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Dietary factors in the modulation of Crohn's disease-like ileitis

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ABSTRACT

Inflammatory bowel diseases (IBD) comprising Crohn's disease (CD) and ulcerative colitis (UC) are chronic relapsing inflammatory disorders of the gastrointestinal tract. Accumulating evidence suggests that a combination of environmental factors such as smoking or diet may contribute to a dysbalanced immune-response against the commensal microbiota in a genetically susceptible host. The worldwide incidence of IBD is rising and a general adaptation to Western lifestyle including a Western diet has been implicated in this observation, especially in developmental countries. Enteral nutritional therapy has been proven successful for treatment in a subset of IBD patients. However, the underlying mechanisms are not completely understood. Thus, we aimed at elucidating the effects of a semi-elemental diet in genetically susceptible animal models of mucosal inflammation. The $TNF^{\Delta ARE/WT}$ mouse resembles a model for CD, which develops chronic ileitis under conventional conditions on a standard Chow diet. By contrast, we could show that pathogenesis of Crohn's disease-like ileitis could be inhibited by early dietary intervention using a semi-elemental experimental diet (Exp). The protective state was associated with decreased expression of proinflammatory cytokines, pattern recognition receptors and homing related addressins in distal ileal tissues. Although, dietary intervention was not associated with a phenotypic change of $CD8^+$ effector IEL/LPL subpopulations, we did observe an overall decrease in infiltrating leukocytes. However, administration of experimental diet was not effective for induction of remission in an already established inflammatory setting. Moreover, supplementation of experimental diet with low concentrations (10%) of Chow was sufficient to induce maximal chronic intestinal inflammation. FT-IR analysis of cecal contents from Chow and Exp treatment groups showed diet-related differences in spectral distance. However, no alteration in antigenicity could be observed in a coculture model of cecal lysate pulsed BM-DCs and $CD4^+$ T-cells. Furthermore, gluten was identified as dietary antigen that plays a role in Crohn's disease-like ileitis. Peptic tryptic digests of gluten induced TNF secretion in total MLNs and gluten-fortified experimental diet could induce chronic ileitis in $TNF^{\Delta ARE/WT}$ mice. The protective effect of experimental diet inhibiting mucosal inflammation could be confirmed in the $IL\text{-}10^{-/-}$ mouse, whereas results in a T-cell transfer model of colitis seemed to be equivocal. In conclusion, we could show that Crohn's disease-like ileitis can be inhibited by dietary intervention using a semi-elemental experimental diet. Unraveling the underlying mechanisms might reveal new concepts for the improvement of nutritional therapy in IBD patients. Moreover, gluten fortification of experimental diet could reverse the protective effect. Thus, the $TNF^{\Delta ARE/WT}$ mouse might serve as a new model for spontaneous gluten intolerance.

1. INTRODUCTION

1.1. Inflammatory bowel disease (IBD)

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders of the gastrointestinal tract with a multifactorial etiology. Crohn's disease (CD) and ulcerative colitis (UC) represent the two major forms of IBD, which typically show disease onset in the second and third decades of life in genetically predisposed individuals [1]. Histologically, ulcerative colitis is characterized by superficial inflammation that is limited to the mucosa and submucosa of the colon. Inflammation usually starts in the rectum, spreads proximally in a continuous fashion and may be associated with cryptitis and crypt abscesses. By contrast, Crohn’s disease most typically involves the terminal ileum but may affect any part of the gastrointestinal tract in a non-continuous fashion. The microscopic features of Crohn's disease include transmural inflammation, a thickened submucosa and commonly associated complications such as strictures, abscesses, fistulas and non-caseating granulomas. [2, 3].
The incidence of IBD is increasing worldwide, and their prevalence is higher than 200 cases per 100,000 inhabitants in Western countries [4]. Several factors that contribute to disease pathogenesis have been identified, including genetic factors, a dysregulated immune system and environmental factors such as diet and the gut microbiota [2, 5] (**Figure I-1**). Balancing the need to respond to pathogens while co-existing with commensal bacteria and luminal antigens is a key challenge of the intestinal immune system [3]. There is accumulating evidence that IBD is associated with an imbalance in the composition of the intestinal microbiota, termed dysbiosis. The concept of substantial microbial impact on disease pathogenesis is supported by various mouse models of IBD in which inflammation is ameliorated by their development in a germ-free environment [6]. Yet, it is not clear whether dysbiosis represents a primary or secondary predisposing factor for IBD, as it may be related to, or intensified by other defects [5, 7]. However, there is strong evidence to support that IBD results from an inappropriate innate and adaptive immune response to commensal microorganisms in genetically susceptible individuals, but the precise etiology remains unclear [8].

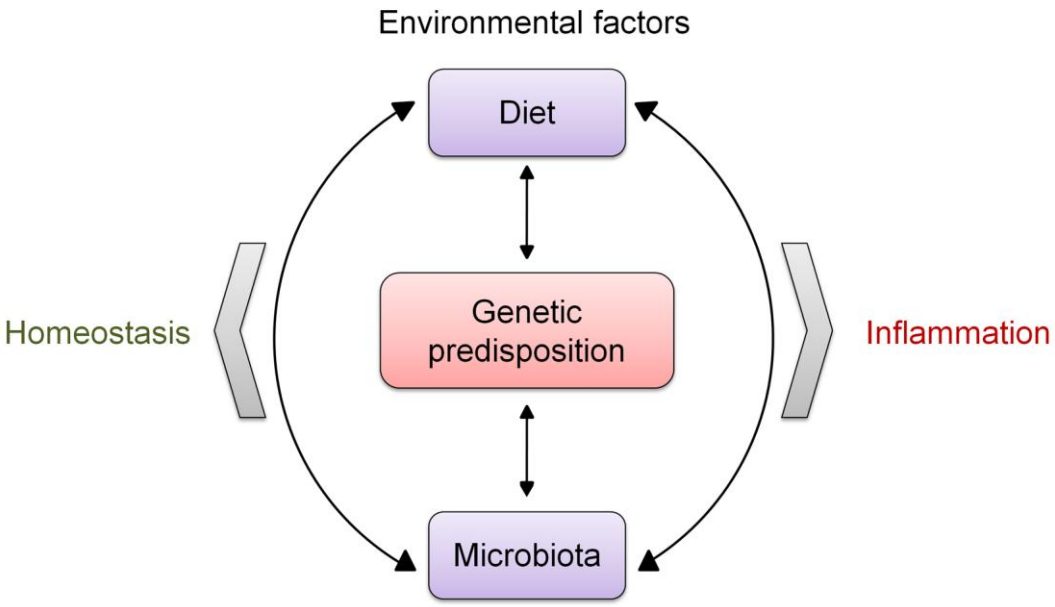


Figure I-1. Interaction of genetic and environmental factors in the pathogenesis of IBD.
The interaction of environmental factors such as diet and the gut microbiota in / with a genetically susceptible host may lead to impaired regulation of intestinal homeostasis associated with chronic mucosal inflammation.

To identify genes that contribute to disease susceptibility, genome-wide association studies (GWAS) have been conducted, successfully revealing 99 non-overlapping genetic risk loci (71 in Crohn's disease and 47 in ulcerative colitis), including 28 that are shared between both types of IBD [9, 10]. In 2001, two groups identified *NOD2* (also designated *CARD15* and *IBD1*), which is an intracellular sensor of bacterial peptidoglycan, as a susceptibility gene in Crohn's disease [11, 12]. Since then, several additional susceptibility loci have been implicated in IBD and confirmed by replication. Analyses of the genes and genetic loci revealed by GWAS show several pathways that are crucial for intestinal homeostasis, including barrier function, epithelial restitution, microbial defence, innate immune regulation, reactive oxygen species (ROS) generation, autophagy, regulation of adaptive immunity, endoplasmic reticulum (ER) stress and metabolic pathways associated with cellular homeostasis [1]. Evidence suggests that there are distinct pathogenic mechanisms related to microbial processing in Crohn's disease and ulcerative colitis, as several genetic risk factors such as *NOD2* and autophagy related *ATG16L1* and *IRGM* are specific to patients with Crohn's disease and are not observed in those with ulcerative colitis. By contrast, multiple genes implicated in the IL-23 pathway, including IL23R, IL12B and STAT3 have been associated with both forms of IBD [13, 14].

Interestingly, more than 50% of IBD susceptibility loci have been found to be associated with other inflammatory and autoimmune diseases as well. Moreover, overlapping genetic risk loci may have contrasting effects in different diseases. For example, protein tyrosine phosphatase, non-receptor type 22 (*PTPN22,* R620W) is associated with altered

responsiveness of T and B cell receptors and is protective against Crohn's disease, while being a strong risk factor for type 1 diabetes and rheumatoid arthritis [15, 16]. These data illustrate the complex interplay between genetic variations and disease phenotypes.

Furthermore, the relatively low concordance rate in monozygotic twins of 10–15% in ulcerative colitis and 30–35% in Crohn's disease suggests that non-genetic environmental factors may have a strong impact on IBD pathogenesis [9, 10]. For example, smoking seems to exacerbate Crohn's disease while being protective against ulcerative colitis, thus being a disease-specific modifier of pathogenesis. Recent evidence suggests that smoking impairs autophagy, a process that seems to play a role especially in Crohn's disease, demonstrating how an environmental factor may mechanistically affect IBD development in a genetically predisposed individual [17]. Apart from smoking, environmental factors such as nonsteroidal anti-inflammatory drugs (NSAIDs), infections and diet have been implicated in the pathogenesis of IBD [18].

Interestingly, over the last several decades, the incidence of IBD has severely increased especially in developing countries with historically low rates for these diseases. Increasing prevalence of IBD has been associated with a spread of “Western lifestyle” and increased status of hygiene in general, which might refer to the Hygiene Hypothesis, postulating that a missing exposure to microbial and/or infectious agents during childhood may negatively affect immune development and predispose to immunological and autoimmune diseases like IBD later in life [19-21]. Yet, the vast majority of studies in this area are impaired by methodological limitations, particularly the reliance on retrospective recall of information making it difficult to determine the importance of the Hygiene Hypothesis in IBD. However, apart from hygiene, an adaptation to “Western diet”, high in fat and protein but low in fruits and vegetables has also been associated with the increasing incidence of IBD in developing countries [22]. There are several biologically relevant mechanisms, by which diet may influence intestinal inflammation, including antigen presentation, prostaglandin imbalance or alteration of the gut microbiota, which may have severe effects on immune and inflammatory responses of the host [23-25]. Accordingly, diet and the effects it has on immune homeostasis are suggested as important factors in the development of IBD.

1.2. The role of nutrition in IBD

1.2.1. Dietary factors in the pathogenesis of IBD

Several studies examined the relationship between specific dietary factors and the risk of IBD, including the macronutrients carbohydrate, fat and protein in general, and subgroups such as refined sugar, fiber, saturated fatty acids, omega-3 and omega-6 fatty acids in specific. Other food groups like fruits, vegetables, meat and certain dairy products have also been investigated [22, 26, 27]. However, only high dietary intake of total fats, PUFAs, omega-6 fatty acids and meat seems to confer to an increased risk of Crohn's disease and ulcerative colitis. By contrast, high intake of fiber and fruits was associated with decreased risk of Crohn's disease and high intake of vegetables pointed at a decreased risk for the development of ulcerative colitis, as demonstrated by a recent meta-analysis of 19 studies comprising 2,609 IBD patients (1,269 Crohn's disease and 1,340 ulcerative colitis patients) and over 4,000 controls [28]. Accordingly, the noticeable rise of Crohn's disease in Japan was analyzed by an epidemiologic study, demonstrating that increased dietary intake of n-6 polyunsaturated fatty acids (PUFAs) and animal protein together with a change towards a more westernized diet were found to be associated with increased risk of developing Crohn's disease [29].

However, in spite of positive results in several clinical settings, the overall findings are equivocal because of insufficient data and methodological limitations, which might reflect that additional environmental and genetic factors modulate the impact of diet on IBD pathogenesis. Though, diet as a source of luminal antigens is still thought to play an important role in the immunopathogenesis of IBD. Whether antibodies against dietary antigens are a primary cause for IBD etiology or secondary to intestinal inflammation is yet to be established [30].

Major efforts have been made to analyze the effects of fatty acids in IBD development and several plausible underlying mechanisms could be revealed. For example, arachidonic acid (AA), derived from omega-6 fatty acids, serves as a precursor for eicosanoids, a family of proinflammatory signaling molecules. By contrast, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which belong to the omega-3 fatty acids, have the ability to antagonize the formation of eicosanoid mediators from AA, which results in suppression of inflammatory cytokines [31]. Omega-3 fatty acids may also decrease the expression of various proinflammatory genes by serving as cofactors for transcriptional factors such as nuclear factor-κB (NF-κB). Furthermore, PUFAs may be integrated into cell membranes and thus can alter membrane structure and function or induce lipoxins and resolvins (signaling molecules involved in the resolution of inflammation) [32]. Effects of fatty acids have also been investigated in animal models of mucosal inflammation. Studies in IL-$10^{-/-}$ and dextran

sodium sulfate (DSS)-induced colitis models showed a reduction in tissue pathology and downregulation of proinflammatory cytokine expression after administration of a diet high in EPA [33, 34]. Moreover, a modulation of intestinal permeability was demonstrated by a study with rats being fed a high-fat diet based on lard (high in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFAs)). Interestingly, these animals showed increased small intestinal permeability due to reduced tight junction protein expression, which was independent of an obese phenotype [35]. Hudert et al. showed that mice, which endogenously biosynthesized omega-3 PUFAs from omega-6 PUFAs produced anti-inflammatory resolvins leading to effective reduction in inflammation and tissue injury in DSS-induced colitis. The protective effect was probably enhanced by higher expression of trefoil factor 3 and zonula occludens-1 (ZO-1) [36].

Less data is available concerning the molecular effects of high protein intake and diets rich in mono-, di- and polysaccharides or total carbohydrates on IBD pathogenesis and studies demonstrated equivocal outcomes [28, 37, 38]. By contrast, clear mechanisms could be attributed to the positive effects of dietary fiber. Fermentable fiber can be metabolized by the microbiota to short-chain fatty acids (SCFAs) (acetate, propionate and butyrate), lactate and gas [39]. These byproducts are suggested to exert anti-inflammatory and anti-carcinogenic functions [40] as exemplified by the SCFA butyrate, which may prevent transcription of proinflammatory cytokines by inhibiting NF-κB. Further, butyrate can enhance peroxisome proliferator-activated receptor (PPAR) γ activation, leading to reduced colonic permeability [41]. PPARγ primarily senses fatty acids and a mutation in PPARγ leading to insufficient activation of toll like receptor (TLR) 4-mediated activation of inflammatory pathways in response to commensal bacteria has recently been found to be associated with a subset of ulcerative colitis patients [42]. Moreover, several studies showed that colonic inflammation could be suppressed via PPARγ induction by dietary supplementation of conjugated linoleic acid (CLA) [43, 44]. This effect seemed to be associated with a CLA-dependent increase of regulatory T-cells [45, 46]. CLA is mainly found in dairy products but can also be produced from linoleic acid by the human gut microbiota [47]. Thus, CLA provides a link between diet, microbial compounds and the control of inflammatory responses related to IBD pathogenesis [48].

Another role for nutrient-specific receptors in the context of IBD is demonstrated by human polymorphisms in the vitamin D receptor (VDR), which is associated with increased susceptibility to IBD development [49, 50]. The active form of vitamin D (1,25-dihydroxycholecalciferol) induces cathelicidin production by specific nutrient-gene interaction, thereby contributing to innate immune defense mechanisms as well as driving immune cell functions towards a regulatory state [51, 52]. Consequently, animal models for IBD deficient

in vitamin D or VDR show increased susceptibility to develop experimental IBD and vitamin D supplementation successfully ameliorated the pathogenesis of colitis in these mice [53, 54].
Apart from direct effects, dietary factors may also have indirect effects on IBD pathogenesis via modulation of the gut microbiota and its metabolites. High-fat diet has been shown to alter the microbiome including a decrease in Bacteroidetes and an increase in both Firmicutes and Proteobacteria independently of an obese phenotype in wild-type and RELMbeta knockout mice [55]. Moreover, it could be demonstrated that dietary fiber and prebiotics not only serve as energy substrates for intestinal bacteria, but may also induce a shift in the microbial composition [56]. Consistently, feeding of infants with breast milk, which besides antimicrobial, anti-inflammatory and immunomodulatory agents contains oligosaccharides that serve as prebiotics, has been shown to decrease the risk of developing Crohn's disease and ulcerative colitis [57-59].

1.2.2. Malnutrition in IBD

Malnutrition is defined by the World Health Organization (WHO) as the cellular imbalance between the supply of nutrients and energy on the one hand, and the body's demand for them to ensure growth, maintenance, and specific functions, on the other [60]. Malnutrition is a common side-effect in patients with IBD, especially in active Crohn’s disease. The most important causes of malnutrition are reduced food intake,[61, 62] presence of active inflammation [63] and enteric loss of nutrients, in periods of disease activity but also during remission [64]. Micronutrient deficiencies are most frequently observed for iron, folate, and vitamin B12, but vitamin E, vitamin A, beta-carotene, magnesium, selenium, and zinc also may be depleted.
Several studies have reported weight loss in 70%-80% of hospitalized IBD patients and in 20%-40% of outpatients with Crohn’s disease [65, 66]. The prevalence of malnutrition is lower in patients with ulcerative colitis, but nutritional deficiencies may develop fast in these patients during periods of active disease [67]. Anaemia is very often seen in patients with IBD, the incidence being up to 80%, [68], most likely because of iron deficiency caused by blood loss through gastrointestinal lesions. Oral iron supplementation seems effective for short periods of time, but intolerance can lead to discontinuation in up to 21% of patients [69]. In addition, a significant number of children with IBD, especially in Crohn’s disease have impaired linear growth. Increased energy expenditure from chronic inflammation, proinflammatory cytokines, such as TNF and IL-1, hormonal imbalances, decreased IGF-1, and exogenous steroids are suggested to be responsible factors. Moreover, as a consequence of malnutrition and malabsorption, protein-energy malnutrition has been found in 20–85% of patients with Crohn’s disease and may further decrease quality of life [62, 70].

1.3. Medical therapy of IBD

1.3.1. Conventional therapy

Several different therapeutics and therapeutic strategies are available for IBD patients. The actual treatment of each patient may however depend on specific goals, such as relief of symptoms, induction of remission in patients with active disease, prevention of relapse, healing of fistulas, or avoidance of emergency surgery. The majority of IBD patients use conventional therapy (namely, aminosalicylates, corticosteroids, antibiotics and immunomodulatory agents) for prolonged periods of time [71]. Classic drug treatments aimed to induce and maintain the patient in remission and ameliorate the disease's secondary effects, rather than modifying or reversing the underlying pathogenic mechanism [72]. However, over the past decade, the goals of therapy for IBD have changed. In addition to induction and maintenance of corticosteroid-free remission, the expectations of treatment have been raised to include mucosal healing, avoidance of surgery and reduced rates of admission to hospital, as well as minimizing adverse effects [73].

Anti-inflammatory 5-aminosalicylic acid (5-ASA, Mesalazine) remains the first-line therapy for both induction and maintenance of remission in ulcerative colitis and is well-tolerated in the majority of patients. However, their use in patients with Crohn's disease remains limited, although some evidence suggests that 5-aminosalicylate therapy could help to prevent recurrence following surgically induced remission [74]. Yet, not all patients respond to 5-ASA therapy within the initial 2–4 weeks and high rates of non-adherence among patients (up to 60%) may affect treatment efficacy [75, 76].

Alternatively, corticosteroids are highly effective agents for inducing remission of active IBD in Crohn's disease and ulcerative colitis patients. Glucocorticoids bind to the glucocorticoid-receptor, which induces a signaling cascade that results in upregulation and downregulation of anti-inflammatory and proinflammatory factors, respectively. However, corticosteroids do not maintain remission and their adverse effects may be severe, which has led to an increased emphasis on limiting the duration and cumulative dose of steroids in IBD [77].

Immunomodulatory drugs may be an alternative therapeutic option for IBD patients. Azathioprine and 6-mercaptopurine have been used for more than 40 years in the treatment of both Crohn's disease and ulcerative colitis. They are effective for both induction [78] and maintenance [79, 80] of remission in both diseases. These thiopurine antimetabolites impair purine biosynthesis and inhibit cell proliferation. Findings from a number of studies highlight the ability of thiopurines to achieve mucosal healing [81, 82], however thiopurine therapy may become ineffective in just over half of all patients and side effects may appear. Although immunosuppressant agents have significant adverse effects, they are safer and better tolerated than long-term corticosteroid therapy [83].

Furthermore, antibiotics may be applied in patients with IBD in specific situations. Antibiotics may be used to induce remission in mild to moderate Crohn's disease. They can also be administered as adjunctive treatment along with other medications for therapy of active IBD and for specific complications or prophylaxis for disease recurrence in postoperative Crohn's disease [84]. Yet, no good evidence supports their use in ulcerative colitis and significant side effects of prolonged systemic antibiotic use must be balanced against their potential benefits in Crohn's disease [85].

1.3.2. Biologicals

The development of the new biologic therapies was made possible by the clarification of the immunopathogenic mechanisms underlying chronic gut inflammation in IBD [86, 87]. In particular, the understanding of the primary role of the potent proinflammatory cytokine tumor necrosis factor (TNF) in mediating the classic lesions of IBD, i.e. ulcers, fistulas, granulomas and intestinal strictures, suggested that targeting this cytokine could offer a better therapeutic chance to IBD patients than conventional therapies [88]. Infliximab belongs to the first generation of anti-TNF antibody-based therapeutics. It is a chimeric immunoglobulin (25% mouse, 75% human) that binds to and neutralizes TNF, representing a new class of therapeutic agents for treating IBD [89]. Although infliximab was specifically designed to target TNF, it also may have more complex actions. Infliximab binds membrane-bound TNF and may cause lysis of these cells by complement- and antibody-dependent cellular cytotoxicity Thus, infliximab may deplete specific populations of subepithelial inflammatory cells [90, 91]. Its long-term role in Crohn's disease is evolving, but emerging evidence supports its efficacy in inducing and maintaining remission, healing mucosa, preventing recurrence of fistulas, reducing hospitalizations and surgical operations [92, 93]. The impact of anti-TNF therapy on mucosal healing has also been investigated in ulcerative colitis. Infliximab achieved mucosal healing in nearly half of ulcerative colitis patients after 54 weeks in randomized double-blind placebo-controlled trials [94]. Unfortunately, about 50% of IBD patients undergo relapse within 1 year after cessation of infliximab [95]. Moreover, loss of response, also known as secondary non-response, occurs in a significant proportion of patients treated with anti-TNF therapy, and its risk has been estimated to be 13% per patient and year (e.g. due to development of anti-drug antibodies (ATI)) [96]. The relatively high costs of anti-TNF antibodies and their potential toxicity, mostly related to opportunistic infections and malignancy, have led to debate regarding the time of starting and the duration of treatment with biologicals [97].

The introduction of immunomodulators such as Azathioprine, and biologicals like antibodies to TNF has shifted the benchmark from clinical remission toward mucosal healing, which could not be accomplished by corticosteroids. However, one-third of patients do not clinically respond to anti-TNF antibodies, and durable remission is achieved in only a minority of the responder patients [96].

1.4. Nutritional therapy in IBD

1.4.1. Maintenance of remission

The European Society for Parenteral and Enteral Nutrition (ESPEN) [98] published guidelines for the role of enteral nutrition (EN) in patients with IBD suggesting oral nutritional supplements including tube feeding in undernourished patients with Crohn's disease or ulcerative colitis to improve nutritional needs. Thus, EN has often been used as an adjunctive therapy to correct or to avoid malnutrition [99-101]. In addition, several authors have considered EN as a strategy to induce and maintain remission in patients with Crohn's disease using supplementary oral formula in combination with a regular diet throughout the day. This approach may also be used adjunctive to medical therapy [99, 102, 103]. There have been several studies assessing the efficacy of EN for maintenance of medically [104-106] or surgically [107-109] induced remission. Four prospective trials compared maintenance of remission between patients who received EN (elemental diet) and patients who did not. All of these studies showed a significantly better clinical remission rate in patients who had been treated with elemental diet, and notably, in one of these studies, [105] elemental diet therapy was associated with an improved endoscopic disease activity index. This observation suggested that EN may alleviate mucosal inflammation and this effect may promote better remission outcome. Thus, long-term EN supplementation may significantly reduce clinical and endoscopic recurrence after resection in Crohn's disease. Moreover, a retrospective review of children with new diagnosed Crohn's disease confirmed the superiority of eight weeks of enteral feeding over treatment with corticosteroids in maintaining remission, improving nutrition status, and recovering linear growth. In addition, mucosal healing was confirmed endoscopically in children on EN [110]. However, in contrast to Crohn's disease, EN therapy has not been adequately evaluated and seems to be less effective in patients with active ulcerative colitis [111, 112].

1.4.2. Induction of remission

1.4.2.1. Parenteral nutrition (PN)

Dudrick et al. [113] were the first to suggest that PN was safe and possibly beneficial to patients with IBD. The use of PN for managing adults with Crohn's disease succeeded in achieving clinical remission and avoiding surgery [113, 114]. However, the remission was often short lived and the number of patients remaining in remission three months later varied between 20% and 79% depending on the length of PN administration, population of patients, definitions of remission or recurrence and simultaneous use of medications [115]. PN was also proven a useful adjunctive therapy for ulcerative colitis patients requiring bowel rest and nutritional support, though not effective in induction of remission [116].

Since EN was shown to be at least as efficient as PN including lower costs and fewer significant side effects, the indications for PN support are restricted to severe malnutrition and for pre- and postoperative nutritional support, in both Crohn's disease and ulcerative colitis [117, 118].

1.4.2.2. Enteral nutrition (EN)

EN was shown to induce clinical remission, improve nutritional status and body composition, induce mucosal healing, decrease pro-inflammatory cytokine levels and reduce serum inflammatory markers in patients with Crohn's disease [119-121]. Three meta-analyses and two Cochrane Database Systematic Reviews published in recent years examined the efficacy of EN compared with corticosteroid therapy in Crohn's disease. The most recent included 192 patients treated with EN and 160 treated with steroids, which yielded a pooled OR of 0.33 favoring steroid therapy (95% CI: 0.21-0.53), and patients in whom remission was achieved, the relapse rates at 12 months were identical (65% and 67%) regardless of the therapy [122-124].

In spite of data suggesting that corticosteroid therapy produces higher efficacy rates as compared with EN in inducing remission, the results of dietary intervention studies should be interpreted with reservations. There are numerous factors that potentially may influence the efficacy of EN, such as population demography, study design, compositions of enteral formulae, route of administration, patient compliance and timing of endpoint assessment as well as definition of clinical remission. Additionally, the long-term efficacy of EN is rarely reported and therefore, is less well-established [30]. Thus, comparison of efficacy alone between EN and corticosteroids is not sufficient, as the two treatment modalities possess entirely different safety profiles. EN has minor, immediate side effects and no known long-term adverse effects, in contrast to corticosteroid therapy that may exhibit severe

complications. Especially for the patients who are steroid-refractory, steroid-dependent or steroid-intolerant, EN should be considered as alternative treatment strategy. As adjunctive therapy, EN is recommended for any malnourished patient or for patients with difficulty in maintaining normal nutritional status [99]. However, there is no evidence to support the use of EN as primary therapy in ulcerative colitis [112].

1.4.2.3. Exclusive enteral nutrition (EEN) in pediatric Crohn's disease

A number of controlled clinical trials conducted in the late 1980s demonstrated that elemental diet was effective in inducing clinical remission in children with active Crohn's disease [125]. A meta-analysis of five randomized clinical trials comprising 147 pediatric patients demonstrated that EEN was as effective as corticosteroids, regarding the induction of remission in children [relative risk = 0.95 (95% CI 0.67-1.34)] [126]. Besides improved growth and nutritional status, treatment with EEN may induce remission in up to 85% of newly diagnosed patients, while avoiding the adverse effects of steroids. These findings make EEN the preferred choice of treatment in children with active Crohn's disease [126, 127]. Thus, in the current European guidelines for the treatment of active Crohn's disease in children, EEN is recommended as the primary therapy [128].

Johnson et al. investigated whether partial enteral nutrition (PEN) might be as effective as EEN in induction of remission in pediatric Crohn's disease. They randomized children with active Crohn's disease to either receive all of their nutrition as elemental diet (EEN) or only 50% (PEN). Total nutritional intake was similar in both groups, but the remission rate was higher in the EEN group (42%) compared with the PEN group (15%) [129]. Regarding the effects of disease location on EEN efficacy, studies in children showed contradictory results. Two studies found no difference in the remission rate of children with ileal *versus* isolated colonic disease [130, 131], in contrast, another report noted a decreased response rate in patients with isolated colonic disease. There is no convincing evidence that the effect of EEN is restricted to small bowel disease only but the impact of disease location and other environmental factors on clinical response to EEN requires further evaluation [132]. In general, EEN is safe and well tolerated with minimal side effects such as nausea, abdominal pain, flatulence, or diarrhea [133]. The only reported severe adverse effect associated with EEN is a single case of re-feeding syndrome [134].

Numerous studies demonstrated that corticosteroids failed to induce mucosal healing in the treatment of Crohn's disease. By contrast, EN showed positive effects of on mucosal integrity in several studies [135, 136] associated with downregulation of mucosal proinflammatory cytokine profiles in both the ileum and the colon. Given that the ultimate goal in the treatment

of Crohn's disease is mucosal healing (in addition to symptomatic improvement) this advantage of EN over corticosteroids seems valuable regarding the choice of a therapeutic strategy [137].

1.5. Celiac disease

In contrast to IBD, in celiac disease, the role of diet as environmental factor seems quite clear and nutritional means are highly efficient for treatment. Celiac disease is one of the most common inflammatory disorders of the small intestine, with an incidence of 1% in western populations. In genetically susceptible individuals (human leukocyte antigen (HLA)-DQ2 or HLA-DQ8), the ingestion of gluten containing cereals trigger an immune-mediated enteropathy [138]. Deamidation of gliadin peptides by tissue transglutaminase (tTG) enables them to be bound with high affinity to HLA-DQ2 or HLA-DQ8 molecules of antigen presenting cells. The inflammatory process can be avoided by a gluten-free diet, which will normalize the clinical manifestations, represented by mucosal inflammation, crypt hyperplasia, small intestinal villous atrophy, increased intestinal permeability and elevated levels of intraepithelial lymphocytes (IELs) [139-141].

Another common disorder of the gastrointestinal tract is irritable bowel syndrome (IBS), a functional disorder that leads to symptoms such as abdominal pain, cramping and changes in bowel movements. Interestingly, in clinical practice, some patients for which celiac disease has been excluded show symptoms of IBS and respond well to a gluten-free diet [142]. Increasing discussion has emerged over the last several years, regarding the topic of gluten sensitivity as a cause of IBS symptoms, termed "non-celiac gluten intolerance" [143-145]. Indeed, wheat has been identified to be one of the most common inducers of gastrointestinal symptoms and gluten is suggested to be the major factor. Yet, it is not known whether gluten is the only responsible agent for enteral irritation in this context [146, 147].

1.6. Animal models of IBD

Understanding the complex interplay between genetic and environmental factors that lead to IBD pathogenesis is a demanding challenge. Animal models are useful tools for this respect, providing fundamental insight into the importance of genetic and immunologic regulatory mechanisms.

1.6.1. Ileitis model ($TNF^{\Delta ARE/WT}$ mouse)

Anti-TNF neutralizing antibodies are efficient in induction of remission in IBD therapy [97]. However, the specific molecular and cellular mechanisms of the pathogenic action of TNF are still not completely understood. Gene targeting of AU-rich elements (ARE) in the untranslated region of the TNF mRNA in mice ($TNF^{\Delta ARE/WT}$ mice) leads to impaired regulation of TNF translation and development of a severe ileitis. The $TNF^{\Delta ARE/WT}$ mouse is one of the very few Crohn's disease-like models leading to transmural inflammation in the terminal ileum. Progression of pathology is accompanied by villous atrophy, crypt hyperplasia and intestinal leukocyte infiltration, closely resembling the clinical manifestations of human Crohn's disease. Intestinal inflammation in $TNF^{\Delta ARE/WT}$ mice is associated with early reduction of CD8αα-expressing intraepithelial lymphocytes and predominance of TNF/IFN-γ producing CD8αβ lymphocytes in the epithelium. Furthermore, lamina propria $CD4^+$ lymphocytes show decreased Th1 and increased Th17 responses and increased αEβ7 integrin expression can be observed in peripheral intestinal-homing CD8αβ lymphocytes [148, 149]. Interestingly, selective chronic overproduction of TNF by intestinal epithelial cells (IEC) suffices to cause full development of intestinal pathology and is associated with early activation of the underlying intestinal myofibroblast, a cell type previously identified as a sufficient target of TNF for disease development in the $TNF^{\Delta ARE/WT}$ mouse model [150]. The pathogenesis of chronic ileitis seems to be $CD8^+$ T cell dependent, as genetic ablation of beta-2 microglobulin or β7 integrin results in complete amelioration of intestinal pathology whereas genetic deletion of CD4 exacerbates Crohn's disease-like ileitis [151].

