Energy Drink Administration Ameliorates Intestinal Epithelial Barrier Defects and Reduces Acute DSS Colitis

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Background: The rise in the prevalence of inflammatory bowel diseases in the past decades coincides with changes in nutritional habits, such as adaptation of a Western diet. However, it is largely unknown how certain nutritional habits, such as energy drink consumption, affect intestinal inflammation. Here, we assessed the effect of energy drink supplementation on the development of intestinal inflammation in vitro and in vivo.

Methods: HT-29 and T84 intestinal epithelial cells and THP-1 monocytic cells were treated with IFNγ in presence or absence of different concentrations of an energy drink. Colitis was induced in C57BL/6 mice by addition of dextran sodium sulfate (DSS) to drinking water with or without supplementation of the energy drink.

Results: Energy drink supplementation caused a dose-dependent decrease in IFNγ-induced epithelial barrier permeability, which was accompanied by upregulation of the pore-forming protein claudin-2. Administration of the energy drink reduced secretion of the pro-inflammatory cytokines interleukin-6 and tumor necrosis factor-α from HT-29, T84, and THP-1 cells. In vivo, energy drink administration reduced clinical symptoms of DSS-induced colitis and epithelial barrier permeability. Endoscopic and histologic colitis scores and expression of pro-inflammatory cytokines were significantly reduced by energy drink co-administration.

Conclusion: Energy drink consumption seems to exert an unexpected anti-inflammatory effect in vitro and in vivo in our experimental setting. However, our experimental approach focuses on intestinal inflammation and neglects additional effects of energy drink consumption on the body (eg, on metabolism or sleep). Therefore, the translation of our findings into the human situation must be taken with caution.

Key Words: IBD, energy drink, claudin-2, occludin, barrier permeability, colitis

INTRODUCTION

Inflammatory bowel diseases (IBDs), with Crohn's disease (CD) and ulcerative colitis (UC) as its main forms, are characterized by chronic and relapsing intestinal inflammation, in addition to extraintestinal manifestations such as inflammation of eyes, joints, or skin.[1] The worldwide incidence of both UC and CD is still on the rise,[2, 3] and in Europe alone, IBD affects an estimated 2.5 to 3 million people.[2] The etiology of IBD is still not fully understood, but a current hypothesis suggests a complex interplay between genetic and environmental factors such as nutrition and gut microbiota, resulting in an aberrant immune response as the driving force for the disease. The role of nutritional factors in the development of IBD has become of more and more interest because nutritional habits have dramatically changed over past decades, and the introduction of a Westernized diet, including increased fast food consumption, is one of the major lifestyle changes in developing countries.[4] Of interest, these countries show the highest and most rapid increase in IBD prevalence.[2,3]

Notably, a well-perceived supplementary treatment option for IBD consists of the implementation of dietary factors such as a low carbohydrate or a high fiber diet, which have been linked with a decreased risk of developing IBD.[5, 6] On the contrary, regular fast food consumption, which results in high

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Abbreviations: DSS, dextran sodium sulfate; FITC, fluorescein isothiocyanate; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IFN/IFNγ, interferon gamma; PTPN2, protein tyrosine phosphatase nonreceptor type 2; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor

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uptake of saturated fatty acids, was associated with a higher risk of developing IBD.[5, 7] Besides the increase of fast food consumption, the consumption of energy drinks (ED) has highly increased in past decades.[4, 8] Energy drinks are high in refined sugar, which might promote intestinal inflammation,[9] but they also contain a variety of additional compounds aimed to promote physical and mental stimulation.[8] Besides 27.5 g of refined sugar, a 250 mL can of the here used energy drink contains 80 mg of caffeine, equivalent to 1 cup of regular coffee, and 1000 mg of taurine. Both caffeine and taurine have previously been shown to decrease experimental colitis.[10–12]

However, processed sugars can affect epithelial barrier function and are linked with an increased risk of developing IBD.[13] Previous studies have shown that an increased sugar consumption can have disruptive effects on epithelial barrier permeability and exacerbate colitis severity.[14] The intestinal epithelium, formed by a single layer of intestinal epithelial cells (IECs), is not only responsible for the absorption of nutrients and water but also acts as a barrier between the gut lumen and the underlying mucosal layer and thus has a central role in mediating the interaction between luminal contents such as the intestinal microbiota, potential pathogens or digested food, and the underlying immune system.[15] Thus, the intestinal epithelium forms the interface between food particles and the human body. Damage in the intestinal epithelium, as observed during CD or UC, seriously affects barrier function and results in malabsorption, chronic inflammation, and diarrhea.[16]

Because energy drinks contain factors that might promote the development of intestinal inflammation and compounds that have been shown to ameliorate colitis, we here investigated the effect of a typical energy drink on intestinal barrier function and intestinal inflammation.

MATERIALS METHODS

Energy Drink Medium Preparation for Cell Culture

Commercially available Red Bull energy drink cans were opened and left to decarbonize at room temperature overnight. Red Bull contains 0.32 mg/mL of caffeine, 0.11 g/mL of sugar, and 4 mg/mL of taurine.[10] The content of the can was transferred to a flask, agitated rigorously to release additional CO_2, and subsequently filtered using a quick release filtration system. Three dilutions, 1:5, 1:10, and 1:50, in appropriate complete cell culture medium were prepared and the pH adjusted to match the corresponding cell culture medium. Before applying to the cells, dilutions were filtered again using a Steriflip filtration system (Merck, Darmstadt, Germany).

Cell Culture and Stimulation

Human intestinal epithelial HT-29 cells (ATCC, Manassas, Virginia, USA) were cultured in low glucose DMEM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% fetal calf serum (FCS, PAN Biotech, Aidenbach, Germany), 0.5% sodium pyruvate (Thermo Fisher Scientific), and 1% nonessential amino acid solution (Thermo Fisher Scientific). The human intestinal epithelial cells T84 (ATCC) were cultured in McCoy's Medium (Thermo Fisher Scientific) supplemented with 10% FCS. The medium RPMI 1640 (Thermo Fisher Scientific), supplemented with 10% FCS, was used to culture THP-1 cells (DSMZ ACC 16, DSMZ, Braunschweig, Germany). The HT-29 and T84 cells were maintained at 10% CO_2, 95% humidity, and 37°C and were subcultured when reaching 70%–80% confluency. The THP-1 cells were kept at a density of 1×10^5–1×10^6 cells/mL in an incubator with 5% CO_2, 95% humidity, and a temperature of 37°C. For experiments, HT-29 and T84 cells were rinsed with phosphate buffered saline (PBS, Thermo Fisher Scientific) and detached with EDTA-trypsin (Thermo Fisher Scientific) from the cell culture flask, and all cells were seeded into 12-mm transwell inserts (MerckMillipore, Burlington, Massachusetts, USA). The THP-1 cells were seeded into 6-well, 12-well, or 24-well plates at a density of 5×10^5–1×10^6 cells/mL. Twenty-four hours hours before the experiment, the media was replaced with medium without FCS. Cells were then treated with different dilutions of the energy drink in DMEM/RPMI and with interferon-γ (IFNγ, 100 ng/mL, Peprotech, London, United Kingdom) for 5 hours before further processing.

Transepithelial Electrical Resistance and FITC-Dextran Permeability Measurement

The HT-29 and T84 IECs were grown on semipermeable inserts, which allow for the formation of a polarized epithelial layer with functional tight junctions. In initial experiments, transepithelial electrical resistance (TEER) was measured every second day and found to reach a plateau between 8 and 13 days after seeding. Therefore for the experiments, after 9 days the medium was replaced with different pH-adjusted dilutions of the energy drink in cell culture medium (control, no energy drink; 1:5, 1:10, and 1:50) for 24 hours. Transepithelial electrical resistance was measured using a chopstick electrode. The reads of an empty insert were subtracted from the reads and values corrected for growth area. For fluorescein isothiocyanate (FITC)-dextran permeability measurement, cells were washed twice with ice-cold PBS (Thermo Fisher Scientific) and 100 µg/mL of FITC-dextran 4 kDa (Sigma-Aldrich, St. Louis, Missouri, USA) applied to the apical side. After a 2-hour incubation time, the basolateral medium was collected, and fluorescence was measured with an excitation wavelength of 488 nm and an emission wavelength for 520 nm on a Synergy HT plate reader (BioTek, Winooski, USA) using the Gene 5.0 software.

