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A potential role for regulatory T-cells in the amelioration of DSS induced colitis by dietary non-digestible polysaccharides

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Abstract

Inflammatory bowel diseases (IBD) including ulcerative colitis (UC) and Crohn's disease (CD) are chronic relapsing inflammatory disorders of the gastrointestinal tract. The interaction between a disturbed microbial composition, the intestinal mucosal barrier and the mucosal immune system plays an important role in IBD and its chronicity. It has been indicated that due to the altered microbial composition the balance between T regulatory cells (Treg) and T helper cells (Th) 17 is disturbed, leading to an inflammatory state.

The present study shows that oral intake of a specific multi fibre mix (MF), designed to match the fibre content of a healthy diet, counteracts IBD-like intestinal inflammation and weight loss in dextran sodium sulphate treated mice. This reduction in inflammation might be brought about, at least in part, by the MF-induced decrease in inflammatory cytokines, increase in IL-10 and the relative increase in Treg cells in the mesenteric lymph nodes (MLN). Moreover, the Treg percentage in the MLN correlates with the percentage of tolerogenic lamina propria derived CD103+RALDH+dendritic cells in the MLN, suggesting that these play a role in the observed effects.

In children with CD exclusive enteral nutrition (EEN) is a widely used safe and effective therapy. Optimizing enteral nutritional concepts with the tested fibre mix, know to modulate the gut microbiota composition, SCFA production and inflammatory status (as indicated by the present study) could possibly further improve efficacy in inducing remission.

Keywords: IBD; Treg; CD103; RALDH; Prebiotic; Multifibre

1. Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic relapsing inflammatory disorders of the gastrointestinal tract caused by multiple genetic and environmental factors [1,2]. Although the detailed mechanism of action is still unclear, the interaction between an abnormal microbial composition, the intestinal mucosal barrier and the mucosal immune system appears to be an important factor in the development of IBD and its chronicity [3,4].

In IBD, including CD, the microbial composition and the balance between intestinal microbiota and mucosal immune responses is disturbed [5]. This dysbiosis has profound effects on the immune system and intestinal health. It is initiated by different mechanisms including alterations in the productions of short chain fatty acids (SCFAs) that have an important effect on colonic homeostasis maintenance, epithelial integrity and immune cell function, migration and apoptosis [6]. The altered microbial composition changes the balance between T regulatory cells (Treg) and T helper cells (Th)17 cells in the lamina propria conditioning an inflammatory state [7,8]. Consequently, increasing the Treg/Th17 ratio will be of benefit in reducing the inflammatory status. A specific lamina propria derived dendritic cell (DC) type, expressing the integrin α chain CD103 and the retinal metabolizing enzyme RALDH2 is indicated to promote Treg cell differentiation and induction of gut-homing receptors [9,10]. A decrease in the number of these DCs results in a loss of Treg cells and an increase in the number of IFN-γ and IL-17 producing T cells [11,12].

Various pharmaceutical IBD treatment options are available. However, they have their limitations in both efficacy and safety [13,14]. Exclusive enteral nutrition (EEN) is widely used as primary therapy in children with CD. It is indicated to be safe inducing remission in up to 80% of the cases [15,16]. EEN, reduces local and systemic inflammation, induces mucosal healing, supports growth and improves the quality of life [17]. Recent data suggest that potential mechanisms of EEN in active CD may include modulation of the intestinal microbiota, direct anti-inflammatory effects and/or correction of the altered epithelial permeability (review by Day et al., 2013 [16]). In view of this, optimizing enteral nutritional concepts with factors which are

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known/suggested to modulate the gut microbiota composition, SCFA production and/or the mucosal immune system could possibly further improve efficacy in inducing remission.

The present study was designed to evaluate the immune modulating (Treg inducing) properties of a distinct prebiotic multifibre (MF) mixture. The specific mixture, including insoluble (cellulose, soy polysaccharide, resistant starch) and soluble (gum arabic, oligofructose, fructo-oligosaccharides and galacto-oligosaccharides) fibres was designed to match the fibre content of a healthy diet and to reduce intestinal inflammation and symptoms associated with IBD. The effects of MF on intestinal inflammation were evaluated in the validated, well-established colonic inflammation model in mice, the dextran sodium sulphate (DSS)-induced colitis [18]. DSS induced colitis was accompanied by a strong Th17-driven response in the mesenteric lymph node (MLN). Oral intake of MF decreased the DSS-induced weight loss and intestinal inflammation. This reduction in inflammation might be brought about, at least in part, by the MF induced decrease in inflammatory cytokines and the relative increase in Treg cells in the MLN. The Treg percentage in the MLN correlates with the percentage of lamina propria derived CD103+RALDH+dendritic cells.