1.6.2. Colitis model ($IL\text{-}10^{-/-}$ mouse)

IL-10 is a well-known suppressor of Th1 cells and macrophage effectors functions. Several *in vitro* studies have shown that IL-10 inhibits IL-12 and TNF production, T cell proliferation and costimulatory B.7.1 and B.7.2 molecule expression. Furthermore, IL-10 may also promote the formation of antigen-specific regulatory T cells [152]. Mice with targeted deletion of the IL-10 gene ($IL\text{-}10^{-/-}$) spontaneously develop a chronic enterocolitis with massive infiltration of

lymphocytes, neutrophils and activated macrophages [153]. IL-$10^{-/-}$ colitis is accompanied by a Th1 cytokine response, which can be ameliorated by neutralizing antibodies to IL-12p40 and to a lesser extent IFN-γ or systemic administration of recombinant IL-10. Colitis in IL-$10^{-/-}$ mice does not develop under germ-free conditions and is thus suggested to be driven by antigens of the mucosal microbiota [154].

1.6.3. T cell transfer model of colitis and ileitis

Adoptive transfer of $CD4^{+}CD45RB^{Hi}$ T cells isolated from the spleens of immunocompetent donor mice in immunodeficient $RAG2^{-/-}$ recipient mice causes a wasting syndrome with transmural intestinal inflammation primarily in the colon. Inflammation starts five to ten weeks after T cell transfer, depending on the microbiota [155-157]. Adoptive transfer of $CD4^{+}$ $IL10^{-/-}$ T cells is also a suitable method to induce experimental colitis and several studies identified IL-10 and TGF-β as central anti-inflammatory factors in this model. Regulatory T cells prevent the onset of gut inflammation and antigen-specific immune responses, when transferred together with pathogenic $CD4^{+}CD45RB^{Hi}$ T cells. Systemic administration of recombinant IL-10 or TGF-β has been shown to inhibit pathogenesis in a similar way. Moreover, bacterial antigens play a crucial role for pathology since treatment with antibiotics or germ-free breeding of recipient mice is associated with significantly less inflammatory response [158]. Another interesting yet underappreciated aspect of this model is the development of small bowel inflammation, which is characterized by leukocyte infiltration and loss of goblet cells and paneth cells most noticeable in the ileum [159].

1.6.4. Elemental diet and animal models of mucosal inflammation

The beneficial effect of enteral nutrition using elemental diet (ED) in the treatment of Crohn's disease has been demonstrated frequently. Alteration of the gut microbiota, low antigenicity and low fat content of the diet as well as improvement of nutritional status are suggested to play a role in the anti-inflammatory effect of ED, however the exact mechanisms remain to be elucidated.

Menezes et al. tested the effects of ED and Chow diet on conventional C57BL/6 wild type mice and compared differences in immunological parameters. The authors demonstrated that ED-fed mice presented an underdeveloped gut-associated-lymphoid tissue (GALT) with lower numbers of $TCR\alpha\beta^{+}$ IELs and lamina propria cells and low levels of secretory IgA when compared to Chow-fed mice. ED-fed mice showed a systemic decrease in the production of IgG and IgA as well as a skewing towards a Th2 profile of cytokine production upon *in vitro* stimulation with increased IL-4 and a reduced IFN-γ and IL-6 secretion [160]. Interestingly,

Shou et al. found significant bacterial translocation to mesenteric lymph nodes but not other organs in conventional C57/BL6 mice being fed an ED. Further experiments suggested that enteral feeding of ED downregulated host oxidative and antimicrobial mechanisms and TNF-dependent cytotoxicity in conventional mice [161]. Furthermore, a rat model of granulomatous enteritis induced by a single intramural injection of peptidoglycan-polysaccharide showed significantly decreased macroscopic and histologic damage scores when being fed ED compared with rats being fed a control diet. ED inhibited the increase in the numbers of macrophages and IL-2R-positive T-cells in Peyer's patches, associated with decreased production of nitric oxide and generation of oxygen free radicals [162]. In IL-10$^{-/-}$ mice, partial replacement of dietary omega-6 fatty acids with medium-chain triglycerides in an experimental diet decreased the incidence of spontaneous colitis. Feeding of the experimental diet resulted in fewer total and apoptotic intraepithelial CD3^{+} and lamina propria CD3^{+}CD4^{+} lymphocytes, as well as reduced expression of IL-6, interferon-y and TLR9 [163]. Accordingly, in a T-cell transfer model of colitis using IL-10$^{-/-}$ donor mice in combination with SCID recipients, ED significantly suppressed intestinal inflammation. The total amount of cecal bacteria decreased in ED-fed mice and the diversity of bacterial species decreased to 60% of that found in the regular diet groups, suggesting a strong impact of ED on the gut microbiota [164]. Another study was performed in TCR α$^{-/-}$ mice to assess the potential role of luminal antigens on the development of chronic colitis. ED-fed TCR α$^{-/-}$ mice showed no pathologic features of colonic inflammation. The protective state was associated with suppressed mucosal B-cell responses and decreased production of Th2-type cytokines in ED-fed mice compared with mice on regular diet. However, rectal administration of *Bacteroides vulgatus* resulted in development of intestinal inflammation. The authors concluded that ED-induced alteration of the intestinal microbiota prevented the development of IBD in TCR α$^{-/-}$ mice [165].

These studies demonstrate that ED may have beneficial effects on intestinal inflammation in several mouse models with diverse genetic backgrounds. A low antigenicity of the diet in combination with indirect immunomodulatory effects through an alteration of the gut microbiota might influence mucosal immune responses and maintain intestinal homeostasis. However, the precise mechanism of action remains to be revealed.

2. AIMS OF THE WORK

Nutritional therapy may be a valuable option for the treatment of IBD patients, with minimal adverse effects compared with conventional therapeutics. However, the underlying mechanisms are not completely understood and data from clinical studies present equivocal results. Animal models may serve as a tool to clarify the complex interactions of genetics, environmental factors and microbial influence on disease pathogenesis.

We aimed at elucidating the impact of dietary intervention therapy on inflammation development in animal models with a strong genetic predisposition. In our study, we focused on the $TNF^{\Delta ARE/WT}$ mouse model to evaluate the effects of a semi-elemental diet on the pathogenesis of Crohn's disease-like ileitis at histological, cellular and transcriptional level in combination with functional analysis of diet-induced alterations of the cecal microbiota. In addition, IL-$10^{-/-}$ mice and a T-cell adoptive transfer model were used to analyze the effects of dietary intervention on colitis and ileitis development with respect to different genetic predispositions.

3. MATERIALS AND METHODS

3.1. Animals

Heterozygous $TNF^{\Delta ARE/WT}$ and WT mice (C57BL/6 background) were conventionally raised at constant room temperature (22 ± 2 °C), air humidity (55 ± 5%), and a light/dark cycle of 12/12 h. Ultrapure water and standard Chow diet (Ssniff R/M-H, Soest, Germany) were provided *ad libitum*. At seven weeks of age, mice were transferred to a semi-synthetic experimental diet (Ssniff E15000, Soest, Germany or Altromin C1000, Lage, Germany) or as indicated in the experiment. Mice were sacrificed by cervical dislocation at the age of 18 weeks or as indicated.

Interleukin-10 deficient ($IL\text{-}10^{-/-}$) mice (129/SvEv background) were raised under specific pathogen-free (SPF) conditions. $IL10^{-/-}$ mice were moved to a conventional animal facility at seven weeks of age being provided ultrapure water and standard Chow (Ssniff R/M-H, Soest, Germany) or experimental diet (Altromin C1000, Lage, Germany) *ad libitum*. Mice were sacrificed by cervical dislocation at the age of 24 weeks.

Lymphocyte-deficient $Rag2^{-/-}$ mice (129/SvEv background) were reconstituted with $5x10^5$ $CD4^+$ $IL\text{-}10^{-/-}$ T-cells suspended in 200µl of PBS by intraperitoneal injection at 7 weeks of age. $CD4^+$ $IL\text{-}10^{-/-}$ T-cells were positively selected from donor $IL\text{-}10^{-/-}$ mice (129/SvEv background, SPF conditions) using FITC-conjugated anti-CD4 antibodies (FITC Rat Anti-Mouse CD4, BD Pharmingen) and anti-FITC MicroBeads (LS columns, Miltenyi Biotec, Bergisch Gladbach, Germany) in combination with magnetic cell separation columns (LS columns, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of isolated $CD4^+$ $IL\text{-}10^{-/-}$ T-cells was determined by flow cytometric analysis using the LSRII flow cytometer in combination with FACSDiva software (BD Bioscience) and was >90% in all experiments. Recipient $Rag2^{-/-}$ mice were moved from SPF to conventional conditions two weeks before transfer. Ultrapure water and standard Chow diet (Ssniff R/M-H, Soest, Germany) or experimental diet (Ssniff E15000, Soest, Germany) were provided *ad libitum*. Body weights were measured once a week and mice were sacrificed by cervical dislocation 10 weeks after transfer.

3.2. Diets

The following diets were used as indicated in the experiments (**Table M1**).

Table M1. Diet description and information.

Description	Name	Manufacturer
Semi-synthetic experimental diet	C1000	Altromin, Lage, Germany
Semi-synthetic experimental diet	E15000	Ssniff, Soest, Germany
Experimental diet fortified with 10g Chow/kg	1% Chow	Ssniff, Soest, Germany
Experimental diet fortified with 100g Chow/kg	10% Chow	Ssniff, Soest, Germany
Experimental diet fortified with 300g Chow/kg	30% Chow	Ssniff, Soest, Germany
Experimental diet fortified with 700g Chow/kg	70% Chow	Ssniff, Soest, Germany
Standard Chow diet	R/M-H V1534-0	Ssniff, Soest, Germany
Autoclavable Chow diet	R/M-H V1534-3	Ssniff, Soest, Germany
Experimental diet fortified with 10g Gluten/kg	Glu	Altromin, Lage, Germany
Wheat depleted Chow diet	Wheat (-W)	Ssniff, Soest, Germany

Gluten was purchased from Sigma-Aldrich (Steinheim, Germany). For details about nutrient composition, see Appendix 1.

3.3. Histological scoring

Tissues were fixed in 4% neutral buffered formalin and embedded in paraffin. Distal ileal or distal colonic sections (5 µm) were stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, Steinheim, Germany). Histological scoring was performed by blindly assessing the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion and architectural distortion in the different gut sections, resulting in a score from 0 (not inflamed) to 12 (massively inflamed), as previously described [166].

3.4. Immunohistochemical labeling

Paraffin embedded tissue was cut into 5µm sections using a Leica RM2255 and applied onto polylysine coated slides, air dried at room temperature for 1h and dried at 37°C over night. Samples were deparaffinized and antigens were unmasked by incubation in boiling 10mM sodium citrate buffer (pH 6.0) (Roth, Karlsruhe, Germany) for 10min. Immunostaining was performed according to the protocol provided by Cell Signaling. Anti-CD3 antibody (Abcam,

Cambridge, UK) was used in combination with AlexaFluor 546 goat anti rabbit IgG (Invitrogen Carlsbad, USA). Briefly, for labeling, slides were washed in PBS (pH 7.4) and blocked with 5% normal goat serum for 60min, followed by incubation with the primary antibody overnight at 4°C. Slides were washed in PBS and incubated with the fluorochrome-conjugated secondary antibody for 90 min at RT. Confocal microscopy was performed using LAS AF Version 2.3.0 (Leica Microsystems).

3.5. RNA isolation, reverse transcription and real-time PCR

Tissue samples (0.5 cm) from distal ileum and distal colon were ground in liquid nitrogen. Total RNA was isolated using the column-based RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentrations and purity (A260/A280 ratio) was determined by spectrophotometric analysis (ND-1000 spectrophotometer, NanoDrop Technologies, Willigton, USA). Reverse transcription was performed using 1µg total RNA. The adequate amount of RNA for 1µg was mixed with 1µl random-hexamer primers (500ng/µl) and filled up to 12µl with PCR-H_2O. After incubation for 5min at 65°C in a thermocycler, 8µl of a solution containing 4µl of 5x First Strand Buffer, 2µl of 0.1M DTT, 1µl of RNase Out (40U/µl) and 1µl MMLV-reverse transcriptase (200U/µl) were added to each sample. The mixture was incubated for 60min at 37°C using a thermocycler, followed by heating to 99°C for 1min.

Real-time PCR was performed using 1µl cDNA in a Light Cycler ® 480 (Roche Diagnostics, Mannheim, Germany) applying the Universal Probe Library system according to the manufacturer's protocol. Relative induction of gene mRNA expression was normalized to GAPDH expression and calculated as fold change against the mean of the control group using the Light Cycler® 480 software. Primer sequences are given in **Table M2**.

Table M2. Primer sequences for real-time PCR.

Targert gene	Forward primer	Reverse primer	UPL probe
Tnf (NM_013693.2)	5'-tgcctatgtctcagcctcttc-3'	5'-gaggccatttgggaacttct-3'	#49
Ifng (ENSMUST00000068592.3)	5'- ggaggaactggcaaaaggat-3'	5'- ttcaagacttcaaagagtctgagg-3'	#21
Tlr2 (AF124741.1)	5'- ggggcttcacttctctgctt-3'	5'- agcatcctctgagatttgacg-3'	#50
Tlr4 (NM_021297.2)	5'-ggactctgatcatggcactg-3'	5'-ctgatccatgcattggtaggt-3'	#2
Tlr5 (NM_016928.2)	5'-ctggagccgagtgaggtc-3'	5'-cggcaagcattgttctcc-3'	#1
Lbp (NM_008489.2)	5'-acctctgacctgcagcctta-3'	5'-ggacattgtcgatctctgctg-3'	#53
Tlr9 (NM_031178.2)	5'-gaatcctccatctcccaacat-3'	5'-ccagagtctcagccagcact-3'	#79
Nod2 (NM_145857.2)	5'-tgtggagtcaccgcaaaac-3'	5'-tcctctgtgcctggaactct-3'	#100
Madcam1 (L21203.1)	5'- ggggaggtgaccaatctgta-3'	5'- ataggacgacggtggagga-3'	#72
Icam1 (NM_010493.2)	5'- cccacgctacctctgctc-3'	5'- gatggatacctgagcatcacc-3'	#81
Ip10 (NM_021274.1)	5'- gctgccgtcattttctgc-3'	5'- tctcactggcccgtcatc-3'	#3
Il15 (NM_008357.1)	5'- cagctcagagaggtcaggaaa-3'	5'- catgaagaggcagtgctttg-3'	#106
Ocln (NM_008756.2)	5'- tccgtgaggccttttgaa-3'	5'- ggtgcataatgattgggtttg-3'	#10
Zo1 (ENSMUST00000102592.2)	5'- cgcggagagagacaagatgt-3'	5'- agcgtcactgtgtgctgttc-3'	#81

3.6. IEL/LPL isolation

Mice were sacrificed and the ileum was removed. Mesenteric fat and peyer's patches were trimmed off. The ileum was cut open longitudinally and washed with wash buffer (HBSS, 2% FBS). Digestion buffer (HBSS, 5mM EDTA, 1mM DTT, 10% FBS) was freshly prepared before use and prewarmed in 37°C water bath until use. The tissue was cut in 0.5 cm pieces and incubated with 5 ml digestion buffer in a horizontal shaker at 220 rpm, 37°C, 30 min. Undigested tissue residues were mashed through a 100 µm cell strainer (BD Falcon). Tissue cell suspensions were combined and centrifuged at 350 g, 4°C, 5 min. For density gradient centrifugation, the pellet was resuspended in 40% isotonic percoll (Sigma-Aldrich, Steinheim, Germany) and put beneath a 20% isotonic percoll layer. Another layer of 80% isotonic percoll was put beneath both layers. Density gradient centrifugation was performed at 720 g, 20 min, 4°C. The IEL/LPL fraction was collected at the interface of 80% and 40% percoll layers. Cells were washed in wash buffer, counted using a Neubauer counting chamber and prepared for flow cytometric analysis.

3.7. Flow cytometry

For flow cytometric analysis, cells were washed in staining buffer (HBSS, 2% FBS), centrifuged at 350 g, 5 min, 4°C and resuspended in 50 µl staining buffer ($1x10^6$ cells in 50 µl). Staining solution (50 µl staining buffer including a mixture of fluorochrome conjugated antibodies (1 µl each) as indicated in the experiments) was added and incubated at 4°C, 20 min. Cells were washed, resuspended in 500 µl staining buffer and analyzed ($1x10^4$ cells were acquired per sample) using the LSRII flow cytometer in combination with FACSDiva software (BD Bioscience). The following antibodies were used: Anti-CD3-APC-Cy7, Anti-CD4-PE-Cy7, Anti-CD8α-PE, Anti-CD8β-FITC (all from BD Pharmingen). Regulatory T-cells were analyzed using the Mouse Th17/Treg Phenotyping Kit (BD Pharmingen) according to the manufacturer's protocol.

3.8. Particle size distribution measurement

Diet pellets (50 g) were dissolved in dH_2O (500 ml) at RT on a magnetic stirrer. Ceca and small intestines were dissected from animals, cecal and ileal content was collected and dissolved in dH_2O (100 mg/ml).
The samples were analyzed by laser diffraction (Malvern Mastersizer MS2000, software version 5.6) with wet analysis using the Hydro 2000S dispersion unit according to the the manufacturer's protocol. Data were compared based on calculated volume based particle size D[4,3].

3.9. FT-IR spectroscopy

Cecal contents were diluted in PBS (1:10), vortexed and centrifuged at 350 g, 5 min, RT. Supernatants were collected and centrifuged at 10000 g, 3 min, RT. Supernatants were discarded and pellets were resuspended in the same volume of dH_2O. Samples (technical duplicates) were transferred onto a ZnSe support material (Bruker) and dried (44°C, 30 min). Spectra were recorded using an IFS-28B FT–IR spectrometer (Bruker) and analyzed using OPUS (Bruker, software version 3.1). The average linkage algorithm was used for cluster analysis on vector normalized first derivatives of the spectra.

3.10. Cecal lysates

Cecal lysates (CL) were prepared from cecal contents of WT and TNF$^{\Delta ARE/WT}$ mice on Chow and experimental diet as indicated in the experiments. CL preparation was performed as previously described [167, 168]. Briefly, the cecum was dissected, cecal content was collected, diluted in sterile PBS (1:1) and vortexed thoroughly. DNAse I (0.1 mg/ml) was added and incubated for 15 min at RT. Cecal suspensions (0.5 ml) were mixed with mg of 0.1 mm glass beads (ROTH) and disrupted in a bead-beater (FastPrep-24, MP Biomedicals LLC) at 6.5 M/s, 0.5 min (four cycles interrupted by 3 min on ice). The glass beads and unlysed cells were removed by centrifugation at 350 *g* for 5 min. Supernatants were collected and centrifuged at 10000 g, 10 min. After centrifugation, the supernatant was filter-sterilized (0.22 µm filter) and the protein concentration was adjusted to 1.0 mg/ml with PBS using a Bradford assay according to the manufacturer's protocol (Roti-Quant, Roth, Karlsruhe, Germany). Cecal lysates were aliquoted and frozen at −80°C.

3.11. Dietary suspensions

Diet pellets were ground under sterile conditions and dissolved in sterile PBS (3 g in 12 ml). Suspensions were vortexed and incubated in a horizontal shaker at 220 rpm, 37°C, 30 min. Dietary suspensions (DS) were vortexed and centrifuged at 350 g, 5 min. Supernatants were collected and centrifuged at 10000 g, 10 min. After centrifugation, the supernatant was filter-sterilized (0.22 µm filter) and the protein concentration was adjusted to 1.0 mg/ml with PBS using a Bradford assay according to the manufacturer's protocol (Roti-Quant, Roth, Karlsruhe, Germany). DS were aliquoted and frozen at −80°C.

3.12. Peptic-tryptic digestion of gluten (PT-gluten)

PT-gluten was prepared as previously described [169]. Briefly, gluten from wheat (Sigma-Aldrich, Steinheim, Germany) was dissolved (1 mg/ml) in 10 mM HCl (pH 1.8) and pepsin (Sigma-Aldrich, Steinheim, Germany) was added (1:100 enzyme:substrate ratio). Gluten was digested for 4 h at 37°C in a water bath. NaOH was added to adjust the ph to 7.8. Trypsin was added at an enzyme/substrate ratio of 1:100 in 100 mM ammonium bicarbonate (pH 8) and the mixture was incubated for 4 h at 37°C. The reaction was stopped by heating (95°C, 10 min). The mixture was centrifuged at 10000 g for 10 min. After centrifugation, the

supernatant was filter-sterilized (0.22 µm filter) and the protein concentration was adjusted to 1.0 mg/ml with PBS using a Bradford assay according to the manufacturer's protocol (Roti-Quant, Roth, Karlsruhe, Germany). PT-gluten was aliquoted and frozen at −80°C.

3.13. Isolation of MLNs and splenocytes

Mesenteric lymph nodes or spleens were dissected from mice and immediately stored in cold T-cell culture medium (RPMI 1640 medium (Invitrogen, Carlsbad, USA) supplemented with 10% FBS (Biochrom, Berlin, Germany), 1% Antibiotic-Antimycotic (Invitrogen, Carlsbad, USA)) and mashed through a 100 µm cell strainer (BD Falcon). Mesenteric lymph node leukocytes (MLNs) or splenocytes were resuspended in 10 ml PBS and centrifuged at 350 g, 5 min, 4°C. The pellet was washed with 10ml PBS, centrifuged, resuspended in 5 ml red blood cell lysis buffer (0.8% NH_4Cl, 0.1% $KHCO_3$, 0.0037% $Na_2EDTA*3H_2O$ in H_2O, pH 7.2) and incubated for 5min at RT. The reaction was stopped by adding 15 ml PBS. After filtering (70µm cell strainer) and centrifugation, the pellet was resuspended in 10 ml PBS. Cells were counted using a Neubauer counting chamber and prepared for flow cytometric analysis or used in cell culture experiments.

3.14. Cell culture and stimulation

Total MLNs were isolated from mesenteric lymph nodes and seeded at $2x10^6$ cells/ml (100 µl/well) in 96-well cell culture plates (Nunc, Roskilde, Denmark) in T-cell culture medium (RPMI 1640 (Invitrogen, Carlsbad, USA), 10% FBS (Biochrom, Berlin, Germany), 1% Antibiotic-Antimycotic (Invitrogen, Carlsbad, USA)). Cells were stimulated with Cecal lysates (CL), Dietary suspensions (DS) or peptic-tryptic digests of gluten (PT-gluten) at different concentrations (10 µg/ml, 50 µg/ml, 100 µg/ml) as indicated in the experiments. MLNs were cultured for 24 h in a humidified 5% CO_2 atmosphere at 37°C. Cell culture plates were centrifuged at 350 g, 4°C, 5 min and supernatants were collected for subsequent ELISA analysis.

3.15. $CD4^+$ T-cell isolation

$CD4^+$ T-cells were positively selected from MLN or splenocyte cell suspensions using FITC-conjugated anti-CD4 antibodies (FITC Rat Anti-Mouse CD4, BD Pharmingen) and anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in combination with magnetic cell separation columns (LS columns, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Briefly, cells were washed in staining buffer (HBSS, 2% FBS), centrifuged at 350 g, 5 min, 4°C and resuspended in 100 µl staining buffer. Staining solution (50 µl staining buffer, 1 µl anti-CD4 antibody) was added and incubated at 4°C, 20 min. Cells were washed, resuspended in 100 µl staining buffer and mixed with 300 µl MicroBeads solution (260 µl staining buffer + 40 µl anti-FITC MicroBeads). Cells were incubated for 10min on ice, washed and resuspended in 1ml staining buffer. Labeled $CD4^+$ T-cells were isolated by magnetic cell separation technology using LS Columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of isolated $CD4^+$ T-cells was determined by flow cytometric analysis using the LSRII flow cytometer in combination with FACSDiva software (BD Bioscience) and was >90% in all experiments.

3.16. Generation of BM-DCs

DC from mouse bone marrow were generated as previously described [170]. Briefly, Wt mice (C57BL/6 background) were sacrificed by cervical dislocation. Femurs and tibiae were removed and cleaned from muscle tissue. Bones were washed in PBS on ice and transferred to 70% EtOH for 3 min at sterile conditions. Bones were washed in sterile PBS and cut at each end with sterile scissors. The bone marrow was flushed through a cell strainer (50 µm) into a 50 ml tube using a syringe (27G needle) and sterile PBS. Cells were pelleted at 350 g, 4°C, 5 min and resuspended in 10 ml PBS. Cells were counted using a Neubauer counting chamber, washed and seeded in petri dishes at $0.2x10^6$ cells/ml in10 ml BM-DC culture medium (RPMI 1640 (Invitrogen, Carlsbad, USA), 10% FBS (Biochrom, Berlin, Germany), 50µM 2-mercaptoethanol (Invitrogen, Carlsbad, USA), 1% Antibiotic-Antimycotic (Invitrogen, Carlsbad, USA), 1% glutamine (Invitrogen, Carlsbad, USA), 15 ng/ml GM-CSF (PeproTech, Offenbach, Germany), 15 ng/ml IL-4 (PeproTech, Offenbach, Germany)). Cells were cultured in a humidified 5% CO_2 atmosphere at 37°C. At day three, 10 ml of BM-DC culture medium was added. At day six, eight and ten, 50% of the cell suspension was removed, centrifuged (350g, 5 min), resuspended in fresh BM-DC culture medium of same volume and given back in culture. BM-DCs were collected between day ten and twelve, when $CD11b^+$ $CD11c^+$

double positive cells were > 90% as assessed by flow cytometry using the LSRII flow cytometer in combination with FACSDiva software (BD Bioscience).

3.17. Coculture of BM-DCs and $CD4^+$ T-cells

BM-DCs were seeded at $0.1x10^6$ cells/ml (100 µl/well) in 96-well cell culture plates (Nunc, Roskilde, Denmark) in coculture medium (RPMI 1640 (Invitrogen, Carlsbad, USA), 10% FBS (Biochrom, Berlin, Germany), 50µM 2-mercaptoethanol (Invitrogen, Carlsbad, USA), 1% Antibiotic-Antimycotic (Invitrogen, Carlsbad, USA), 1% glutamine (Invitrogen, Carlsbad, USA), 15 ng/ml GM-CSF (PeproTech, Offenbach, Germany)). BM-DCs were pulsed by adding 50 or 100 µg/ml of cecal lysates, dietary suspensions or PT-gluten as indicated in the experiments. In MHCII blocking experiments, 12 µg/ml or 25 µg/ml of anti-MHCII blocking antibody (BD Pharmingen) was added together with the antigens. BM-DCs were incubated for 6 h in a humidified 5% CO_2 atmosphere at 37°C. Cells were washed twice with PBS and isolated $CD4^+$ T-cells from mesenteric lymph nodes of $TNF^{\Delta ARE/WT}$ mice were added at $2x10^6$ cells/ml (100 µl/well). BM-DCs and $CD4^+$ T-cells were cocultured for 72 h in a humidified 5% CO_2 atmosphere at 37°C. BM-DCs and $CD4^+$ T-cells that were cultured alone served as a control. Cell culture plates were centrifuged at 350 g, 4°C, 5 min and supernatants were collected for subsequent ELISA analysis.

3.18. ELISA

Cytokine protein concentrations of TNF, IFN-γ and IL-12p40 in supernatants of cell culture and coculture experiments were determined by mouse-specific ELISA kits according to the manufacturer's instructions (Ready-Set-Go! ELISA kits, eBioscience, San Diego, CA). Briefly, NuncMaxiSorp flat-bottom 96 well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated with the appropriate capture antibody overnight at RT. Plates were washed, blocked and incubated with cell culture supernatants for 1.5 h at RT. Plates were washed and incubated with the appropriate detection antibody for 1.5 h at RT. Plates were washed and incubated with a detection enzyme. Plates were washed and incubated with a substrate solution. Protein concentration was determined by photometrical analysis of the reaction of substrate and detection enzyme.

3.19. Statistical analysis

Statistical analysis was performed using SigmaPlot 11.0 (Systat Software). Data comparing two groups were analyzed using the unpaired t test. Data comparing more than two groups were analyzed using One-Way or Two-Way ANOVA followed by an appropriate multiple comparison procedure. Differences between groups were considered significant if P values were < 0.05.

4. RESULTS

4.1. Effects of dietary intervention on Crohn's disease-like ileitis

4.1.1. Semi-synthetic diet ameliorates ileitis in TNF$^{\Delta ARE/WT}$ mice

Genetically susceptible TNF$^{\Delta ARE/WT}$ mice being kept under conventional conditions on standard Chow diet developed a severe chronic intestinal inflammation in the distal ileum after 18 weeks of age. In contrast, TNF$^{\Delta ARE/WT}$ mice being transferred to a semi-synthetic, experimental diet (Exp) showed no or only mild symptoms of inflammation ($P < 0.001$). The observed protective effect was not specific for one diet manufacturing company and could be reproduced using semi-synthetic diet of an alternative manufacturer. Neither Chow diet nor experimental diet induced any signs of tissue pathology in wild type (WT) mice (**Figure 1**).

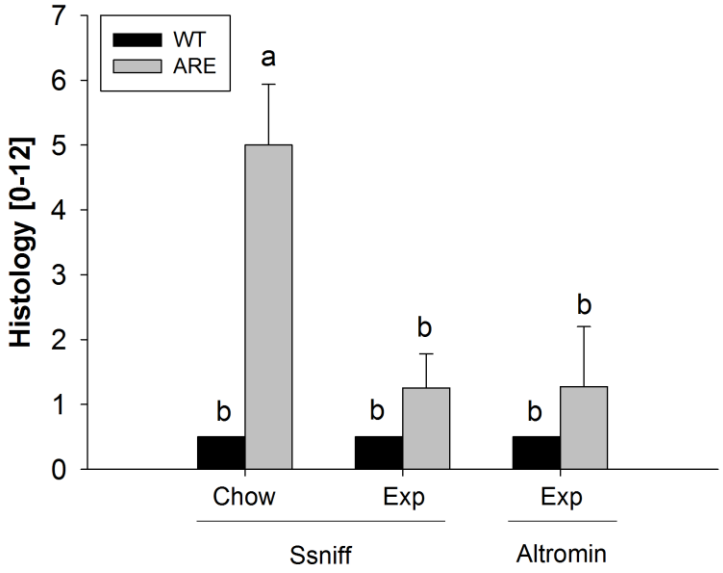


Figure 1. Tissue pathology of WT and TNF$^{\Delta ARE/WT}$ mice on Chow diet and experimental diet.
Tissue pathology assessed by histological examination (score 0-12) of distal ileal sections from WT and TNFΔARE/WT (ARE) mice (18 weeks of age) on standard Chow diet (manufacturer Ssniff) and experimental diet (Exp) (manufacturer Ssniff or Altromin, as indicated). Values are means ± SD, n = 6 per group. ^{a,b,c} Within each graph, means without a common letter differ, $P < 0.001$.

WT mice had significantly ($P < 0.05$) higher body weights compared to TNF$^{\Delta ARE/WT}$ mice and showed no differences according to diet. Inflamed TNF$^{\Delta ARE/WT}$ mice on Chow did not differ from the non-inflamed Exp group (**Figure 2A**).

The reversed pattern could be observed, when we compared spleen weights of the distinct trial groups. TNF$^{\Delta ARE/WT}$ mice showed significantly ($P < 0.001$) increased spleen weights compared with WT mice. Interestingly, although TNF$^{\Delta ARE/WT}$ mice on Exp showed only very

mild tissue pathology, spleen weights were not different from highly inflamed TNF$^{\Delta ARE/WT}$ mice (**Figure 2B**).

We observed strong differences in cecum size and weight depending on genotype and diet. Inflamed TNF$^{\Delta ARE/WT}$ mice on Chow showed highest cecum weights ($P < 0.001$), followed by WT on Chow and TNF$^{\Delta ARE/WT}$ mice on Exp. WT mice on Exp revealed the lowest cecum weights. A strong diet dependent effect could be observed in the WT and TNF$^{\Delta ARE/WT}$ group. Animals on Chow showed significantly increased cecum weights compared to animals on Exp of the same group. Interestingly, cecum weights of protected TNF$^{\Delta ARE/WT}$ mice on Exp were comparable with those of WT mice (**Figure 2C**).

Mesenteric lymph nodes (MLN) play a pivotal role in mucosal immune responses. Therefore, we were also interested in MLN weights and associations with genotype and diet. Inflamed TNF$^{\Delta ARE/WT}$ mice on Chow showed significantly increased MLN weights compared with the WT Chow and ARE Exp group. TNF$^{\Delta ARE/WT}$ mice on Exp showed a higher variance in MLN weight but ranked between highly inflamed TNF$^{\Delta ARE/WT}$ and completely healthy WT mice ($P < 0.001$). No differences could be seen, comparing WT mice on Chow and Exp diet (**Figure 2D**).

