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***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 2×10^8 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.

4.1. 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].

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 RegIII γ 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 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. 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 μ L of either 1) anaerobic PBS or 2) 2×10^8 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/dexdomitor/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 μ l 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 CCTTGCGTTGGCTTCAGAT 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 Amebocyte 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 5×10^5 or 1×10^6 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 $\pm$ 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 2×10^8 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.

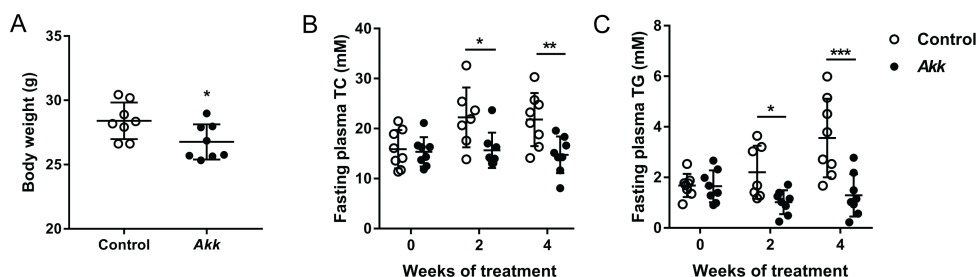


Figure 4.1: *A. muciniphila* decreased body weight and plasma lipid levels. (A) Body weight (after 4 weeks) as well as fasting plasma (B) total cholesterol (TC) and (C) triglyceride (TG) levels were determined at the indicated times after daily oral administration of hyperlipidemic E3L.CETP mice with *A. muciniphila* (Akk) or vehicle (control). Data are means $\pm$ 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.

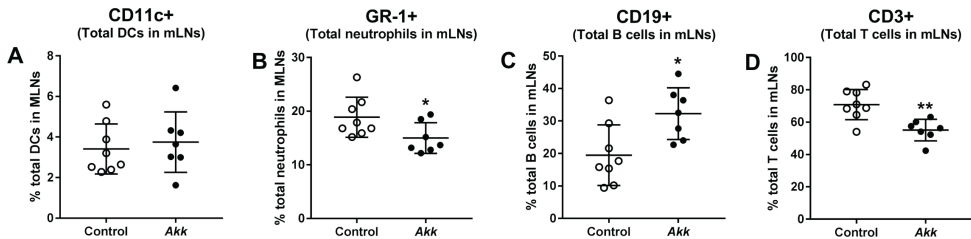


Figure 4.2: *A. muciniphila* modulated immune cell populations in mesenteric lymph nodes. Mesenteric lymph nodes (MLNs) were isolated from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control), and the percentage of total (A) dendritic cells, (B) neutrophils,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

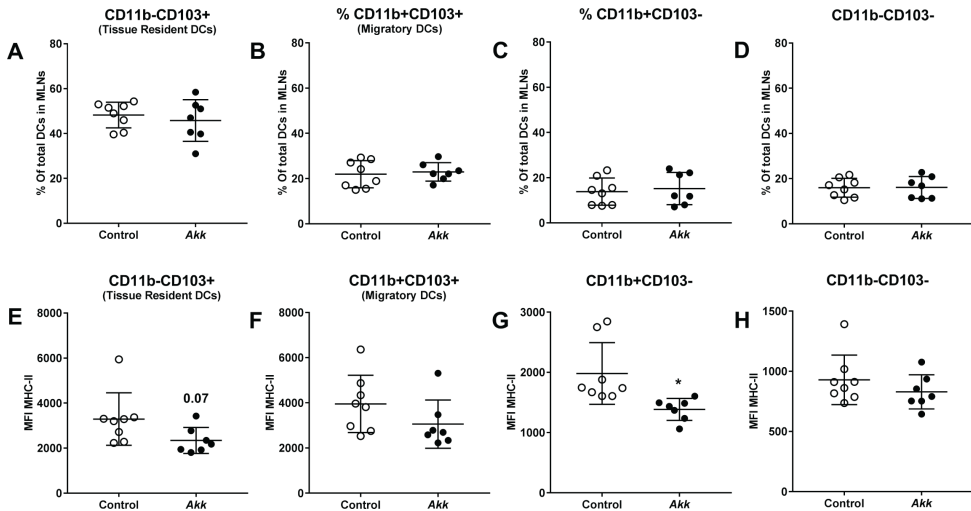


Figure 4.3: *A. muciniphila* did not affect percentage of dendritic cell subpopulations but decreased expression of MHC-II on DCs in mLNs. Mesenteric lymph nodes (MLNs) were isolated from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control), and subsets 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 $\pm$ 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*.

