modestly and reduced portal vein LPS levels tremendously. Therefore, intestinal microbiota form an interesting and new target for modulation of the host peripheral immune system and might be useful in prevention or treatment of immunometabolic diseases. To this end, longitudinal oral administration of known beneficial single or mixed bacterial cultures can be used for diminishing the immunological effects of a dysbiosed microbiome.

6

6.3.3. IMPLICATIONS OF INTESTINAL MICROBIOTA FOR HOST PATHOPHYSIOLOGY

In Chapter 2 and Chapter 3 we applied co-housing, a rodent analogue of FMT, for modulation of intestinal microbiota after BMT. Experimental BMT in mice is relatively easy, effective and cost-efficient tool to study the role of immune cells in many immune-associated disorders [39]. However, the procedure of BMT, which combines use of broad range antibiotics and ionizing radiation induces acute and chronic side effects like decreased body weight gain [40, 41], intestinal damage [42] and , disturbs the intestinal microbiota and causes dysbiosis [43, 44]. Similar findings have been recently reported in humans [35]. We hypothesized that co-housing could restore the dysbiosed microbiome to a eubiotic microbiome and as a consequence ameliorate the pathophysiological side effects of BMT in mice. Chapter 2 described that the decreased total body weight gain after BMT is a consequence of decreased weight gain of multiple organs and alterations in the adipose tissue cell pool composition as well as to a decreased pancreatic secretion of the anabolic hormone insulin. Co-housing with healthy, non-BMT mice, did not prevent nor restore this decreased total body weight gain (Body weight data of co-housed groups is not shown in this thesis). A mouse study examing the effect of FMT post BMT, showed that FMT preserved the intestinal bacterial composition, improved gastrointestinal tract function, intestinal epithelial integrity and prevented decreased body weight gain after

BMT [45]. A clinical study showed that oral supplementation of glutamine, fibre and oligosaccharides (GFO) from seven days prior and up to twenty-eight days post stem cell transplantation, decreased the severity of mucosal injury and prevented weight loss in humans compared with the control group [46]. Another clinical study reported beneficial clinical outcomes like mitigated mucosal injury and decreased incidence of acute Graft Versus Host Disease after oral administration of dietary fibres during the procedure of BMT [47]. These animal and clinical studies indicate that intestinal microbiota might serve at least partially as a target in prevention of decreased body weight gain after BMT and restoration of intestinal ephitelial intergity might play a role in this.

In Chapter 3 we show that splenocytes of BMT treated mice show higher cytokine release as compared to healthy controls, when stimulated with various pathogenic stimuli. This hyper-responsive phenotype of splenocytes is transferred by FMT from BMT treated mice to healthy control mice. By inoculation of germ free mice with cecum content of BMT treated mice, we showed the causal role of intestinal microbiota in hyperresponsiveness of splenocytes post BMT, as a measure of peripheral immunity. Interestingly, the FMT experiment showed the dominance of a dysbiosed microbiome over a healthy one and showed that the balance of a healthy microbiome can be overruled. The observation of a dominant dysbiosed microbiome has previously been described in different immune models. Elinav *et al.* showed that NLRP6 inflammasome deficient mice have altered intestinal microbiota composition, showed spontaneous intestinal inflammatory cell recruitment and were more susceptible for chemically induced colitis. Co-housing experiments revealed that the colitogenic intestinal microbiota of these NLRP6 inflammasome deficient mice were transferable by FMT [48]. It seems that the homeostasis of a healthy microbiome is based on the balance between the many different members of that community, the host and its environment. However, this balance might be more fragile and easier to perturb by endogenous or exogenous effectors, than was previously thought. In our specific experimental design, FMT perturbed this balance and drove the intestinal microbiota community of the recipient mice into a dysbiosis and affected host immunity as we have shown in Chapter 3.

Regarding the recent findings in literature, we can conclude that intestinal microbiota are able to ameliorate both the physiological and immunological side effects of BMT. However, it seems extremely important to choose the right strategy for modulation of intestinal microbiota in order to modulate host metabolism and immunity in a favourable way.

