Dietary Magnesium Alleviates Experimental Murine Colitis Through Upregulation of the Transient Receptor Potential Melastatin 6 Channel

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Background: Magnesium (Mg) is essential for human health and is absorbed mainly in the intestine. In view of the likely occurrence of an Mg deficit in inflammatory bowel disease (IBD) and the documented role of Mg in modulating inflammation, the present study addresses whether Mg availability can affect the onset and progression of intestinal inflammation.

Methods: To study the correlation between Mg status and disease activity, we measured magnesemia by atomic absorption spectroscopy in a cohort of IBD patients. The effects of dietary Mg modulation were assessed in a murine model of dextran sodium sulfate (DSS)–induced colitis by monitoring magnesemia, weight, fecal occult blood, diarrhea, colon length, and histology. Expression of the transient receptor potential melastatin (TRPM) 6 channel was assessed by real-time reverse transcription polymerase chain reaction and immunohistochemistry in murine colon tissues. The effect of Mg on epithelial barrier formation/repair was evaluated in human colon cell lines.

Results: Inflammatory bowel disease patients presented with a substantial Mg deficit, and serum Mg levels were inversely correlated with disease activity. In mice, an Mg-deficient diet caused hypomagnesemia and aggravated DSS-induced colitis. Colitis severely compromised intestinal Mg^{2+} absorption due to mucosal damage and reduction in TRPM6 expression, but Mg supplementation resulted in better restoration of mucosal integrity and channel expression.

Conclusions: Our results highlight the importance of evaluating and correcting magnesemia in IBD patients. The murine model suggests that Mg supplementation may represent a safe and cost-effective strategy to reduce inflammation and restore normal mucosal function.

Key Words: colon, dextran sodium sulfate, hypomagnesemia, inflammatory bowel disease, tumor necrosis factor–α

INTRODUCTION

The pathogenesis of inflammatory bowel disease (IBD) results from the interplay of multiple factors, among which genetic predisposition, the gut microbiome, the immune response, and the environment play a leading role.[1] The contribution of environmental factors, diet foremost, is supported by the globally growing incidence rate, in parallel with industrialization and urbanization.[2] Clinical studies ascertained frequent multifactorial nutritional deficiencies in IBD patients and demonstrated the efficacy of nutrition therapy either as sole therapy or as adjunctive therapy.[3] The main determinants of specific nutrient deficits include disease location and extension, disease activity, alimentation, nutritional support, and medications used for IBD.[4] Most common are micronutrient and vitamin deficiencies, which are associated with prolonged and complicated course of disease.[5] Few studies have documented a deficit of magnesium (Mg; Mg refers to both bound and free forms of magnesium, whereas Mg^{2+} refers to the ionized form), especially in active Crohn's disease (CD) patients.[6]

Mg is essential for human health, as it is involved in virtually every cell function, including enzymatic processes, phosphorylations, energy homeostasis, protein synthesis, and DNA stability.[7] Disturbances of Mg homeostasis seem to underlie the pathophysiology of a variety of diseases.[8] In particular, Mg deficiency has been associated with the development and maintenance of diverse inflammation-driven chronic conditions, such as diabetes, metabolic syndrome, cardiovascular disease, and cancer.[9, 10] Both deficient Mg intakes (<250 mg/d)

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and reduced serum Mg concentrations (≤0.75 mM) have been linked to elevated serum C-reactive protein levels.[9] Vice versa, a recent meta-analysis indicated that Mg supplementation reduces CRP levels among individuals with inflammation.[11] These findings strongly suggest that even a subclinical Mg deficiency may predispose to chronic inflammatory stress and that Mg supplements may have a beneficial role as an adjuvant for the management of low-grade chronic systemic inflammation.[9]

Systemic Mg homeostasis primarily depends on the collaborative actions of the intestine, responsible for Mg uptake from food, and the kidneys, regulating urinary Mg excretion.[8] Mg is absorbed in the gut through 2 separate pathways: paracellular transport, which is driven by the electrochemical gradient, is responsible for bulk Mg^{2+} absorption, and takes place mostly in the small intestine, whereas fine-tuning occurs in the cecum and colon via transcellular transport.[8] Transient receptor potential melastatin (TRPM) 6 and 7 cation channels mediate Mg^{2+} transcellular transport on the luminal side of the enterocytes. Although TRPM7 is ubiquitously expressed, TRPM6 expression is restricted to specific tissues; the highest levels are found in the distal small intestine and colon, in murine and human tissues.[12, 13] The latest findings have demonstrated that TRPM6 is required in the intestine to maintain systemic Mg^{2+} balance[14] and that TRPM6 function cannot be replaced by other ion channels, including the highly homologous TRPM7 channel.[15] Dietary Mg intake is known to affect intestinal Mg^{2+} absorption, largely by increasing paracellular transport.[12, 13, 16, 17]

The documented role of Mg in modulating inflammation and the likely occurrence of this nutritional deficit in IBD patients prompted us to investigate whether Mg availability can affect the onset and progression of intestinal inflammation. In this paper, we tested the following questions: (1) Is magnesemia significantly altered in IBD patients? (2) Can dietary Mg influence the activity of dextran sodium sulfate (DSS)–induced colitis in a murine model? (3) Does inflammation affect the expression of TRPM6/7 channels? Vice versa, does TRPM6/7 expression play a role in modulating inflammation? Our results show that Mg deficiency aggravates the activity of the disease, and that Mg supplementation can be an effective strategy to protect the colonic mucosa by promoting its healing and restoring its physiological function.

METHODS

Selection of Patients

This project was approved by the internal Ethics Committee at Università Cattolica del Sacro Cuore, Rome (protocol number P/491/CE/2011), and all participants signed an informed consent form. Thirty patients with a well-established diagnosis of CD (13 subjects) or ulcerative colitis (UC; 17 subjects) were enrolled together with 26 healthy controls. All patients were treated at the outpatient clinic at the "Agostino Gemelli" University Hospital, and their clinical disease activity was calculated according to the Harvey-Bradshaw index (HBI)[18] or the Colitis Disease Activity Index (CDAI)[19] for CD or UC patients, respectively. Table 1 reports demographics and main characteristics for all subjects at the time of enrollment. Patients were considered in an active stage of disease with HBI ≥5 or CDAI >3, or in remission otherwise. For each patient, blood samples were collected at 2 different time points, that

TABLE 1: Demographics and Main Baseline Characteristics of Enrolled Subjects

	Active Phase[a] (n = 18)	Remission[b] (n = 12)	Healthy (n = 26)
Sex (male/female)	12/6	3/9	15/11
Age, mean (range), y	44 (22–82)	40 (21–60)	33.8 (24–61)
Disease (UC/CD)	12/6	5/7	NA
Disease duration, mean (range), y	5.03 (0.4–16)	5.95 (1–23)	NA
HBI, mean (range)	9.5 (5–15)	2.7 (1–4)	NA
CDAI, mean (range)	7.6 (4–10)	2.2 (1–3)	NA
CRP, mean (range), mg/L	22.3 (3–67.1)	5.6 (0.5–12)	NA
Disease location			
Jejunum	3	1	NA
Ileum	5	6	NA
Right colon	7	4	NA
Left colon	8	4	NA
Rectum	8	2	NA

[a]Classified by HBI ≥5 or a CDAI >3 for CD or UC patients, respectively.
[b]Classified by HBI <5 or CDAI ≤3 for CD or UC patients, respectively.

is, upon enrollment and during a follow-up visit, when disease activity was reevaluated.