2. Materials and methods

All experimental procdures using laboratory animals were approved by an independent animal experiments committee (DEC Consult, Bilthoven, The Netherlands).

Ex vivo cell incubations were performed at 37°C in a humidified environment containing 5% CO_2.

2.1. Diet

All animals received semi-purified AIN-93G-based diets (Research Diet Services, Wijk bij Duurstede, The Netherlands). The control diet (C) was described previously [19]. The multifibre mix diet (MF) consisted of 1.5% (w/w) specific fibres including: 48.8% galacto-oligosaccharides (Vivinal GOS, Borculo Domo, Zwolle, The Netherlands), 5.5% fructo-oligosaccharides (Raftiline HP, Orafti, Wijchen, The Netherlands), 13.0% oligofructose (Raftilose P95, Orafti, Wijchen, The Netherlands), 14.3% gum arabic (Willy Benecke GmbH, Hamburg, Germany), 10% soya polysaccharides (LN IP non-GM, The Solae Company, St. Louis, USA), 7.5% cellulose (Vitacel L 600-20, J. Rettenmaier & Söhne, Ellwangen, Germany), and 1% resistant starch (Novelose 330, National Starch and Chemical Company, Neustadt, Germany). The amount of added carbohydrates from the MF was exchanged for the same amount of carbohydrates (cellulose and dextrose) from the control diet in such a way that the caloric intake, fibre intake and taste are minimal effected. The oligosaccharides were mixed into the AIN-93G diet and pressed into pellets. The different study groups received the control diet or the diet including the MF starting 14 days before and during the colitis induction.

2.2. Induction of colitis

Male C57Bl/6J mice (Charles River, Maastricht, The Netherlands), aged 6 weeks at arrival (day -14), were randomly assigned to a control or to the different test groups (n=6). All animals had free access to the control or the MF diet and tap water. Acute colitis was induced at 8 weeks of age (day 0) by administration of 2.5% DSS (w/w, MW 36-50 KD, MP Biomedicals, Aurora, OH, USA) in tap water for 7 constitutive days. Disease progress was evaluated daily by monitoring body weight, food consumption, faecal blood (Haemacult-sensa Test, Beckman Coulter, Woerden, The Netherlands) and stool consistency. At day 7, mice were bled under terminal anaesthesia (isolfurane/N_2O/O_2) and sacrificed. The colon and the MLN were isolated for cytokine analysis and fluorescence-activated cell sorting (FACS), respectively.

2.3. Blood collection

Blood was collected in Lithium Heparin MiniCollect tubes (Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands) and preserved at room temperature awaiting whole blood analysis. The remaining blood was centrifuged (5 minutes at 6000 rpm in an Eppendorf centrifuge) and plasma was stored at −80°C until serum amyloid A and cytokine detection.

2.4. Colon homogenisation

Colons were dissected and parts were stored at −80°C until homogenization. Frozen colons were weight and dissolved in 500-µl lysis buffer (1% Triton X-100 (Sigma, Zwijndrecht, The Netherlands) and enzyme inhibitor-mix (Roche Diagnostics, Almere, The Netherlands) in PBS). The homogenates were centrifuged at 13500 rpm in an Eppendorf® centrifuge (Eppendorf, 5810-R, VWR, Roden, The Netherlands). The supernatant was collected and stored at −80°C until cytokine analysis.