Acute DSS-colitis and Mouse Endoscopy

Female C57BL/6J wild-type (WT) mice (Taconic, Rensselaer, New York, USA) within a weight range of 20 to 25 g were used for all experiments. Mice were kept in

a pathogen-free environment with water and food (regular mouse and rat chow #3436, Kliba AG, Kaiseraugst, Switzerland) ad libitum. Water intake did not differ between the groups and was approximately 4 mL/mouse/day (Supplementary Fig. 1A). Diet was the same throughout the acclimatization period and the experiment, and food intake was between 3.5 to 4 g/mouse/day in all groups, except the dextran sodium sulfate (DSS) and DSS + energy drink groups in which food intake was reduced to approximately 2.5 g/mouse/day on the last 2 days of the experiment (Supplementary Fig. 1B). For acute colitis induction, 2.5% dextran sodium sulfate (MP Biomedicals, Illkirch-Graffenstaden, France) was added to the drinking water for 7 days. The energy drink was diluted 1:2 with drinking water; glucose and sucrose (5.5 g/100 mL each; Sigma-Aldrich), representing possible confounders, were added in control groups to match the content found in energy drink. Mice were randomized into 4 groups and treated for 7 days with 2.5% DSS + glucose/fructose, 2.5% DSS + energy drink, energy drink, or glucose/fructose as control. Weight changes were recorded daily. On day 8, mice were anesthetized with 120 mg/kg body weight ketamine (Vétoquinol, Bern, Switzerland) and 8 mg/kg xylazine (Bayer, Lyssach, Switzerland) intraperitoneally. Mouse colonoscopy was performed with the Karl Storz Tele Pack Pal (Karl Storz Endoskope, Tuttlingen, Germany) and scored using the murine endoscopic index of colitis severity (MEICS).[17] After colonoscopy, mice were sacrificed by cervical dislocation. The distal 1.5 cm of the colon was removed and fixed in 4% formalin (Formafix AG, Hittnau, Switzerland) overnight; the rest of the colon was divided into 1-cm pieces, placed in liquid nitrogen, and stored at −80°C until further processing.

Histologic Assessment of Mouse Colon

Formalin-fixed colon pieces were dehydrated using ascending alcohol series, transferred to Histoclear (Chemie Brunschwig, Basel, Switzerland), and then embedded in paraffin. Using a microtome, the paraffin blocks were cut into 5-µm sections, placed onto a glass slide, and left to air-dry for 24 hours. The slides were deparaffinized in Histoclear and rehydrated using a descending alcohol series, then placed in water and transferred to hematoxylin (Chemie Brunschwig) for 10 minutes. Next, slides were differentiated for 2 seconds in 1% HCl-EtOH solution and then placed under running, lukewarm water for 10 minutes. Slides were dipped for 10 seconds in eosin (2% w/v, Sigma-Aldrich) and dehydrated using an ascending alcohol series, ending with Histoclear. Coverslides were mounted using Pertex mounting medium (International Medical Products, Brussels, Belgium).

Colitis severity was assessed as previously described using the following parameters: immune cell infiltration (0, no infiltration; 1, infiltration around crypt area; 2, infiltration reaching mucosal layer; 3, infiltration reaching submucosal layer; 4, infiltration reaching muscularis mucosae) and epithelial damage (0, no damage; 1, partial loss of goblet cells; 2, major loss of goblet cells; 3, loss of crypts; 4, extended crypt loss).[18]

For immunofluorescence, after rehydration the section were heated (98°C) in antigen retrieval buffer pH 6 (Dako, Glostrup, Denmark) for 30 minutes, blocked in blocking buffer (10% goat serum, 10% bovine serum albumin (BSA) in PBS) for 1 hour and incubated 1 hour with a rat anti-mouse F4/80 (Cell Signaling Technologies, Danvers, Massachusetts, USA) and a rabbit anti-mouse CD206 (Abcam, Cambridge, Massachusetts, USA). The slides were washed 3 times with PBS, incubated with AlexaFluor594-anti-rat, AlexaFluor647 anti-rabbit secondary antibodies, and DAPI nuclear stain for 1 hour, washed again 3 times, and mounted with fluorescence mounting medium. Images were captured using a Leica SP8 confocal microscope and quantification performed using ImageJ/FIJI software (NHI Image, Bethesda, Maryland).

Protein Isolation and Western Blot

Cell culture samples were homogenized using Mammalian Protein Extraction reagent (M-PER, Thermo Fisher Scientific), supplemented with Complete Mini protease inhibitor cocktail (Merck). Colon samples were dissociated in M-PER using a GentleMac tissue homogenizer (Miltenyi Biotech, Bergisch Gladbach, Germany), centrifuged and the supernatant mixed with loading buffer (NuPAGE 4× LDS Sample Buffer, Life Technologies, Carlsbad, California, USA) and 50 mM of dithiothreitol, and boiled for 10 minutes at 95°C. Equal amounts of protein for each sample were loaded onto polyacrylamide gels, separated by gel electrophoresis (SDS-PAGE), and subsequently transferred onto nitrocellulose membranes (Millipore, Billerica, Massachusetts, United States). Membranes were blocked for 1 hour with a combination of 1% bovine serum albumin (PAN Biotech) and 3% milk powder (Carl Roth, Karlsruhe, Germany) in washing solution (Tris buffered saline containing 1% Tween 20; for occludin, protein tyrosine phosphatase nonreceptor type 2 [PTPN2], claudin-2, signal transducer and activator of transcription [STAT]3, STAT1, phospho-p65, total p65, phospho-Akt, total Akt, β-actin), 5% BSA (pSTAT3) or 2% milk powder and 1% BSA (pSTAT1). Primary antibody (1:1000 dilution, except for pSTAT1 1:2000) was diluted in the corresponding blocking solution, and membranes were incubated overnight at 4°C. Membranes were then washed 3 times with washing solution and incubated with the appropriate horse radish peroxidase (HRP)-linked secondary antibody (Santa Cruz Biotechnologies, Dallas, Texas, USA) for 1 hour in corresponding blocking solution. After washing 3 times for 10 minutes in washing solution, membranes were developed using a Western Blot detection kit (Western Bright Sirius or ECL, Advansta, Menlo Park, California, USA). Protein bands were detected using a Fusion Solo S imager (Vilber Lourmat, Witec AG, Littau,

Switzerland). Densitometry values were normalized to β-actin. For phospho-proteins, values were normalized to the corresponding total protein. Anti-pSTAT1 (#9172S), anti-STAT1 (#9167S), anti-pSTAT3 (#9131S), anti-STAT3 (#9139S), anti-p-p65 (#3033S), anti-p65 (#8242S), anti-pAkt (#4060S), anti-Akt (#4691S), and the secondary anti-Rabbit-IgG HRP-linked antibody (#7074) were obtained from Cell Signaling Technologies. Anti-claudin2 (#ab53032) and anti-occludin (#ab31721) were obtained from Abcam, anti-β-Actin (LOT: 2951837), anti-mouse-IgG HRP-linked (LOT:2951837) from EMD Millipore, anti-PTPN2 (MAB1930) from R&D systems (Minneapolis, Minnesota, USA) and goat-anti-mouse HRP-linked (lot#C0316) from Santa Cruz Biotechnologies.

Myeloperoxidase Assay

Colon samples of 0.5 cm were homogenized in a 50-mM phosphate buffer solution (pH 6.04) containing 0.5% hexadecy ltrimethylammoniumbromid using a GentleMac tissue homogenizer (Miltenyi Biotech). After 3 freeze-thaw cycles in liquid nitrogen, the supernatant was mixed with a 50-mM phosphate buffer solution containing 0.02% dianisidine and 0.0005% H_2O_2 (both Sigma-Aldrich). After 20 minutes of incubation time, absorbance at 460 nm was measured using a plate reader. Myeloperoxidase activity (MPO, arbitrary unit) was calculated as mean absorbance (460 nm) per incubation time (min) per protein content (g).