Animal Model

All procedures involving animals and their care conformed to the Directive 2010/63/EU of the European Parliament. The studies were approved by the Italian Ministry of Health, and by the internal Ethics Committee for Animal Research Studies at Università Cattolica del Sacro Cuore, Rome (protocol number SF46350/13(NN42)). Female 7- or 8-week-old C57BL/6 mice (Charles River Laboratories Italia) were housed in a controlled environment (23°C, 12-hour/12-hour light/dark, 50% humidity, ad libitum access to food and water). After a 2-week acclimation period, mice were randomized into 3 groups and fed a semipurified, mineral-adjusted diet containing different amounts of Mg: Hypo-Mg diet (30 mg/kg Mg), CTRL diet (1000 mg/kg Mg), and hyper-Mg diet (4000 mg/kg Mg), as verified by atomic absorption spectroscopy (see Table 2 for detailed diet composition). Experimental colitis was induced in each diet group following a well-established protocol.[20, 21] Briefly, 2.5% w/v DSS (36–44 kDa molecular weight, MP Biomedicals) was dissolved in de-ionized water and administered ad libitum in water bottles from day 0 until day 5. At day 5, half of the untreated and DSS-treated animals in each diet group were killed (acute phase). From day 5, all the remaining mice received de-ionized water without DSS to allow recovery until death at day 12 (recovery phase). Eighty mice were used overall; the full experimental design and group composition are reported in Supplementary Figure 1. For each animal, weight, fecal consistency, and fecal occult blood were evaluated daily, and a disease activity index (DAI) was derived as reported.[22] On the day of killing, animals were anesthetized by intraperitoneal injection, and blood was collected by puncturing the heart. Mice were then killed through cervical dislocation. The colon and kidneys were excised from the ileo-cecal junction to the proximal rectum, bisected longitudinally, and processed for histological analysis and mRNA analysis. Before bisection, the entire colon length was recorded.

TABLE 2: Composition of Semipurified Mg-Adjusted Diets[a]

	Hypo-Mg,[b] g/kg	CTRL,[c] g/kg	Hyper-Mg,[d] g/kg
Casein	200	200	200
Wheat starch	650	650	650
Fiber alphacel	50	50	50
Corn oil	50	50	50
Mineral mix[e]	35	35	35
Vitamin mix[f]	10	10	10
DL-methionine	3	3	3
Choline bitartrate	2	2	2
MgO	-	1.67	6.68

[a]Final Mg content in each diet was determined by atomic absorption spectroscopy.
[b]Containing 30 mg/kg Mg.
[c]Containing 1 g/kg Mg.
[d]Containing 4 g/kg Mg.
[e]Mineral mix composition (g/kg): calcium phosphate dibasic 500, sodium chloride 74, potassium citrate monohydrate 220, potassium sulfate 52, manganese carbonate 3.5, ferric citrate 6, zinc carbonate 0.53, cupric carbonate 0.1, sodium selenite 0.0033, chromium potassium sulfate 0.55, sucrose 135.3.
[f]Vitamin mix composition (mg/kg): thiamine hydrochloride 600, riboflavin 600, pyridoxine hydrochloride 700, nicotinic acid 3000, D-calcium panthothenate 1600, folic acid 200, D-biotin 20, cyanocobalamin (vitamin B_{12}) 1, retinyl palmitate (vitamin A) premix 1600, DL-α-tocopherol-calcium acetate 20,000, cholecalciferol (vitamin D_3) 250, menaquinone (vitamin K_2) 50, sucrose, finely powdered, 972.9 g.

Atomic Absorption Measurements

After collection, blood was centrifuged at 1500*g* for 15 minutes, and the supernatant (serum) was stored at −80°C until analysis. Ion extraction was performed by diluting serum 1:10 in 1 N HNO_3. Following incubation at room temperature overnight, samples were vortexed and centrifuged at 14,000 rpm for 5 minutes. Supernatants were further diluted (1:100) in 0.25 N HNO_3 + 0.5% $LaCl_3$ and analyzed at an AAnalyst 200 Atomic Absorption Spectrometer (Perkin Elmer). Each sample was measured in triplicate, and Mg^{2+} concentration was derived from a calibration curve obtained with Mg^{2+} concentration standards ranging from 10 to 100 µg/L.

Histological Examination

Colon tissue was fixed in 4% formalin, embedded in paraffin, and sliced into 3-mm-thick sections. Hematoxylin and eosin (H&E)–stained sections were assessed blindly on the basis of severity and extent of inflammation, and presence and extent of ulceration.[23] Scores ranged from 0 to 4 for each parameter; single values were summed up to obtain the overall histological score (maximum possible value, 16).

Immunohistochemical Analysis

Immunohistochemical staining was performed as previously described.[22, 24] Mouse monoclonal anti-TRPM7 (Novus Biologicals) and rabbit polyclonal anti-TRPM6 (Biorbyt) primary antibodies were used at a 1:1000 and 1:250 dilution, respectively. Photomicrographs were taken with 40× magnification on a Nikon E400 Eclipse microscope.

Real-time Reverse Transcription Polymerase Chain Reaction

Colon and kidney tissues for RNA analysis were snap-frozen and stored at –80°C until processed. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA quantitation and quality analysis were performed using a NanoDrop spectrophotometer (ThermoFisher Scientific). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied

Biosystems), following the manufacturer's instructions. Quantitative real-time reverse transcription polymerase chain reaction (PCR) was performed in duplicate using iQ SYBR Green Supermix (BioRad) on an iCycler iQ5 (BioRad) with the following scheme: an initial denaturation at 95°C for 3 minutes, 40 cycles of PCR amplification, each consisting of a denaturing step of 95°C for 10 seconds, annealing at 55°C for 30 seconds, and a final step at 72°C for 1 minute. The primer pairs used were for *TRPM6*: forward 5'-GCTCCTGTGGTGGTGAGAAT-3' and reverse 5'-CTGGAGTGCATCTGTGAGGG-3'; for *TRPM7*: forward 5'-GCTCCATGGGGAGTGATAGA-3' and reverse 5'-CTCAGTCTGACTTCTGCCCC-3';

for *β-actin*: forward 5'-TGTTACCAACGTGGACGACA-3' and reverse 5'-CTGGGTCATCTTTTCA-3'. *β-actin* was used as an endogenous reference gene to normalize the expression of *TRPM6* and *TRPM7*. Comparison between experimental groups was done using the $2^{-\Delta\Delta CT}$ method[25] and expressed as fold increase with respect to the untreated animals on the CTRL diet.

Cell Culture

Human colon carcinoma HT29 and HCT116 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, containing 0.8 mM $MgSO_4$) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and L-glutamine. Human colon carcinoma CaCo2 cells were grown in the same medium with 20% FBS. In given experiments, cells were grown in Mg-free DMEM (Invitrogen) supplemented as for routine culture plus the specified amount of $MgSO_4$ for 48 hours, and then treated with 25 ng/mL tumor necrosis factor (TNF)–α (Peprotech) for the indicated time.

Scratch Assay

CaCo2 cells were seeded in culture inserts (ibidi GmbH, 70,000 cells/insert well) and cultured for 24 hours to allow attachment. Culture media were changed to complete media containing the specified Mg concentration with or without TNF-α (25 ng/mL), and the inserts were removed, which created a 500-μm-wide cell-free gap between 2 confluent cell monolayers. Wound closure was monitored with a Nikon Eclipse TE2000-S microscope, and images were analyzed using ImageJ software (National Institutes of Health [NIH], http://imagej.nih.gov/ij/). Results were expressed as the percentage of the initial gap area that was covered by cells after 24 hours.