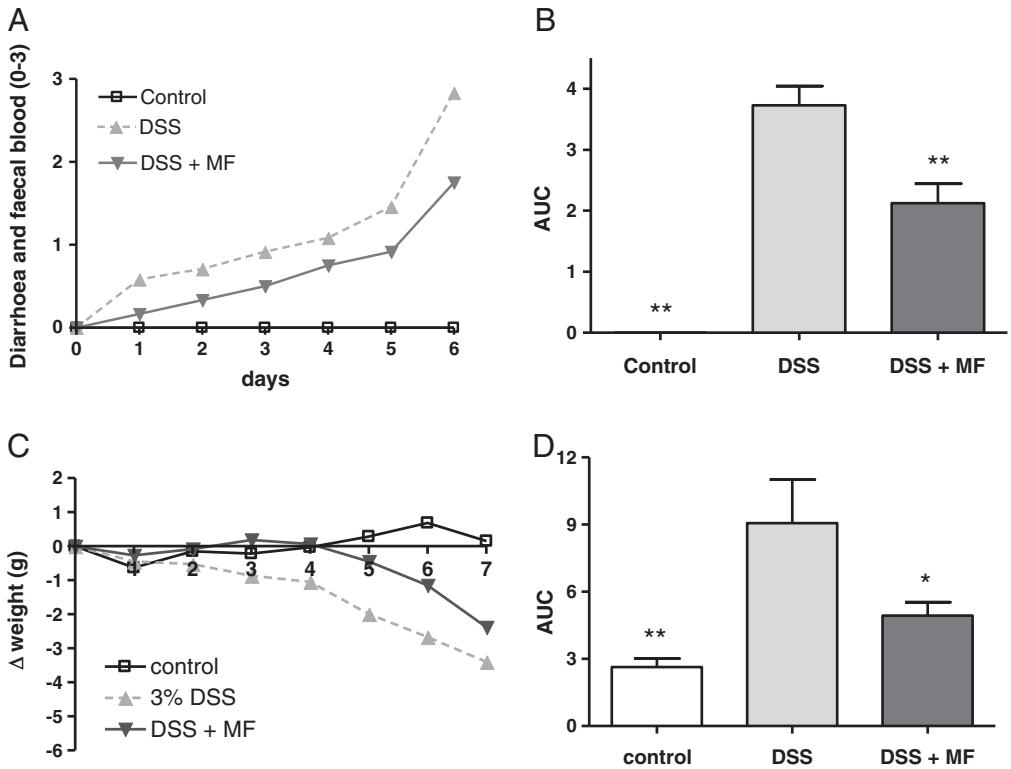

Fig. 1. Mice were fed control AIN-93G diet or AIN-93G diet supplemented with 1.5% (w/w) MF starting 14 days before 7 days DSS treatment. MF feeding decreased disease severity as detected by faecal score (A and B) and weight loss (C+D). Results are depicted as mean±S.E.M. *P<.05, **P<.01, n=6.

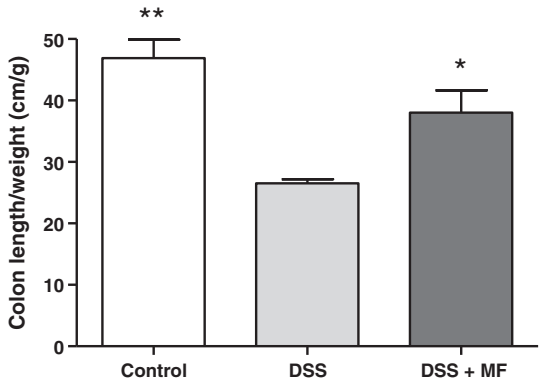

Fig. 2. Mice were fed control AIN-93G diet or AIN-93G diet supplemented with 1.5% (w/w) oligosaccharides MF starting 14 days before 7 days DSS treatment. Colon length and weight were detected and the length/weight ratio was calculated. Results are depicted as mean±S.E.M. *P<.05, **P<.01, n=6.

2.5. Cytokine detection

Cytokine levels in plasma and the colon homogenates were detected using a commercial Multiplex Bead immunoassay (BioRad, Veenendaal, The Netherlands) including IL-10, IL-17, TNF-α, IFNγ and IL-1β according to the manufacturers protocol.

The analyses were performed by using the Bio-Plex system (BioRad). Results were calculated using Bio-Plex Manager Software 3.1 (BioRad).

2.6. Serum amyloid A detection

Levels of the acute phase protein serum amyloid A (SAA) were detected in the thawed plasma samples using a commercial solid-phase sandwich ELISA kit SAA (Invitrogen, Fisher Scientific, Landsmeer, The Netherlands), according to the manufacturers' protocol.