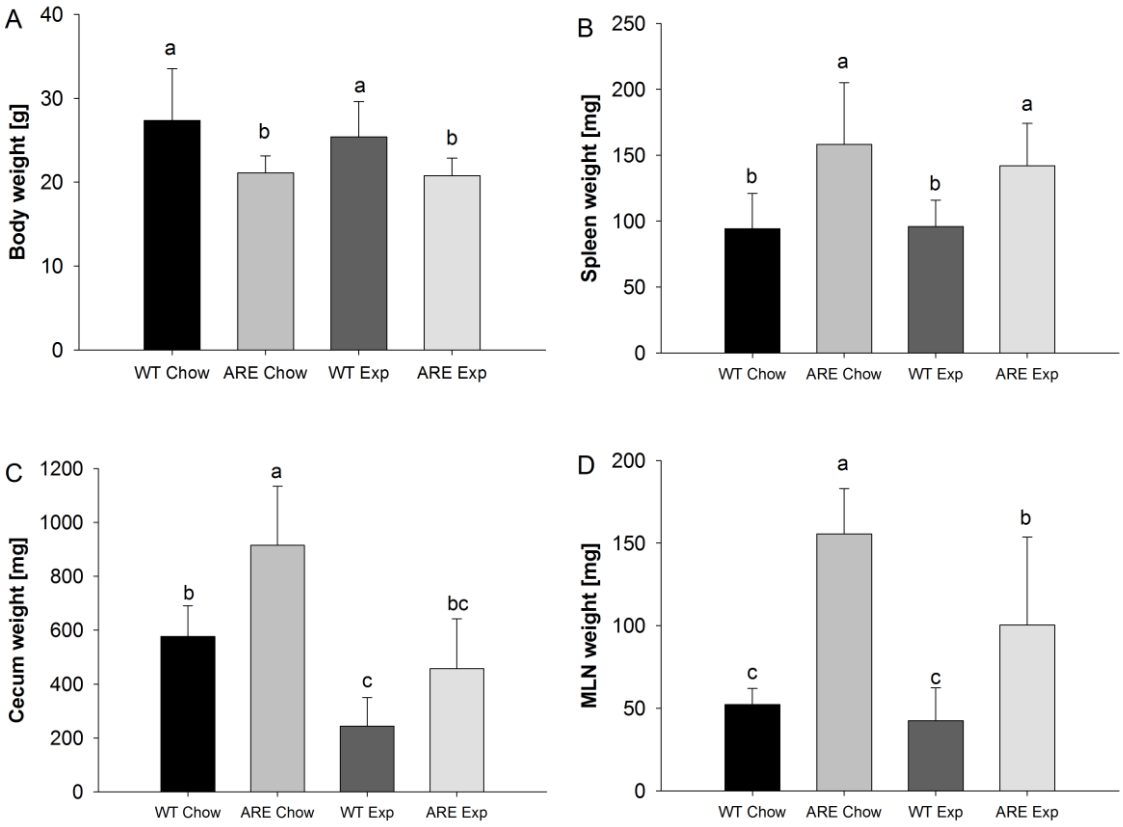


Figure 2. Body and organ weights of WT and TNF$^{\Delta ARE/WT}$ mice.

Body weight, spleen weight, cecum weight and mesenteric lymph node (MLN) weight of WT and TNFΔARE/WT mice on Chow and experimental diet (Exp), sacrificed after 18 weeks of age by cervical dislocation. Values are

means ± SD, n = 6 per group. [a,b,c] Within each graph, means without a common letter differ, P < 0.05 and P < 0.001 for (A) and (B, C, D), respectively.

We next wanted to evaluate whether the differences in splenocyte and MLN weights could be confirmed at total cell numbers. Splenocytes and MLNs were isolated and counted using a Neubauer counting chamber. Although TNF$^{\Delta ARE/WT}$ mice on Chow and Exp had different inflammatory conditions as shown above, total splenocyte numbers were not different. The WT Chow and WT Exp group did also not differ in total splenocytes. Though, both ARE groups showed significantly (P < 0.05) increased cell numbers, compared with the WT groups, independently of the diet (**Figure 3A**).

Inflamed TNF$^{\Delta ARE/WT}$ mice on Chow showed highest (P < 0.001) total MLN leukocyte numbers. The ARE Exp group counted significantly (P < 0.001) less but significantly (P < 0.001) more total cells than the ARE Chow and both WT groups, respectively. No difference was found comparing WT mice on different diets (**Figure 3B**).

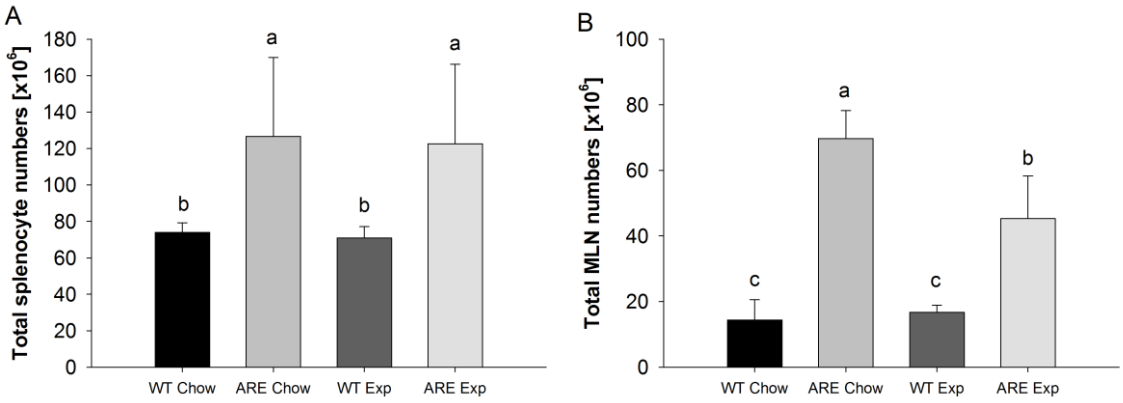


Figure 3. Leukocyte counts in spleens and mesenteric lymph nodes.

Total leukocyte numbers of (A) spleens and (B) MLNs from WT and TNFΔARE/WT mice on Chow diet and experimental diet (Exp). Values are means ± SD, n = 6 per group. [a,b,c] Within each graph, means without a common letter differ, P < 0.05 and P < 0.001 for (A) and (B), respectively.

4.1.2. Quantitative real-time PCR analysis of TNF$^{\Delta ARE/WT}$ and WT mice

TNF$^{\Delta ARE/WT}$ mice that develop a chronic ileitis show strongest disease manifestation in the distal ileum. We therefore assessed the level of proinflammatory gene expression in affected tissue regions. Cytokine expression in the distal ileum was measured by qRT-PCR and revealed significantly ($P < 0.001$) elevated levels of pro-inflammatory cytokines TNF and IFN-y in TNF$^{\Delta ARE/WT}$ mice on Chow diet. Cytokine expression in TNF$^{\Delta ARE/WT}$ mice on experimental diet did not differ from WT mice, suggesting that pathogenesis of Crohn's disease-like ileitis was inhibited by the experimental diets (**Figure 4**).

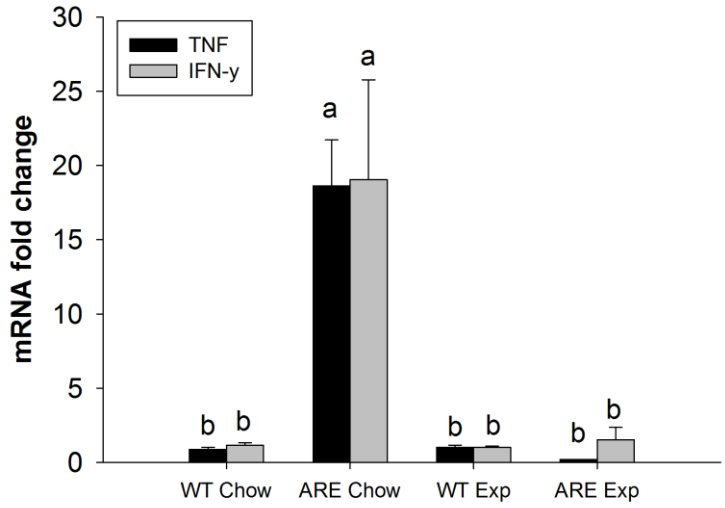


Figure 4. Quantitative real-time PCR analysis of proinflammatory cytokines.
Relative mRNA expression of TNF and IFN-y in distal ileal tissue from WT and TNFΔARE/WT mice on Chow diet and experimental diet (Exp). Tumor necrosis factor (TNF); interferon-y (IFN-y). Values are means ± SD, n = 6 per group. [a,b,c] Within each graph, means without a common letter differ, $P < 0.001$.

The expression of pattern recognition receptors TLR-2 and TLR-4 as well as LBP was highly elevated in distal ileal tissues of inflamed TNF$^{\Delta ARE/WT}$ mice on Chow diet ($P < 0.05$), whereas mice on experimental diet did not show increased expression compared to WT mice. Interestingly, expression of TLR-2, TLR-4 and LBP was also increased in WT mice on Chow diet compared with WT mice on experimental diet, although no tissue pathology could be detected. In contrast, flagellin related TLR-5 showed no regulation under any condition (**Figure 5**).

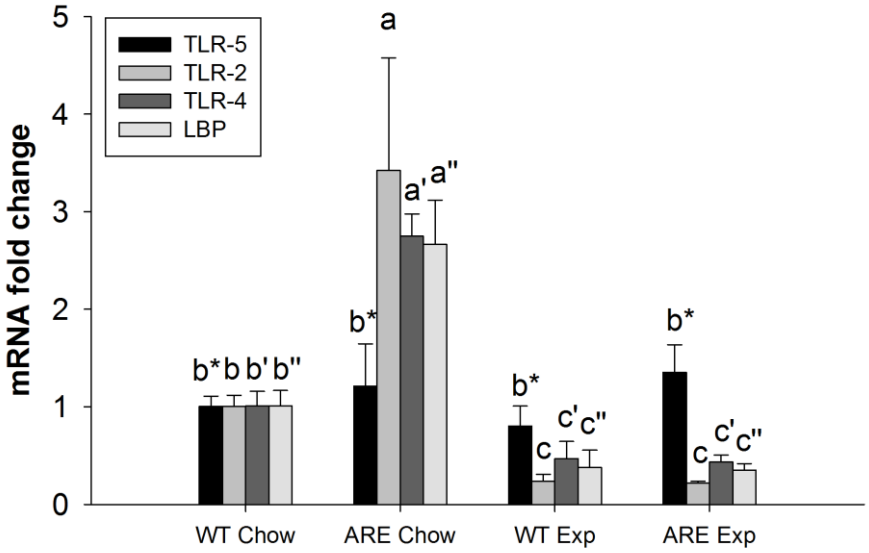


Figure 5. Quantitative real-time PCR analysis of pattern recognition receptors.

Relative mRNA expression of pattern recognition receptors in distal ileal tissue from WT and TNFΔARE/WT mice on Chow diet and experimental diet (Exp). Toll like receptor 2 (TLR-2); toll like receptor 4 (TLR-4); toll like receptor 5 (TLR-5); LPS binding protein (LBP). Values are means ± SD, n = 6 per group. [a,b,c] Within each graph, means without a common letter differ, P < 0.001.

TLR-9 and NOD-2 recognize CpG rich motifs in bacterial or viral DNA and muramyl dipeptide (MDP) moieties of peptidoglycan, respectively. Both are expressed intracellularly and play a crucial role in immune activation and are associated with autoimmune inflammatory disorders.

The inflamed ARE Chow group showed increased (P < 0.01) expression of TLR-9 and NOD-2, whereas $TNF^{\Delta ARE/WT}$ mice on experimental diet showed no difference in mRNA expression compared with WT mice on Chow or experimental diet (**Figure 6**).

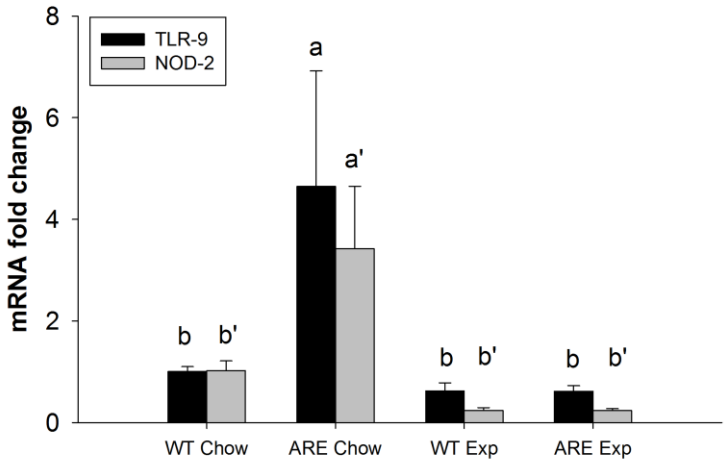


Figure 6. Quantitative real-time PCR analysis of pattern recognition receptors.

Relative mRNA expression of pattern recognition receptors in distal ileal tissue from WT and TNFΔARE/WT mice on Chow diet and experimental diet (Exp). Toll like receptor 9 (TLR-9); Nucleotide-binding oligomerization domain-containing protein 2 (NOD-2). Values are means ± SD, n = 6 per group. [a,b,c] Within each graph, means without a common letter differ, $P < 0.01$.

Infiltration of leukocytes into sites of inflammation is a pivotal step in Crohn's disease-like ileitis. IP-10 has been attributed to several roles, such as chemoattraction of antigen presenting cells (APCs) and promotion of T-cell adhesion to endothelial cells. The cell adhesion molecules MAdCAM-1 and ICAM-1 are also important factors for leukocyte recruitment and extravasation in inflamed tissues.

Distal ileal sections of inflamed $TNF^{\Delta ARE/WT}$ mice on Chow showed significantly ($P < 0.01$) increased expression of IP-10, MAdCAM-1 and ICAM-1 compared to all other groups. WT Chow, WT Exp and ARE Exp groups had similar mRNA expression levels, suggesting that tissue activation had not taken place in $TNF^{\Delta ARE/WT}$ mice on experimental diet (**Figure 7**).

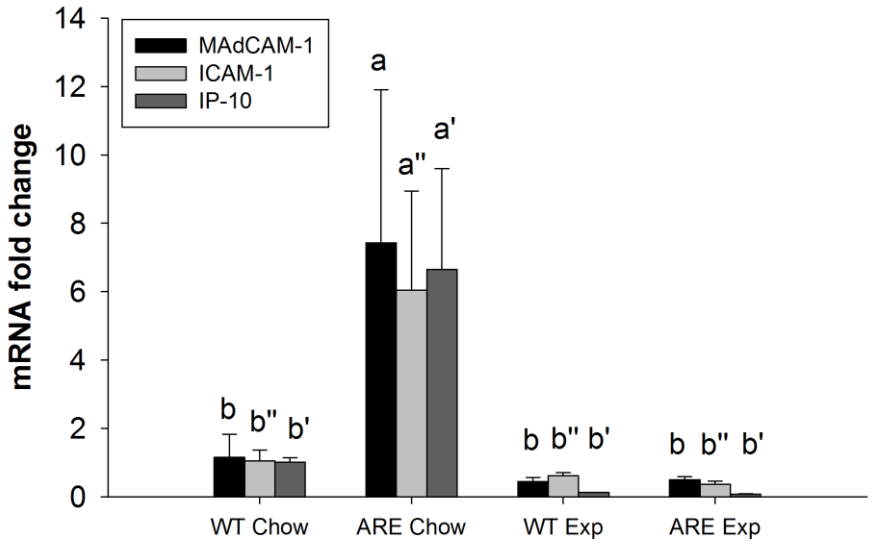


Figure 7. Quantitative real-time PCR analysis of cell adhesion molecules and IP-10.
Relative mRNA expression of cell adhesion molecules MAdCAM-1 and ICAM-1 and proinflammatory chemokine IP-10 in distal ileal tissue from WT and TNFΔARE/WT mice on Chow diet and experimental diet (Exp). Mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1); intercellular adhesion molecule 1 (ICAM-1). Values are means ± SD, n = 6 per group. ^{a,b,c} Within each graph, means without a common letter differ, P < 0.01.

4.1.3. Cellular analysis of TNF$^{\Delta ARE/WT}$ and WT mice

Leukocyte infiltration in distal ileal tissues of TNF$^{\Delta ARE/WT}$ mice was visualized by immunohistochemical labeling of CD3^{+} T-cells. Distal ileal sections of TNF$^{\Delta ARE/WT}$ mice on Chow diet showed increased infiltration of CD3^{+} T-cells and impaired intestinal architecture as compared with TNF$^{\Delta ARE/WT}$ mice on experimental diet (**Figure 8**).

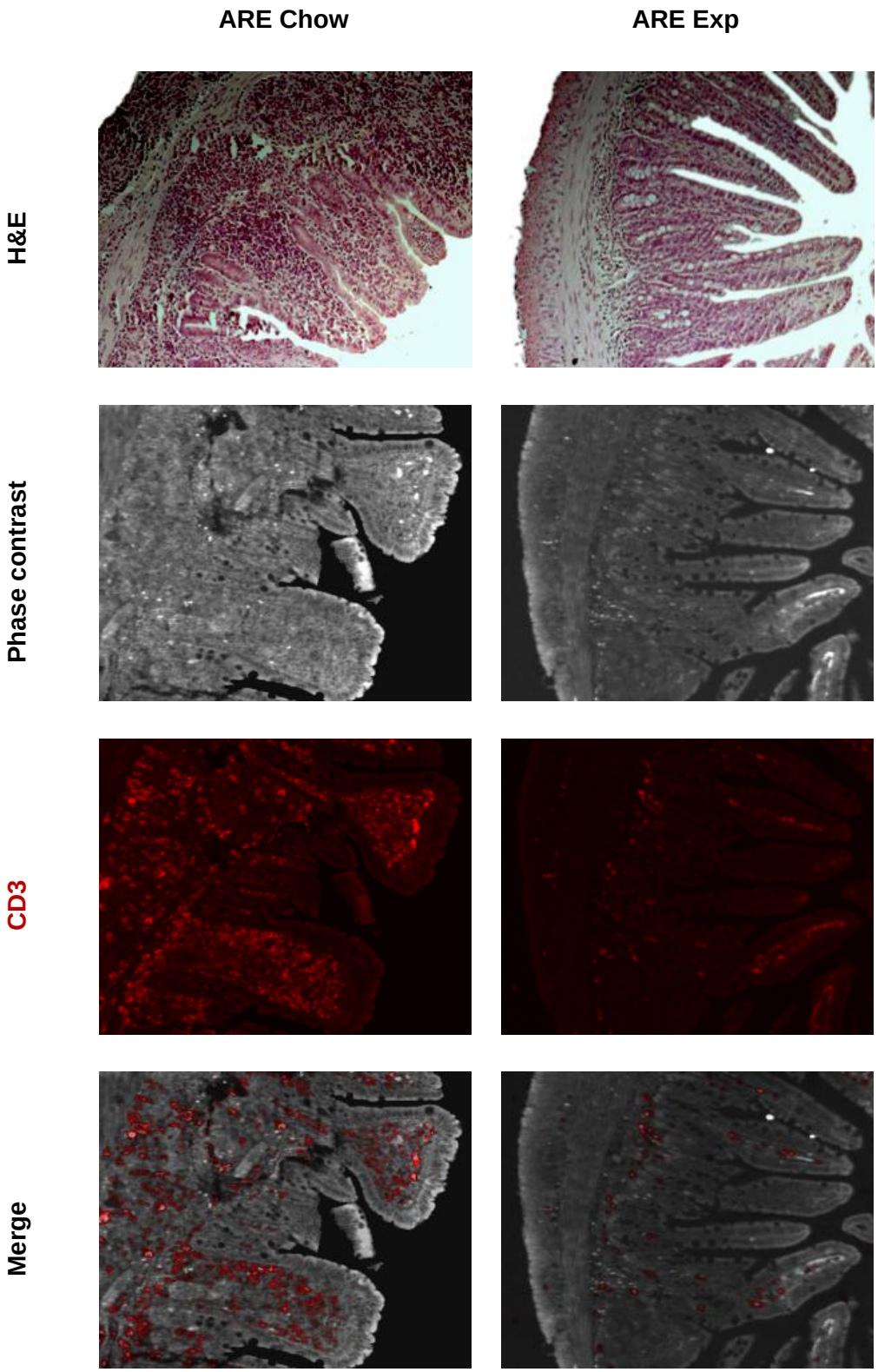


Figure 8. Fluorescence microscopy of distal ileal tissues from TNF$^{\Delta ARE/WT}$ mice.

Representative H&E staining (upper panels) and immunohistochemical labeling (CD3+ T-cells) of distal ileal sections from inflamed and non-inflamed TNFΔARE/WT mice (18 weeks of age) on Chow diet (left column) and experimental diet (right column). Fluorescence microscopy is visualized by phase contrast, fluorescence and merged images as indicated (100x magnification).

We determined total ileal leukocyte cell numbers after isolation from ileal tissue by density gradient centrifugation. Inflamed TNF$^{\Delta ARE/WT}$ mice on Chow showed significantly ($P < 0.001$) higher leukocyte numbers compared with all other groups. TNF$^{\Delta ARE/WT}$ mice on Exp revealed a non significant trend for elevated leukocyte numbers compared with the WT groups. Dietary intervention had no effects on total cell numbers in WT mice (**Figure 9**).

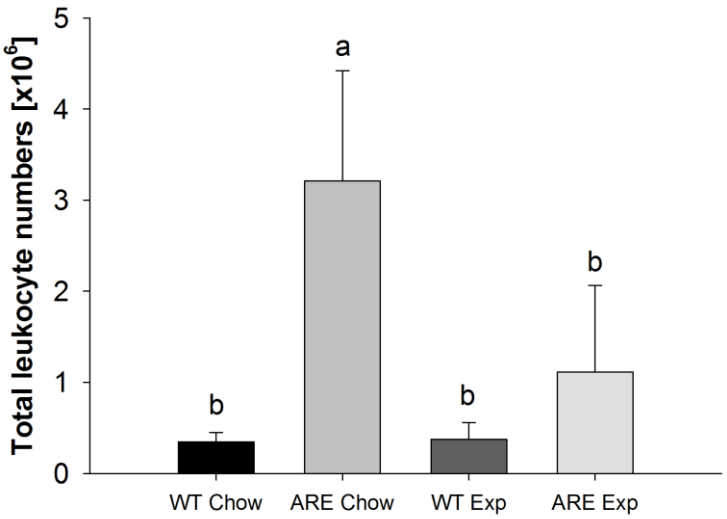


Figure 9. Leukocyte counts in ileal tissues of WT and TNF$^{\Delta ARE/WT}$ mice.

Total leukocyte numbers in ileal tissues from WT and TNFΔARE/WT mice on Chow diet and experimental diet (Exp) were determined using a Neubauer counting chamber after density gradient centrifugation. Values are means ± SD, n = 5-6 per group. ^{a,b,c} means without a common letter differ, $P < 0.001$.

Infiltrating lamina propria lymphocyte (LPL) and intraepithelial lymphocyte (IEL) populations were further characterized by flow cytometric analysis. CD8αβ$^+$ T-cells are suggested to be a major effector T-cell population in the TNF$^{\Delta ARE/WT}$ mouse model. CD8αα$^+$ T-cells represented the dominant phenotype in the ileum of WT mice, whereas TNF$^{\Delta ARE/WT}$ mice showed a significant ($P < 0.01$) increase of CD8αβ$^+$ T-cells associated with a significant decrease of CD8αα$^+$ T-cells ($P < 0.01$), irrespective of the different diets (**Figure 10**).

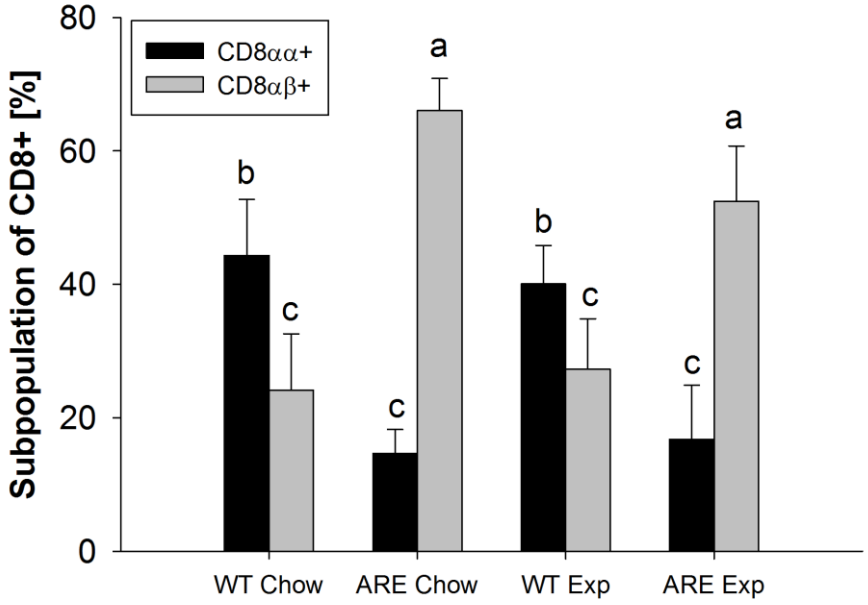


Figure 10. Flow cytometric analysis of IEL and LPL in WT and TNF$^{\Delta ARE/WT}$ mice.

Flow cytometric analysis of CD8αα+ and CD8αβ+ subpopulations of CD8+ IEL and LPL from ileal tissues of WT and TNFΔARE/WT mice on Chow diet and experimental diet (Exp). Values are means ± SD, n = 4-6 per group. ^{a,b,c} means without a common letter differ, P < 0.01. Intraepithelial lymphocytes (IEL); lamina propria lymphocytes (LPL).

Regulatory T-cells (Tregs) play a crucial role in immune homeostasis and regulation of inflammatory responses. To assess the role of Tregs in the distinct treatment groups, we analyzed IEL/LPL fractions for CD4+ Foxp3+ Tregs. There was no difference between the inflamed ARE Chow group and the non-inflamed ARE Exp group. Both ARE groups showed a trend to have higher proportions of Tregs compared to the WT Chow group, but values did not reach significance. WT mice on Exp had significantly (P < 0.01) less Tregs than both ARE groups but did not differ from WT mice on Chow (**Figure 11**).

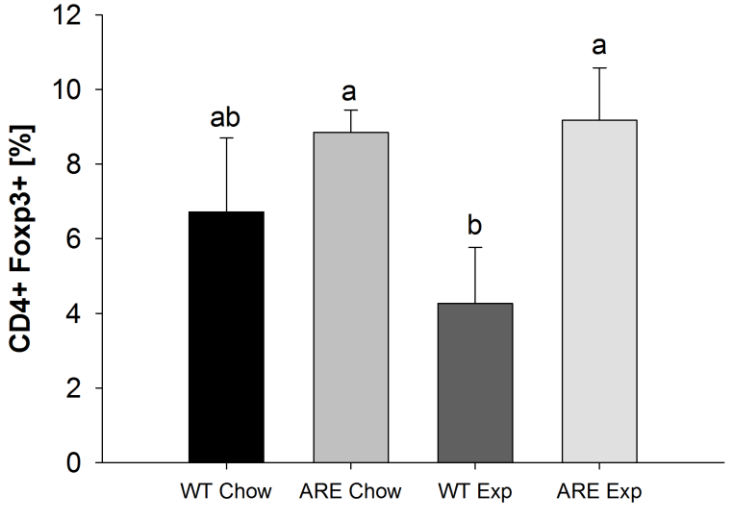


Figure 11. Flow cytometric analysis of IEL and LPL in WT and TNF$^{\Delta ARE/WT}$ mice.

Flow cytometric analysis of CD4+ Foxp3+ subpopulations of CD4+ IEL and LPL from ileal tissues of WT and TNFΔARE/WT mice on Chow diet and experimental diet (Exp). Values are means ± SD, n = 4-6 per group. ^{a,b,c} means without a common letter differ, P < 0.01. Intraepithelial lymphocytes (IEL); lamina propria lymphocytes (LPL); forkhead box P3 (Foxp3).

We measured significant differences in MLN weights from WT and TNF$^{\Delta ARE/WT}$ mice with dietary effects in the latter group. Next we wanted to investigate, whether these changes were associated with alterations of T-cell phenotypes.

Flow cytometric analysis of total mesenteric lymph node cells revealed no difference in CD4+/CD8+ T-cell ratios comparing WT and ARE groups. Although a significant difference in MLN weights was observed, no phenotypical difference could be detected in T-cell populations of TNF$^{\Delta ARE/WT}$ mice on different diets (**Figure 12**).

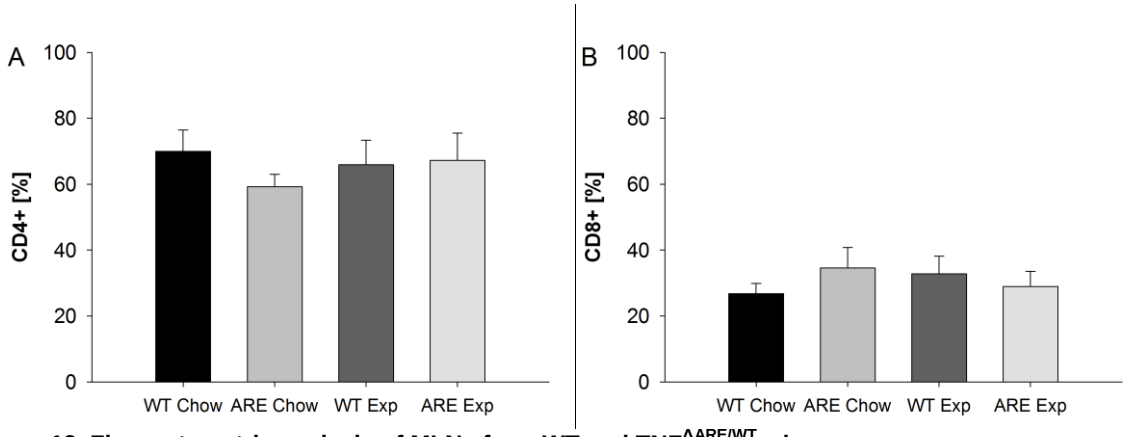


Figure 12. Flow cytometric analysis of MLNs from WT and TNF$^{\Delta ARE/WT}$ mice.

Flow cytometric analysis of (A) CD4+ and (B) CD8+ T-cell populations of total mesenteric lymph node leukocytes (MLNs) from WT and TNFΔARE/WT mice on Chow diet and experimental diet (Exp). Values are means ± SD, n = 4-6 per group.

4.1.4. Time-dependent effects of dietary intervention

We next aimed at characterizing the protective effect of the experimental diet in a time-dependent manner. Figure 13 represents a schematic illustration of the feeding experiment. TNF$^{\Delta ARE/WT}$ mice being transferred to an experimental diet with seven weeks of age did not develop pathology ($P < 0.001$). In contrast, dietary transfer in week 10 or 14 of age did not inhibit the development of full intestinal inflammation after 18 weeks of age compared with TNF$^{\Delta ARE/WT}$ mice on Chow diet *ad libitum*. These results suggest a small time frame between weeks seven and ten for effective dietary prevention of ileitis in TNF$^{\Delta ARE/WT}$ mice (**Figure 13**).

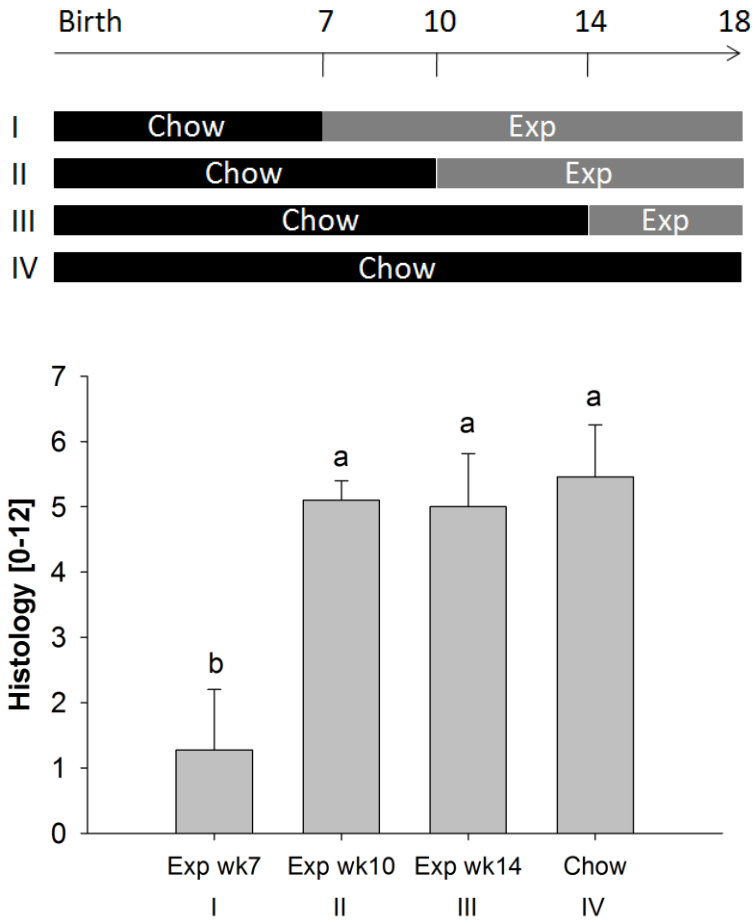


Figure 13. Tissue pathology of TNF$^{\Delta ARE/WT}$ mice after time-dependent transfer to experimental diet.
Tissue pathology assessed by histological examination (score 0-12) of distal ileal sections from TNFΔARE/WT mice on different diets as indicated. TNFΔARE/WT mice were transferred from Chow diet to experimental diet at 7, 10 or 14 weeks of age as indicated. Mice were sacrificed at 18 weeks of age. Upper graphic, schematic representation of the feeding experiment. Values are means ± SD, n = 6 per group. ^{a,b} means without a common letter differ, $P < 0.001$.