Immunoblot

Cells were lysed in RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.05% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (10 μg/mL leupeptin, 20 μg/mL aprotinin, 1 mM phenylmethanesulfonyl fluoride, 1 mM $NaVO_4$, 100 mM NaF). Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Cell extracts (50 μg) were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with rabbit monoclonal anti-TRPM7 (1:1000, Abcam), rabbit polyclonal anti-TRPM6 (1:500, Biorbyt), rabbit polyclonal anti-occludin (1:500, ThermoFisher), and rabbit polyclonal anti-actin (1:1000, Sigma-Aldrich) primary antibodies. Horseradish peroxidase–conjugated secondary antibodies (GE Healthcare) were detected by use of the ECL Prime Western Blotting Detection Reagent (GE Healthcare) and the ChemiDoc XRS system (Bio-Rad). Densitometric analysis was performed by using ImageJ software (NIH, http://imagej.nih.gov/ij/).

Statistical Analysis

In vitro experiments were repeated independently 3 times. GraphPad Prism, version 5.01, was used for all statistical analyses. The statistical significance of differences between data from the 2 groups was evaluated using an unpaired Student *t* test. Statistical significance between more than 2 groups was assessed by 1-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Spearman's correlation analysis was performed to determine the association between magnesemia and clinical indicators. Differences were considered statistically significant for a P value <0.05, and significance levels were assigned as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Activity of Disease Is Associated With Hypomagnesemia in IBD Patients

Table 1 shows demographics and main baseline characteristics of enrolled subjects. Atomic absorption spectroscopy was used to measure serum Mg^{2+} concentration. Overall, the mean magnesemia in IBD patients was significantly lower than the magnesemia in controls (0.80 ± 0.03 mM vs 0.94 ± 0.05 mM, respectively; $P < 0.05$, Student *t* test) (Fig. 1A). Both UC and CD patients had reduced serum Mg^{2+} levels (0.83 ± 0.04 mM and 0.76 ± 0.04 mM, respectively) in comparison with controls, and the difference reached statistical significance for CD patients ($P < 0.05$, Tukey's multiple comparison test) (Fig. 1B).

The average serum Mg^{2+} concentration in patients was above the threshold that defines clinical hypomagnesemia (0.70 mM). Upon stratification of patients according to their disease activity, we observed that magnesemia in remitting patients was significantly higher than in active patients (0.86 ± 0.03 mM vs 0.74 ± 0.03 mM, respectively) and was statistically similar to control magnesemia (Fig. 1C). Notably, 57% of patients in an active phase of the disease (85% of CD patients) were frankly hypomagnesemic (serum $[Mg^{2+}] <$ 0.70 mM); in 2 subjects, magnesemia was <0.50 mM, which corresponds to symptomatic hypomagnesemia. Pairing magnesemia values in acute or remitting phases for the same patient highlighted a striking inverse correlation between serum Mg^{2+} concentration and disease activity (Fig. 1D), which was further confirmed by a correlation analysis between magnesemia and clinical score (Spearman's correlation coefficient $r = -0.4766$;

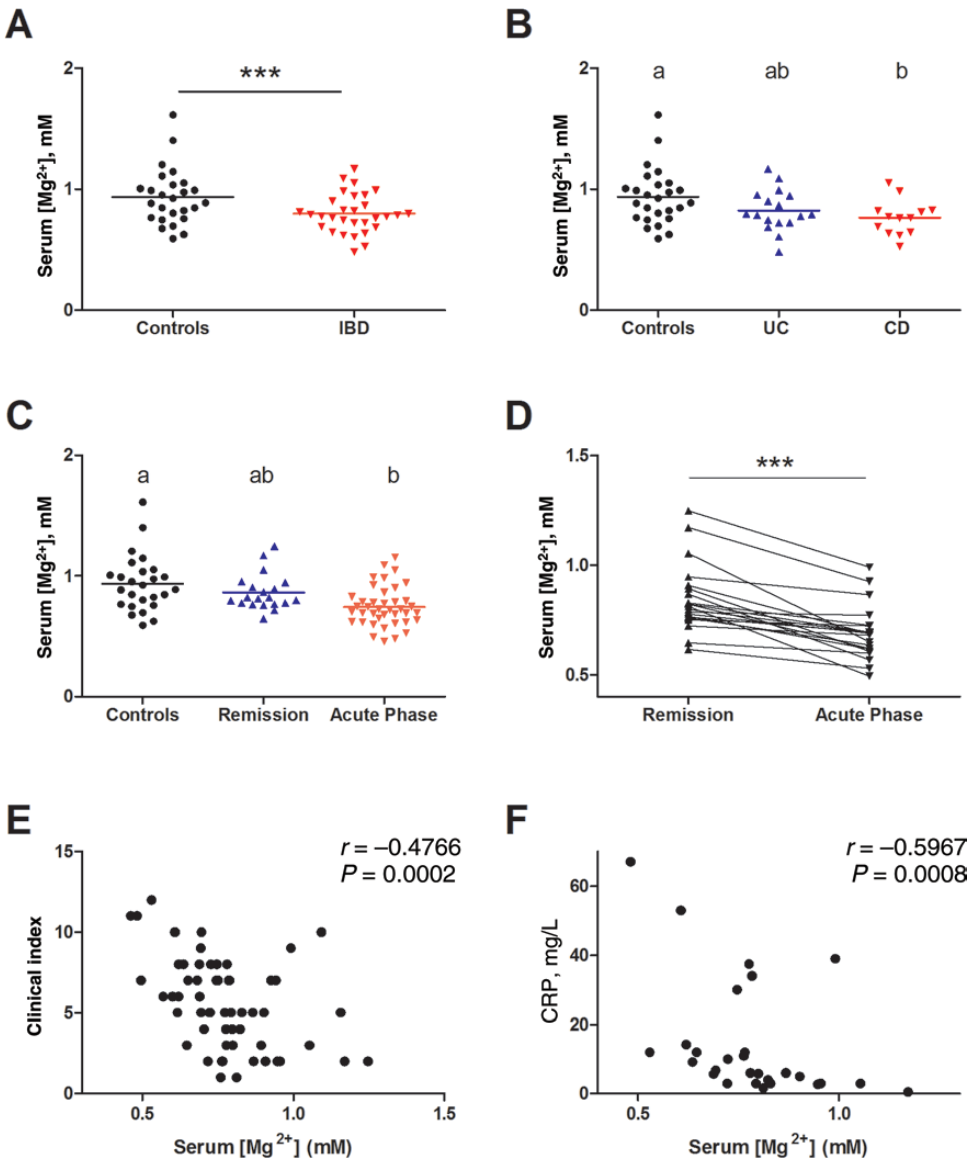

FIGURE 1. Activity of disease is associated with hypomagnesemia in IBD patients. A, Mean serum Mg^{2+} concentration in 26 healthy controls and 30 IBD patients. *$P < 0.05$ by unpaired Student *t* test. B, Mean serum Mg^{2+} concentration in UC vs CD patients. Letters indicate statistical significance by ANOVA, followed by Tukey's post hoc test; data sharing the same letter are not statistically different. C, Mean serum Mg^{2+} concentration after stratification of patients according to their clinical disease activity. Letters indicate statistical significance by ANOVA, followed by Tukey's post hoc test; data sharing the same letter are not statistically different. D, Mean serum Mg^{2+} concentration during remission or in an acute phase of the disease in the same patient; lines connect paired values; ***$P < 0.001$ by paired Student *t* test. E, Scatterplot showing the relationship between clinical index values and magnesemia assessed for each patient at 2 different time points. Spearman's correlation coefficient and *P* value are reported. F, Scatterplot showing the relationship between CRP values and magnesemia assessed for each patient at enrollment. Spearman's correlation coefficient and *P* value are reported. Serum Mg^{2+} concentration was measured by atomic absorption spectroscopy. Disease was considered active when HBI ≥5 or CDAI >3, for CD or UC, respectively.