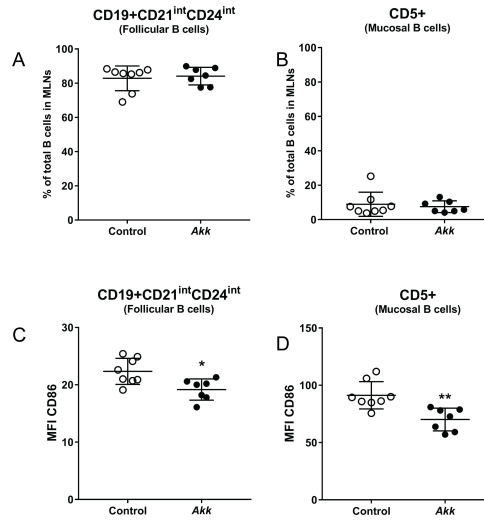


Figure 4.4: *A. muciniphila* did not affect abundance of follicular and mucosal B cells in mLNs, but decreased their CD86 expression. Mesenteric lymph nodes (MLNs) were isolated from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control), and subsets of 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 $\pm$ 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 mLN, 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.

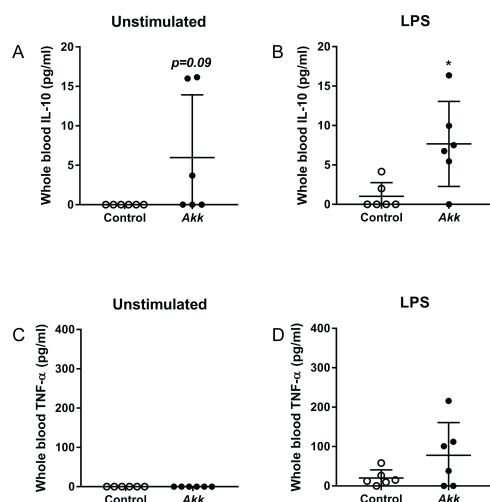


Figure 4.5: *A. muciniphila* increased IL-10 secretion in *ex vivo* LPS-stimulated blood. Whole blood was collected from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control) and, subsequently, stimulated *in vitro* (A, C) without or (B, D) with 10 pg/ml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

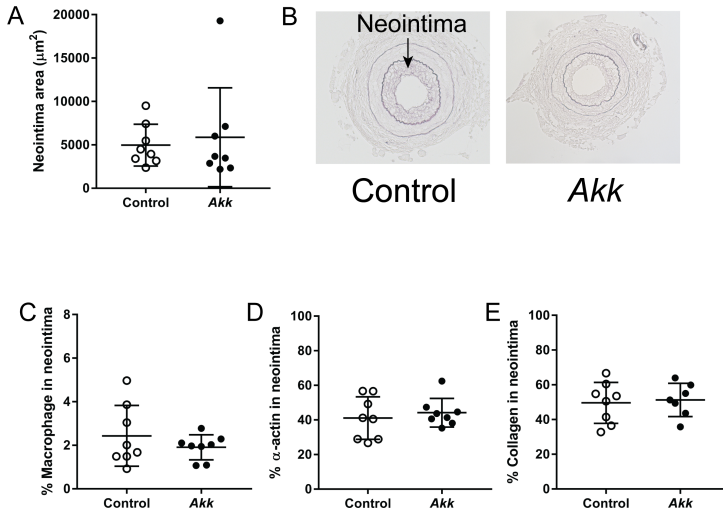


Figure 4.6: *A. muciniphila* did not affect neointima formation and composition. (A) The neointimal area in the cuffed femoral artery was determined in E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control) and (B) representative pictures are shown. In addition, neointimal content of (C) macrophages, (D) vascular smooth muscle cells and (E) collagen was determined. Data are means $\pm$ 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-1 β (IL-1 β) [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 2x10⁹ 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

