Wissenschaftszentrum Weihenstephan
Lehrstuhl für Ernährung und Immunologie

Impact of high-fat diet on inflammatory and metabolic phenotypes in animal models of ileitis and colitis

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. M. Klingenspor

Prüfer der Dissertation:

1. Univ.-Prof. Dr. D. Haller
2. Univ.-Prof. Dr. H. Daniel

Die Dissertation wurde am 30.10.2013 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 13.01.2014 angenommen.

“If we knew what it was we were doing,
it would not be called research, would it?”
— Albert Einstein

ZUSAMMENFASSUNG

Die Rolle der Ernährung bei der Entstehung chronisch entzündlicher Darmerkrankungen (CED) ist nicht hinreichend geklärt. Übergewicht geht statistisch mit einem schwereren Verlauf der Erkrankung einher, und epidemiologische Daten deuten darauf hin, dass eine fettreiche Ernährung die Pathogenese von CED begünstigt. Tierstudien konnten bereits eine durch fettreiche Diät (FD) induzierte Beeinträchtigung der Darmgesundheit, besonders auf der Ebene der Darmepithelzellen, beobachten. Diese Arbeit untersucht den Einfluss einer FD auf die Pathogenese von CED in verschiedenen Mausmodellen für Ileitis und Colitis, mit Fokus auf den Zusammenhang zwischen der Pathogenese und FD-induzierten metabolischen Veränderungen.

$IL10^{-/-}$ und $TNF^{\Delta ARE/WT}$ Mäusen, Mausmodelle für Colitis und Morbus Crohn, wurde für verschiedene Zeitspannen eine Palmöl-basierte FD (48%kJ aus Fett) verabreicht, welche mit einer Kontrolldiät (12%kJ aus Fett) verglichen wurde. Die Fütterung der FD verstärkte den Schweregrad der frühen Ileitis und Colitis in $TNF^{\Delta ARE/WT}$ Mäusen und weiblichen $IL10^{-/-}$ Mäusen. Sie führte hingegen nicht zur Ausprägung von Übergewicht oder typischerweise damit assoziierten Begleiterkrankungen in den entsprechenden Mäusen. In beiden Suszeptibilitätsmodellen sowie der Mauslinie SWR/J, die resistent gegenüber Nahrungs-induziertem Übergewicht ist, reduzierte die FD die Expression des Tight-Junction-Proteins Occludin im distalen Ileum. $TNF^{\Delta ARE/WT}$ und die entsprechenden, nichtentzündeten, Wildtyp-Mäuse wiesen unter FD erhöhte Endotoxinspiegel im Pfortaderblut auf, sowie eine gesteigerte Expression von entzündungsassoziierten Faktoren in den Epithelzellen, Rekrutierung von $CD11c^{+}$ Dendritischen Zellen via CCL20, und eine Th17-geprägte Immunantwort in der Lamina Propria. *In-vitro*-Studien zeigten eine verstärkte Rekrutierung von Dendritischen Zellen und Th17-Prägung der Immunantwort aufgrund des Einflusses luminaler Faktoren auf intestinale Epithelzellen.

FD-Fütterung begünstigt die Entstehung intestinaler Entzündung in den CED-suszeptiblen Tiermodellen $TNF^{\Delta ARE/WT}$ und $IL10^{-/-}$, unabhängig von Übergewicht oder damit assoziierten Pathologien, wahrscheinlich über Mechanismen der erhöhten intestinalen Permeabilität und veränderten luminalen Faktoren.

ABSTRACT

Obesity has been associated with a more severe disease course in inflammatory bowel disease (IBD) and epidemiological data recently identified dietary fats but not obesity as risk factor for the development of IBD. Animal studies have demonstrated an impairment of intestinal health by high-fat diets (HFD), especially at the level of epithelial cells. The present work aimed to assess the impact of a HFD on intestinal pathogenesis in different mouse models susceptible for ileal or cecal/colonic inflammation, with focus on interrelations of promoted pathogenesis and metabolic alterations during HFD feeding.

$IL10^{-/-}$ mice and $TNF^{\Delta ARE/WT}$, models for colitis and Crohn's Disease-like ileitis respectively, were fed HFD (palm oil based, 48%kJ from fat) compared to control diet (12%kJ from fat) for different durations. HFD aggravated ileal as well as colonic inflammation in early phase in $TNF^{\Delta ARE/WT}$ mice as well as in female $IL10^{-/-}$ mice, but did not result in significant overweight or typically associated co-morbidities in these mice. The distal ileum exhibited markedly reduced levels of the tight junctional protein Occludin in both susceptibility models as well as in the obesity-resistant mouse strain SWR/J. Under HFD, $TNF^{\Delta ARE/WT}$ and the corresponding wildtype mice showed enhanced endotoxin levels in hepatic portal blood, as well as increased expression of inflammation-related activation markers in the ileal epithelial cells, along with recruitment of $CD11c^{+}$ dendritic cells via CCL20, and Th17-biased lymphocyte infiltration into the ileal lamina propria. Accordingly, *in vitro* studies were used to highlight that dendritic cell recruitment and Th17-commitment are possibly driven by HFD-associated luminal factors and their effect on intestinal epithelial cells, rather than the pathophysiological state of obesity.

In summary, HFD feeding, independently of obesity and its associated pathologies, accelerated onset of intestinal inflammation in the IBD-susceptible mouse models $IL10^{-/-}$ and $TNF^{\Delta ARE/WT}$ likely through mechanisms that involve increased intestinal permeability and altered luminal factors.

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1. INTRODUCTION

1.1. Inflammatory Bowel Disease

1.1.1. Epidemiology and etiology of Inflammatory Bowel Disease

The prevalence of inflammatory bowel disease (IBD), spontaneously relapsing immunologically mediated disorders of the gastrointestinal tract, has increased considerably over the last decades in both Western and developing countries. IBD comprises the two major idiopathic pathologies Ulcerative Colitis (UC) and Crohn's Disease (CD), the latter named after Dr. Burrill Crohn who first described the disease together with his colleagues Dr. Gordon Oppenheimer and Dr. Leon Ginzburg in 1932. In the 1990s and 2000s, incidence rates for CD in Western countries were reported to vary between 4,[1] and 8 cases,[2] per 100.000 person-years. Globally seen, UC is more prevalent than CD, with North America and northern Europe exhibiting the highest incidence rates ranging from 9,[2] to 20 cases,[3] per 100.000 person-years. CD in tendency displays higher rates in women compared to men, and an incidence peak among the 15-30-year-olds,[1,4,5,6,7] whereas UC shows a similar age distribution but without major gender differences. Despite the lack of data for many areas (especially the African continent), there seems to be a north-south axis of IBD prevalence within continents or even countries.[8,9,10] In Asia, incidence rates of IBD have been low in the past, but today there is evidence for rising incidence rates in this area as well.[11,12] These data emphasize that IBD may emerge as a global disease in the future.

Even though collectively described by the term IBD, UC and CD are two distinct entities with different symptoms and quite distinct pathogenesis. CD potentially extends to the submucosal layers and may occur anywhere along the gastrointestinal tract - with a predominantly ileal phenotype referred to as *ICD*, and a colonic phenotype as *CCD*. In UC, in contrast, inflammation is restricted to the mucosa and only involves the large intestine. Interestingly, UC displays features of autoimmunity, with detectable antibodies against self-antigens.[13] Alternating phases of active disease (relapse) and freeness of symptoms (remission) are characteristics for both etiologies.

IBD is regarded as a multifactorial disease in which, according to the 'second-hit-theory', a combination of a given host susceptibility and a certain microbial aggressiveness leads to inappropriate host immune reactions toward the microbiota. The interrelations of the contributing factors, depicted in Figure 1, complicate the elucidation of IBD pathogenesis: the host genetic

background establishes a certain disease susceptibility prerequisite related to intestinal barrier function and/or immune response. This can result in an aberrant response toward intestinal microbiota. At the same time the host condition and genetic background also shape the intestinal microbial communities. Environmental triggers such as diet or drugs can affect both intestinal barrier and immune functions of the host, and composition as well as functionality of intestinal microbiota.

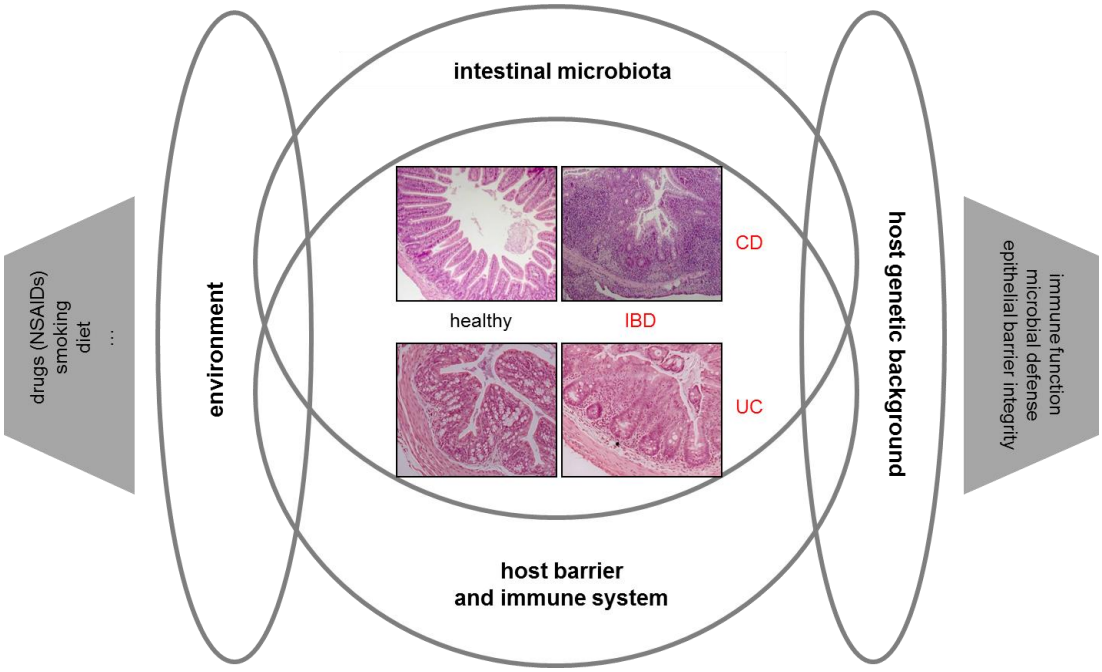

Figure 1: IBD is a multifactorial disease, with diverse interrelations of the contributing factors: host genetic background, host barrier and immune system characteristics, environment, intestinal microbiota.

The host genetic background establishes a certain susceptibility regarding epithelial barrier function, microbial defense and immune response. At the same time the host condition and genetic background also shape the intestinal microbiota. Environmental triggers, such as drugs, smoking or diet can affect both barrier and immune functions of the host, and composition as well as functionality of intestinal microbiota. IBD: inflammatory bowel disease, CD: Crohn's Disease, UC: Ulcerative Colitis, NSAIDs: non-steroid anti-inflammatory drugs. The histological pictures show distal ileum tissues of $TNF^{\Delta ARE/WT}$ mice displaying CD-like ileitis and the corresponding wildtype mouse, as well as proximal colon tissues of $IL10^{-/-}$ mice developing UC-like colitis and the corresponding wildtype mouse.

The factors genetic susceptibility, host barrier and immune system, intestinal microbiota as well as environmental triggers are described in more detail in the following.

1.1.2. Genetic factors in IBD

The contribution of genetic factors to disease development has been first highlighted by the use of twin studies. These found that the concordance rate for CD in monozygotic twins is up to 50% in Northern Europe, representing a highly increased relative risk for individuals with an affected twin compared to the general population.[14,15,16,17] With rates of 4-20%, concordance is lower for UC than CD.[18,19,20]

There are a large number of genetic associations for IBD, far more than for other complex diseases. Up to date there are 163 gene loci reported in genome-wide association studies (GWAS), with 110 genes implicated for both etiologies.[21] It is noteworthy that along with differences in IBD prevalence, the identification of relevant susceptibility genes varies in Asians versus Caucasians,[22,23,24] and in Jewish versus Non-Jewish populations.[25,26,27] In Caucasians, 13.6% of the disease variance in CD can be explained by the means of variations in these genes, and 7.5% in UC.[21] Thus, the disease variance that can be explained by these known susceptibility genes is higher in CD than UC, probably indicating that genetic factors might contribute less significantly to UC than to CD development, or that environmental and lifestyle triggers are overwriting genetics more efficiently in UC.

It is believed today that IBD results from aberrant host-microbe interaction and disrupted tolerance to non-pathogenic microbiota, leading to aberrant inflammatory response. IBD is therefore considered to be a consequence of loss of intestinal homeostasis. The concept of *homeostasis* in this context not necessarily means an absence of inflammatory responses, but the ability of the host to mount an appropriate response toward any changes concerning the interaction with microbial community. Confining the microbiota without overreaction toward non-pathogenic organisms needs fine-balanced host-microbe interactions at various levels. The results of GWAS in IBD highlight the impact of the host immune status and its responsiveness toward microbiota and microbial components. A substantial number of genes are implicated in both major IBD etiologies, mostly involved in barrier function, primary defense mechanisms such as sensing and processing of bacteria, as well as cytokine signaling and adaptive immunity.[28,29] The stratification of these functions and processes in the network of intestinal cells including examples for respective genes associated with IBD is depicted in Figure 2.

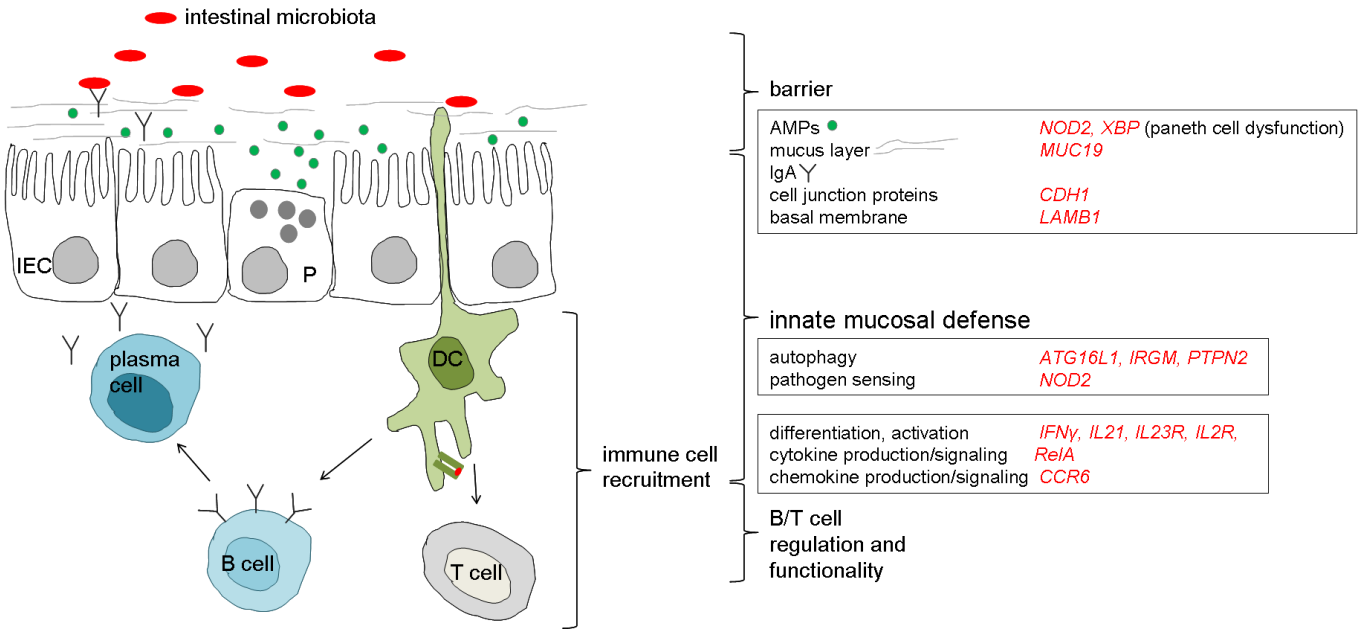

Figure 2: Maintenance of homeostasis at the host-microbe interface by confining the microbiota without overreaction toward non-pathogenic organisms needs fine-balanced host-microbe interactions at various levels.
A compromised barrier function of the host, whether resulting from impaired AMP production (for example due to Paneth cell dysfunction), mucus layer aberrations, inadequate IgA production, loss of cell junction proteins or disorganized basal membrane structure, may lead to intestinal inflammation as observed in IBD. Adequate innate mucosal defense mechanism, which involve autophagy and pathogen sensing, ensure appropriate responses of the host immune system. Immune cell recruitment and lymphocyte regulation and functionality are regulated by a complex network of cytokines, chemokines and differentiation processes. GWAS reveal a critical role of these processes of microbe-host-interactions in IBD pathogenesis, with examples of identified susceptibility loci given in red. IEC: intestinal epithelial cell, P: Paneth cell, DC: dendritic cell, AMPs: anti-microbial peptides, IgA: immunoglobulin A

1.1.3. Characteristics of intestinal barrier and immune system in IBD

The given genetic predisposition associated to IBD pathogenesis impacts on the host's ability to balance host-microbe-interactions. IBD is characterized by impaired barrier integrity, aberrant microbial recognition and processing, as well as overshooting immune responses. In the following chapters, the clinical characteristics as well as the genetic factors that might contribute to these phenotypes are summarized.

Barrier integrity. Functional integrity of the intestinal barrier can be seen as a prerequisite for maintaining mucosal homeostasis. An increase in intestinal permeability is considered a major hallmark of IBD.[30,31,32,33] The mucosal barrier is built up by various components: commensal microbiota block niches or compete with pathogens, immunoglobulin (Ig) A and anti-microbial peptides (AMPs) are secreted to the lumen by the intestinal epithelial cell (IEC) layer,

mucins cover the epithelium on the luminal side and protect it from direct contact to microbial organisms, and barrier proteins seal the host IEC layer. As indicated in Figure 2, several IBD associated loci that occur throughout the layer of this stratification emphasize the critical role of barrier integrity in IBD susceptibility. GWAS report a significant association of CDH1 with CD, resulting in truncated forms of E-Cadherin.[34] Patients carrying the mutated alleles display defective E-Cadherin localization in the intestinal epithelium.[34] MUC19, encoding a secreted protein forming a chemical barrier together with other mucins and embedded AMPs, is also implicated in IBD susceptibility.[35,36,37] Further, paneth cell function is critical in maintaining barrier integrity, as Paneth cells are the producers of AMPs. Paneth cell dysfunction is a common consequence of mutations associated to IBD development, such as nucleotide-binding oligomerization domain 2 (NOD2), autophagy-related protein 16-1 (ATG16L1), or x-box binding protein 1 (XBP1).[38] Extracellular matrix proteins such as the lamin-encoding LAMB1 are also implicated in IBD, predominantly in UC.[21,29] As laminins are the major non-collagenous constituent of basement membranes, defective variants could allow the penetration of microbes.[39]. Indeed, analysis of biopsies of UC patients showed abrogation of laminin in the epithelial basement membranes surrounding the crypts in affected tissues.[40]

Pathogen sensing and processing. Bacteria that have already penetrated the epithelial barrier have to be sensed and processed for the induction of defense mechanisms. When intruded into various kinds of cells such as Paneth cells or antigen-presenting cells (APCs), bacteria are lysed in autophagolysosomes, and their structures are sensed by NOD-like receptors. NODs contain caspase recruitment domains (CARD) which mediate downstream signaling pathways such as nuclear factor κB, and thus activate immune response, resulting in clearance of the infection.[41] Various polymorphisms of NOD2/CARD15 have been identified as more prevalent in Caucasian CD patients compared to healthy controls.[42,43,44] NOD2 dysfunction leads to inefficient clearance of intruded bacteria, likely in part mediated by reduced expression of Paneth cell-derived defensins.[45,46] NOD2-deficient mice do not spontaneously develop IBD, but studies reveal increased susceptibility toward infectious bacteria.[47,48] Besides NOD2, ATG16L is regarded as one of the strongest genetic contributor to CD.[37] ATG16L1 is critical for formation of autophagosomes and thus for degradation and processing of microbial proteins.[49] Patients with ATG16L1 or NOD2 mutations display ineffective induction of autophagy and bacterial processing.[50,51] Further factors involved in autophagic processes, such as T cell protein tyrosine phosphatase (PTPN2),[52,53,54] and interferon-inducible protein 1 (IRGM), [21,44,54,55] are implicated in IBD. Inefficient bacterial killing in macrophages leads to increased susceptibility toward infections in mice deficient of IRGM ($IRGM^{-/-}$),[56] while $PTPN2^{-/-}$

mice exhibit compromised T cell functions,[57,58] and increased susceptibility to chemically-induced colitis.[59] This convergence of several strong genetic risk factors highlights the importance of pattern recognition and autophagic processes in the clearance of bacteria and maintenance of intestinal homeostasis.

T Helper cell immune response. A complex network of signals is established for the induction of tolerance or immune response toward the sensed and processed organisms. Upon antigen-recognition and appropriate co-stimulation by APCs, T cells undergo lineage commitment for different T helper cell (Th) lines. Depending on the contact co-stimulation by the APCs and the surrounding cytokines and other stimulatory factors, the T cell will acquire a specific phenotype and functionality. As depicted in Figure 3, the phenotypes of adaptive immunity vary substantially between the two major IBD etiologies. While CD is associated with a Th1 and Th17 immune responses, dominated by the expression of the cytokines interleukin (IL) 12, IL23, interferon (IFN) γ and tumor necrosis factor (TNF), UC displays predominantly Th2 phenotype, associated with cytokines such as IL5 and transforming growth factor (TGF) β. Nevertheless, many of the genetic susceptibility loci are implicated in the pathogenesis of both etiologies, as indicated in Figure 3. In general, genes associated with the regulation of cytokine production are especially overrepresented in IBD GWAS, particularly regarding IFNγ, IL12, TNF and IL10 signaling.[21,60] IFNγ, IL12 and TNF are the predominant cytokines in Th1 immune response in general, and are also characteristic for CD. The commitment of T cells to Th1 is mediated by the transcription factor T-box transcription factor TBX21 (Tbet). IL10 is a major anti-inflammatory cytokine and its signaling is required for generation of regulatory T cell responses via the transcription factor forkhead box P3 (FoxP3).[61] The impact of IL10 functionality on intestinal health is emphasized by the fact that IL10 deficient mice develop colitis when colonized and have therefore become a widely used model of IBD (see Chapter 1.3.2).[62,63] Along with the Th17 phenotype described for CD, genes annotated to the ontology term ‘regulation of interleukin-17 production’ are strongly enriched in GWAS data. Among these are genes driving the differentiation toward Th17 (RAR-related orphan receptor gamma t [RORγt], STAT3, IL23R, IL12B, IL6ST) and genes related to Th17 signaling (IL21) and chemotaxis (C-C chemokine receptor 6 [CCR6]).[21,29,35,44,60,64] As a subunit of IL12 driving Th1 differentiation also serves as a subunit of IL23, IL12 can be regarded as interface between Th1 and Th17 directed cell commitment,[65] as can be TYK2 which processes signaling from both IFNγ/IL12 and IL23. Further Th1 associated genes identified as susceptibility loci include gene encoding for factors driving Th1 differentiation (STAT1) and mediating Th1 signaling such as IL2 or IFNG with respective receptor forms. A broad range of receptor and signaling proteins related to TNF are

reported as IBD loci as well: the TNF ligand superfamily (TNFSF15, TNFSF18), the TNF receptor superfamily (TNFRSF4, TNFRSF6B, TNFRSF14, TNFRSF14), TNF receptor-associated factors (TRAF3IP2) as well as TNF-induced proteins (TNFAIP3) are represented with several members. [21,29]

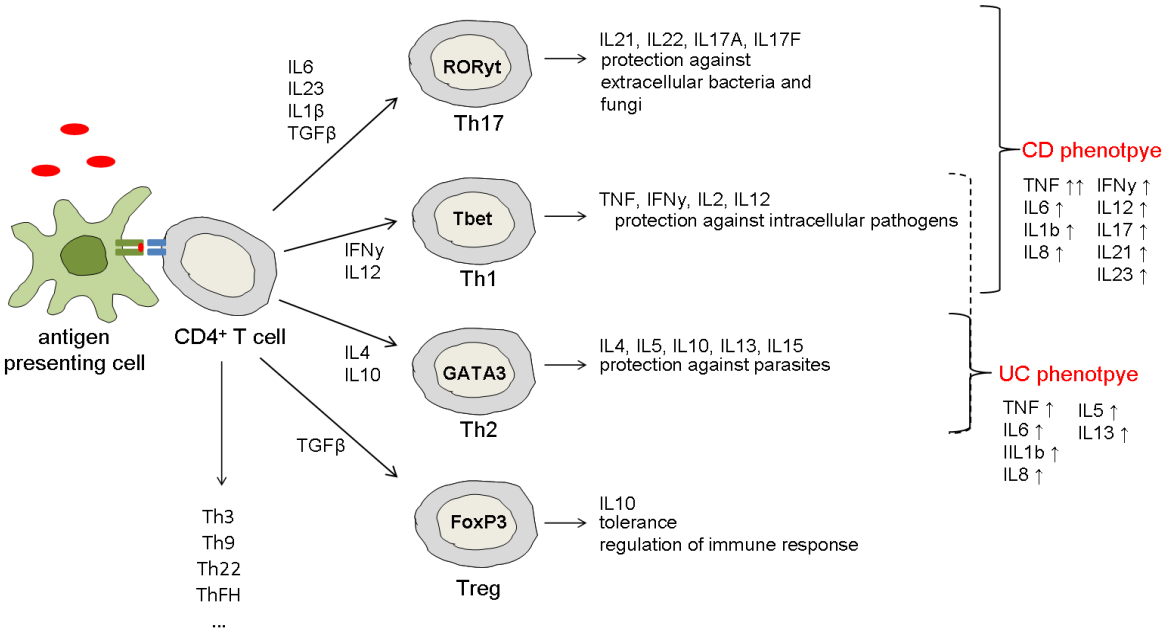

Figure 3: Antigen-presenting cells can induce T cell differentiation into one of the major T helper cell types: Th1, Th17, Th2 and Treg.

Differentiation depends on co-stimulatory factors of the antigen-presenting cell surface or by the network of cytokines present, that are sensed by receptors on the T cell surface. Lineage-commitment then requires induction of transcription factors, the most important of them are outlined in bold in the figure. Printed in red are reported susceptibility genes for the development of CD or UC. Association with both etiologies is indicated by *.

Clinical as well as experimental studies emphasize a role of specific IBD-associated cytokines in the modification of barrier functions, especially for TNF and IFNγ in the case of CD, and IL13 in the case of UC.[66,67] It is therefore likely that single initial host conditions can create a vicious circle and enhance IBD pathogenesis. This furthermore highlights the interrelations of the different characteristics described for IBD.

1.1.4. Microbial factors in IBD development

The common consequence of disturbances of either barrier integrity, pathogen sensing and processing as well as cellular immune response is a failure to mount appropriate host responses toward the intestinal microbiota or microbial components. There is substantial evidence for intestinal microbiota and host tolerance toward it playing essential roles in the pathogenesis of both IBD etiologies.[28,68] Inflammation is prevented by surgical ileostomy in patients with active CD, and inflammation then reoccurs rapidly after reinfusion of the bypassed segments.[69] In addition, antibiotic treatment may result in amelioration or even abrogation of inflammation in CD patients.[70,71,72] Treatment with broad spectrum antibiotics as well as housing under germ-free conditions also ameliorates or abrogates inflammation in many genetically engineered rodent models of intestinal inflammation.[63,73,74,75,76] In their entirety these studies provide substantial evidence for a combined effect of genetic host susceptibility and microbiota in the development of chronic intestinal inflammatory diseases. However, there is no specific microorganism described which can be consistently isolated in each IBD case and is absent in healthy people. Hypotheses considering particular bacterial agents as causative for IBD etiology can therefore not be supported. Nevertheless several bacterial species are reported to correlate with the disease. IBD may in general be caused by overall changes in the composition and functionality of the intestinal microbiota, a state termed *dysbiosis.*

Reduced diversity of the intestinal ecology has been demonstrated as a hallmark in CD,[77,78,79,80] and UC,[66,78] compared to non-affected control patients. This reduction in diversity is connected to a reduced abundance of the dominant members of the human gut microbiota in IBD patients. In general, a decrease of *Firmicutes,*[31,66,78,79,81,82] and often *Bacteroidetes,*[78,81,83] with concomitant increase of *Proteobacteria,*[80,81,82,84] and *Actinobacteria,*[80,81] was reported for mucosal biopsy samples or feces of IBD patients. Among the phylum *Firmicutes* there is substantial evidence for a loss of abundance of *Clostridia IXa* and *IV* members.[77,81,85,86,87] Decreased abundance of *Faecalibacterium,* particularly *F. prausnitzii,* seems a common feature in IBD patients, especially in CD.[81,84,88,89,90,91] Interestingly, supplementation of live *F. prausnitzii* or conditioned supernatant ameliorated chemically induced colitis in animal studies and tended to correct the connected dysbiosis.[90] The findings regarding *Bacteroidetes* abundance are not as consistent as for the *Firmicutes*. While some comparisons find *Bacteroidetes* numbers decreased in IBD,[78,81,83] others do not observe significant changes,[92] or even report increased abundance.[79] Of note, within the phylum *Bacteroidetes*, *Bacteroides vulgatus* and *Bacteroides thetaiotaomicron* isolated from inflamed mouse intestine were introduced into antibiotic-pretreated mice, upon which it colonized

transgenic IBD-susceptible and –non-susceptible mice equivalently, but induced disease in the susceptible mouse strain exclusively.[93] Despite an overall tendency to relative increases in members of the *Actinobacteria* phylum, reduced populations of *Bifidobacteria* have also been reported for UC patients.[94] It has been observed that the abundance of *Bifidobacteria* inversely correlates to abdominal pain in healthy subjects.[95]

Concerning the increased abundance of *Proteobacteria* in IBD, there is particular evidence for overgrowth of facultative anaerobe *Enterobacteriaceae*.[79,80,82,83,86] This family was not found to be associated with disease in a causative way however, as *E. coli* isolates did not induce colitis in antibiotic-pretreated susceptible mice despite robust colonization.[93] This example emphasizes that IBD-associated alterations in microbial composition do not necessarily reflect a causative relation. It cannot be ruled out that *dysbiosis* may be a consequence of inflammation rather than a cause, with adaptation of the microbiota to a changed conditions, may they be derived from host genetics, inflammatory status or treatment. Interestingly, the growth of *Enterobacteriaceae* has been shown to be fostered by an inflammatory state *per se* or genetic background of the host in animal models,[96,97] and overgrowth of introduced *Salmonella typhimurium* was also observed in an adoptive transfer model, in which colitis primarily is caused by cytotoxic T cells destroying the epithelium.[98] This highlights the impact of the host genotype and phenotypic condition on the microbial composition. Strikingly, genotypic effects on bacterial composition have also been reported for humans: unaffected twins from UC patients showed lower bacterial diversity than healthy unrelated individuals, including similar tendencies concerning compositional changes.[83] It is also noteworty that the species with increased abundance in IBD are largely facultative anaerobe bacteria. The microbial composition associated to active IBD might therefore in part result from adaptation to the changed redox potential due to a loss of epithelial barrier integrity.[99]

Many of the studies investigating dysbiosis in IBD reveal ICD as very distinct from healthy and UC state, whereas CCD or UC are often not distinguishable from each other or from healthy state. ICD is more likely to be treated with immunosuppressant therapy and less likely to be treated with mesalamine or antibiotics than colonic phenotypes,[100] and these confounders might strongly affect the correlations seen between microbial composition and disease state. Only an increase in *Enterobacteriaceae* abundance for CD, *Faecalibacterium* for ICD specifically, and *Clostridia* abundance in both etiologies, could be substantiated after correction for all identified covariates.[100]

1.1.5. Environmental factors in IBD development: highlights on diet

There are relatively low overall concordance rates for IBD, and all susceptibility loci together only can account for a small percentage of the disease variance. These findings, together with the rapid increase in the prevalence of IBD in societies with a 'Western lifestyle' during the last decades, indicate environmental triggers to have a strong impact on IBD pathogenesis.[28,101] Among the environmental factors implicated are smoking, non-steroidal anti-inflammatory drugs (NSAIDs), infections and diet.[101,102]

Dietary components have long been suspected as major factors in IBD etiology but studies aiming to identify dietary factors associated with the risk to develop IBD based on epidemiological observations give equivocal results.[103] The field of so-called 'nutrigenetics' in the context of IBD aims at identifying nutrient-gene interactions associated with disease-conditioning and disease-modulation. Two main aspects have come into particular focus of IBD-related nutrigenetics: 1) the regulation of cellular energy homeostasis and 2) nutrient-activated transcription factors.