4.1.5. Rapid development of chronic intestinal inflammation in TNF$^{\Delta ARE/WT}$ mice

We performed another feeding experiment to analyze the kinetics of disease development. TNF$^{\Delta ARE/WT}$ mice were kept on experimental diet until week 12 of age. Animals were then transferred to Chow diet for two or six weeks.

After two weeks on Chow diet, TNF$^{\Delta ARE/WT}$ mice showed a significant ($P < 0.01$) increase in histopathology. Animal housing on Chow diet for six weeks resulted in severe ileitis with low standard deviation (**Figure 14**).

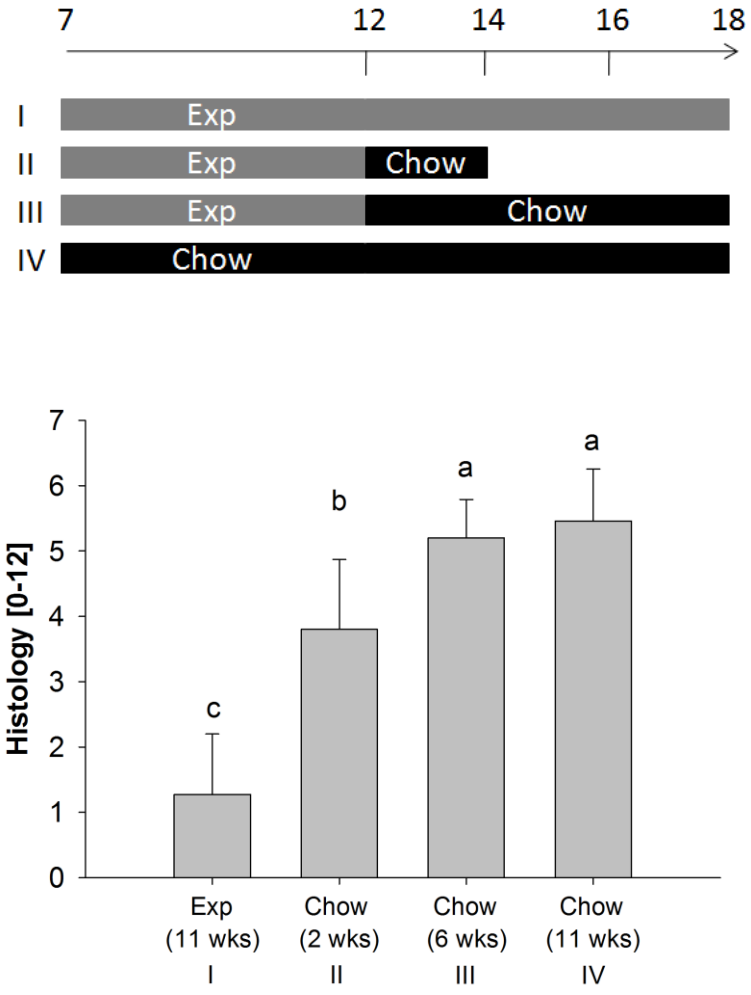


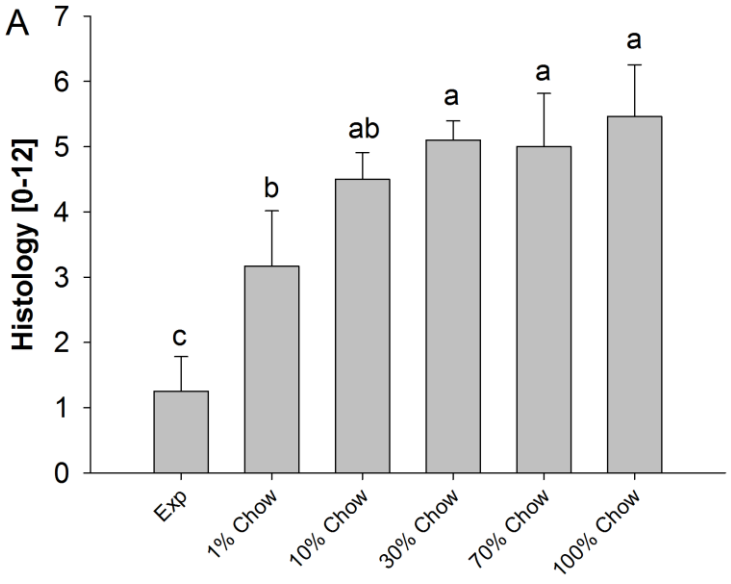
Figure 14. Tissue pathology of TNF$^{\Delta ARE/WT}$ mice after transfer to Chow diet.

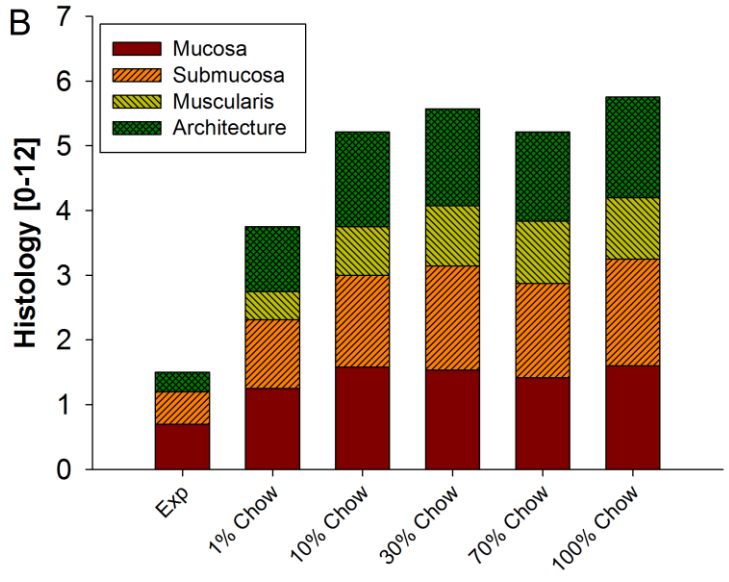
Tissue pathology assessed by histological examination (score 0-12) of distal ileal sections from TNFΔARE/WT mice on different diets as indicated. TNFΔARE/WT mice were transferred from experimental diet to Chow diet at 7 or 12 weeks of age. Mice were kept on the different diets for 2, 6, or 11 weeks, as indicated in bracket. Upper graphic, schematic representation of the feeding experiment with endpoints after 14 (II) or 18 weeks of age (I, III, IV). Values are means ± SD, n = 3-6 per group. ^{a,b} means without a common letter differ, $P < 0.01$.

4.1.6. Low concentrations of Chow diet induce chronic ileitis in TNF$^{\Delta ARE/WT}$ mice

In order to characterize disease pathogenesis of TNF$^{\Delta ARE/WT}$ mice on Chow diet, we designed a Chow titration experiment. Customized diets differing in Chow content were prepared and fed *ad libitum*.

Tissue pathology assessed by histological examination (score 0-12) of distal ileal sections revealed moderate inflammation (score 3.17 ± 0.85) in TNF$^{\Delta ARE/WT}$ mice being fed 1% Chow diet. Experimental diets with a Chow content of 10%, 30% or 70% induced strong ileal inflammation and showed no difference compared to 100% Chow diet. TNF$^{\Delta ARE/WT}$ mice on pure experimental diet showed very mild tissue pathology (**Figure 15A**). We apportioned the mean histological scores according to infiltration of leukocytes into mucosa, submucosa and muscularis as well as condition of intestinal architecture. There was a steady increase of scores in all categories, starting lowest in the Exp group, increasing in the 1% Chow group and reaching its maximum in the 10% Chow group with no difference to 30%, 70% and 100% Chow groups. Scoring criteria that differed the most from the Exp group were infiltration of leukocytes into the muscularis and destruction of intestinal architecture via crypt loss and villous atrophy (**Figure 15B**). Increase of pathology is visualized by representative H&E staining (**Figure15C**).


A
Histology [0-12]
0
1
2
3
4
5
6
7
c
b
ab
a
a
a
Exp
1% Chow
10% Chow
30% Chow
70% Chow
100% Chow


B
Histology [0-12]
Mucosa
Submucosa
Muscularis
Architecture
0
1
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3
4
5
6
7
Exp
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70% Chow
100% Chow

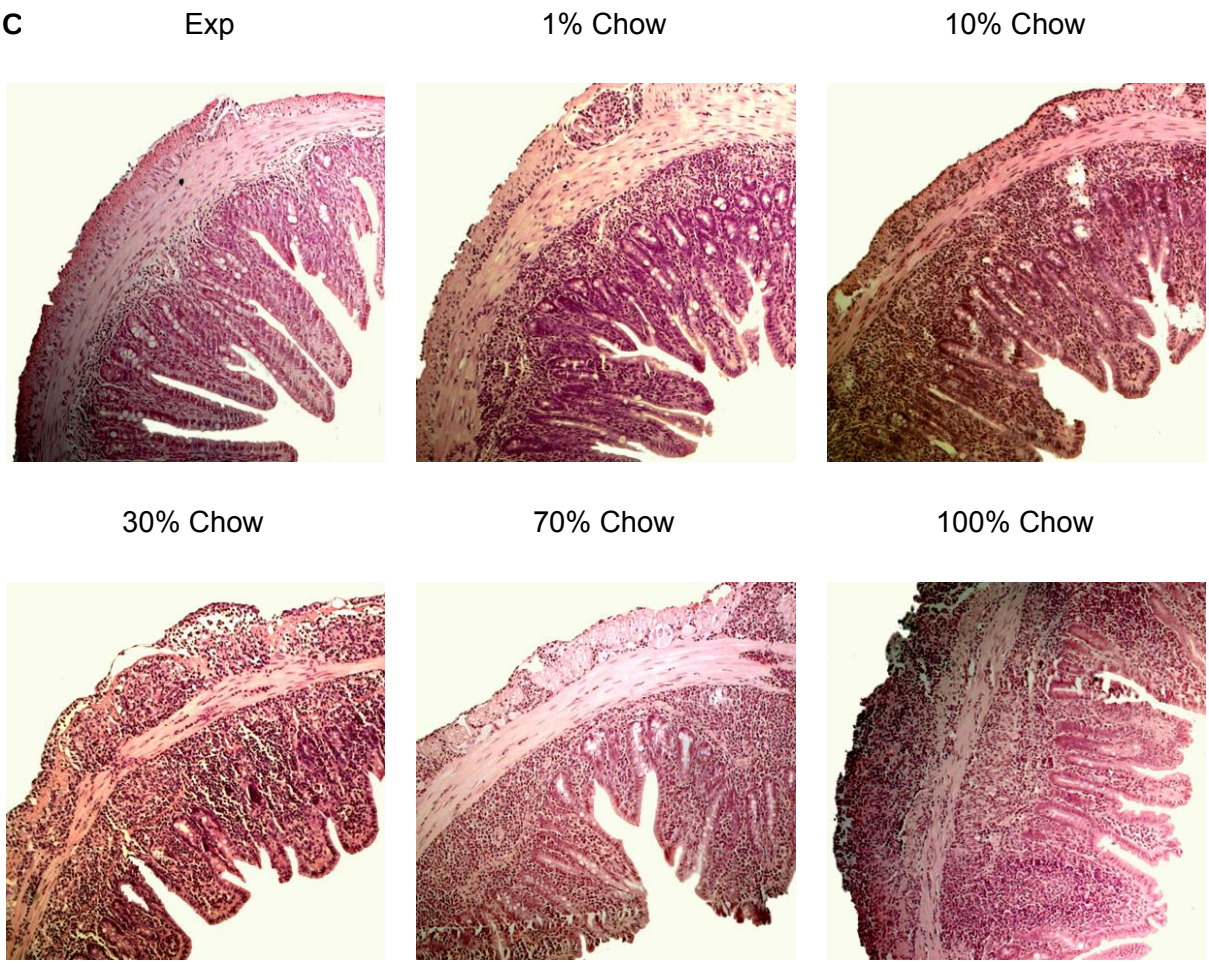


Figure 15. Tissue pathology of TNF$^{\Delta ARE/WT}$ mice being kept on diets with increasing Chow content.
(A) Tissue pathology assessed by histological examination (score 0-12) of distal ileal sections from TNFΔARE/WT mice on experimental diet, 1%, 10%, 30%, 70% and 100% Chow. (B) Apportioned mean histological scores according to leukocyte infiltration into mucosa, submucosa, muscularis and appearance of intestinal architecture. (C) Representative H&E staining of distal ileal sections from TNFΔARE/WT mice (18 weeks of age) on diets as indicated (100x magnification). Values are means ± SD, n = 6 per group. ^{a,b} means without a common letter differ, $P < 0.001$.

4.1.7. Particle size distribution measurements

Standard Chow diet seemed to have a coarse-grained appearance with higher variations in particle size compared with the homogenous grain structure of the experimental diet. Inflammatory responses might be triggered by shear forces of non-digested coarse-grained particles that might induce mechanic stress on distal ileal tissue, where the intestine forms a bottleneck before entering the cecum. To confirm our macroscopical observations of different diet structures, we performed a particle size distribution measurement by laser diffraction analysis.

The mean particle size of Chow was significantly ($P < 0.001$) bigger, than the mean particle size of the experimental diet. Thus, we ordered a modified Chow diet with increased grinding state (Chow fine). Particle size distribution measurement revealed that the mean particle size was significantly decreased compared with standard Chow diet, although it was still more coarse-grained ($P < 0.001$) than the experimental diet (**Figure 16**).

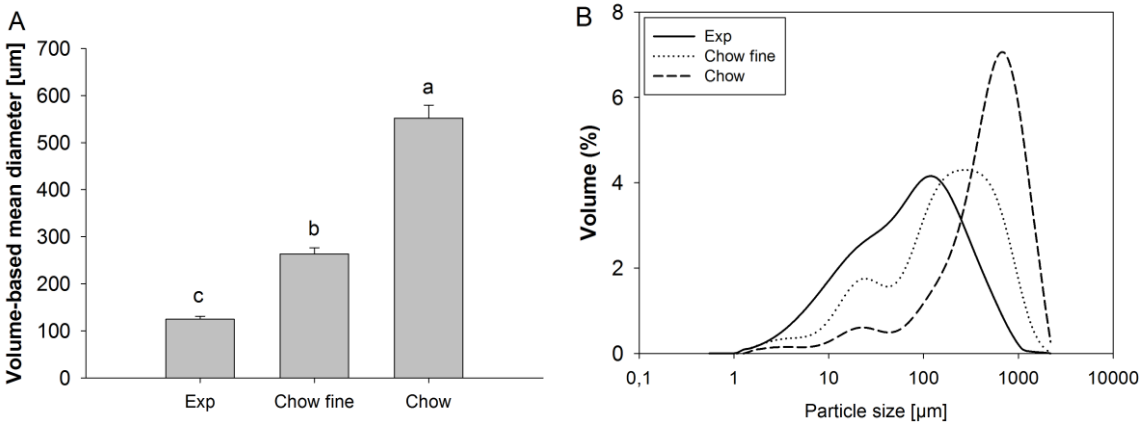


Figure 16. Laser diffraction analysis of experimental (Exp), fine Chow and standard Chow diet.

(A) Volume-based mean diameter size of particles, calculated upon equivalent sphere theory [D(4,3)]. (B) Mean particle size distribution diagram after wet analysis. Values are means ± SD of three independent experiments. ^{a,b,c} means without a common letter differ, $P < 0.001$.

We next measured the particle size of ileal and cecal contents in $TNF^{\Delta ARE/WT}$ mice on Exp, Chow fine and standard Chow diets to analyze the effect of dietary graining state on luminal content. Ileal contents of the Chow groups showed no difference in particle size, suggesting that standard Chow diet had been comminuted and digested to the size of Chow fine diet. Interestingly, particle sizes of Chow fine and Exp diets were not further decreased during digestion (289 ± 18 µm and 121 ± 9 µm, respectively) and had approximately the same values as the original diets (263 ± 13 µm and 125 ± 6 µm, respectively). A significant ($P < 0.001$) difference in particle size was still visible, comparing ileal contents of Exp with each of the Chow groups (**Figure 17**).

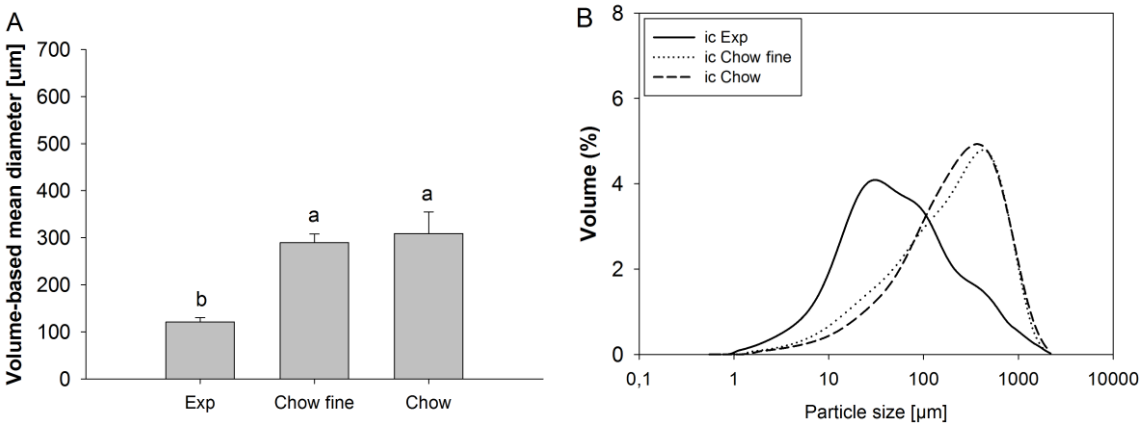


Figure 17. Laser diffraction analysis of ileal contents from TNF$^{\Delta ARE/WT}$ mice being kept on experimental (Exp), fine Chow and standard Chow diet.

(A) Volume-based mean diameter size of particles, calculated upon equivalent sphere theory [D(4,3)]. (B) Mean particle size distribution diagram after wet analysis. Values are means ± SD, n = 5 per group. ^{a,b,c} means without a common letter differ, P < 0.001.

Particle size determination of cecal contents showed the same pattern as observed in ileal content analysis. There was no difference in cecal content particle size distribution of the Chow fine and standard Chow group. Yet, each of these groups significantly (P < 0.001) differed from the Exp group (**Figure 18**). These findings corroborated our results from ileal content measurements.

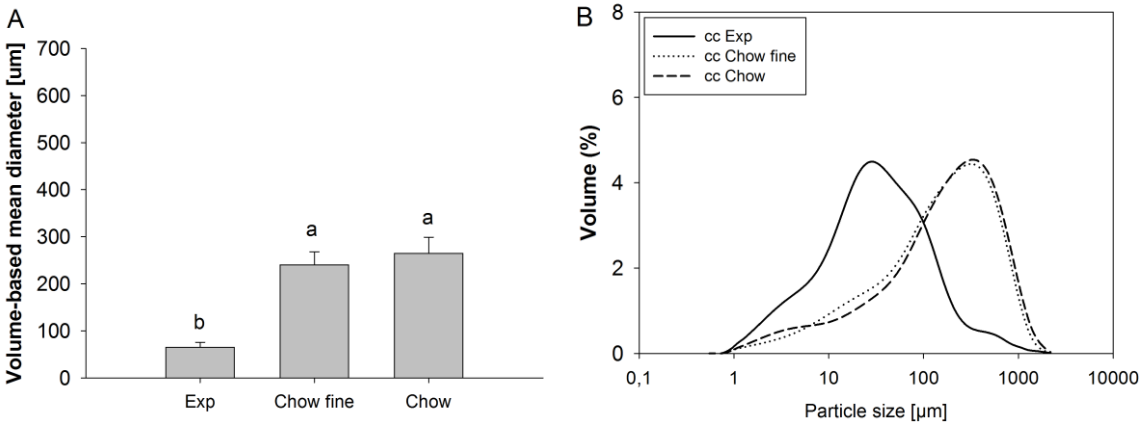


Figure 18. Laser diffraction analysis of cecal contents from TNF$^{\Delta ARE/WT}$ mice being kept on experimental (Exp), fine Chow and standard Chow diet.

(A) Volume-based mean diameter size of particles, calculated upon equivalent sphere theory [D(4,3)]. (B) Mean particle size distribution diagram after wet analysis. Values are means ± SD, n = 5 per group. ^{a,b,c} means without a common letter differ, P < 0.001.

We analyzed grinding state associated effects on pathology development using histological scoring after 18 weeks of age. The increased grinding state of Chow fine diet had no effects on chronic ileitis compared with coarse-grained standard Chow diet (**Figure 19**).

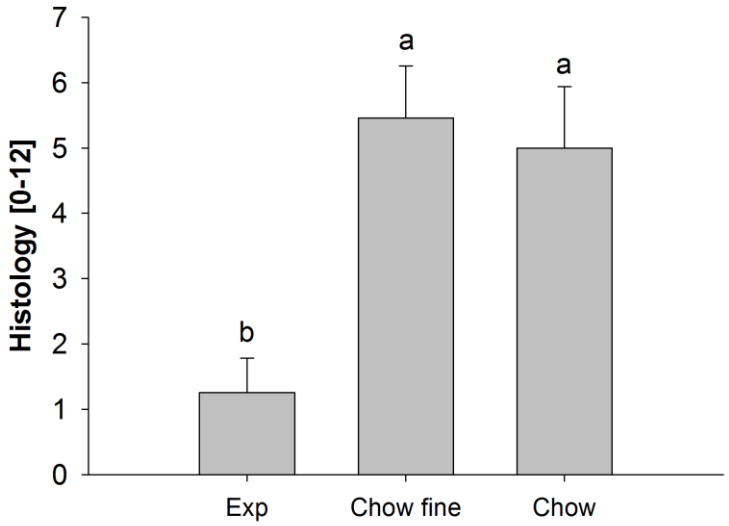


Figure 19. Tissue pathology of TNF$^{\Delta ARE/WT}$ mice being kept on experimental (Exp), fine Chow and standard Chow diet.

Tissue pathology assessed by histological examination (score 0-12) of distal ileal sections from TNFΔARE/WT mice on different diets as indicated. Values are means ± SD, n = 6 per group. ^{a,b} means without a common letter differ, P < 0.001.

4.1.8. Fourier transform infrared spectroscopy (FT-IR) analysis

We prepared bacterial suspensions of cecal content from TNF$^{\Delta ARE/WT}$ mice on Chow diet (Chow) and experimental diet (Exp) to evaluate possible alterations in the cecal microbial composition by Fourier transform infrared spectroscopy (FT-IR).

Figure 20 shows the dendrogram, generated by average linkage algorithm for cluster analysis of vector normalized first derivatives of total range FT-IR absorption spectra (4000 – 600 cm $^{-1}$). Cecal bacterial samples showed a clear cluster formation according to the different diets used in the feeding experiment. Exp samples were related closely to each other and exhibited increased spectral distance to cecal bacterial samples derived from the Chow group. “Chow a” showed least spectral relation to all other groups, even regarding the Chow group itself.

Figure 20. FT-IR analysis of cecal bacterial suspensions from TNF$^{\Delta ARE/WT}$ mice being kept on experimental diet (Exp) and Chow diet (4000 – 600 cm $^{-1}$).

Dendrogram, generated by average linkage algorithm for cluster analysis of vector normalized first derivatives of total range FT-IR absorption spectra (4000 – 600 cm $^{-1}$). Cecal bacterial suspensions of three (a, b, c) female TNFΔARE/WT mice per group (Chow and Exp) were prepared and measured in duplicates.

To analyze the observed cluster formation of cecal bacterial samples in more detail, we focused on macronutrient specific regions. Strong cluster formation according to dietary treatment was observed in the carbohydrate specific region (1240-900 cm $^{-1}$). Cecal bacterial samples of experimental diet exhibited close internal spectral distance and clearly separated from cecal bacterial samples of the Chow feeding (**Figure 21A**). The associated absorption spectra showed a clear difference in the fingerprint region (~ 1000 cm $^{-1}$), probably responsible for the strong clustering (**Figure 21B**).

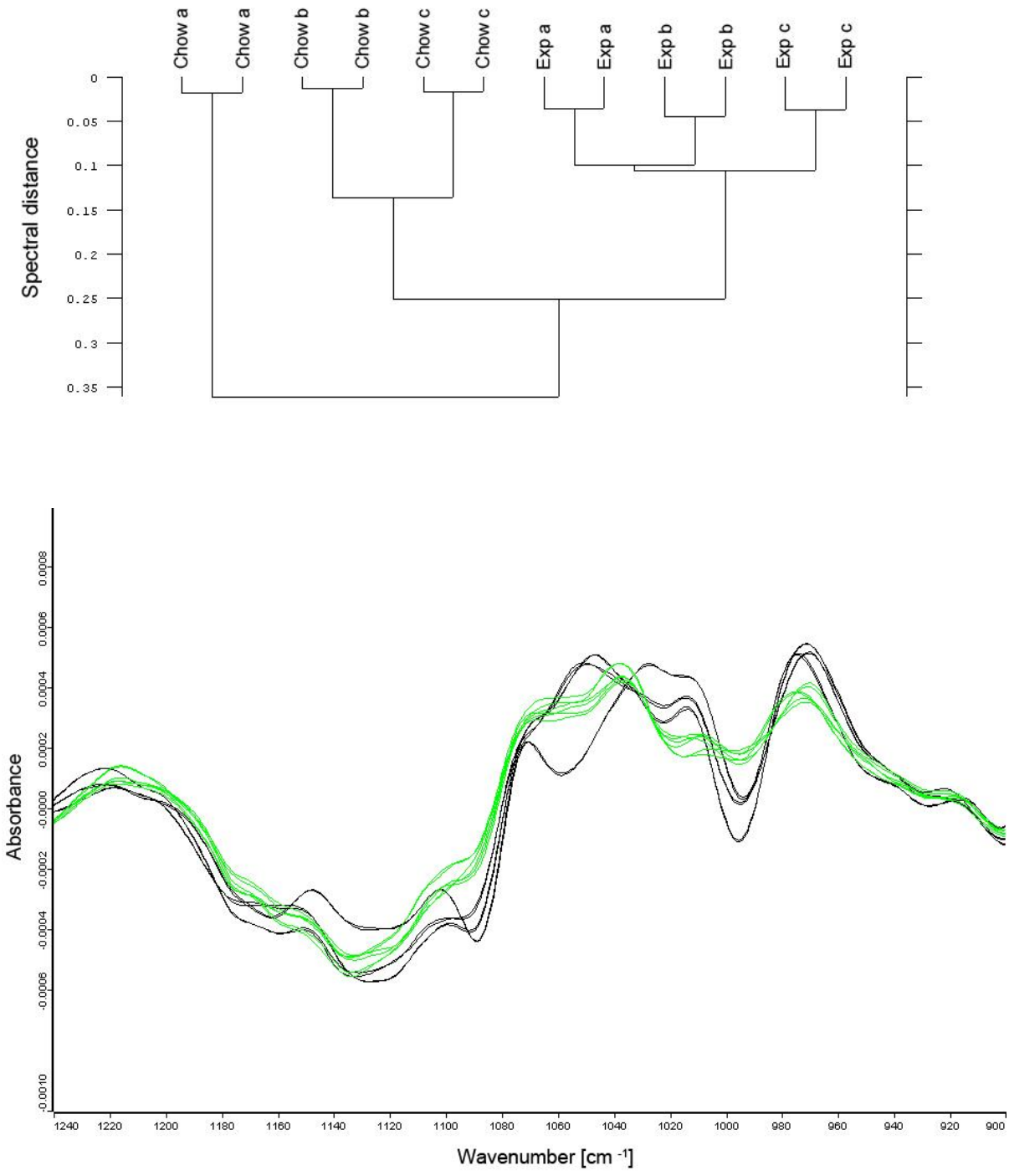


Figure 21. FT-IR analysis of cecal bacterial suspensions from TNF$^{\Delta ARE/WT}$ mice being kept on experimental diet (Exp) and Chow diet (1240-900 cm $^{-1}$).

(A) Dendrogram, generated by average linkage algorithm for cluster analysis of vector normalized first derivatives of carbohydrate specific FT-IR absorption spectra (1240-900 cm $^{-1}$). Cecal bacterial suspensions of three (a, b, c) female TNFΔARE/WT mice per group (Chow and Exp) were prepared and measured in duplicates. (B) Vector normalized first derivatives of carbohydrate specific FT-IR absorption spectra (1240-900 cm $^{-1}$). Black (Chow), green (Exp).

No clear cluster formation according to dietary treatment could be found in the protein specific region (1720 – 1480 cm $^{-1}$) (**Figure 22**).

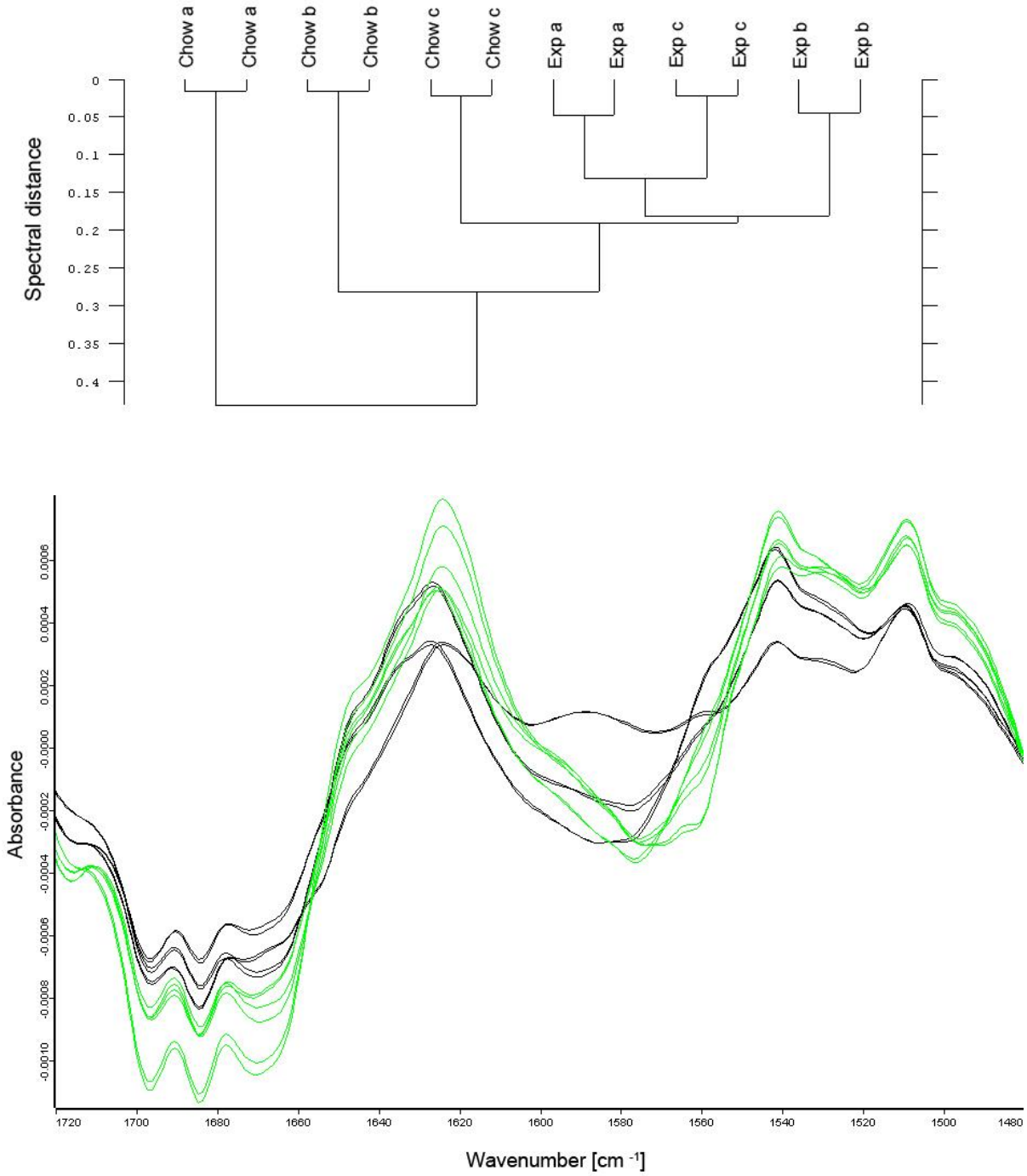


Figure 22. FT-IR analysis of cecal bacterial suspensions from TNF$^{\Delta ARE/WT}$ mice being kept on experimental diet (Exp) and Chow diet (1720-1480 cm $^{-1}$).