2.7. Flowcytometry

Cells from the MLNs were isolated by gently pressing the organs through nylon mesh filters (Falcon cell strainer, Becton Dickinson (BD), Alphen a/d Rijn, the Netherlands). After counting, a total of 1×10^6 cells were washed with PBS containing 2% FBS/0.4% EDTA and incubated for 15 min with anti-mouse CD16/CD32 antibody (BD) blocking the Fc receptors. The cells were then stained with different combinations of anti-CD4-FITC (BD), anti-CD4-PerCp-Cy5 (BD 1/400), anti-CD25-PE (Beckman Coulter 1/50), anti-CD69-APC (eBioscience 1/100), anti CCR-6-APC (R&D systems 1/100) for 30 minutes. Intracellular staining was performed according manufacturer's protocol (eBioscience, Foxp3 staining set, Bio Connect, The Netherlands). For intracellular staining the antibodies anti-Foxp3-FITC (eBioscience 1/100) or anti-Foxp3-PerCpCy5.5 (eBioscience 1/200), anti-Gata-3-PE (eBioscience 1/100), anti-RORyt-Pe (eBioscience 1/800), anti-tBet-Alexa fluor (BD 1/100) were used in combination with above mentioned surface markers. Matching Isotype controls were used for all staining protocols to correct for the influence of nonspecific binding,for proper gate setting and background corrections.

RALDH activity in individual cells was measured using a Aldefluor staining kit (StemCell Technologies, Grenoble, France), according to the manufacturer's protocol with modifications. Briefly, 10^6 cell suspended in 100 µl Aldefluor assay buffer containing the Aldefluor substrate with or without the RALDH inhibitor diethylaminobenzaldehyde (DEAB) were incubated for 45 minutes at 37°C. For flow cytometric analysis the Aldefluor-reacted cells were subsequently stained with anti-CD11c-V450 (1:100, BD Horizon), and anti-CD103-PE (1:100, BD Horizon) mPDCA-APC (1:25).

All staining procedures were performed on ice (unless stated different) and protected from light. Cells were counted and analyses were performed using FACSCanto II and FACSDiva software (BD Biosciences), respectively.

2.8. Statistical analysis

All data are expressed as mean±S.E.M. All readout conditions were compared to the DSS control using the analysis of variance (one-way ANOVA). The overall