Cellular energy supply. Chronic intestinal inflammation is considered to be an energy deficiency disease characterized by altered mitochondrial functionality in IECs.[104] IBD-associated polymorphisms in the carnitine transporter OTCN2 impair β-oxidation,[35] as carnitine is needed for the transport of long-chain fatty acids (FA) into mitochondria. Ablation of OCTN2 as well as pharmacological inhibition of intestinal FA β-oxidation results in experimental colitis, supporting the role of OCTN2 dysfunction as a primary cause of IBD.[105,106] In addition, butyrate oxidation is reportedly reduced in the inflamed mucosa of UC patients,[107,108] and in murine colitis models.[109] The monocarboxylate transporter MCT1, responsible for butyrate uptake in colonocytes,[110] is reportedly downregulated in active IBD,[111] possibly accounting for decreased butyrate availability and thus oxidation. However, it is not clear whether this deficiency in butyrate oxidation is rather a consequence of mucosal abnormalities during tissue inflammation than a primary cause of IBD.

Nutrient-induced cellular signaling. Providing the closest interface of diet and regulation of gene expression, nutrient-induced transcription factors are of particular interest in IBD-related nutrigenetics (Figure 4). Diet- and microbiota-derived xenobiotics are actively removed from IEC via the multi-drug resistance protein (MDR1). Variants of MDR1 are associated with UC pathogenesis.[112,113] Xenobiotic substances remaining in the host cell may then activate the transcription factor Aryl hydrocarbon receptor (Ahr).[114] Fatty acids activate peroxisome

proliferator-activated receptors (PPAR) while derivatives of diet-derived vitamins A and D bind to the transcription factors retinoid-X-receptor (RXR) and Vitamin-D-receptor (VDR) respectively. VDR polymorphisms are associated with IBD,[115] and also contribution of PPARs to IBD susceptibility is discussed.[114,116] PepT1, a major brush border transporter of di- and tripeptides, is implicated as IBD susceptibility gene.[117] It fosters uptake of muramyl dipeptide which activates NOD2 and thus cellular inflammatory signaling pathways via NFκB.[118,119] The activated transcription factors Ahr, RXR, VDR, PPAR and NFκB modulate the host immune response and thus in turn also modulate composition and activity of the microbiota, emphasizing the manifold interactions of host, dietary factors and microbiota.

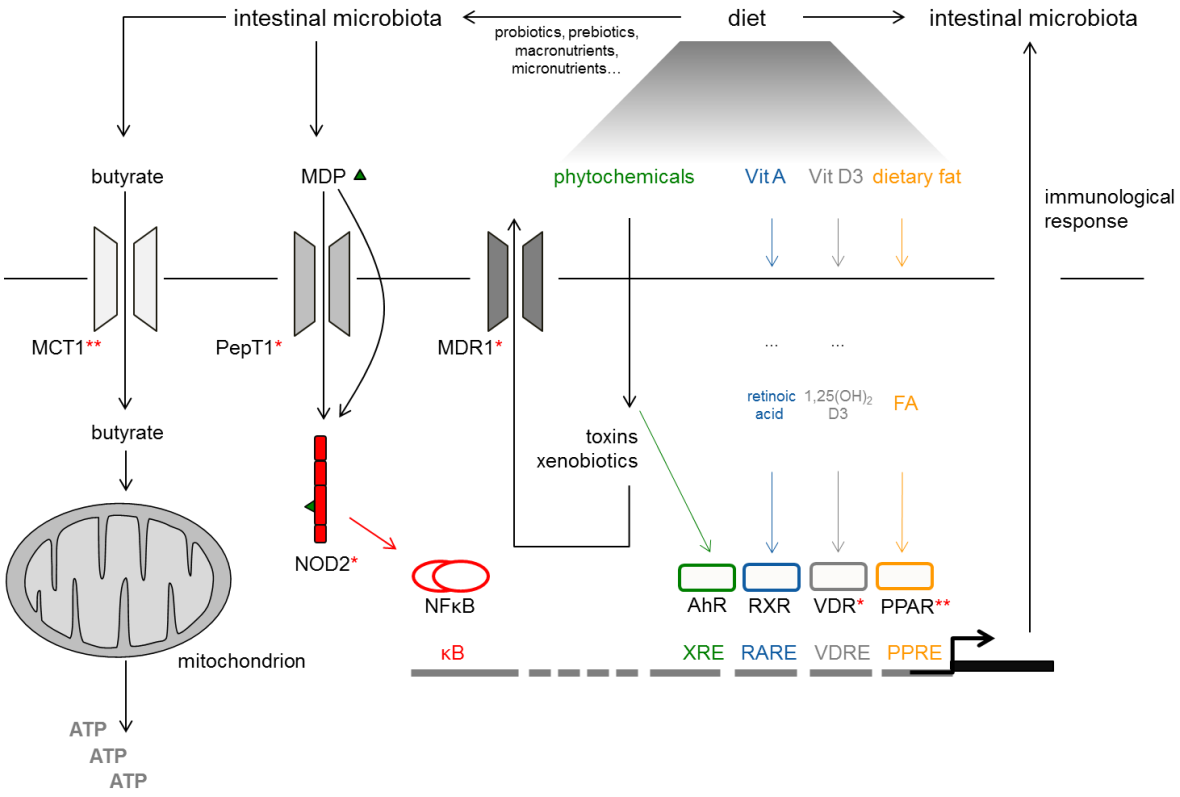

Figure 4: Nutrigenetics in the context of IBD focus on nutrient-gene interactions associated with disease-conditioning and disease-modulation.

MCT1: monocarboxylate transporter 1; ATP: adenosine triphosphate; PepT1: peptide transporter 1; MDP: muramyl dipeptide; NOD2: nucleotide-binding oligomerization domain 2; NFκB: nuclear factor κB; MDR1: multidrug resistance protein 1; AhR: aryl hydrocarbon receptor; Vit A: vitamin A; XRE: xenobiotic response element; RXR: retinoid X receptor; RARE: retinoic acid response element; Vit D: vitamin D; VDR: vitamin D receptor; VDRE: VDR responsive element; FA: fatty acid; PPAR: peroxisome proliferator-activated receptor; PRE: PPAR responsive element; * IBD susceptibility gene; ** regulated in IBD

Besides these putative direct effects of dietary components on the cellular signalling, diet may also impact on IBD in a more indirect way, e.g. via modulation of intestinal microbial composition and function.

Dietary iron. Administration of dietary but not systemic iron, both in physiological doses, aggravated the disease in a mouse model of CD-like ileitis, probably via the modulation of intestinal microbial composition in combination with induction of endoplasmic reticulum stress and apoptosis in IECs.[120] This provides evidence for a role of dietary iron during IBD pathogenesis. These findings may impact on IBD treatment in the future, considering that IBD patients are often anemic and therefore receive oral iron-supplements.

Enteral diet in IBD therapy. Enteral diet has been considered as efficacious in inducing remission during CD therapy, particularly in pediatric CD cases where more aggressive therapies are eschewed.[121,122,123] Relapse prevention can be achieved by enteral nutrition in both children,[124] and adults.[125] Elemental diets, meaning diets based on easily assimilated nutrients, have also been reported as beneficial in CD.[126,127] When tolerated by the CD patients, enteral and elemental diets can in the short term be as effective regarding induction and maintenance of remission as steroid treatment.[127] However, both strategies seem largely ineffective in UC, and the mechanisms of action of these diets are not fully elucidated. As IBD is characterized by a reduced bacterial diversity as described above, it might seem contradictory that both enteral nutrition and elemental diets are reported to reduce bacterial diversity.[128,129] The reduction of diversity might correlate to a reduced antigenic load of the microbiota in this case. A liquid diet results in enhanced intestinal transit, reducing the time of exposure to ingested antigens. A reduction of mucosal antigen exposure could also result from the very low presence of bacterial antigens in semi-synthetic enteral nutrition formulas. In addition, enteral nutrition might also be able to modulate the metabolic activity of the present microbiota,[130] for example by altering the nutrient availability.

Dietary fat in IBD therapy. In some studies addressing the feasibility of enteral nutrition as therapeutic means for IBD, modifications of enteral diet composition have been evaluated. It has been revealed that FA amounts and composition of the triglycerides (TG) seems to have a great impact on the efficacy of the enteral diet. Enteral diets with a very low fat content (0.6 kcal% - 3 kcal%) have been associated with more favorable results in comparison to diets with a high fat content (12 kcal% - 30kcal),[131,132,133] and meta-analyses indicate enteral diets with very low fat content and/or very low amounts of long-chain TG to be more effective in CD.[121,134] Besides this hint on long-chain TG, there are few indications for specific FA structures in the

modulation of disease activity. Medium-chain TG, considered as an easily absorbed energy source, may contribute to the therapeutic effect of enteral nutrition.[134] Despite their often reported anti-inflammatory effects, ω3 FA have so far not been reported as beneficial in IBD therapy.[135,136,137] Thus, indications for dietary fat as a modulatory factor in established IBD can be derived from the use of therapeutic strategies, but larger studies systematically investigating the effect of fat content and TG composition on treatment outcomes are lacking.

Dietary fat in IBD pathogenesis. As indicated by several epidemiologic studies listed in Table 1, dietary fat may also play a role in human IBD development.[138,139] Statistical significance for an effect of total dietary fat intake, or of subgroups of dietary fat, was reached only in few of the studies. However, a recent meta-analysis confirmed a positive association between high intake of fat, polyunsaturated FA (PUFAs), ω6 FA and meat with CD as well as UC risk,[140] even after adjusting for total energy intake and lifestyle confounders. The latter report indicates that dietary fat might increase IBD development independent of its role as an energy source. A recent prospective cohort study reported indeed a lack of association of body mass index or total energy intake with IBD development.[141] Although IBD patients have historically been reported as lean, an increasing number of studies report rising prevalence of obesity among IBD patients,[142,143] and associate obesity with a more severe disease course.[142,144,145] It is therefore crucial to investigate in the future whether dietary fat plays a causative role in IBD development and whether this is related to or independent of its obesogenic effect.

Table 1: Evidence from epidemiologic studies for an effect of dietary fat on IBD risk.

Various retro- and prospective studies analyzed the effect of dietary fat and/or dietary fat composition on the incidence of CD and UC in different populations. Within the dietary components, studies are grouped regarding the investigated etiology, the outcome and the type of study.

component	etiology	effect on disease risk	type of study	references
overall fat intake	CD	+	retrospective CC	[146]
		+ (ns)	retrospective CC	[147]
		+	population-based correlation	[148]
	UC	+	retrospective CC	[146]
		+ (ns)	retrospective CC	[149,150]
		+ (ns)	prospective CC	[151]
		+ (ns)	prospective cohort	[152]
total SFAs	CD	+ (ns)	retrospective CC	[146,147]
	UC	+ (ns)	retrospective CC	[146,149,150]
		+ (ns)	prospective CC	[151]
total MUFAs	CD	+	retrospective CC	[146]
		+ (ns)	retrospective CC	[147]
	UC	+	retrospective CC	[146,149,150]
		+	prospective CC	[151]
total PUFAs	CD	+ (ns)	retrospective CC	[146,147]
	UC	+	retrospective CC	[149,150]
		+ (ns)	retrospective CC	[146]
		+ (ns)	prospective CC	[151]

total ω6-PUFAs	CD	+	retrospective CC	[146,147]
		+	population-based correlation	[148]
	UC	+ (ns)	retrospective CC	[146,149]
		+ (ns)	prospective CC	[153]
linoleic acid	UC	+	prospective CC	[153]
total ω3-PUFAs	CD	+	retrospective CC	[146]
		+ (ns)	retrospective CC	[147]
	UC	+ (ns)	retrospective CC	[146]
		+ (ns)	prospective CC	[153]
long-chain ω3-PUFAs	CD	-	retrospective CC	[147]
	UC	- (ns)	prospective CC	[154]
		- (ns)	prospective cohort	[155]
DHA	UC	-	prospective CC	[153,154]
trans-unsaturated FA	UC	+ (ns)	prospective cohort	[155]

+: increase of risk; -: decrease of risk; (ns): not significant; CC: case-control study
SFAs: saturated fatty acids; MUFAs: mono-unsaturated- fatty acids; PUFAs: poly-unsaturated fatty acids; DHA: docosahexaenoic Acid;

1.2. Obesity and dietary fat in intestinal health

1.2.1. Obesity as a state of low-grade inflammation

Obesity is one of the most important public health issues, and one of the most important risk factors contributing to the overall disease burden worldwide.[156] It is associated with a chronic low-grade inflammatory state of the white adipose tissue and activated immune response. In the state of obesity, the white adipose tissue increases in mass largely due to hypertrophy of adipocytes, and also due to infiltration of immune cells. This is accompanied by increased expression of adipokines, including leptin or resistin, and classical cytokines and chemokines such as IL6, TNF, IL1β and monocyte chemotactic protein 1 (MCP1) or macrophage inflammatory proteins (MIP) respectively.[157,158,159] Both adipocytes and the infiltrating recruited immune cells express these pro-inflammatory factors. Concomitantly elevated systemic plasma levels of the respective parameters are also reported. Obese subjects also display elevated plasma levels of free FA,[160] which activate pro-inflammatory pathways. Especially the visceral fat depots are considered as metabolically active and as contributing to the increased risk for comorbidities.[161] Consequences of obesity are typically insulin resistance and impaired glucose tolerance leading to type 2 diabetes, as well as vasculopathy, atherosclerosis and cardiovascular disease.

1.2.2. Effects of DIO on intestinal physiology

Human obesity is in most cases driven by a mere energetic imbalance due to insufficient energy expenditure versus overnutrition. In western countries and modern times, besides other obesogenic eating habits (such as eating frequency and sugar-sweetened beverages), this overnutrition is often characterized by a high intake of dietary fats.[162,163] As intervention trials mainly use diets rich in fat to generate obesity, the effects of diet-induced obesity (DIO) and associated physiologic changes can hardly be separated from sheer dietary fat-induced effects. Although it is not yet clear if dietary fat contributes to the risk of IBD pathogenesis, there is evidence for its ability to impact on gastrointestinal physiology regarding various molecular mechanisms from both human trials and animal studies.

The epithelial cell layer seems to be affected by DIO in numerous animal studies, though some of the observed effects are small compared to effects of inflammation or other stimuli. Several studies report activation of the NF-κB pathway upon high-fat diet (HFD) feeding. HFD induces

the activation of NF-κB and the expression of pro-inflammatory cytokines (TNF, IL6, IL1β) and of TLR4 in the colon,[164,165] dependent on initial TLR4 activation.[165] HFD induces ileal TNF mRNA expression, preceeding the development of obesity,[166] in conventionally raised but not in germfree mice. This indicates a crucial role for microbiota as mediator for HFD-induced effects. Regarding the intestinal microbial community, characteristics during obesity include decreased diversity,[167] decreased abundance of members of the phylum *Bacteroidetes*,[167,168] and increased abundance of *Firmicutes,*[168]

Clinical trials have reported impaired barrier integrity in obese patients and patients with metabolic syndrome, even with correlation to body-mass-index as well as other anthropometric data.[169,170] Similar effects are observed in diet-induced obese rodents, with decreased transepithelial resistance and reduced expression or altered localization of tight-junction (TJ) proteins such as Zonula Occludens 1 (ZO1) or Occludin (Ocln).[164,165,171,172,173,174] Cytokines, in particular TNF, IFNγ and IL6 which are all also implicated in obesity, are suspected as major contributors to the observed increase in intestinal permeability.[66,169,175,176,177] Impaired barrier function can contribute to the phenomenon of ‘metabolic endotoxemia’, meaning elevated levels of lipopolysaccharide (LPS), associated with obesity or increased energy intake in humans,[178,179] and mouse models.[165,174,180,181] LPS is a component of the outer membrane of gram-negative bacteria, and exerts inflammatory function via activation of TLR 4. Translocated LPS leads to the expression of LPS-binding and sensing factors, and acute-phase proteins such as serum amyloid A (SAA) and C-reactive protein (CRP) in the liver, mediated mainly by IL1β and IL6.[182]

1.2.3. Immunometabolism in the context of DIO and intestinal health

The term ‘immunometabolism’ describes the merge of mechanisms historically assigned exclusively to either the field of immunology or the field of metabolism, and also the merge of the respective areas of research. It can be considered as the investigation of metabolic-immune crosstalk. Adipose tissue constitutes a major site of immune responses, becoming evident in the role of infiltrating immune cells and cytokines during metabolic disorders such as obesity and insulin resistance as described above. These metabolic diseases are now considered as states of chronic low-grade inflammation. It has now been understood that obesity affects immune responses and promotes inflammatory processes also outside the adipose tissue. On the other hand, immunological diseases are often accompanied by metabolic disturbances, such as

wasting during IBD. [183,184] In addition, typically metabolic mechanisms appear to be critical in immune cell generation and functionality, such as the sensing and the availability of certain nutrients.

Integration of nutrient sensing and immune signaling. Fatty acids, and medium-chain FA in particular, provide an example for the convergence of microbial recognition mechanisms and nutrient sensing pathways. For example, PPARγ senses FA and is a major player in the regulation of key metabolic functions but may also be a potent anti-inflammatory transcription factor as it opposes the actions of the pattern recognition receptor toll-like receptor (TLR) 4.[185] The pathway downstream of TLR4 integrates sensing of bacterial products and of nutrient-related structures, LPS and certain FA respectively. A polymorphism of *TLR4* is discussed to be associated with human IBD.[186,187,188] It is suggested that the inconsistency of previous findings is a matter of under-powered studies, and meta-analyses reveal statistical significance for the association of *TLR4* polymorphisms with both CD and UC.[189,190]

Energy source butyrate as anti-inflammatory agent. As described above, butyrate oxidation is reduced in inflamed intestinal tissue. Low levels of butyrate and butyrate-producing bacteria have also been reported for type 2 diabetes patients,[191] and administration of butyrate reduces HFD-induced insulin resistance in mice.[192,193] Interestingly, butyrate is not only a major energy source for IEC, but it also acts upon immune cell functionality. G-protein coupled receptor GRP43, expressed by IEC, immune cells, liver and adipose tissue [194,195,196,197,198] has been identified as a target of SCFA.[199,200] $Grp43^{-/-}$ mice show exacerbated inflammation in murine chronic models of arthritis and colitis, dependent on immune cells. While SCFA-administration could ameliorate colitis in wildtype mice, it was ineffective in GRP43-deficient mice.[199]

Interplay of mitochondria in innate immune response. Besides their role in cellular energy supply, mitochondria integrate cellular signaling pathways. Interrelations between activation of TLRs, Retinoic acid-inducible gene-I (RIG-I)-Like Receptors (RLR) and NOD-like receptor (NLR) on the one hand, and energy metabolism on the other hand have recently been described. In principle, mitochondria can evoke oxidative stress,[201,202] unfolded-protein response,[203] and mtDNA release,[204,205] acting as a host danger signal during inflammation, bacterial and viral infection and cellular damage. These mitochondrial mechanisms seem to interact with innate TLR, RLR and NLR signaling by regulating gene expression cytokines and chemokines, as well as caspase-1-dependent maturation of IL1β and IL18,[201,205] mediated by the inflammasome.

Inflammasome unites sensing of microbial products and metabolic stress. The term ‘inflammasome’ describes a structured assembly of proteins including pattern recognition receptors, mostly CARD domain-containing sensors, and caspases.[206] These cytoplasmic complexes respond to a variety of stimuli of both microbial and metabolic nature,[207] upon which they process cytokines, induce inflammatory cell death or modulate cell energy homeostasis.[208] The inflammasome constitutes a unique immunometabolic structure in myeloid cells, and has important functions in obesity and insulin resistance (namely the control of energy expenditure and adipogenic gene expression),[209,210] while it is at the same time a crucial player in innate immunity pathways.[211]

In conclusion, inflammatory conditions in different tissues share a variety of molecular mechanisms with metabolic diseases. Emphasizing these overlaps between typical metabolic processes and immune responses, immunometabolic processes might be tools to understand how diet and/or DIO tentatively affect gut physiology in the context of IBD.

1.3. Mouse models of IBD-like pathologies

Animal models, in particular rodent models, of IBD or IBD-like etiologies have become indispensable for the study of molecular mechanisms as well as for testing treatment strategies. In general one can distinguish between genetically engineered models (knock-out or transgene models), chemically induced models and cell-transfer models. In addition several infection models are commonly used which contribute to the understanding of general mechanisms in bacteria-induced inflammation.[212] As this study investigates the potential interaction of environmental triggers and host genetic susceptibility during IBD pathogenesis, animal models with a genetically introduced immune abnormality also implicated in human IBD were chosen.

1.3.1. $TNF^{\Delta ARE/WT}$ mouse model of ileitis

The $TNF^{\Delta ARE/WT}$ mouse model is one of the very few models of CD-like ileitis. With a deletion of the AU-rich element in the untranslated region of the TNF mRNA, this mouse model exhibits increased TNF mRNA stability resulting in increased TNF expression.[213] While the homozygous genotype leads to severe inflammation and premature death before 12 weeks of age, heterozygous mice display a spontaneous T-cell driven small intestinal inflammation resembling clinical manifestation of human IBD, as well as arthritis. The progression of the ileal

pathology is accompanied by leukocyte infiltration, villous atrophy, crypt hyperplasia and transmural inflammation (ulcer formation). Interestingly, IEC specific overproduction of TNF suffices for the full establishment of intestinal pathology and is associated with early activation of intestinal myofibroblasts. Accordingly, the myofibroblasts have been previously identified as a sufficient target of TNF for disease development in the $TNF^{\Delta ARE/WT}$ mouse model.[214]

There are marked alterations in lymphocyte phenotypes in the $TNF^{\Delta ARE/WT}$ mouse model regarding $CD4^+$ and $CD8^+$ T cells. Despite the observation that deletion of CD4 exacerbates disease,[215] $CD4^+$ lymphocytes in the lamina propria (LP) of $TNF^{\Delta ARE/WT}$ mice also show enhanced Th17-associated responses.[216] The pathogenesis of chronic ileitis in this mouse model seems to be largely $CD8^+$ T cell dependent, as genetic deletion of beta-2 microglobulin or β7 integrin leads to abrogation of intestinal inflammation.[215] Interestingly, gene expression profiling of $CD8^+$ but not $CD4^+$ T cells was useful to predict prognosis in both UC and CD patients at the point of diagnosis.[217] An early reduction of CD8αα-expressing intraepithelial lymphocytes and predominance of TNF/IFN-γ producing CD8αβ lymphocytes in the epithelium is observed during intestinal inflammation in $TNF^{\Delta ARE/WT}$ mice,[218] while αEβ7 integrin expression in peripheral intestinal-homing CD8αβ lymphocytes is reportedly increased.[148, 149]

At present there are no data published on germ-free $TNF^{\Delta ARE/WT}$ mice or the effect of antibiotic-treatment on inflammation in this model, so the impact of microbial composition on the pathology is not elucidated yet. The $TNF^{\Delta ARE/WT}$ mouse has been reported to display reduced body weight and decreased adipose tissue depots and adipocyte sizes under normal feeding conditions. This reduction in bodyweight precedes a decrease in energy uptake which occurs at later age presumably due to intestinal symptoms and the development of arthritis.[219]

The $TNF^{\Delta ARE/WT}$ disease phenotype can be described as Th1 and Th17 dominant, with an increase in IFNγ, IL6, IL1β and KC (also named C-X-C motif ligand [CXCL] 1, and corresponding to the human IL8).[219] TNF signaling is also strongly implicated in IBD pathogenesis by GWAS, as described above. Together with the cytokine profiles and the histology similar to human CD, this points to a certain transferability of this mouse model to the human situation.

1.3.2. $IL10^{-/-}$ mouse model of colitis

The $IL10^{-/-}$ mouse model exhibits colonic inflammation, similar to the human UC phenotype. The lack of the anti-inflammatory cytokine IL10 leads to a disruption of immune homeostasis and to

excessive pro-inflammatory processes,[220] as the absence of IL10 as a suppressor of IL12 and TNF leads to a Th1-mediated inflammation.[221] Conventionally housed homozygous IL10-deficient mice spontaneously develop a chronic enterocolitis with massive infiltration of lymphocytes, neutrophils and activated macrophages,[62] including involvement of the cecum. This colitis is characterized by a Th1 cytokine response, which can be ameliorated by neutralization of IL12p40 and to a lesser extent IFNγ, or by systemic administration of IL10.[222] It has to be kept in mind that the $IL10^{-/-}$ mouse model differs in this respect from the Th2-associated human UC phenotype. Interestingly, mouse models developing Th2-mediated colonic inflammation are limited.

IL10 is also implicated in human IBD pathogenesis by GWAS.[21] Remarkably $IL10^{-/-}$ mice do not develop disease when kept under germ-free conditions,[63] or when treated with antibiotics before the onset of the disease.[73,74] These findings highlight the necessity of the microbial triggers for inflammation development in this mouse model.

2. AIMS OF THE WORK

While epidemiologic data suggest a role of dietary fact in IBD pathogenesis, experimental evidence for this effect and the potential underlying mechanisms is scarce. Typical metabolic diseases such as obesity and immunologic pathologies such as IBD share molecular mechanisms. In fact, dietary fat-induced obesity has been reported to modulate gut physiology, immune responses and intestinal microbial composition in both humans and mouse models.

Hence, the present work aimed to assess the impact of a dietary fat on intestinal pathogenesis in mouse models exhibiting ileal or cecal/colonic inflammation. A major interest of the present study was to investigate the interrelations of promoted pathogenesis and metabolic alterations during high-fat feeding. In addition, possible molecular mechanisms for an effect of dietary fat on IBD etiopathogenesis were to be elucidated.

3. MATERIAL AND METHODS

Animals and experimental setup. Heterozygous male $TNF^{\Delta ARE/WT}$ mice on C57BL/6N background, wildtype $TNF^{WT/WT}$ C57BL/6 littermates (WT), AKR/J and SWR/J were raised at constant room temperature (22 ± 2 °C), air humidity (55 ± 5 %), and a light/dark cycle of 12/12 h. Water and diets (ssniff, Soest, Germany) were provided ad libitum. At 4 weeks of age, animals were divided into feeding groups and received either control diet or palm oil-based HFD until the ages of 8, 12 or 16 weeks. Disease Activity Index (DAI) was assessed weekly as a combined score of weight loss, stool consistency and rectal bleeding, adapted from Cooper et al. [223]. Homozygous male and female $IL10^{-/-}$ mice on 129svev background as well as WT 129svev mice were raised under specific-pathogen free (SPF) housing at the conditions described above. At the age of 4 weeks all mice were fed the control diet diets (ssniff, Soest, Germany) ad libitum. At 12 weeks of age, animals were divided into feeding groups and received either control diet or palm oil-based HFD until the age of 16 weeks. Any invasive animal experiments were performed upon approval by the Bavarian Animal Care and Use Committee with the ethical approval number the application number AZ 55.2-1-54-2531-130-09 and respective amendments.

Experimental diets. Control diet (12 kcal% fat) and palm oil-based high-fat diet (HFD; 48 kcal % fat) were purchased from ssniff, Soest Germany as semi-synthetic diets. Both diets were γ-irradiated. Detailed information on the composition of the diets is given in Table 2. To verify the FA profile of the diets, FA concentrations were assessed by biochemical analysis according to the standard protocol DGF C-VI 11e and German standard guidelines [224] at the BIOANALYTIK Weihenstephan, Alte Akademie 10, Freising Germany. The HFD was largely dominated by C16:0 and C18:1 FA (Figure 5).