6.4. Studying microbial communities by 16s sequencing.

The emergence of the microbiome field became possible because of rapid evolution of Next Generation Sequencing technology (NGS). NGS developments have made it possible to sequence billions of nucleotides from multiple samples in a single sequencing run with reduction of time and cost [49]. Currently, a frequently used method for studying microbial community composition and dynamics is 16s sequencing. Despite the fact that the cost of sequencing per nucleotide rapidly decreased over the past decade, down to several cents for each base pair, using NGS as an experimental read-out is still expen-

sive. The commercial price of 16s sequencing for a single stool sample ranges from € 16-50, depending on a.o. sample size, sequencing depth requirements and sequencing platform [50, 51]. This price mostly excludes DNA isolation and NGS data analysis costs, so the actual cost per sample is higher. For a sizeable mouse experiment with an intervention and multiple stool samples over the study period, NGS sequencing costs can still be prohibitive.

Another limitation of 16s sequencing is the resolution of 16s sequencing, which is limited to the amplicon sequence and availability of reference data in 16s databases. In 16s sequencing, variation in specific regions of the bacterial 16s rRNA gene is used as a marker gene for taxonomic analysis and distinguishing different taxa within the sample [52, 53]. A short amplicon of the 16s rRNA gene or its entire 16s rRNA gene can be sequenced and used for taxonomic analysis. However, in most cases a short amplicon of the 16s rRNA gene for e.g. the V4 region or a combination of two or more amplicons like the V3 and V4 regions is sequenced, rather than sequencing the full-length 16s RNA gene. Sequencing a short amplicon of 16s rRNA gene results a higher number of reads per sample and a higher sequencing depth in comparison with full-length 16s sequencing. However, it should be noted that the maximal resolution of 16s sequencing is predominantly at the genus level [54].

As described in Chapter 5, sequencing the full-length 16s rRNA gene provided a different view regarding bacterial relative abundance, in-sample diversity and in in-between-sample diversity, as compared to V4 sequencing regardless of sequence analysis platform. Thereby, the improvement of resolution in terms of taxonomic analysis was in our particular experimental setting marginal. In a recent study, bacterial communities in wastewater were characterized using PacBio full-length 16s rRNA gene sequencing and compared to *in silico* extracted V3-V4 short sequences from the same PacBio data [55].The authors claimed that analysing full-length 16s rRNA gene provided more refined and reliable taxonomic assignment, even to the species level. Detailed examination of their findings revealed that they only made this comparison for the genus *Acinetobacter* and not for their entire dataset. In this particular study, using full-length 16 rRNA gene only assigned six additional OTUs to species level compared to short V3-V4 reads. In our study, described in Chapter 5, we see comparable results on species level OTU assignment by full-length 16s rRNA gene (data not shown) and refer to these differences as a marginal improvement in resolution of taxonomic analysis. Another study using mouse gut samples, compared full-length 16s rRNA gene sequenced by Oxford Nanopore sequencing platform with short V3-V4 region reads sequenced by Illumina platform. The authors reported that full-length 16s rRNA gene data had better resolution than the short-read sequencing data, at the species level [56]. The results of these two studies are in line with our findings described in Chapter 5.

It has to be mentioned that both studies [55, 56] mainly focused on the differences in taxonomic assignment of OTUs on species level between full-length 16s rRNA gene and a short amplicon of the 16s rRNA gene. In contrast, in Chapter 5 we also took into account in-sample and in-between-sample diversity analysis to show the difference between using full-length 16s rRNA gene and a short amplicon of this marker gene. We used UniFrac distance as a measure for in-between-sample diversity. Full-length 16s rRNA sequenced samples had a different UniFrac distance compared to V4 sequenced

sample, showing that the full-length 16s rRNA data resembled a different bacterial phylogeny. Furthermore, using full-length 16s rRNA resulted in a more robust UniFrac distance, as the jack-knifing variance was smaller compared to the V4 data. These observations are not revealed by studying taxonomic assignment only. Therefore, focusing on taxonomic assignment of OTUs only and not taking into account differences in-sample and in–between-sample diversity does not utilise the full potential of using full-length 16s rRNA gene sequencing.