RNA Isolation, Complementary DNA Synthesis, and Real-time Polymerase Chain Reaction

RNA was isolated using the Maxwell RSC simply RNA kit from Promega Corporation (Madison, Wisconsin, USA) according to the manufacturer's instructions. In brief, 0.5-cm pieces from the distal colon or cell culture samples were disrupted in 1-thioglycerol containing homogenization solution (Promega Corporation). Mouse tissue was additionally homogenized using a GentleMac tissue homogenizer (Miltenyi Biotech). Further, automated processing was performed with a Maxwell RSC Instrument (Promega Corporation). Assessment of RNA concentration was performed measuring absorbance at 260 on a NanoDrop 1000 (Thermo Fisher Scientific). For complementary DNA (cDNA) synthesis, the HighScribe cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California, USA) was used according to manufacturer`s instruction. Real-time polymerase chain reaction (RT-PCR) was performed using FAST qPCR Master Mix for Taqman Assays (Life Technologies) on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) with QuantStudio Real Time PCR software (v.1.3). For each sample, triplicates were measured, and results were analyzed using the ΔΔCt method. The *ACTB* gene (encoding β-Actin) served as endogenous control. Gene expression assays were all obtained from Thermo Fisher Scientific. Steps for real-time PCR consisted of an enzyme activation step (5 min, 95°C) and 45 cycles of denaturing (95°C, 15 sec) and annealing/extending (60°C, 1 min).

Statistical Analysis

Data are presented as ±SEM for a series of n experiments. Statistical analysis was performed (GraphPad Software, Inc., San Diego, California, USA) by 1-way analysis of variance (ANOVA) followed by Mann-Whitney *U* or Student *t*-test as appropriate. Tukey multiple comparison correction was used to correct for multiple testing as appropriate. *P* values of <0.05 were considered significant.

Ethical Considerations

All experiments involving mice were conducted according to Swiss animal welfare law and approved by the local authorities (veterinary office of the canton Zürich, ZH121/17).

RESULTS

Energy Drink Decreases IFNγ-induced Barrier Permeability in Intestinal Epithelial Cells

To assess whether energy drink supplementation might affect intestinal health, we first investigated its effect on intestinal epithelial permeability and tight junction integrity using HT-29 and T84 IECs grown as monolayers. To assess the effect of the energy drink upon inflammatory insults, 1 set of cells was additionally cotreated with IFNγ for 24 hours to induce an inflammatory response. As expected, IFNγ promoted FITC-dextran permeability, but energy drink supplementation alone had no effect on permeability to 4 kDa FITC-dextran (Fig. 1A). However, IFNγ-induced barrier permeability was clearly reduced in a dose-dependent manner upon cotreatment with the energy drink (Fig. 1A).

To further investigate the effect of energy drink addition on epithelial barrier function, we measured TEER across HT-29 and T84 IEC monolayers treated with IFNγ and supplemented with or without energy drink. As expected, in both cell types, IFNγ induced a significant drop in TEER after 24 hours, an effect that was reversed in IECs treated with the energy drink, again in a dose-dependent manner (Fig. 1B), indicating that the energy drink might reduce IFNγ-induced barrier defects.

To assess whether the observed changes in epithelial barrier function were due to alterations in tight junction proteins, we performed Western Blot analysis of claudin-2 and occludin in HT-29 cells treated for 24 hours with IFNγ and/or energy drink. Interferon-γ-induced expression of the pore-forming molecule claudin-2 was reverted upon energy drink treatment in a dose-dependent manner (Fig. 1C, D), which is well in line with the rescue in TEER we observed upon energy drink treatment. Further, consistent with a decrease in IFNγ-induced FITC-dextran permeability, IECs treated with energy drink and IFNγ showed higher expression of the tight junction protein occludin than cells treated with IFNγ alone (Fig. 1C, D).

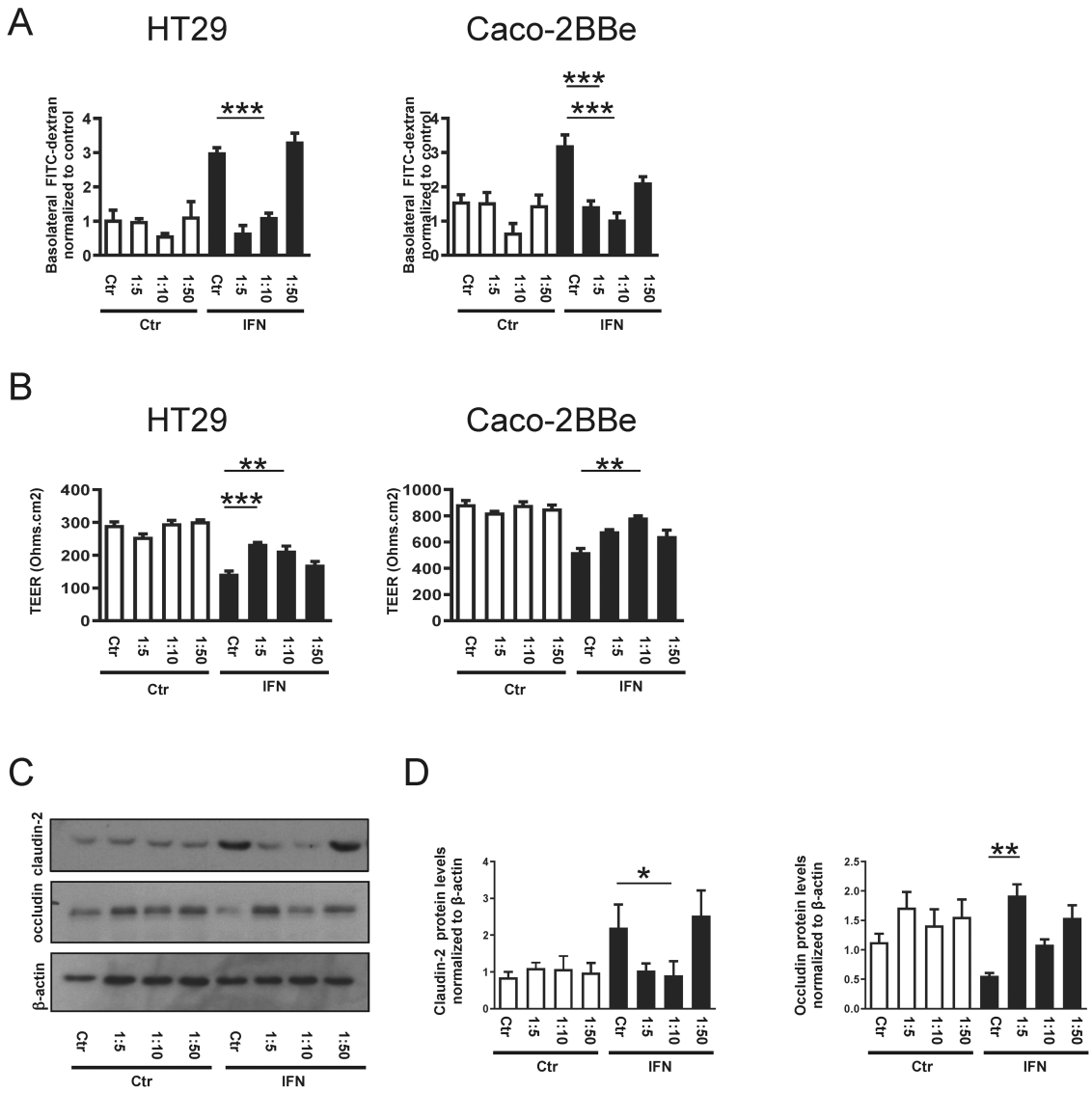

FIGURE 1. Energy drink reduces IFNγ-induced barrier defects and claudin-2 expression in IEC. The HT-29 and T84 cells were treated with the indicated dilutions of the energy drink (Control/Ctr, 1:5, 1:10; 1:50) and stimulated with IFNγ (IFN) for 24 hours. A, 4 kDa fluorescein isothiocyanate dextran was added to the apical side, and FITC-fluorescence was measured in the basolateral medium 2 hours later. B, Transepithelial electrical resistance. C, Representative pictures from Western Blot analyses for the tight junction proteins claudin-2 and occludin. D, Densitometry analyses for claudin-1 and occludin normalized to β-actin. Asterisks indicate significant differences (n = 3 separate experiments per condition, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; 1-way ANOVA, post hoc Tukey multiple comparisons test).

