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Dual effects of a high-protein diet on DSS-treated mice during colitis resolution phase

Annaïg Lan,[1] Anne Blais,[1] Desire Coelho,[2] Juliette Capron,[1] Manar Maarouf,[1] Robert Benamouzig,[1,3] Antonio H. Lancha, Jr.,[2] Francine Walker,[4] Daniel Tomé,[1] and François Blachier[1]

[1]*UMR PNCA, Nutrition Physiology and Ingestive Behavior, AgroParisTech, INRA, Université Paris-Saclay, Paris, France;*
[2]*Laboratory of Applied Nutrition and Metabolism, School of Physical Education and Sports, University of São Paulo, Brazil;*
[3]*Department of Gastroenterology, Hôpital Avicenne, Université Paris 13, Bobigny, France; and* [4]*Service d'Anatomie et Cytologie Pathologiques, Hôpital Bichat-Claude Bernard, Paris, France*

Lan A, Blais A, Coelho D, Capron J, Maarouf M, Benamouzig R, Lancha AH Jr, Walker F, Tomé D, Blachier F. Dual effects of a high-protein diet on DSS-treated mice during colitis resolution phase. *Am J Physiol Gastrointest Liver Physiol* 311: G624–G633, 2016. First published August 25, 2016; doi:10.1152/ajpgi.00433.2015.—The impact of the dietary protein level on the process of colonic mucosal inflammation and subsequent recovery remains largely unknown. In this study, we fed DSS-treated mice with either a normoproteic (NP) or a high-protein (HP) isocaloric diet from the beginning of the 5-day dextran sulfate sodium (DSS) treatment to 14 days later. Measurements of colitis indicators (colon weight:length ratio, myeloperoxidase activity, cytokine expressions) showed a similar level of colonic inflammation in both DSS groups during the colitis induction phase. However, during the colitis resolution phase, inflammation intensity was higher in the DSS-HP group than in the DSS-NP group as evidenced by higher inflammatory score and body weight loss. This coincided with a higher mortality rate. In surviving animals, an increase in colonic crypt height associated with a higher number of colon epithelial cells per crypt, and TGF-β3 content was observed in the DSS-HP vs. DSS-NP group. Moreover, colonic expression patterns of tight junction proteins and E-cadherin were also different according to the diet. Altogether, our results indicate that the HP diet, when given during both the induction and resolution periods of DSS-induced colitis, showed deleterious effects during the post-induction phase. However, HP diet ingestion was also associated with morphological and biochemical differences compatible with higher colonic epithelium restoration in surviving animals, indicating an effect of the dietary protein level on colonic crypt repair after acute inflammation. These data highlight the potential impact of the dietary protein amount during the colitis course.

high-protein diet; colitis induction; colitis resolution; DSS; colonic crypt repair

NEW & NOTEWORTHY

A high-protein diet exerts a biphasic effect on dextran sulfate sodium (DSS)-induced colitis by worsening inflammation during the induction-resolution phase, but also by helping colonic crypt repair after acute inflammation likely through modulation of colonic epithelial proliferation, differentiation, and expression of intestinal barrier proteins. This study points out the potential impact of the dietary protein amount during the colitis course and raises the possibility of a causal link between colonic epithelial repair and survival in our irritable bowel diseases (IBD) model.

Address for reprint requests and other correspondence: A. Lan, UMR PNCA, Nutrition Physiology and Ingestive Behavior, AgroParisTech, INRA, Université Paris-Saclay, 16, rue Claude Bernard, 75005 Paris, France (e-mail: annaig.lan@agroparistech.fr).

CHRONIC inflammatory bowel diseases (IBD), mainly Crohn's disease and ulcerative colitis (UC) (27), are characterized by an inflammation of intestinal parts with alternating remission and relapse episodes, resulting from inappropriate mucosal immune responses against luminal intestinal components in genetically predisposed individuals (17). Etiology of IBD relates to numerous factors including genetic and environmental factors, among which unhealthy diets with high sugar and fat content are associated with an increased risk of IBD (12, 30, 32). In addition, studies on the impact of high protein (HP) diet consumption on IBD onset and clinical course showed both an increased risk of IBD in women (14) and an increased relapse rate for UC patients (15).

An HP diet provides an increased amount of amino acids, some of them being beneficial for mucosal healing (19, 21), and also increases the quantity of undigested protein reaching the large intestine. This nitrogenous material is subjected to microbial metabolism, resulting in the production of numerous amino acid-derived bacterial metabolites (5–7, 13, 23, 35). Some of them, including p-cresol (2), hydrogen sulfide (26), and ammonia (1), exert detrimental effects on the intestinal mucosa at high concentrations, notably by inhibiting colonic epithelial cell oxygen consumption, while others appear beneficial (3, 11). These changes in the large intestine luminal composition may therefore affect mucosal physiology and immunity (31), and then intestinal colitis resolution. Interestingly, in healthy rats, HP diet ingestion has resulted in a lower number of goblet cells at the epithelial surface, but has increased the goblet cell number in colonic crypts together with an increased *Muc3* and a slight reduction in *Il-6* gene expression (18), suggesting that the amount of dietary protein impacts the colonic mucosal barrier.

The aim of present work was to evaluate the potential impact of an HP diet on the colitis induction and resolution phases in the widely used dextran sulfate sodium (DSS)-treated mouse model of IBD (36, 37). Mice receiving DSS orally can develop colitis with numerous analogies with UC, which is associated with weight loss, shortening of the gut, and leukocyte infiltration (34). The C57BL/6 strain is a good responder to a single course of the DSS chemical irritant and develops a robust disease, most severe in the distal colon and with acceptable reproducibility (24). This study compared the impact of an HP lower carbohydrate diet to an isocaloric normoproteic (NP) diet on acute colitis and during the inflammation resolution/mucosal healing phase in the C57BL/6 IBD mouse model.

Table 1. *Composition of the experimental diets*

	NP	HP
Ingredients, g/kg		
Whole milk proteins	140	530
Corn starch	622.7	287
Sucrose	100	45.7
Cellulose	50	50
Soybean oil	40	40
Mineral Mixture, AIN 93-M	35	35
Vitamin Mixture, AIN 93-V	10	10
Choline	2.3	2.3
Metabolizable energy, kJ/g	14.6	14.6

NP, normoproteic diet; HP, high-protein diet.

MATERIALS AND METHODS

Animals. Seven-week-old male C57BL/6J mice (Harlan, Gannat, France) were acclimated for 1 wk with free access to standard mouse chow and tap water. Each mouse was maintained in a cage under controlled conditions of temperature (23°C), humidity (55 ± 10%), and light (12:12-h light-dark cycle). Before inclusion in experiments, mice were allowed to acclimate to the diet (individual ball of fresh NP diet prepared daily, Table 1) for 3 days after 3 days of a standard mouse chow/fresh NP diet. All aspects of the present protocol are in accordance with the guidelines of the French Committee for Animal Care and the European convention of vertebrate animals used for experimentation under European council directive, and received written agreement from the local animal ethical committee (COMETHEA at Jouy-en-Josas, France N°12/133).