Another drawback of focusing on taxonomic assignment only is based on the fact that currently available 16s reference databases like the Greengenes and SILVA database [57, 58] mainly rely on 16s rRNA gene sequences of cultured taxa. Therefore, a certain proportion of the sequences in a typical 16s sequencing dataset can not be assigned, because of the lack of a reference sequence in these databases. Although, recently, uncultured taxa are being included in the databases, we can not yet understand the full potential of an uncultured taxon, because of the lack of information about the in vitro and in vivo properties of these uncultured bacteria.

Taken together, 16s sequencing is a useful tool for studying microbial community changes and dynamics globally, but less useful for studying individual members of the community. The scientific community, using this technology as a read-out tool for experimental outcome, should be aware of the inherent limitations and biases of NGS technology and take these limitation and biases in account for the interpretation of their experimental results.

6.5. Conclusion

The studies described in this thesis addressed the causal role of intestinal microbiota in host metabolism, immunity and pathophysiology of atherosclerosis. We showed that oral administration of the bacterial species *A. muciniphila* affected host lipid metabolism. In addition, our co-housing experiments after BMT indicated that intestinal bacteria play a crucial role in systemic immune responses. Furthermore, we provided critical insight in two generally accepted and widely applied research tools in cardiometabolic research and microbiome research, BMT and 16s rRNA sequencing. After BMT mice clearly suffer from a multitude of abnormalities that should be taken into account when designing and interpreting results from BMT experiments. Similarly, the choice of amplicon for 16S rRNA sequencing affects interpretation of the results and should be taken into account when designing and interpreting results from experiments which affect the microbiota.

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Summary

Chapter 1 serves as a general introduction illustrating the role of intestinal microbiota in cardiometabolic diseases. The main underlying cause of cardiometabolic diseases is atherosclerosis, which is mainly caused by dyslipidemia and inflammation. The role of intestinal microbiota on the local and systemic alternation of the immune system is discussed in this chapter. Furthermore, this chapter focusses on the technical aspects of cardiometabolic and microbiota research. Therefore, bone marrow transplantation (BMT), a widely used tool to study gene function in atherogenesis, and Next Generation Sequencing, a tool for profiling the intestinal microbiota, are discussed in detail.

Experimental bone marrow transplantation is a relatively easy and effective strategy for assessing the role of specific immune related genes in pathophysiological mechanisms in mice. Remarkedly, the application of BMT in obesity research is hampered by the significant reduction in high-fat diet (HFD)-induced obesity, which is caused by the BMT procedure. In **Chapter 2** we characterized the metabolic tissues that may be affected by the BMT procedure and that may impair the HFD-induced response. Taken together, we concluded that the BMT procedure has multilevel effects on the organism and affects metabolically important organs. These multi-level effects are associated with metabolic abnormalities that involve both white adipose tissue (WAT) and pancreas dysfunction in response to a HFD. Therefore, the metabolic effects of BMT cannot simply be neglected and should be seriously considered in study design and interpretation of the data.

In addition to the metabolic side effects of BMT as characterized in **Chapter 2,** conditioning regimens of BMT acutely induce systemic inflammation, intestinal damage and result in shifts in the gut microbial composition. As alterations in the intestinal microbiota community are associated with both obesity and immunity, this could explain the metabolic side effects of BMT. In **Chapter 3,** we aimed to investigate whether, post-BMT, the peripheral immune system is modulated as a direct consequence of BMT induced alterations in the gut microbiota. We investigated in mice the effect of BMT on the response patterns of splenocytes and peritoneal macrophages to various pathogenic stimuli as markers of the extra-intestinal immune system. Splenocytes are a mixed population of immune cells and thus represent responses from both the adaptive and innate immune system, whereas peritoneal macrophages represent the innate immune system. The potential role of microbiota in the response patterns of splenocytes and peritoneal macrophages was investigated by co-housing control mice with BMT-treated mice and by transfer of cecum content to germfree mice. We show that 24 weeks post-BMT, splenocytes but not peritoneal macrophages display increased cytokine response patterns upon ex-vivo stimulation with various pathogens as compared to untreated controls. The pattern of BMT-induced cytokine responses was transferred to splenocytes, and not to peritoneal macrophages, of healthy controls via co-housing and transferred to germfree mice via transplantation of cecum content. Thus, gut microbiota

increase the cytokine responses pattern of splenocytes after BMT. This phenotype can be transferred to splenocytes of healthy controls by co-housing or to germfree mice via transfer of cecum content, indicating that they are independent of BMT-induced intestinal damage and microbial leakage.