Table 2: Composition of the diets.

	control diet (S575-E712)	high-fat diet (S575-E702)
Metabolizable energy [MJ/kg]	15.5	19.7
Protein [kJ%]	23	18
Fat [kJ%]	12	48
Carbohydrates [kJ%]	65	34
Casein [weight%]	24	24
Corn starch [weight%]	47.8	27.8
Soy oil [weight%]	5	5
Palm oil [weight%]	-	20
Cellulose [weight%]	5	5
Maltodextrin [weight%]	5.6	5.6
Sucrose [weight%]	5	5
Vitamin mix [weight%]	1.2	1.2
Mineral / trace element mix [weight%]	1.2	1.2
Calcium [weight%]	0.92	0.92
Phophate [weight%]	0.63	0.63
Sodium [weight%]	0.19	0.19
Magnesium [weight%]	0.21	0.21
Lysine [weight%]	1.71	1.71
Methionine [weight%]	0.75	0.75
Methionine + cysteine [weight%]	1.04	1.04
Threonine [weight%]	0.93	0.93
C14:0 [weight%] *	0.03	0.24
C16:0 [weight%] *	0.76	9.34
C16:1 [weight%] *	0.01	0.04
C18:0 [weight%] *	0.24	1.02
C18:1 [weight%] *	1.32	8.57
C18:2 [weight%] *	2.78	4.28
C18:3 [weight%] *	0.02	0.38
C20:0 [weight%]	0.03	0.13
C20:1 [weight%] *	0.01	0.04
C22:0 [weight%] *	0.03	0.04

* as analyzed by BIOANALYTIK Weihenstephan (ZIEL, Technische Universität München)

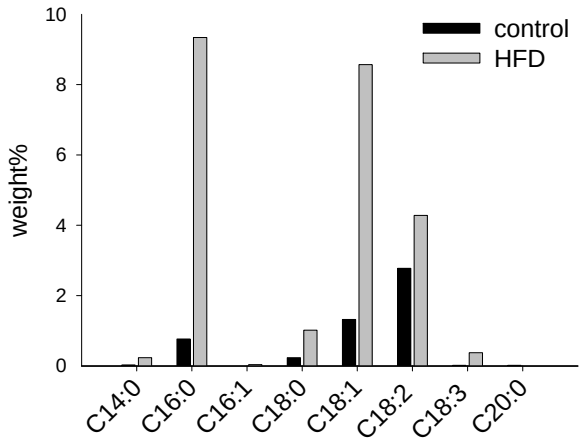

Figure 5: The fatty acid profile of the palm-oil based HFD is dominated by C16:0 and C18:1.
Biochemical analysis of the feed reveals the fatty acid composition of the HFD to be dominated by fatty acids characteristic for palm oil, the fat basis of the HFD. Only the eight most abundant fatty acids are shown.

Sampling procedures. Animals were killed by application of CO_2. EDTA-blood was collected from the *vena cava inferior* or the *vena portae hepatis*, centrifuged at 3000 g for 10 min and plasma stored at -80 °C until analysis. Tissue samples were removed, snap-frozen using liquid nitrogen or dry ice and stored immediately at -80 °C. Samples for histological processing were fixed in 4% formalin immediately.

Histological scoring. Intestinal samples fixed in formalin for at least 24 h were dehydrated, embedded in paraffin and cut in 5 µm sections followed by hematoxylin and eosin (H&E) staining. Histological scoring was performed by blindly assessing the degree of LP mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion and architectural disruption in the different gut sections, resulting in a score from 0 (not inflamed) to 12 (highly inflamed), as previously described.[225]

Adipocyte size measurements. Formalin fixed intestinal samples of mesenteric adipose tissue (MAT) and epididymal adipose tissue (EAT) or perigonadal adipose tissue (PAT) were embedded in paraffin and cut in 5 µm-thick sections followed by H&E staining. The mean adipocyte size was determined in the stained fat sections at a magnification of 200-fold and the size of adipocytes was calculated using AxioVision 4.6 (Carl Zeiss AG, Jena, Germany).

Body composition measurement. Development of body composition was monitored using a minispec TD-NMR analyzer (Bruker Optics, Ettlingen, Germany) in the SPF facility. The mice

were placed in a red Plexiglas tube with vent holes to restrain mobility. The tube was inserted in the minispec for app. 1.5 min of measurement. Calibration had been performed using lean chicken meat for lean mass and mouse adipose tissue for fat mass.

Food/energy uptake measurement. Food uptake was assessed by daily weighing of the feed of individual animals across one week. Energy uptake was then calculated using the energy content of the diets as stated by the manufacturer (see Table 2).

Glucose tolerance test. Oral glucose tolerance tests were performed as follows: 6-h-fasted mice were gavaged with glucose solution (2 g glucose/kg body weight, 20 % glucose solution in 0.9 % NaCl). Blood glucose concentration was determined before and 15 min, 30 min, 60 min and 120 min after injection with a glucose meter (FreeStyle Lite, Abbot Diabetes Care, Alameda, CA, USA) on a drop of blood collected from the tip of the tail. The area under the curve was calculated using the trapezoidal rule.

Analysis of energy content of feces. Fecal samples were collected from individual mice at indicated time points, dried at 60 °C for 3 days, and stored airtight at RT until being analyzed. Fecal pellets were manually ground and pressed into pellets and the gross energy content was determined using a 6300 Calorimeter (Parr Instrument Company, Moline, IL, USA). Fecal composition was analyzed using Fourier transform infrared spectrometry (FT-IR) on a HTS-XT spectrophotometer (Bruker Optics) with diffuse reflection technology. Spectra were obtained in the range of 6000 cm-1 to 600 cm-1. Data were analyzed and calculation of first deviation, vector normalization and cluster analysis according to Ward’s algorithm were performed using the OPUS Software version 6.5 (Bruker).

Cytokine and chemokine quantification. Plasma specimen or cell culture supernatants were used to quantify CCL20 by Quantikine ELISA kit (R&D Systems Europe, Abingdon, UK). SAA was quantified in plasma by ELISA (ICL Inc., Portland, OR, USA). Leptin, IL6, TNF and MCP1 were quantified in plasma using a bioplex system (BioRad, München, Germany).

Metabolite analysis. For LC-MS analysis 1 cm of distal ileum was excised and flushed with 1 ml of phosphate buffered saline using a sterile syringe and then preserved at -80 °C. EDTA-blood was centrifuged at 3000 g for 10 min and plasma stored at -80 °C until analysis. Further analyses were performed at the Nestlé Research Center, Lausanne, Switzerland. The Biocrates Life Sciences AbsoluteIDQ kit, originally designed for plasma samples, was adapted as previously published,[219] to be used for intestinal tissue. The tissue was homogenized in 300 µl of EDTA (ethylenediaminetetraacetic acid; 0.292 mg/ml PBS) and 10 µl of BHT-buffer (butylated

hydroxytoluene; 79.2 mg/ml PBS) using the FastPrep 24 system (MP Biomedicals LLC; Illkirch, France). Afterward, well plate preparation and sample application and extraction were carried out according to the manufacturer's instructions. A final volume of 10 µl of tissue homogenate or plasma was loaded onto the provided 96-well plate. Liquid chromatography was realized on a Dionex Ultimate 3000 ultra-high pressure liquid chromatography (UHPLC) system (Dionex AG, Olten, Switzerland) coupled to a 3200 Q TRAP mass spectrometer (AB Sciex; Foster City, CA) fitted with a TurboV ion source operating in electrospray ionization (ESI) mode. Sample extracts (20µl) were injected two times (in positive and negative ESI modes) via direct infusion using a gradient flow rate of 0-2.4 min: 30 µl/min, 2.4-2.8 min: 200 µl/min, 2.9-3 min: 30 µl/min. MS source parameters were set at: esolvation temperature: 200 °C, high voltage: -4500 V (ESI -), 5500 V (ESI +), curtain (CUR) and nebulizer gases: nitrogen; 20, 40, and 50 psi; respectively, nitrogen collision gas pressure: 5 mTorr. MS/MS acquisition was realized in scheduled reaction monitoring mode with optimized declustering potential values for the 163 metabolites screened in the assay. Raw data files (Analyst software, version 1.5.1; AB Sciex, Foster City, CA, USA) were imported into the provided analysis software MetIQ to calculate metabolite concentrations. List of all detectable metabolites is available from Biocrates Life Sciences, Austria (http://biocrates.com). To maximize the separation between the sample groups the supervised methods Partial Least Squares (PLS) and Orthogonal PLS (O-PLS) discriminant analyses (PLS-DA and O-PLS-DA) were further applied. All models were generated using one predictive and one orthogonal component to discriminate between two groups of animals. The model robustness was assessed using the standard 7-fold cross validation method (repeatedly leaving out a seventh of the samples and predicting them back into the model). Also R^2X and R^2Y values were computed to show how much of the variation in the data set X (metabolite data) and in the data set Y (group information) was explained by the model. The validity of the model against overfitting was monitored by computing the cross-validation parameter Q^2 which represents the predictability of the models and relates to the statistical significance. Negative or very low values of the Q^2 indicated that no statistically significant differences were observed. In addition, Mann-Whitney U tests were performed on variables to assess significant differences of metabolite concentrations, respectively.

Isolation of mouse intestinal epithelial cells. Primary intestinal epithelial cells (IEC) from the ileal epithelium were purified as previously described.[220] Briefly, the fresh ileal tissue was opened longitudinally, washed in PBS and incubated at 37 °C in DMEM containing 10 % FCS, 1 % Glutamine, 1 % antibiotic-antimycotic (Invitrogen, Carlsbad, USA) and 1 mM DTT for 20 min, followed by vortexing for 1 min. The remaining tissue was incubated in 30 ml of PBS containing

1.5 mM EDTA for additional 20 min, followed by 1 min of vortexing. The supernatants were centrifuged for 5 min at 350 g and the cell pellets were re-suspended in DMEM containing 5% FCS. Finally, the primary IEC suspension was purified by centrifugation through a 20 %/40 % discontinuous Percoll gradient at 600 g for 30 min. Purified IEC were washed in PBS, lysed for further processing and stored at -80 °C.

Gene expression analysis. Total RNA was isolated from IEC, adipose tissue or isolated lymphocytes using a phenol based extraction (Qiagen, Hilden, Germany). RNA yield and quality were assessed by absorbance using a Nanodrop ND-1000 spectrophotometer (LabTech International, Brampton, ON, Canada). A total of 1 µg RNA was used for reverse transcription using M-MLV point mutant system (Promega, Madison, USA). Primers and probes were designed using the universal probe library (Roche, Mannheim, Germany). RNA-expression profiles were analyzed using the LightCycler (Roche) with 400 nM primers, 200 nM probe and 1 µl cDNA (QuantiTect Probe RT-PCR Master Mix buffer) in a real time quantitative polymerase chain reaction (RT-qPCR).The relative induction of mRNA expression was calculated using the equation $2^{-\Delta\Delta Cp}$ and normalized for the expression of GAPDH. Data were expressed as fold change against WT on control diet.

Western blotting analysis. Purified primary IEC of 12-week-old mice were lysed in lysis buffer, homogenized using ultrasound and protein concentration was quantified by Bradford method (Carl Roth, Karlsruhe, Germany). Protein lysates were boiled in Laemmli buffer at 95 °C for 10 min. Equal amounts of proteins were resolved on 7-20% SDS-polyacrylamide gels and transferred by electroblotting to a nitrocellulose membrane. Rabbit anti-Ocln (Invitrogen), mouse anti-E-Cadherin (Abcam, Cambridge, UK), rabbit anti-CCL20/MIP-3α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-β-Actin (MP Biomedicals, Illkirch, France) were used to bind immunoreactive proteins of interest. For detection, appropriate HRP-coupled secondary antibodies goat anti-rabbit and goat anti-mouse (both Dianova, Hamburg, Germany) were applied, using an enhanced chemiluminescence light-detecting kit (Amersham, Arlington Heights, USA).

Immunefluorescence staining of tissue. Formalin fixed samples of 12-week-old mice were embedded in paraffin and cut in 5 µm sections, cooked in citrate buffer and blocked with 5% serum (from host species of secondary antibody) in PBS including 0.3% Triton X-100. Staining was performed with mouse anti-CD3 (Santa Cruz Biotechnology), rabbit anti-Ocln, mouse anti-ZO-1, rabbit anti-β-Catenin (all Invitrogen, Carlsbad, CA, USA), mouse anti-E-Cadherin intracellular (Abcam) or a combination or rabbit anti-E-cadherin intracellular (Cell Signaling,

Beverly, MA, USA) and mouse anti-E-Cadherin extracellular (Santa Cruz Biotechnology), dissolved in PBS containing 0.3% Triton X-100 and 1% BSA, to localize immunoreactive proteins of interest. As secondary antibodies, anti-mouse and anti-rabbit antibodies coupled to Alexa-488, Alexa-594 (both Invitrogen) or Alexa-647 (Dianova) were used. For staining of $CD11c^+$ cells, distal ileum specimen were cryoembedded in O.C.T. compound (Sakura Finetek, Torrance, CA, UDA) on dry ice. Sections of 5 µm were fixed in dried methanol for 15 min on dry ice, washed in PBS three times for 5 min and then blocked and stained using the buffers described above, with anti-CD11c (BD Biosciences) and Cy3-conjugated anti-hamster antibody (Dianova). DAPI (Invitrogen) was used to stain nuclei. Microscopic pictures were acquired at a magnification of 200- to 600-fold as indicated, using the confocal microscope FluoView FV10i (Olympus, Hamburg, Germany) and processed using Volocity 3D Image Analysis Software (Perkin Elmer, Rodgau, Germany). Background fluorescence of the epithelial area varied between 350 A.U. and 1300 A.U. and was not substracted from the fluorescence intensity values.

Endotoxin quantification. Endotoxin levels in hepatic portal vein plasma of 12-week-old mice were quantified using LAL Chromogenic Endpoint Assay (Hycult biotech, Uden, Netherlands). Protocol was optimized according to recovery rates. Briefly, plasma was diluted 1:10 in endotoxin-free water, heated to 70 °C for 10 min, and measured with addition of 1:100 Pyrosperse (Lonza, Walkersville, MD, USA). Recovery rates were assessed to validate the results.

Polyethylene glycol permeability assay. Polyethylene glycols (PEG) of the average molecular sizes 1500 and 3000 Da were applied solubilised in PBS to 16 week old mice, at final amount of 1.5 mg and 5 mg per mouse respectively, and urine was collected for 24 h. Urine samples were diluted to an osmolarity of 500 mosmol in acetonitrile/H_20 (1:9) for subsequent analysis via LC/MS. Samples were analyzed in duplicates. MS spectra were obtained from a hybrid triple-quadrupole/linear ion trap mass spectrometer (API 4000 QTrap; Applied Biosystems Inc., Foster City, CA, USA). The ion source (Turbo Ion Spray) was operated in the positive electrospray ionization (ESI) mode. Settings were as follows: curtain gas (CUR): 20 psi, (IS): 5500 V, temperature (TEM): 450 °C, ion source gas 1 (GS1): 45 psi, ion source gas 2 (GS2): 55 psi. For LC-MS measurements, the mass spectrometer was operated in the SIM (single ion monitoring) mode. Data acquisition was carried out using Analyst 1.5 software (Applied Biosystems). HPLC separation prior to mass spectrometric detection was performed on a Shimadzu LC-20A prominence HPLC system (Shimadzu, Kyoto, Japan). As stationary phase a 150 mm x 3.0 mm i.d. 3 µm Gemini-NX (Phenomenex, Aschaffenburg, Germany) was used. The mobile phase was

mixed water (A) and acetonitrile (B) following a linear binary gradient as follows: Initial conditions were 15% B and 85% A. The content of solvent B was linearly raised during the next 10 min to obtain 30% B and 70% A. During the next 15 min the content of B was linearly raised to 40% and during the next minute to 50%. These conditions were held for 3 min, before the content of solvent B reached initial conditions after 1 min. Injection volume was 10 µl, flow rate was 0.2 ml/min and equilibration time was between two runs 10 min. PEG were calculated as sum of the sodium adducts.

Isolation of lamina propria lymphocytes. Lamina propria lymphocytes (LPLs) of 12-week-old mice were isolated by 1 h collagenase digestion (1 mg/ml in PBS with $Ca2^+$ and $Mg2^+$) of ileal tissue after removal of epithelial cells. The cells were filtered through a 70µm cell strainer (Corning Incorporated, Corning, NY, USA) before further processing for gene expression analysis or flow cytometry.

Flow cytometry. LPLs or bonemarrow-derived dendritic cells were pre-treated with Fc receptor block (Milteny Biotec, Bergisch Gladbach, Germany) and stained as indicated in the results section, using anti-CD11c, anti-CD11b, anti-CD86, anti-MHCII, anti-CD3e, anti-CD4 (all BD Biosciences, Franklin Lakes, NJ, USA) or anti-CD8α (AbD Serotec, Düsseldorf, Gemany). A total of 10,000 cells was acquired from the LSR II (BD Biosciences) and analyzed using BD FACSDiva software. Gating methods have been described elsewhere.[218]

Stimulation of PTK6 cells. The murine large intestinal epithelial cell line PTK6 (passages 45-48) was kindly provided by R. Whitehead Vanderbilt University, Nashville, Tennessee). The cells were cultured in T75 flasks in RPMI-1640 (Sigma Aldrich, Taufkirchen, Germany) containing 5% FCS (Biochrom, Berlin, Germany), 1 µg/ml Insulin-Transferrin-Selenium A (Gibco, Carlsbad, CA, USA), 10 Units/ml murine IFNγ (Biosource life technologies, Carlsbad, CA, USA) and 1% antibiotic-antimycotic (Sigma Aldrich), in humidified 5% CO_2 atmosphere at 37 °C. For stimulation and measurement of transepithelial resistance in vitro, $3x10^5$ cells/ml were grown on 0.4 µm polyester Transwell filters (Costar Corning, Corning, NY, USA) in cell culture medium without IFNγ at 37°C. The transepithelial electrical resistance (TER) was measured using a Volt-Ohm-meter (Millipore, Schwalbach/TS, Germany). TER development was examined and cells were stimulated as indicated in the results section when TER values had reached a constant level. Stimulation was performed with cecal water at final concentration of 50 µg protein/ml for 48 h. Barrier break was induced by apical application of 50 ng/ml recombinant mouse TNF (Invitrogen) in combination with 20 ng/ml IFNγ for 48 h.

Stimulation of Mode K cells. The murine small intestinal epithelial cell line Mode K (passages 15-27) was cultured in 6-well cell culture dishes in DMEM-F12 (Sigma Aldrich) containing 10 % FCS (Biochrom, Berlin, Germany), 1 % Glutamine and 1 % antibiotic-antimycotic (both Sigma Aldrich), in humidified 5% CO_2 atmosphere at 37 °C. At the state of early confluency Mode K cells were stimulated for 24 h with different preparations as described below (50 µg protein/ml) or 20 ng/ml recombinant mouse TNF (Invitrogen). Mode K cell conditioned media (CM) were harvested and stored at -80 °C.

Cecal lysates. Cecal lysates were prepared from cecal contents of WT mice receiving the different experimental diet as indicated in the experiments. The preparation was adapted from a previously described protocol.[226] Briefly, the cecum was dissected, the cecal content was collected and snap-frozen. It was stored at -80 °C until usage, then diluted in sterile PBS (1:1) and vortexed thoroughly. After centrifugation at 8000 g for 5 min, cecal water was collected. At this step, the samples were split into two aliquots, one undergoing preparation for a whole lysate, one for a bacterial lysate. For generation of whole cecal lysates, cecal suspensions (0.5 ml) were mixed with PBS and one volume of 0.1 mm glass beads (Roth, Karlsruhe, Germany) and disrupted in a bead-beater (FastPrep-24, MP Biomedicals LLC) at 5000 Hz for 0.5 min, at total six cycles interrupted by 2 min on ice each. The glass beads and unlysed cells were removed by centrifugation at 350 g for 5 min. Supernatants were collected and centrifuged at 8000 g, 5 min. After centrifugation, the supernatant was filtered through a prewashed 0.45 µm filter. For generation of bacterial lysates, cecal content was mixed with PBS (1:1), vortexed and centrifuged at 300 g for 1 min. The supernatant was then processed with glass beads as described above. The protein concentration in each fraction was assessed using a Bradford assay according to the manufacture's protocol (Roti-Quant, Roth, Karlsruhe, Germany). Cecal lysates were aliquoted and frozen at −20 °C.

Dietary suspensions. Diet pellets were ground under sterile conditions and dissolved in sterile PBS (1.5 g in 12 ml). Suspensions were vortexed and incubated in a horizontal shaker at 300 rpm, 37 °C, 60 min. Dietary suspensions (DS) were centrifuged at 350 g, 5 min. Supernatants were collected and centrifuged at 8000 g, 10 min. After centrifugation, the supernatant was filter-sterilized (0.4 µm filter) and the protein concentration was assessed using a Bradford assay according to the manufacture's protocol (Roti-Quant, Roth, Karlsruhe, Germany). PBS was used to prepare dilutions.

Dendritic cell transmigration assay and stimulation. Dendritic cells (DCs) were generated from bone marrow of C57BL/6N mice (BM-DCs). Briefly, femurs and tibiae were removed,

cleaned thoroughly, cut open on both sides under sterile conditions and flushed with PBS to receive bone marrow. After lysis of red blood cells, remaining cells were cultivated in DMEM with 10 % FCS, 1 % Pen/Strep, 2 mM β-ME, 15 ng/ml IL4 and 15 ng/ml GM-CSF (both PeproTech, London, UK) for 7 days. Suspension cells were phenotyped by cell cytometry before performing migration assays, using anti-CD11c, anti-CD11b, anti-MHCII and anti-CD86 (all BD Biosciences) as described above. For migration assays, BM-DCs were used at a density of $5x10^4$ cells/ml in a 3 µm pore size transwell permeable support system (Corning Incorporated), applying different Mode K CM as chemoattractants (see above) for 1 h. The potential of a CM to result in migration of BM-DCs through the membrane was normalized to spontaneous migration (chemotactic index). For stimulation BM-DCs at a density of $2.5x10^5$ cells/ml were stimulated with the different Mode K CM for 24 h. BM-DCs were then lysed and RNA was isolated for subsequent qPCR analysis as described above.

Bile acid analysis. Frozen cecal content of 12-week-old mice was lyophilized and dried matter was homogenized in methanol to extract metabolites of interest. An aliquot of the extract was then combined with the isotope standards, diluted with water (1:1), filtered and injected (10 µl) in the HPLC system. A gradient with 0.2% formic acid in water and acetonitrile (solvent B going from 30 % at the start of the run up to 100 % in 20 min) at 0.6 ml/min was used to separate the sample through a packed column (Phenomenex LunaC18(2) 150 x 4,6 mm; 5 µ), kept at 40 °C. The mass spectrometer (ABSciex 5500) was operated in negative mode and the mass spectra of the eluents recorded using the multiple reaction-monitoring mode. Quantitation was made using Analyst Software (ABSciex). A dilution row of normal (no isotope) standards of bile acids was processed and analyzed in a similar manner as described above. Data are shown as mean±SEM.

Statistical analysis. Statistical tests were performed using two-way ANOVA by the Holm-Sidak method when comparing more than two groups, or unpaired Student's t-test or Mann-Whitney-U rank sum test comparing dietary groups within the same genotype and age. Differences were considered significant if p-values were <0.05(*), <0.01(**), or <0.001(***). Data are shown as mean±SD or as median with lower and upper quartile (box-whisker-plot), unless stated otherwise.

4. RESULTS

4.1. HFD promoted onset of pathogenesis in TNF$^{\Delta ARE/WT}$ mice independently of obesity

4.1.1. HFD aggravated early ileitis in TNF$^{\Delta ARE/WT}$ mice

Feeding with the 48%kcal-fat diet (HFD) compared to the control diet from the age of 4 weeks on aggravated ileal histopathology in TNF$^{\Delta ARE/WT}$ mice at the age of 8 weeks and 12 weeks. At 16 weeks of age the histopathological grade of ileitis reached a plateau and did not differ any longer between HFD and control (Figure 6). WT mice did not develop histopathological signs of inflammation in the distal ileum.

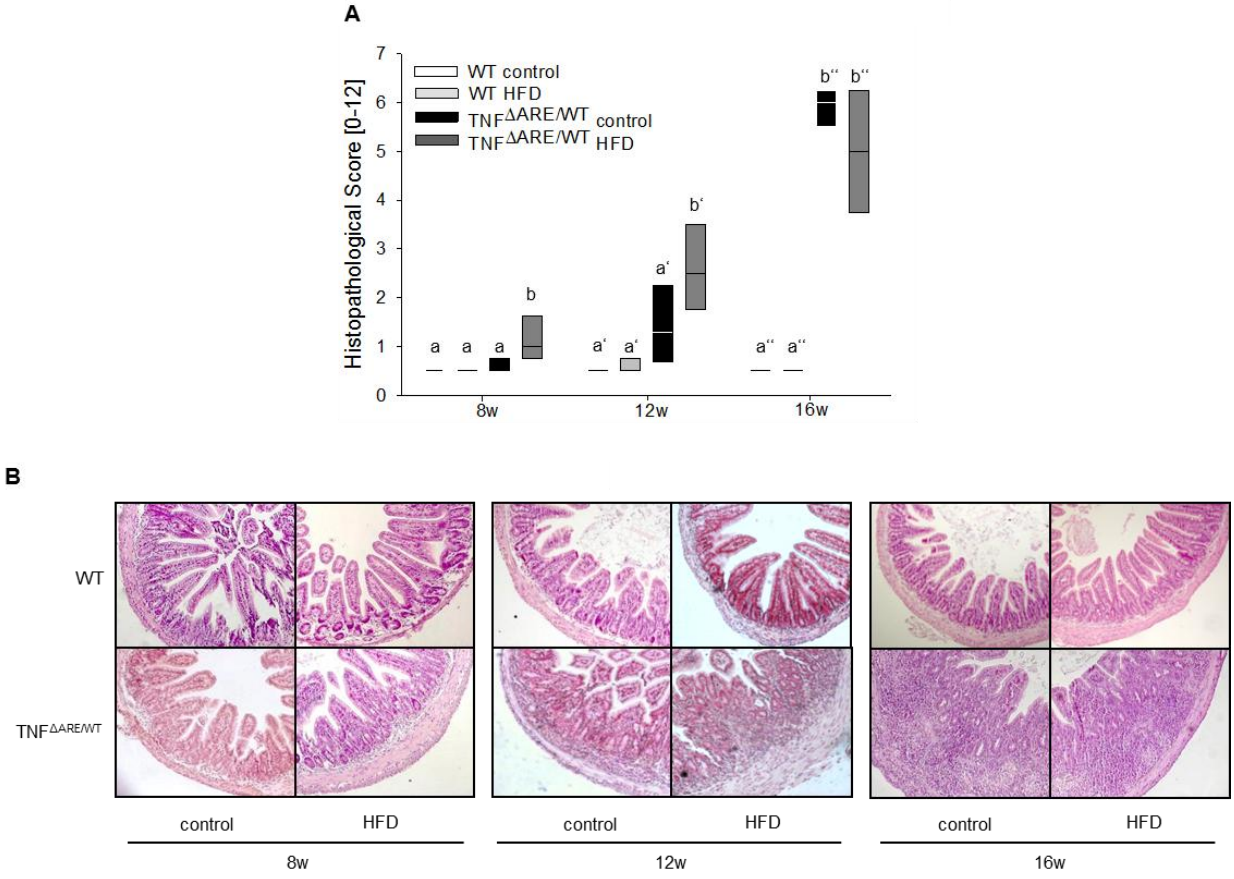

Figure 6: HFD accelerates onset of ileitis in TNF$^{\Delta ARE/WT}$ mice.

At 8 and 12 weeks of age, ileal histopathology in the TNF$^{\Delta ARE/WT}$ mice is significantly aggravated by HFD (A). At 16 weeks of age, HFD does not affect the histopathological score in the distal ileum. WT mice do not display histological signs of inflammation. H&E stained sections were used to assess the grades of inflammation and reveal infiltration of immune cells as well as architectural disruption of the tissue with progressing inflammation (100fold magnification) (B). n=6 per group for 8 and 12 weeks of age, n=10 per group for 16 weeks of age. a,b: data sets with different superscript letters differ significantly from each other (within age groups) according to two-way ANOVA

4.1.2. HFD aggravated ceco-colitis in TNF$^{\Delta ARE/WT}$ mice

The secondarily affected proximal colon of TNF$^{\Delta ARE/WT}$ mice was more severely inflamed under HFD at the age of 16 weeks, whereas no histopathological inflammation of the proximal colon (pCo) was observed at earlier time points (Figure 7 A/B). WT mice did not develop histopathological inflammation along the colon. The disease activity index, a combined evaluation of weight loss, stool consistency and blood in the stool, was significantly higher in TNF$^{\Delta ARE/WT}$ mice on HFD compared to control diet from week 11 on, underlining the finding of aggravated colonic pathology (Figure 7 C).