Experimental design. Two types of experimental isocaloric diets (14.6 kJ/g) were used in this study: an NP (140 g/kg whole milk protein) and an HP low carbohydrate (530 g/kg whole milk protein) isocellulose diet with similar energy content (Table 1). Mice were divided into 4 groups with the same mean body weight and fed with either the NP or HP diet, and treated or not with DSS to induce colitis, thus leading to the groups DSS-NP, DSS-HP, NP and HP (Fig. 1). Colitis was induced by the addition of DSS [3.5% (wt/vol), 36,000–50,000 MW, MP Biomedicals Illkirch-Graffenstaden, France] to the drinking water for 5 days, given from *day 1* to *day 5* (fresh DSS solution being prepared daily). Healthy control animals received fresh tap water, only, daily for 14 days.

To study the effects of an HP diet associated with DSS on the colon, 2 experiments were performed. In the first one, mice were euthanized after colitis induction at *day 6* (n = 6 per DSS-group), while in the second experiment, mice were dissected under anesthesia 14 days after the beginning of DSS treatment (n = 11 in control groups and n = 14 in DSS-treated groups at the beginning of the experiment). Drink and food were provided ad libitum and food consumption as well as body weight were measured daily. Mice were euthanized if they lost ≥ 30% body weight, as per approved animal protocol guidelines, to meet the end point criteria. Colitis was quantified with clinical activity, as described elsewhere (37) using the parameters of stool consistency and fecal blood, which were determined daily for each mouse.

Tissue collection. At euthanasia, mice were anesthetized by inhalation of isoflurane and blood was withdrawn by intracardiac puncture. Blood was collected in EDTA tubes and plasma was frozen and kept at −80°C for measurement of cytokine and acute phase protein concentrations. The entire colon was removed and measured. After two flushes with PBS, the colon was weighed and 4 segments of distal colon were processed for further analysis: two segments of 1 cm in the middle of each intestinal segment were immediately frozen in liquid nitrogen and stored at −80°C for myeloperoxidase (MPO) and cytokine assays; a 1-cm segment was harvested for RNA analysis, immediately frozen in Trizol and stored at −80°C until further processed; the last 1-cm intestinal segment was fixed in 4% buffered formaldehyde for histological analysis. The liver, spleen, and kidneys were resected and weighed. A sample of each colonic content (~0.1 g) was precisely weighed and kept at 55°C for 48 h. After water evaporation, dry colonic content was weighed and relative water content was calculated using the formula: (initial weight − final weight) × 100/initial weight.

Analysis of local and systemic inflammatory markers. The content in TNFα, IL-6, IL-β, and the three isoforms of TGFβ were analyzed in colon tissue homogenates from dissected tissues homogenized with an ultrathurax in buffer containing PBS, supplemented with complete miniproteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) on ice. The homogenized solution was centrifuged for 10 min at 18,000 rpm, and the supernatant was separated into aliquots and frozen at −80°C until analysis. The concentration was determined using Bioplex Multiplex Suspension Array System kits, according to the manufacturer's instructions (Bio-Rad, Marnes-la-Coquette, France), and subsequently analyzed using the Bioplex Manager 3.0 software (Bio-Rad). The colonic contents of cytokines were expressed as picograms per milligram of total colonic protein after protein quantification with Bio-Rad DC Protein assay kit. Plasma concentrations of the acute-phase protein serum amyloid A (SAA) and IL-6 were determined with commercial solid-phase sandwich ELISA from Tridelta Development (Maynooth, Ireland) for SAA and eBioscience SAS (Paris, France) for IL-6.

Histological and biochemical assessment of colitis, colon epithelial proliferation, and mucosecretant cells. Histological assessment of colonic damage was double-blind performed on microscopic hematoxylin-and-eosin stained sections using histological scoring based on severity of inflammation, and extent of injury, regeneration, and crypt damage. Each of these changes was multiplied by the percentage quantifying the disease involvement on cross sections of colon (8). The final score was calculated by the sum of the scores for all parameters. Colonic crypt length was determined by analysis of 30 well-oriented crypts per animal using the image analysis software Calopix (TRIBVN, Châtillon, France). Neutrophil infiltration into the intestine was quantified by measuring MPO activity using an *O*-dianasidine dihydrochloride assay as described previously (18). Proliferation was evaluated by Ki 67 immunohistochemistry labeling, carried out on 4-μm transversal colon sections at the Cochin HistIM
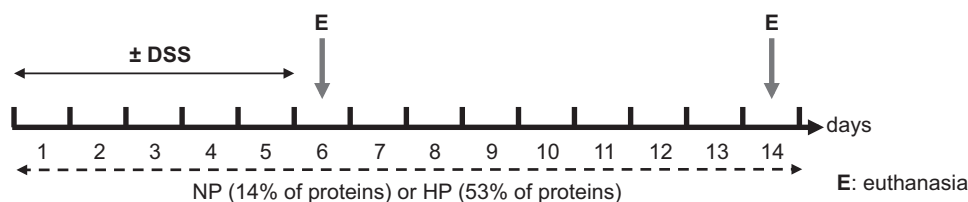

Fig. 1. Schematic representation of the experimental design. Mice received either normoproteic (NP) or high-protein (HP) low-carbohydrate isocaloric diets from the beginning of the 5-day dextran sulfate sodium (DSS) treatment (3.5% DSS in drinking water) to 14 days. Mice were euthanized (E) after colitis induction at *day 6* (n = 6 per DSS-group) or 14 days after the beginning of DSS treatment (n = 11 and n = 14 in control and DSS-treated groups, respectively, at the experiment start).

Facility. After antigen unmasking in sodium citrate buffer 10 mM pH 6.0, proliferating cells were detected using a rabbit polyclonal antibody to anti-Ki 67 (ab15580, 1/500, Abcam, Cambridge, UK) and counterstained with hematoxylin. Periodic acid-Schiff (PAS) staining was used to visualize mucus-producing cells on 4-μm transversal colon sections counterstained with hematoxylin.

Quantitation of gene expression by real-time PCR. Colon tissue was lysed in Trizol and total RNA was extracted and cleaned up with the RNeasy kit and RNase-free DNase I (Qiagen SAS, CourtabŒuf, France) digestion based on the manufacturer's protocol. qRT-PCR was performed with mouse-specific primers (sequences available on demand) with SYBR-Green PCR master mix. cDNA samples were assayed in triplicate and gene expression levels for each sample were normalized relative to HPRT with $2^{-\Delta\Delta Ct}$ calculation.

Expression analysis of tight junction and adherent junction proteins by Western blotting and immunohistochemistry. Total proteins (30 μg) from total colon tissue homogenates were loaded onto 4–12% Criterion XT (Bio-Rad) and run using 1× MOPS buffer. After transfer onto nitrocellulose membrane and incubation in blocking solution [TBS pH 7.5, 0.05% Tween 20, and 5% (wt/vol) nonfat dry milk], membranes were incubated overnight (4°C) with a rabbit polyclonal antibody to occludin (ab31721, 1/250, Abcam), to ZO-1 (61–7300, 1/250, Invitrogen, Camarillo, CA), to claudin-1 (71–7800, 1/250, Invitrogen) or with a mouse monoclonal antibody to E-cadherin (ab76055, 1/1000, Abcam) diluted in blocking solution. After incubation with the appropriate secondary antibody, the immune complexes were detected by enhanced chemiluminescence (ECL system, Pierce Biotechnology, CourtabŒuf, France), and were quantified by densitometry using the FluorChem FC2 device and the AlphaView software (Cell Biosciences, Santa Clara, CA) with normalization by subtraction of lane background values above and below the corresponding band. Histone H3 expression (ab76307, 1/1,000, Abcam) was used to ensure consistent protein loading and transferring. Immunohistochemistry E-cadherin labeling was carried out on 4-μm transversal colon sections at the Cochin HistIM Facility. After antigen unmasking in sodium citrate buffer 10 mM, pH 6.0, expression of E-cadherin was detected using a mouse monoclonal antibody to E-cadherin (ab76055, 1/200, Abcam) and counterstained with hematoxylin.