$P < 0.001$) (Fig. 1E). Finally, we observed a significant correlation also between magnesemia and C-reactive protein (CRP) levels (Spearman correlation coefficient $r = –0.5967$; $P < 0.001$) (Fig. 1F). These findings indicate that hypomagnesemia commonly occurs in IBD patients, especially during active phases of the disease and in CD patients. Most importantly, we confirm that decreased serum Mg^{2+} levels are associated with an inflammatory state and show for the first time that they also correlate with exacerbation of disease.

Dietary Mg Alleviates DSS-Induced Murine Colitis

Colitis was induced by DSS administration in mice fed with a control, Mg-deficient, or Mg-enriched diet (CTRL,

hypo-Mg, or hyper-Mg groups, respectively) (Supplementary Fig. 1). During modeling, we observed no significant difference in body weight between CTRL and hyper-Mg mice, whereas hypo-Mg animals exhibited a trend toward reduced weight gain (Supplementary Fig. 2).

As expected,[12, 13, 16, 17] dietary Mg intake influenced serum Mg^{2+} levels. At day 5, hypo-Mg mice had significantly lower magnesemia than CTRL animals (0.77 ± 0.11 mM vs 1.64 ± 0.04 mM, respectively), whereas hyper-Mg mice displayed a slight increase in serum Mg^{2+} levels (1.77 ± 0.09 mM), which, however, did not reach statistical significance (Fig. 2A, left panel, solid bars). At day 12, magnesemia remained at approximately the same values for each diet group (Fig. 2A, compare solid bars in left vs right panel). In addition, we confirmed that colon inflammation affects systemic Mg^{2+} levels, as DSS-treated mice showed reduced magnesemia in comparison with untreated animals in all diet groups at day 5 (Fig. 2A, left panel, checkered vs solid bars). However, Mg supplementation resulted in serum Mg^{2+} values comparable to normal levels even in colitic animals, as magnesemia in DSS-treated hyper-Mg mice was not significantly different than in either untreated hyper-Mg or untreated CTRL mice at day 5 (Fig. 2A, left panel, checkered green vs solid green and black bars). After the recovery phase (day 12), all DSS-treated mice returned to serum Mg^{2+} levels comparable to untreated animals, but both control and treated hypo-Mg mice remained severely hypomagnesemic (Fig. 2A, right panel, checkered and solid red bars).

Dietary Mg content also modulated the course of experimental colitis. In DSS-treated hypo-Mg mice, weight loss was more pronounced (Fig. 2B, left panel) and DAI remained consistently higher than in CTRL animals at all time points (Fig. 2B, right panel). On the other hand, DSS-treated hyper-Mg animals had a more favorable clinical course, as demonstrated by their weight loss and DAI similar to CTRL animals. In particular, at the end of the experiment (day 12), hyper-Mg mice scored a significantly reduced DAI in comparison with hypo-Mg mice and, to a lesser extent, in comparison to CTRL mice (Fig. 2B, right panel). The exacerbation of acute disease caused by Mg deficiency was also apparent from the significant decrease in colon length in DSS-treated vs untreated hypo-Mg mice at day 5 (Fig. 2C, left panel). The recovery phase (day 12) evidenced that, in the longer term, dietary Mg content seemed to correlate with colon span, which measured significantly longer in hyper-Mg mice than in hypo-Mg mice (Fig. 2C, right panel). Finally, we examined the histological preservation of colon mucosa. H&E-stained colorectal sections showed that DSS caused the expected mucosal disruption in all diet groups, with distortion of crypts, loss of goblet cells, severe epithelial injury, and inflammatory cell infiltration in the mucosa and submucosa at day 5 (not shown). However, the recovery phase evidenced an exacerbating trend associated with the hypo-Mg diet, whereas control and hyper-Mg mice seem to display a better architecture of the glandular epithelium, as shown by the inflammatory score. Indeed, at day 12, although neither single nor total inflammatory scores reached statistical significance, hypo-Mg mice scored the highest index, whereas hyper-Mg mice showed a slightly lower score in comparison with CTRL animals (Fig. 2D).

Altogether, these results are consistent with the hypothesis that an Mg-deficient diet exacerbates colitis, whereas Mg supplementation, by keeping systemic Mg up to physiological levels, exerts a protective effect with regard to mucosal inflammation and overall activity of disease.

Experimental Colitis Modulates Expression of the TRPM6 Mg^{2+} Channel

We then analyzed murine colon tissues by real-time RT-PCR to evaluate whether dietary Mg content and/or experimental colitis could affect the expression of *TRPM6* and *TRPM7*. As a positive control, we assessed *TRPM6* expression in the kidney of all diet groups. As expected,[8] dietary Mg content strongly modulated *TRPM6* expression in kidney tissues: Mg-deficient diet upregulated *TRPM6* expression, whereas Mg-enriched diet downregulated *TRPM6* expression. This trend started to appear at day 5 and became statistically significant at day 12 (Supplementary Fig. 3). Renal *TRPM6* mRNA expression in DSS-treated mice did not significantly differ from their diet-matched controls, either at day 5 or day 12. In the colon, diet alone did not seem to significantly alter *TRPM6* mRNA levels at either day 5 or day 12 (Fig. 3A, B). On the other hand, DSS acute treatment showed a trend toward diminished colon *TRPM6* expression in both CTRL and hypo-Mg mice, as opposed to hyper-Mg animals (day 5) (Fig. 3C). The recovery phase confirmed this trend and evidenced a striking increase in colon *TRPM6* expression in hyper-Mg mice (day 12, $P < 0.05$ hyper-Mg vs CTRL and hypo-Mg, Tukey's post hoc test) (Fig. 3D). *TRPM7* expression was not significantly altered by either diet or DSS treatment (not shown).

Next, we studied the localization and abundance of the TRPM6 protein by immunohistochemistry (Fig. 4). TRPM6 expression was evident mainly in the apical part of the colon epithelium in CTRL mice, and diet did not significantly modify expression levels, in accordance with real-time RT-PCR analysis. DSS-induced colitis caused a marked decrease in TRPM6 expression in all diet groups, mainly due to massive mucosal damage visible at day 5; however, hyper-Mg mice maintained patches of well-preserved mucosal staining. As shown in photomicrographs at day 12, the recovery phase allowed restoration of mucosal architecture in all group diets, but recovery of TRPM6 expression was visible only in CTRL and hyper-Mg mice.