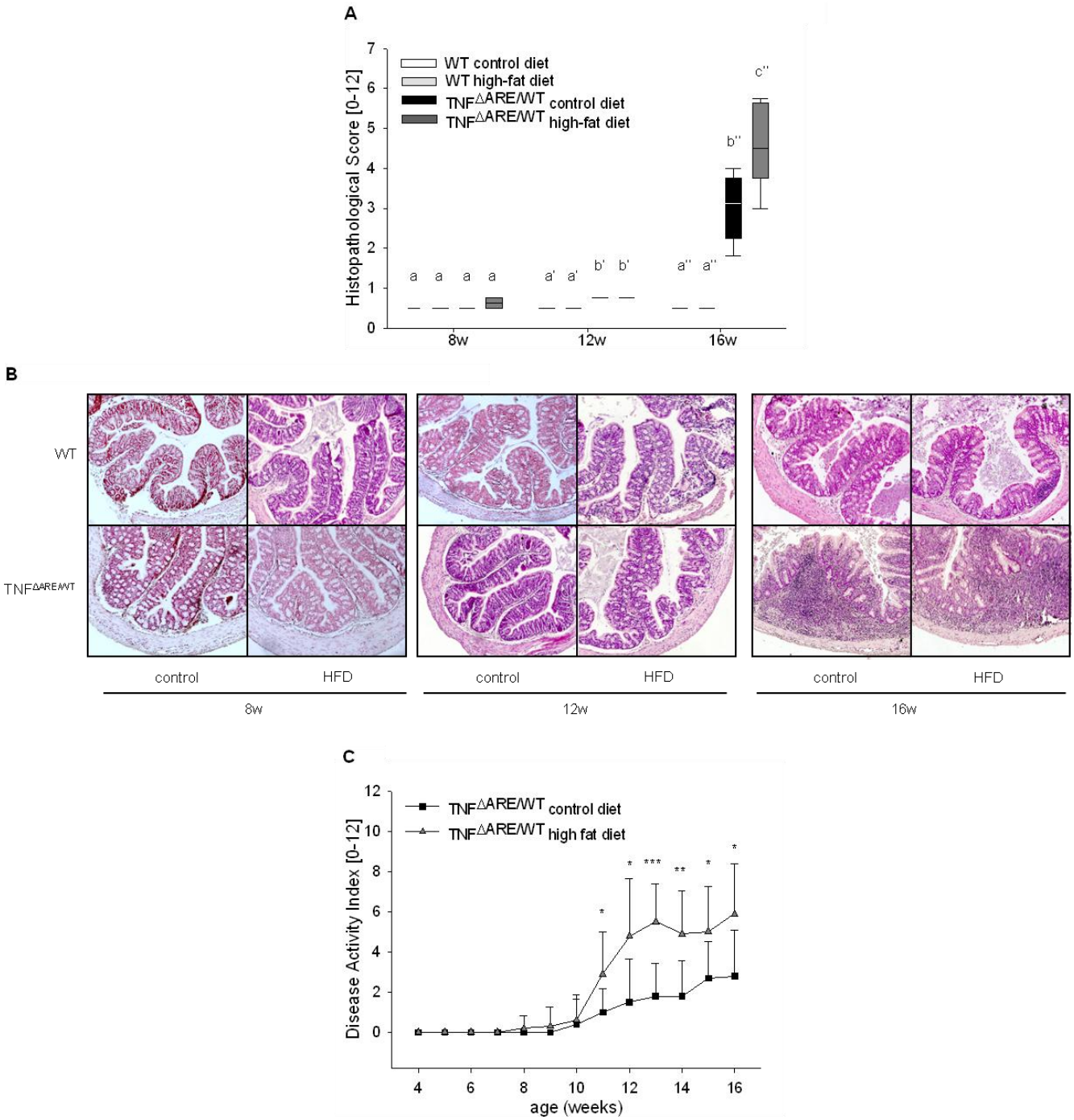

Figure 7: HFD aggravates colonic inflammation in TNF$^{\Delta ARE/WT}$ mice.

At 16 weeks of age, HFD significantly aggravates inflammation in the proximal colon in TNF$^{\Delta ARE/WT}$ mice (A/B). WT mice do not display histological signs of inflammation (100fold magnification) (A/B). Disease Activity Index of TNF$^{\Delta ARE/WT}$ mice was significantly increased by HFD from the age of 11 weeks onwards. n=6 per group for 8 and 12 weeks of age, n=10 per group for 16 weeks of age. a,b,c: data sets with different superscript letters differ significantly from each other (within age groups) according to two-way ANOVA; * p<0.05 ** p<0.01 *** p<0.001 within age and genotype according to Student's t-test

At 12 weeks of age, TNF$^{\Delta ARE/WT}$ mice exhibited significantly elevated energy content of feces under HFD feeding compared to control diet according to bomb calorimetric analysis (Figure 8 A). FT-IR analysis revealed a spectral region characteristic for FA (wavelength app. 2820 cm^{-1}) as mainly discriminating this experimental group from the others (Figure 7 B/C). These findings specify the observed diarrhea as steatorrhea.

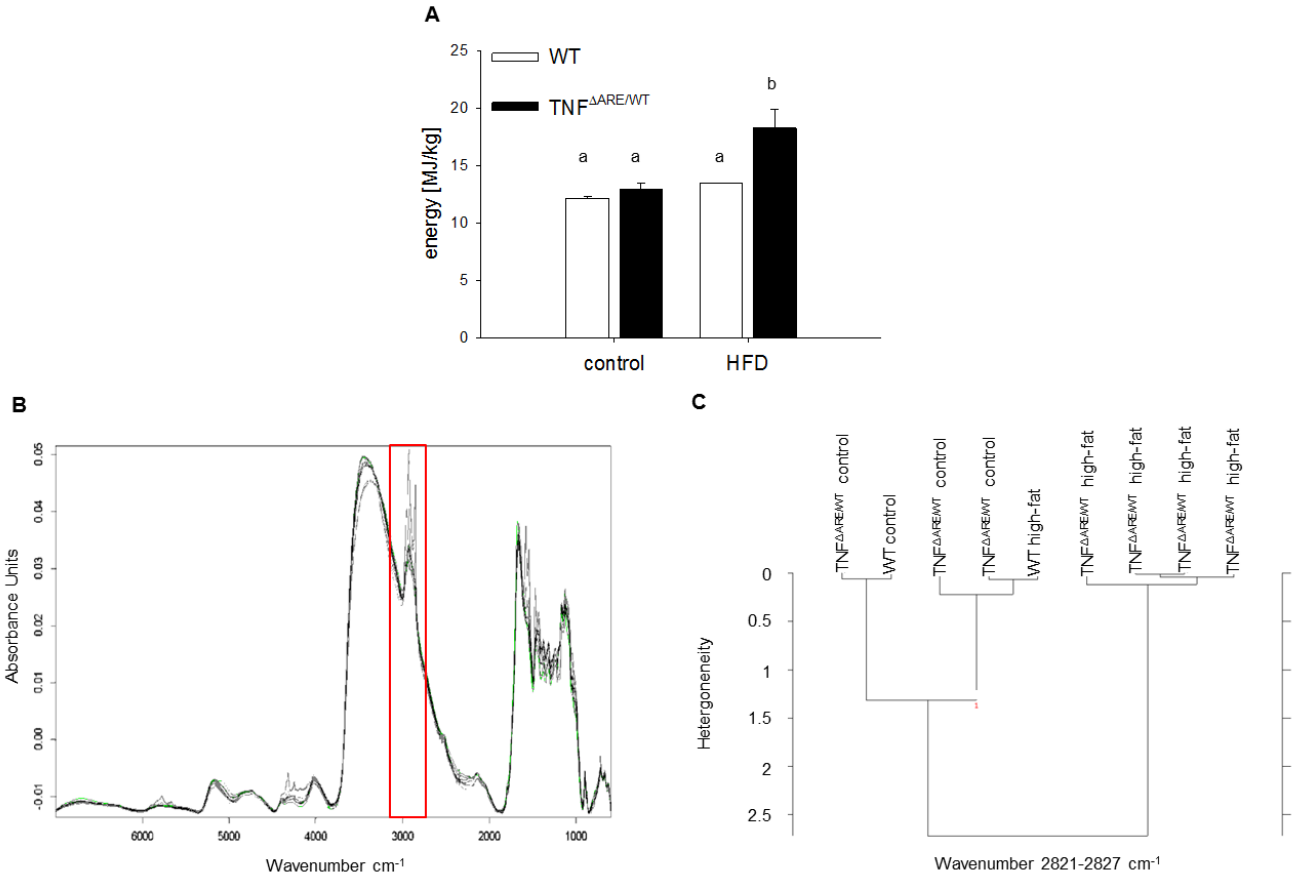

Figure 8: TNF$^{\Delta ARE/WT}$ mice develop steatorrhea under HFD feeding.

HFD significantly increases the energy content of feces in TNF$^{\Delta ARE/WT}$ mice (A) (n=4-6 per group). FT-IR analysis revealed clustering of the feces samples in a spectral range around 2825 cm^{-1} (B,C). This range was identified as characteristic for fatty acids by spiking a mixture of stearic and palmitic acid into a WT mouse feces sample (data not shown). a,b: data sets with different superscript letters differ significantly from each other (within age groups) according to two-way ANOVA

4.1.3. HFD did not induce obesity or associated parameters in TNF$^{\Delta ARE/WT}$ mice

To test the hypothesis whether this aggravation of intestinal inflammation is associated with obesity and metabolic disorders, body weight development was investigated. From the age of 12 weeks on, HFD induced significant overweight in WT mice, whereas TNF$^{\Delta ARE/WT}$ mice did not gain weight to considerable extend (Figure 9 A).

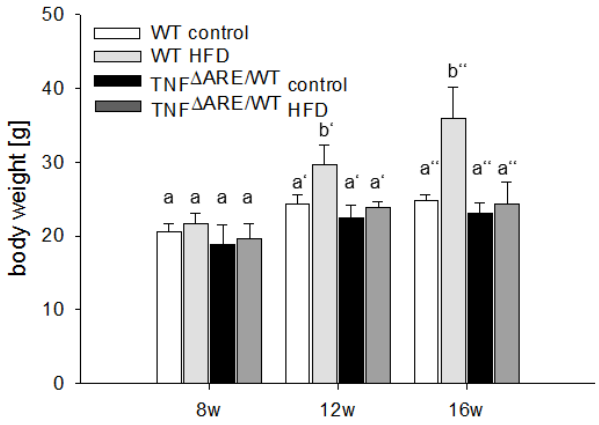

Figure 9: HFD induces overweight in WT mice, but fails to do so in TNF$^{\Delta ARE/WT}$ mice.
At 12 and 16 weeks of age, HFD fed WT mice display significantly increased body weight, whereas the body weight of TNFΔARE/WT mice is not significantly affected. n=6 per group for 8 and 12 weeks of age, n=10 per group for 16 weeks of age. a,b: data sets with different superscript letters differ significantly from each other within age groups

Next, the morphology of both mesenteric adipose tissue (MAT) and epididymal adipose tissue (EAT) were analyzed using H&E-stained tissue sections. Again, in contrast to WT mice, TNF$^{\Delta ARE/WT}$ mice did not show increased MAT or EAT (Figure 10 A/C), nor increased adipocyte sizes (Figure 10 B/D). In addition, adipocyte size in TNF$^{\Delta ARE/WT}$ mice was significantly decreased compared to WT mice at 12 and 16 weeks of age in both dietary groups ($p<0.05$) in both these compartments.

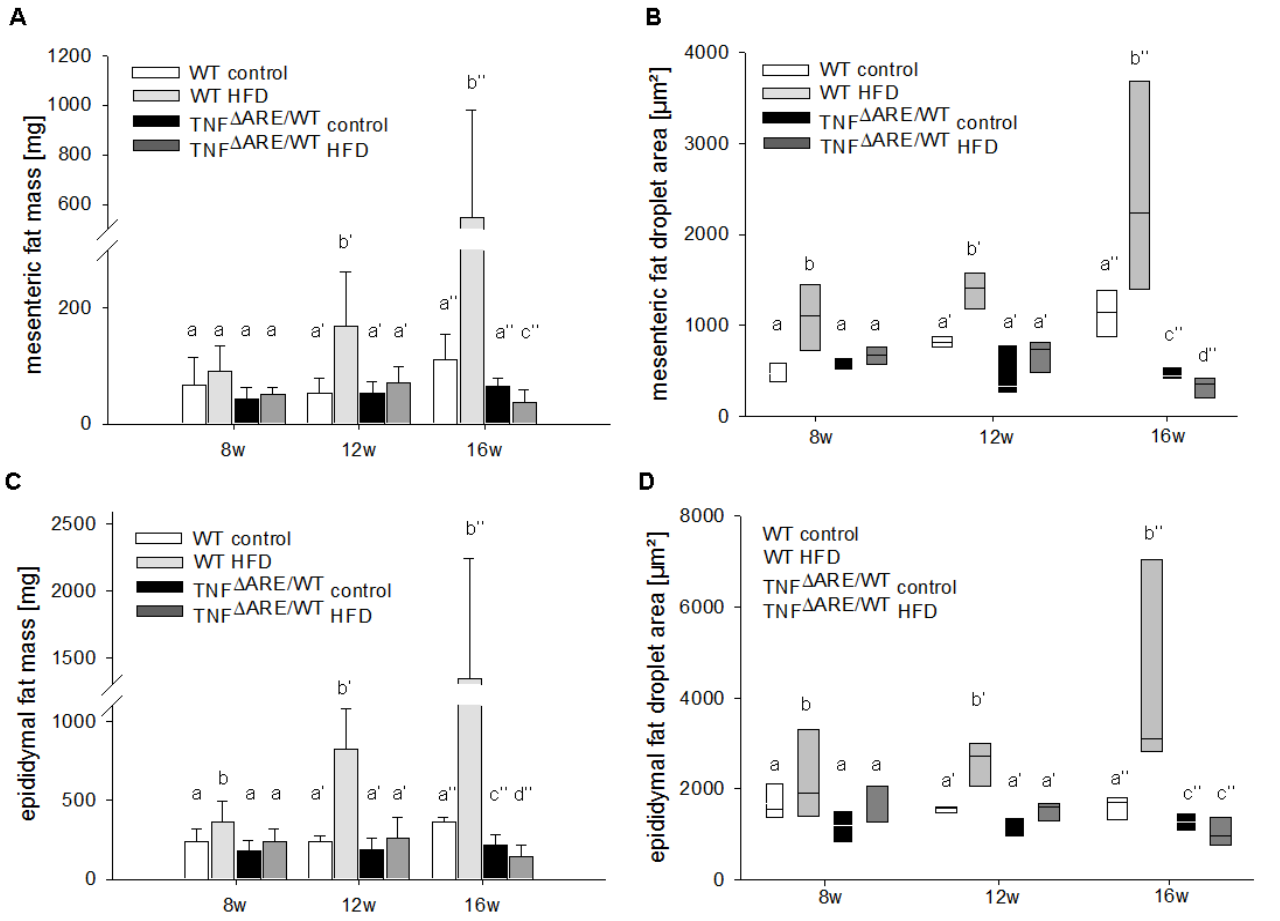

Figure 10: In TNF$^{\Delta ARE/WT}$ mice, HFD neither affects mesenteric (A) nor epididymal adipose tissue mass (C), and does not increase adipocyte size in neither adipose tissue (B/D).

At 8, 12 and 16 weeks of age, HFD fed WT mice display increased mesenteric and epididymal fat mass and adipozyte size, whereas these parameters are not significantly affected by diet in TNF$^{\Delta ARE/WT}$ mice. n=6 per group for 8 and 12 weeks of age, n=10 per group for 16 weeks of age. a,b,c,d: data sets with different superscript letters differ significantly from each other (within age groups) according to two-way ANOVA

Local mRNA expression and systemic levels of adipokines typically associated with low grade inflammation in obesity or HFD were analyzed using qPCR on MAT tissue RNA, and ELISA technique on blood samples respectively. At 12 weeks of age the gene expression of TNF in MAT was app. 9-fold upregulated in TNF$^{\Delta ARE/WT}$ compared to WT mice, together with significant increase of TNF plasma levels, but with no further regulation due to HFD (Figure 11 A). Expression of MCP1, recruiting macrophages as an early event of adipose tissue inflammation, as well as of cytokine IL6 were regulated in a similar manner, displaying no induction due to HFD in the TNF$^{\Delta ARE/WT}$ mouse model (Figure 11 B,C). The expression and plasma levels of leptin were strongly induced during HFD in WT mice, but not in TNF$^{\Delta ARE/WT}$ mice (Figure 11 D).

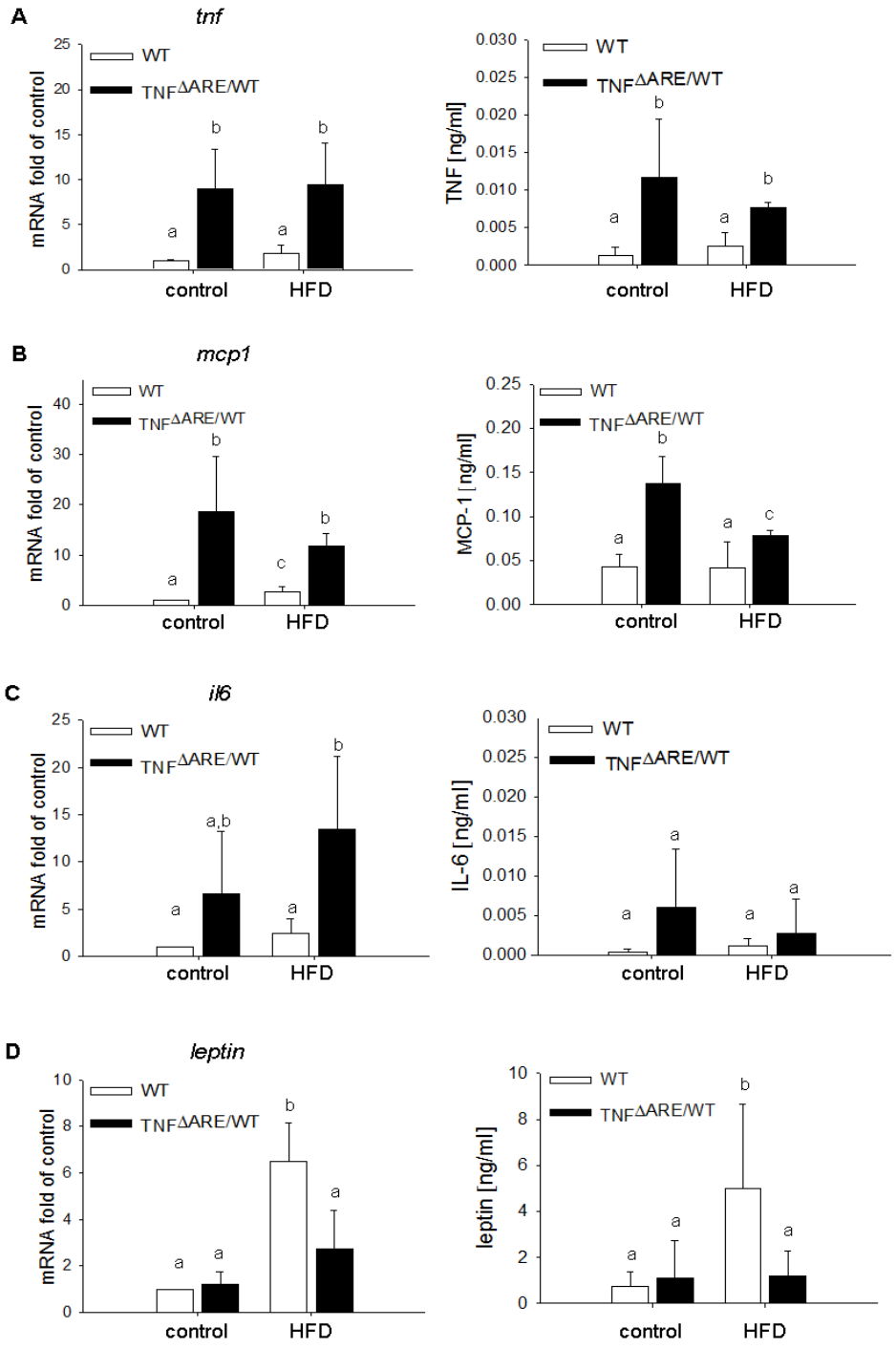

Figure 11: HFD does not induce mesenteric adipose tissue gene expression and plasma levels of selected cytokines and adipokines in TNF$^{\Delta ARE/WT}$ mice.

TNF mRNA expression in mesenteric fat tissue and TNF plasma concentrations are increased in TNF$^{\Delta ARE/WT}$ mice, but not affected by diet (A). Expression and plasma levels of MCP1 (B) and IL6 (C) are increased in TNF$^{\Delta ARE/WT}$ mice, but not significantly elevated by diet. Leptin gene expression and plasma concentrations are significantly upregulated by HFD in WT but not TNF$^{\Delta ARE/WT}$ mice (D). n=6 per group. a,b,c: data sets with different superscript letters differ significantly from each other according to two-way ANOVA

As type 2 diabetes is one of the most prominent co-morbidities of obesity, the mice were tested for a diabetic phenotype after the different feeding periods. Fasting blood glucose concentrations were significantly elevated in WT mice under HFD. Glucose tolerance was impaired in WT mice under HFD according to a standard oral glucose tolerance test, in contrast to apparently unaffected glucose homeostasis in TNF$^{\Delta ARE/WT}$ mice (Figure 12).

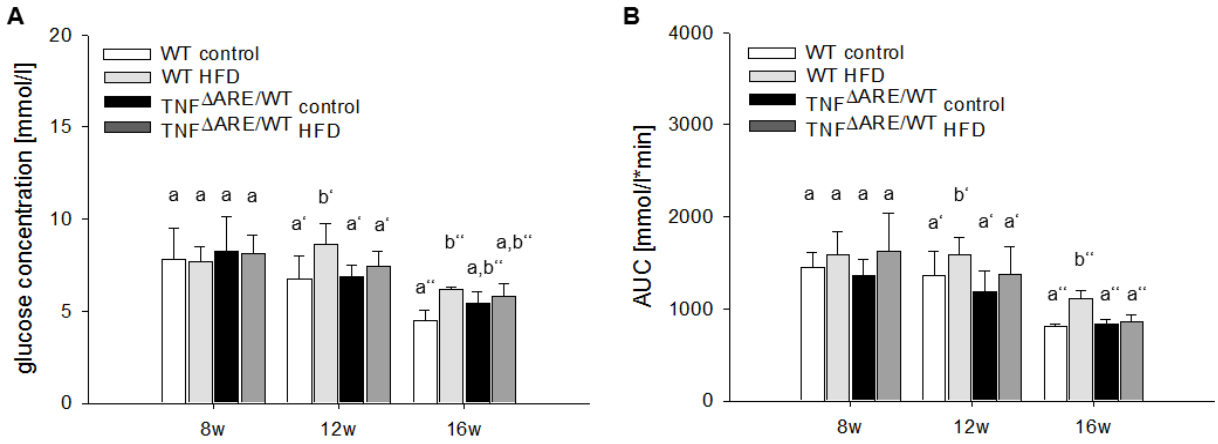

Figure 12: HFD does not increase fasting blood glucose levels (A) in TNF$^{\Delta ARE/WT}$ mice, and did not affect glucose tolerance (B).

At 12 and 16 weeks of age, WT mice exhibit elevated fasting blood glucose levels (A) as well as impaired glucose tolerance in an oral glucose tolerance test using 2 g glucose/kg body weight as 20 % glucose solution in 0.9 % NaCl (B). In contrast, TNF$^{\Delta ARE/WT}$ mice do not show such metabolic changes. n=6 per group for 8 and 12 weeks of age, n=10 per group for 16 weeks of age. a,b: data sets with different superscript letters differ significantly from each other according to two-way ANOVA

Energy intake of the mice was assessed by repeatedly measuring the amount of feed consumed over a period of 24 h at 12 weeks of age, then taking into account the different energy contents of the diets stated by the manufacturer as given in Table 2. Remarkably, TNF$^{\Delta ARE/WT}$ mice on HFD exhibited rates of daily energy intake similar to WT mice on HFD, and significantly higher energy intake than either genotype on control diet despite their reduced body weight (Figure 13).

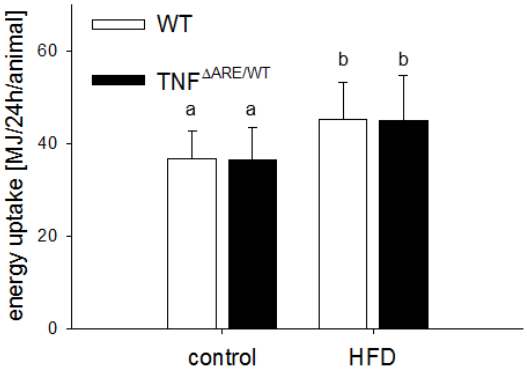

Figure 13: Energy uptake of both WT and TNF$^{\Delta ARE/WT}$ mice is elevated by HFD feeding.

Daily energy uptake per animal was calculated from daily feed consumption during one week at 12 weeks of age, and the energy contents of the diets as stated by the manufacturer. n=6 per group. a,b: data sets with different superscript letters differ significantly from each other according to two-way ANOVA

4.1.4. HFD altered metabolomic composition in plasma but not ileal tissue

To assess the impact of the diet on systemic and tissue metabolism, plasma and ileal tissue were subjected to targeted metabolomics analysis using the Biocrates Life Sciences AbsoluteIDQ kit. The ileal tissue was not affected regarding metabolite composition neither by diet nor genotype (Figure 14). This held true for both time points investigated (8 and 12 weeks of age), indicating no significant compositional restructuring of the intestinal tissue.

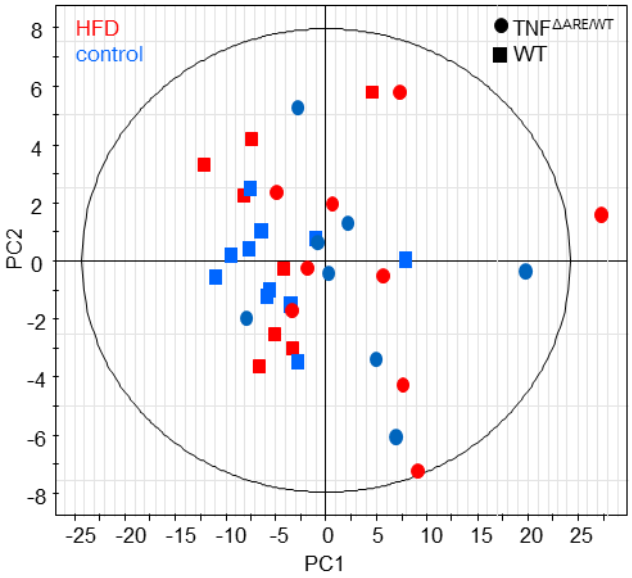

Figure 14: Metabolite composition of the distal ileal tissue is not significantly changed at 12 weeks of age, visualized as PCA plot.

Targeted metabolomics by LC-MS/MS reveals a stable lipophilic metabolite composition of the distal ileum, irrespective of genotype or diet. n=6 -8 per group.

While there were no significant differences between the groups at the age of 8 weeks (data not shown), the lipophilic plasma metabolome was significantly changed by the diet at 12 weeks of age, with Q^2=79% according to O-PLS-DA analysis (Figure 15). In total 46 metabolites were significantly regulated between the groups. Genotype did not have a major impact on the plasma metabolome (Q^2=41%).

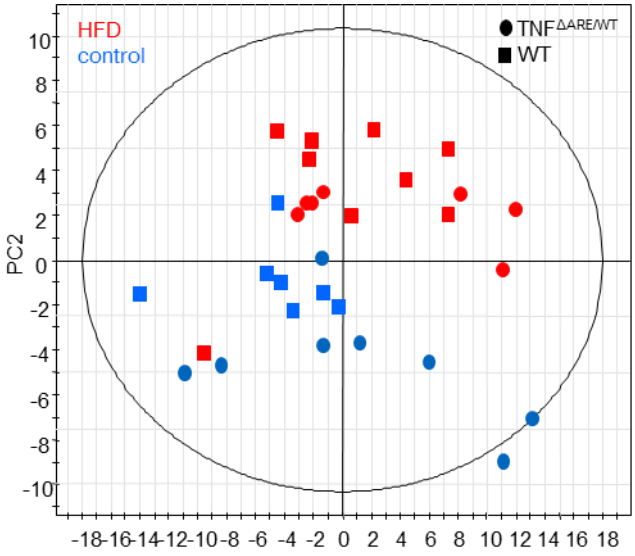

Figure 15: Metabolite composition of the plasma is significantly altered by HFD at 12 weeks of age, visualized as PCA plot. Targeted metabolomics by LC-MS/MS reveals a diet-dependent change of lipophilic metabolites, whereas genotype does not significantly influence the metabolite composition. n= 6-8 per group

The most regulated metabolites in the plasma are listed in Table 3. Mainly C16, C18 and C18:1 FA and their respective choline glycerophospholipids and lysophosphatidylcholine derivates were affected by the diet, with regulation factors ranging from 1.4 to 2.7. These changes in plasma metabolome reflected the FA profile of the palm-oil based HFD, largely dominated by the acylcarnitins C16 and C18:1, as depicted in Figure 5.

Table 3: HFD-regulated metabolites in plasma at 12 weeks of age.

metabolite	regulation by HFD
C16	2.7±0.8 ***
C18	2.1±0.7 ***
C18:1	2.6±1.0 ***
PC aa C36:1	2.0±0.6 ***
lysoPC a C20:3	1.4±0.3 ***
lysoPC a C16:0	1.6±0.3 ***
PC aa C34:1	1.9±0.7 ***
lysoPC a C18:1	1.7±0.4 ***

C: acylcarnitine, PCaa: choline glycerophospholipid, lysoPC: lysophosphatidylcholine. Only the eight strongest regulated metabolites are shown. *** p<0.001 between dietary groups according to two-way ANOVA

4.1.5. HFD modified ileal tight junction protein expression and LPS translocation

To assess the impact of HFD on intestinal permeability, studies regarding expression of barrier proteins in the distal ileum, translocation rate of bacterial components and of permeability markers *in vivo* and *ex vivo* were performed.

Immunefluorescence staining using anti-E-Cadherin (red) and anti-Ocln (green) revealed a pronounced reduction of Ocln protein while intracellular E-Cadherin remained stably expressed in the ileal epithelium (Figure 16 A). As discussed, background fluorescence was not subtracted from the fluorescence intensity values. Western blotting technique was used to confirm these results with lysates of ileal IEC (Figure 16 B). Despite the reduced protein levels of Ocln, mRNA levels were affected for neither Ocln nor E-Cadherin (Figure 16 C/D).