Statistical analysis. Data are expressed as means ± standard error of mean (SE). Statistical evaluations were performed by ANOVA and Sidak-Bonferroni's test for multiple comparisons, and by *t*-test with Welch's correction in case of unequal variances when appropriate using StatView version 5.0 software (SAS Institute, Cary, NC). *P* values < 0.05 were considered statistically significant.

RESULTS

Effect of dietary protein level on clinical symptom in DSS-treated mice. To study the effect of dietary protein level on acute colitis and on the consecutive intestinal wound healing, mice were subjected to a 5-day 3.5% DSS treatment and fed with either the HP or the NP diet for 6 and 14 days. Survival was significantly lower in the DSS-HP group than in the DSS-NP group (43% vs. 86%, Fig. 2*A*). Mortality rate increased during recovery phase and was associated with body weight loss (Fig. 2*B*). Weight loss began after DSS arrest at *day 6* and reached 23% of body weight by *day 8* in the DSS-HP mice compared with 12% in the DSS-NP mice. Mice began to regain weight by *day 9* in DSS-NP group, whereas DSS-HP mice regained weight only from *day 11* but never caught up to DSS-NP mouse weight (final body weight: 18.5 ± 1.7 g vs. 21.7 ± 0.7 g in DSS-HP and DSS-NP groups, respectively, *P* < 0.01). The body weight of both DSS-treated group animals remained markedly lower (*P* < 0.0001) when compared with respective untreated control groups even though energy intake, markedly decreased in the two days following the arrest of DSS, went up again in the days that followed (Fig. 2*C*).

Systemic inflammation was detected in DSS-treated mice, as evidenced by the plasmatic acute-phase protein SAA and IL-6 concentration increases, but with some differences depending on the diet and time (Table 2). Measurement of SAA revealed a high systemic inflammatory state in all DSS-treated animals. Interestingly, HP diet ingestion increased plasmatic SAA concentration to a higher extent at *day 6* compared with the DSS-NP group. However, the plasmatic IL-6 concentration was significantly lower in DSS-HP animals than in the DSS-NP group at *day 14* (*P* < 0.05).

Furthermore, DSS treatment not only affected colonic mucosa but induced an increase in the spleen and liver/body weight ratios (Table 2) after 14 days of diet. HP diet ingestion induced a higher increase in the liver relative weight at *day 14* compared with NP animals (*P* < 0.0001). That coincided with the higher plasmatic SAA concentration observed at *day 6*, suggesting a differential effect of the diet associated with DSS on the liver. In addition, HP diet ingestion increased kidney/body weight ratio, both in DSS-treated and -untreated control animals.
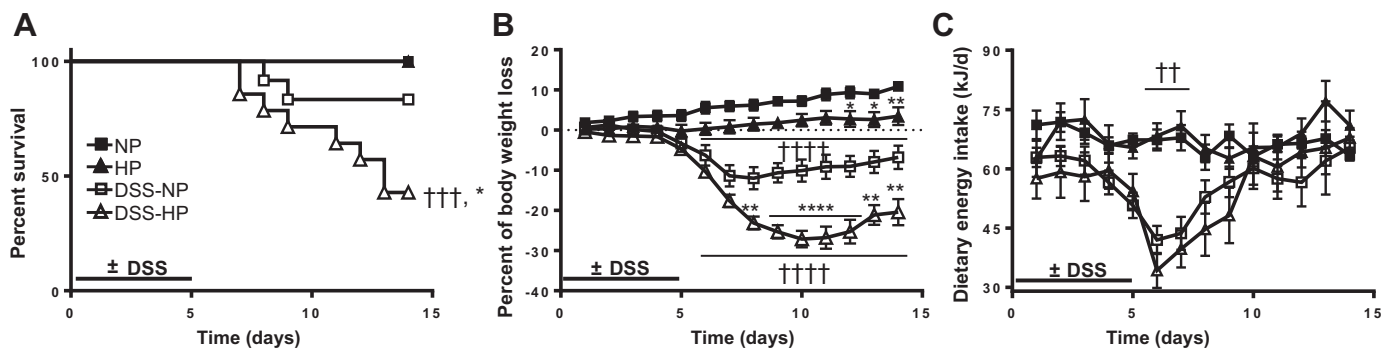

Fig. 2. Effects of HP diet compared with NP diet on animal survival, body weight, and food intake. Mice drank water containing 3.5% DSS (DSS-NP and DSS-HP) for 5 days (as indicated by the bar) or water without DSS (NP and HP), and then water without DSS in all groups. Survival (*A*), body weight loss (*B*), and dietary energy intake (*C*) were monitored daily for up to 14 days in DSS-treated groups compared with control groups. *n* = 11–14 at the start of the experiment; ††*P* < 0.01, †††*P* < 0.001, ††††*P* < 0.0001 vs. control untreated group; **P* < 0.05, ***P* < 0.01, *****P* < 0.0001, DSS-HP mice vs. DSS-NP mice at the same time point.

Table 2. *Effect of HP diet on systemic inflammatory markers and anatomical parameters*

	NP	HP	DSS-NP (6 days)	DSS-HP (6 days)	DSS-NP (14 days)	DSS-HP (14 days)
IL-6, pg/ml	1.9 ± 1.0	4.3 ± 2.1	13.5 ± 4.7	23.2 ± 1.5	49.2 ± 9.0††††‡‡‡	25.4 ± 3.0*
SAA, µg/ml	15.0 ± 3.9	11.4 ± 0.5	56.8 ± 16.6††††	>100	>100	>100
Liver/BW	43.2 ± 1.8	47.3 ± 1.3	41.5 ± 1.3	42.1 ± 2.3	49.3 ± 1.2†‡	64.4 ± 3.0††††‡‡‡****
Spleen/BW	2.81 ± 0.09	2.63 ± 0.08	3.22 ± 0.25	4.10 ± 0.32†	6.34 ± 0.4††††‡‡‡‡	5.61 ± 0.62††††‡
Kidney/BW	11.3 ± 0.3	13.2 ± 0.4**	12.0 ± 0.3	13.1 ± 0.3	12.1 ± 0.4	13.9 ± 0.3*

Data (means ± SE) are expressed as weight ratio relative to body weight (BW) at euthanasia ($n = 6$–12). Plasma concentrations of IL-6 and the acute-phase protein SAA as well as organ weights were determined after 6 and 14 days of normo- (NP) or high-protein (HP) diet ingestion. NP and HP control were determined at *day 14*. †$P < 0.05$, ††††$P < 0.0001$ vs. corresponding control untreated group; ‡$P < 0.05$, ‡‡‡$P < 0.001$, ‡‡‡‡$P < 0.0001$ vs. corresponding same treatment at different time point; *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, DSS-HP mice vs. DSS-NP mice at the same time point.