(A) Dendrogram, generated by average linkage algorithm for cluster analysis of vector normalized first derivatives of protein specific FT-IR absorption spectra (1720-1480 cm $^{-1}$). Cecal bacterial suspensions of three (a, b, c) female TNFΔARE/WT mice per group (Chow and Exp) were prepared and measured in duplicates. (B) Vector normalized first derivatives of protein specific FT-IR absorption spectra (1720-1480 cm $^{-1}$). Black (Chow), green (Exp).

The lipid specific region ranges from 3000 - 2780 cm $^{-1}$. No clear cluster formation according to dietary treatment could be observed in this region (**Figure 23**).

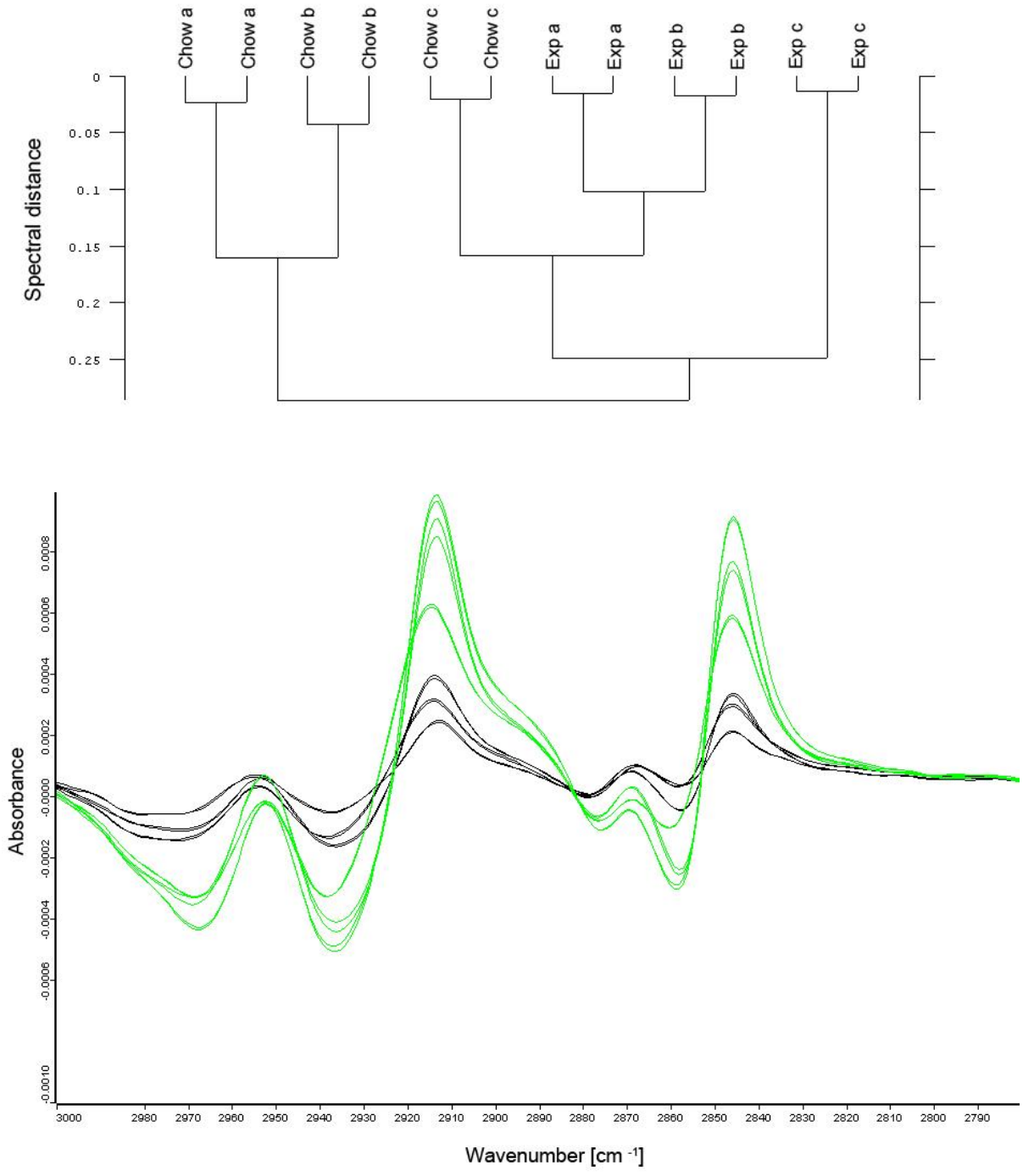


Figure 23. FT-IR analysis of cecal bacterial suspensions from TNF$^{\Delta ARE/WT}$ mice being kept on experimental diet (Exp) and Chow diet (3000-2780 cm $^{-1}$).

(A) Dendrogram, generated by average linkage algorithm for cluster analysis of vector normalized first derivatives of lipid specific FT-IR absorption spectra (3000-2780 cm $^{-1}$). Cecal bacterial suspensions of three (a, b, c) female TNFΔARE/WT mice per group (Chow and Exp) were prepared and measured in duplicates. (B) Vector normalized first derivatives of lipid specific FT-IR absorption spectra (3000-2780 cm 1). Black (Chow), green (Exp).

4.1.9. Antigenicity of cecal lysates is not altered by experimental diet

The commensal microbiota is suggested to play a pivotal role in the development of Crohn's disease-like ileitis of $TNF^{\Delta ARE/WT}$ mice [7]. Experimental diet might have an impact on the commensal bacterial composition compared to the microbiota of mice on Chow diet, thus leading to dysbiosis and an altered immune response.

We tested the antigenicity of cecal lysates originating from cecal content of WT and $TNF^{\Delta ARE/WT}$ mice on Chow and experimental diet. Therefore, BM-DCs were cultured and differentiated *in vitro* and stimulated with 10 ug/ml and 100 ug/ml of cecal lysates.

Cecal lysate mediated activation of BM-DCs triggered a dose dependent secretion of TNF ($P < 0.001$). But no difference could be observed regarding the type of cecal lysate (**Figure 24A**). BM-DCs were differentiated *in vitro* and therefore had not experienced any tolerance or priming mechanisms which might be important for a differentiated response. Thus, we isolated total mesenteric lymph node cells (MLNs) from WT and $TNF^{\Delta ARE/WT}$ mice on Chow diet and experimental diet for cecal lysate stimulation.

MLNs from $TNF^{\Delta ARE/WT}$ mice showed significantly ($P < 0.001$) higher TNF secretion after cecal lysate stimulation compared with MLNs from WT mice, irrespective of the diet. Though, no difference in stimulation capacity could be observed regarding the type of cecal lysate (**Figure 24B**).

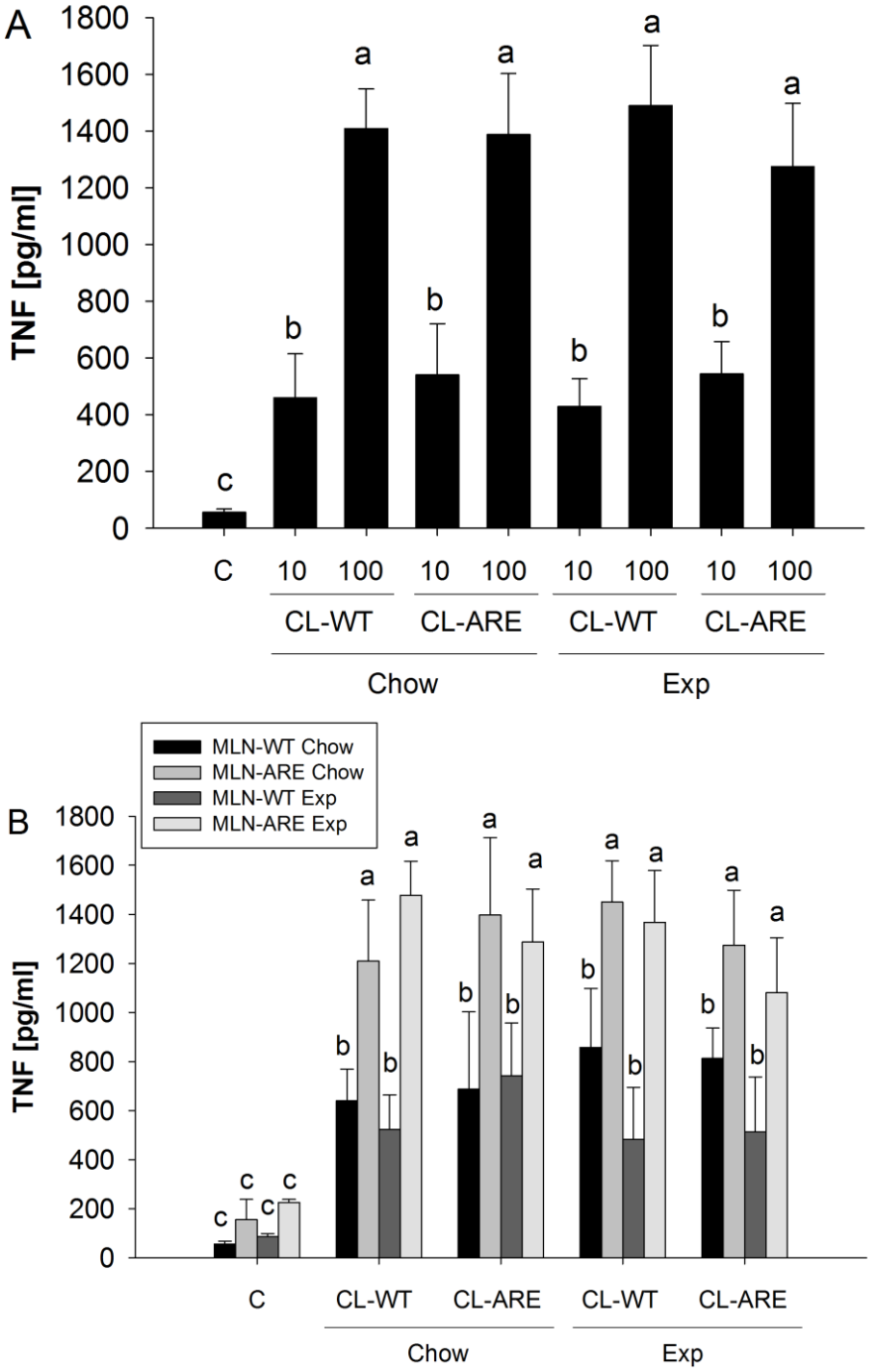
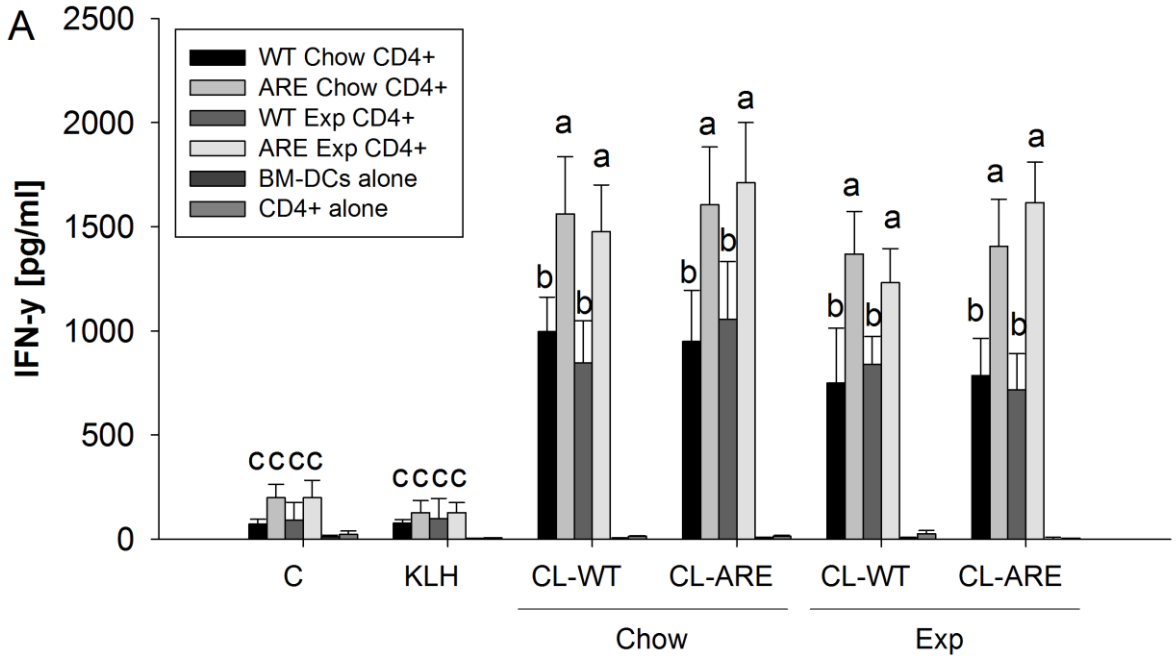


Figure 24. Cell culture analysis of cecal lysates (CL) prepared from WT and TNF$^{\Delta ARE/WT}$ mice being kept on experimental diet (Exp) and Chow diet.

Concentration of TNF in cell culture supernatants, measured by ELISA. (A) Bone marrow derived dendritic cells (BM-DCs) were cultured in vitro for 10-12 days until CD11b+ CD11c+ cells were > 95%. BM-DCs were stimulated with 10 ug/ml or 100 ug/ml cecal lysate from WT (CL-WT) or TNFΔARE/WT mice (CL-ARE) on Chow diet or experimental diet, as indicated, for 24 h. (B) Mesenteric lymph node cells from WT (MLN-WT) and TNFΔARE/WT (MLN-ARE) mice on Chow diet (Chow) and experimental diet (Exp) were stimulated with 100 ug/ml cecal lysate, as indicated, for 24 h. Values are means ± SD of three independent experiments. ^{a,b,c} means without a common letter differ, $P < 0.001$.

T-cell specific responses were analyzed using a coculture system of isolated CD4+ T-cells and BM-DCs which were pulsed with the appropriate cecal lysates. CD4+ T-cells from $TNF^{\Delta ARE/WT}$ mice showed significantly higher IFN-y secretion compared with WT mice ($P < 0.05$). No difference regarding the type of cecal lysate could be detected (**Figure 25A**).

A dose-dependent inhibition ($P < 0.001$) of IFN-y secretion could be achieved by using an anti-MHCII blocking antibody. CD4+ T-cells and BM-DCs alone did not respond to cecal lysate stimulation, suggesting that IFN-y secretion was dependent on MHCII:peptide complex presentation (**Figure 25B**). MHCII blocking had no effect on BM-DC activity as shown by secretion of IL-12 (**Figure 25C**).



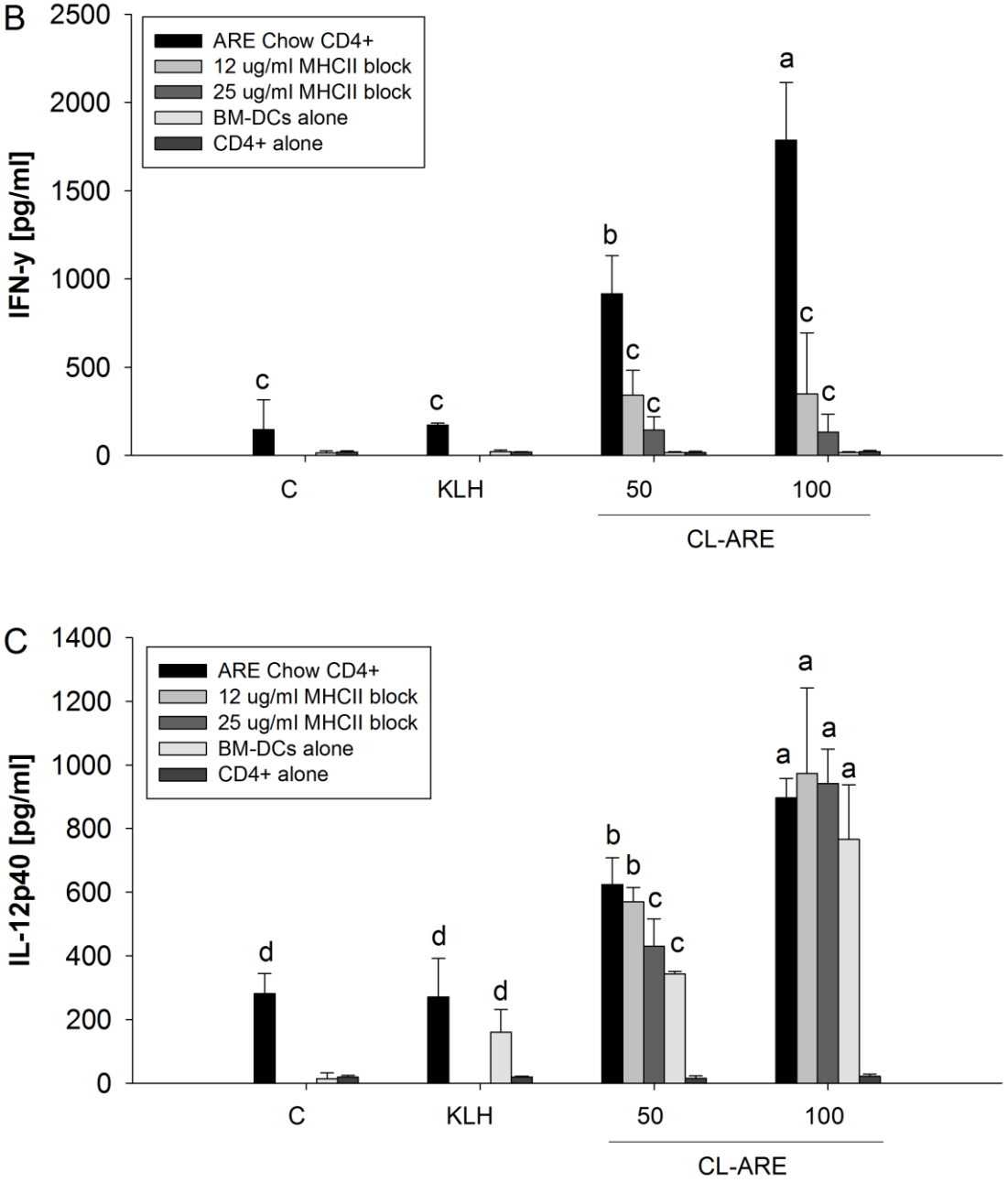


Figure 25. Coculture analysis of cecal lysates (CL) prepared from WT and TNF$^{\Delta ARE/WT}$ mice being kept on experimental diet (Exp) and Chow diet.

Concentration of IFN-y in coculture supernatants, measured by ELISA. (A) Bone marrow derived dendritic cells (BM-DCs) were pulsed with cecal lysates from WT (CL-WT) or TNFΔARE/WT mice (CL-ARE) on Chow diet (Chow) and experimental diet (Exp) for 6 h. CD4+ T-cells were isolated from mesenteric lymph nodes of WT and TNFΔARE/WT mice on Chow diet and experimental diet. BM-DCs and CD4+ T-cells were cocultured in 96-well plates for 72 h. (B) Coculture blocking experiment. Antigen presentation of cecal lysate (50 ug/ml or 100 ug/ml) pulsed BM-DCs was blocked, using 12 ug/ml or 25 ug/ml of anti-MHCII blocking antibody. BM-DCs and CD4+ T-cells were cocultured for 72 h. (C) Coculture blocking experiment. Determination of IL-12p40 in coculture supernatants after 72 h. In each experiment, keyhole limpet hemocyanin (KLH, 100 ug/ml) was used as a negative control. Values are means ± SD of three independent experiments. ^{a,b,c,d} means without a common letter differ, $P < 0.001$.

4.1.10. Chow suspensions have immunogenic potential

TNF$^{\Delta ARE/WT}$ mice being kept on autoclaved Chow diet under specific pathogen free (SPF) conditions showed scattered disease pathogenesis after 18 weeks of age, ranging from non-inflamed over moderately inflamed to highly inflamed distal ileal tissues (unpublished data of E. Berger and I. Sava, data not shown). Apart from airborne germs, diet-resident bacteria might have an effect on disease development in TNF$^{\Delta ARE/WT}$ mice.

We compared the endotoxin content of experimental and Chow diet and observed highly increased ($P < 0.001$) amounts of endotoxin in the Chow diet, whereas almost no endotoxins could be measured in the experimental diet (**Figure 26**). These data suggest microbial contamination of Chow and very low bacterial burden in the experimental diet.

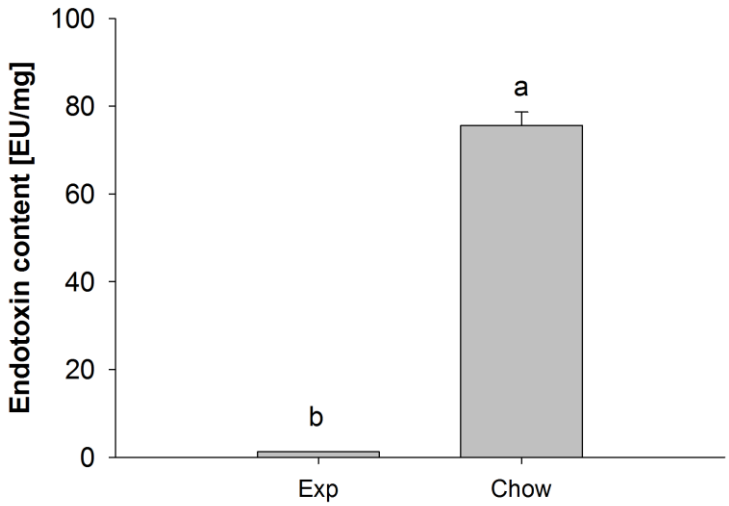


Figure 26. Endotoxin content in experimental diet (Exp) and Chow diet.
Endotoxin unit (EU). Values are means ± SD of three independent experiments. ^{a,b} means without a common letter differ, $P < 0.001$.

To investigate possible effects of live bacteria and microbial compounds in the diet, we prepared dietary suspensions from Chow (DS Chow), autoclaved Chow (DS Chow (A)) and experimental diet (DS Exp). Culture of DS Chow on unspecific blood agar plates for 24 hours resulted in dense colony formation, whereas DS Exp developed only sporadic or no colonies. No colonies could be detected after culture of DS Chow (A) (data not shown).

Immunostimulatory properties of dietary suspensions were evaluated by total MLN culture. DS Chow significantly ($P < 0.001$) induced cytokine secretion, whereas DS Exp had no significant effect on TNF secretion. The DS Chow (A) group showed significantly less ($P < 0.001$) TNF secretion compared with the standard Chow group, but still more ($P < 0.001$) TNF secretion than unstimulated controls. The difference in supply of diet (Chow vs Exp) during animal housing of TNF$^{\Delta ARE/WT}$ mice had no influence on TNF secretion by total MLNs (**Figure 27A**).

Accordingly, CD4+ T-cells from TNF$^{\Delta ARE/WT}$ mice on Chow and experimental diet showed significantly ($P < 0.001$) increased IFN-y secretion in coculture experiments using DS Chow pulsed BM-DCs compared to DS Exp and DS Chow (A) pulsed BM-DCs. (**Figure 27B**).

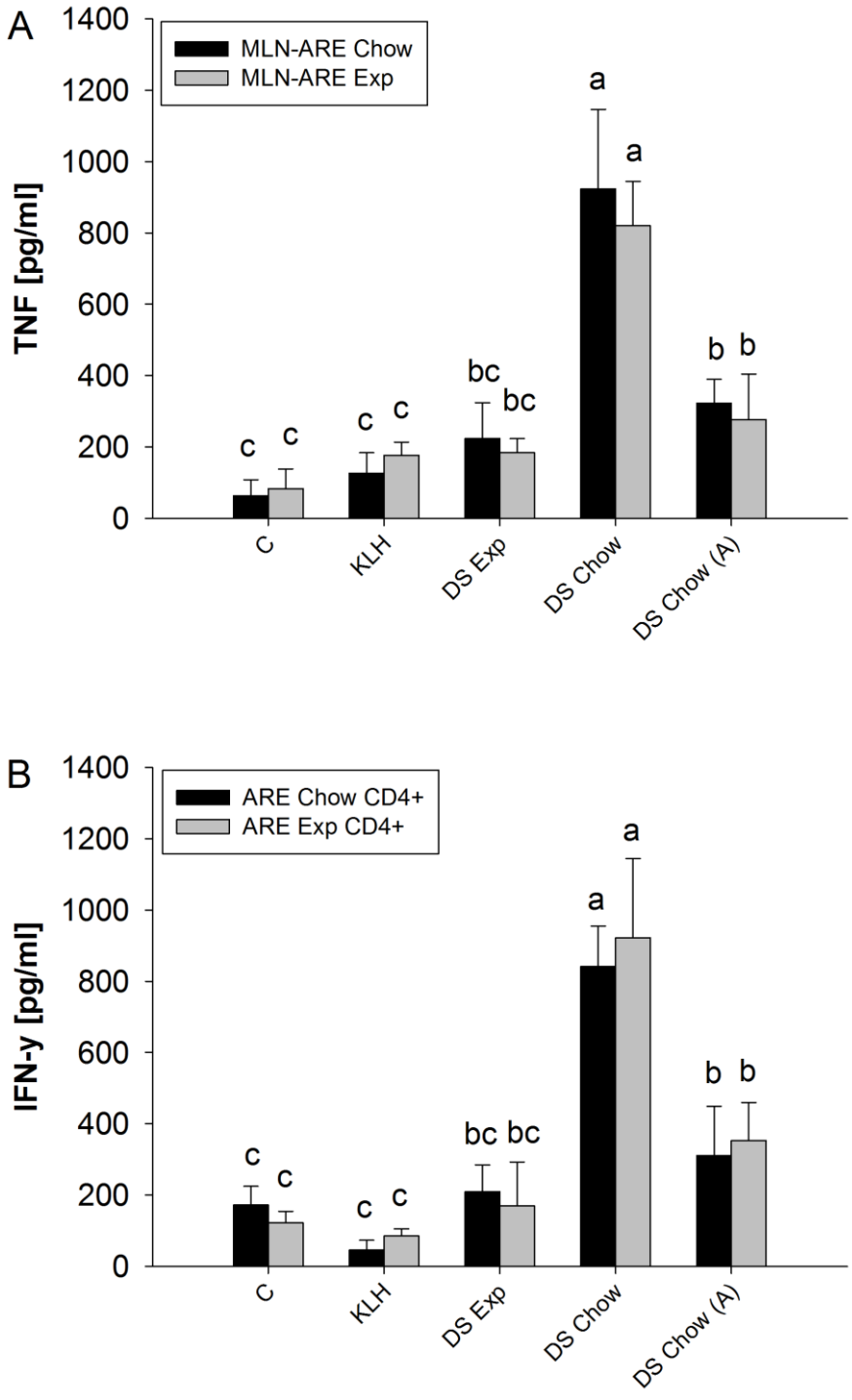


Figure 27. Cell culture analysis of dietary suspensions (DS) from experimental diet (DS Exp), Chow diet (DS Chow) and autoclaved Chow diet (DS Chow (A)).

Concentration of TNF and IFN-y in cell culture supernatants, measured by ELISA. (A) Concentration of TNF in cell culture supernatants. Mesenteric lymph node cells from TNFΔARE/WT mice (MLN-ARE) on Chow diet (Chow) and experimental diet (Exp) were stimulated with dietary suspensions of Chow diet (DS Chow, 100 ug/ml)

autoclaved Chow diet (DS Chow (A), 100 ug/ml) or experimental diet (DS Exp, 100 ug/ml) for 24 h. (B) Concentration of IFN-γ in coculture supernatants, measured by ELISA. Bone marrow derived dendritic cells (BM-DCs) were pulsed with DS Chow (100 ug/ml), DS Chow (A) (100 ug/ml) or DS Exp (100 ug/ml) for 6 h. CD4+ T-cells were isolated from mesenteric lymph nodes of TNFΔARE/WT mice on Chow diet (MLN-ARE Chow) and experimental diet (MLN-ARE Exp). BM-DCs and CD4+ T-cells were cocultured in 96-well plates for 72 h. In each experiment, keyhole limpet hemocyanin (KLH, 100 ug/ml) was used as a negative control. Values are means ± SD of three independent experiments. ^{a,b} means without a common letter differ, $P < 0.001$.

We designed a feeding experiment, where we transferred TNF$^{\Delta ARE/WT}$ mice to autoclaved Chow diet in week seven of age. To assess possible differences in progression of pathology, we sacrificed mice after 12 and 18 weeks of age. Tissue pathology assessed by histological examination of distal ileal sections could show no differences in disease development and severity compared with standard Chow fed TNF$^{\Delta ARE/WT}$ mice (**Figure 28**). These results suggest that live microbial contamination of the Chow diet seems to have no effect on histopathology in the TNF$^{\Delta ARE/WT}$ mouse model.

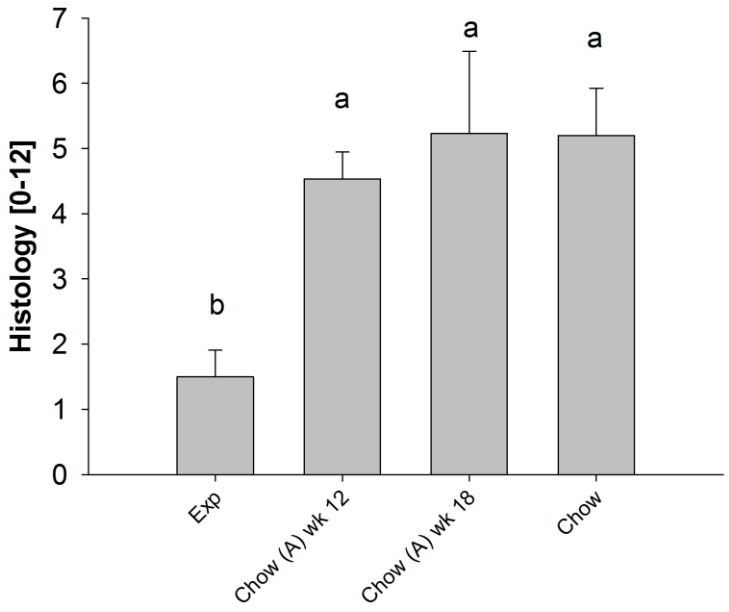


Figure 28. Tissue pathology of TNF$^{\Delta ARE/WT}$ mice being kept on experimental diet (Exp), Chow diet and autoclaved Chow diet (Chow (A)).

Tissue pathology assessed by histological examination (score 0-12) of distal ileal sections from TNFΔARE/WT mice on different diets as indicated. Animals have been sacrificed after 18 or 12 weeks of age. Values are means ± SD, n = 3-6 per group. ^{a,b} means without a common letter differ, $P < 0.001$.

4.1.11. Gluten-fortified experimental diet induces chronic ileitis in TNF$^{\Delta ARE/WT}$ mice

Chow diet is a standard diet that consists of natural components with wheat as a major ingredient. Thus, Chow diet is rich in gluten proteins, whereas experimental diet is gluten-free. We next wanted to investigate, whether there was an effect of gluten in the genetically susceptible TNF$^{\Delta ARE/WT}$ mouse model. For this purpose, we designed a gluten-fortified experimental diet (Glu). **Table 1** illustrates the nutritional composition of Chow, Exp and Glu.

Table 1. Nutritional composition of Chow diet (Chow), experimental diet (Exp) and gluten-fortified experimental diet (Glu).

	Chow	Exp	Glu
Energy [MJ ME/kg]	12,8	14,7	14,7
Crude protein [%]	19,0	17,6	17,6
Crude fat [%]	3,3	5,1	5,1
Crude fiber [%]	4,9	4,1	4,1
Crude ash [%]	6,4	5,5	5,5
Starch [%]	36,5	47,2	47,2
Sugar [%]	4,7	11,3	11,3

Wheat and wheat products account for 50% and 0% of Chow and Exp ingredients, respectively. Glu has been fortified with 100 g gluten per kg diet. For a more detailed illustration of nutrients, see Appendix 1.

In order to assess the inflammatory potential of gluten in the pathogenesis of Crohn's disease-like ileitis, we set up a feeding experiment using the gluten-fortified experimental diet (Glu). Activation of total MLNs from TNF$^{\Delta ARE/WT}$ mice, being fed Chow, Exp and Glu, with peptic-tryptic digested gluten (PT-gluten) significantly ($P < 0.001$) induced secretion of TNF (**Figure 29A**). In contrast, CD4+ T-cell specific IFN-y secretion in coculture experiments with PT-gluten pulsed BM-DCs could not be detected, suggesting an antigen-independent co-activation. In contrast, DS Chow pulsed BM-DCs induced high secretion ($P < 0.001$) of IFN-y (**Figure 29B**).