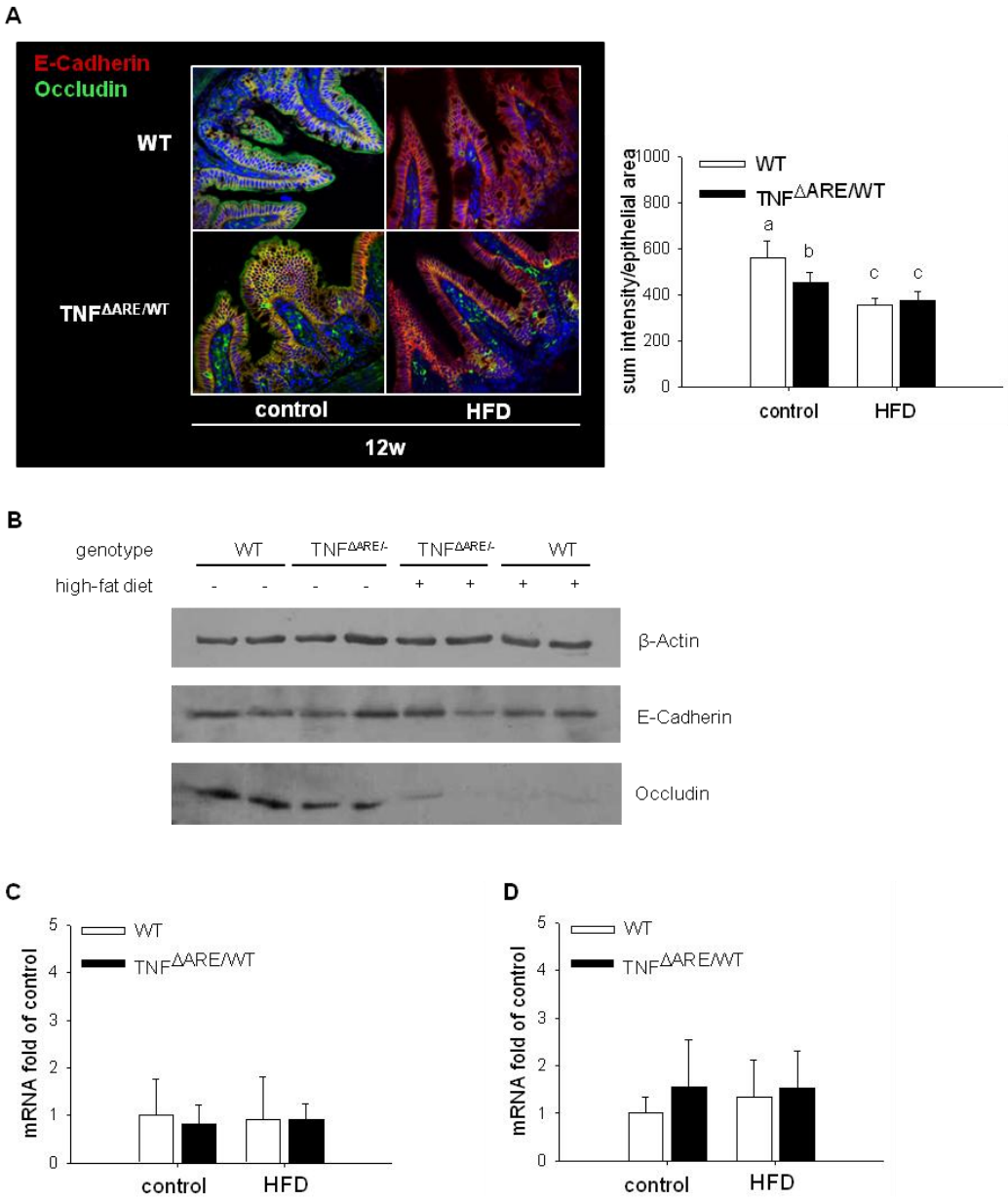

Figure 16: HFD reduces ileal Occludin expression on protein level, but does not affect E-Cadherin expression at 12 weeks of age.

Immunefluorescence staining (600fold magnification) and Western blot analysis confirm a reduced presence of the protein Ocln in HFD groups regardless of genotype (A/B), whereas E-Cadherin protein levels are not affected (A/B). Quantification of immunefluorescence staining for Ocln is based on the sum intensity normalized to epithelial area (n=4 per group). Accoring to qPCR mRNA expression of neither Ocln (C) nor E-Cadherin (D) is different between the groups (n=6 per group). a,b,c: data sets with different superscript letters differ significantly from each other according to two-way ANOVA

Of note, this reduction in Ocln levels proved to be established at the time point of 8 weeks already (Figure 17), at which histopathological inflammation of the distal ileum tissue was not yet pronounced.

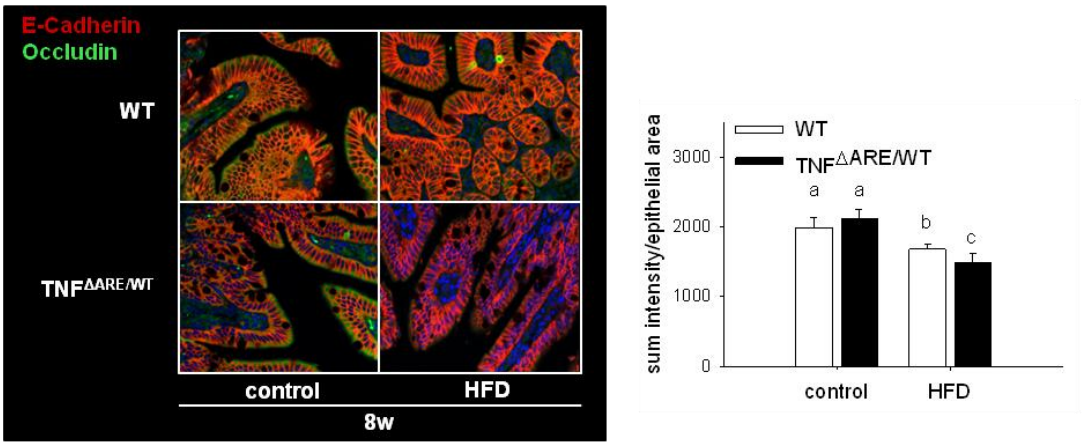

Figure 17: Reduction of Ocln protein occurs as an early event at 8 weeks of age, before the onset of inflammation.
Immunefluorescence staining reveals reduced presence of the protein Ocln in HFD groups, whereas E-Cadherin protein levels are not affected (600fold magnification). Quantification of staining for Ocln is based on the sum intensity normalized to epithelial area. n=4 per group. a,b,c: data sets with different superscript letters differ significantly from each other according to two-way ANOVA

Along with the HFD-promoted colonic inflammation, a reduction of Ocln expression (green) assessed via immunefluorescence staining was observed in the epithelium of the pCo at 16 weeks of age in both genotypes (Figure 18 B), but not at 12 weeks where there was no colonic inflammation present (Figure 18 A). E-Cadherin expression (red) in the pCo epithelium remained unaffected by diet throughout the feeding periods (Figure 18 A/B).

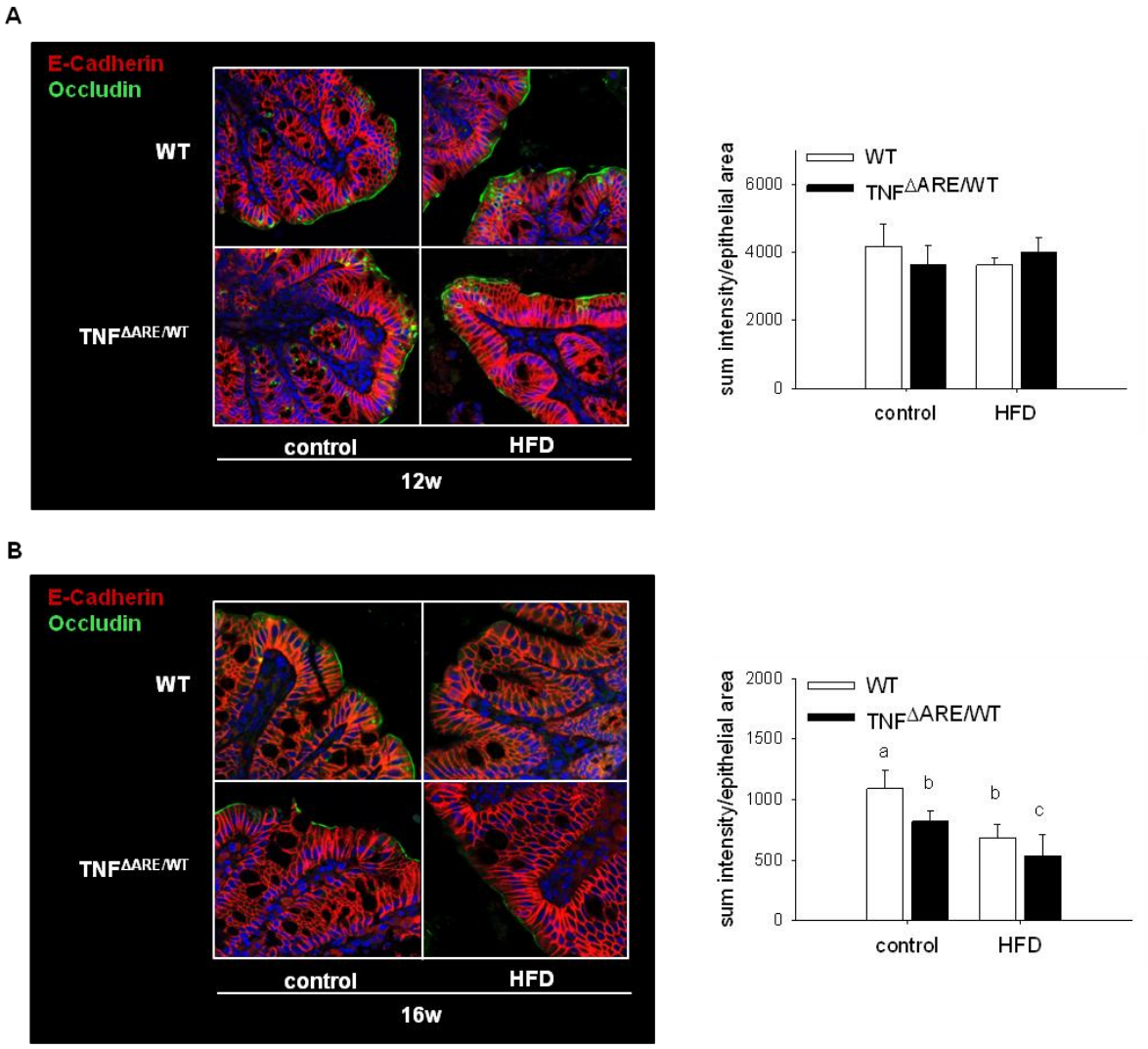

Figure 18: HFD reduces colonic Occludin expression on protein level at 16 weeks of age (B) but not 12 weeks of age (A). Immunefluorescence staining (600fold magnification) revealed unaffected Ocln levels at 12 weeks of age (A) and reduced presence of Ocln in HFD groups regardless of genotype at 16 weeks of age (B), while E-Cadherin protein levels are not affected (A/B). Quantification of immunefluorescence staining for Ocln is based on the sum intensity normalized to epithelial area. n=4 per group. a,b,c: data sets with different superscript letters differ significantly from each other according to two-way ANOVA.

Further proteins associated to intestinal permeability were not found to be significantly affected in the ileum. Protein expression of ZO-1 and β-Catenin remained stable throughout the dietary and genotype groups (data not shown). In addition, mRNA levels of *ZO-1*, *claudin 2* and *claudin 4* were unaffected by diet or genotype (data not shown).

Portal plasma endotoxin levels were increased in HFD animals simultaneously to the reduced presence of Ocln (Figure 19 A), indicating a facilitated translocation of these molecules. This selective loss of barrier was not reflected in the recovery of orally applied PEG of different sizes which translocated at extends dependent on genotype, not diet (Figure 19 B). In addition, Ussing-Chamber measurements revealed no significant differences in TER or translocation of NaF between the dietary groups (data not shown).

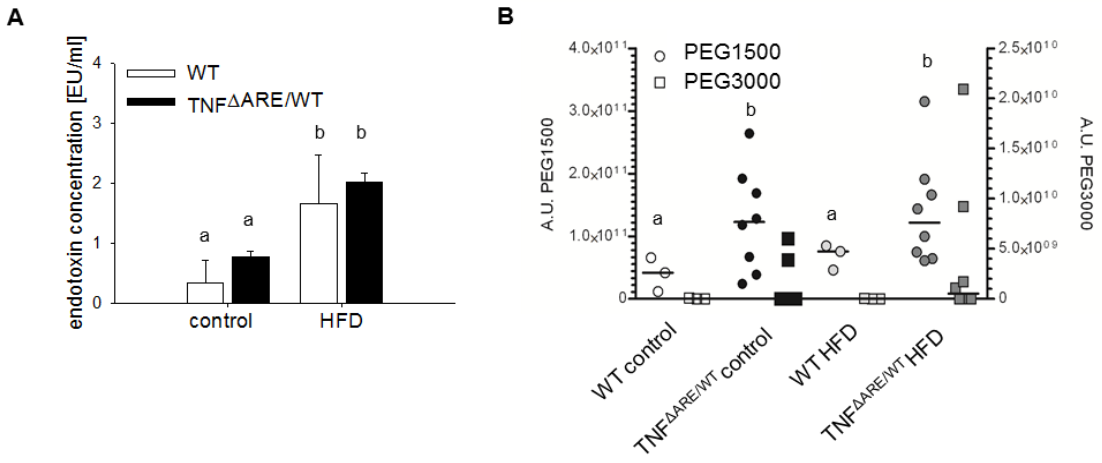

Figure 19: HFD increases concentration of LPS in the hepatic portal vein plasma (A), but does not affect urinary recovery of orally applied PEG species (B).

Plasma endotoxin concentrations in hepatic portal vein EDTA-plasma were assessed using an LAL Chromogenic Endpoint Assay (A) (n=5 per group). Translocation of orally applied PEG of the molecular sizes 1500 and 3000 Da were determined by assessing recovery in the urine via LC/MS (B) (n=3-8 per group). a,b: data sets with different superscript letters differ significantly from each other according to two-way ANOVA

Loss of the tight junction protein Ocln and increased portal vein LPS concentrations suggest an enhanced translocation of LPS from the lumen to the liver. Isolated liver tissue was therefore analyzed for the expression of LPS-stimulated pro-inflammatory mechanisms. Again, TNF expression was only determined by the genotype, with app. 10-fold upregulation in $TNF^{\Delta ARE/WT}$ mice compared to WT mice regardless of genotype (A). IL6 and TLR4 expression were upregulated in $TNF^{\Delta ARE/WT}$ mice on HFD, whereas expression of these genes was largely unaffected by HFD in livers of WT mice (B/C).

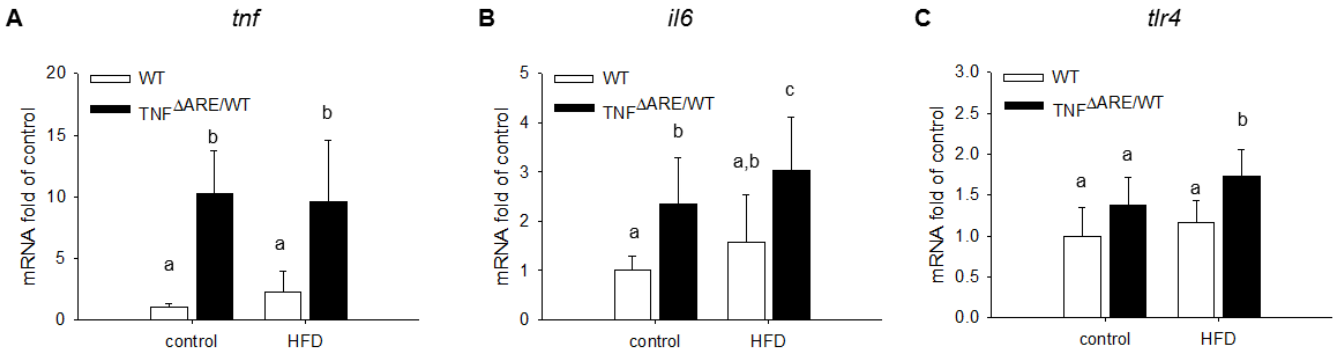

Figure 20: Cytokine expression in the liver is affected by genotype and diet.

mRNA expression of tnf (A), il6 (B) and tlr4 (C) in liver tissue was assessed by qPCR. n=6 per group. a,b,c: data sets with different superscript letters differ significantly from each other according to two-way ANOVA

To elucidate possible mechanisms underlying the HFD-associated modulation of intestinal barrier in a lean mouse, the putative influence of luminal factors on intestinal permeability was assessed. Cecal water of wildtype mice fed the different diets was applied to PTK6 cells, whereupon HFD-derived cecal water was more potent in decreasing TER compared to control diet (Figure 21).

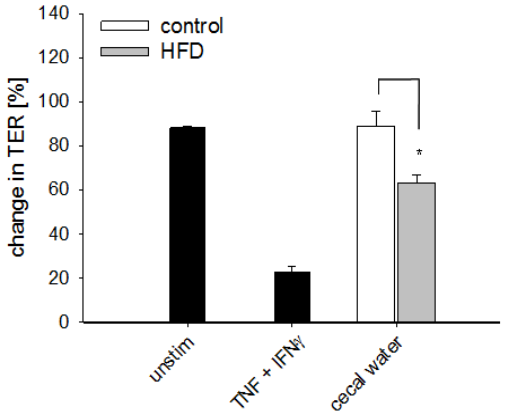

Figure 21: PTK6 epithelial cells display reduced transepithelial resistance after stimulation with HFD-derived compared to control-derived cecal water.

PTK6 cells were stimulated with cecal water (50 µg protein/ml) derived from HFD-fed mice compared to control mice for 48 h at 37 °C. TNF Stimulations were performed in triplicates. Data are representative for two independent experiments. * $p<0.05$ according to Student's t-test

To investigate potential factors among the cecal luminal metabolites that might contribute to the effect, the cecal content was analyzed for the concentration of different bile acid (BA) species. While the overall BA concentration was not significantly different throughout the experimental groups (Figure 22 A), tauro-conjugated BA species were increased in the HFD groups, and especially the TNF$^{\Delta ARE/WT}$ mice on HFD (Figure 22 B). Among the regulated BA species were tauromurocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid, taurocholic acid and tauroursodeoxycholic acid (Figure 22 D). The only non-taurogonjugated BA species found to be affected by HFD feeding was ursodeoxycholic acid, a tertiary BA (Figure 22 C).

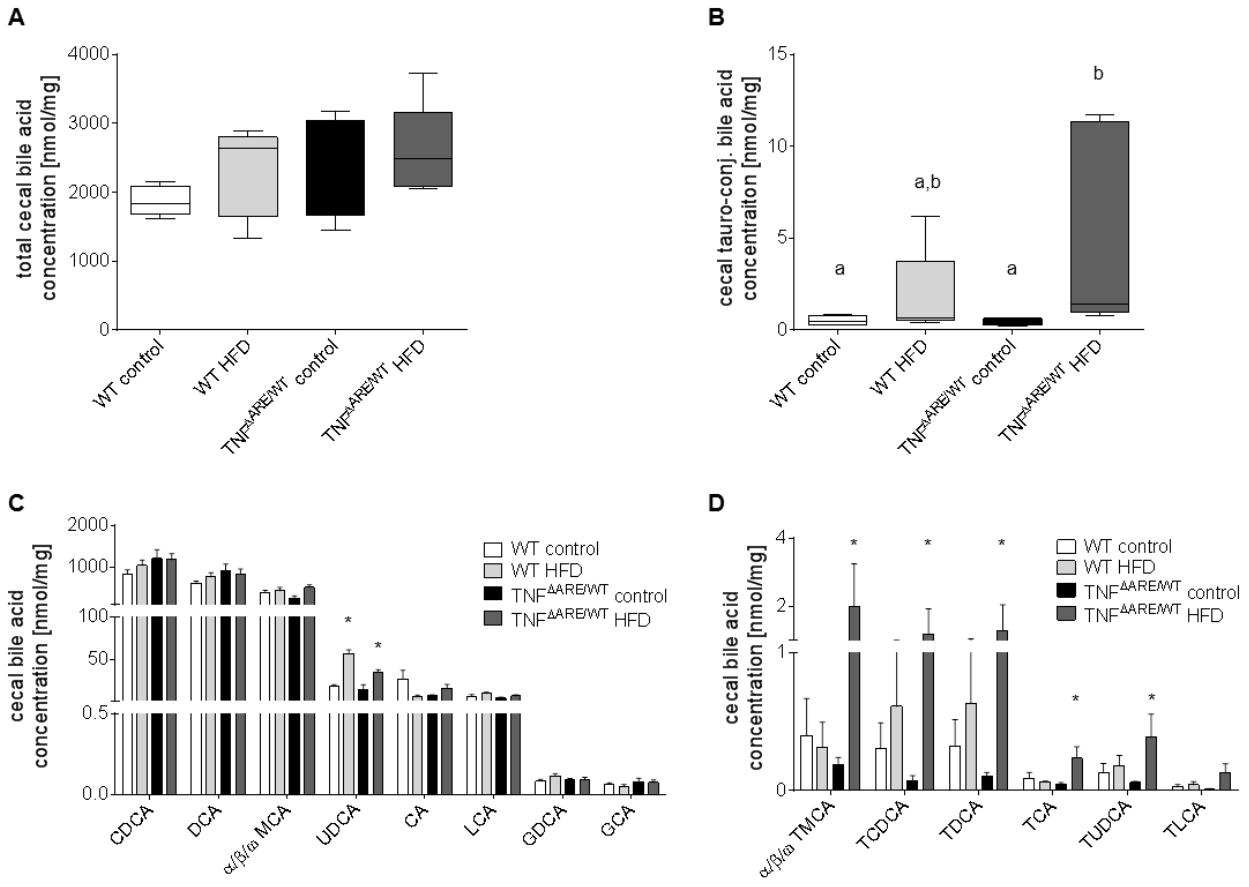

Figure 22: HFD modifies cecal luminal concentrations of BA species.

While total bile acid concentration remains unaffected throughout the experimental groups (A), the concentration of tauro-conjugated bile acids is increased significantly by HFD (B). HFD increases the concentration of ursodeoxycholic acid (UDCA) among the non-tauro-conjugated bile acid species (D), and tauromurocholic acid (TMCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), taurocholic acid (TCA) and tauroursodeoxycholic acid (TUDCA) among the tauro-conjugated bile acid species (D). Cecal content was freeze dried and bile acid concentrations were analyzed using LC-MS. a,b: data sets with different superscript letters differ significantly from each other according to two-way ANOVA; n=5 per group. * $p<0.05$ within age group and genotype according to Student's t-test

4.1.6. HFD affected ileal epithelial cell activation status and induces recruitment of dendritic cells to the lamina propria

We were next aiming to elucidate the molecular mechanisms underlying the aggravated intestinal inflammation. Epithelial cell homeostasis is critical for the maintenance of a healthy gut. We therefore investigated the effect of HFD on the expression of stress markers and chemokines in ileal IEC. With an intrinsic overexpression in the TNF$^{\Delta ARE/WT}$ model (4-fold increase compared to WT), TNF expression was not further influenced by HFD in neither WT nor TNF$^{\Delta ARE/WT}$ mice (Figure 23 A). The expression of intercellular cell adhesion molecule 1 (*icam1*), functioning as a facilitator for lymphocyte transmigration, was upregulated under HFD (Figure 23 B). The expression of mucin 2 was increased by HFD on mRNA level (Figure 23 C).

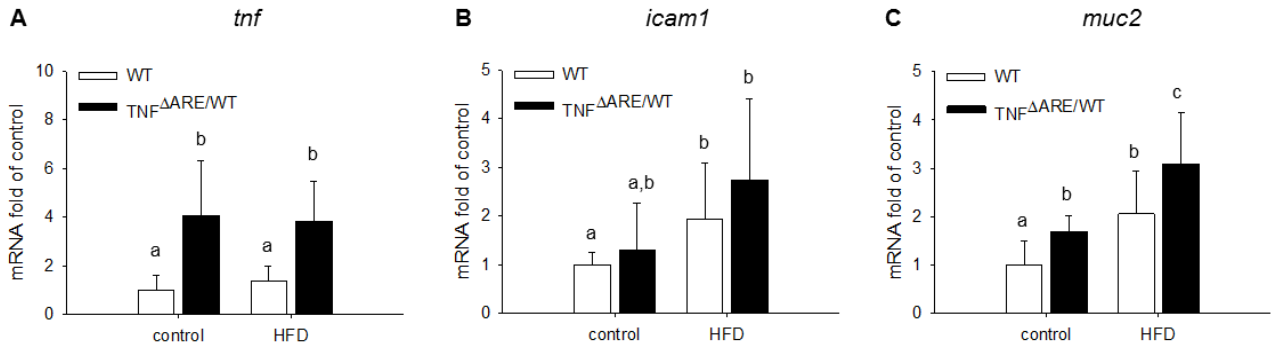

Figure 23: Gene expression in intestinal epithelial cells is affected by genotype and diet.
mRNA expression of *tnf* (A), *icam-1* (B) and *muc2* (C) was assessed by qPCR. n=6 per group. a,b,c: data sets with different superscript letter differ significantly from each other according to two-way ANOVA

In addition, expression of the chemokine (C-C motif) ligand (*ccl*) *20*, targeting DCs, was induced by HFD in IEC of both genotypes on mRNA (A) as well as protein level (B). Notwithstanding the regulation of *ccl20*, the expression levels of other chemokines such as *cxcl 2* (C) or *cxcl10* (D), primarily recruiting neutrophils and T cells respectively, were not affected.

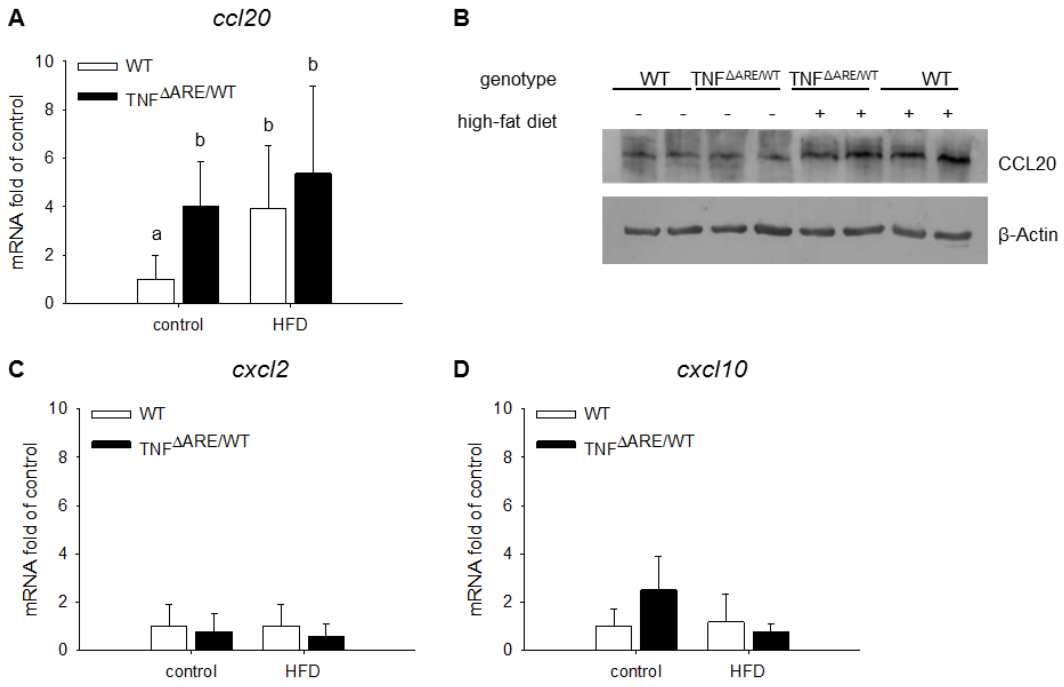

Figure 24: HFD increases *ccl20* expression in intestinal epithelial cells on both mRNA (A) and protein (B) level, but does not affect *cxcl2* (C) or *cxcl10* (D) expression.

At 12 weeks of age mRNA expression of *ccl20* was increased by HFD feeding in wildtype mice, and non-significantly in TNF$^{\Delta ARE/WT}$ mice (A). Western blot technique confirmed upregulation of CCL20 expression under HFD (B). Expression of c*xcl2* and *cxcl10* is unaffected (C/D) by diet and genotype. n=6 per group. a,b: data sets with different superscript letter differ significantly from each other according to two-way ANOVA

In addition to the upregulated expression of CCL20 in IEC, CCL20 plasma levels were significantly increased by HFD feeding at 12 weeks of age (Figure 25 A), but not at 8 weeks of age (data not shown). We tested whether enhanced chemokine expression at 12 weeks of age would result in increased recruitment of DCs to the ileal lamina propria. Quantification of CD11c^{+} cells by cell cytometry and immunefluorescence staining in tissue revealed that the number of CD11c^{+} cells in the ileal LP was significantly increased due to HFD in both genotypes, with a stronger presence in TNF$^{\Delta ARE/WT}$ mice (Figure 25 B). In addition, CD11c^{+} cells were visualized in ileal tissue using immunefluorescence staining, and were identified as dendritic cells according to their morphology (Figure 25 C).