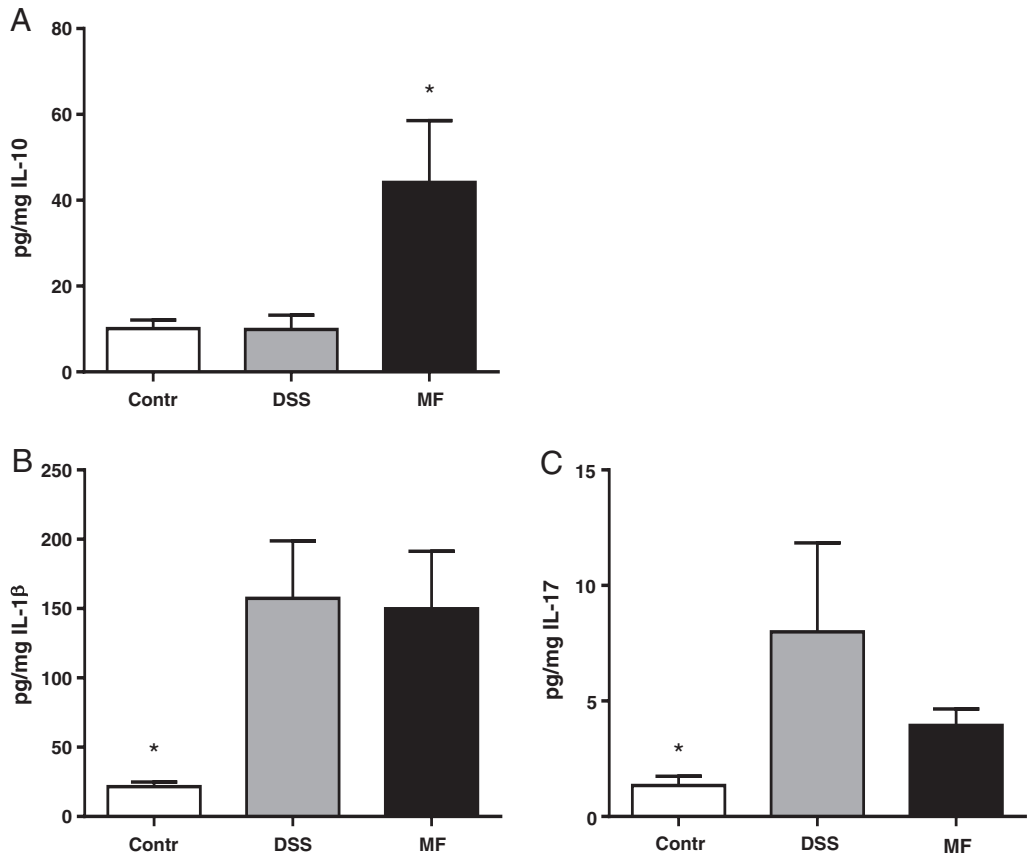

Fig. 3. AIN-93G diet or AIN-93G supplemented with 1.5% (w/w) MF feeding was started 14 days before DSS treatment. Different immune parameters, IL-10 (A), IL-1β (B) and IL-17 (C), were analysed in colon homogenates after 7 days DSS treatment. Results are depicted as mean±S.E.M. *P<.05, **P<.01, n=6.