Effects of the HP diet on the severity of inflammation at the end of colitis induction and resolution phases. The clinical inflammatory score (addition of fecal consistency score and fecal blood score) of DSS-treated animals reached its maximum at *days 6* and *7* in animals receiving the NP and the HP diet, respectively (Fig. 3*A*). The clinical inflammatory score was significantly higher in the DSS-HP group throughout the course of the experiment from *day 7* to *day 12*. Indeed, visible fecal blood could no longer be detected in DSS-NP mice from *day 8*, whereas the stool consistency score remained high. The relative water content in the luminal bulk was actually higher at *day 14* in DSS-treated animals, suggesting luminal hyperosmolarity and/or water malabsorption, but was not different between DSS-HP and DSS-NP groups (Fig. 3*B*). Upon eutha-

nasia, colon length was measured to determine the severity of colitis and shown to be shorter in DSS-treated mice at both time points, but without significant difference among groups (data not shown). The colon weight:length ratio, which increased in DSS-treated mice and is indicative of colon thickening, was higher in DSS-HP mice at the end of the DSS treatment than in the HP control group ($P < 0.05$). It remained similar, though, in both DSS groups at *day 14* (Fig. 3*C*). MPO activity was higher after DSS removal than at the end of the experiment as it returned to near its basal level when measured at 14 days (Fig. 3*D*). This demonstrated that mice treated for 5 days with DSS were at an active, acute stage of colon inflammation, these results being concordant with the colonic expression of the proinflammatory cytokine TNFα (Fig. 4 and Table
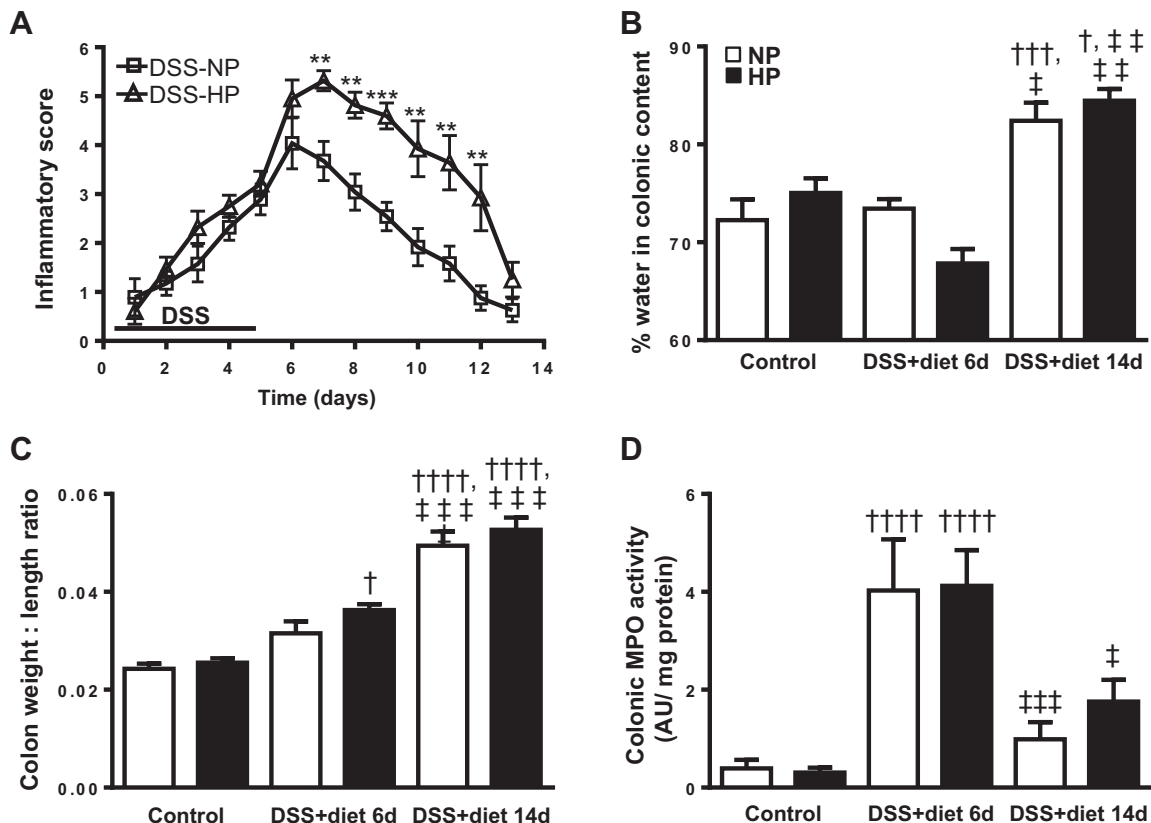

Fig. 3. Effects of HP diet compared with NP diet on clinical inflammatory score and colonic parameters after DSS challenge. Mice were treated with DSS to induce colitis for 5 days and received concomitantly either an NP or an HP diet. Mice were euthanized 6 or 14 days after DSS treatment start. *A*: clinical inflammatory score (fecal blood score added to stool consistency score). *B*: relative water content in the colonic luminal bulk. *C*: colon weight:length ratio. *D*: MPO activity in distal colon. $N = 6$–12; †$P < 0.05$, †††$P < 0.001$, ††††$P < 0.0001$ vs. corresponding control untreated group; ‡$P < 0.05$, ‡‡$P < 0.01$, ‡‡‡$P < 0.001$ vs. corresponding same treatment at different time point; **$P < 0.01$, ***$P < 0.001$, DSS-HP mice vs. DSS-NP mice at the same time point.
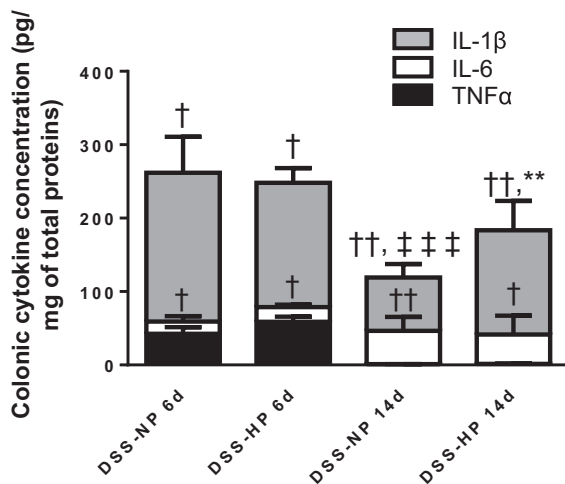
Fig. 4. Effect of HP diet compared with NP diet on colonic proinflammatory cytokine content of DSS-treated mice. Cytokine protein contents (pg/mg of total colonic proteins) in colonic homogenates were analyzed by Luminex technology after 6 and 14 days of NP or HP diet ingestion ($N = 6$–12 animals per group). †$P < 0.05$, ††$P < 0.01$ vs. corresponding control untreated group; ‡‡‡$P < 0.001$ vs. corresponding same treatment at different time point; **$P <$ 0.01, DSS-HP mice vs. DSS-NP mice at the same time point.