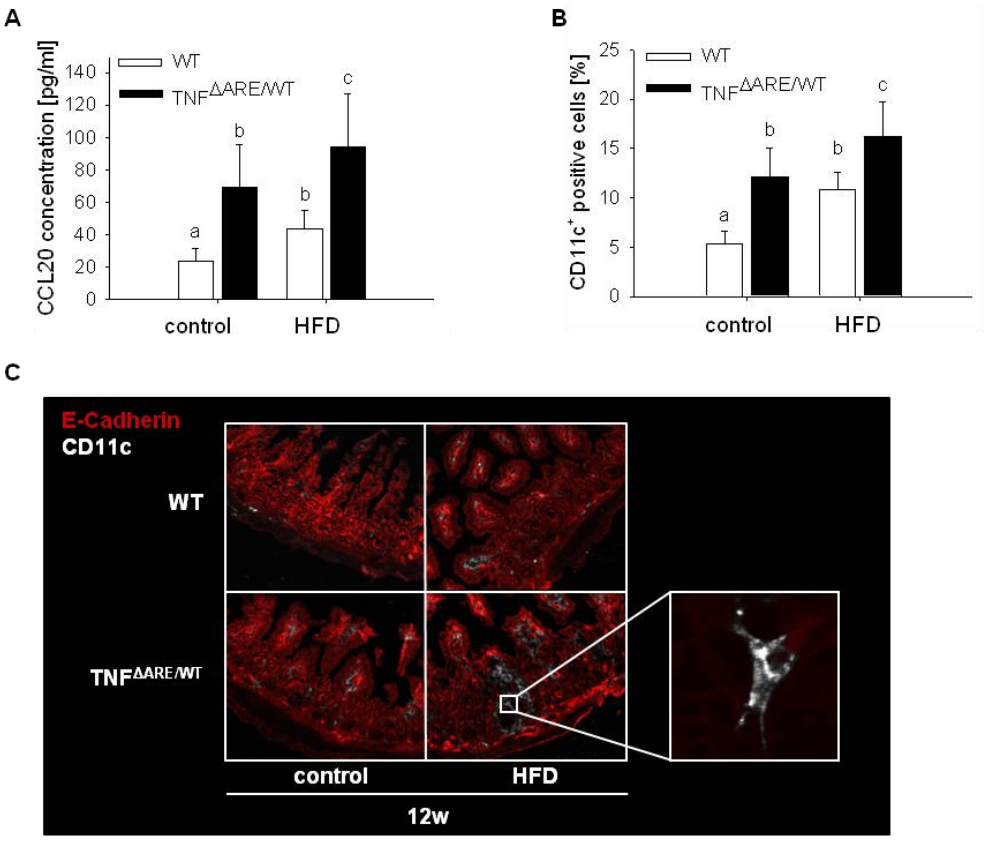

Figure 25: HFD increases CCL20 plasma concentration (A) and enhances presence of CD11c+ cells in the ileum, quantified by cell cytometry (B) immunefluorescence staining (C).

At 12 weeks of age CCL20 plasma concentration, quantified by ELISA, was increased by HFD feeding in both genotypes (A) (n=5 per group). At the same time, quantification of $CD11c^+$ cells among isolated lamina propria lymphocytes showed increased numbers due to HFD feeding (B) (n=5 per group), confirmed by immunefluorescence staining of $CD11c^+$ cells (200fold magnification and ~1200fold magnification for close-up view) (C) (n=4 per group). a,b,c: data sets with different superscript letter differ significantly from each other according to two-way ANOVA

To investigate potential underlying effects, the small intestinal cell line Mode K was stimulated with dietary suspensions and cecal contents of wildtype mice fed the different diets. CCL20 production upon treatment with suspensions of the pure diets was not increased under HFD conditions (Figure 26 A). The Mode K cells secreted significantly more CCL20 when treated with HFD-derived whole cecal lysate compared to control diet-derived whole cecal lysate (B). Similar effects were observed when stimulating Mode K cells with bacterial lysates derived from the cecal contents, whereas cecal water, e.g. metabolites present in the cecum lumen, did not affect CCL20 production (Figure 26 A). Reflecting the regulation of the CCL20 production, the CM of Mode K cells stimulated with HFD derived whole cecal lysate was much more potent to recruit bone marrow-derived DCs compared to control diet-derived lysate (Figure 26 B).

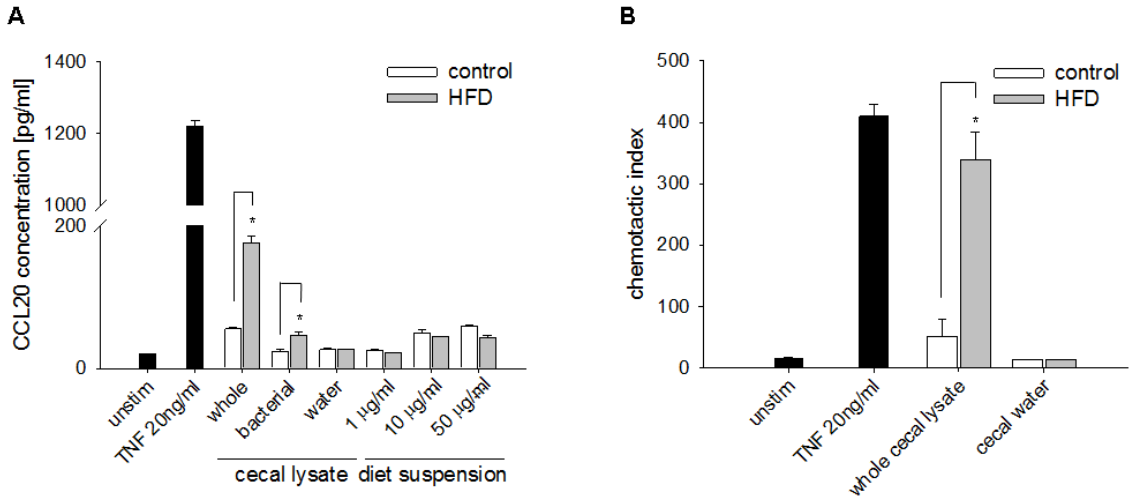

Figure 26: Mode K ileal epithelial cells express more CCL20 upon stimulation with HFD-derived cecal lysates (A), together with enhanced ability to recruit BM-DCs (B).

Mode K cells express more CCL20 upon stimulation with whole or bacterial cecal lysate (50 µg protein/ml) derived from HFD-fed mice compared to control mice, while the cecal waters or diet suspensions do not differentially induce CCL20 expression (A). Media from unstimulated Mode K cells, or Mode K cells stimulated with TNF (20 ng/ml) were used as controls. CCL20 was measured via ELISA after 24 h of incubation at 37 °C with the respective solutions. Stimulations were performed in triplicates. Data are representative for three independent experiments except for stimulation with diet suspensions.

The conditioned media generated by Mode K cells under the influence of HFD-derived cecal lysate was more potent to recruit BM-DCs compared to control diet-derived cecal lysate (B). Pure Mode K cell conditioned media generated as in (A) were used to stimulate migration of BM-DCs in a transwell system. The proportion of transmigrated cells was assessed by counting after 1 h of incubation at 37 °C. Stimulation was performed in triplicates. Data are representative for two independent experiments. * $p<0.05$ according to Student's t-test

4.1.7. HFD-derived cecal lysate stimulated IEC to promote Th17-committed immune response

To further elucidate the effects of luminal factors on immune activation, we analyzed the effect of cecal lysates on epithelial cell-mediated activation of DCs. The activation status of BM-DCs, assessed as expression of co-stimulatory factor CD80 and antigen-presenting major histocompatibility complex MHCII was induced by CM of stimulated Mode K cells, but was not different between treatments with CM derived from the stimulation with cecal lysates originating from the different diets (Figure 27).

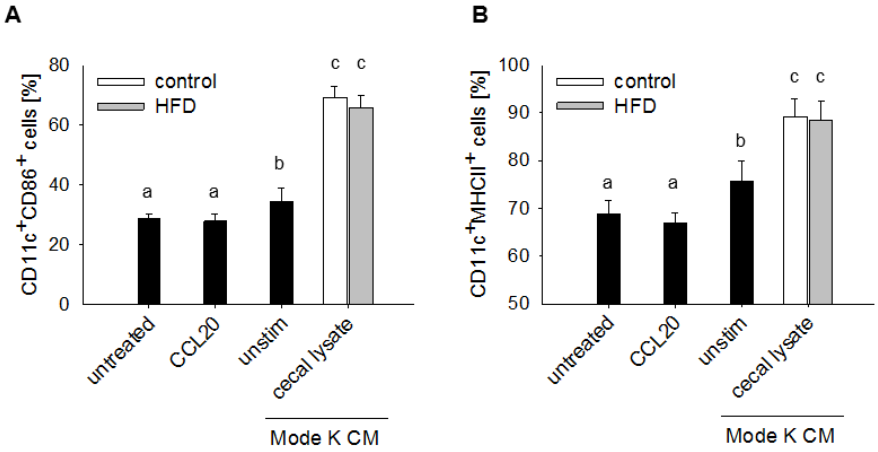

Figure 27: Epithelial cell conditioned media do not differentially stimulate CD86 (A) or MHCII (B) expression in BM-DCs. Epithelial cell conditioned Mode K cells were stimulated with cecal lysates (50 µg protein/ml) of mice fed the different diets for 24 h. Media from unstimulated Mode K cells, or Mode K cells stimulated with CCL20 (200 pg/ml) were used as controls. The proportion of CD11c+ cells coexpressing CD86 (A) or MHCII (B) was assessed by cell cytometry after 24 h of stimulation with the respective controls or Mode K cell conditioned media. Stimulation was performed in triplicates. Data are representative for three independent experiments. a,b,c: data sets with different superscript letters differ significantly from each other.

However, CM from Mode K cells stimulated with HFD–derived cecal lysate primed BM-DCs towards increased mRNA expression of Th17-conditioning *il23p19* (Figure 28 A), but not toward Th1 conditioning *il12p35* (Figure 28 B).

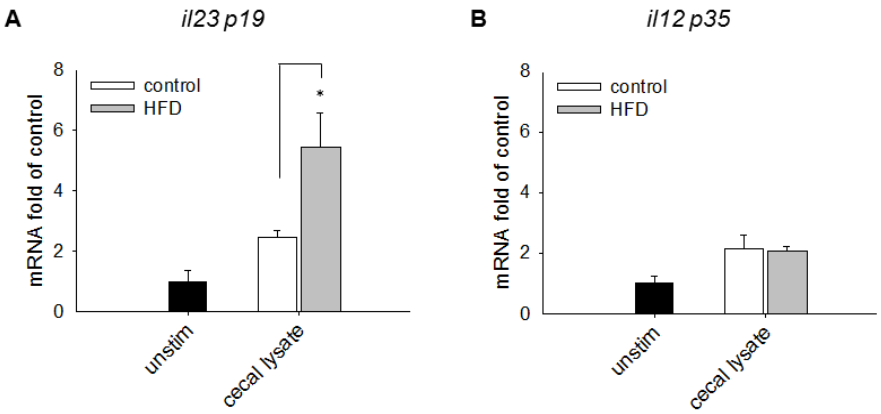

Figure 28: Cecal lysates from HFD fed mice stimulates *il23p19* (A) but not *il12p35* (B) mRNA expression of BM-DCs.
Dendritic cell mRNA expression was quantified by qPCR after 24h stimulation with Mode K cell conditioned medium. Mode K cells were stimulated with cecal lysates (50µg protein/ml) of mice fed the different diets for 24h. Results are representative for two independent experiments. Stimulation was performed in triplicates. Data are representative for three independent experiments. * $p<0.05$ according to Student's t-test

Reflecting the *in vitro* results, mRNA expression analysis of isolated ileal mouse LPLs revealed an induction of *il23p19* expression (Figure 29 A) due to HFD, without significant alterations in *il12p35* expression (Figure 29 B) in HFD-fed WT or $TNF^{\Delta ARE/WT}$ mice.

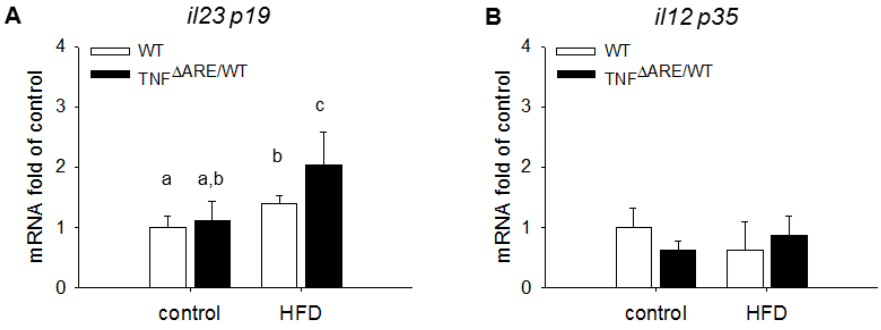

Figure 29: HFD upregulates *il23p19* (A) but not *il12p35* (B) mRNA expression in the ileal lamina propria.
Expression of the transcription factors *rorgt* (A), *tbet* (B) and *foxp3* (C), and the effector cytokines *il17a* (D), *ifng* (E) and *il10* (F) were quantified in isolated lamina propria lymphocytes using pPCR. n=5 per group. a,b,c: data sets with different superscript letters differ significantly from each other according to two-way ANOVA

Along this line, significantly up-regulated expression of the Th17-associated transcription factor-encoding *rorgt* (Figure 30 A), but not Th1-associated *tbet* (Figure 30 B) or Treg-associated *foxp3* (Figure 30 C) was observed in the ileal LP compartment. Expression analysis of the respective effector cytokines revealed an increase in *il17a* expression (Figure 30 D) under HFD, whereas *ifng* (Figure 30 E) as well as *il10* (Figure 30 F) expression remained unaltered in the ileal LP compartment. In addition, expression of Th2-associated transcription factor *gata3* as well as of the signature cytokine *il4* was not affected by diet or genotype (data not shown).

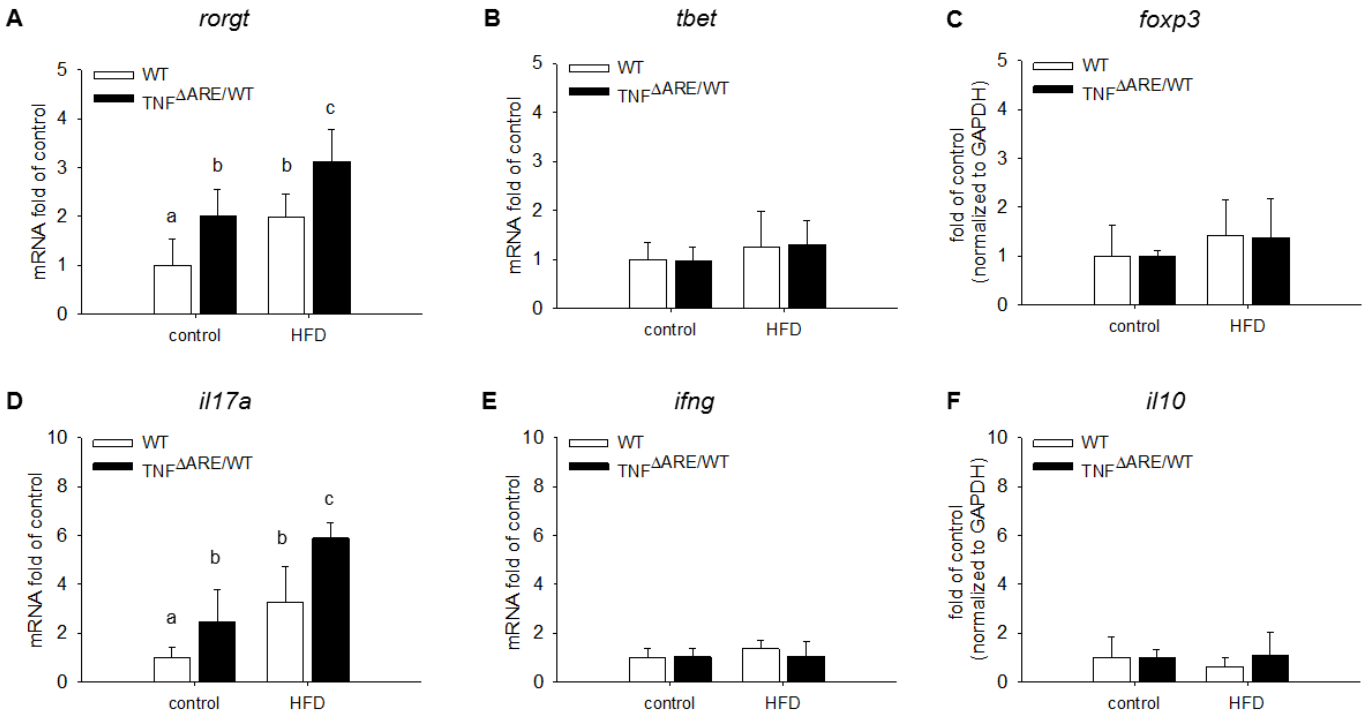

Figure 30: HFD is associated with Th17 biased immune response in the lamina propria.

Expression of the transcription factors *rorgt* (A), *tbet* (B) and *foxp3* (C), and the effector cytokines *il17a* (D), *ifng* (E) and *il10* (F) were quantified in isolated lamina propria lymphocytes using pPCR. n=5 per group. a,b,c: data sets with different superscript letters differ significantly from each other according to two-way ANOVA

4.2. HFD aggravated colonic inflammation in IL10$^{-/-}$ mice in a gender-specific manner dissociated from obesity

To further investigate the effects of HFD on colonic inflammation, male and female IL10$^{-/-}$ mice, a mouse model of colitis, were fed the palm-oil based diet for 4 weeks until the age of 16 weeks. WT 129svev males and females from an independent breeding scenario were used as non-susceptible control.

Until the age of 16 weeks, most animals did not develop histopathological inflammation in the cecum or colon, but sporadic inflammation was observed in IL10$^{-/-}$ females under the influence of HFD only (Figure 31 A-C). When defining the presence of inflammation as histological score above or equal 1, this concerned three out of 13 mice in the HFD group (23%). None of the mice displayed ileal involvement (Figure 31 A/B). 129svev WT mice did not exhibit histopathological signs of inflammation in any of the investigated intestinal segments.

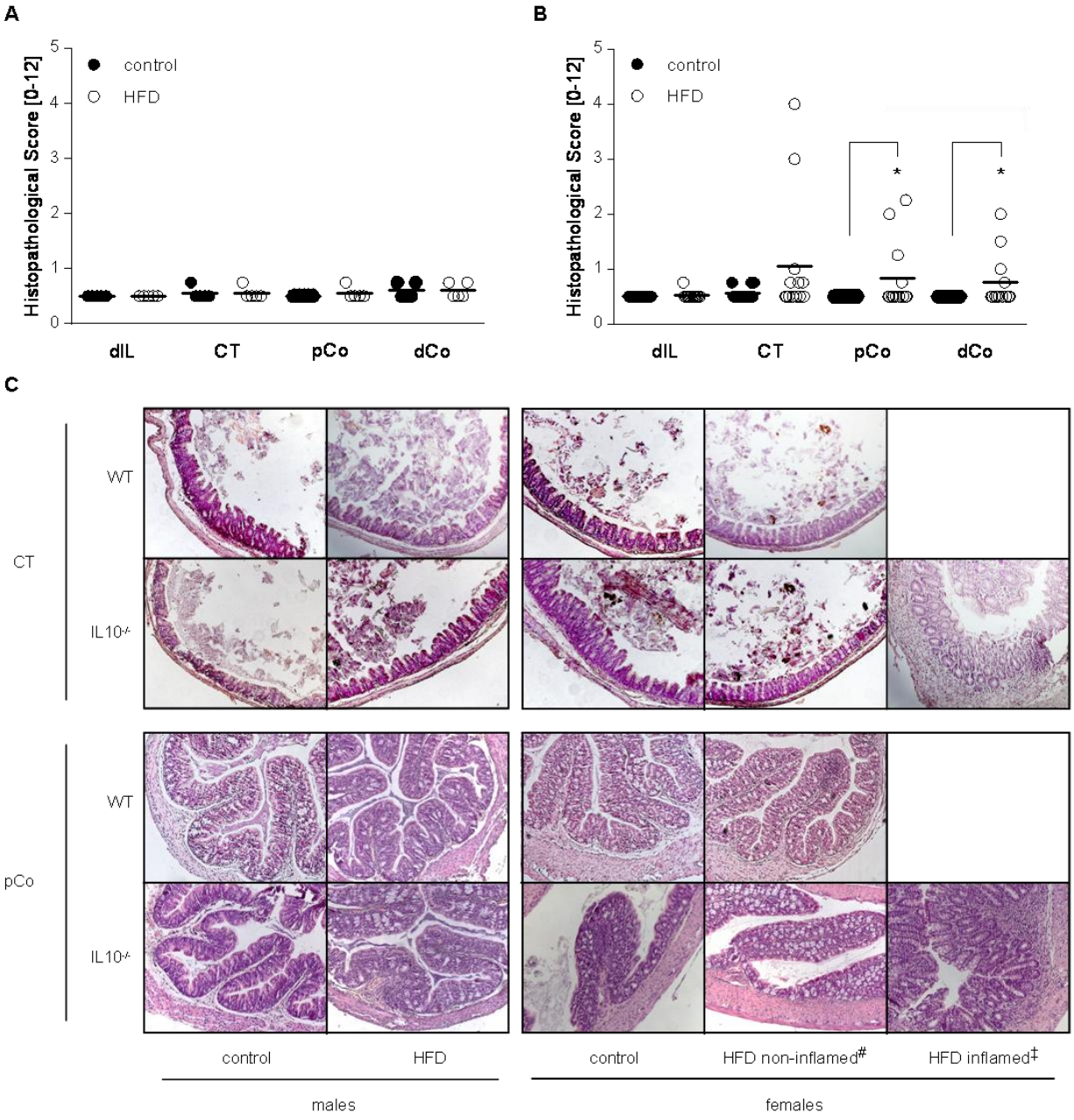

Figure 31: HFD induces sporadic inflammation in the large intestine of female IL10$^{-/-}$ mice at 16 weeks of age under specific pathogen free housing.

Male mice did not exhibit histopathological inflammation in any part of the intestine investigated (A). Female mice displayed sporadic inflammation in cecum tip, proximal colon as well as distal colon under influence of HFD only (B). H&E stained sections were used to assess the grades of inflammation and reveal infiltration of immune cells as well as architectural disruption of the tissue with progressing inflammation in IL10$^{-/-}$ mice in the cecum tip and proximal colon (100fold magnification) (C). dIL: distal ileum, CT: cecum tip, pCo: proximal colon, dCo: distal colon. A,B: inflammation was defined as histopathological score above or equal 1, therefore # comprises 10 CT and pCo tissues, and ‡ comprises 3 CT and pCo tissues. n=5-per group for males, n=5-13 per group for females. * $p<0.05$ according to Mann-Whitney-U-test

To further validate this finding of enhanced inflammatory status, the size of secondary lymphoid organs was assessed as a surrogate marker. Mesenteric lymphe nodes (Figure 32 A/B) were significantly increased in weight under HFD feeding in IL10$^{-/-}$ females only. Spleen weight was increased in females of both genotypes upon HFD feeding (Figure 32 C/D).

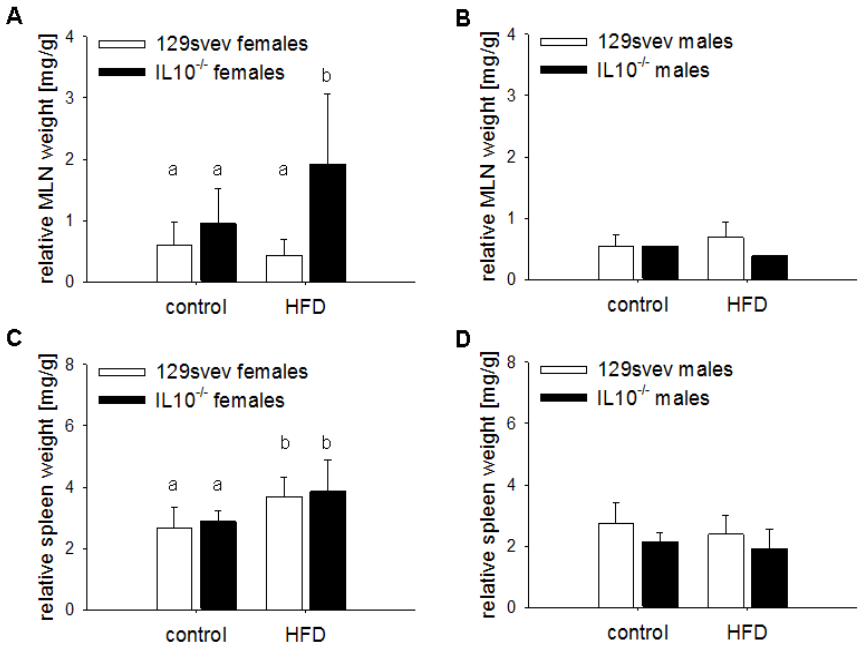

Figure 32: HFD induces enlargement of secondary lymphoid organs in female, but not male, IL10$^{-/-}$ mice.
Female, but not male, IL10$^{-/-}$ mice display increased relative MLN weight upon HFD (A/B). Female, but not male, mice of both genotypes display increased spleen weight upon HFD (C/D). n=5-per group for males, n=5-13 per group for females. a,b: data sets with different superscript letters differ significantly from each other according to two-way ANOVA.

In contrast, male but not female IL10$^{-/-}$ mice gained weight upon HFD feeding (Figure 33 A/B), and exhibited significant increase in the weight of adipose tissue depots of both MAT (Figure 33 C/D) and EAT (Figure 33 E/F). This finding was supported by the adiposity index calculated from NMR-based body composition measurements in IL10$^{-/-}$ mice during the experiment (Figure 33 G).

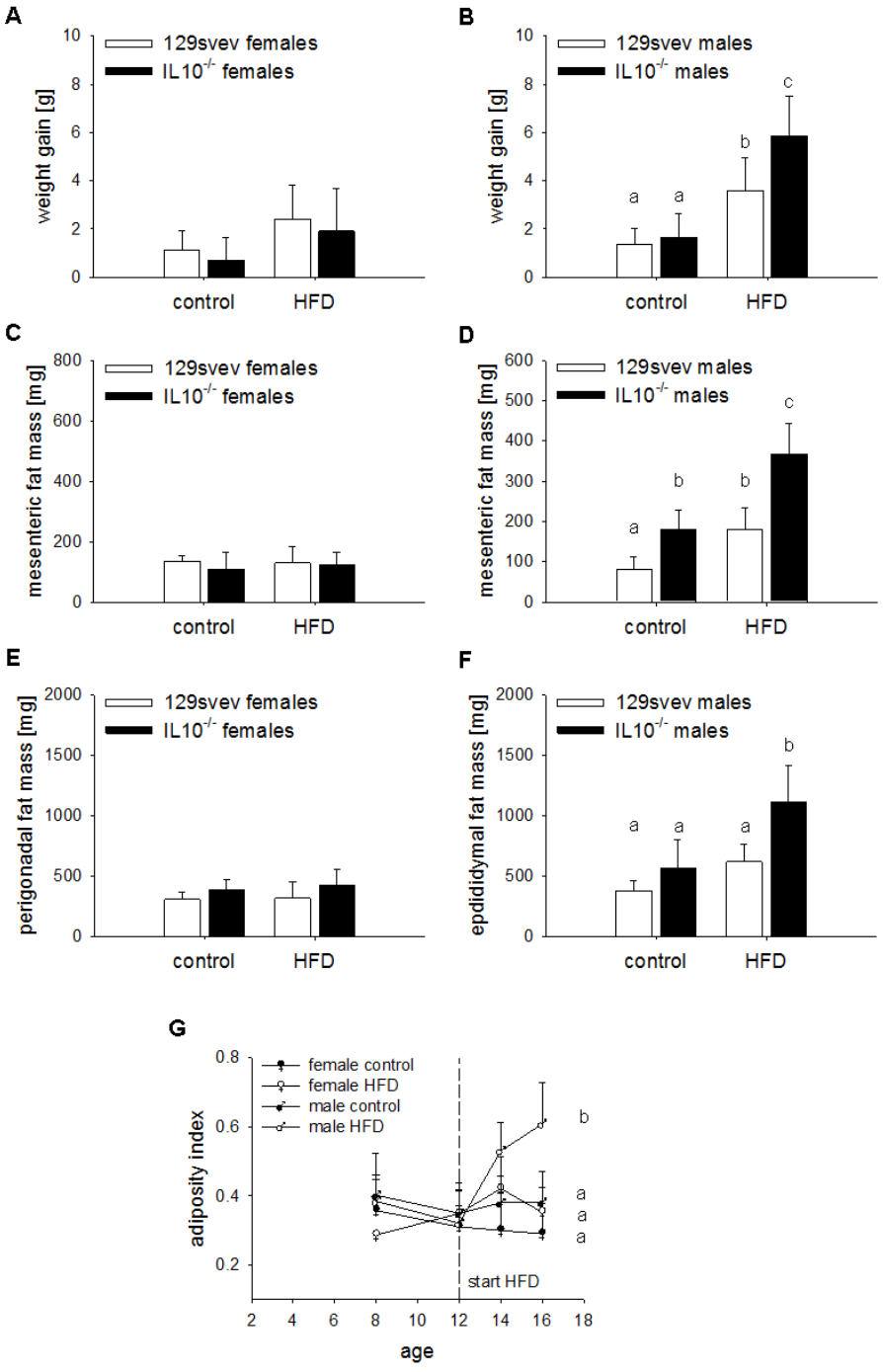

Figure 33: HFD induces overweight in male, but not female, IL10-/- mice.

Male but not female HFD fed IL10$^{-/-}$ mice display increased body weight (A/B), mesenteric (C/D) and perigonadal fat mass (E/F) as well as increased adiposity index (G). n=5-per group for males, n=5-13 per group for females. a,b,c,d: data sets with different superscript letters differ significantly from each other according to two-way ANOVA

HFD impaired glucose tolerance regardless of genotype and gender (Figure 34 A/B), and plasma SAA concentrations assessed by ELISA were increased significantly by HFD throughout all groups (Figure 34 C/D).