Mg Improves Healing of the Intestinal Epithelium

Our in vivo data showed that Mg supplementation contributes to attenuating DSS-induced colitis, which causes

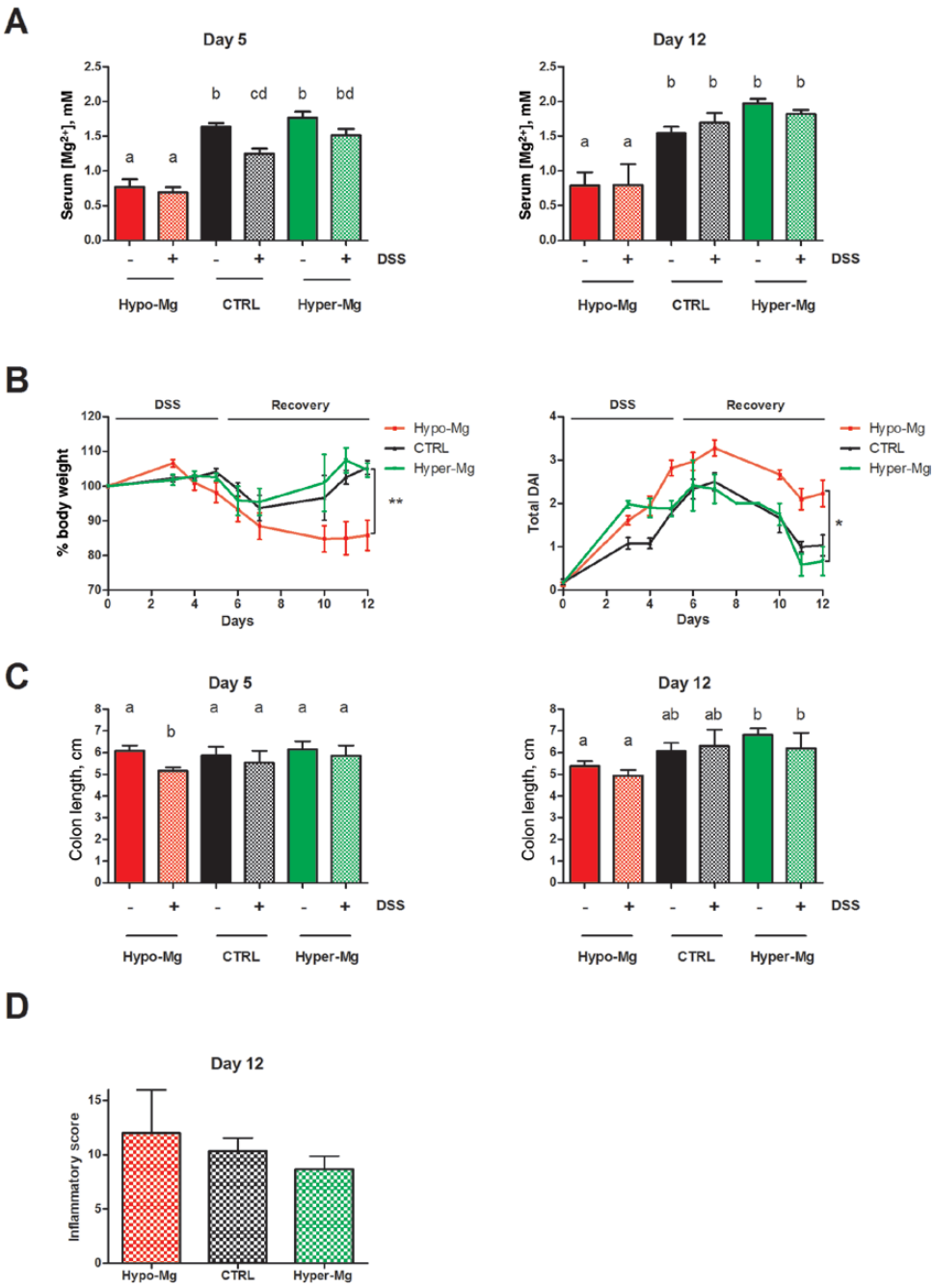

FIGURE 2. Dietary Mg alleviates experimental murine colitis. Mice were fed an Mg-deficient (red), normal (black), or Mg-enriched (green) diet, and colitis was induced by 2.5% DSS administration for 5 days, followed by a recovery period until day 12. A, Mean serum Mg^{2+} concentration, as measured by atomic absorption spectroscopy in control (solid bars) and DSS-treated (checkered bars) mice on the 3 diets at days 5 (left panel) and 12 (right panel). B, Time course of relative weight (left) and disease activity index (right) in DSS-treated mice on the 3 Mg-adjusted diets. Statistical significance at the end of the experiment (day 12) is reported. C, Colon length after the acute phase (day 5, left panel) and after the recovery period (day 12, right panel). D, Inflammatory score, reflecting inflammation grade and extension, and ulcer number and depth, in DSS-treated mice at the end of the experiment (day 12). Statistical significance was assessed by 1-way ANOVA, followed by Tukey's post hoc test. Data sharing the same letter are not statistically different; $^{*}P < 0.05$; $^{**}P < 0.01$.

nonspecific damage to the epithelium. We sought to determine whether this might be due to an effect of Mg on mucosal barrier repair. Therefore, we performed a scratch assay on human colon CaCo2 cells to assess their wound-healing capacity in conditions of different Mg availability, and upon exposure to TNF-α, a pivotal pro-inflammatory cytokine implicated in the

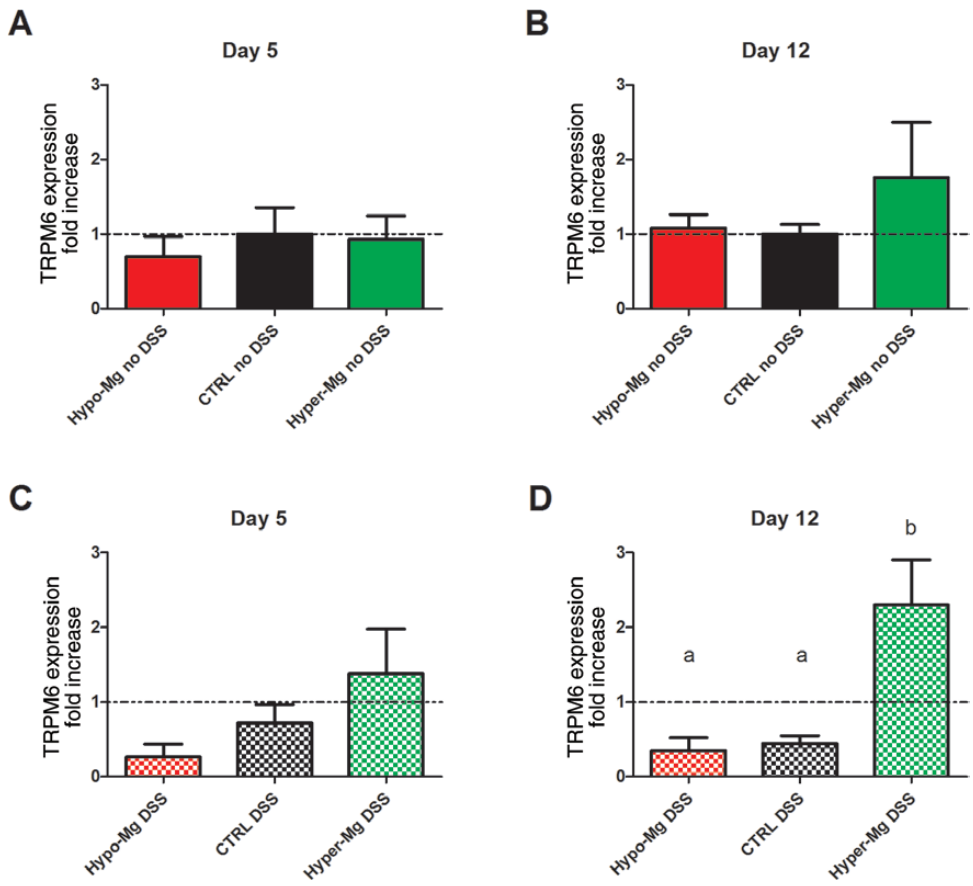

FIGURE 3. Experimental colitis modulates *TRPM6* mRNA expression. Real time RT-PCR was used to analyze *TRPM6* expression in colon tissues from untreated (solid bars) and DSS-treated (checkered bars) mice fed a Mg-deficient (red), normal (black), or Mg-enriched (green) diet. *β-actin* was used as an endogenous reference gene. Comparison between experimental groups was done by the $2^{-\Delta\Delta CT}$ method and expressed as fold increase with respect to the untreated animals on the CTRL diet (indicated by the dotted line). A, Effect of the diet after 5 days: *TRPM6* expression in untreated animals. B, Effect of the diet after 12 days: *TRPM6* expression in untreated animals. C, Effect of experimental colitis after the acute phase: *TRPM6* expression in DSS-treated animals. D, Effect of experimental colitis after the recovery period: *TRPM6* expression in DSS-treated animals. Statistical significance was assessed by 1-way ANOVA, followed by Tukey's post hoc test. Data sharing the same letter are not significantly different.