Taken together, these results show a dose-dependent effect of the energy drink in preventing the IFNγ-induced barrier defect in HT-29 and T84 IEC monolayers.

Energy Drink Ameliorates the Inflammatory Response in HT-29 Cells Upon Stimulation With IFNγ

Because IFNγ is known to activate the JAK-STAT-pathway, which affects the expression of tight junction molecules, we performed Western Blot analysis of STAT1 and STAT3 phosphorylation. Upon addition of the diluted energy drink, we observed a decrease in STAT1 and STAT3 phosphorylation, indicative for activation, compared with the nonenergy drink–treated cells (Fig. 2A, B, Supplementary Fig. 1). We have previously demonstrated that PTPN2 regulates IFNγ-induced inflammatory responses, including direct de-phosphorylation of STAT molecules and suppression of claudin-2,[19, 20] two effects also observed upon energy drink treatment. Therefore, we hypothesized that energy drink addition might affect PTPN2

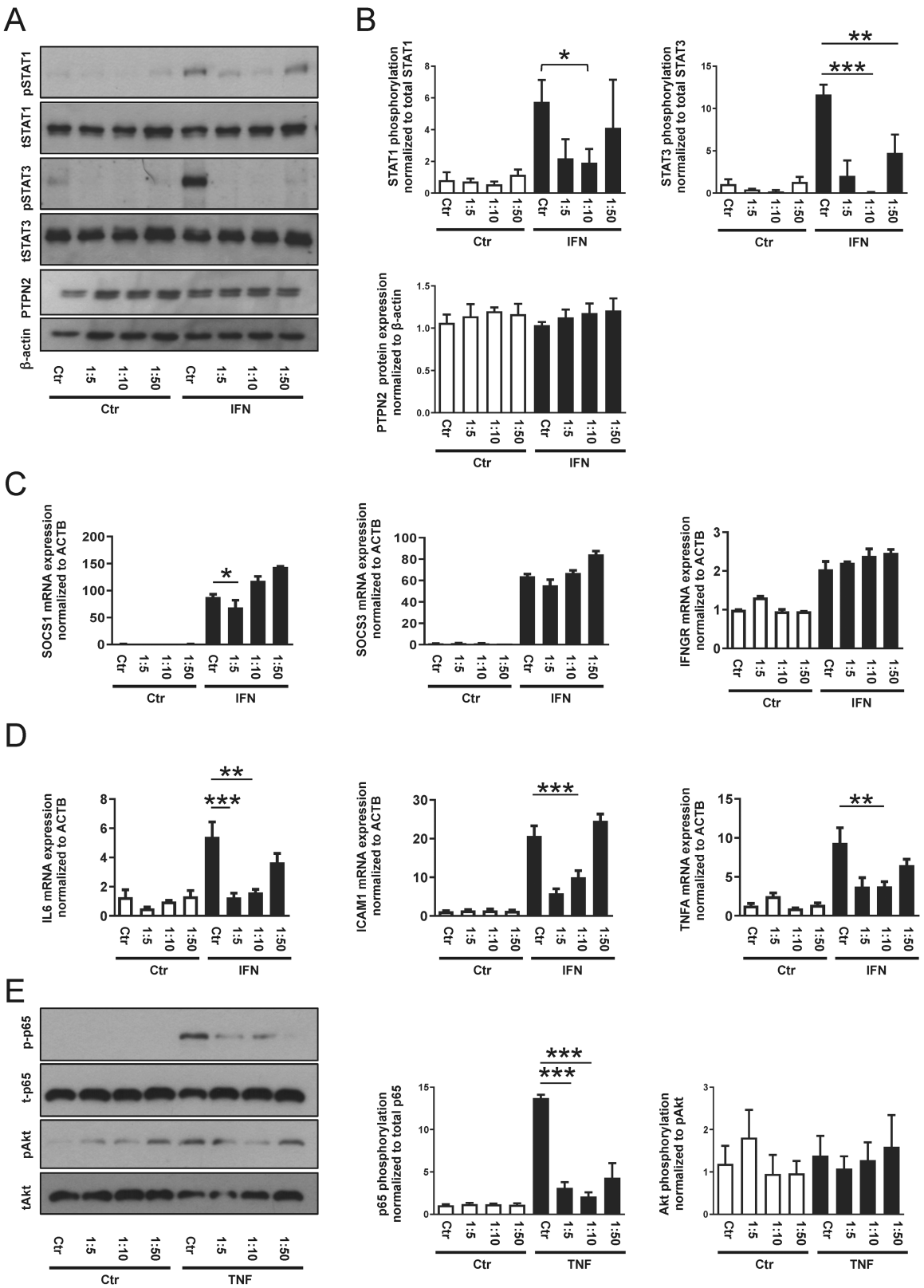

FIGURE 2. Reduction in pro-inflammatory gene expression in IEC upon addition of energy drink. A–D, HT-29 cells were treated with the indicated dilutions of the energy drink (Control/Ctr, 1:5, 1:10; 1:50) and stimulated with IFNγ (IFN) for 30 minutes (A, B) or 24 hours (C, D). A, Representative

expression. However, Western Blot analysis showed no differences in PTPN2 protein levels (Fig. 2A, B). Further prominent regulatory molecules of IFNγ-induced signaling include SOCS1 and SOCS3. Energy drink treatment reduced the expression of SOCS1, a trend also visible in SOCS3 (Fig. 2C), thus not providing an explanation for the observed anti-inflammatory effect. However, IFNγ-receptor expression was not altered (Fig. 2C).

Upon inflammatory insults, IECs express a number of inflammatory cytokines and genes that further promote inflammation and the recruitment of immune cells. To assess the effect of energy drink treatment on inflammatory responses in IEC upon IFNγ stimulation, we performed mRNA analysis of the pro-inflammatory molecules IL-6, TNF-α, and ICAM-1. Interferon-γ induced a significant increase in the expression of all 3 molecules, an effect that was significantly decreased upon energy drink treatment (Fig. 2D, Supplementary Fig. 1). The effect was comparable in cells treated with 1:5 and 1:10 energy drink dilutions but less pronounced in cells treated with 1:50 diluted energy drink solutions, again indicating a dose-dependent effect. Furthermore, energy drink addition also reduced TNF-α-induced NF-κB p65 activation but did not affect Akt phosphorylation (Fig. 2E, Supplementary Fig. 1).

Energy Drink Addition Ameliorates IFNγ-Induced Pro-inflammatory Responses in THP-1 Cells

To assess whether the effect of energy drink addition was limited to IECs or whether it also influences innate immune cell responses, we investigated its effect on IFNγ-induced effects in human monocytic THP-1 cells. We incubated THP-1 cells with different energy drink dilutions (Ctr, 1:5, 1:10, and 1:50) and additionally treated them with IFNγ for 24 hours. We observed a dose-dependent decrease in IFNγ-induced STAT1 and STAT3 phosphorylation (Fig. 3A), which was similar to the effects in IECs cells. In contrast to HT-29 cells, we observed a clear amelioration of IFNγ-induced expression of the regulatory proteins SOCS1 and SOCS3 (Fig. 3B), whereas the expression of the IFNγ-receptor was enhanced in the 1:5 dilution (Fig. 3B). Next we assessed the mRNA expression of the pro-inflammatory cytokines IL-6, TNF-α, and ICAM-1, in addition to the IFNγ-induced transcription factor T-bet (encoded by TBX21). As with IECs, there was a clear dose-dependent effect observed in preventing the IFNγ-induced induction of all of those molecules (Fig. 3C). Again, energy drink application also reduced TNF-α-induced activation of NF-κB p65 but not Akt activation (Fig. 3D). Altogether, we observed comparable anti-inflammatory effects upon energy drink addition in THP-1, HT-29, and T84 cells, indicating that the effect of the energy drink was not limited to a specific cell type or signaling mechanism.