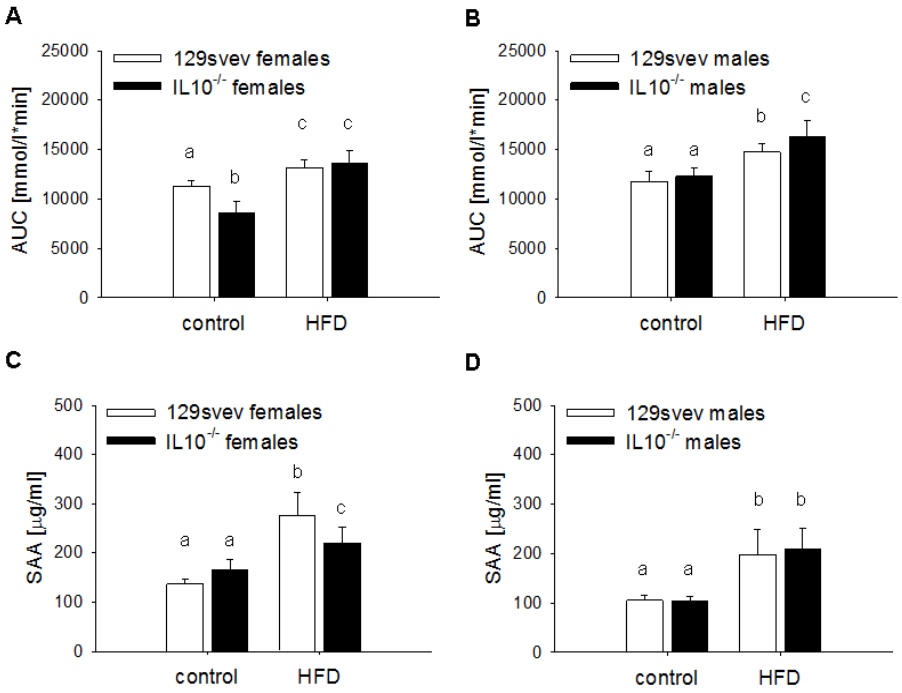

Figure 34: HFD induces impaired glucose tolerance (A/B) and elevated plasma SAA levels (C/D) independently of gender in IL10$^{-/-}$ and 129svev mice.

HFD fed female (A) and male (B) mice display increased area under the curve after an oral glucose tolerance test (n=4-7 per group). HFD fed female (C) and male (D) mice display elevated plasma SAA concentrations, measured via ELISA (n=5-per group for males, n=5-13 per group for females). a,b,c,d: data sets with different superscript letters differ significantly from each other according to two-way ANOVA.

To identify a potential mechanism underlying the aggravation of the intestinal pathology and stimulation of immune activation, expression of the intestinal tight junction protein Ocln was investigated via immunefluorescence staining using anti-Ocln (green). While Ocln levels were markedly decreased in the ileal epithelium (Figure 35 A), Ocln levels were not affected by HFD in the pCo (Figure 35 B). Ussing chamber measurements revealed a trend (p=0.09) in distal ileum toward decreased TER in female IL10$^{-/-}$ mice on HFD compared to control diet (Figure 35 C). In contrast to the findings in the experiments with TNF$^{\Delta ARE/WT}$ mice, CCL20 plasma levels were unchanged by diet and/or genotype (data not shown).

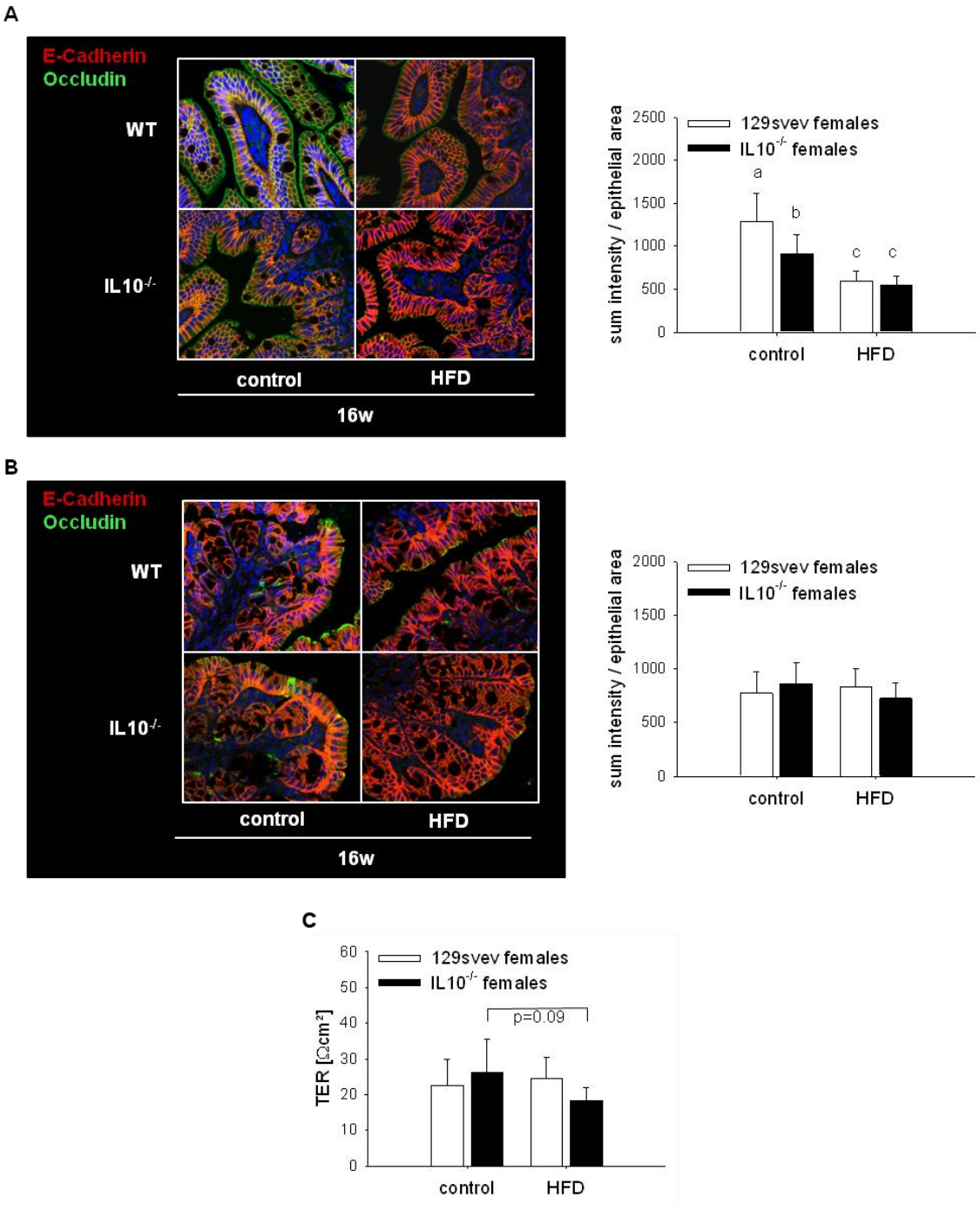

Figure 35: HFD reduces Ocln protein levels in the distal ileum (A) but not colon (B) of IL10$^{-/-}$ and corresponding 129svev mice.

Immunefluorescence staining reveals reduced presence of the protein Ocln in the distal ileum of HFD mice (600fold magnification) (A), while HFD does not affect Ocln expression in the proximal colon (B). Quantification of staining for Ocln is based on the sum intensity normalized to epithelial area. Ussing Chamber measurements suggest a trend toward impaired transepithelial resistance ($p<0.09$) in the ileum of female IL10$^{-/-}$ under HFD, while ileal transepithelial resistance is unaffected in 129svev mice. n=4-5 mice per group (C). a,b,c: data sets with different superscript letters differ significantly from each other according to two-way ANOVA.

4.3. Metabolic and disease-conditioning effects of HFD dissociate in mouse models with different susceptibility for obesity

In TNF$^{\Delta ARE/WT}$ mice as well as IL10$^{-/-}$ mice, HFD accelerated the onset of disease but did not induce obesity in the same individuals. In order to further study the potential interrelation between obesity and enhancement of the intestinal disease, we studied the effect of HFD on metabolic and disease-related parameters in AKR/J and SWR/J mice. While AKR/J mice are reported to be prone to obesity, SWR/J mice are rather obesity resistant.[227] Using the same experimental setup as for TNF$^{\Delta ARE/WT}$ mice, AKR/J and SWR/J mice were fed the different diets from 4 weeks until the age of 12 weeks.

While AKR/J mice developed significant obesity, SWR/J mice displayed only marginal, yet significant overweight (Figure 36 A). In accordance with this finding, adipose tissue mass of both EAT and MAT were significantly increased in AKR/J mice under HFD conditions, but not in SWR/J mice (Figure 36 B/C).

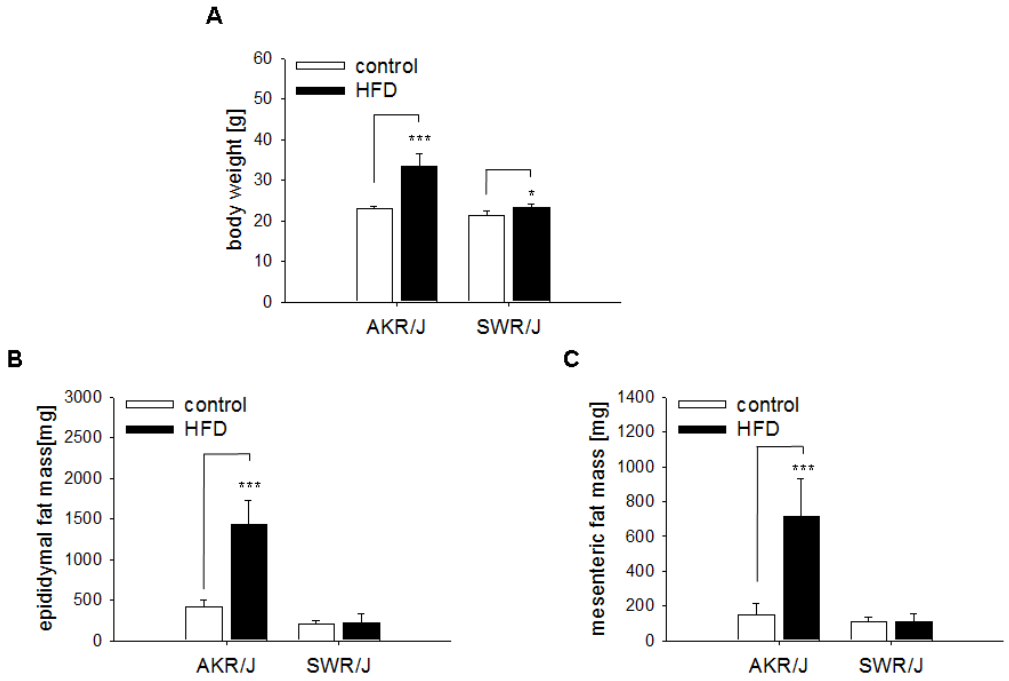

Figure 36: HFD induces overweight (A) and increase of EAT (B) and MAT (C) mass in AKR/J mice but not SWR/J mice.
At 12 weeks of age, bodyweight of AKR/J mice was significantly increased by HFD, while it was only slightly increased in SWR/J mice (A). (B). n=5 per group; * p<0.05 *** p<0.001 within genotype according to Student's t-test

While HFD feeding did not induce histopathological signs of inflammation in any of the genotypes (data not shown) at the investigated time point, the spleen weight was significantly increased in SWR/J mice but not AKR/J mice.

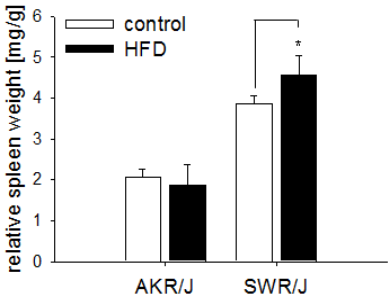

Figure 37: HFD increased spleen weight in SWR/J mice but not AKR/J mice.

At 12 weeks of age, spleen weight of SWR/J mice was significantly increased by HFD, while it was not affected in AKR/J mice. n=5 per group; * p<0.05 within genotype according to Student's t-test

As in TNF$^{\Delta ARE/WT}$ and IL10$^{-/-}$ mice, Ocln protein expression was analyzed in the ileal epithelium via immunefluorescence staining using anti-Ocln (green). HFD induced a significant reduction of Ocln protein expression in SWR/J mice and in trend lower Ocln levels (p=0.07) in AKR/J mice (Figure 38). E-Cadherin levels (red) were not affected by the diet.

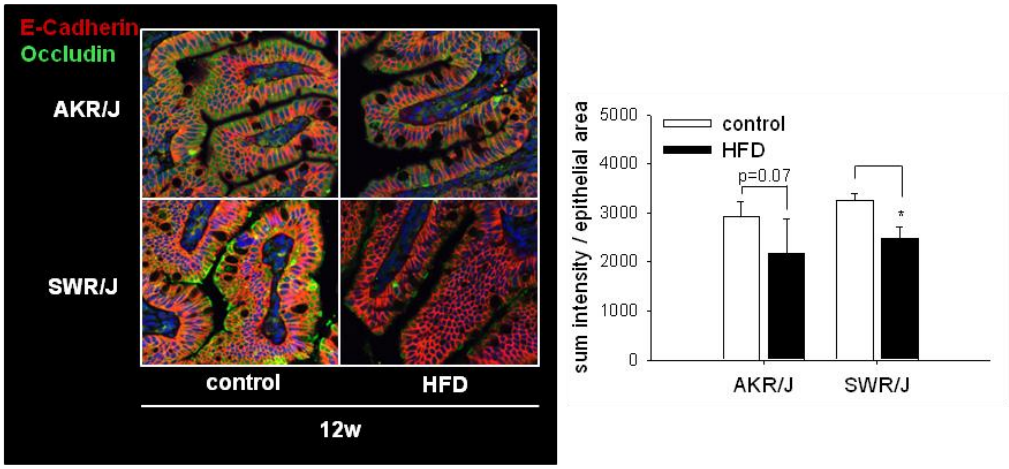

Figure 38: HFD reduces Ocln protein levels in the distal ileum of lean SWR/J mice.

Immunefluorescence staining reveals reduced presence of the protein Ocln in the distal ileum of SWR/J mice under HFD, and a tendency toward reduced levels in AKR/J mice (600fold magnification). Quantification of staining for Ocln is based on the sum intensity normalized to epithelial area. n=3-5 mice per group. * p<0.05 within genotype according to Student's t-test

5. DISCUSSION

The aim of this work was to characterize the effect of HFD feeding on the pathogenesis of IBD in mouse models, with an emphasis on interrelations with metabolic changes. The study revealed aggravated pathogenesis of ileitis in a mouse model for CD-like ileitis upon feeding of a palm-oil enriched diet. This effect was associated with enhanced translocation of endotoxin due to selectively increased intestinal permeability, as well as activation of pro-inflammatory processes in the ileal epithelium. Colonic inflammation was aggravated by the HFD feeding in both the $TNF^{\Delta ARE/WT}$ mice and in female $IL10^{-/-}$ mice. In both genotypes, the group of mice that displayed amplified inflammatory response did not develop obesity.

5.1. Synergism of genetic susceptibility and dietary factors in IBD

In the present study, neither C57BL/6 ($TNF^{WT/WT}$) nor 129svev mice exhibited histopathological inflammation in the investigated intestinal segments. HFD was able to evoke aggravation of inflammation only in combination with genetic susceptibility. While several studies report increased expression of pro-inflammatory cytokines in IEC upon HFD feeding,[164,165] this was not observed in the present study. It has to be stressed that many of the respective animal studies used diets with 60kcal% fat,[164,165] or up to even 72kcal% fat in a carbohydrate free diet.[171] It is therefore questionable if the observed effects in non-susceptible wildtype mice are of physiological relevance and transferable to a human situation.

Nevertheless, WT mice of both genetic backgrounds displayed reduced Ocln levels in the distal ileum in this study, together with enhanced translocation of LPS. Interestingly, Ocln levels were significantly decreased in $TNF^{\Delta ARE/WT}$ but not in WT mice at 8 weeks of age, indicating a synergistic effect of diet and host genetic background at this point. Elevated CCL20 plasma levels together with enhanced recruitment of $CD11c^{+}$ cells were observed in WT mice as well. While these changes apparently do not impact hepatic gene expression, cytokine plasma levels or histopathology in WT mice, systemic inflammation is evoked in the $TNF^{\Delta ARE/WT}$ mouse model, probably due to the combined effect of intrinsic pro-inflammatory potential derived from TNF over-expression and the additional stimulus provided by LPS and other yet not elucidated triggers. Likely, similar mechanisms underlie the aggravation of colitis in $IL10^{-/-}$ mice, with the lack of IL10-induced counteraction driving further inflammatory processes. It can therefore be

speculated, that HFD does not impair intestinal health to a critical extent without a given genetic susceptibility.

5.2. Temporal resolution of HFD-associated alterations in TNF$^{\Delta ARE/WT}$ mice

Assessment of metabolic and inflammation-associated parameters at different points of time during disease progression, as was performed in TNF$^{\Delta ARE/WT}$ mice, helps to identify mechanisms underlying the aggravation of intestinal disease. An overview of the temporal resolution of major observed HFD-induced biological alterations in TNF$^{\Delta ARE/WT}$ mice is shown in Figure 39, which at the same time sums up major findings of the present work. The aggravation of ileal pathology was not associated with the development of obesity at any investigated point of time. A decrease of ileal Ocln expression was already observed at the early stage of disease aggravation at 8 weeks of age, whereas increased CCL20 plasma levels were not observed at 8 weeks but at 12 weeks of age. This indicates that impairment of barrier function might contribute to disease pathology as an initial factor.

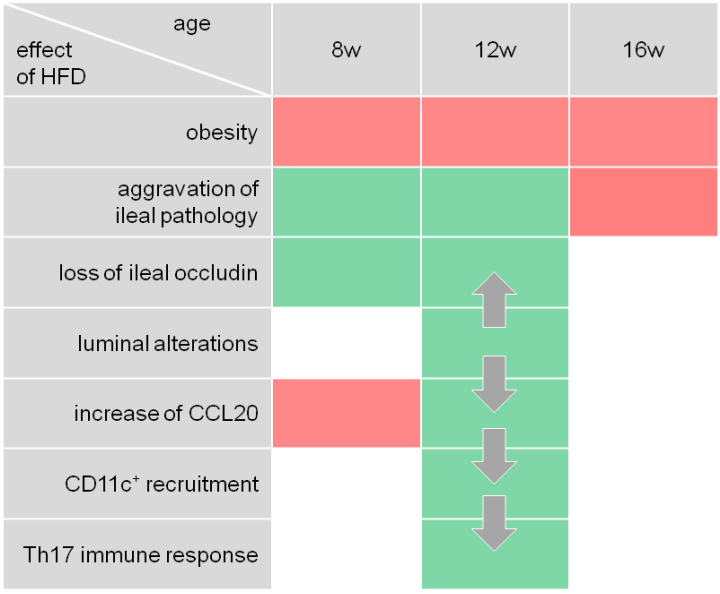

Figure 39: Temporal resolution of the main observed HFD-associated biological alterations in TNF$^{\Delta ARE/WT}$ mice.
red: effect not observed, green: effect observed, white: not investigated; possible interrelation investigated

Potential interrelations of different parameters have been assessed, largely using *in vitro* approaches (Figure 39). Luminal alterations were linked to an increase in CCL20 expression in the epithelium, and CCL20 expression was in turn linked to enhanced $CD11c^+$ DC recruitment. DC stimulation by IEC-derived factors was associated to Th17-commited immune response. While luminal factors were found to decrease TER in a cell culture system using colonic cells, the effect of these factors on Ocln expression could not be demonstrated. Identifying whether luminal alterations occur in parallel to the reduction of Ocln expression might further elucidate the biological associations of the findings.

Major considerations that can be derived from this temporal resolution of effects and the studied interrelations are i) the dissociation of the metabolic and disease-conditioning effects of HFD in this model, and ii) the critical role of luminal factors in HFD-associated aggravation of intestinal inflammation.

5.3. Dissociation of HFD-associated metabolic and disease-conditioning effects

A major drawback of both human trials and animal studies investigating the effect of dietary fat on gastrointestinal health is the concomitant development of obesity upon diets rich in fat. This hinders the separation of sheer diet-induced effects from systemic effects of obesity and associated physiologic changes. For many of the effects associated with DIO, the question whether they arise from dietary factors or from the (patho-)physiological state of obesity, is currently on vivid debate. In the present study HFD-induced inflammatory effects were not associated with obesity development in both mouse models. Male $TNF^{\Delta ARE/WT}$ mice on C57BL/6 background seem to be protected from HFD-induced obesity and associated metabolic parameters and co-morbidities such as impaired glucose tolerance compared to their wildtype littermates. This is in line with our previous observation that $TNF^{\Delta ARE/WT}$ mice exhibit reduced body weight and adipocyte size on Chow diet compared to WT mice.[219] The intrinsic overexpression of TNF, also shown here for the MAT, is likely to account for this effect. Suppression of lipogenesis,[228,229,230] and a promoted fat oxidation are reported actions of TNF,[231,232] and have been observed in this mouse model before.[219] In contrast to the frequently reported MAT hypertrophy and inflammation in CD patients, a phenomenon termed ‘creeping fat’,[233,234] $TNF^{\Delta ARE/WT}$ mice display smaller adipocytes when compared to WT cells in the mesenteric compartment at 12 and 16 weeks of age, regardless of the diet.

The IL10$^{-/-}$ mouse model used in this study is of a 129svev genetic background. Mice of this background exhibit higher metabolic rate and diet-induced thermogenesis compared to C57BL/6,[235,236] and are less prone to HFD-induced obesity.[235] While it was initially published that IL10–deficiency is associated with reduced body weight,[62] this was not observed in the present study. While Siegmund et al. reported that IL10-deficiency reduces obesity in *ob/ob* mice, they did not observe reduced body weight of IL10$^{-/-}$ mice compared to wildtype mice,[237] supporting the finding in the present study. Interestingly, in this study only mice of male gender displayed pronounced development of obesity, especially in combination with IL10-deficiency, whereas weight gain and adipose tissue weight of female mice were largely unaffected regardless of genotype. In general there are reports on the contribution of sex as factor in obesity development and the interaction of diet and sex in mice.[238,239] Protection of female C57BL/6 mice from obesity compared to male mice is abrogated with ovariectomy,[240] with a likely effect of estradiol or its derivates.[241] To my knowledge there are currently no studies reporting this gender dimorphism in HFD-induced obesity in IL10$^{-/-}$ mice. It is possible, that gender differences in basal and acute IL10 expression account for these differences in obesity susceptibility.[242] Remarkably, in the IL10$^{-/-}$ mouse model, metabolic and inflammatory responses toward HFD were differentially affected by gender: male mice displayed obesity, whereas female mice exhibited reduced barrier integrity, enhanced immune activation status as well as increased susceptibility toward ceco-colonic inflammation. A recently published study also reported this gender effect regarding intestinal inflammation, with female IL10$^{-/-}$ mice exhibiting significantly higher histopathological scores compared to IL10$^{-/-}$ males.[243]

These findings highlight the independency of inflammatory mechanisms from the metabolic effects of HFD in both mouse models used. The independency of the decrease in Ocln protein expression was further confirmed in a non-inflamed host background using SWR/J mice. While these mice did not develop HFD-induced obesity or impaired glucose tolerance at 12 weeks of age during the same experimental setup as used for TNF$^{\Delta ARE/WT}$ mice, protein expression of Ocln in the distal ileum was strongly reduced.

As both metabolic effects and a restructuring of the distal ileum tissue were found to be absent in the mice that displayed amplified inflammatory response, and thus unlikely contribute to the observed aggravation of disease, other factors were to be identified. More and more studies indicate that the intestinal alterations observed in diet-induced obese mice are not due to the state of obesity, but due to luminal alterations directed by fat ingestion.

5.4. Luminal alterations and their potential role in HFD-associated aggravation of IBD

The hypothesis that luminal alterations are a driving force in mediating HFD-associated effects in the intestine is supported by several animal studies. Most importantly, HFD induced ileal TNF mRNA expression in conventionally raised but not in germfree mice, indicating a crucial role for intestinal microbiota.[166] Recent work of Devkota et al. identified milk fat-induced blooming of the pathobiont *Bilophila wadsworthia* as the crucial factor during diet-associated aggravation of colitis in $IL10^{-/-}$ mice.[244] The HFD-associated promotion of sulfate-reducing bacteria (SRB), to which also *Bilophila wadsworthia* belongs, might be of particular importance regarding the effect of HFD on IBD pathogenesis. The presence of SRB is reportedly increased in IBD, in particular UC, with concomitant increase of sulfidogenesis rates.[245,246,247] Comprising about 23 genera, the largest group of SRB is found among the *δ-proteobacteria,* for example within *Desulfovibrionales and Desulfobacterales.*[248] An increase in relative amounts of SRB likely contributes to the often observed increases in *Proteobacteria* abundance as described in Chapter 1.1.4. SRB can metabolize sulfate via dissimilatory reduction and use it as the terminal electron acceptor for the generation of energy. Sulfate or elemental sulfur can also be microbially converted into hydrogen sulfide, which impacts on IEC by inhibiting butyrate utilization,[249] as well as proliferation.[250] *Bilophila wadsworthia,* for example, utilizes the sulfur-containing organic acid taurine for energy production.[251] Another important aspect of the possible involvement of SRB in colitis has to be considered: Dextran sodium sulfate (DSS) is often used to chemically induce colitis in rodent models. Colonotoxic effects of sulfur compounds were initially reported with the observation that DSS-treatment induces colitis and colorectal tumors.[252,253] Probably, the microbial reduction of sulfate in the DSS molecule to the inflammatory, barrier-breaking hydrogen sulfide is an initial trigger for development of colitis.[254] Noteworthy, DSS alters the microbial community already prior to the onset of intestinal pathology,[255] probably due to selection of bacteria that can degrade DSS or metabolize sulfur such as *Proteus mirabilis*,[256] or *Akkermansia muciniphila* (*Verrucomicrobiae*), the latter only detectable in DSS-treated mice.[255] This implies that certain SRB species might be initiators of IBD development and not only bystanders, emphasizing the relevance of HFD-associated bloom of SRB.

The bloom of *Bilophila wadsworthia* in the above-mentioned study was mainly determined by the BA composition influenced by HFD [244]. BA composition was found to be significantly altered in the present study as well. With BA described profoundly shaping the intestinal microbial composition overall,[244,257,258] it can be speculated that microbiota was affected by HFD in

this study as well. Phylogenic profiling of the intestinal microbial ecosystem using adequate samples and techniques should follow to address this question.

In the present study, associations between luminal alterations and the observations associated with HFD-induced aggravation of IBD are provided by *in vitro*-modeling linking i) HFD-derived cecal water to a modulation of small intestinal barrier integrity and ii) HFD-derived cecal lysates to enhanced CCL20 expression and concomitant DC recruitment and differentiation. The cecal lysates used for the studies were derived from WT animals, allowing the conclusion that the observed effects are independent of the presence of tissue inflammation but rather dependent on diet-induced luminal alterations.

5.4.1. Barrier integrity during HFD-associated aggravation of IBD, and potential role of luminal factors

HFD-induced decrease of Ocln protein expression in the distal ileum was observed in this study, together with increase of LPS concentrations in the portal vein blood, indicating modifications in small intestinal barrier homeostasis. *In vitro* experiments show that HFD-associated cecal water decreased TER in PTK6 cells and thus point to a crucial role of factors present in the cecal water regarding modulation of the intestinal barrier.