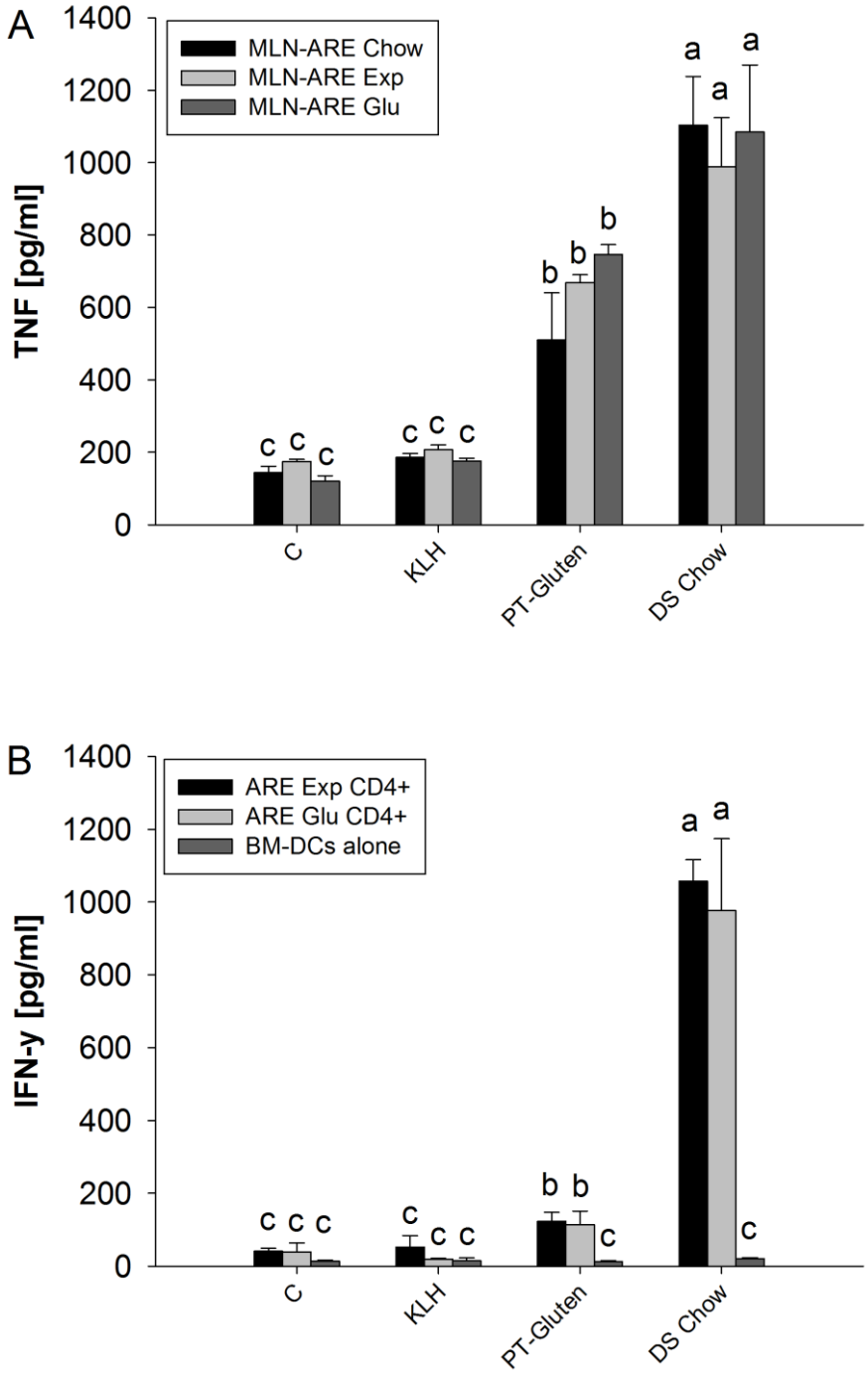


Figure 29. Cell culture analysis of peptic-tryptic digested gluten (PT-gluten).

Concentration of TNF and IFN-y in cell culture supernatants, measured by ELISA. (A) Concentration of TNF in cell culture supernatants. Mesenteric lymph node cells from TNFΔARE/WT mice (MLN-ARE) on Chow diet (Chow), experimental diet (Exp) and gluten fortified experimental diet (Glu) were stimulated with peptic-tryptic digests of gluten (PT-Gluten, 100 ug/ml) or dietary suspensions of Chow diet (DS Chow, 100 ug/ml) for 24 h. (B) Concentration of IFN-y in coculture supernatants, measured by ELISA. Bone marrow derived dendritic cells (BM-DCs) were pulsed with PT-Gluten (100 ug/ml) or DS Chow (100 ug/ml) for 6 h. CD4+ T-cells were isolated from mesenteric lymph nodes of TNFΔARE/WT mice on experimental diet (ARE Exp CD4+) or gluten fortified experimental diet (ARE Glu CD4+). BM-DCs and CD4+ T-cells were cocultured in 96-well plates for 72 h. In each

experiment, keyhole limpet hemocyanin (KLH, 100 ug/ml) was used as a negative control. Values are means ± SD of three independent experiments. [a,b] means without a common letter differ, P < 0.001.

We performed qPCR analysis of distal ileal tissues to analyze changes in inflammation-related gene expression. $TNF^{\Delta ARE/WT}$ mice being kept on Glu showed significantly (P < 0.01) increased expression of TNF and IFN-y compared with $TNF^{\Delta ARE/WT}$ mice on experimental diet. There was no difference in expression levels of IFN-y as compared with animals on Chow diet. Interestingly, IL-15 expression was significantly (P < 0.01) elevated in $TNF^{\Delta ARE/WT}$ mice on Glu but not on Chow diet (**Figure 30A**). A similar expression pattern could be observed regarding TLR2 and TLR4. $TNF^{\Delta ARE/WT}$ mice on Glu showed significantly induced expression of both pattern recognition receptors and Chow fed mice showed even higher values (P < 0.01) (**Figure 30B**).

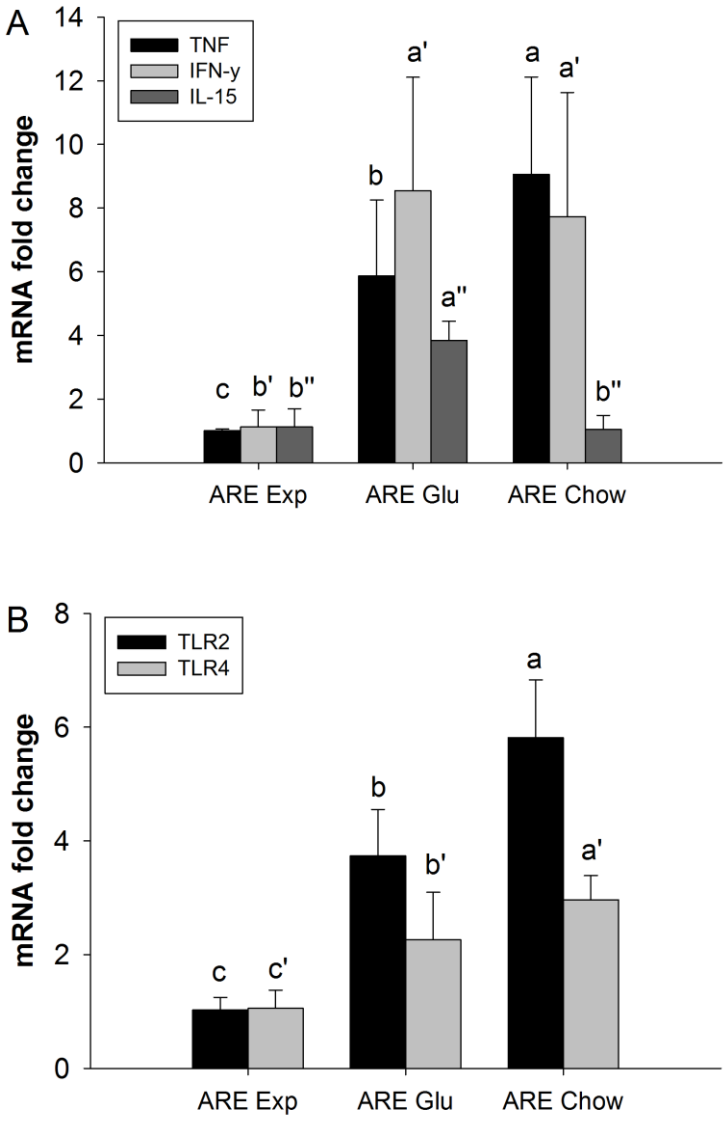


Figure 30. Quantitative real-time PCR analysis of proinflammatory cytokines and pattern recognition receptors.

Relative mRNA expression of (A) TNF, IFN-y and (B) TLR2, TLR4 in distal ileal tissue from TNFΔARE/WT mice being kept on Exp, Chow and Glu (sacrificed at 18 weeks of age). Values are means ± SD, n = 6 per group. [a,b,c] Within each graph, means without a common letter differ, P < 0.01.

Tight junction-related occludin (OCLN) was significantly (P < 0.01) decreased in $TNF^{\Delta ARE/WT}$ mice with chronic ileitis on Glu and Chow diet. By contrast, zonula occludens-1 (ZO-1) expression was not changed in any condition (**Figure 31**).

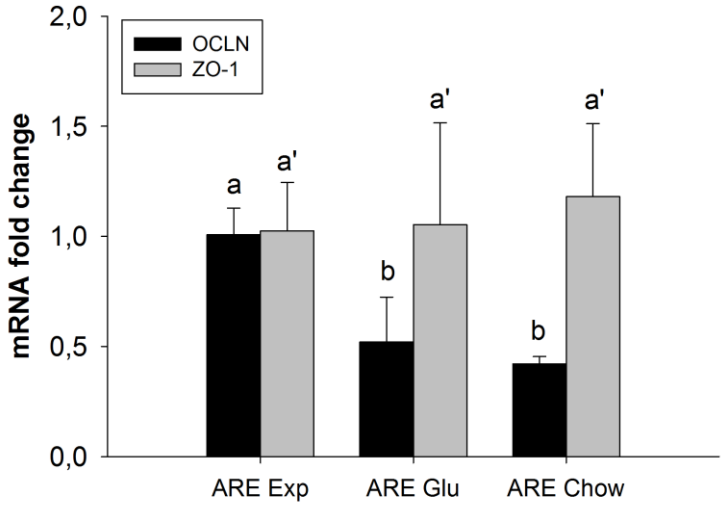


Figure 31. Quantitative real-time PCR analysis of tight junction molecules.
Relative mRNA expression of occludin (OCLN) and zonula occludens-1 (ZO-1) in distal ileal tissue from TNFΔARE/WT mice being kept on Exp, Chow and Glu. Values are means ± SD, n = 6 per group. [a,b,c] Within each graph, means without a common letter differ, P < 0.01.

Glu significantly (P < 0.001) induced tissue pathology in the distal ileum after 18 weeks of age as assessed by histological scoring of distal ileal sections **(Figure 32**). Tissue pathology was accompanied by strong diarrhea of TNF$^{\Delta ARE/WT}$ mice on Glu diet.

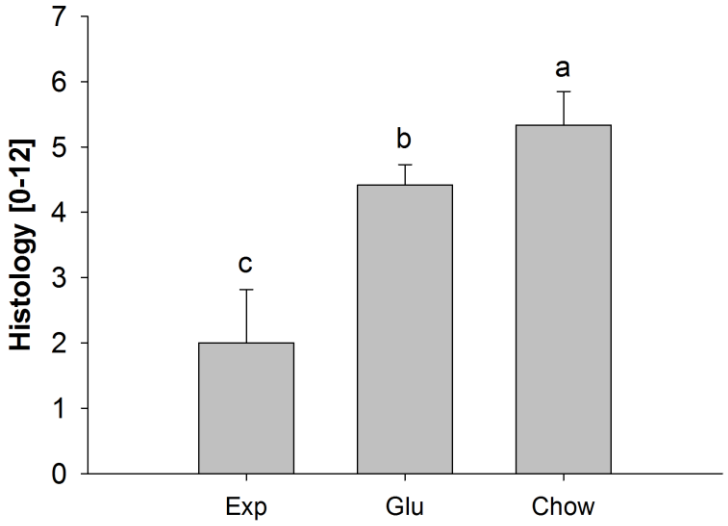


Figure 32. Tissue pathology of TNF$^{\Delta ARE/WT}$ mice being kept on experimental diet (Exp), gluten-fortified experimental diet (Glu) and Chow diet.

Tissue pathology assessed by histological examination (score 0-12) of distal ileal sections from TNFΔARE/WT mice (ARE, 18 weeks of age) on experimental diet (Exp), gluten fortified experimental diet (Glu) and Chow diet (Chow). Values are means ± SD, n = 6 per group. ^{a,b} means without a common letter differ, P < 0.001.

Leukocyte infiltration in distal ileal tissues of TNF$^{\Delta ARE/WT}$ mice was visualized by immunohistochemical labeling of CD3^{+} T-cells. Distal ileal sections of TNF$^{\Delta ARE/WT}$ mice on Glu and Chow diet showed increased infiltration of CD3^{+} T-cells and impaired intestinal architecture as compared with TNF$^{\Delta ARE/WT}$ mice on experimental diet (**Figure 33**), corroborating the histological findings. These results suggest gluten as a potential trigger of Crohn's disease-like ileitis in the TNF$^{\Delta ARE/WT}$ mouse model.

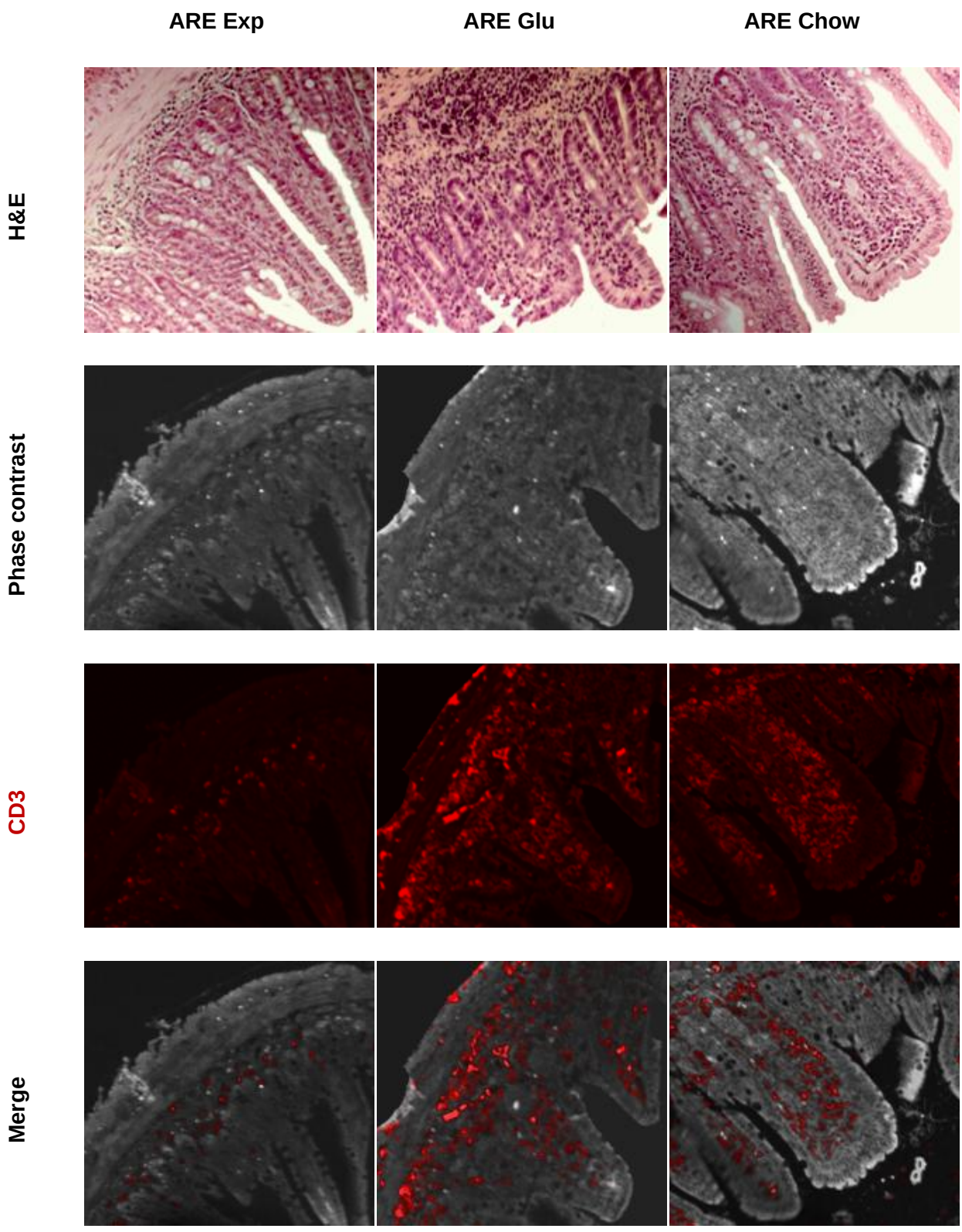


Figure 33. Fluorescence microscopy of distal ileal tissues from TNF$^{\Delta ARE/WT}$ mice.

Representative H&E staining and immunohistochemical labeling (CD3+ T-cells) of distal ileal sections from TNFΔARE/WT mice (18 weeks of age) on experimental diet (Exp), gluten fortified experimental diet (Glu) and Chow diet (Chow). Fluorescence microscopy is visualized by phase contrast, fluorescence and merged images as indicated (100x magnification).

4.1.12. Effects of a wheat depleted Chow diet on tissue pathology in TNF$^{\Delta ARE/WT}$ mice

We could show that gluten-fortified experimental diet induced chronic ileitis in TNF$^{\Delta ARE/WT}$ mice, in contrast to gluten-free experimental diet, which inhibited the development of tissue pathology. Next we wanted to analyze the effects of a modified Chow diet (Chow –W), which was depleted from wheat products (**Table 2**).

Table 2. Natural ingredients in standard Chow diet and wheat depleted Chow diet (Chow -W).

	Chow	Chow (-W)
Wheat & wheat products [%]	50,0	/
Babycorn, pre-treated [%]	/	30,6
Puffed rice [%]	/	16
Soybean products [%]	22,7	27,4
Barley [%]	16,0	16,0

Wheat and wheat products have been substituted by corn and rice products in Chow (-W). For a more detailed illustration of nutrients, see Appendix 1.

Activation of total MLNs from TNF$^{\Delta ARE/WT}$ with dietary suspensions of Chow (DS Chow) or wheat depleted Chow (DS Chow (-W)) significantly ($P < 0.001$) induced secretion of TNF. No difference in immunostimulatory potential of DS Chow and DS Chow (-W) could be detected (**Figure 34A**).

CD4+ T-cell specific IFN-y secretion in coculture experiments reached comparable levels using DS Chow and DS Chow (-W) pulsed BM-DCs. (**Figure 34B**).

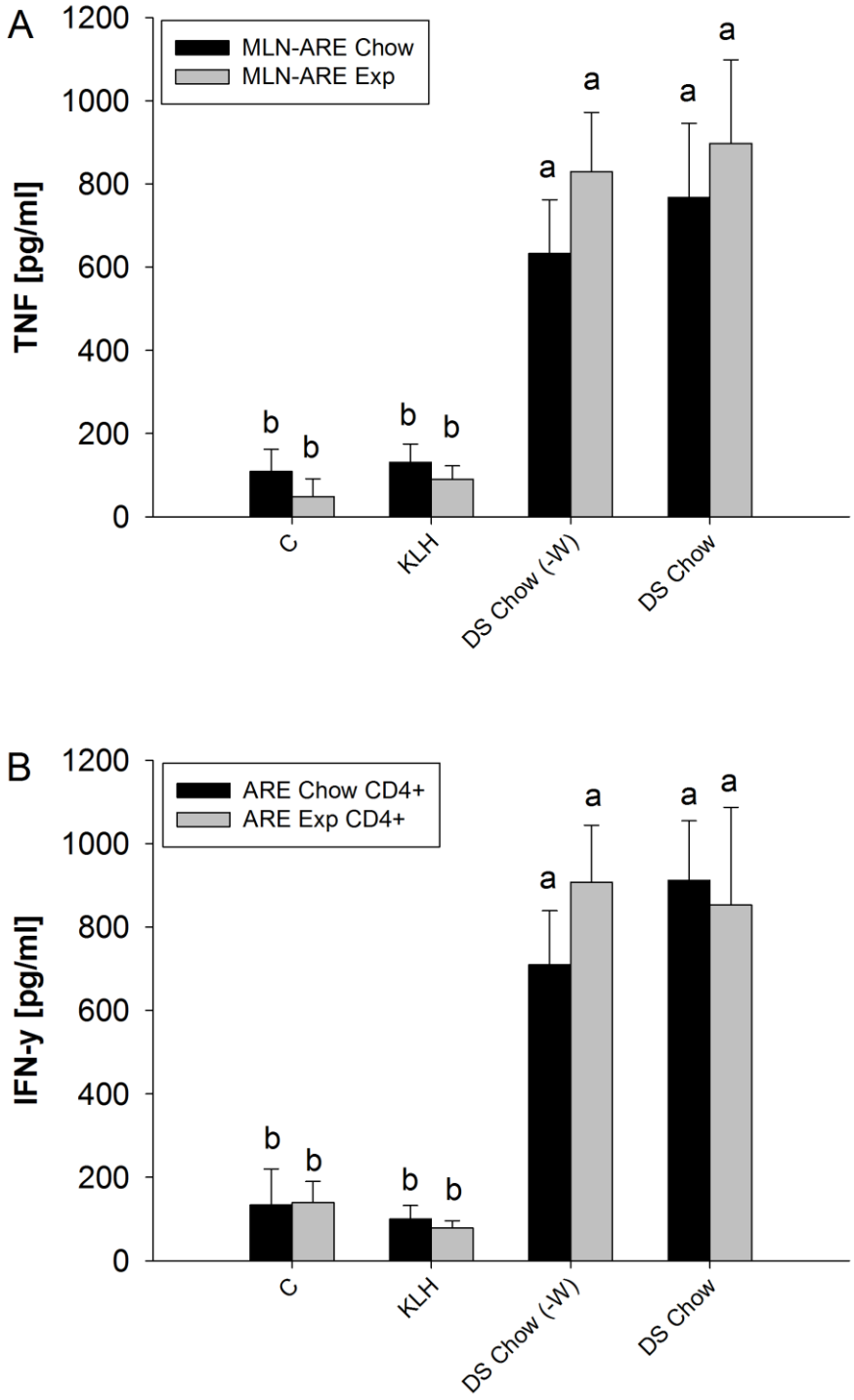


Figure 34. Cell culture analysis of dietary suspensions (DS) from Chow diet (DS Chow) and wheat depleted Chow diet (DS Chow (-W)).

Concentration of TNF and IFN-y in cell culture supernatants, measured by ELISA. (A) Concentration of TNF in cell culture supernatants. Mesenteric lymph node cells from TNFΔARE/WT mice (MLN-ARE) on Chow diet (Chow) and experimental diet (Exp) were stimulated with dietary suspensions of Chow diet (DS Chow, 100 ug/ml) or wheat depleted Chow diet (DS Chow (-W), 100 ug/ml) for 24 h. (B) Concentration of IFN-y in coculture supernatants, measured by ELISA. Bone marrow derived dendritic cells (BM-DCs) were pulsed with DS Chow (100 ug/ml) or DS Chow (-W) (100 ug/ml) for 6 h. CD4+ T-cells were isolated from mesenteric lymph nodes of TNFΔARE/WT mice on Chow diet (ARE Chow CD4+) and experimental diet (ARE Exp CD4+). BM-DCs and

CD4+ T-cells were cocultured in 96-well plates for 72 h. In each experiment, keyhole limpet hemocyanin (KLH, 100 ug/ml) was used as a negative control. Values are means ± SD of three independent experiments. [a,b] means without a common letter differ, P < 0.001.

To evaluate the effects of the wheat depleted Chow diet on disease pathogenesis, we started a feeding experiment with seven weeks old $TNF^{\Delta ARE/WT}$ mice and kept them on Chow (-W) until 18 weeks of age. No differences in ileitis severity could be observed after histological examination of distal ileal sections (**Figure 35**).

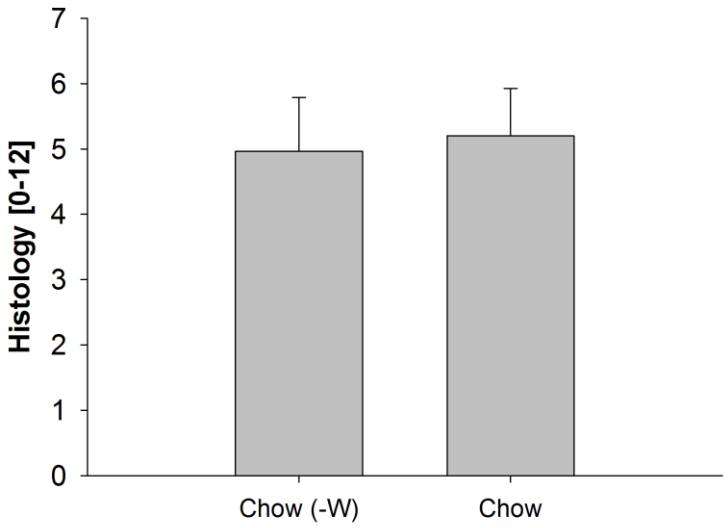


Figure 35. Tissue pathology of $TNF^{\Delta ARE/WT}$ mice being kept on Chow diet and wheat depleted Chow diet.
Tissue pathology assessed by histological examination (score 0-12) of distal ileal sections from TNFΔARE/WT mice (18 weeks of age) on Chow diet and wheat depleted Chow diet (Chow (-W). Values are means ± SD, n = 6 per group.

4.2. Dietary modulation of IL-10$^{-/-}$ colitis by experimental diet

In contrast to TNF$^{\Delta ARE/WT}$ mice, which develop a Crohn's disease-like ileitis, IL-10$^{-/-}$ mice develop spontaneous colitis. It has been shown that IL-10$^{-/-}$ mice being kept under germ-free conditions do not develop pathology. Thus, the commensal microbiota is suggested to play a crucial role in disease development.

Based on our findings regarding the protective potential of experimental diet in feeding experiments with TNF$^{\Delta ARE/WT}$ mice, we wanted to assess possible effects of experimental diet on colitis development in IL-10$^{-/-}$ mice. Animals were transferred from specific pathogen free (SPF) to conventional housing conditions at six weeks of age. IL-10$^{-/-}$ mice were kept on Chow or experimental diet and sacrificed after 24 weeks of age.

Dietary treatment had no effect on body weight development. Spleens of IL-10$^{-/-}$ mice on Chow and Exp were of comparable size and weight (**Figure 36A and B**). Spleen to body weight ratios showed no significant difference either (data not shown). Colons of animals in the Exp and Chow group were of comparable length (**Figure 36C**).

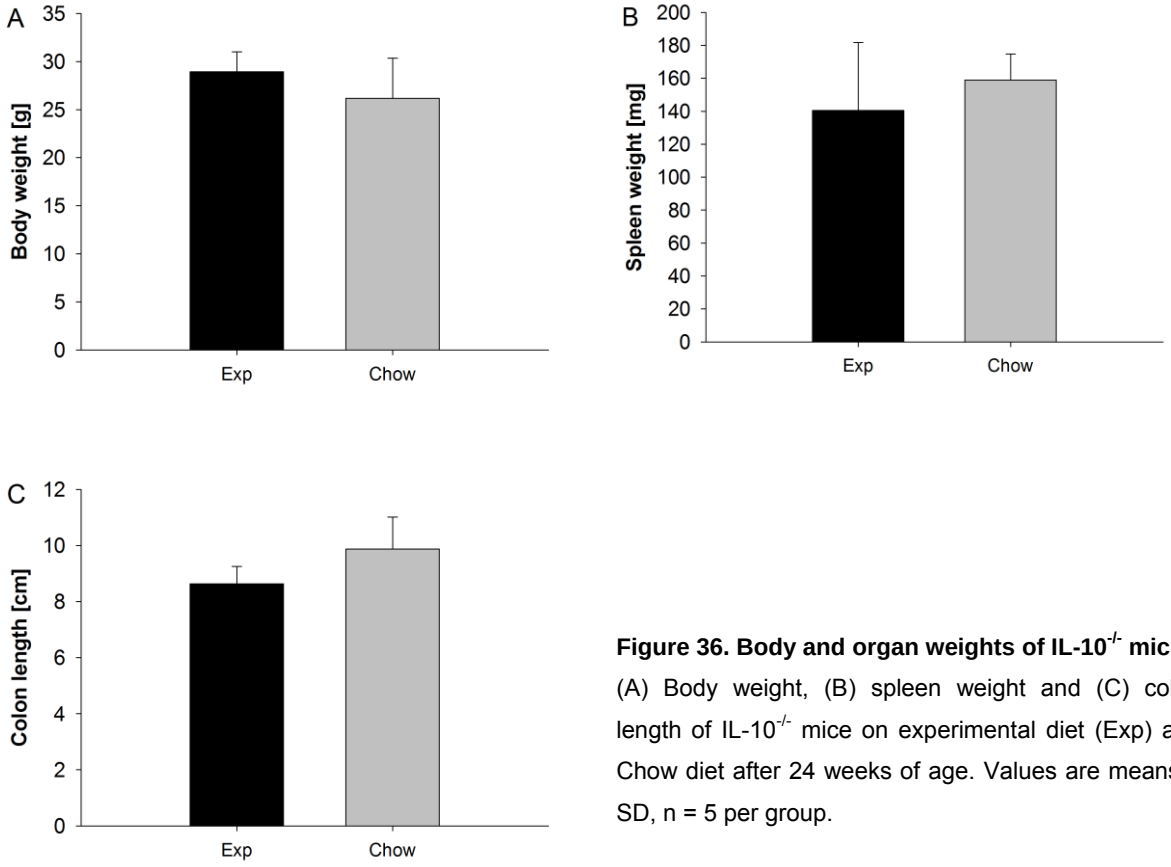


Figure 36. Body and organ weights of IL-10$^{-/-}$ mice. (A) Body weight, (B) spleen weight and (C) colon length of IL-10$^{-/-}$ mice on experimental diet (Exp) and Chow diet after 24 weeks of age. Values are means ± SD, n = 5 per group.

Histological scoring of the distal colon revealed strong tissue pathology in IL-10$^{-/-}$ mice being fed Chow, whereas animals on experimental diet showed only mild colitis ($p < 0.05$) (**Figure**

37A). We next tried to confirm our findings by serum amyloid A (SAA) measurement in plasma samples. SAA values of the Chow group were significantly ($P < 0.01$) increased, compared with plasma samples of animals on experimental diet and did highly correlate with histology scores (**Figure 37B**). These data might direct to an interchangeable protective effect in Crohn's disease-like ileitis and IL-10$^{-/-}$ colitis by dietary intervention using experimental diet.

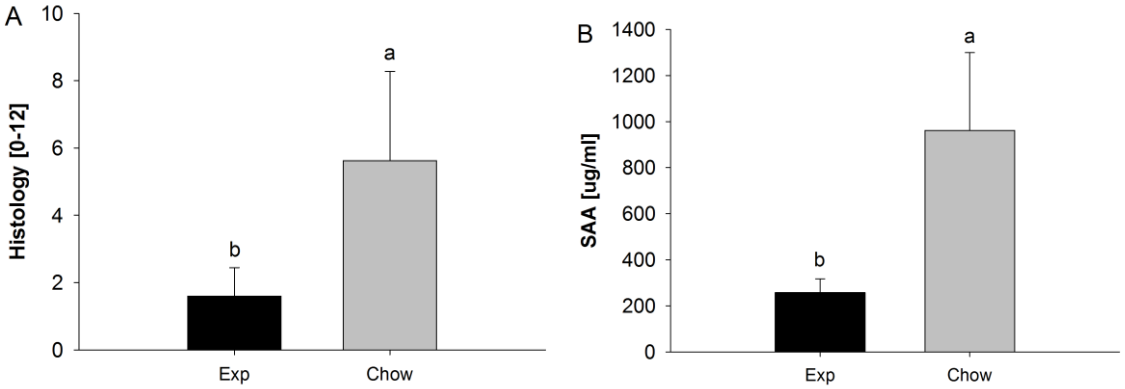


Figure 37. Tissue pathology and serum amyloid A (SAA) levels of IL-10$^{-/-}$ mice.

(A) Tissue pathology assessed by histological examination (score 0-12) of distal colonic sections from IL-10$^{-/-}$ mice (24 weeks of age) on experimental diet (Exp) and Chow diet (Chow). (B) Serum amyloid A (SAA) values in plasma samples of IL-10$^{-/-}$ mice. Values are means ± SD, n = 5 per group. ^{a,b} means without a common letter differ, $P < 0.05$ and $P < 0.01$ for (A) and (B), respectively.