3). However, no difference regarding TNFα was observed between the DSS-HP and the DSS-NP groups. After 14 days of diet, levels of the proinflammatory cytokines IL-6 and IL-1β were still high in colons of DSS-treated animals ($P < 0.01$ compared with control animals), and colonic IL-1β concentration was doubled in DSS-HP mice ($P < 0.01$) compared with the DSS-NP group (Fig. 4). Gene expression of other cytokines related to colitis such as Ifnγ, Il-17a, and Il-13 were not statistically different from untreated control groups (Table 3). IL-10, IFNγ, and GM-CSF colonic content was not affected by diet or DSS in colonic homogenate after DSS removal (not shown).

Effects of the HP diet on crypt repair during colitis resolution phase. Hematoxylin- and eosin-stained sections of the distal colon (Fig. 5A) showed that at the end of the DSS treatment (*day 6*), changes in the surface epithelium were noticed with loss of the superior third of crypts and surface epithelium (Fig. 5Ac), crypt abnormalities (Fig. 5Ad) and moderate to severe infiltration of inflammatory cells into the mucosa (Fig. 5A, c and d) compared with untreated-DSS mice (Fig. 5A, a and b). These changes were generally more marked in DSS-NP animals than in DSS-HP (Fig. 5B), although the

histological score of colitis was similar between DSS-treated animals at the same time point (Fig. 5B). DSS treatment seemed, however, to differently alter epithelium according to the diet (Fig. 5C) as the mean size of colonic crypts of DSS-NP mice was lower at the end of colitis induction (*day 6*) compared with DSS-HP animals.

At *day 14*, the histological score of colitis was ameliorated according to attenuation of the inflammatory score (Fig. 3A) and cell infiltration (in particular neutrophils as confirmed above by MPO activity measurement, Fig. 3D), and to epithelial regeneration (Fig. 5A, e and f) even if cryptic distortion was frequently noticed. Thickening of the colon was observed at *day 14* (as shown by the colon weight:length ratio, Fig. 3C) in both DSS-animals receiving an HP or NP diet, and this was associated with an increase in crypt size, which was much more marked in DSS-HP mice than in DSS-NP (Fig. 5C, $P <$ 0.0001). These architectural modifications were concomitant with an increase in the Tgfβ cytokine gene expression (Table 3), which is known to be involved in mucosal healing. Accordingly, the colonic content of the wound healing factor TGFβ-3 was increased by a 2-fold factor in DSS-treated mice fed with the HP diet (Fig. 5D) compared with the DSS-NP group ($P <$ 0.05); by a 3-fold factor compared with control groups ($P <$ 0.001); and finally by a 5-fold factor compared with DSS-treated animals at *day 6* ($P < 0.0001$), while the two other isoforms of TGFβ were undetectable (not shown).

Histological evaluation of cell proliferation in colonic crypts by Ki67 positive expression (Fig. 6A) revealed a marked colonic epithelial hyperproliferation in DSS-HP animals at *day 14* (Fig. 6Ad) compared with all other groups. The total number of colonic epithelial cells per crypt was indeed higher in DSS-HP animals than in DSS-NP at *day 14*, reaching $69.2 \pm$ 1.5 vs. 60.2 ± 2.7 colonocytes per crypt, respectively, $P <$ 0.0001 (mean of 6 animals/group of 100 well-oriented crypts, data not shown). At the same time, the number of PAS-positive cells per 100 colonocytes was significantly higher after DSS-treatment than in the control groups, whatever the dietary supplementation (Fig. 6, B and C). However, less PAS-positive cells per 100 colonocytes were enumerated in the DSS-HP group compared with the DSS-NP group ($P < 0.0001$).

Effects of the HP diet on the expression of epithelial junction proteins during colitis resolution phase. No measurable differences were detected between NP- and HP-control animals regarding the expression of occludin, ZO-1, claudin-1, and E-cadherin (data not shown). In contrast, expression analysis of the tight junction proteins (Fig. 7, A and B) measured at *day 14*

Table 3. *Effect of HP diet on colonic cytokine gene expression*

	NP	HP	DSS-NP (6 days)	DSS-HP (6 days)	DSS-NP (14 days)	DSS-HP (14 days)
Tnfα	0.96 ± 0.13	0.78 ± 0.12	6.28 ± 1.69†	7.50 ± 2.64†††	4.07 ± 0.75	4.20 ± 0.91
Il-6	1.91 ± 0.81	0.88 ± 0.23	7.44 ± 2.86	23.3 ± 8.78	48.5 ± 21.0†	30.9 ± 14.3
Il-1β	0.99 ± 0.26	0.66 ± 0.27	6.46 ± 2.04	8.03 ± 2.70	12.9 ± 5.48	28.7 ± 14.2†
Ifn-γ	1.48 ± 0.43	0.60 ± 0.08	3.21 ± 0.84	1.22 ± 0.54	5.88 ± 1.97	2.86 ± 1.29
Il-17a	1.01 ± 0.05	0.88 ± 0.05	0.72 ± 0.13	0.71 ± 0.12	1.48 ± 0.28	1.51 ± 0.49
Il-13	1.20 ± 0.23	1.40 ± 0.29	1.46 ± 0.26	0.93 ± 0.36	0.70 ± 0.10	0.63 ± 0.15
Tgfβ	1.05 ± 0.10	0.96 ± 0.07	1.39 ± 0.17	1.79 ± 0.43	1.47 ± 0.21	2.78 ± 0.75†††*
Il-22	1.03 ± 0.08	1.03 ± 0.07	nd	nd	2.63 ± 0.42†††	1.76 ± 0.40