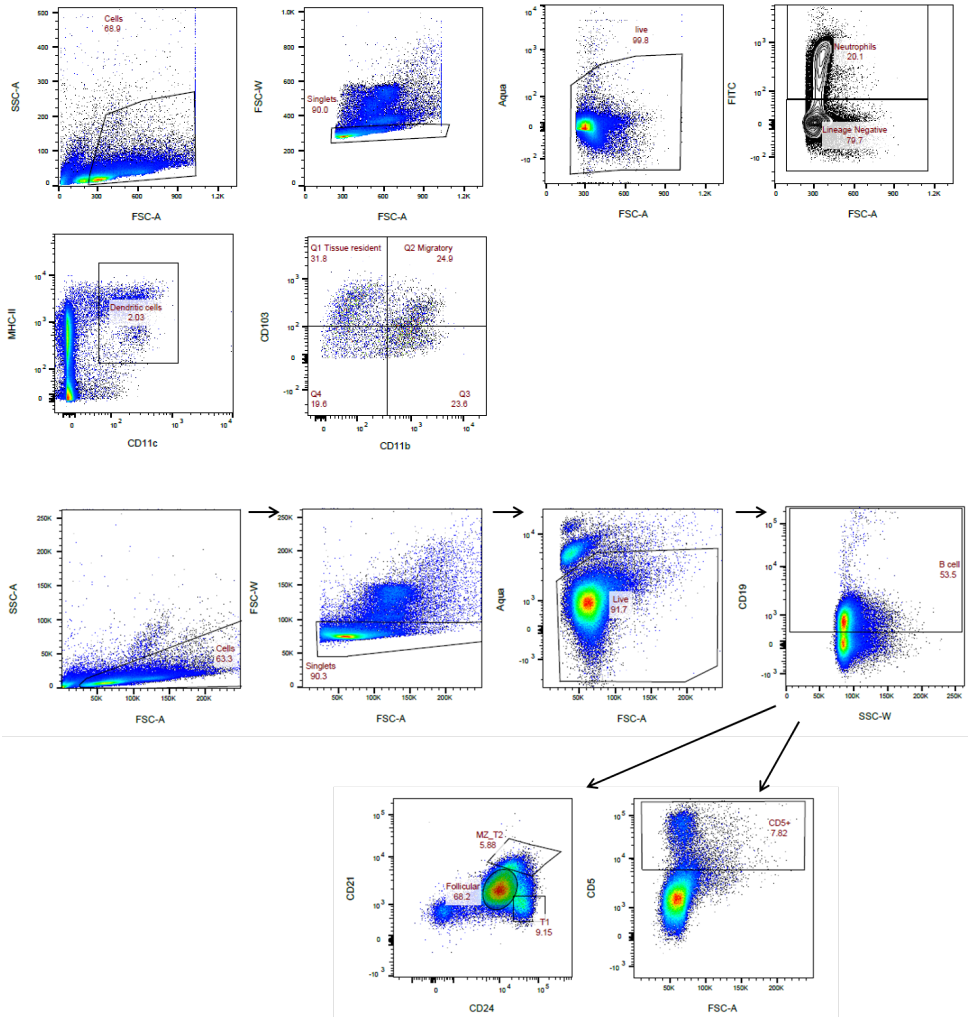