Energy Drink Ameliorates Intestinal Inflammation in a Mouse Colitis Model

Having shown a potent anti-inflammatory effect of energy drink in reducing IFNγ-induced barrier defects and inflammatory responses in vitro, we next investigated the effect of energy drink in the in vivo setting. For this aim, we induced acute colitis by administration of 2.5% DSS in the drinking water with or without addition of the energy drink. Mice receiving energy drink only did not significantly differ from the water controls (H_2O), which is visible in similar weight curves, colonoscopy, and histology scores, MPO scores, and colon length, showing that supplementation of energy drink did not significantly affect gut physiology. Notably, energy drink supplementation increased the overall activity of the mice (both in water control and DSS-treated mice), whereas we did not observe effects on the sleeping pattern. As expected, we observed weight loss and diarrhea in all DSS-treated mice (Fig. 4A), an effect significantly less pronounced in the mice receiving DSS + energy drink (Fig. 4A).

Similar effects were observed when assessing colitis severity at the end of the experiment: endoscopy revealed bleeding, loose stool, loss of transparency, thickening of the colon wall, and abnormal vascularization patterns in DSS-only treated mice and histology demonstrated epithelial erosion and inflammatory infiltrates (Fig. 4B). These changes were clearly reduced in mice treated with DSS + energy drink, resulting in a lower total colonoscopy score (MEICS score, Fig. 4C), decreased immune cell infiltration, and reduced epithelial erosion in DSS + energy drink compared with DSS-only treated mice (Fig. 4D), indicating attenuated intestinal inflammation. Further, we detected a trend toward reduced myeloperoxidase levels in the distal colon upon energy drink treatment (Fig. 4E), which is a good approximation for estimating the amount neutrophil granulocyte infiltration and activity. The strong inflammation during experimental colitis resulted in shortening of the colon; however, despite the overall anti-inflammatory effect, DSS-induced colon shortening was not altered upon energy drink treatment (Fig. 4F), and spleen weight was elevated in the DSS + energy drink group compared with the DSS-only group (Fig. 4G). Furthermore, energy drink application significantly reduced DSS-induced increase in FD4 permeability (Fig. 4H).

Western Blot pictures for pSTAT1, pSTAT3, STAT1, STAT3 and for the regulatory protein PTPN2. B, Densitometry analyses for phosphorylated and total STAT1 and STAT3 and for PTPN2. C, mRNA expression of SOCS1, SOCS3, and IFNγ receptor (IFNGR) normalized to *ACTB1*. D, mRNA expression of IL-6, ICAM-1, and TNF-α normalized to *ACTB*. E, HT-29 cells were treated with the indicated dilutions of the energy drink (Control/Ctr, 1:5, 1:10; 1:50) and stimulated with TNF-α (TNF) for 30 minutes. The pictures show representative Western Blot pictures for pNF-kB p65, pAkt, total p65, and total Akt and the respective densitometry analyses. Asterisks indicate significant differences (n = 3 separate experiments per condition, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; 1-way ANOVA, post hoc Tukey multiple comparisons test).

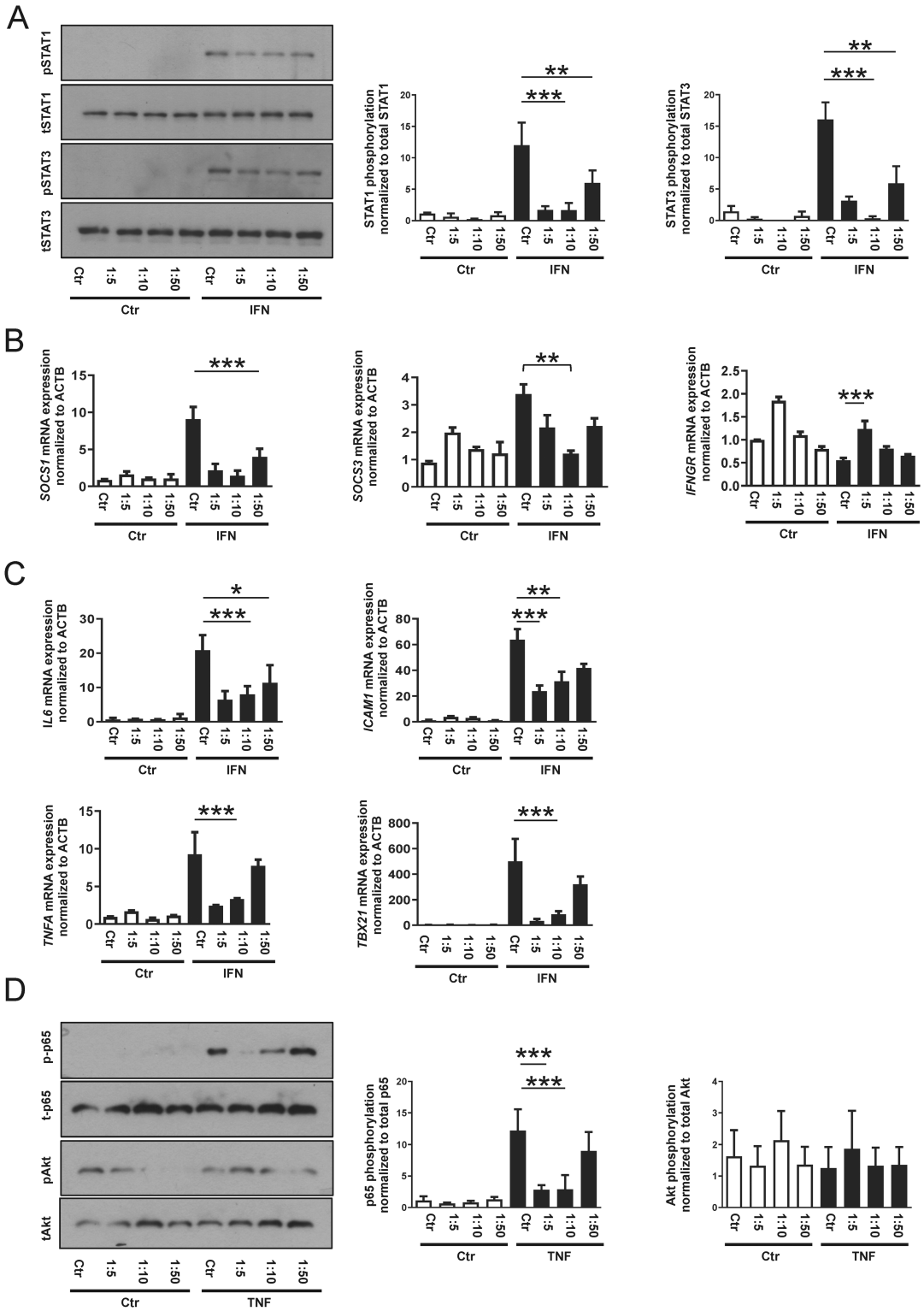

FIGURE 3. Reduced SOCS1 and SOCS3 expression in IFNγ-stimulated THP-1 cells upon addition of energy drink. THP-1 cells treated with different dilutions of the energy drink (Ctr, 1:5, 1:10; 1:50) and stimulated with IFNγ (A–C) or TNF-α (D) for 30 minutes (A, D) or 24 hours (B, C). A, Representative pictures of Western Blot and respective densitometry analyses for phosphorylated and total STAT1 and STAT3. B, mRNA expression of the regulatory proteins SOCS1 and SOCS3, IFNγ receptor and (C) the inflammatory molecules IL6, ICAM1, TNFA, and TBX21 normalized to *ACTB*. D, Representative Western Blot pictures and respective densitometry for phosphorylated and total NF-kB p65 and Akt. Asterisks indicate significant differences (n = 3 separate experiments per condition, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; 1-way ANOVA, post hoc Tukey multiple comparisons test).