mRNA expression relative to control NP-fed animals is expressed as mean $2^{-\Delta\Delta Ct} \pm$ SE ($n = 6$–12 animals per group). Cytokine mRNA expression in colonic homogenates of control (at *day 14*) and DSS-treated mice were analyzed by qRT-PCR after 6 and 14 days of normo- (NP) or high-protein (HP) diet ingestion. NP and HP control were determined at *day 14*. †$P < 0.05$, †††$P < 0.001$ vs. corresponding control untreated group; *$P < 0.05$ DSS-HP mice vs. DSS-NP mice; nd, not detected.
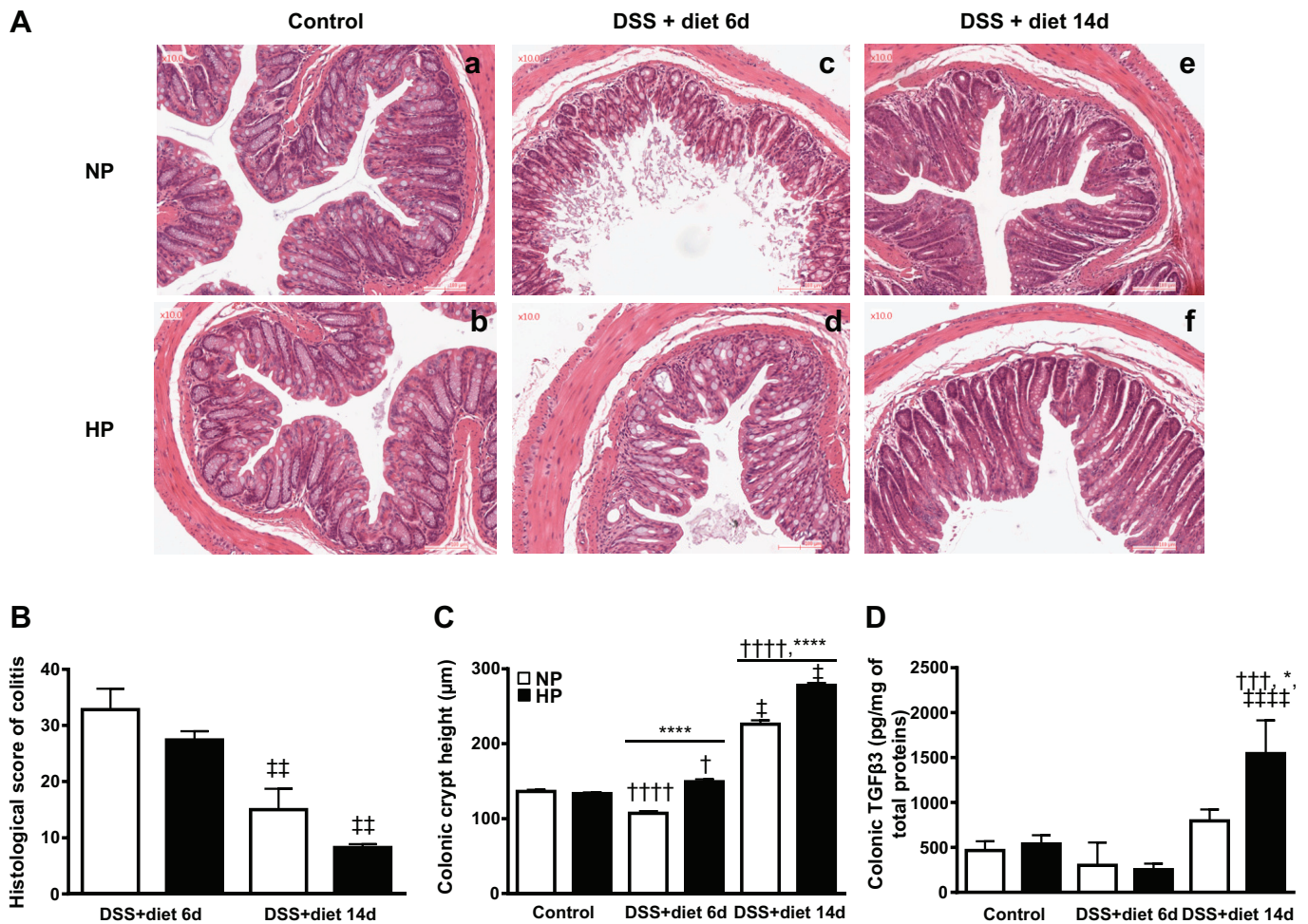
A

B **C** **D**

Fig. 5. Effects of HP diet compared with NP diet on colonic morphology, colitis histological score, colonic crypt height, and TGF-β3 content after DSS challenge. *A*: histological examination of colonic sections stained with hematoxylin-eosin in control (*a* and *b*) and DSS-treated mice (*c–f*) after 6 (*c, d*) or 14 (*e, f*) days of an NP (*a, c, e*) or an HP diet (*b, d, f*). *B*: histological assessment of colonic damages. *C*: colonic crypt height. *D*: colonic content of TGF-β3 (pg/mg of total colonic protein). Images are representative of 6 mice per group; †$P < 0.05$, †††$P < 0.001$, ††††$P < 0.0001$ vs. corresponding control untreated group; ‡$P < 0.05$ ‡‡$P < 0.01$, ‡‡‡$P < 0.001$ vs. corresponding same treatment at different time point; *$P < 0.05$, ****$P < 0.0001$, DSS-HP mice vs. DSS-NP mice at the same time point.

in the colon of DSS-treated mice revealed an overexpression of occludin following HP diet ingestion. Indeed, occludin was increased almost twofold in the DSS-HP group (189 ± 23%, $P = 0.0011$) compared with DSS-NP mice. However, claudin-1 expression was decreased in the colon of DSS-HP (65 ± 8%, $P = 0.0027$) compared with DSS-NP mice. A similar situation was observed regarding the expression of the cell-cell adhesion protein, E-cadherin (heavy form), which was expressed 3 times less in DSS-HP (36 ± 5%, $P < 0.0001$) compared with DSS-NP mice. Interestingly, E-cadherin immunolabeling on colonic sections showed that the level of expression of this cell-cell adhesion protein was similar between HP and NP groups (Fig. 7*C*), but with some difference in E-cadherin regarding cellular localization in DSS-treated groups, this protein being strongly associated with epithelial membranes in DSS-HP animals (Fig. 7*Cd*).

DISCUSSION

The present study showed that a high-protein diet exerts dual effects in the C57BL/6 IBD mouse model, being noxious

during the post-induction phase but helpful in repairing the colonic epithelium in surviving animals when compared with DSS-treated animals receiving a control, isocaloric normo-protein diet.