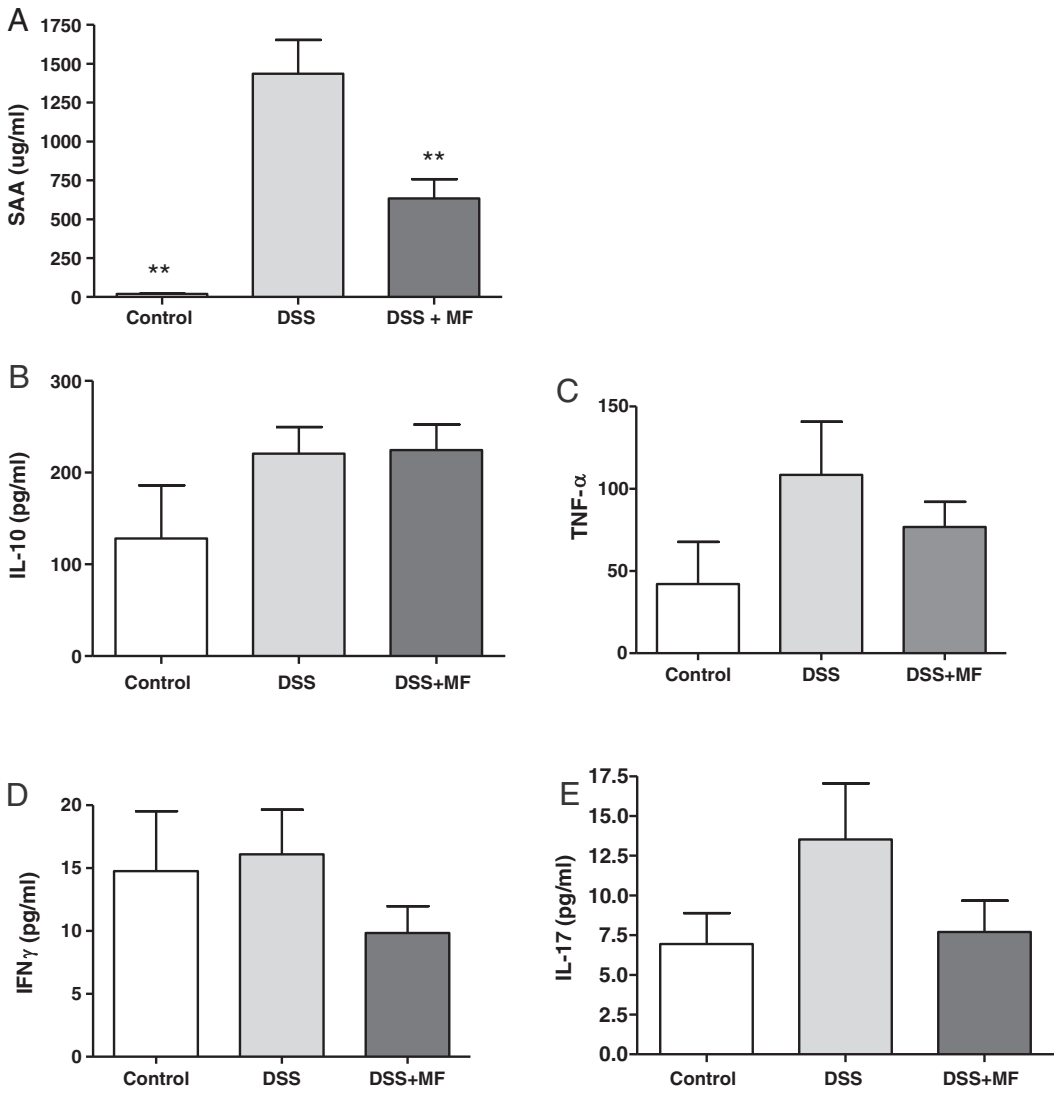

Fig. 4. AIN-93G diet or AIN-93G supplemented with 1.5% (w/w) MF feeding was started 14 days before DSS treatment. Different inflammatory parameters, SAA (A), IL-10 (B), TNF-α (C), IFNγ (D), IL-17 (E), were analysed in plasma after 7 days DSS treatment. Results are depicted as mean±S.E.M. *P<.05, **P<.01, n=6.

significance of differences for all calculations was tested using the post hoc Dunnett's test. Correlations were calculated using the Pearson's linear regression model.

3. Results

3.1. Clinical indices of inflammation

Control mice receiving water showed no clinical signs of spontaneous intestinal inflammation. In the DSS treaded mice disease activity increased while body weight decrease in time. MF feeding significantly counteracted these effects (Fig. 1).

3.2. Local indices of inflammation

After applying DSS in the drinking water for 7 days, the animals were sacrificed and the colon was dissected after which length and weights were measured. MF feeding significantly counteracted the DSS-induced decrease in colon length/weight ratio (Fig. 2). In colon homogenates it was found that IL-10 cytokine levels in the colon increased in the MF group, IL-1β and Il-17 levels were not significantly affected by MF (Fig. 3).

3.3. Systemic indices of inflammation

DSS treatment significantly increased SAA blood levels (Fig. 4A). The DSS induced increase in other plasma markers including IL-10, TNF-α and IL-17 was not significant (Fig. 4). MF feeding counteracted the DSS induced increase in the different inflammatory plasma levels, these effects are except for SAA not significant (Fig. 4).

3.4. MLN Treg cell composition

DSS treatment increased the absolute number of Treg cells in the spleen and MF feeding counteracted this effect (Fig. 5A). However, the percentage of Treg cells in the CD4 population was not influenced by DSS (Fig. 5B). The ratio of the percentage Treg (CD4+CD25+Treg+) to Th1 (CD4+Tbet+), Th2 (CD4+GATA+) or Th17 (CD4+CCR6+RORγT) was calculated. Treg/Th1 and Treg/Th2 ratios were not influenced by DSS treatment (Fig. 5C+D). The Treg/Th17 ratio decreased after DSS treatment pointing to a DSS induced relative increase in Th17 cells. MF feeding increased the Treg/Th2 and Treg/Th17 ratio significantly, which indicates a relative increase in the number of Treg cells (Fig. 5D+E).