immune-pathogenesis of IBD.[26] Wound closure was evaluated after 24 hours and expressed as the percentage of the initial gap area that was eventually covered by cells. Extracellular Mg concentration did not appear to affect wound healing by untreated cells (Fig. 5A, upper panels); however, in the presence of 25 ng/mL TNF-α, Mg-supplemented cells seemed to close the wound much better than cells grown in low or normal Mg concentrations (Fig. 5A, lower panels). Image analysis and quantification proved that although TNF-α exposure significantly decreased the area covered by cells grown in either 0.2 or 0.8 mM Mg, Mg-supplemented TNF-α-treated cells filled the cell-free gap as efficiently as untreated cells (Fig. 5B). We conclude that Mg supplementation improves epithelial monolayer recovery upon TNF-α insult.

Mg Modulates Occludin Status

As tight junction (TJ) proteins are key players that modulate mucosal barrier integrity and permeability,[27] we assessed the effect of Mg availability and/or TNF-α treatment on occludin expression in CaCo2 cells by immunoblot. Occludin is a 65 kDa protein that can exist in a variety of phosphorylated forms, ranging up to approximately 85 kDa.[28] Previous reports have shown that phosphorylation of occludin is a key step in tight junction assembly: hypophosphorylated (lower molecular weight) occludin is distributed on the basolateral membranes, whereas highly phosphorylated (higher molecular weight) occludin is selectively concentrated at TJs.[29] Accordingly, in our hands, TNF-α treatment markedly increased expression of the 65 kDa form, while decreasing expression of the 85 kDa form at all Mg concentrations (Fig. 6A). Interestingly, however, the 85 kDa/65 kDa ratio, although reduced by TNF-α treatment, remained significantly higher in Mg-supplemented cells than in control or Mg-deficient cells (Fig. 6B). These results suggest that Mg protects against inflammation-driven epithelial damage via modulation of the TJ component occludin.

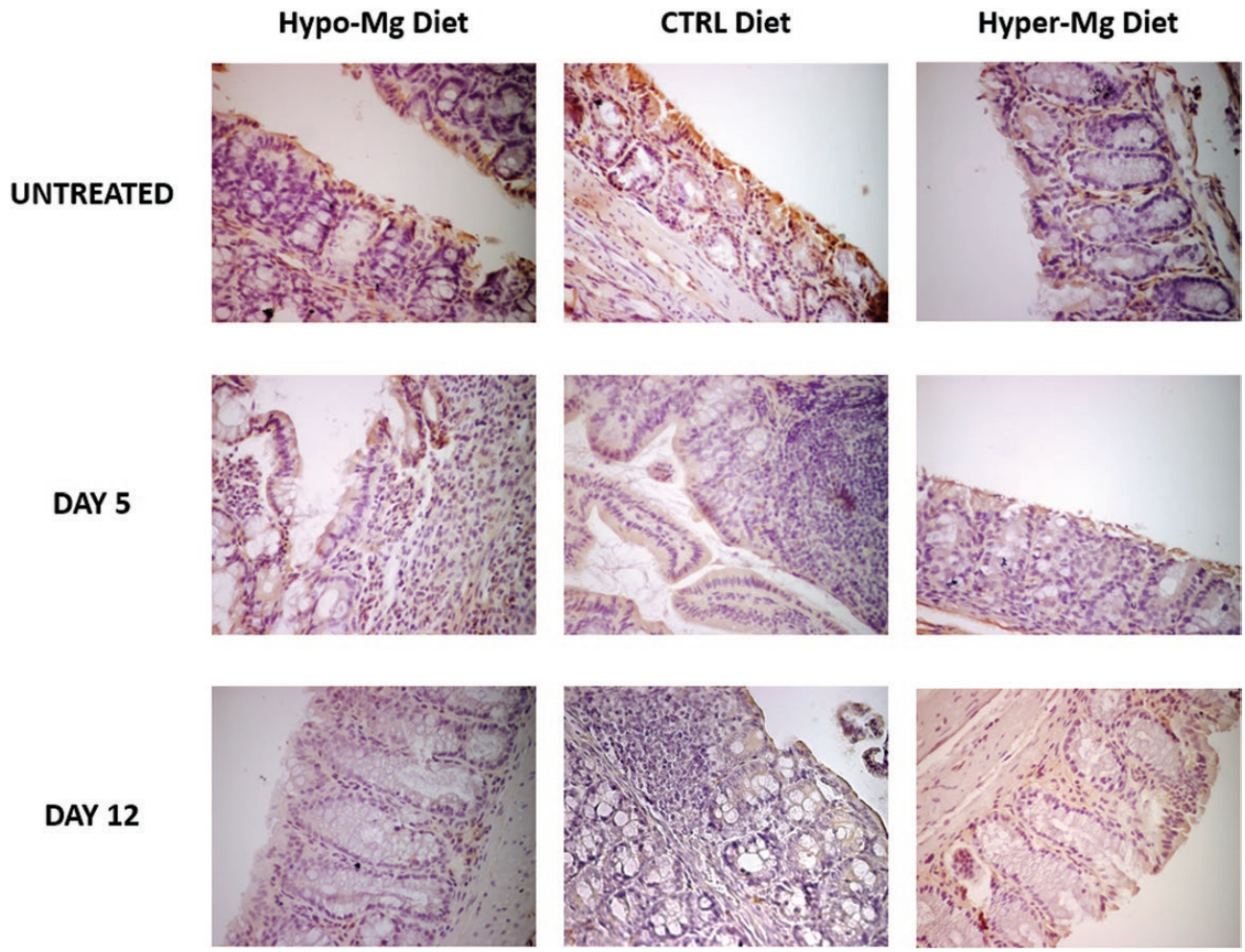

FIGURE 4. Experimental colitis modulates TRPM6 protein expression. Colon tissues from untreated and DSS-treated mice on the different Mg-adjusted diets were analyzed by immunohistochemistry with an anti-TRPM6 specific antibody. Tissues were excised at day 5, after acute DSS treatment, and at day 12, after the recovery period.

Mg Attenuates Inflammation-Induced TRPM6 Downregulation

Finally, we sought to identify the possible molecular mechanisms underlying the downregulation of TRPM6 that occurred in our murine colitis model. Therefore, we assessed the effects of a pro-inflammatory stimulus on TRPM6 channel expression by immunoblot analysis of human colon cell lines. Exposure to 25 ng/mL TNF-α caused a drastic plunge in TRPM6 protein expression (Fig. 7A). Notably, Mg supplementation significantly attenuated the effect of TNF-α treatment on TRPM6 expression (Fig. 7B). Thus, inflammatory cytokines such as TNF-α can directly affect TRPM6 expression in the colon, and Mg supplementation exerts a protective effect.