When given during the colitis course induced by DSS, HP diet ingestion had a deleterious effect on mouse general health status compared with animals receiving an NP diet. Importantly, this deleterious effect occurred after DSS removal. Indeed, while the inflammatory score still increased within one day following the arrest of DSS treatment in the animals fed with the NP diet, the inflammatory score rose during one additional day in DSS-HP animals. It remained higher from *day 7* to *day 12* in DSS-HP than in DSS-NP animals, indicating that a high level of dietary proteins enhances the intensity of intestinal inflammation after the DSS challenge. At *day 6*, thus one day after the arrest of DSS treatment, the MPO activity was equally high no matter the diet consumed by the mice, indicating that neutrophil infiltration in the colonic mucosa was equivalent in mice consuming normal- or high-protein diets. This was associated with a modest increase in the colon
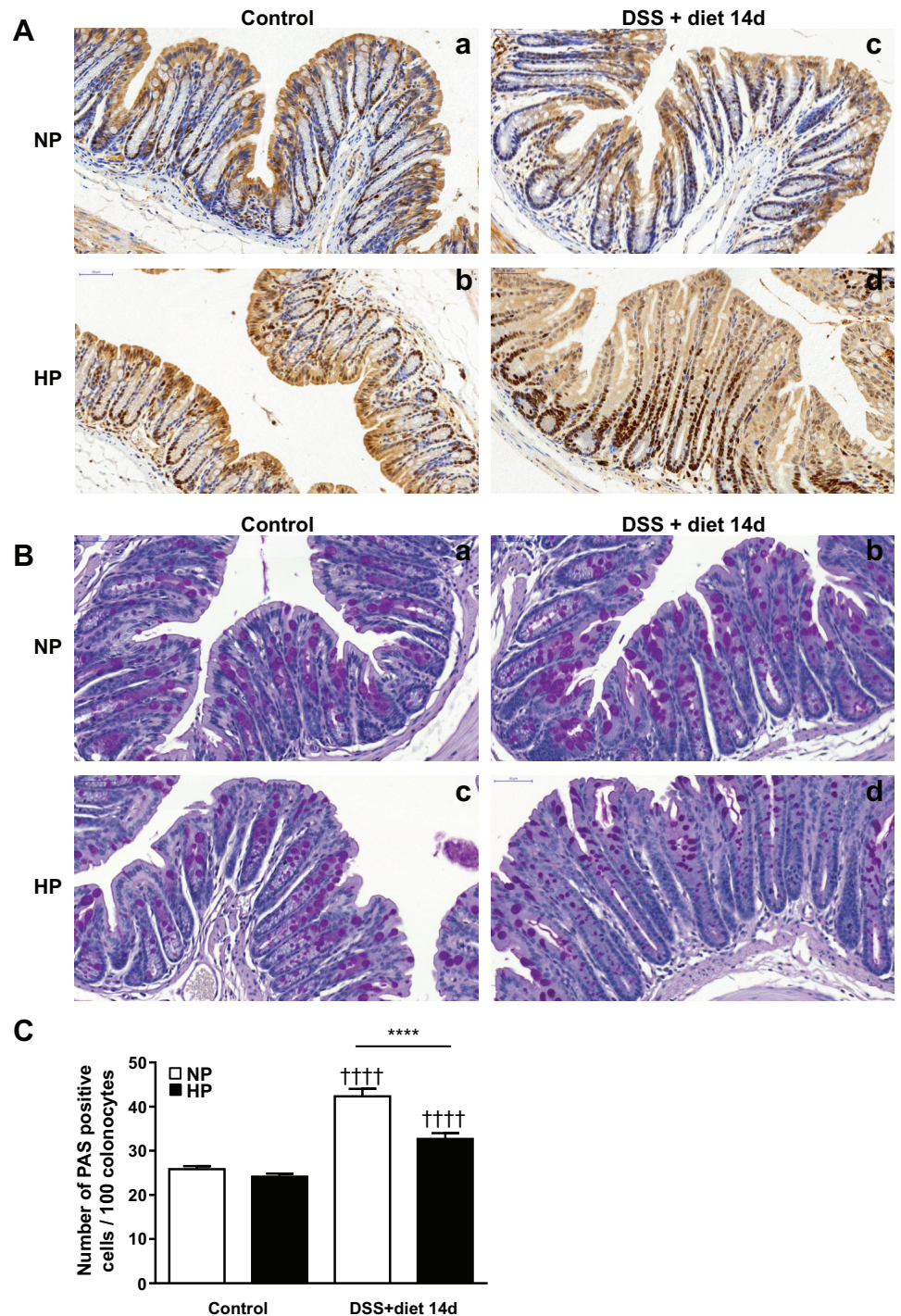
Fig. 6. Effects of HP diet compared with NP diet at *day 14* on epithelial proliferation and mucus-producing cells after DSS challenge. *A*: Ki67 immunolabeling of colonic sections in control (*a, b*) and DSS-treated mice (*c, d*) after 14 days of an NP (*a, c*) or an HP diet (*b, d*). *B*: histological examination of colonic sections stained with PAS in control (*a, b*) and DSS-treated mice (*c, d*) after 14 days of NP (*a, c*) or HP diet (*b, d*). Images are representative of 6 mice per group. *C*: number of muco-secretant cells after 14 days of an NP or an HP diet. ††††$P < 0.0001$ vs. corresponding control untreated group; ****$P < 0.0001$, DSS-HP mice vs. DSS-NP mice at the same time point.

weight:length ratio, which was not modulated by the diet, reinforcing the view that the level of protein ingestion has little impact on the severity of mucosal inflammation at that time. From *day 6* in DSS-NP mice and *day 7* in DSS-HP animals, the inflammatory score gradually decreased until *day 14* when the basal inflammatory score was close to that recorded in control untreated animals.

Importantly, there was a marked and unexpected increase in the mortality of DSS-treated mice receiving the HP diet in the meantime. This correlated with a major body weight loss, whereas no difference in dietary energy intake was observed between DSS-HP and DSS-NP groups. Interestingly, there was no animal mortality in the whole phase of colitis induction, demonstrating that the HP diet was exerting its deleterious effect mainly in the inflammation resolution phase. Although the more intense severity of colitis in DSS-HP animals, and the delayed resolution of inflammation may play a role in this increased mortality, the severe body weight loss in the DSS-HP group is also likely partly responsible for such an effect. It cannot be excluded that other deleterious effects of an HP diet far from the intestine may also play a role in the increased mortality in the colitis context. Indeed, the relative kidney
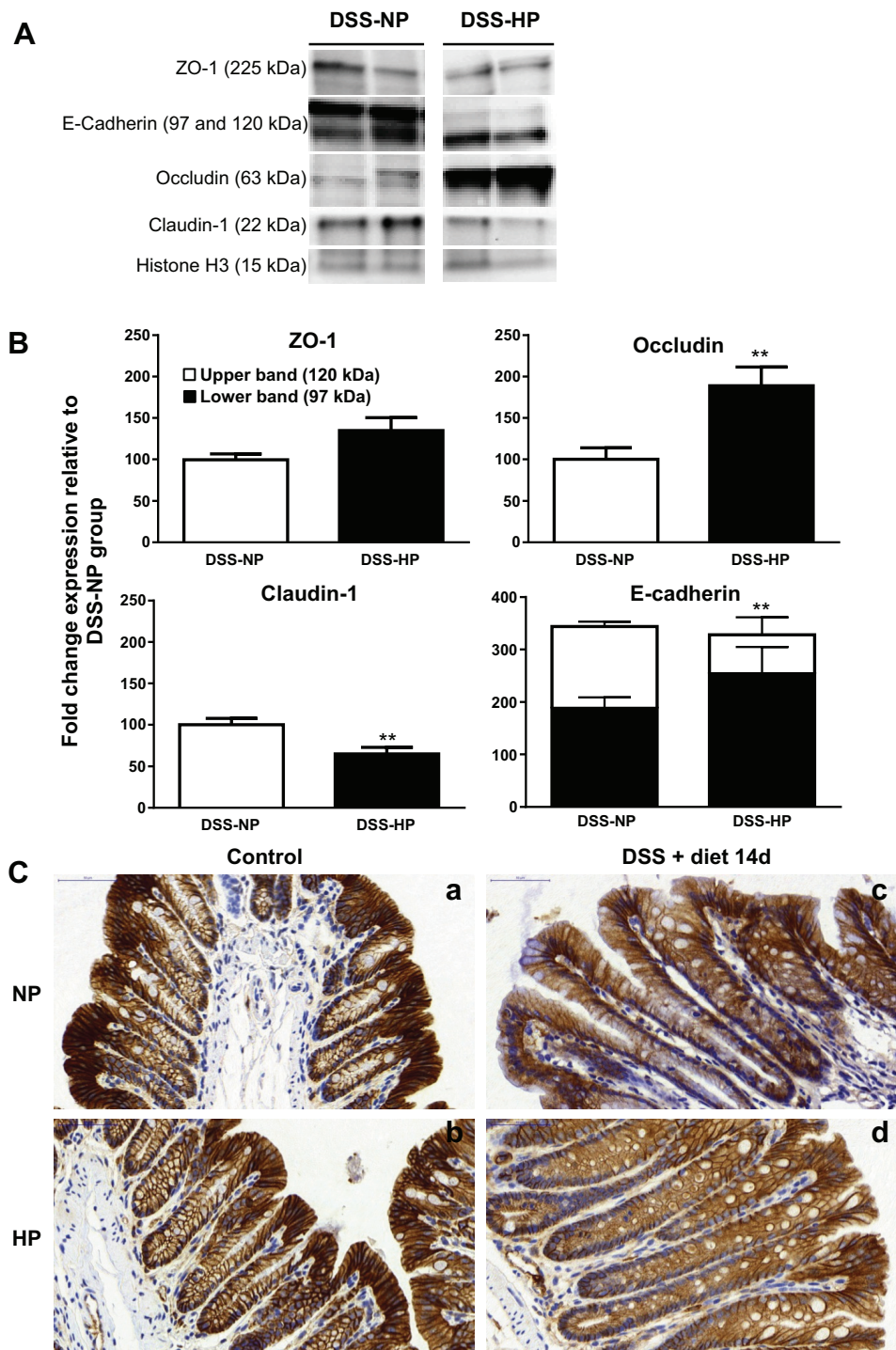
Fig. 7. Effect of HP diet compared with NP diet on the colonic expression of epithelial junction and adherent proteins in DSS-treated mice. *A*: Western blot analysis of tight junction proteins ZO-1, occludin, claudin-1, and the adherens junction protein E-cadherin was performed in colonic homogenates of DSS-treated mice recovered at *day 14*. Histone H3 was used as loading control. Image is representative of 8 Western blots. *B*: data are expressed as relative band density normalized to background (mean of 8 Western blots, $n = 6$ animals per group) and then further normalized to the corresponding expression in DSS-NP group. **$P < 0.05$, DSS-HP mice vs. DSS-NP mice at *day 14*. *C*: immunolabeling of the adherens junction protein E-cadherin on mouse colonic sections in control (*a, b*) and DSS-treated mice (*c, d*) after 14 days of NP (*a, c*) or HP diet (*b, d*). Images are representative of 6 mice per group.