Impaired intestinal barrier function is a hallmark and causal factor in IBD. Despite the disputability of many techniques used for the study of putative barrier function, also DIO has been associated with increased intestinal permeability. Many publications, especially those investigating subtle changes such as under the influence of DIO or HFD, focus on expression studies of permeability-associated proteins such as TJ proteins or claudins.[171,181,259] Ocln expression has become one of the major read-outs regarding assessment of putative intestinal permeability, and several studies report Ocln expression to be reduced under conditions of HFD feeding.[165,172]

The impairment of barrier integrity in our models is independent of obesity. It was also reported that genetically obese *ob/ob* mice do not exhibit increased permeability.[260] The scientific discussion about the mechanisms underlying impaired intestinal integrity during HFD has resulted in cumulating evidence for a crucial role of diet-associated BA profiles in the recent years.[172,173,260] Interestingly certain luminal BA decrease barrier function,[172,173,261] probably via epidermal growth factor receptor and/or modulation of the nuclear farnesoid X

receptor in IEC.[262] One study reported that the anti-inflammatory effect potentially exerted by secondary BA is abolished upon sulfation of the respective species.[263] Analysis of the cecal content revealed shifts in BA composition toward tauro-conjugated species and thereby underpinned the hypothesis that a modulation of the BA profile may contribute to impaired barrier function, as indicated by our *in vitro* studies. Interestingly, targeted quantification has revealed particular implication of tauro-conjugated BA species in the lumen of UC patients.[263]

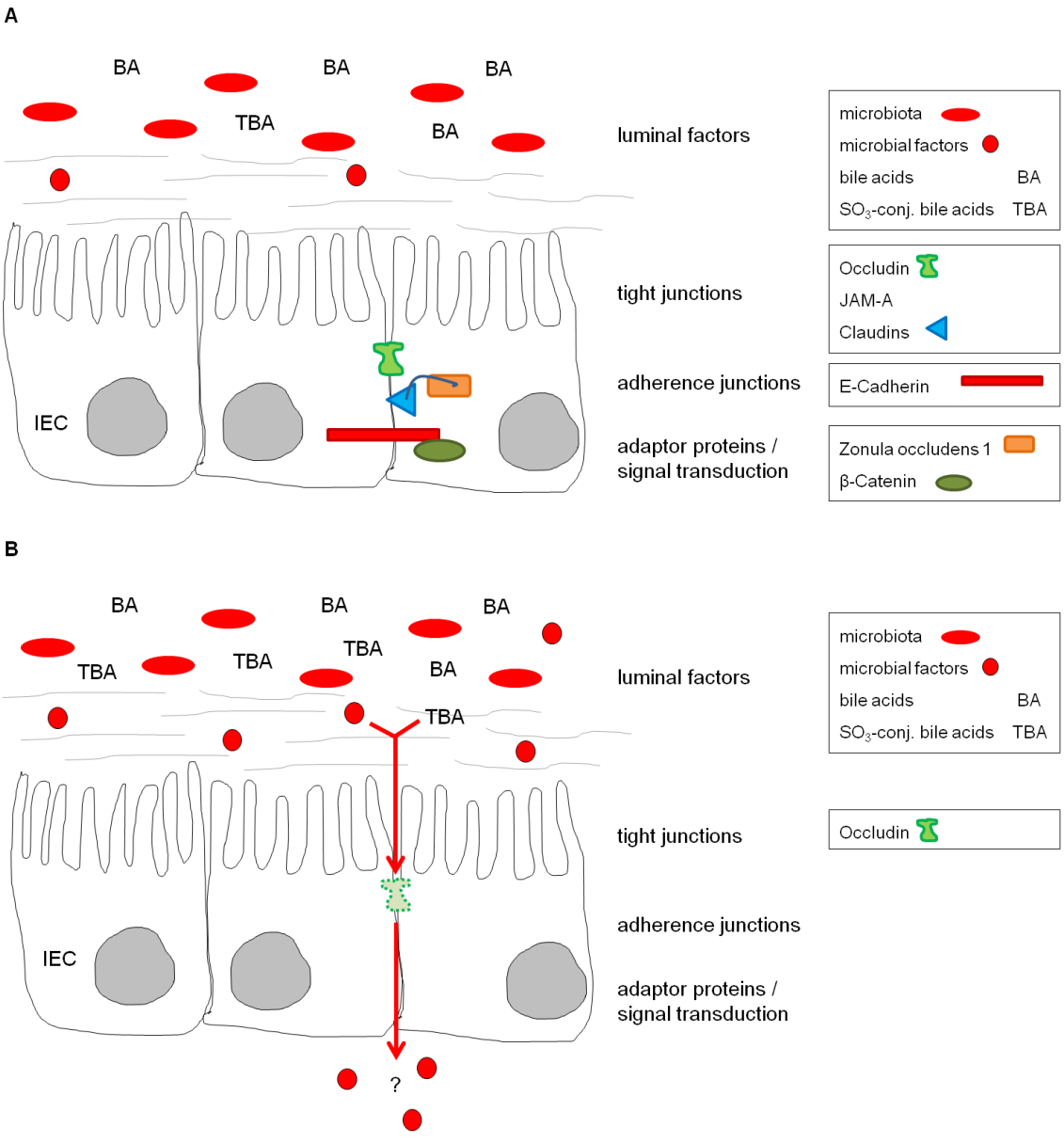

Figure 40: Alteration of luminal factors and perturbation of bile acid composition might lead to decreased barrier integrity. Under normal conditions, tight junction proteins such as Occludin seal the epithelial layer (A). HFD-associated alterations of bile acid composition, in particular high abundance of tauro-conjugated bile acids, and microbial factors might impair barrier integrity (B).

It is under debate to what extent certain assessment methods of permeability reflect actual gut barrier functionality *in vivo*. This discrepancy is also reflected by the results of the present study in $TNF^{\Delta ARE/WT}$ mice, showing HFD-enhanced translocation of LPS together with reduced Ocln levels, but genotype-dependent PEG-translocation as well as unchanged *ex vivo* TER and NaF translocation in Ussing chamber experiments. An infiltration-induced massive thickening of the small intestinal tissue may account for the inability of the latter method to reflect subtle alterations in permeability, as the tissue was not deprived of the submucosal compartments. In fact, besides the often observed effect of endotoxemia, there is little persuasive data on functional disturbances of barrier integrity during DIO or HFD feeding. The translocation of LPS and its recovery in the hepatic portal vein blood is probably the most valuable of the assessed permeability parameters, as it reflects an *in vivo* effect with initiation of pro-inflammatory responses as physiological consequence. Interestingly, a state of metabolic endotoxemia is observed postprandially after meals with a high fat content.[264,265] A concomitant raise in the soluble LPS-sensing sCD14, TNF, IL6 and CRP levels has been observed.[265,266,267,268] Also dietary fat composition can modulate both cytokine expression and LPS-associated factors in mice. In comparison to milk fat, rapeseed oil or sunflower oil, a palm-oil based diet resulted in the highest plasma level of IL6 together with the highest expression of IL1β and of LPS-sensing TLR4 and CD14 in adipose tissue.[269] Of note, the routes by which LPS reaches the blood stream are not fully known. It is suggested that besides paracellular passage, one major translocation route of LPS occurs via transport in chylomicrons.[265,270,271] As chylomicrons are not reaching the portal venous system, but are passed on from chyle to the systemic circulation via the thoracic duct, the observed increase in LPS levels in portal vein blood is likely not due to an enhanced uptake of LPS in chylomicrons but due to direct translocation of LPS to the blood stream. Translocated LPS encounters liver and MLNs first, decreasing the amount of LPS that reaches the systemic circulation and activating inflammatory gene expression in both these tissues.

It is possible that a change in microbial composition contributes to increased LPS translocation in the state of obesity. Bifidobacteria are reportedly decreased or even absent in HFD.[272] Mice fed a HFD supplemented with oligofructose exhibited restored Bifidobacteria levels and decreased LPS plasma levels.[273] Being one of the most important butyrate-producing bacteria (BPB), *F. prausnitzii* levels negatively correlate with plasma LPS concentrations in obesity.[274] Of note, butyrate was reported to modulate TJ functionality,[275,276] and rectal butyrate enemas have the ability to strengthen barrier integrity in mice,[277] and humans.[278,279] These findings may link endotoxemia to bacterial composition in the state of DIO. The population

of BPB is affected by fiber, lowering the colonic pH and thus fostering growth of BPB.[280] While it is possible, that the abundance of BPB is altered by a fostering of other bacterial groups under HFD conditions, it is not assessed if intake of dietary fat might directly affect BPB populations.

Of note, small intestinal permeability seems crucial in inflammation pathogenesis also in UC patients,[281,282] and mouse models of colitis.[283] Clinical studies report increased small intestinal permeability, often not accompanied by alterations regarding large intestinal permeability.[281] Indeed, promoting small intestinal barrier function by either drug or probiotic treatment is sufficient to reduce colitis severity in $IL10^{-/-}$ mice.[259,284] It is therefore likely that HFD-induced impairment of barrier integrity, assessed as Ocln expression, also contributes to aggravation of disease in $IL10^{-/-}$ mice in the present study.

5.4.2. Effects of luminal alterations during HFD on immune response

In vitro-modeling could also provide a link between intestinal luminal changes due to HFD feeding and enhanced CCL20 expression in the ileal epithelium, leading to recruitment of dendritic cells, as it was observed in the ileum of the animals.

Epithelial cell signals are crucial for driving the recruitment and directing the differentiation of DCs. The migrating DC subsets that are recruited as a result of pathogenic insults on the intestinal epithelium then target T cells and initiate further immune responses. CCL20 is the most potent inducer for DC progenitor migration known to date,[285] and IEC have been identified as the main producers of CCL20 in inflamed epithelium in different inflammatory conditions.[285] CCL20 expression is reportedly increased in colonic biopsies of UC and CD, but not in non-IBD colitis patients.[286,287,288] C-C chemokine receptor type (CCR) 6, the sole receptor known for CCL20, is expressed on immature DCs. Upon stimulation by CCL20, $CCR6^{+}$ DCs migrate to sites of infection or inflammation, where they suppress CCR6 expression with concomitant upregulation of CCR7 for migration to the lymph nodes.[289] This mechanisms also contributes to the differential positioning of particular murine DC subsets in epithelial and lymphoid tissues, as $CD11b^{+}$ myeloid DCs mostly express CCR6, whereas $CD8\alpha^{+}$ lymphoid DC are negative for CCR6.[290] Of note, CCR6 is implicated in IBD development according to GWAS.[21]

It is reported in the present study that ileal IEC can promote DC recruitment by CCL20 expression, and that this mechanism is amplified during HFD feeding. While stimulation of the

Mode K cell line with lysates of the different pure diets fails to do so, the lysed cecal content of mice fed these diets are able to differentially promote CCL20 production. This emphasizes the importance of luminal factors others than dietary lipids as such for the aggravation of disease. We suggest a link between diet-induced luminal changes and increased CCL20 expression with concomitantly promoted recruitment of $CD11c^+$, and presumably $CCR6^+$, DCs (Figure 41 A). The driving force might be enhanced pathogenic potential of the microbiota, or reduced barrier integrity allowing microbial factors to stimulate or even penetrate the epithelial cell layer, or a combination.

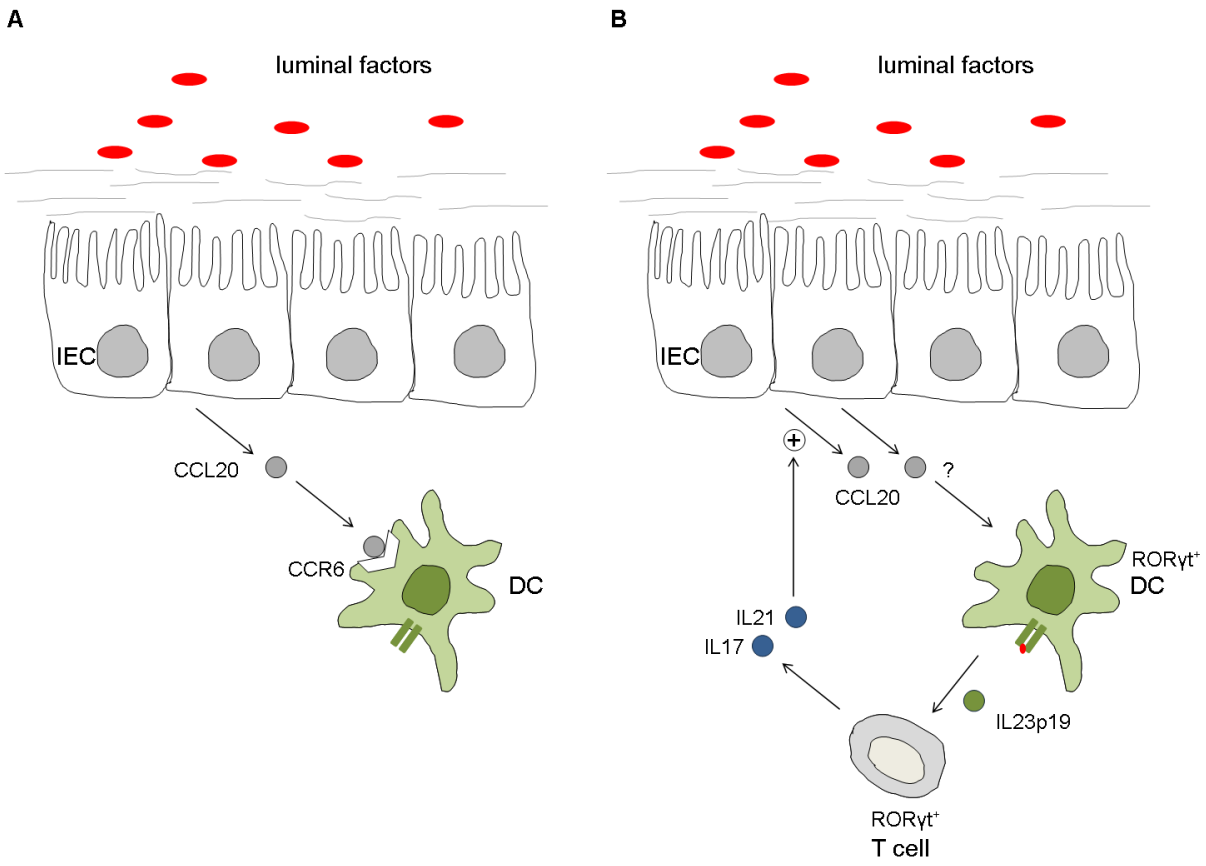

Figure 41: Epithelial cell derived factors mediate the effect of luminal factors on immune cells.

Upon stimulation by HFD-associated luminal factors, ileal intestinal epithelial cells express the chemokine CCL20 and recruit dendritic cells (A). Other factors generated by epithelial cells condition dendritic cells to produce IL23 which prime T cells to Th17-commitment and to produce effector cytokines such as IL17 and IL21 (B).

IEC: intestinal epithelial cell, DC: dendritic cell, IL: interleukin, CCL20: (C-C motif) ligand 20, CCR6: C-C chemokine receptor type 6, RORyt: RAR-related orphan receptor gamma t

The expression of CCL20 is induced upon LPS or TNF/IL1β stimulation,[285] whereas IL10 was reported to suppress CCL20 production.[291] A synergistic effect of TNF overproduction in the transgenic mouse model and luminal factors under HFD might underlie the observed regulation of CCL20 production in this study. A potential increase in TNF concentration in the cecal lumen is unlikely to account for the observed effect, as treatment of Mode K cells with cecal water did not result in CCL20 production.

Besides DCs, CCR6 is expressed on Th17 cells specifically,[292] and regulates Th17 cell migration to the gut.[293] Disruption of CCR6/CCL20 by administration of an anti-CCL20 neutralizing antibody reduced Th17 cell infiltration in a mouse model of dry eye disease, and significantly decreased the generation of Th17 cells.[294] Anti-CCL20 neutralizing antibody also reduced histological damage in a mouse model of trinitrobenzenesulfonic acid-induced colitis, together with marked decrease in $CCR6^+$ lamina propria T cells.[295] In line with these findings, we here demonstrate that unspecified epithelial-derived factors can drive the differentiation of DCs toward a Th17-conditioning phenotype under HFD conditions (Figure 41 B). The expression of Th17-directing *il23p19* is enhanced by luminal conditions under HFD, promoting the generation of Th17 committed $ROR\gamma t^+$ cells. As we investigated the expression of cytokines and lineage commitment-related transcription factors in total LPLs in this study, these $ROR\gamma t^+$ cells could be of both DC and Th cell origin, the differentiation of both cell types into a Th17 direction being driven by RORγt. During the last years, Th17 immune response has been recognized as crucial during the development of autoimmune diseases. Blocking antibodies against IL17 or IL23 have proven to be beneficial in the treatment of psoriasis,[296] collagen-induced arthritis,[297] and Crohn's disease.[298] Mice deficient for either IL23p19 or IL17 are protected against arthritis and IBD.[299,300,301] The finding that genes annotated to the ontology term 'regulation of interleukin-17 production' are strongly enriched in GWAS datasets, as described in Chapter 1.1.3. further highlights the importance of this Th subset during IBD pathogenesis.[21,29,35,64] Of note, Th17-associated cytokines such as IL23 and IL21 are able to drive CCL20 production in the intestinal epithelium,[288,302] thus further promoting recruitment of $CCR6^+$ cells (Figure 41 B). Besides, as the $TNF^{\Delta ARE/WT}$ mouse model also develops arthritis, the effect of promoted Th17 response on this peripheral pathology could be interesting to study.

It has been reported before that HFD-induced obesity promotes expansion of the Th17 T cell lineage in mice.[303] Our present results indicate that these findings are related to luminal alterations and epithelium-derived signals rather than the state of obesity. Several particular bacteria and microbial patterns with the potential to promote Th17 immune responses,

presumably via conditioning of both IECs and DCs, have already been described. Segmented filamentous bacteria,[304,305] and commensal bacteria-derived adenosine 5′-triphosphate have been found to promote Th17.[306] In addition, bacteria-derived flagellin triggers the DC-dependent differentiation of Th1 and Th17 cells.[307] Zielinski et al. suggested that different microbial triggers may promote different Th17 cell subsets in humans, as they found *Candida albicans* to induce Th17 cells of a different phenotype compared to *Staphylococcus aureus*, dependent on the balance between mainly IL1β and IL12 as polarizing cytokines.[308]

Besides Th17 committed T cells, also innate lymphoid cells (ILC) can be RORγt$^+$. One subgroup of ILC critically depends on RORγt for development, and expresses IL22 upon IL23 stimulation. The role of these cells in maintaining gut homeostasis has been largely unknown, until recent studies indicated that RORγt$^+$ ILC interact with CD4$^+$ T cells via MHCII-dependent mechanisms to regulate adaptive immune cell responses toward commensal bacteria.[309] The fact that a lack of functional MHCII in RORγt$^+$ ILC leads to CD4$^+$ T cell expansion and spontaneous development of antibiotics-sensitive colitis, demonstrates the crucial role of MHCII$^+$RORγt$^+$ ILC in limiting inflammatory responses toward commensals. This subset of lymphocytes might be regulated in the present study as well, but as MHCII$^+$RORγt$^+$ ILC are not capable of producing IL17 upon IL23 stimulation,[309] the observed increase of IL17 expression in the lamina propria must result from other cell types, most likely T cells as indicated.

5.5. Conclusion and perspective

In summary, the present data support the hypothesis that HFD feeding, independently of obesity and its associated pathologies, accelerates onset of intestinal inflammation in an IBD-susceptible host through mechanisms that involve increased intestinal permeability and altered luminal factors. After analysis of the impact of HFD on microbial composition, several experiments could be conducted to further characterize the luminal components responsible for aggravation of disease, i) applying the respective diets to $TNF^{\Delta ARE/WT}$ and $IL10^{-/-}$ mice and investigating the change of intestinal microbial composition and ii) transplantation of cecal content of WT mice into germfree $TNF^{\Delta ARE/WT}$ and $IL10^{-/-}$ mice, followed by iii) transplantation of selected bacterial strains regulated by diet into germfree $TNF^{\Delta ARE/WT}$ and $IL10^{-/-}$ mice.

Although results from mouse models are not necessarily transferable to the human situation, the findings presented here may support epidemiological data indicating dietary fat but not obesity as risk factor for the development of IBD. Although recommendations for dietary measures in already established pathology cannot be drawn from this study, personalized nutrition recommendations taking dietary fat restriction into account might be useful for patients with susceptible background, for example for first-grade relatives of IBD patients.

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ABBREVIATIONS

AMP	antimicrobial peptide
APC	antigen-presenting cell
ATG16L1	autophagy related protein 16-1
BA	bile acid
BSA	bovine serum albumin
BM-DC	bone marrow-derived dendritic cell
CARD	caspase recruitment domain
CC	case-control study
CCD	Crohn’s disease with colonic phenotype
CCLx	C-C chemokine ligand
CCRx	C-C chemokine receptor
CXCLx	C-X-C motif ligand
CD	Crohn’s disease
CDH1	E-Cadherin gene
CDx	cluster of differentiation x
CRP	C-reactive protein
CT	cecum tip
DC	dendritic cell
dCo	distal colon
dIL	distal ileum
DIO	diet-induced obesity
DMEM	Dulbecco’s modified Eagle medium
DSS	dextran sodium sulfate
EAT	epididymal adipose tissue
FA	fatty acids
FD	fettreiche Diät
FoxP3	forkhead box P3
FT-IR	fourier transform infrared spectrometry
GWAS	genome-wide association studies
IBD	inflammatory bowel disease
ICAM1	intercellular cell adhesion molecule 1
ICD	Crohn’s disease with ileal phenotype
IEC	intestinal epithelial cell

IFNy/IFNG	interferon y
IgA	immunoglobulin A#
ILC	innate lymphoid cells
ILx	interleukin x
IRGM	interferon-inducible protein
HFD	high fat diet
HPLC	high performance liquid chromatography
LP	lamina propria
LPL	lamina propria lymphocyte
LPS	lipopolysaccharide
MAT	mesenteric adipose tissue
MCP1	monocyte chemotactic protein 1
MIPx	macrophage inflammatory protein
MUFA	mono-unsaturated fatty acid
NaF	sodium fluorescein
NLR	nucleotide-binding oligomerization domain -like receptor
NSAID	non-steroidal anti-inflammatory drug
NOD	nucleotide-binding oligomerization domain
Ocln	Occludin
PBS	phosphate buffered saline
pCo	proximal colon
PEG	polyethylene glycol
PTPN2	T cell protein tyrosine phosphatase 2
PUFA	poly-unsaturated fatty acid
RLR	Retinoic acid-inducible gene-I (RIG-I)-Like Receptors
RORyt	RAR-related orphan receptor gamma t
SAA	serum amyloid A
SFA	saturated fatty acid
SRB	sulfate-reducing bacteria
TBA	tauro-conjugated bile acids
Tbet	T-box transcription factor TBX21
SD	standard deviation
SEM	standard error of means
SPF	specific pathogen free
TER	transepithelial electrical resistance

TGFβ	transforming growth factor β
Thx	T helper cell type x
TNF	tumor necrosis factor
TLRx	toll-like receptor x
TG	triglycerides
UC	Ulcerative colitis
WT	wildtype mouse/mice
XBP1	x-box binding protein 1
ZO-1	zonula occludens 1

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ACKNOWLEDGEMENTS

First of all, I want to thank Prof. Dirk Haller for making this thesis and the studies behind it possible, for his support and mentoring.

Sincere thanks to Prof. Hannelore Daniel who is a role model for many students of nutritional science including me, who succeeds in encouraging generations of students and young scientists and who is able to vitalize any discussion however ‘somniferous’ the topic may be. I also thank her for her time and energy spent on reading this thesis and to act as examiner.

Thank you to Prof. Martin Klingenspor for very fruitful collaboration, friendly words, empathy, and for agreeing to act as a chairman for the PhD examination.

Special thanks to those people who contributed to the experiments and analyses: Pia Lichti for the metabolomics data, Thomas Clavel for microbial analysis even though it was not included, Stephanie May for help with sampling and for data sharing, Veronika Müller and Jarlei Fiamoncini from the Chair for Nutritional Physiology for Ussing Chamber measurements and bile acid analysis respectively, Sigrid Kisling for never-ending scoring and ethical approval proofreading; Brita Sturm and Nico Gebhardt for making our daily life work at all. There are several people from other departments and institutes whom I would like to thank for collaboration during the last years: Caroline Kless from the Chair for Molecular Nutritional Medicine; François-Pierre Martin from the Nestlé Research Center; François Reichardt and especially Prof. Dr. Michael Schemann from the Chair of Human Biology.

I am deeply thankful to all my colleagues and friends at the Chair of Nutrition and Immunology, for their scientific and especially for their mental support. As time goes by, colleagues will become friends even beyond the time of collaboration: Emanuel Berger, Pia Lichti, Anja Petzet, Marie-Anne von Schillde, Jana Hemmerling, Natalie Steck, Gabriele Hörmannsperger, Thomas Clavel, Nico Gebhardt, Job Mapesa – thank you all for saving me from social isolation during the last four years…

Ich danke meinen Eltern, meiner Schwester Karin und meinem ‚tollen Mann‘ Lars für ihre Liebe und ihre stetige Unterstützung.

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Peer-reviewed original manuscripts and reviews in course of doctoral thesis

- **Gruber L.**, Kisling S., Lichti P., Martin F.P., May S., Klingenspor M., Lichtenegger M., Rychlik M., Haller D.
  High Fat Diet Accelerates Pathogenesis of Murine Crohn's Disease-Like Ileitis Independently of Obesity.
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- **Gruber L**.,* Reichardt F.,* Baudry C.,* Mazzuoli G.,* Moriez R., Scherling C., Kollmann P., Daniel H., Kisling S., Haller D., Neunlist M., Schemann M.
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- Wagner S., Schmidt A., Effenberger M., **Gruber L.**, Danier J., Haller D.
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Published abstracts in course of doctoral thesis

- **Gruber L.**, Fiamoncini J., Müller V., Lichtenegger M., Rychlik M., Daniel H., Clavel T., Haller D.
  High fat diet aggravates Crohn's Disease-like ileitis independently of obesity via alterations of epithelial barrier homeostasis.
  Journal of Crohn's and Colitis. 2013 Feb; 7(s1), Supplement 1:22

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- **Gruber L.**, Haller D.
  High-fat feeding increases recruitment of ZO1+Ocldn+CD11c+ dendritic cells to the lamina propria and accelerates disease onset in a murine model of Crohn's Disease-like ileitis.
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Oral presentations

- **Gruber L.**, Kisling S., May S., Haller D.
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  United European Gastroenterology Week (UEGW) 2013
  Berlin, Germany, October 12-16, 2013

- **Gruber L.**, Fiamoncini J., Müller V., Daniel H., Clavel T., Haller D.
  High fat diet aggravates Crohn's Disease-like ileitis independently of obesity via alterations of epithelial barrier homeostasis.
  50th Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung
  Bonn, Germany, March 20-22, 2013

- **Gruber L.**, May S., Lichti P., Haller D. High-fat feeding accelerates disease onset in a murine model of Crohn's Disease-like ileitis.
  2nd German-Russian Week of the Young Scientist "Health and Society"
  Ekaterinburg, Russia, September 16-21, 2012

- **Gruber L.**, Baur P., Haller D. Metabolic assessment of the development of Crohn's like IBD in the $TNF^{\Delta ARE}$ mouse model.
  48th Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung
  Potsdam, Germany, March 16-18, 2011

- **Gruber L.**, Schmidt S., Pitariu S., Haller D. The effect of high-fat feeding in a mouse model of inflammatory bowel disease.
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Poster presentations

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  High Fat Diet Aggravates Crohn's Disease-Like Ileitis Independently of Obesity.
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- **Gruber L.**, Clavel T., Fiamoncini J., Müller V., Lichtenegger M., Rychlik M., Daniel H., Haller D.
  High fat diet aggravates Crohn's Disease-like ileitis independently of obesity via alterations of epithelial barrier homeostasis.
  8th Congress of ECCO (European Crohn's and Colitis Organisation)
  Vienna, Austria, February 14-16, 2013 (Poster of Distinction)

- **Gruber L.**, Nürnberger C., Schüppel V., Kunde M., Lichtenegger M., Müller V., Rychlik M., Daniel, H., Haller D.
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  High-fat feeding increases recruitment of dendritic cells to the lamina propria and accelerates disease onset in a murine model of Crohn's Disease-like ileitis.
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- **Gruber L.**, Haller D.
  High-fat feeding increases recruitment of $ZO1^{+}Ocldn^{+}CD11c^{+}$ dendritic cells to the lamina propria and accelerates disease onset in a murine model of Crohn's Disease-like ileitis.
  Digestive Disease Week (DDW 2012)
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- **Gruber L.,** May S., Haller D.
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  49th Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung
  Freising, Germany, March 14-16, 2012

- **Gruber L.**, Baur P., Schmidt S., Martin F.P., Collino S., Guy P., Thorimbert A., Rezzi S., Haller D.
  The effect of high-fat feeding in a mouse model of inflammatory bowel disease.

8th Nestlé International Nutrition Symposium 'Nutrition & the Immune System'
Lausanne, Switzerland, October 19-21, 2011

- **Gruber L.**, Baur P., Martin F-P., Collino S., Guy P., Thorimbert A., Rezzi S., Kochar S., Benyacoub J., Kollias G., Haller D.
  Metabolic phenotyping of a Crohn's like IBD Etiophatology in the TNF$^{\Delta ARE}$ mouse model.
  NuGOweek 2011 'Measuring Health: How to apply nutrigenomics for measuring metabolic health'
  Wageningen, Netherlands, September 6-9, 2011

- **Gruber L.**, Kless C., Bolze F., Rychlik M., Klingenspor M., Haller D.
  Assessment of barrier integrity and metabolic changes in the context of high-fat feeding.
  2nd Statusseminar of the Federal Ministry of Education and Research (BMBF)
  Potsdam, Germany, May 16-18, 2011

- **Gruber L.**, Baur P., Schmidt S., Martin F.P., Collino S., Guy P., Thorimbert A., Rezzi S., Haller D.
  The effect of high-fat feeding in a mouse model of inflammatory bowel disease.
  4th Seeon Conference 'Microbiota, Probiota and Host' of the of the German Society of Hygiene and Microbiology
  Seeon, Germany, April 15-17, 2011

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Presentation/Poster Co-Authorships

- Hemmerling J., Heller K., **Gruber L.**, Haller D.
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  16th International Congress of Mucosal Immunology (ICMI 2013)
  Vancouver, Canada, July 17-20 (Presentation)

- Hemmerling J., Heller K., Fischer I., **Gruber L.**, Haller D.
  Fetal gut programming by maternal inflammation and maternal obesity in the context of offspring's susceptibility to intestinal inflammation
  50th Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung
  Bonn, Germany, March 20-22, 2013

- Hemmerling J., Heller K., **Gruber L.**, Haller D.
  Fetal gut programming by maternal inflammation and maternal diet- induced obesity in context of offspring's susceptibility to chronic intestinal disease.
  NuGOweek 2012 'Nutrition, lifestyle and genes in the changing environment'
  Helsinki, Finland, August 28-31, 2012 (Presentation)

- Wagner S.**, Gruber L.**, Haller D.
  Semi-synthetic diet prevents chronic intestinal inflammation in a mouse model for Crohn's disease like ileitis.
  49th Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung
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- Baur P., **Gruber L.**, Martin F.P., Collino S., Bosco N., Guy P., Rezzi S., Kochhar S., Benyacoub J., Kollias G., Haller D.
  Metabolic phenotyping of Crohn's disease-like IBD etiophatology in the $TNF^{\Delta ARE/WT}$ mouse model.
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  Metabolic phenotyping of Crohn's disease-like IBD in the $TNF^{\Delta ARE}$ mouse model.
  7th Nestlé International Nutrition Symposium 'Nutrition and Epigenetics'
  Lausanne, Switzerland, October 27-29, 2010

- Baur P., **Gruber L.**, Martin F.P., Collino S., Guy P., Thorimbert A., Rezzi S., Haller D.
  Metabolic phenotyping of Crohn's disease-like IBD in the $TNF^{\Delta ARE}$ mouse model.
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  Seeon, Germany, June 18-20, 2010

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Grants and Awards

1. Oral Free Paper Prize at the United European Gastroenterology Week in Berlin, Germany, October 12-16, 2013

2. Travel Grant for Basic Scientists from United European Gastroenterology for the United European Gastroenterology Week in Berlin, Germany, October 12-16, 2013

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5. International Travel Grant from Institut Danone Ernährung für Gesundheit e.V. for the annual congress of the European Nutrigenomics Organisation NuGOweek 2010 'Metabolic Health' in Glasgow, United Kingdom, August 31 – September 3, 2010