4.3. Experimental diet in the modulation of adoptive transfer colitis and ileitis

To evaluate the effects of dietary intervention on disease location, we performed an adoptive transfer of $CD4^+$ IL-$10^{-/-}$ T cells into $Rag2^{-/-}$ recipient mice. Recipient mice were kept on Chow or experimental diet for 10 weeks after transfer. The T cell transfer model is a valuable tool to investigate development of chronic enterocolitis as well as ileitis in the same animal.

Body weights were measured every week to generate a weight curve. There was no difference in weight development comparing $Rag2^{-/-}$ recipient mice on Chow and experimental diet (**Figure 38**).

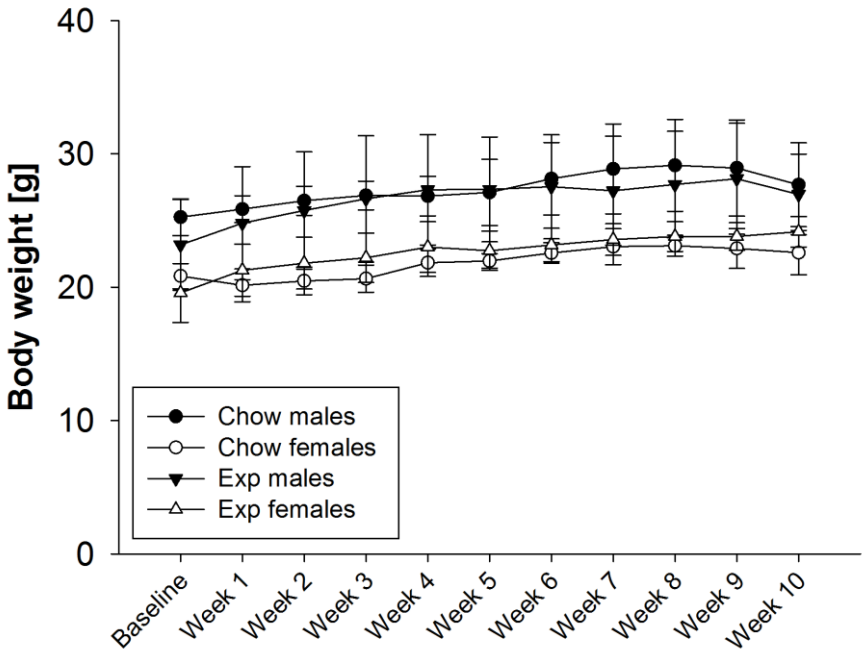


Figure 38. Body weight curves of $Rag2^{-/-}$ mice.
Body weights of male and female $Rag2^{-/-}$ mice on experimental diet (Exp) and Chow diet (Chow) were measured every week starting at the day of T-cell transfer (Baseline). Values are means ± SD, n = 3 per group.

We measured organ weights and noticed a significantly ($P < 0.05$) increased colon weight-to-length ratio of $Rag2^{-/-}$ mice in the Chow group. Accordingly, cecum weights of $Rag2^{-/-}$ mice were significantly higher ($P < 0.001$) when kept on Chow. However, no significant difference could be found comparing the spleen weights (**Figure 39**).

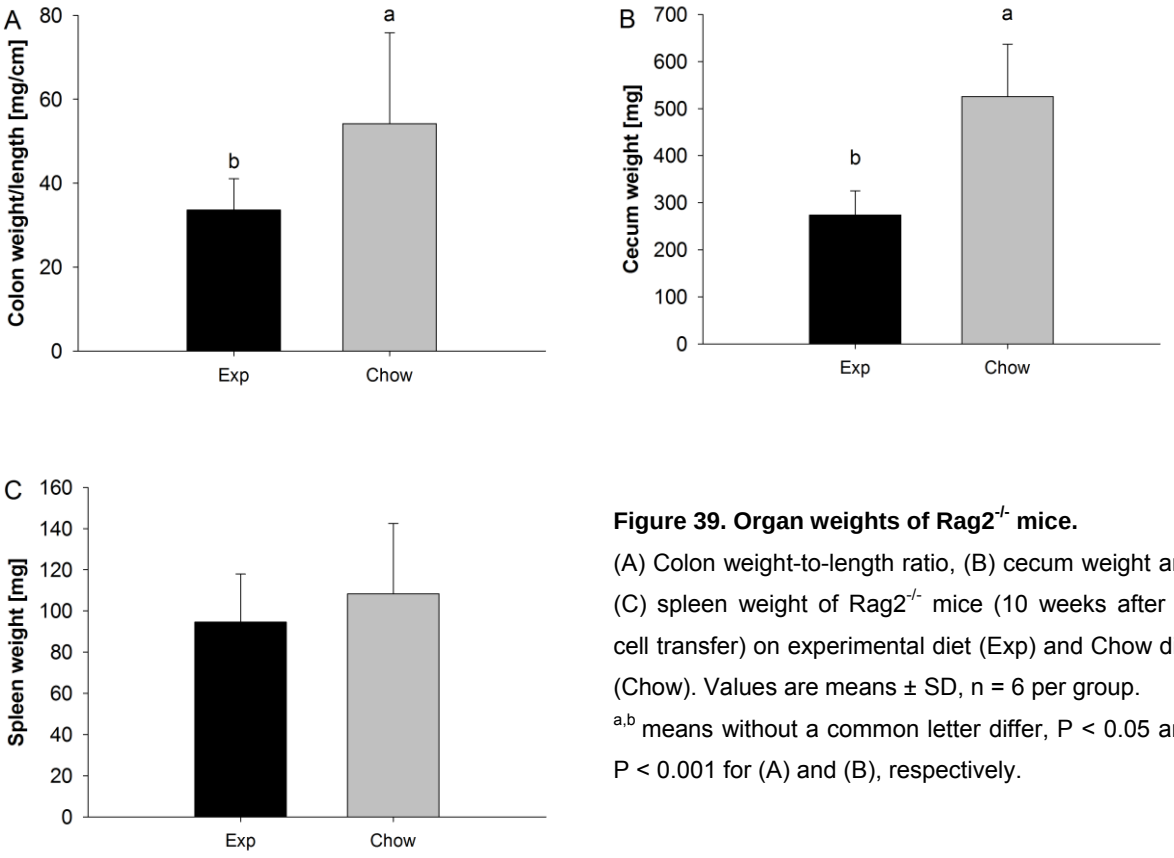


Figure 39. Organ weights of $Rag2^{-/-}$ mice.
(A) Colon weight-to-length ratio, (B) cecum weight and (C) spleen weight of $Rag2^{-/-}$ mice (10 weeks after T-cell transfer) on experimental diet (Exp) and Chow diet (Chow). Values are means ± SD, n = 6 per group.
^{a,b} means without a common letter differ, $P < 0.05$ and $P < 0.001$ for (A) and (B), respectively.

We next aimed at evaluating the effects of dietary intervention on colitis compared with ileitis pathogenesis. Tissue pathology in distal ileal and distal colonic tissues was assessed by histological scoring of H&E stained sections. Surprisingly, no difference in histopathology could be observed in moderately inflamed distal ileal tissues of Rag2$^{-/-}$ recipient mice kept on Chow or experimental diet (**Figure 40A**). There was a trend to stronger tissue pathology in distal colonic sections of Rag2$^{-/-}$ mice on Chow compared with Rag2$^{-/-}$ mice on experimental diet, yet values did not reach significance (**Figure 40B**).

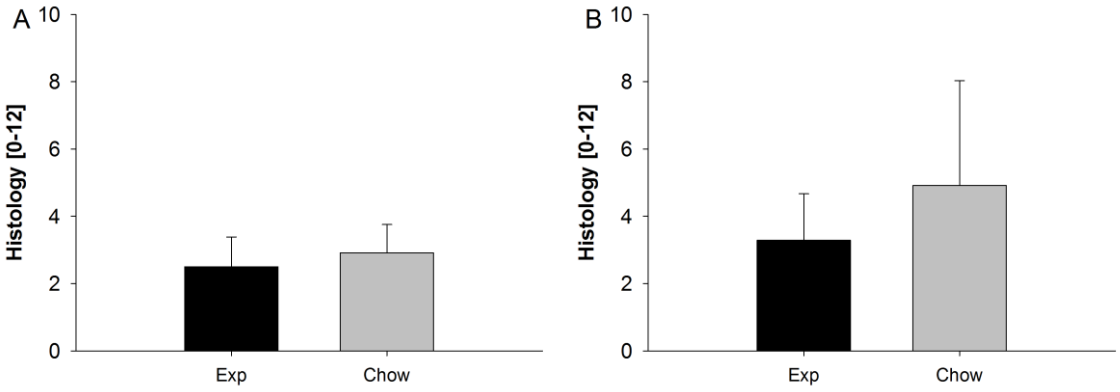


Figure 40. Tissue pathology in the ileum and colon of Rag2$^{-/-}$ mice.

(A) Tissue pathology assessed by histological examination (score 0-12) of (A) distal ileal and (B) distal colonic sections from Rag2$^{-/-}$ mice (10 weeks after T-cell transfer) on experimental diet (Exp) and Chow diet (Chow).

We next performed qPCR analysis of distal ileal and colonic tissue sections. Interestingly, TNF and IFN-y levels were significantly ($P < 0.05$) higher in distal ileal tissues of Chow fed Rag2$^{-/-}$ mice compared with Exp fed Rag2$^{-/-}$ mice (**Figure 41A**). By contrast, no difference in TNF and IFN-y expression could be seen in colonic tissue mRNA (**Figure 41B**).

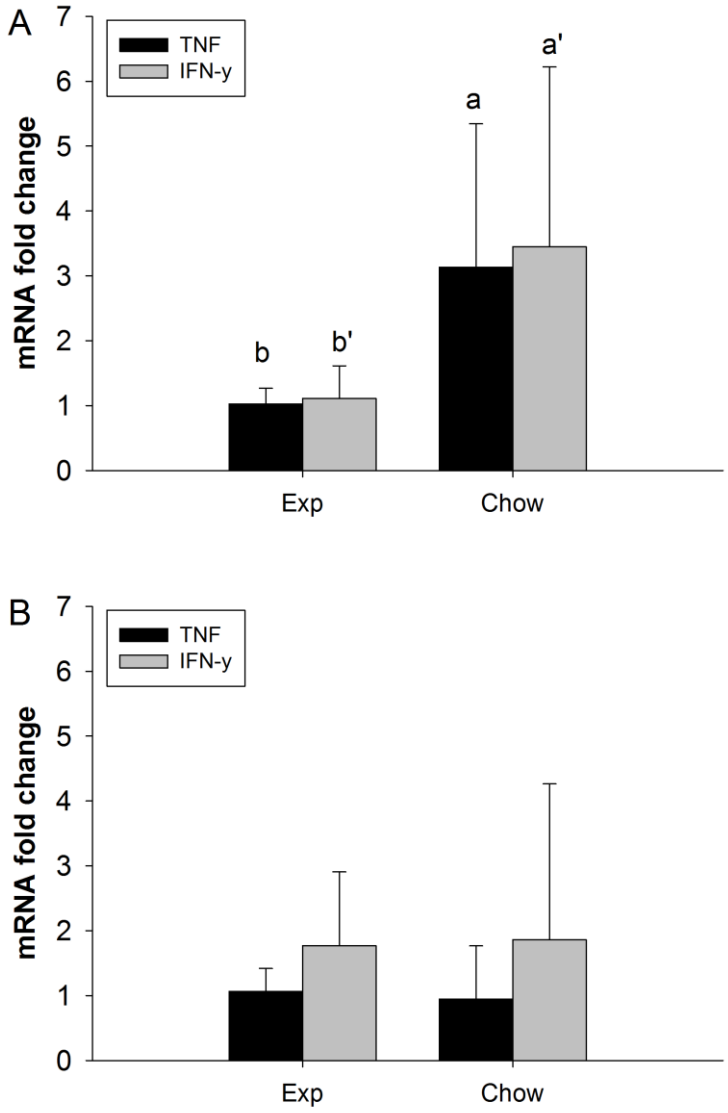


Figure 41. Quantitative real-time PCR analysis of proinflammatory cytokines in the ileum and colon.
Relative mRNA expression of TNF in (A) distal ileal and (B) distal colonic tissue from Rag2$^{-/-}$ mice (10 weeks after T-cell transfer) on experimental diet (Exp) and Chow diet (Chow). Values are means ± SD, n = 6 per group.
[a,b] Within each graph, means without a common letter differ, $P < 0.05$.

Systemic inflammation was assessed by serum amyloid A (SAA) measurement in plasma samples of all animals. SAA values were significantly ($P < 0.01$) elevated in the Chow group, suggesting an increased systemic inflammatory response in T-cell transfer recipient mice on Chow diet compared with those on experimental diet (**Figure 42**).

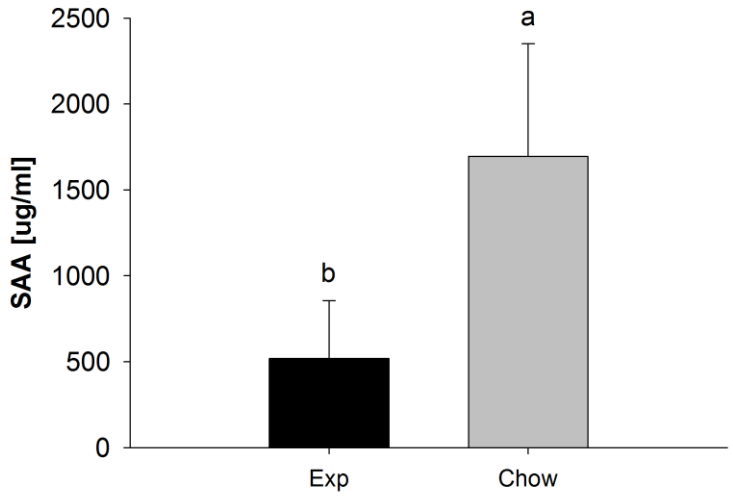


Figure 42. Serum amyloid A (SAA) levels of Rag2$^{-/-}$ mice.

Serum amyloid A (SAA) values in plasma samples of Rag2$^{-/-}$ mice (10 weeks after T-cell transfer) on experimental diet (Exp) and Chow diet (Chow). Values are means ± SD, n = 6 per group. ^{a,b} means without a common letter differ, $P < 0.01$.

5. DISCUSSION

In clinical studies, nutritional therapy was shown to be efficacious in inducing and maintaining remission in Crohn's disease patients, associated with improved nutritional status, decreased proinflammatory cytokine levels and mucosal healing [119-121]. Even though meta-analysis reviews comparing therapeutic efficacy have yielded clinical data that favor corticosteroid therapy in adult Crohn's disease patients [122, 124, 171], therapeutic outcome may be influenced by several factors like patients demography, nutritional adherence, disease state and time of intervention [30]. Interestingly, nutritional therapy in the form of EN seems to be much more promising in Crohn's disease patients and less effective in ulcerative colitis patients, the reasons for which are as yet unknown [112, 172]. It is noteworthy that EEN is recommended as primary therapy in pediatric Crohn's disease with clinical response rates as high as in corticosteroid therapy, while avoiding its severe adverse effects [126, 128]. Moreover, children with newly diagnosed Crohn's disease showed highest responsiveness (up to 85%) to EEN. By contrast, in children on relapse, the clinical response rate fell to 50%, comparable to the response rate of 53% achieved in the European Cooperative Crohn's Disease Study IV, including 107 adults [127, 173]. Thus, timing and IBD subtype seem to be important factors for therapeutic outcome.

Several studies have been performed to elucidate to role of EN formula on therapeutic efficacy. Meta-analysis of ten trials comprising 334 patients demonstrated no difference in the efficacy of elemental versus non-elemental formulas (OR 1.10; 95% CI 0.69 to 1.75). Subgroup analyses performed to evaluate the different types of elemental and non-elemental diets (elemental, semi-elemental and polymeric) showed no statistically significant differences [122]. A semi-synthetic diet was used in our dietary intervention studies, with close resemblance to semi-elemental diet, as applied in clinical settings. However, animals were kept on diets provided in a pelleted form instead of liquid diets, as used in clinical studies. We used different animal models of mucosal inflammation with focus on the $TNF^{\Delta ARE/WT}$ model for Crohn's disease-like ileitis, regarding the fact that EN yields highest clinical response in Crohn's disease patients. Tissue pathology was selected as main experimental endpoint and results were compared with experimental outcome on a standard Chow diet.

5.1. Effects of dietary intervention on Crohn's disease-like ileitis

The semi-synthetic experimental diet exhibited a clear protective effect on tissue pathology development in $TNF^{\Delta ARE/WT}$ mice, demonstrated by histological examination of distal ileal sections after 18 weeks of age. This effect was accompanied by a significant reduction of leukocyte infiltration, MLN weights and total MLN leukocyte numbers. We observed reduced tissue activation in the distal ileum with decreased expression of pattern recognition receptors, proinflammatory cytokines, as well as reduction of homing associated addressins. These findings suggest that chronic intestinal inflammation was inhibited in $TNF^{\Delta ARE/WT}$ mice on experimental diet.

Interestingly, dietary intervention seemed to have no effect on spleen attributes. $TNF^{\Delta ARE/WT}$ mice on Chow and experimental diet did not differ concerning spleen weight and yield of splenocyte numbers and showed increased values compared with WT animals on each of the diets. The spleen plays a crucial role in initiating immune reactions to blood-borne antigens and for filtering the blood of foreign material and old or damaged red blood cells. Increased spleen size may be associated with several immunologic conditions such as viral, parasitic or bacterial infections, lymphoma or chronic inflammatory diseases [174, 175]. Besides Crohn's-like intestinal inflammation, constitutive expression of TNF in the joints of $TNF^{\Delta ARE/WT}$ mice results in erosive polyarthritis, which has also been associated with increased spleen size [176]. We did not investigate the effects of dietary intervention on polyarthritis pathogenesis in detail but macroscopic and functional observations regarding impaired grabbing and climbing ability suggested no dramatic changes of disease phenotype. Thus, joint inflammation in addition to constitutive TNF expression in the spleen might be the reason for unaltered spleen weights in $TNF^{\Delta ARE/WT}$ mice on Chow and experimental diet.

We performed flow cytometric analysis to further characterize ileitis development in $TNF^{\Delta ARE/WT}$ mice. Crohn's disease-like ileitis is suggested to be $CD8\alpha\beta^+$ T-cell dependent [149] and we could confirm an increased ratio of $CD8\alpha\beta^+$ to $CD8\alpha\alpha^+$ T-cells in ileal tissues of $TNF^{\Delta ARE/WT}$ mice. However, inhibition of chronic ileitis did not modify ratios of $CD8^+$ T-cell subpopulations, suggesting that a relative increase of $CD8\alpha\beta^+$ T-cells is not necessarily associated with intestinal inflammation in $TNF^{\Delta ARE/WT}$ mice. Apostolaki et al. [149] have shown that development of intestinal inflammation in this mouse model is critically dependent on β7 integrin-mediated T-lymphocyte recruitment, suggesting that upregulation of homing mechanisms in immunoactivated tissues, associated with increased infiltration of leukocytes in general might be more important for ileitis development than dominance of the $CD8\alpha\beta^+$ T-cell subtype. Moreover, it has been shown that selective chronic overproduction of TNF by IEC suffices to cause full development of Crohn's-like pathology [177]. Epithelial TNF

overexpression leads to early activation of underlying intestinal myofibroblasts, which are a sufficient target of TNF for disease development in the $TNF^{\Delta ARE/WT}$ model.

Analogous to flow cytometric results of intestinal tissues, in this study no phenotypic difference could be seen in MLN leukocyte populations after dietary intervention, although clear differences in MLN weight and leukocyte numbers could be detected. MLNs play a crucial role in the induction of mucosal immune responses. Intravascular *naive* T-cells home to the inductive sites of the gut-associated lymphoid tissue (GALT), including Peyer's patches (PPs), isolated lymphoid follicles as well as the gut-draining MLNs to mount a protective immune response to enteric pathogens. It is suggested that MLNs may function as primary gut-associated inductive site where *naive* T-cells first encounter enteric antigens, which are transported by antigen-loaded dendritic cells (DCs) from the intestinal lamina propria and PPs via afferent lymphatics [178]. When *naive* T-cells encounter their cognate antigens, antigen-driven priming/activation, polarization, and expansion takes place to yield specific effector T-cells [179, 180]. Results from several different animal studies suggest that in the absence of appropriate regulatory mechanisms, this same sequence of events may occur in response to commensal bacteria resulting in enteric antigen-dependent induction of chronic intestinal inflammation [181-183]. Thus, we suggest that increased MLN weights of $TNF^{\Delta ARE/WT}$ mice on Chow were associated with increased effector T-cell generation and presence of enteric antigens required for the induction of chronic intestinal inflammation.

On Chow diet, $TNF^{\Delta ARE/WT}$ mice develop a moderate inflammation in the distal ileum within eight weeks of age and severe pathology can be observed after 12 weeks of age [184]. Dietary intervention using experimental diet was successful in the inhibition of intestinal inflammation in $TNF^{\Delta ARE/WT}$ mice. To find out whether experimental diet could also be used to induce remission in a state of progressed inflammation, we transferred $TNF^{\Delta ARE/WT}$ mice to experimental diet at different time points. Histological findings showed that early dietary intervention seems to be crucial to maintain gut homeostasis, whereas induction of remission at later time points (10 and 14 weeks of age) was not possible. These data suggest that progression of pathology could be inhibited by experimental diet at an early stage. However, an established inflammatory setting could not be reversed. There seems to be a critical time frame between week 7 and 10 with regards to efficacy of the experimental diet. Severe intestinal inflammation is associated with reduced barrier function, increased intestinal permeability and bacterial translocation, leading to direct contact of luminal antigens and gut resident immune cells [185]. A proinflammatory environment in $TNF^{\Delta ARE/WT}$ mice with dysregulated TNF biosynthesis might dominate regulatory mechanisms. Under these conditions, direct contact with luminal antigens might be the inflammatory point of no return, when dietary intervention becomes ineffective. Yet, these are speculations and further experiments need to be done to elucidate the underlying mechanisms. Interestingly, when

we transferred mature mice from experimental diet to Chow diet after 12 weeks of age, inflammation was already found to be moderately and strongly expressed after two and six weeks on Chow, respectively. Histological scores describing tissue pathology resembled those being reported by Baur et al. [184] after eight and 12 weeks, respectively, pointing at an accelerated onset of pathology in mature $TNF^{\Delta ARE/WT}$ mice. To investigate the pathogenesis of Crohn's disesase-like ileitis on Chow diet in more detail, we designed customized diets with increasing concentrations of Chow content on the basis of a semi-synthetic diet. A Chow content of only 1% was sufficient to produce a scattered picture of inflammation and 10% induced maximal pathology in the treatment groups. These observations may suggest pure experimental diet to maintain a homeostatic state, which is protective but labile and susceptible for modulation.

It is noteworthy that we could not detect any effect of Chow compared to experimental diet in WT mice regarding the histological appearance of the distal ileum. However, we identified a small but significant diet-related effect on mRNA expression of the pattern recognition receptors TLR-2 and TLR-4, which were less expressed in WT mice on experimental diet compared with Chow diet. But we did not analyze TLR signaling and thus may only speculate about modified TLR sensing and activity. Nonetheless, there was a significant diet-related effect on cecum weight in WT and $TNF^{\Delta ARE/WT}$ mice. Cecum weights were clearly increased on Chow diet and this effect was even pronounced under inflammatory conditions. The cecum is a pouch, connecting the ileum with the ascending colon of the large intestine. It hosts a large number of bacteria, which aid in the enzymatic breakdown of nutrients. Thus, dietary intervention may have a big impact on cecal microbial composition and related metabolite production [186, 187]. Certain indigenous microorganisms may play an essential role in maintaining the integrity of sodium chloride associated water-transport mechanism in the intestinal epithelium [188-190]. Water was shown to accumulate in the cecal lumen in mice given antibacterial drugs in their drinking water after the first 24 to 48 hours of the treatment leading to increased cecal size. The microorganisms most likely to be involved, affecting cecal physiology are anaerobic bacteria that predominate in the cecal microbiota [189, 191]. Germ-free rodents show also enlarged cecal proportions corroborating the impact of cecal microbes on cecum physiology [190]. Dietary effects on cecum size, thus, may be related to changes in the cecal microbiota and might also be associated with altered expression and recognition of enteric antigens. Moreover, inflammation in $TNF^{\Delta ARE/WT}$ mice is not only restricted to the distal ileum, but may also affect the cecum and proximal colon. Thus, inflammatory processes may play an additional role in the modulation of cecal proportions. In this study, we could not detect an accumulation of cecal water but an increase in total mass of cecal content. Thus, we suggest that differences in cecum weight originated

from the interplay between an altered microbial composition and metabolic activity in combination with dysregulated tissue physiology due to inflammatory processes.

Furthermore, we observed Chow pellets to have a coarse-grained particle structure, in contrast to the homogenous grained pellets of experimental diet. Bowel activity was speculated to be enhanced in animals being kept on Chow diet, which was associated with a general increase of intestinal thickening in $TNF^{\Delta ARE/WT}$ and WT mice. However, achievement of induction of remission in nutritional therapy is in part suggested to be associated with decreased bowel activity [192]. The intestinal mucosal layer is confronted with numerous forces. For example, mucosal cells may experience pressure and shear stress from interaction with relatively non-compressible endoluminal chyme and increasing evidence suggests that such forces may substantially influence intestinal mucosal cell biology [193]. In pathologic conditions such as chronic inflammatory states, intraluminal pressures are often elevated and may adversely affect gut physiology and healing [194]. Contraction of the muscular layers thus may result in mucosal compression, as the mucosa is squeezed between the contracting musculature and the non-compressible chyme [195]. Although Chow fine had a decreased grinding state as measured by laser diffraction analysis, particle size distribution in ileal and cecal contents did not differ and no reduction of tissue pathology could be observed in distal ileal sections.

To analyze the composition of cecal contents in more detail, we prepared cecal bacterial suspensions and performed Fourier-transform infrared spectroscopy (FT-IR) analysis. FT-IR is a physiochemical technique, considered to be a powerful method for characterizing chemical compositions of complex probes [196]. In FT-IR, all cellular components contribute to the spectral pattern generated, and thus represents an adequate tool to differentiate and identify microorganisms in situations, where environmental conditions affect the whole cell. In addition, a number of recent studies have shown that FT-IR spectroscopy can help to understand microbial responses, when exposed to stress conditions in the environment such as inflammation or dietary intervention [197]. Spectral distances of bacteria-associated clusters between Chow and experimental diet groups were most striking in dendrograms of whole range (4000-600 cm^{-1}) FT-IR spectra and spectra focusing on the fingerprint region in the carbohydrate specific area between 1200 and 900 cm^{-1}. Carbohydrate specific differences in the absorption spectra might occur due to the effects of a distinct sugar composition of the complex Chow diet on cecal bacteria. Indeed, alterations of glycoprotein structures in bacterial cell walls or variations in carbohydrate metabolite profile may be factors that contribute to differences in this region. Consequently, we next aimed at characterizing functional alterations in the cecal microbiota with effects on microbial antigenicity.

Coculture analyses with cecal lysate pulsed BM-DCs in combination with $CD4^+$ T-cells showed strong immunostimulatory effects of the cecal lysates, yet no difference in antigenicity could be found. Kim et al. [198] described that colitis initiated by inoculation of nonpathogenic bacteria (*Enterococcus faecalis* and *Escherichia coli*) into germ-free IL-$10^{-/-}$ mice exhibited distinct patterns of intestinal inflammation in different anatomical locations. In a coculture system they could show that $CD4^+$ T-cells from *Enterococcus faecalis* or *Escherichia coli* monoassociated interleukin $10^{-/-}$ mice selectively produced higher levels of IFN-γ and IL-4 when stimulated with APCs pulsed with the bacterial species that induced disease. Thus, the authors suggested that different cytokine levels could be explained by bacterial-antigen specific responses. In contrast to our study, Kim et al. used T-cell depleted splenocytes as APCs that were mainly composed of B-cells (88%–95%) [198]. Regulatory mechanisms of primed APCs compared with *in vitro* generated BM-DCs may have an influence on experimental outcome. Strong activation of T-cells by highly activated BM-DCs might overshadow cecal lysate associated specific effects. Thus, modifications of the coculture system regarding the use of primed APCs isolated from local tissues might identify diet induced differences in antigenicity. Furthermore, the pre-purification of cecal lysates using endotoxin-removal systems or targeted culture of bacterial candidates from cecal content on selective agar plates followed by bacterial lysate preparation might be an additional approach. Yet, another possibility is that there is no antigen specific effect of the experimental diet on the cecal microbiota and that the protective state of $TNF^{\Delta ARE/WT}$ mice on semi-synthetic diet is independent of the cecal microbial antigenicity.

$TNF^{\Delta ARE/WT}$ mice under conventional conditions invariably developed chronic ileitis. By contrast, $TNF^{\Delta ARE/WT}$ mice raised in a specific pathogen free environment partly did not show tissue pathology, indicating that modulation of pathogenesis seems to be possible also on Chow diet (personal communication). Apart from absence of certain microbial species and an increased level of general hygiene, Chow diet was autoclaved for SPF conditions to avoid contamination with food-borne microbial agents. In contrast to experimental dietary suspensions, we could show that dietary suspensions of Chow pellets had immunostimulatory properties in cell culture experiments. However, autoclaving of the Chow diet did not have a disease preventative effect on ileitis development *in vivo*, although autoclaved Chow lost its immunostimulatory effects in cell culture experiments. Several animal models of intestinal inflammation do not develop pathology under germ-free conditions, indicating microbial interactions to play a crucial role in the modulation of gastrointestinal disorders [199]. The effects of a germ-free environment on Crohn's disease-like ileitis have not yet been investigated. However, the invariable expression of chronic ileitis in Chow fed $TNF^{\Delta ARE/WT}$ mice under conventional conditions in contrast to the scattered

occurrence of inflammation in SPF animal housing and disease-ameliorating effects of antibiotic treatment (personal communication) may suggest that the gut microbiota plays a crucial role in the TNF$^{\Delta ARE/WT}$ mouse model. Interestingly, we have noticed that dietary intervention using experimental diet was not exclusively protective for ileitis development. Approximately 10% of TNF$^{\Delta ARE/WT}$ mice showed moderate and less often severe inflammation despite being kept on experimental diet. This phenomenon did not seem to be related with caging or breeding effects, because it could also be observed between siblings in the same cage. These observations corroborated the theory of a protective but labile state of intestinal homeostasis on experimental diet.

Earlier experiments suggested iron deficient experimental diet to be protective against ileitis development in TNF$^{\Delta ARE/WT}$ mice, whereas iron adequate experimental diet seemed to induce inflammation. It could be shown that iron-depleted experimental diet induced significant changes in cecal microbial composition and that abundance of certain bacterial genera correlated with histology scores of distal ileal sections [200]. These findings might seem to be contradictory to our present observations at first glance, though, at the same time, they might reflect the susceptibility of experimental diet related intestinal homeostasis to external triggers and variations in the gut microbiota. It is noteworthy that microbiota dependent animal models, being bred in different animal facilities may have different characteristics and experimental outcomes [201]. This circumstance further corroborates the strong impact microbial variations may have on disease phenotype. It is reasonable that the microbial ecology may be subject to variations over time and that animal models may adapt to environmental factors with increased numbers of breeding generations which might also shift the narrow time frame where experimental diet can exhibit a preventive effect on chronic ileitis development in TNF$^{\Delta ARE/WT}$ mice. However, further analyses need to be performed to reveal putative correlations between distinct microbial agents and Crohn's disease-like pathogenesis. Pyrosequencing technology will be used to characterize the microbial composition of TNF$^{\Delta ARE/WT}$ mice on different diets associated with intestinal homeostasis and inflammation. Comparison of pyrosequencing data from earlier and recent experiments showing equivocal results will reveal changes of the microbial composition over time and may lead to the identification of microbial agents critical for Crohn's disease-like pathogenesis.