DISCUSSION

In this paper, we report several important novel findings: (1) we confirm that IBD patients present with a substantial Mg deficit and demonstrate for the first time that serum Mg^{2+} levels inversely correlate with disease activity; (2) we prove that dietary Mg intake modulates the clinical course of experimentally induced murine colitis; (3) we establish that colitis severely compromises intestinal transcellular Mg^{2+} absorption by reducing expression of the key TRPM6 channel via action of inflammatory cytokines; (4) we indicate that the protective effect of Mg is likely due to enhancement of mucosal barrier formation/repair via modulation of TJs.

In contrast to several well-documented micronutrient and vitamin deficiencies in IBD patients, the prevalence of Mg deficiency is unclear, with reported rates varying between 13% and 88% of patients.[30] In our cohort, magnesemia in IBD patients was significantly lower than in healthy controls; this was especially evident in CD patients (Fig. 1A, B). Though average serum Mg^{2+} levels in patients were above the threshold for clinical hypomagnesemia (0.70 mM), the majority of active patients fell below this value, and, in 2 of them, magnesemia

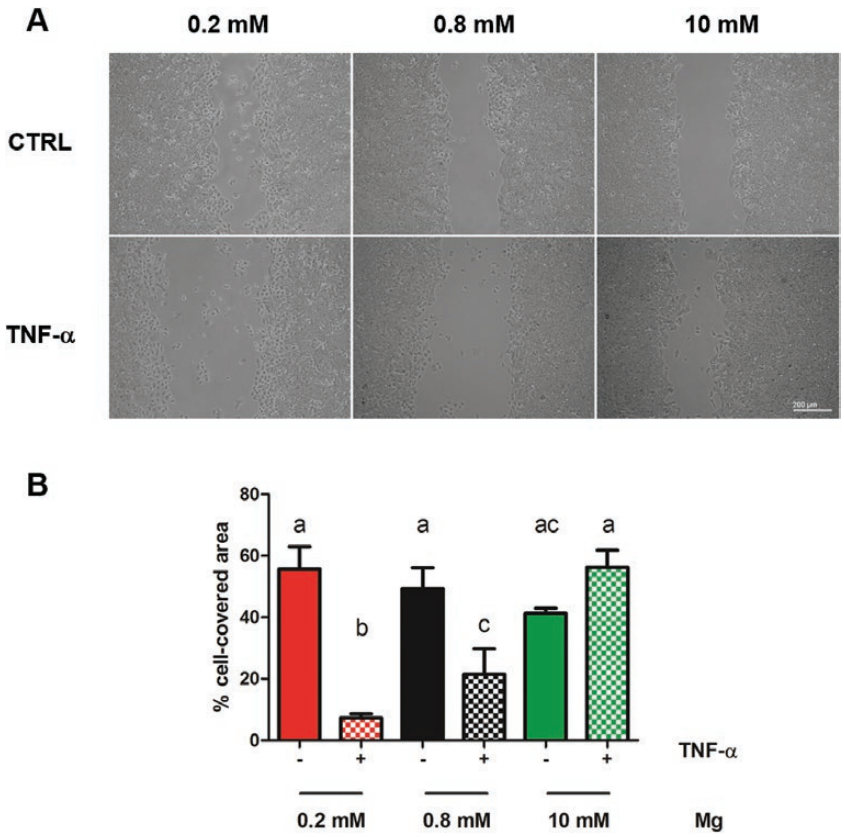

FIGURE 5. Mg improves healing of the intestinal epithelial monolayer. Human colon CaCo2 cells were grown to confluence in inserts that were subsequently removed, leaving a 500-µm-wide cell-free gap. Wound closure was monitored by microscopy for the following 24 hours. A, Representative images of the wound after 24 hours in conditions of different Mg availability without (upper panels) or with 25 ng/mL TNF-α (lower panels). B, Quantification of the cell-covered area from 3 independent experiments; results are expressed as the percentage of the initial gap area that was covered by cells after 24 hours. Statistical significance was assessed by 1-way ANOVA, followed by Tukey's post hoc test. Data sharing the same letter are not significantly different.

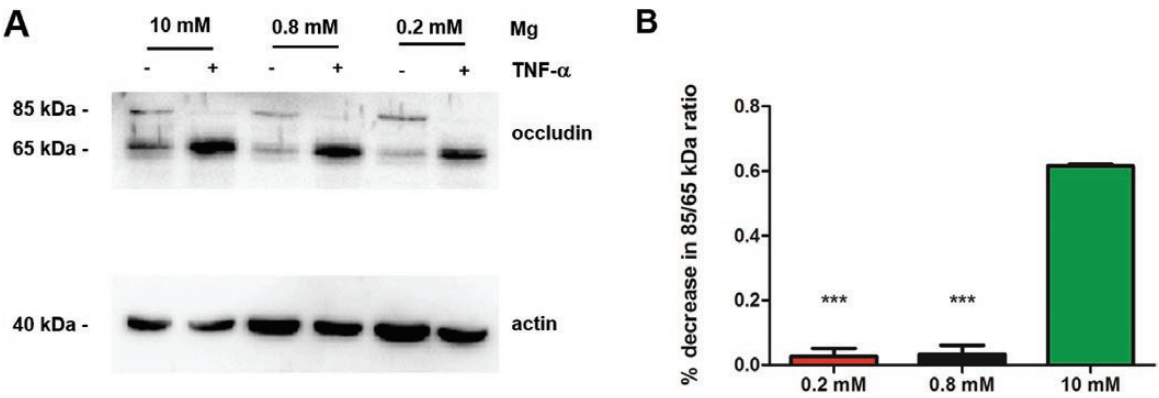

FIGURE 6. Mg modulates occludin status. Human colon CaCo2 cells were grown in Mg-deficient (0.2 mM $MgSO_4$), normal (0.8 mM $MgSO_4$), or Mg-supplemented (10 mM $MgSO_4$) DMEM for 48 hours and then exposed to TNF-α (25 ng/mL) for a further 24 hours. Occludin protein expression was evaluated by immunoblot analysis. A, A representative immunoblot is shown. B, Densitometric analysis of the 85 kDa/65 kDa ratio; results are reported as the percent decrease after TNF-α treatment at each concentration (mean ± SE, n = 3). Statistical significance was assessed by 1-way ANOVA, followed by Tukey's post hoc test. $^{***}P < 0.001$.

was low enough to be symptomatic. Symptoms of hypomagnesemia include neuromuscular, cardiovascular, and metabolic manifestations that can significantly hamper the patient's quality of life and can even be life-threatening. Our findings confirm that Mg deficiency in IBD patients calls for careful reconsideration. The correlation between exacerbation of the