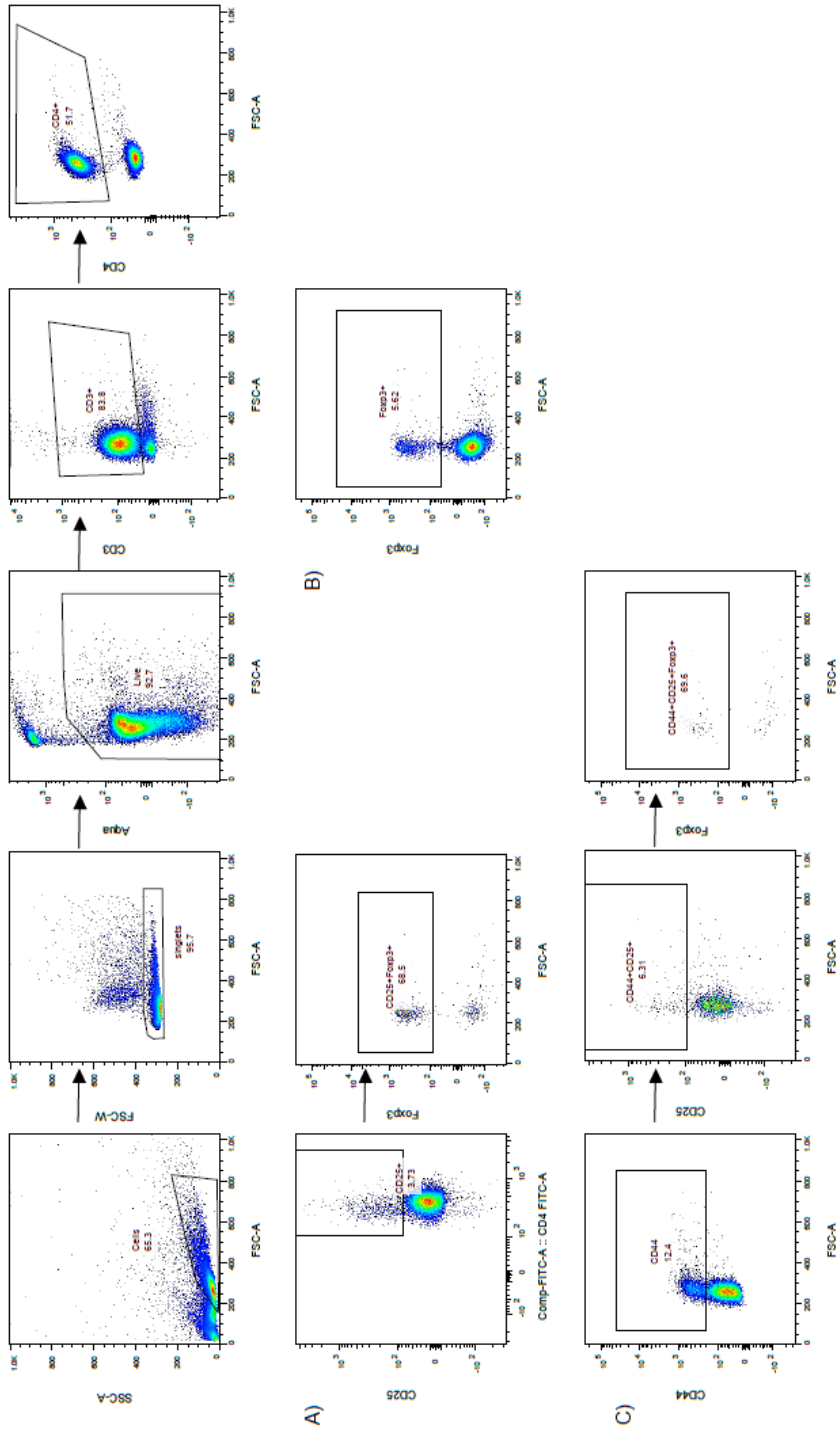