weight was increased in DSS-HP mice, which may be related to an impact of excessive nitrogenous supply on renal function (28). In addition, DSS treatment may likely affect liver physiology through peripheral action of IL-6, originating from the colon, on acute phase protein production such as SAA. Indeed, alteration of liver function by DSS has been reported (16) and hepatic DSS-induced inflammation has been shown to be worsened by dietary parameters (9). The marked increase in liver/body weight ratio in DSS-HP animals observed at *day 14* is likely a consequence of the catabolic action of proinflam-

matory cytokines on skeletal muscle (25). Further work outside the scope of the present study would be necessary to further decipher the extra-intestinal effects of DSS in this classical model of colitis induction.

In addition, systemic disorders such as systemic inflammation and spleen weight increase could be the consequence of higher bacterial translocation following an increase in intestinal barrier permeability in DSS-HP animals compared with DSS-NP group at *day 6* and the following days. Although no measure of intestinal barrier integrity was performed in this

study, differences were indeed observed regarding the expression of proteins involved in intestinal barrier function depending on the diet ingested. This included a modulation of E-cadherin expression, the major component of adherens junction, in the colon of DSS-HP animals at *day 14*. Although the interpretation of the differential E-cadherin isoform expression and localization in the DSS-HP vs. DSS-NP animals is somewhat difficult, it is worth noting that E-cadherin has been shown to exert a key role in intestinal homeostasis by ensuring mechanical integrity (33). In addition, upon DSS treatment, mice deficient in E-cadherin lose more weight, are more severely dehydrated and display blood in the feces more frequently (10).

Several interesting observations were made at *day 14* in a limited number of surviving DSS-treated animals fed with an HP compared with NP diet. First, the increase in colon weight: length ratio was associated with a marked increase in the crypt height, this increase being prominent in the surviving DSS-HP mice. Second, the number of colonic epithelial cells per crypt was higher in DSS-HP animals than in DSS-NP, and this observation was reinforced by a higher histological labeling of proliferating cells in colonic crypts. Last, at the same time, the number of mucus-producing cells per 100 colonocytes was lower in DSS-HP animals than in DSS-NP, suggesting that the program of proliferation/differentiation of colonic epithelial cells is modulated at the end of the colitis resolution phase according to the amount of dietary protein consumed. Such changes may rely on the increase in amino acid availability needed for the mucosal healing process (19). Providing more amino acids by the means of an HP diet would then be beneficial for the repair of intestinal damage, as amino acids might be used by the colonic mucosa as substrates for nucleotide and protein synthesis, for energy metabolism and/or as a precursor of endogenous bioactive peptides (e.g., for glutathione synthesis) and signal molecules. In support of this proposition, it has been shown that the mucosal healing process is associated with increased protein synthesis (21), thus presumably requiring an increased supply of amino acids. Lesions in colonic tissue of UC patients have indeed displayed reduced levels of amino acids (29).

Furthermore, a significant increase in colon IL-1β and TGF-β3 contents was observed in DSS-HP animals. TGF-β3 is known to promote intestinal epithelial wound repair (25) without fibrosis (36), and its expression has been shown to be modulated by proinflammatory agents such as IL-1β in intestinal epithelial cells (16). Thus, by increasing TGF-β3, an HP diet would more efficiently restore epithelial integrity than an NP diet. However, the signals (amino acids, bacterial metabolites) responsible for IL-1β production increase, and the types of cells responsible for TGF-β3 production (myofibroblasts, colonocytes) remain to be elucidated. These observations thus require further study regarding the consequences of providing an HP diet after an inflammatory flare and the long-term benefits of TGF-β3-induced production for colonic mucosal healing.

Incidentally, these results likely raise the possibility of a causal link between increased colonic epithelial repair and survival in our model. In other words, the results are compatible with a heterogenic response among animals. The animal, unable to mount a protective mucosal response in a context of robust colonic inflammation and HP diet consumption, will die; while those that take advantage of HP diet ingestion to develop protective mechanisms (including mucosal healing) will survive. Deciphering characteristics at the basis of such heterogeneity among individuals would obviously be of prime interest.

Last, the composition of the diets in terms of quantity and quality of the macronutrients used should be considered. Since the amount of carbohydrates (corn starch and sucrose) was reduced in the HP diet to provide an isocaloric diet, it is not possible to exclude the role of such a decrease regarding the effects observed. However, these carbohydrates are digested and absorbed in the small intestine and thus are not believed to reach the colon lumen to be used by the colon microbiota. From a previous study, we know that the HP lower-carbohydrate diet used in the present work does not change either short-chain fatty acid concentrations in the colonic luminal content of healthy rats or colonocyte oxidative capacity for oxidation of butyrate, L-glutamine, and D-glucose compared with the isocaloric NP diet (22). Furthermore, the quality of the protein used might also modulate the HP diet effects observed, since different dietary proteins are associated with different characteristics in terms of digestibility and amino acid composition. Such differences may result in different amino acid availability through the bloodstream and also in different transfer of protein from the small to the large intestine. Finally, the extent of such transfer may impact the production of the amino acid-derived bacterial metabolites with different effects on the colonic epithelium renewal (4).

In conclusion, the HP diet appears to exert its severe deleterious effects mainly in the resolution phase following colitis induction in our DSS model, while also appearing to positively intervene in post-colitis epithelial repair in surviving animals. This study therefore points out not only the potential importance of dietary factors in IBD (20), but also the difficulty in fixing the optimal amounts and kinetics of dietary protein supply in such a context.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

A.L. conception and design of research; A.L., A.B., D.C., J.C., and M.M. performed experiments; A.L., D.C., J.C., M.M., and F.W. analyzed data; A.L. interpreted results of experiments; A.L. prepared figures; A.L. and F.B. drafted manuscript; A.L., A.B., R.B., A.H.L.J., D.T., and F.B. edited and revised manuscript; A.L., A.B., D.C., J.C., M.M., R.B., A.H.L.J., F.W., D.T., and F.B. approved final version of manuscript.

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