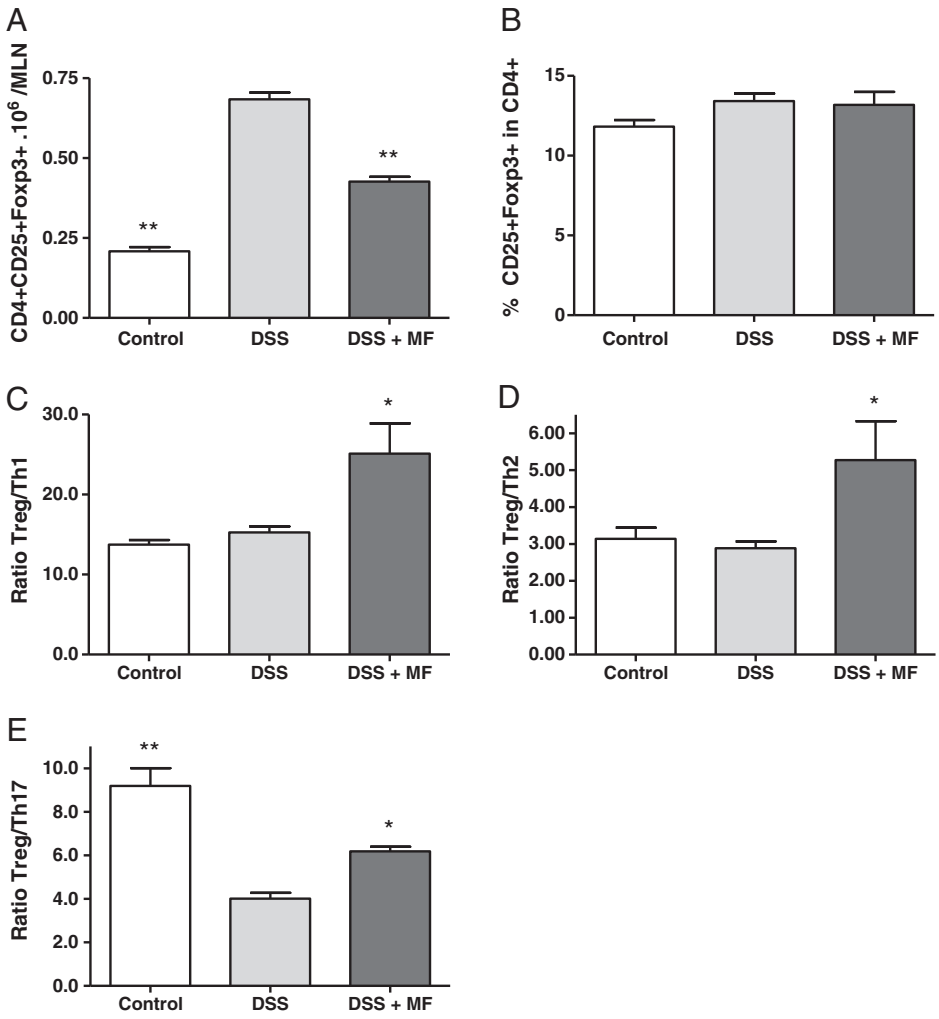

Fig. 5. Mice were fed control AIN-93G diet or AIN-93G diet supplemented with 1.5% (w/w) MF starting 14 days before 7 days DSS treatment. MLN cells were isolated, counted and, stained with cell markers identifying the different T cell subsets. The total number (A) and the percentage of Treg cells out of the CD4+ population (B) were calculated. Ratios between the Treg cells and Th1 (C), Th2 (D) and Th17 (E) in the MLN are shown. Results are depicted as mean±S.E.M. *P<.05, **P<.01, n=6.

3.5. MLN DC cell composition

The DSS-induced decrease in the percentage of conventional DCs (CD11c+CD103+) in the MLN was counteracted by MF feeding (Fig. 6A). The percentage RALDH+cells in this population increased by DSS treatment, this increase was not influenced by the MF intervention (Fig. 6B).

3.6. MLN Treg DC correlation

It has been reported that the number of Treg cells in the MLN is influenced by CD103+RALDH+cDCs. The collective data confirm this positive correlation between these specific cDCs and Tregs (Fig. 7). The percentages of CD11c+CD103+RALDH- DCs and Tregs in the MLN did correlate in a negative way (Pearson r=−0.56, P=.0053).

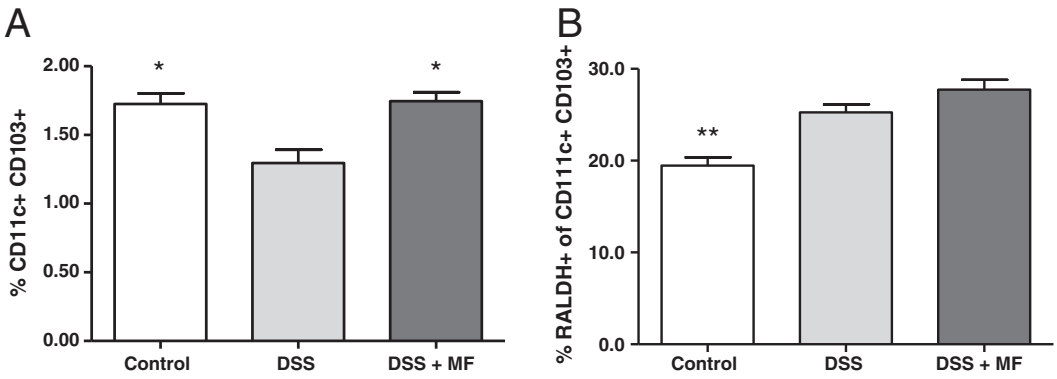

Fig. 6. Mice were fed control AIN-93G diet or AIN-93G diet supplemented with 1.5% (w/w) MF starting 14 days before 7 days DSS treatment. MLN cells were isolated and stained with CD11c, CD103 and RALDH. The % CD11c+CD103+ cells (A) and CD11c+CD103+RALDH+cells (B) were calculated. Results are depicted as mean±S.E.M. *P<.05, **P<.01, n=6.