Figure S4.1: Gating strategies for the various cell populations. The gating strategies that were used for analysing (A) dendritic cells (DCs) and neutrophils, (B) B cells, and (C) T cells are shown.



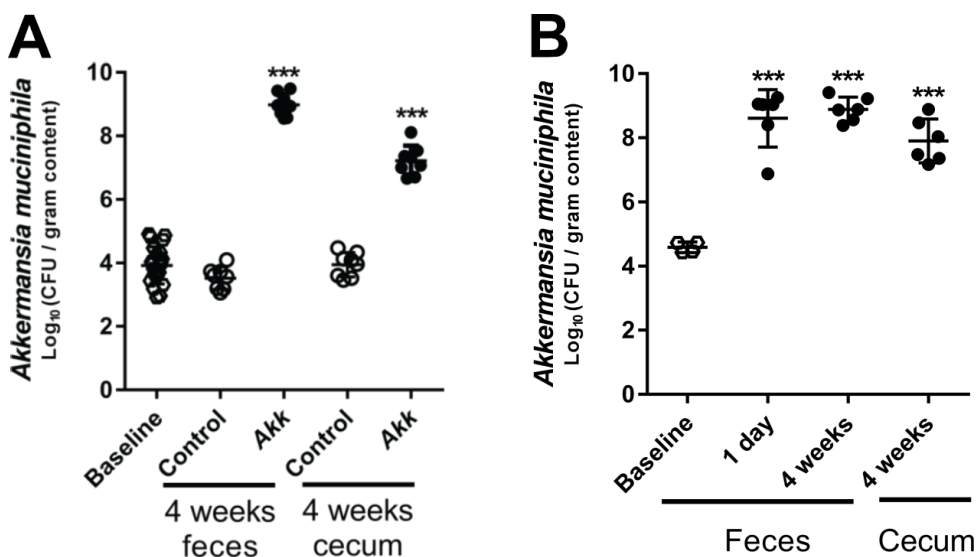


Figure S4.2: *A. muciniphila* colonized E3L.CETP mice efficiently. (A) The concentration of *A. muciniphila* was determined by qPCR in feces and cecum of E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control); n=8 per group. (B) The concentration of *A. 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. *** $p < 0.001$.

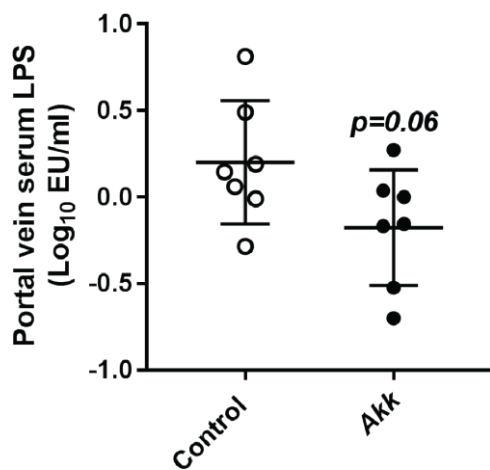


Figure S4.3: *A. muciniphila* tended to lower portal vein serum LPS levels. Portal vein blood was collected from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control). LPS levels were determined in portal vein serum. Data are means ± SD; n=7 per group.

Regulatory T cells

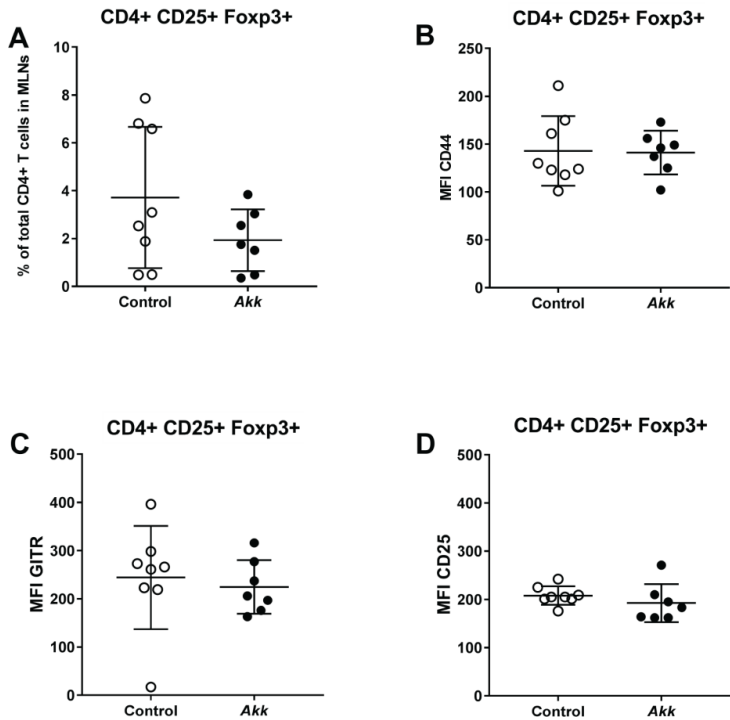


Figure S4.4: *A. muciniphila* did not influence regulatory T cells. Mesenteric lymph nodes (mLNs) were isolated from E3L.CETP mice after 4 weeks of daily oral administration of *A. muciniphila* (Akk) or vehicle (control), and (A) CD4+CD25+Foxp3+ (regulatory T cells) were studied using flow cytometry. 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 $\pm$ SD; n=7-8 per group.

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