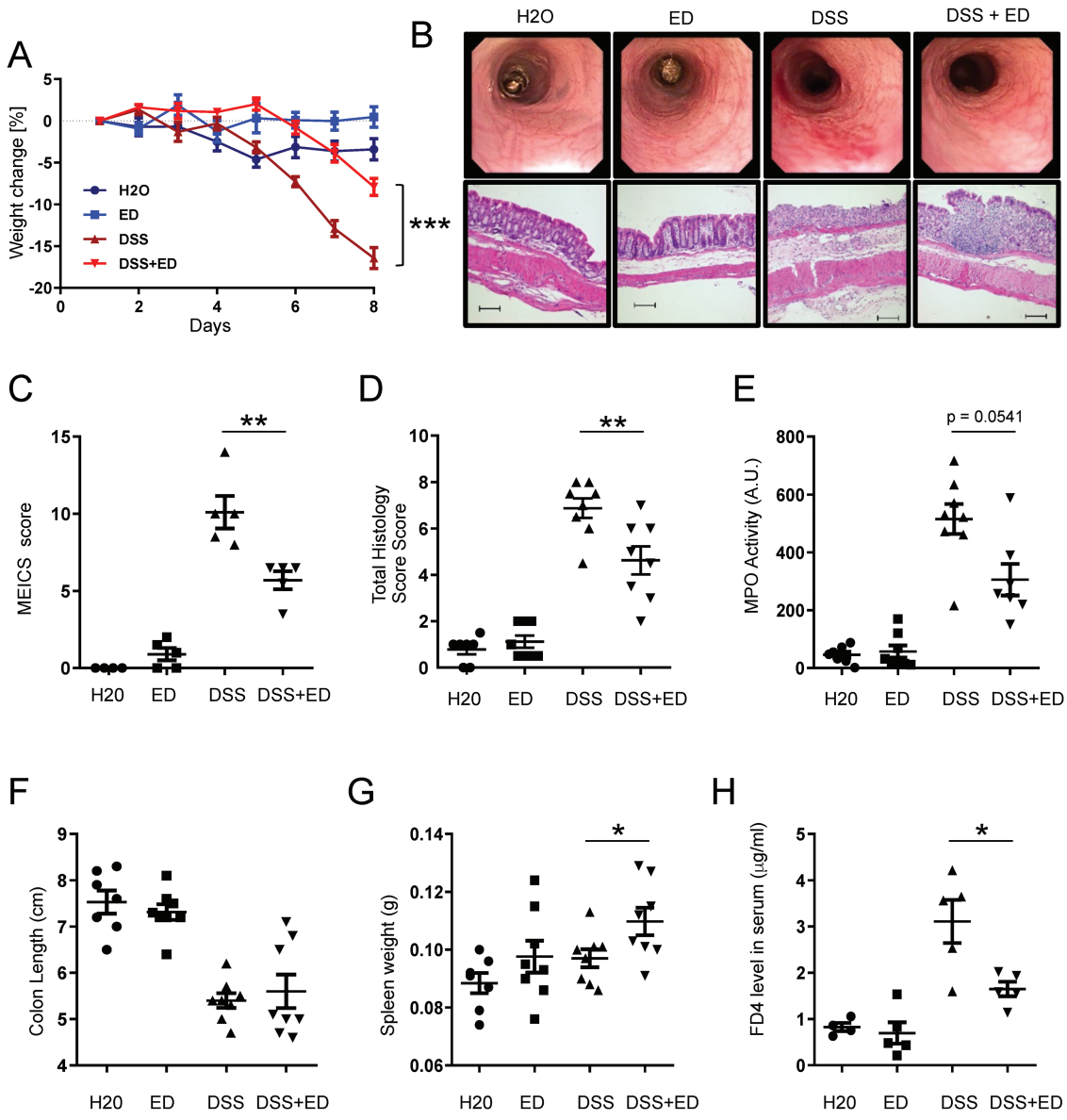

FIGURE 4. Reduced weight change, colonoscopy, and histology score in energy drink–treated mice. C57BL/6 mice were randomly distributed into 4 groups (n = 7 mice in H2O group and 8 mice in the rest of the groups) and treated with a combination of water, energy drink, DSS, or DSS + energy drink for 7 days. Shown are (A) percentage weight change, (B) representative colonoscopy and histology pictures of terminal colon, (C) colonoscopy score, (D) total histology score, (E) myeloperoxidase (MPO) activity, (F) colon length, and (G) spleen weight. H, Mice were orally gavaged with 80 mg/mL of 4kDa Fitc-Dextran (FD4) and serum levels of FD4 analyzed 5 hours later. Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$; Mann-Whitney *U* test). Scale bar delineates 100 µm.

Taken together, energy drink supplementation clearly reduced disease severity in a DSS-induced acute colitis mouse model.

Reduction of Claudin-2 in Mice Treated With Energy Drink

To assess whether the energy drink had similar effects on epithelial permeability and the expression of tight junction proteins as observed in our in vitro cell culture model, we analyzed the expression of occludin and claudin-2 in the whole colon tissue from DSS and DSS + energy drink–treated mice. Dextran sodium sulfate treatment alone had no impact on protein levels of claudin-2 or occludin. However, in line with reduced epithelial permeability observed in IFNγ-stimulated HT-29 cells upon energy drink treatment, there was a clear trend toward reduced protein expression of claudin-2 in the DSS + energy drink group, although this did not reach statistical significance, whereas occludin expression was unaltered (Fig. 5A, Supplementary Fig. 3).