In **Chapter 4,** we aimed to investigated the effects of oral administration of a single bacterial species on metabolic and immunological markers of atherogenesis in mice. Previous mouse studies showed that oral administration of the intestinal commensal bacterium, *A. muciniphila*, protected against HFD-induces obesity and improved metabolic and immunological markers such as glucose tolerance, and hypercholesterolemia and cytokine secretion by different immune cells. However, the effects of *A. muciniphila* on atherogenesis were not investigated yet. We investigated the effects of *A. muciniphila* on lipid metabolism, immunity, and cuff-induced neointima formation in hyperlipidemic APOE*3-Leiden (E3L).CETP mice. We determined the effect of 4 weeks oral *A. muciniphila* administration in E3L.CETP mice on 1) plasma lipid levels, 2) the immune response by measuring portal vein lipopolysaccharide (LPS) levels, mesenteric lymph node (mLN) immune cell composition and ex vivo responses of circulating leukocytes to LPS, and 3) neointima formation and composition. We found that administration of *A. muciniphila* lowered hyperlipidemia in hypercholesterolemic E3L.CETP mice and had immunomodulatory properties. As both hyperlipidemia and immune responses are involved in the pathogenesis of atherosclerosis, these observations suggest that *A. muciniphila* has anti-atherogenic potential. However, in contrast to our hypothesis, *A. muciniphila* was unable to ameliorate atherosclerosis in our cuff-induced neointima formation model, suggesting that the anti-atherogenic effects of *A. muciniphila* were not sufficiently strong in this mouse model.

In several chapters of this thesis, monitoring the composition of intestinal microbiota and compositional shifts in intestinal microbial communities after an experimental intervention were important experimental read outs. In the field of microbiota research, sequencing a relative small part of the 16s rRNA gene, the so called V4 region, is a widely used method for monitoring the microbial composition. In **Chapter 5,** we aimed to assess whether sequencing the full-length 16S rRNA gene affected the results and interpretation of a dietary intervention compared to sequencing only the V4 region of this gene. To compare the effects of the dietary intervention measured on either the PacBio platform for sequencing the full-length 16S rRNA gene or Illumina MiSeq platform for sequencing the V4 region, we performed taxonomic analysis and diversity analysis. To test this, mice were fed a diet without and with the prebiotic inulin. From cecum samples, two primary data sets were generated: 1) a 16S rRNA full-length data set generated by the PacBio platform; 2) a 16S rRNA V4 region data set generated by the Illumina MiSeq platform. A third derived dataset was generated by in silico extracting the 16S rRNA V4 region data from the 16S rRNA full-length PacBio data set. Analyses of the primary and derived 16S rRNA V4 region data indicated similar bacterial abundances, and α- and β-diversity. However, comparison of the 16S rRNA full-length data with the primary and derived 16S rRNA V4 region data, revealed differences in relative bacterial abundances, and α- and β-diversity. We conclude that sequencing the full-length 16S rRNA gene provides a different view regarding bacterial relative abundance, in-sample diversity and in in-between-sample diversity, as compared to V4 sequencing regardless of sequence

analysis platform. This clearly has implications for interpretation of biological data after a dietary intervention.

In **Chapter 6,** we describe the causal role of intestinal microbiota in host metabolism, immunity and pathophysiology of atherosclerosis. The studies described in this thesis showed that oral administration of the bacterial species *A. muciniphila* affected host lipid metabolism. In addition, our cohousing experiments after BMT indicated that intestinal bacteria play a crucial role in systemic immune responses. Furthermore, we provided critical insight in two generally accepted and widely applied research tools in cardiometabolic research and microbiome research, BMT and 16s rRNA sequencing, which should be taken into account when designing and interpreting results from experiments using these tools.