5.2. Gluten and Crohn's disease-like ileitis

A striking difference between Chow diet and experimental diet regarding nutrient composition is the presence of wheat and wheat derived products that account for 50% of Chow components, whereas experimental diet is free of wheat compounds. Wheat related gastrointestinal disorders are of common incidence. Apart from wheat allergies, gluten intolerance seems to be one of the most prevalent reasons [202]. It is to note that recent studies could show gluten to have effects not only in genetically predisposed people. HLA DQ2 or DQ8 haplotypes are found in over 90% of celiac disease patients, leading to an inappropriate T-cell mediated immune response against ingested, deamidated gliadin peptides [203, 204]. Several studies reported that celiac disease is a condition in which paracellular permeability is enhanced and the integrity of the tight junction system is compromised [205, 206]. However, accumulating clinical evidence supports the existence of an irritable bowel syndrome (IBS)-like form of gluten sensitivity named non-celiac gluten intolerance [207, 208]. Drago et al have investigated the effect of gliadin on permeability of celiac and non-celiac intestinal mucosa. Their results provided evidence that gliadin activates zonulin signaling, resulting in immediate reduction of intestinal barrier function and passage of gliadin into the subepithelial compartment irrespective of a genetic predisposition [209].
Moreover, Biesiekierski et al. conducted a double-blind, randomized, placebo-controlled rechallenge trial in patients with irritable bowel syndrome in whom celiac disease was excluded and who were symptomatically controlled on a gluten-free diet. Compared with the gluten group, patients who remained gluten free reported significant improvements in pain, bloating, satisfaction with stool consistency and tiredness. The authors concluded that gluten is associated with overall IBS symptoms, in a subset of patients [142]. Another group suggested that several forms of gluten intolerance might occur frequently not only in patients with gastrointestinal symptoms, but also in first- and second-degree relatives and patients with numerous common disorders even in the absence of gastrointestinal symptoms [210]. The negative effects of gluten administration might have even more impact in a setting where intestinal homeostasis is disturbed and proinflammatory mechanisms dominate like in the $TNF^{\Delta ARE/WT}$ mouse. In support of this hypothesis, it has been shown that the persistent presence of inflammatory mediators such as TNF and IFN-y may increase the permeability across the endothelial and epithelial layers [211, 212].
To test this hypothesis, we designed a gluten-fortified experimental diet, which was identical with pure experimental diet except that parts of the casein fraction were replaced by gluten. The gluten-fortified experimental diet induced intestinal inflammation in AREs *in vivo*, which was associated with increased levels of TNF, IFN-y and IL-15 as well as TLR-2 and TLR-4 expression in distal ileal tissues. Interestingly, PT-gluten stimulation of MLNs from Chow,

experimental and gluten- fortified experimental diet treatment groups resulted in comparable TNF secretion, whereas T-cell dependent IFN-y secretion did not occur. These findings suggest an antigen-independent co-activation of immune cells rather than T-cell specific antigen recognition. Several studies have been performed to identify the antigenic agent, responsible for gluten intolerance in celiac patients. Gluten consists of gliadin and glutenin fractions. Specific gliadin peptides that are deamidated by tissue transglutaminase (e.g., alpha-gliadin P57-68) bind to HLA DQ2 and/or DQ8 molecules and may induce an adaptive Th1 proinflammatory response in a genetically susceptible host [213]. However, early innate immunity has been considered as another possible key element in celiac disease [214]. In the case of the innate immune response, alpha-gliadin P31-43, which is not recognized by T cells, induces IL15 production, which in turn is thought to cause expansion of intra epithelial lymphocytes (IEL) and induction of epithelial apoptosis that is independent of TCR specificity [215-217]. In addition, imbalances in the intestinal microbial composition have been associated with celiac disease, suggesting a role of intestinal microbiota in this pathology as well [218]. Mouse models for celiac disease are rare and need to have genetically humanized modifications regarding TCR and HLA genes to develop pathogenesis [219]. To our knowledge, no spontaneous celiac disease model exists to date and gluten responsiveness of the $TNF^{\Delta ARE/WT}$ mouse could especially contribute to the understanding of non-celiac gluten intolerance. Further experiments need to be performed to describe the fundamental mechanisms in more detail, including the analysis of gluten related effects on intestinal homeostasis in WT mice.

Adherence to a gluten-free diet is efficient for induction of remission and recover of intestinal homeostasis in patients with celiac disease and gluten intolerance [202]. However, wheat depleted, gluten-free Chow diet neither had reduced immunostimulatory properties in cell culture experiments, nor was there any positive effect on ileitis development in $TNF^{\Delta ARE/WT}$ mice *in vivo*. Yet, wheat depleted Chow still is a complex mixture of natural ingredients, with barley accounting for 16% of content. Indeed, it has been shown that gluten-like proteins in barley and rye may also trigger immune responses in celiac patients [220], thus, Chow (-W) may not be an adequate equivalent to a gluten-free diet.

5.3. Impact of experimental diet on IL-10$^{-/-}$ mice and T-cell transfer colitis

Dietary intervention using experimental diet was successful for prevention of chronic ileitis in TNF$^{\Delta ARE/WT}$ mice. To investigate the effects of experimental diet on colitis pathogenesis, we performed a feeding experiment with IL-10$^{-/-}$ mice. In contrast to the TNF$^{\Delta ARE/WT}$ mouse model, where proinflammatory mechanisms dominate due to impaired regulation of TNF translation, imbalance of immune homeostasis in the IL-10$^{-/-}$ colitis model originates from impaired anti-inflammatory mechanisms that lead to the development of enterocolitis [153]. Although no differences could be found in body weight, spleen weight and colon length, histological examination of colonic sections clearly showed increased tissue pathology in the Chow group, which was confirmed by increased SAA plasma levels. The high standard deviations of the Chow group derived from two outliers, one that did not develop colitis and another that showed extreme tissue pathology. Interestingly, IL-10$^{-/-}$ mice on experimental diet showed only mild signs of inflammation with low interindividual variations, suggesting that the efficacy of dietary intervention with experimental diet was not restricted to Crohn's disease-like ileitis. Generally, IL-10$^{-/-}$ mice raised in a SPF or conventional environment develop colitis marked by epithelial cell hyperplasia and transmural inflammation [153]. Yet, colitis does not develop under germ-free conditions and thus is suggested to be driven by antigens of the mucosal microbiota [221, 222]. Moreover, IL-10$^{-/-}$ mice exhibit increased intestinal permeability even prior to the development of overt colitis. This change in barrier function may lead to increased contact with or stimulation by antigens in the mucosal microbiota and thus might be a factor that facilitates the development of inflammation [223]. The break in normal "tolerance" to commensal antigens thus might be modified by dietary intervention using experimental diet. Though, whether the underlying mechanism, which inhibits mucosal inflammation in IL-10$^{-/-}$ colitis is similar to the prevention of Crohn's disease-like ileitis could not be resolved and remains to be elucidated.

The T-cell transfer model of colitis represents another model of inadequate regulatory response, which is driven by microbial antigens and leads to mucosal inflammation. In addition to naïve CD4^{+} CD45RB^{hi} T-cells, CD4^{+} IL-10$^{-/-}$ T-cells may be used for the induction of T-cell transfer colitis in immunocompromised Rag2$^{-/-}$ recipient mice. It seems to be underappreciated that apart from colitis, these animals develop small intestinal inflammation, especially in the distal part [159]. Thus, the T-cell transfer model may be used as a tool to study the effects of dietary intervention on colitis and ileitis development at the same time. Although we could not detect a significant difference in body or spleen weight, comparison of Chow and experimental diet treatment groups revealed significant differences in cecum weight and colon weight-to-length ratios, which have been found to correlate with histological scores in T-cell transfer colitis [224]. However, in TNF$^{\Delta ARE/WT}$ mouse experiments, we have

observed that significant differences in cecum weights may also occur among healthy WT mice due to dietary effects. Interestingly, histological examination of distal ileal and distal colonic sections exhibited no pathological difference comparing Chow and experimental diet groups.

In T-cell transfer models, increased tissue pathology usually is associated with a loss of body weight [224]. As we could not see differences in weight over time in any group (apart from gender-specific differences), dietary effects on mucosal inflammation might have been visible with prolonged experimental time. Furthermore, molecular characteristics of the transferred effector T-cells and their interaction with the commensal microbiota seem to be crucial factors for disease pathogenesis. Animals fail to develop disease under germ free conditions and co-transfer of regulatory T-cells can inhibit colitis development [225]. Moreover, Strauch et al. have shown that SCID recipients, which received naive T-cells from germ free donors, developed an earlier onset of colitis compared with mice reconstituted with lymphocytes from conventionally housed animals [226]. Earlier experiments showed that T-cell transfer colitis exhibited a late onset of inflammation associated with a scattered inflammatory phenotype of the immunodeficient recipient mice when conducted in our SPF animal facility (personal communication). In the T-cell transfer experiment described above, donor $CD4^{+}$ IL-$10^{-/-}$ T-cells were derived from SPF, whereas $Rag2^{-/-}$ recipient mice were kept under conventional conditions. Therefore, it would be interesting to repeat the experiment with both donors and recipients being kept under conventional conditions to avoid differences in the initial microbiota. Nevertheless, proinflammatory cytokines TNF and IFN-y were increased in distal ileal tissues of Chow fed animals, although no difference in regulation could be observed in distal colonic tissues. Moreover, T-cell transfer recipient mice on experimental diet showed clearly reduced levels of SAA compared with mice on Chow diet, suggesting a lower state of systemic inflammation and thus a positive effect of experimental diet on T-cell adoptive transfer colitis in Rag2 -/- mice.

5.4. Conclusion and outlook

We have shown that pathogenesis of Crohn's disease-like ileitis can be inhibited by early dietary intervention using an experimental diet. However, administration of experimental diet could not be used for induction of remission in an already established inflammatory setting. Interestingly, clinical studies of nutritional therapy have shown that EEN is effective for inducing remission in pediatric Crohn's disease especially in newly diagnosed patients [172]. In conclusion, these observations might suggest that early administration of nutritional therapy could be associated with a favored therapeutic outcome

Strong efforts have been made to elucidate the mechanisms underlying EN therapy. Several aspects have been suggested to be involved such as induction of bowel rest, decreased intestinal permeability, modulation of the microbiota and associated increase and decrease of anti-inflammatory and proinflammatory responses, respectively [227-229]. Dietary antigens in the protein fraction were assumed to be another factor contributing to immune dysbalance, though evaluation of polymeric diets showed no difference in efficacy compared with elemental diets. Apart from being solid, the semi-synthetic experimental diet used throughout the experiments has a similar composition to semi-elemental diets used for EN therapy. Consequently, the observed protective effects in our feeding experiments and its underlying mechanisms might also reveal interesting concepts, relevant for the improvement of EN therapy.

In general, intestinal homeostasis on experimental diet seems to be labile and Chow contents of only 1% were sufficient to induce moderate ileitis. In addition, transfer of mature $TNF^{\Delta ARE/WT}$ mice from experimental diet to Chow diet resulted in rapid disease development. It is strongly suggested that the microbial composition and microbe-host interactions play a crucial role in the modulation of Crohn's disease-like ileitis in $TNF^{\Delta ARE/WT}$ mice. To dissect the contribution of microbial interactions in more detail, transfer of $TNF^{\Delta ARE/WT}$ mice to a germ free environment in combination with monoassociation studies may be a promising approach. A comprehensive analysis of pyrosequencing data from different feeding experiments with different inflammatory outcomes may yield microbial candidates for further monoassociation studies. Apart from ileitis prevention, it would be interesting whether experimental diet may also be effective in maintenance of remission. Anti-TNF antibody or antibiotic therapy might be tested to induce remission in $TNF^{\Delta ARE/WT}$ mice. Subsequent administration of Chow or experimental diet in absence of medical intervention might yield important knowledge about the role of experimental diet in maintaining remission. In clinical practice, responsiveness of Crohn's disease patients to EN is variable and the relapse rate is about 65% at 12 months in adult studies [122]. Moreover, EN therapy seems to be not efficient in ulcerative colitis

patients. The reasons for this variability are not known but might be useful for the improvement of therapeutic strategies.

Profound understanding of the modulation of microbial ecologies using nutritional therapy and the immunologic consequences caused by altered microbe host interactions would strongly contribute to the development of improved therapeutic strategies for IBD treatment. Cutting edge technologies such as pyrosequencing analysis, metabolomics and nutrigenomics may provide deeper insights into the effects of dietary intervention on intestinal homeostasis and interindividual variations. A combination of these technologies might have strong impact on the future development of highly efficient, personalized strategies for nutritional therapy.

6. APPENDIX

Appendix 1. Nutrient composition of the diets used in the project. Detailed information about diet ingredients is provided on the following pages in form of the original manufacturer's data sheets.

Ingredients		Percentage
Ssniff Exp (Control diet EF R/M (E15000))		
Casein, acid	%	24.0
Corn starch, pre-gelatinized	%	30.0
Maltodextrin	%	19.6
Glucose (Dextrose)	%	10.0
Cellulose powder	%	5.0
L-Cystine	%	0.2
Vitamin premix, w/o choline Cl	%	1.0
Mineral & tace element premix	%	6.0
Choline Chloride	%	0.2
Soybean oil	%	4.0
Altromin Exp (Control diet C1000)		
Casein, purified	%	20.0
Corn starch, purified	%	53.0
Saccharose,refined	%	10.0
Cellulose powder,purified	%	4.0
Vit. Form. C 1000 with Saccharose	%	2.0
Minerals and trace elements.Form. C 1000	%	6.0
Sunflower oil,refined.	%	5.0

Altromin Glu (Control diet C1000 + 10% gluten)

Casein, purified	%	10.6
Corn starch, purified	%	52.4
Saccharose,refined	%	10.0
Cellulose powder,purified	%	4.0
Vit. Form. C 1000 with Saccharose	%	2.0
Minerals and trace elements.Form. C 1000	%	6.0
Sunflower oil,refined.	%	5.0
Gluten from wheat (Sigma-Aldrich)	%	10.0

Ssniff Chow (R/M-H (V1534))

Wheat & wheat products	%	50.0
Barley	%	16.0
Soybean products	%	22.7
Amino acids	%	0.2
Vitamins & trace elements	%	1.3
Minerals	%	3.4
Fiber sorces (Ø ~19 % CF)	%	6.4

Ssniff Chow (-W) (S5745-S020)

Babycorn, pre-treated	%	30.6
Puffed rice	%	16.0
Barley	%	16.0
Soybeanmeal	%	19.4
Soybean concentrate	%	8.0
Lignocellulose	%	1.0
L-Lysine HCl	%	0.1
DL-Methionine	%	0.1
Vitamin & trace element premix	%	1.0
Monocalcium phosphate	%	1.4
Calcium carbonate	%	1.0
Calcium propionate	%	0.5
Salt (NaCl)	%	0.6
Sorbic acid	%	0.1
Choline Chloride	%	0.3
Sugar beet pulp. dehydrated	%	3.0
Soybean oil	%	1.0

ssniff® R/M-H

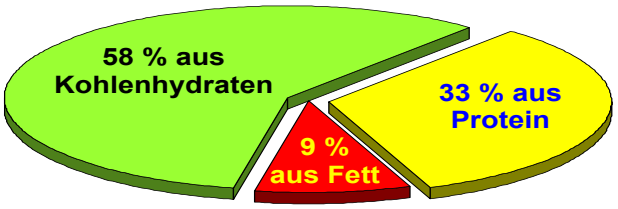
Alleinfuttermittel für die Haltung von Ratten und Mäusen

Beschreibung

Dieses Futtermittel ist für Ratten und Mäuse im Erhaltungsstoffwechsel vorgesehen. Aufgrund der ausgewogenen Nährstoffkonzentrationen bei mittlerer Energiedichte und niedrigem Nitrosamin-Gehalt ist es auch als Basisfutter für Langzeitstudien gut geeignet.

Rohnährstoffe	[%]
Trockensubstanz	87,7
Rohprotein (N x 6,25)	19,0
Rohfett	3,3
Rohfaser	4,9
Rohasche	6,4
N-freie Extraktstoffe	54,1
Stärke	36,5
Zucker	4,7

Energie	[MJ/kg]
Bruttoenergie (GE)	**16,3**
Umsetzbare Energie (ME) *	**12,8**



Mineralstoffe	[%]
Calcium	1,00
Phosphor	0,70
Natrium	0,24
Magnesium	0,22
Kalium	0,91

Fettsäuren	[%]
C 14:0	0,01
C 16:0	0,47
C 16:1	0,01
C 18:0	0,08
C 18:1	0,62
C 18:2	1,80
C 18:3	0,23
C 20:0	0,01
C 20:1	0,02
C 20:5	----
C 22:6	----

Aminosäuren	[%]
Lysin	1,00
Methionin	0,30
Met+Cys	0,65
Threonin	0,68
Tryptophan	0,25
Arginin	1,14
Histidin	0,44
Valin	0,88
Isoleucin	0,76
Leucin	1,30
Phenylalanin	0,85
Phe+Tyr	1,43
Glycin	0,80
Glutaminsäure	3,90
Asparaginsäure	1,61
Prolin	1,25
Alanin	0,79
Serin	0,89

Vitamine	per kg	
Vitamin A	15.000	IE
Vitamin D_3	1.000	IE
Vitamin E	110	mg
Vitamin K (als Menadion)	5	mg
Thiamin (B_1)	18	mg
Riboflavin (B_2)	23	mg
Pyridoxin (B_6)	21	mg
Cobalamin (B_{12})	100	µg
Nicotinsäure	135	mg
Pantothensäure	43	mg
Folsäure	7	mg
Biotin	525	µg
Cholin-Cl	2.990	mg
Inositol	100	mg

Spurenelemente	per kg	
Eisen	179	mg
Mangan	69	mg
Zink	94	mg
Kupfer	16	mg
Iod	2,2	mg
Selen	0,3	mg
Cobalt	2,1	mg

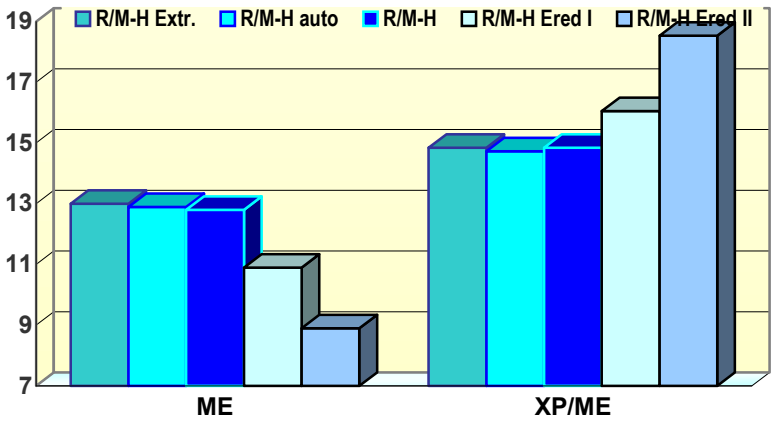
Futterzusammensetzung

absteigende Reihenfolge der Gruppen (FMV)

Getreide und Getreidenebenprodukte, Ölsaatprodukte, Mineralstoffe, pflanzliche Öle, Vitamine, Spurenelemente.

** ME berechnet nach der Schätzformel für Schweine, Anlage 4 der Futtermittelverordnung*

Energiedichte [MJ ME/kg] und Protein-/Energie-Verhältnis [g XP/MJ ME]



Hauptprodukte

V1530-0 Mehl, einfach vermahlen
V1534-0 10 mm Pellets
V1535-0 15 mm Pellets

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ssniff Spezialdiäten GmbH
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E-Mail mail@ssniff.de
www.ssniff.de

Status: 06/2005

ssniff® EF R/M Kontrolle

Experimentalfuttermittel für Ratten und Mäuse

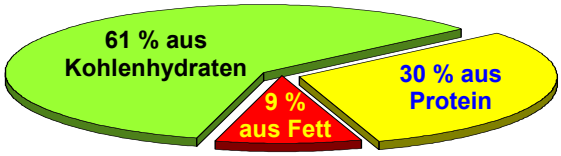
Beschreibung

Dieses Futtermittel basiert auf hoch gereinigten Ausgangserzeugnissen (purified diet) und ist daher für Studien vorgesehen, die eine besonders genaue Einstellung der Nährstoffkonzentrationen erfordern; dabei weist die Experimentaldiät eine hohe Nährstoffverfügbarkeit auf, sodass der Bedarf von Ratten und Mäusen im Erhaltungsstoffwechsel sowie im Wachstum in vollem Umfang gedeckt wird. Diese Futtermischung dient zugleich als Basis für nahezu sämtliche gereinigten bzw. halb-synthetischen ssniff® Diäten.

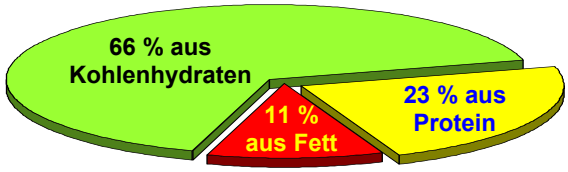
Rohnährstoffe	[%]
Trockensubstanz	95,2
Rohprotein (N x 6,25)	20,8
Rohfett	4,2
Rohfaser	5,0
Rohasche	5,6
N-freie Extraktstoffe	59,4
Stärke	46,8
Zucker	10,8

Energie	[MJ/kg]
Bruttoenergie (GE)	18,0
Umsetzbare Energie (ME)	

15,4 [1)]



15,0 [2)]



[1)] *Berechnungsgrundlage: Schätzformel für Schweine nach Anlage 4 der FMV*

[2)] *Berechnet mit Atwater-Faktoren*

Mineralstoffe	[%]
Calcium	0,90
Phosphor	0,63
Natrium	0,19
Magnesium	0,21
Kalium	0,97

Fettsäuren	[%]
C 8:0	----
C 10:0	----
C 12:0	----
C 14:0	0,02
C 16:0	0,45
C 16:1	0,02
C 18:0	0,19
C 18:1	1,07
C 18:2	2,12
C 18:3	0,26
C 20:0	0,02
C 20:1	----
C 20:5	----
C 22:6	----

Aminosäuren	[%]
Lysin	1,71
Methionin	0,73
Met+Cys	0,82
Threonin	0,93
Tryptophan	0,27
Arginin	0,76
Histidin	0,66
Valin	1,42
Isoleucin	1,09
Leucin	2,05
Phenylalanin	1,11
Phe+Tyr	2,22
Glycin	0,43
Glutaminsäure	4,69
Asparaginsäure	1,55
Prolin	2,39
Alanin	0,68
Serin	1,24

Vitamine	per kg	
Vitamin A	15.000	IE
Vitamin D_3	1.500	IE
Vitamin E	150	mg
Vitamin K (als Menadion)	20	mg
Vitamin C	30	mg
Thiamin (B_1)	16	mg
Riboflavin (B_2)	16	mg
Pyridoxin (B_6)	18	mg
Cobalamin (B_{12})	30	µg
Nicotinsäure	49	mg
Pantothensäure	56	mg
Folsäure	19	mg
Biotin	310	µg
Cholin-Cl	1.040	mg
Inositol	80	mg

Spurenelemente	per kg	
Eisen	166	mg
Mangan	98	mg
Zink	65	mg
Kupfer	14	mg
Iod	1,2	mg
Selen	0,14	mg
Cobalt	0,15	mg

Zusammensetzung

Auf Anfrage

Hauptprodukte

E15000-00 Mehl, einfach vermahlen
E15000-04 10 mm Pellets

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Status: 06/2005

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E-Mail: info@altromin.de - http://www.altromin.de

Nummer **Lfd. Nummer** Änderung 05.05.2009 13:07:04

10100000 100000 C 1000 Kontrolldiät Ratte/Maus | Control Diet Rats/Mice

Inhaltsstoff	Einheit	Bedarf	Gehalt	Differenz
Rohprotein / Crude Protein	mg/kg		176115,000	
Rohfett / Crude Fat	mg/kg		50830,000	
Rohfaser / Crude Fibre	mg/kg		40450,000	
Rohasche / Crude Ash	mg/kg		54943,225	
Feuchtigkeit / Moisture	mg/kg		81735,625	
Disaccharide(s)	mg/kg		110960,500	
Polysaccharide(s)	mg/kg		471700,000	
Umsetzb. Energie/Metab. Energy	kcal/kg		3518,055	
Lysin / Lysine	mg/kg		17400,970	
Methionin / Methionine	mg/kg		10688,000	
Cystin / Cystine	mg/kg		3196,180	
Threonin / Threonine	mg/kg		7154,170	
Tryptophan	mg/kg		1976,960	
Arginin / Arginine	mg/kg		9828,790	
Histidin / Histidine	mg/kg		5275,790	
Isoleucin / Isoleucine	mg/kg		7222,820	
Leucin / Leucine	mg/kg		14762,770	
Phenylalanin / Phenylalanine	mg/kg		7171,970	
Valin / Valine	mg/kg		3296,140	
Alanin / Alanine	mg/kg		2528,000	
Asparaginsäure / Aspartic acid	mg/kg		3583,140	
Glutaminsäure / Glutamic acid	mg/kg		23674,970	
Glycin / Glycine	mg/kg		3136,000	
Prolin / Proline	mg/kg		12762,980	
Serin / Serine	mg/kg		5267,800	
Tyrosin / Tyrosine	mg/kg		9285,010	
Vitamin A	I.E./kg		15000,000	
Vitamin D3	I.E./kg		500,000	
Vitamin E	mg/kg		163,900	
Vitamin K3 als/as Menadion(e)	mg/kg		10,000	
Vitamin B1	mg/kg		20,040	
Vitamin B2	mg/kg		20,322	
Vitamin B6	mg/kg		15,034	
Vitamin B12	mg/kg		0,030	
Nikotinsäure / Nicotinic acid	mg/kg		50,170	
Pantothensre./Pantothenic acid	mg/kg		50,106	
Folsäure / Folic acid	mg/kg		10,00240	
Biotin	mg/kg		0,201	
Cholinchlorid/Choline chloride	mg/kg		1011,500	
P-Aminobenzoesre./Benzoic acid	mg/kg		100,000	
Inosit / Inositol	mg/kg		111,000	
Vitamin C	mg/kg		20,000	
Calcium	mg/kg		9310,506	
Ges.Phosphor / Phosphorus	mg/kg		7522,765	
Verd.Phosphor/Digest.Phosporus	mg/kg		7199,565	
Magnesium	mg/kg		683,506	
Natrium / Sodium	mg/kg		2488,262	
Kalium / Potassium	mg/kg		7088,682	
Schwefel / Sulfur	mg/kg		2791,540	
Chlor / Chlorine	mg/kg		3630,000	

Inhaltsstoff	Einheit	Bedarf	Gehalt	Differenz
Eisen / Iron	mg/kg		178,579	
Mangan / Manganese	mg/kg		100,888	
Zink / Zinc	mg/kg		29,299	
Kupfer / Copper	mg/kg		5,751	
Jod / Iodine	mg/kg		0,514	
Molybdän / Molybdenum	mg/kg		0,198	
Fluor / Fluorine	mg/kg		4,170	
Selen / Selenium	mg/kg		0,334	
Kobalt / Cobalt	mg/kg		0,147	
Caprinsäure C-10:0	mg/kg		2,500	
Laurinsäure C-12:0	mg/kg		2,500	
Myristinsäue C-14:0	mg/kg		2,500	
Pentadecansäure C-15:0	mg/kg		2,500	
Palmitinsäure C-16:0	mg/kg		2700,000	
Palmitoleinsäure C-16:1	mg/kg		2,500	
Margarinsäure	mg/kg		2,500	
Stearinsäure C-18:0	mg/kg		1250,000	
Ölsäure C-18:1	mg/kg		10950,000	
Linolsäure C-18:2	mg/kg		35050,000	
Linolensäure C-18:3	mg/kg		150,000	
Arachinsäure C-20:0	mg/kg		250,000	
Eicosaensäure C-20:1	mg/kg		250,000	
Eicosadiensäure C-20:2	mg/kg		250,000	
Arachidonsäure C-20:4	mg/kg		2,500	
Eicosapentaensäure C-20:5	mg/kg		2,500	
Behensäure C-22:0	mg/kg		250,000	
Docosahexaensäure C-22:6	mg/kg		2,500	
Tricosansäure	mg/kg		2,500	
Nervonsäure C-24:1	mg/kg		2,500	
Erucasäure C-22:1	mg/kg		2,500	
Aluminium	mg/kg		3,706	
Volumen / Volume	kg		1000,000	

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

5-ASA	Anti-inflammatory 5-aminosalicylic acid
APC	antigen presenting cell
ARE	TNFΔARE/WT mouse
BM-DC	bone marrow-derived dendritic cell
CD	Crohn´s disease
Chow	standard Chow diet
CL	cecal lysate
DC	dendritic cell
DS	dietary suspension
DSS	dextran sodium sulfate
ED	elemental diet
EEN	exclusive enteral nutrition
ELISA	enzyme-linked immunosorbent assay
EN	enteral nutrition
Exp	experimental diet
FT-IR	Fourier-transform infrared spectroscopy
GALT	gut-associated lymphoid tissue
Glu	gluten-fortified experimental diet
GWAS	genome-wide association studies
H&E	hematoxylin and eosin
H&E	hematoxylin and eosin
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
ICAM-1	intercellular adhesion molecule-1
IEC	intestinal epithelial cell
IEL	intraepithelial lymphocyte
IFN-y	interferon-y
IL	interleukin
KLH	keyhole limpet hemocyanin
LBP	lipopolysaccharide binding protein
LPL	lamina propria lymphocyte
LPS	lipopolysaccharide
MAdCAM-1	mucosal vascular addressin cell adhesion molecule-1
MLN	mesenteric lymph node

NF-κB	nuclear factor-κB
NOD	nucleotide-binding oligomerization domain
NSAID	nonsteroidal anti-inflammatory drug
OCLN	occluding
PAMP	pathogen-associated molecular pattern
PN	parenteral nutrition
PP	Peyer's patch
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
PUFA	polyunsaturated fatty acid
RAG	recombination activating gene
ROS	reactive oxygen species
SAA	serum amyloid A
SCFA	short-chain fatty acid
SPF	specific pathogen-free
TGF-β	transforming growth factor β
TLR	toll-like receptor
TNF	tumor necrosis factor
tTG	tissue transglutaminase
UC	ulcerative colitis
WHO	World Health Organization
WT	wild type mouse
ZO-1	zonula occludens-1

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PUBLICATIONS AND PRESENTATIONS

Peer-reviewed original manuscripts and reviews

Wagner SJ, Schmidt A, Effenberger MJP, Haller D.
Semi-synthetic diet ameliorates Crohn's disease-like ileitis in $TNF^{\Delta ARE/WT}$ mice.
Inflamm Bowel Dis, accepted for publication.

Werner T, **Wagner SJ**, Martínez I, Walter J, Chang JS, Clavel T, Kisling S, Schuemann K, Haller D.
Depletion of luminal iron alters the gut microbiota and prevents Crohn´s disease-like ileitis. *Gut.* 2011; 60:325-333

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Eisen.
In *Praxishandbuch Functional Food*. Erbersdobler HF, Mayer AH, Behr's Verlag, 2011:1-39

Werner T, **Wagner SJ**, Haller D.
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In *Bauchredner: Ernährung - Freund und Feind bei CED*. DCCV-Journal, Gerd Rieker Verlag, Neckartailfingen, 1/2010

Published abstracts

Wagner SJ, Werner T, Martínez I, Walter J, Chang JS, Clavel T, Kisling S, Schuemann K, Haller D.
Depletion of Dietary Iron Alters the Gut Microbiome and Prevents Crohn's Disease-Like Ileitis
Z Gastroenterol. 2011; 49:A2

Werner T, **Wagner SJ**, Martínez I, Walter J, Chang JS, Kisling S, Schuemann K, Haller D.
Impact of Luminal and Systemic Iron on the Development of Chronic Ileitis Targeting the Gut Microbial Composition and Stress Response in Intestinal Epithelial Cells.
Gastroenterology. 2010; 138:S-413

Oral presentations

Wagner SJ, Werner T, Martínez I, Walter J, Chang JS, Clavel T, Kisling S, Schuemann K, Haller D.
Depletion of Dietary Iron Alters the Gut Microbiome and Prevents Crohn's Disease-Like Ileitis
III. Kloster Arnsburg - Meeting Intestinale Mucosafunktion, July 9-10, 2010, Lich, Germany

Wagner SJ, Werner T, Martínez I, Walter J, Chang JS, Clavel T, Kisling S, Schuemann K, Haller D.
Impact of Luminal and Systemic Iron on the Development of Chronic Ileitis Targeting the Gut Microbial Composition and Stress Response in Intestinal Epithelial Cells.
3rd Seeon Conference (German Society for Hygiene and Mikrobiology, DGHM), June 18-20, 2010, Kloster Seeon, Germany

Wagner SJ, Werner T, Martínez I, Walter J, Chang JS, Kisling S, Schuemann K, Haller D.
Effects of dietary and systemic iron on chronic ileitis: Impact on stress response mechanisms in intestinal epithelial cells (IEC).
47. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung (German Nutrition Society), March 11-12, 2010, Jena, Germany

Wagner SJ, Werner T, Martínez I, Walter J, Chang JS, Kisling S, Schuemann K, Haller D.
Effects of dietary and systemic iron on chronic ileitis targeting endoplasmic reticulum stress responses and apoptosis in the epithelium.
5th Congress of the European Crohn's and Colitis Organisation (ECCO), February 25-27, 2010, Prague, Czech Republic

Poster presentations

Wagner SJ, Schmidt A, Haller D.
Dietary Factors in the Regulation of Crohn's Disease-Like Ileitis.
49. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung (German Nutrition Society), March 14-16, 2012, Freising-Weihenstephan, Germany

Wagner SJ, Werner T, Martínez I, Walter J, Chang JS, Kisling S, Schuemann K, Haller D.
Impact of Luminal and Systemic Iron on the Development of Chronic Ileitis Targeting the Gut Microbial Composition and Stress Response in Intestinal Epithelial Cells.
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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbstständig angefertigt habe. Es wurden nur die in der Arbeit genannten Quellen und Hilfsmittel benutzt. Wörtlich oder sinngemäß übernommenes Gedankengut habe ich als solches kenntlich gemacht.

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