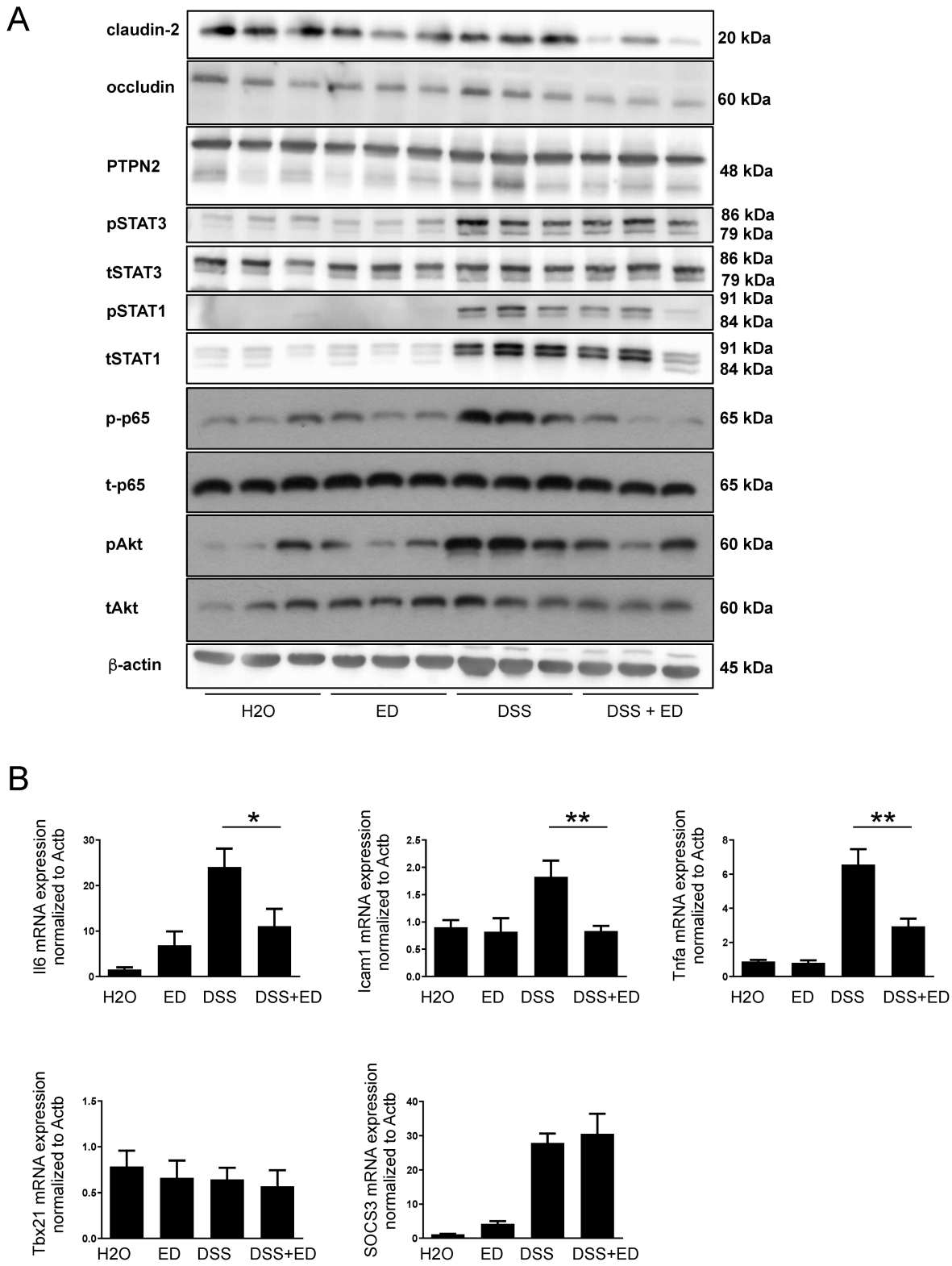

FIGURE 5. Reduced NF-kB p65 activation and trend toward reduced claudin-2 expression upon DSS-induced colitis in energy drink-treated mice. Colitis was induced in C57BL/6 mice as in in Figure 3. A, Representative Western Blot analysis of the tight junction proteins occludin and claudin-2 and the phosphorylated and total forms of STAT1 and STAT3 and expression levels of PTPN2. B, mRNA analysis of Il-6, ICAM1, TNF-α, TBX21, and SOCS3 normalized to *ACTB*. Asterisks indicate significant differences (n = 7 mice in H2O group and 8 mice in the rest of the groups; *$P < 0.05$ and **$P < 0.01$; Mann-Whitney *U* test).

Reduced Molecular Inflammatory Response in Mice Treated With Energy Drink

To elucidate a possible involvement of IFNγ-induced transcription factors and the JAK-STAT-pathway in energy drink–mediated effects, we performed Western Blot analysis of the phosphorylated and total forms of STAT1 and STAT3. The phosphorylated (active) form of STAT1 showed a trend toward decreased expression in the DSS + energy drink group, whereas STAT3 remained the same when compared with mice treated with DSS only (Fig. 5A). Furthermore and in line with our cell culture data, energy drink administration reduced DSS-mediated increase of NF-kB p65 phosphorylation (Fig. 5A, Supplementary Fig. 3). In addition, DSS-induced Akt activation was reduced upon energy drink coadministration (Fig. 5A, Supplementary Fig. 3). Of note, energy drink treatment did not result in a difference in the expression of the 2 important negative regulators of JAK-STAT signaling, SOCS3 (Fig. 5B) and PTPN2 (Fig. 5A). Consistent with our findings in the culture experiments, mRNA expression of Il-6, ICAM1, and TNF-α were decreased in the DSS + energy drink group compared with the DSS only group, whereas TBX21 (encoding T-bet) did not differ between any of the groups (Fig. 5B). Though energy drink administration did not affect the abundance of macrophages in the intestine, it prevented the DSS-induced reduction of alternatively activated (CD206+) macrophages (Fig. 6).

Our results indicate that administration of the energy drink ameliorated inflammation-induced intestinal permeability defects and suppressed the inflammatory response. These effects were not only visible in cell lines but also observed in DSS-treated mice that received the energy drink.

DISCUSSION

In our study, we investigated the effect of a typical energy drink as a component of a Western diet on intestinal barrier integrity and intestinal inflammation in vitro and in vivo. In contrast to our expectation, we found that addition of the energy drink reduced epithelial barrier defects and secretion of inflammatory cytokines upon IFNγ- and TNF-α treatment in vitro and ameliorated the severity of DSS-induced colitis in vivo. These data are somewhat contradictory to the wide-spread assumption that energy drinks would be rather unhealthy, particularly due to the high content of refined sugar and other ingredients that contribute to their advertised effects. However, energy drinks also contain a significant amount of caffeine and taurine, 2 compounds that have been shown to exert beneficial roles in experimental colitis.[10–12]

Caffeine, a central nervous system stimulant derived from coffee beans, is mostly known for its effect to reduce fatigue, prevent sleep, and increase cognitive functions.[21] However, it has also been associated with anti-inflammatory, antiapoptotic, antioxidant, and antimutagenic effects.[22] Previous studies have shown that caffeine consumption has a protective effect in ulcerative colitis patients.[5] Additionally, intestinal epithelial cells treated with caffeine showed decreased levels of chitinase 3-like-1 (CHI3L1), a glycoprotein with increased expression in the intestine of IBD patients that facilitates bacterial attachment and invasion.[12] In different mouse models, oral caffeine administration has been shown to ameliorate colitis severity, reduce bacterial translocation, and decrease tumor incidence by reducing oxidative DNA damage.[23] Thus, the unexpected beneficial effects of the energy drink might partially be mediated via its high caffeine content.

Further, taurine (2-aminoethanesulfonic acid), another prominent ingredient of energy drinks, is involved in calcium homeostasis, osmoregulation, and bile acid conjugation and acts as an antioxidant, antiapoptotic, and anti-inflammatory agent.[24] During inflammation, taurine exerts an inhibitory effect on pro-inflammatory mediators such as nitric oxide, TNF-α, interleukins, and prostaglandins.[24, 25] Thus, the high amounts of taurine might also contribute to the observed anti-inflammatory effects of the energy drink in our experimental settings. Nevertheless, additional studies (eg, using caffeine or taurine-depleted energy drink) would be necessary to delineate the exact molecular mechanism of how energy drink supplementation affects intestinal inflammation.

To prevent unlimited passage of luminal contents across the epithelial barrier, IECs are connected by tight junctions, which are multiprotein structures located at their apical side.[26] The primary proteins forming tight junctions include junctional adhesion molecules (JAMs), occludin and claudins.[16, 26] Addition of IFNγ to IEC monolayers disrupts the organization of tight junctions via promoting the expression of the pore-forming molecule claudin-2 and internalization of barrier-sealing molecules such as occludin.[27–29] Here, we demonstrated that energy drink supplementation reduced the effects of IFNγ, preventing epithelial barrier damage. This was of clear physiological relevance, as in mice that were subjected to DSS—which induces severe colitis by detrimental barrier disruption[30]—energy drink supplementation attenuated weight loss and protected the epithelial barrier, which translated in reduced histological colitis scores. It must be mentioned that energy drink administration reduced claudin-2 levels below baseline levels because DSS treatment did not cause an increase in claudin-2. Of interest, Zhao et al and Lee et al observed similar effects when treating mice either with taurine or caffeine in a DSS-induced acute colitis model.[11, 12] This indicates that the anti-inflammatory effects we observed upon energy drink administration might be a consequence of high levels of caffeine and taurine in the energy drink.