Samenvatting

Hoofdstuk 1 dient als algemene introductie en licht de rol van de darmmicrobiota in cardiometabole aandoeningen toe. De hoofdoorzaak van cardiometabole aandoeningen is atherosclerose, die voornamelijk veroorzaakt wordt door dyslipidemie en inflammatie. In dit hoofdstuk wordt beschreven hoe de darmmicrobiota het immuunsysteem lokaal en systemische beïnvloedt. Daarnaast richt dit hoofdstuk zich op de technische aspecten van cardiometabool en microbiota onderzoek. Beenmergtransplantatie (BMT), een veelgebruikte methode bij het bestuderen van gen-functies bij atherogenese, en Next Generation Sequencing, een methode voor het profileren van de darmmicrobiota worden uitvoerig besproken.

Experimentele beenmergtransplantatie is een relatief eenvoudige en effectieve strategie voor het bestuderen van de rol van specifieke immuungerelateerde genen bij pathofysiologische mechanismen in muizen. Opvallend genoeg wordt de toepassing van BMT bij obesitasonderzoek belemmerd door een significante vermindering van hoog vet dieet (HFD) geïnduceerde obesitas, dit als direct gevolg van de BMT-procedure. In **hoofdstuk 2** hebben wij de metabole organen die door de BMT-procedure mogelijk beïnvloed kunnen worden en die de HFD-geïnduceerde respons kunnen verstoren uitvoerig bestudeerd. Wij hebben geconcludeerd dat de BMT-procedure verschillende effecten op het organisme heeft en metabool belangrijke organen beïnvloedt. Deze effecten zijn geassocieerd met metabole afwijkingen die betrekking hebben op zowel het wit vetweefsel (WAT) als op pancreasdisfunctie in reactie op een HFD. Daarom kunnen de metabole effecten van BMT niet genegeerd worden en moeten deze serieus in acht genomen worden bij het onderzoeksontwerp en bij de interpretatie van de onderzoeksdata.

Naast de metabole bijwerkingen van BMT zoals beschreven in **hoofdstuk 2**, veroorzaakt de conditionering van BMT acute systemische inflammatie, darmschade en resulteert in veranderingen in de samenstelling van de darmmicrobiota. Aangezien veranderingen in de intestinale microbiota composities geassocieerd zijn met zowel obesitas als ontregeling van het immuunsysteem, kan dit de metabole bijwerkingen van BMT verklaren. In **hoofdstuk 3** hebben wij de regulatie van het perifere immuunsysteem onderzocht, als direct gevolg van veranderingen in de darmmicrobiota veroorzaakt door BMT. Wij hebben in muizen het effect onderzocht van BMT op de reactiepatronen van splenocyten en peritoneale macrofagen op verschillende pathogene stimuli als markers van het extra-intestinale immuunsysteem. Splenocyten zijn een gemengde populatie van immuuncellen en vertegenwoordigen daarmee reacties van zowel het aangeleerde als het aangeboren immuunsysteem, terwijl peritoneale macrofagen alleen het aangeboren immuunsysteem vertegenwoordigen. De potentiële rol van de darmmicrobiota in de reactiepatronen van splenocyten en peritoneale macrofagen werd onderzocht door controlemuizen samen te huisvesten met BMT-behandelde muizen en door overdracht van cecum-inhoud naar germfree muizen. Wij hebben laten zien dat 24 weken na BMT, splenocyten, maar niet peritoneale macrofagen, een verhoogde cytokinereactie laten

zien bij *ex-vivo*-stimulatie met verschillende pathogenen in vergelijking met onbehandelde controles. Het patroon van door BMT-geïnduceerde cytokinereacties werd overgedragen op splenocyten, en niet op peritoneale macrofagen, van gezonde controles via samen-huisvesting en overgedragen op germfree muizen via transplantatie van cecum-inhoud. Concluderend, de darmmicrobiota verhoogt de cytokinereacties van splenocyten na BMT. Dit fenotype kan op splenocyten van gezonde controles door samen-huisvesting of op germfree muizen via overdracht van cecum-inhoud worden overgedragen, wat impliceert dat dit fenotype onafhankelijk van BMT-geïnduceerde darmschade en lekkende darm syndroom is.