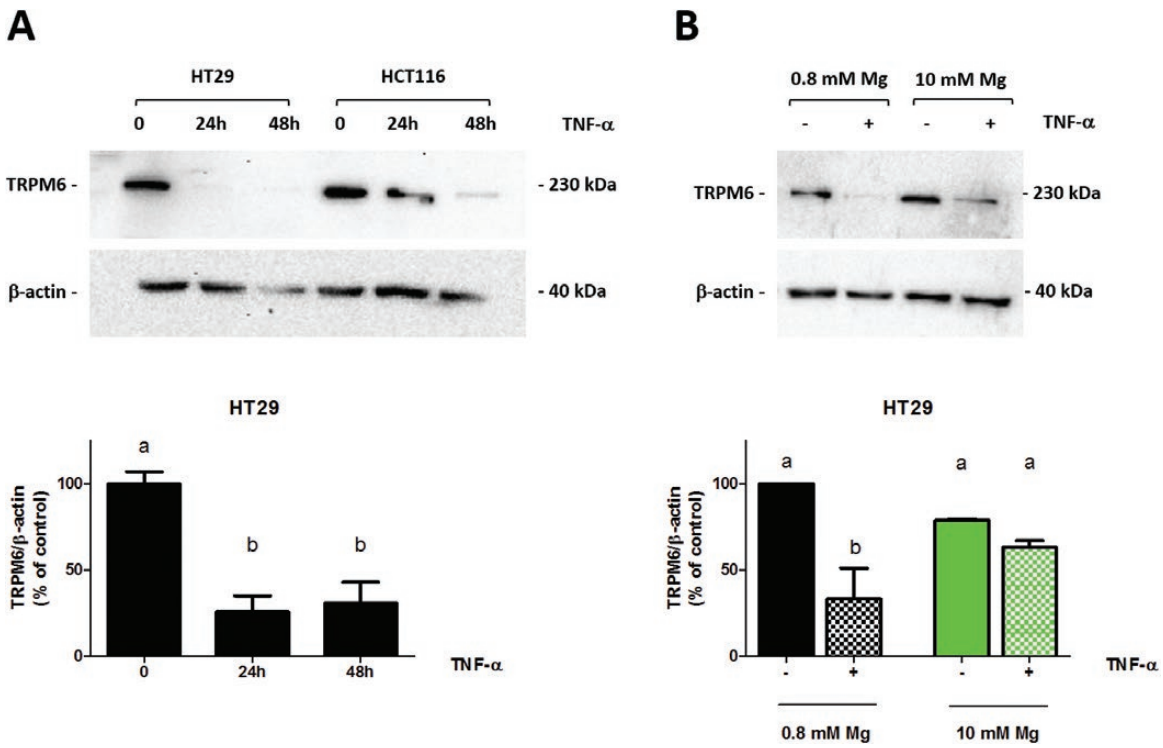

FIGURE 7. Mg attenuates TNF-α-induced TRPM6 downregulation. Human colon cells were exposed to 25 ng/mL TNF-α, and TRPM6 protein expression was evaluated by immunoblot analysis. A representative immunoblot and densitometric evaluation of TRPM6 expression normalized to actin are shown (mean ± SE, n = 3). A, HT29 and HCT116 cells were exposed to TNF-α for the indicated time. B, HT29 cells were grown in normal (0.8 mM $MgSO_4$) or Mg-supplemented (10 mM $MgSO_4$) DMEM for 48 hours and then exposed to TNF-α for a further 24 hours. Statistical significance was assessed by 1-way ANOVA, followed by Tukey's post hoc test. Data sharing the same letter are not significantly different.

disease and hypomagnesaemia that here we document for the first time (Fig. 1C–E) is primarily a consequence of common factors, including decreased food intake and increased enteric loss from chronic diarrhea or fistula output,[4] which result in a diminished concentration gradient for paracellular Mg uptake. Moreover, inflammatory processes cause destruction of enterocytes and, consequently, loss of the active transcellular transport component of Mg absorption, as we discuss later.

We believe that hypomagnesemia creates a vicious circle, whereby disease activity reduces systemic Mg availability and, on the other hand, reduced Mg availability affects the course of the disease. In support of this hypothesis, we present convincing evidence that Mg-deficient mice exhibit a more severe disease, whereas mice on an Mg-enriched diet display an overall more positive course (Fig. 2). We are aware of 2 main limitations in our model. First, we observed a limited beneficial effect of Mg supplementation in comparison with the CTRL diet, which can be ascribed to the comparatively high Mg content in CTRL diet (1g/kg vs the estimated nutrient requirement of 0.5 g/kg[31]). Nevertheless, our data show indisputably that dietary Mg intake, and consequently systemic Mg status, correlates with disease activity. A second limitation of our study is that DSS-induced colitis mainly models acute damage to the epithelium. However, upon loss of barrier integrity, bacteria rapidly penetrate the epithelium and mediate both direct and inflammation-associated toxicity. From this standpoint, the DSS model effectively allows assessing responses mediated by intestinal epithelial cells and innate immune cells, and evaluating therapeutic interventions to improve epithelial regeneration.[32] In the quest for the molecular mechanism(s) underlying the protective action of Mg, we provide support to the hypothesis that Mg enhances epithelial recovery upon an inflammatory insult (Fig. 5), probably through modulation of tight junction complexes (Fig. 6). Although little explored, there is evidence that Mg plays an important role in TJ formation and maintenance of TJ barrier function.[33] The precise mechanisms are currently under investigation in our lab, but phosphorylation of TJ components is likely involved, as indicated by our results on occludin status and supported by the established role of Mg in the active ATP molecule.[8] At the moment, our proposed mode of action does not rule out other explanations, including a modulation of the immune response, as suggested by previous reports,[34,35] and/or gut microbiota composition, as described by Pachikian et al.[36] In-depth analysis of such issues is beyond the scope of the present paper, and we reserve dealing with them for an in vivo model better suited to characterizing the immunological and inflammatory processes involved in the pathogenesis of IBD.

To our knowledge, no reports have so far investigated the molecular mechanisms leading to hypomagnesemia in IBD patients. Here we demonstrate that colitis markedly jeopardizes the active transcellular component of intestinal Mg^{2+} absorption, which is mediated by the TRPM6 channel. In our model, DSS treatment led to a marked reduction in TRPM6 expression, both at the mRNA and protein levels (Figs. 3 and 4). These findings are in line with the results of a whole-genome expression profile analysis performed during the development of DSS-induced colitis, which demonstrated *TRPM6* downregulation over time.[37] Relevantly, *TRPM6* downregulation has also been reported in human IBD biopsy samples compared with control samples.[38] In the present paper, we establish the novel notion that Mg supplementation is effective in preventing channel loss and restoring normal magnesemia (Figs. 2 and 3).

In addition to the destruction of the colonic mucosa, we also identify TNF-α as a key molecular player that mediates the downregulation of TRPM6 and prove that Mg supplementation attenuates the effects of TNF-α on TRPM6 expression (Fig. 7). Altogether, our results are consistent with the hypothesis that a higher Mg concentration switches off inflammation, and, in turn, recovery from inflammation reinstates physiological functions, including active Mg^{2+} absorption. This loop could also hold true in a cluster of chronic inflammatory disorders, for which numerous reports have described an association between Mg deficiency and disease incidence and/or course, without identifying causative connections.[9] We limited our study to the effects of Mg supplementation on the course of experimental colitis, but it would be noteworthy to investigate whether Mg deficiency may represent a risk factor for developing IBD, as suggested by an early report.[39]

In conclusion, our results highlight the importance of evaluating magnesemia in IBD patients, and correcting it when necessary. Mg levels are still not determined routinely in daily clinical practice, and novel parameters for a more accurate determination of status and dietary requirements for Mg are needed.[40] We demonstrate that Mg supplementation may represent a safe and cost-effective strategy to reduce inflammation and restore normal mucosal function, but appropriate supplementation protocols, compatible with acute disease symptoms, have to be defined. Elucidating the role of Mg and Mg^{2+} channels in modulating immune function, and thus maintaining a healthy colon, may promote a deeper understanding of the pathogenesis not only of IBD, but also of inflammation-associated colon cancer, for which a protective role of Mg has already been proven.[34, 41] Ultimately, this may lead to the development of novel pharmacological approaches stretching from disease treatment to health promotion.

SUPPLEMENTARY DATA

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

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