On a molecular level, the energy drink affected tight junction molecules and most prominently the expression of claudin-2. In healthy individuals, claudin-2 expression is typically restricted to the proximal nephron and the small intestine, with little or no expression in the other parts of the gastrointestinal tract.[28, 29] In IBD patients, however, claudin-2 is highly

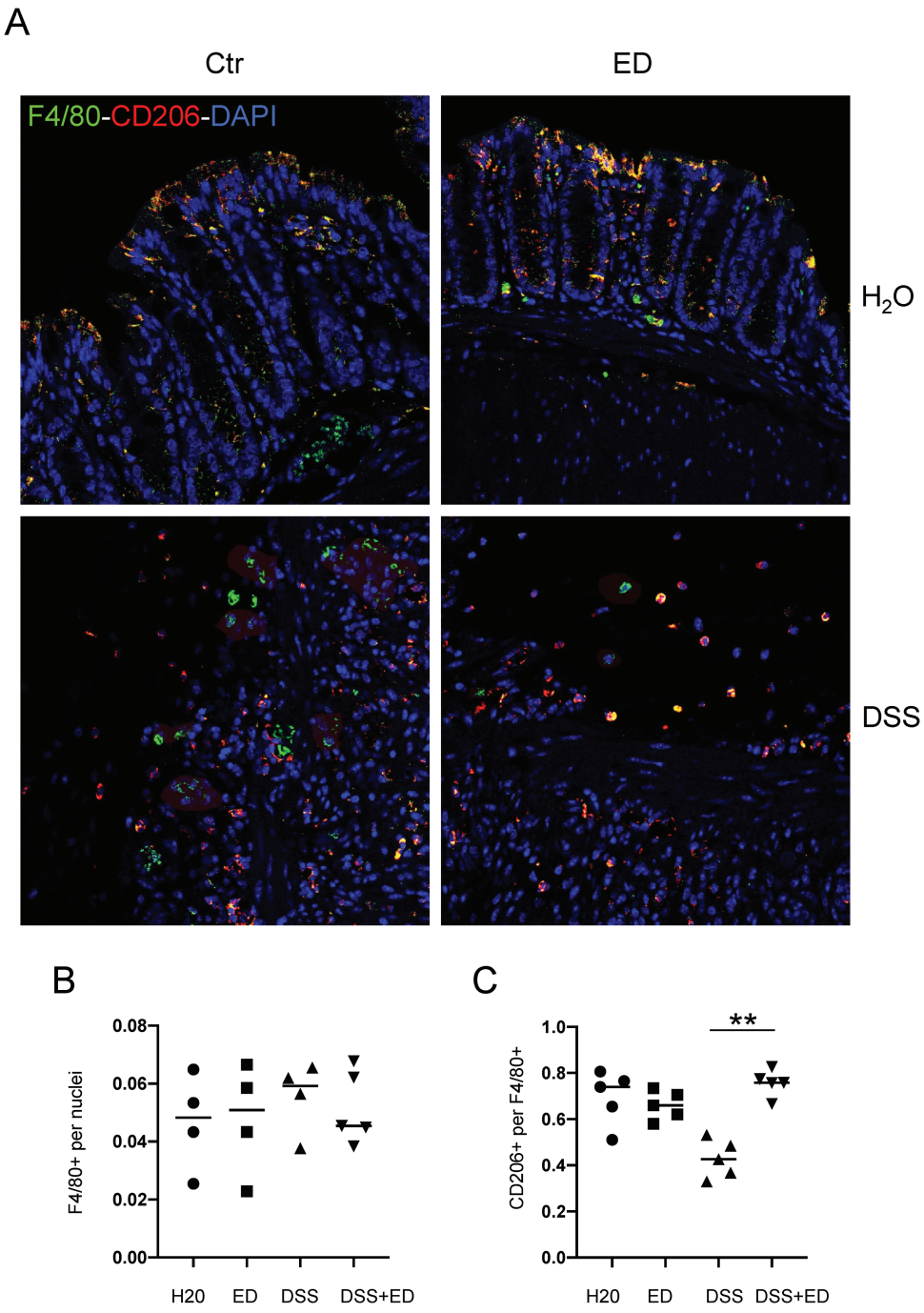

FIGURE 6. Energy drink supplementation prevents DSS-induced reduction in alternatively activated macrophages. Colitis was induced in C57BL/6 mice as in in Figure 3. A, Representative immunofluorescence images of the terminal colon, (B) number of F4/80+ cells normalized to the number of DAPI + nuclei, (C) number of CD206+ cells normalized to F4/80+ cells. Asterisks indicate significant differences (n = 5 mice in all groups *$P < 0.005$; Mann-Whitney *U* test).

increased in the whole gastrointestinal tract, and its expression correlates positively with disease activity.[28, 31] Consistent with previous reports,[32] we found highly elevated claudin-2 expression upon IFNγ stimulation, an effect clearly reduced upon supplementation of the energy drink. In contrast, the energy drink promoted expression of the barrier-sealing molecule occludin. In IBD patients, expression and tight junction localization of occludin are disturbed, resulting in increased epithelial barrier permeability,[27] and the increased expression of occludin in energy drink–supplemented HT-29 and T84 cells might mediate

the reduction of IFNγ-induced 4kDa-FITC-dextran translocation. Pro-inflammatory cytokines, such as IL-6, IFNγ, and TNF-α, not only affect tight junction molecule expression, (ie, promote claudin-2 expression[28]) but also promote the expression of inflammatory cytokines themselves and upregulate the cell-adhesion molecule ICAM-1, which promotes inflammatory cell infiltration into the intestine. Addition of the energy drink reversed IFNγ-induced upregulation of these factors, indicating an anti-inflammatory effect. However, the effect on ICAM-1 is of interest because it has been demonstrated that upregulation of ICAM-1 is required for intestinal healing upon inflammatory insults.[33] Despite its clear anti-inflammatory effect during development of acute, epithelial damage–induced colitis, it might thus be of interest to assess whether energy drink has effect on gut healing in more chronic settings.

Furthermore, energy drink supplementation affected inflammatory markers in IECs and upregulation of the cell-adhesion molecule ICAM-1 that facilitates infiltration of immune cells, and it reduced the expression of inflammatory markers in THP-1 monocytes, indicating a direct effect on IECs and infiltrating immune cells. Of interest, energy drink–treated THP-1 cells expressed increased levels of IFNγ receptor mRNA, suggesting an enhanced ability to respond to IFNγ. Nevertheless, monocytes treated with energy drink showed reduced activation of IFNγ-induced signaling cascades, indicating that the effect of energy drink is downstream of the receptor and not via affecting IFNγ receptor expression. Thus, increased expression of IFNγ receptor might be the result of compensatory effects to overcome reduced IFNγ signaling. Somewhat surprising, energy drink–treated mice showed clearly reduced levels of inflammatory markers, and they presented with slightly elevated spleen weight. Inasmuch as spleen weight tends to increase upon presence of ongoing inflammatory responses, the increase in spleen weight cannot be explained with our data and clearly warrants further investigation. In line with the anti-inflammatory effect of energy drink, energy drink–treated monocytes expressed reduced levels of IL-6 and TNF-a, and levels of CD206 expressing macrophages were higher in the intestines of DSS + energy drink–treated mice when compared with DSS-treated animals. This clearly indicates that energy drink supplementation affects intestinal inflammation in multiple ways, from amelioration of barrier functions to affecting immune cell infiltration and prevention of pro-inflammatory differentiation.

CONCLUSION

In conclusion, we investigated the effect of an energy drink on IFNγ-induced inflammatory responses in vitro and in an acute, DSS-induced in vivo colitis mouse model. Counterintuitively, the energy drink exerted beneficial effects on intestinal epithelial barrier function, possibly due to the high levels of taurine and/or caffeine. Our results strongly indicate that those beneficial effects were mediated by regulating claudin-2 expression. In addition to this barrier protective effect, the energy drink normalized expression of pro-inflammatory mediators (ie, IL-6, ICAM-1, and TNF-α) in vitro and in vivo, indicating decreased inflammatory responses. However, these effects are observed under experimental conditions and without taking into consideration additional effects of energy drink consumption (eg on carbohydrate or lipid metabolism). Notably in vivo, the control groups were supplemented with the same amount of sugars as found in the energy drink, thus our study neglects effects of refined sugars during the development of intestinal inflammation. Furthermore, the amount of energy drink was rather high and might not entirely reflect the typical energy drink ingestion of people. Thus, the here-demonstrated beneficial effects of energy drink supplementation have to be considered with great caution. Nevertheless, we can clearly conclude that energy drink consumption does not have detrimental effects on the intestinal epithelial barrier during the development of colitis and that energy drink consumption does likely not contribute to the development of intestinal inflammation.

SUPPLEMENTARY DATA

Supplementary data is available at *Inflammatory Bowel Diseases* online.

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