In **hoofdstuk 4** hebben wij de effecten van orale toediening van een enkele bacteriesoort op metabole en immunologische markers van atherogenese in muizen onderzocht. Voorgaande muisstudies hebben aangetoond dat orale toediening van de darmbacterie *A. muciniphila* bescherming bood tegen HFD-geïnduceerde obesitas en het verbeterde de metabole en immunologische markers zoals glucose tolerantie, hypercholesterolemie en cytokinesecretie door verschillende immuun cellen. De effecten van *A. muciniphila* op atherogenese waren echter nog niet onderzocht. Wij hebben de effecten van *A. muciniphila* op lipide metabolisme, immuun systeem en cuff-geïnduceerde neointima vorming in hyperlipidemische APOE * 3-Leiden.CETP (E3L.CETP) muizen onderzocht. Wij hebben het effect onderzocht van 4 weken orale *A. muciniphila*-toediening bij E3L.CETP muizen op 1) plasma lipidenniveaus, 2) de immuunrespons door het meten van poortader lipopolysaccharide (LPS) niveaus, samenstelling van immuuncellen in mesenterische lymfeknopen (mLN) en *ex vivo* responsen van circulerende leukocyten op LPS en 3) neointima vorming en samenstelling. Wij bevonden dat de toediening van *A. muciniphila* hyperlipidemie verlaagde in de hypercholesterolemische E3L.CETP muizen en immuun modulerende eigenschappen heeft. Aangezien zowel hyperlipidemie als het immuunsysteem betrokken zijn bij de pathogenese van atherosclerose, suggereren deze waarnemingen dat *A. muciniphila* anti-atherogene potentie heeft. In tegenstelling tot onze hypothese was *A. muciniphila* echter niet in staat om atherosclerose te verbeteren in ons cuff-geïnduceerde neointima vormingsmodel, wat suggereert dat de anti-atherogene effecten van *A. muciniphila* in dit muismodel niet voldoende sterk waren.

Het monitoren en inzichtelijk maken van de darmmicrobiota compositie en diens veranderingen als gevolg van een experimentele interventie, is in verschillende hoofdstukken van dit boek een belangrijke parameter. In het veld van microbioom onderzoek is het sequensen van een relatief klein deel van het 16S rRNA-gen, de zogenaamde V4-regio, een veelgebruikte methode om de microbiële compositie te monitoren. In **hoofdstuk 5** hebben wij onderzocht of het sequensen van het volledige 16S rRNA-gen de resultaten en interpretatie van een dieetinterventie beïnvloedt, in vergelijking met sequensen van slechts de V4-regio van dit gen. Om de effecten van de dieetinterventie te vergelijken, is het PacBio-platform voor sequensen van het volledige 16S rRNA-gen en het Illumina MiSeq-platform voor sequensen van de V4-regio gebruikt. Vervolgens hebben wij taxonomische en diversiteitsanalyse uitgevoerd. Hiertoe werden muizen een dieet met èn zonder de prebioticum inuline toegediend. Cecum-inhoud van deze muizen is gebruikt om twee primaire datasets te generen: 1) een volledige 16S rRNA dataset gegenereerd door het PacBio-platform; 2) een 16S-rRNA V4-regio dataset gegenereerd door

het Illumina MiSeq-platform. Door het 16S rRNA V4-regio uit de volledige 16S rRNA PacBio dataset in silico te extraheren, is een derde (afgeleide) dataset gegenereerd. Analyse van de primaire en afgeleide 16S rRNA V4-regio datesets vertoonde vergelijkbare bacteriële abundantie, en α- en β-diversiteit. Echter, vergelijking van de volledige 16S rRNA dataset met de primaire en afgeleide 16S rRNA V4-regio datasets, onthulde verschillen in relatieve bacteriële abundantie en α- en β-diversiteit. Wij concluderen dat sequensen van het volledige 16S rRNA-gen ongeacht het sequencingplatform een ander beeld geeft ten aanzien van de relatieve bacteriële abundantie, in-monster-diversiteit en inter-monsterdiversiteit, in vergelijking met V4-regio sequensing. Deze bevinding heeft duidelijke implicaties voor de interpretatie van biologische data na een dieetinterventie.