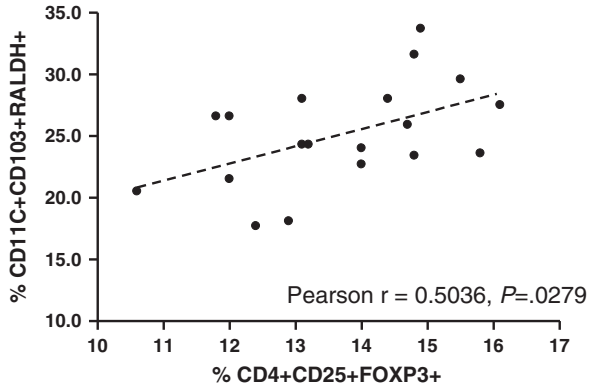

Fig. 7. MLN cells were isolated and stained with different cell markers for FACS analysis. The correlation between the % of Treg cells (CD4+CD25+Foxp3+) and the % of CD11+CD103+RALDH+DCs is depicted. All study groups have been included in the graph.

4. Discussion

IBD patients display a shift in commensal bacterial populations towards increased Proteobacteria and decreased Firmicutes compared to healthy controls, whereas their production of SCFA is impaired [20–23]. The tested MF, designed to match the fibre content of a healthy diet, consists of fibres with different chain lengths, structures and linkages types causing a continuous fermentation rate along the colon. This fermentation process generates mainly the SCFAs acetate, propionate and butyrate (unpublished data).

During the onset of colitis the amount of SCFA is significantly reduced [24] and dietary fibre or SCFA intake has been shown to be clinically beneficial in the treatment of various forms of colitis [25–27]. It has been indicated that SCFAs in interaction with their receptor, the G-protein coupled receptor 43 (GPR43) are required to control inflammatory responses during experimental colitis [7]. Dietary factors, modulating gut microbiota composition, SCFA production and/or the mucosal immune system, represent a promising opportunity for interfering in IBD. Dependent on their type and concentration, SCFAs have been shown to influence chemotaxis and cell adhesion. Especially butyrate, but also acetate and propionate seem to have an anti-inflammatory effect. This effect is mediated by different signalling pathways including nuclear factor-κB and inhibition of histone deacetylase [28–30].

In the present study MF counteracted the clinical symptoms of DSS induced colitis (diarrhoea, weight loss). It modulated inflammatory mediators/markers both local colon (IL-10 induction) and systemically (serum amyloid A and inflammatory cytokine decrease).

The protective effects of MF on DSS induced colitis were accompanied by a relative increase in MLN Treg cells, compared to Th1, Th2 and Th17 cells. Treg are able to suppress, via different mechanisms, many aspects of the immune responses [31]. They have been indicated to modulate the severity of inflammatory colitis [32–36]. Furthermore, in commensal bacteria-colonized mice it has been shown that particularly the IL-10 production by Treg cells plays a critical role in maintaining intestinal homeostasis. Blocking of IL-10 signals skews intestinal CD4+ T-cells towards the Th1 and Th17 lineages [37].

The commensal microbial composition actively modulates the immune system. It influences the interplay between IECs and DCs resulting in, amongst others, the induction of non-inflammatory (tolerogenic) or inflammatory DCs [38–40].

CD103+RALDH+DCs have been shown to drive Treg development in mouse and human [41]. The positive correlation between CD11c+CD103+RALDH+and Treg cells in the MLN, as indicated in the present study, confirms these previous findings. Literature indicates that gut microbiota-derived SCFAs might also directly regulate Treg number and function [7,42]. This direct interaction mechanism might, independent from DC involvement, also have contributed to the relative increase of Treg cells.

Various pharmaceutical IBD treatment options are available. However, they have their limitations in both efficacy and safety [13,14]. Enteral nutrition (EN) is considered a good first-line therapy for active CD in children. It induces mucosal healing, improves quality of life and shows no differences in terms of efficacy as compared to corticosteroids [43,44]. A fibre mix, representing a complete nutritional composition, is attractive as potential therapeutic agents as add-on to enteral nutrition. More detailed studies, including clinical trials are warranted to confirm the potential of this promising immune-modulatory mixture high in prebiotics.

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