In **hoofdstuk 6** beschrijven wij de causale rol van de darmmicrobiota in gastheer metabolisme, immuunsysteem en pathofysiologie van atherosclerose. De studies die in dit proefschrift beschreven zijn, tonen aan dat orale toediening van de bacterie *A. muciniphila* lipidenmetabolisme van de gastheer beïnvloedt. Bovendien gaven onze samen-huisvesting-experimenten na BMT aan dat darmbacteriën een cruciale rol spelen bij systemische immuunreacties. Wij hebben kritische inzichten verschaft over twee algemeen aanvaarde en breed toegepaste onderzoeksmethoden in cardiometabole en microbioom-onderzoek, namelijk BMT en 16S rRNA sequencing. Deze inzichten zouden in acht genomen moeten worden bij het onderzoeksontwerp en bij de interpretatie van de onderzoeksdata, wanneer deze methoden toegepast worden.

Curriculum Vitæ

Saeed Katiraei

Saeed Katiraei werd op 30 september 1984 in Kermanshah, Iran, geboren. Na het afronden van de basisonderwijs in Iran emigreerde hij op 10 jarige leeftijd met zijn familie naar Nederland. Hij behaalde in 2004 zijn Gymnasium diploma aan de Terra College, te Den Haag. In datzelfde jaar begon hij de studie Life Science & Technology (LST) aan de TU Delft en Universiteit Leiden, die hij in 2013 afrondde.

Daarnaast heeft hij in deze periode vele extra curriculaire en nevenwerkzaamheden verricht. In 2006 startte hij een onderneming in de personenvervoerbranche en werd politiek actief bij een politieke jongeren organisatie. In 2009 combineerde hij zijn politieke interesse met zijn studie in de vorm van een stage bij de Tweede Kamer fractie van Partij van de Arbeid, daar deed hij onderzoek naar politieke standpuntvorming rondom medisch ethische zaken. In datzelfde jaar nam hij samen met een aantal medestudenten deel aan de internationale synthetische biologie competitie iGEM, georganiseerd door Massachusetts Institute of Technology (Boston, VS). Dit project werd met twee internationale prijzen bekroond: een gouden medaille en Best Information Processing Award. Bij de gemeenteraadsverkiezingen van 2010 en 2014 was Saeed kandidaat gemeenteraadslid in Den Haag.

Na het behalen van zijn MSc diploma Life Science & Technology begon hij aan een lang gekoesterde droom. Een promotieonderzoek op de afdeling Humane Genetica van het Leids Universitair Medisch Centrum in Leiden, onder begeleiding van Prof.dr.ir. Ko Willems van Dijk, Dr. Jimmy Berbée en Dr. Vanessa van Harmelen. Tijdens zijn promotieonderzoek heeft hij zich ook intensief bezig gehouden met leren programmeren en een verdiepingsslag in statistiek gemaakt. Dit was het fundament van zijn huidige carrière als Data Scientist. Het promotieonderzoek, waarvan de resultaten staan beschreven in dit proefschrift, werd afgerond in 2018 en verdedigd in maart 2023.

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De studies uit hoofstukken drie en vier van dit boekwerk zijn dusdanig complex en multidisciplinaire van aard geweest, dat het opzetten en uitvoeren ervan zonder hulp van collega's van andere onderzoeksinstituten simpelweg onmogelijk was geweest. Ik heb daarbij de mogelijkheid gehad om met een aantal vooraanstaande wetenschappers mogen werken, voor wiens werk ik veel ontzag heb. Deze samenwerking reikten van Wageningen, Nijmegen en België tot aan Brazilië toe. Mijn dank voor deze leerzame samenwerkingen gaat uit naar Prof. Willem de Vos, Prof. Mihai Netea, Prof. Patrice Cani, Prof. Mauro Teixera en hun toegewijde teamleden.

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