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Indoleamine 2,3-dioxygenase-dependent expansion of T-regulatory cells maintains mucosal healing in ulcerative colitis

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Abstract

Background: Dendritic cell (DC)-derived indolamine 2,3-dioxygenase (IDO) degrades tryptophan to kynurenine, which promotes conversion of inflammatory T cells in immunosuppressive regulatory T cells (Tregs). We analyzed the significance of the IDO:Treg axis for inducing and maintaining mucosal healing in ulcerative colitis (UC).

Methods: Dextran sodium sulphate (DSS)-induced colitis in BALB/c mice (model for mucosal healing) and C57BL/6 mice (model for persistent disease) was used. Serum, fecal samples and colon-infiltrating immune cells of 65 patients with UC with mucosal healing or persistent colitis were analyzed.

Results: Significantly higher serum levels of kynurenine and downregulated inflammatory cytokines were noticed in DSS-treated BALB/c mice compared with C57BL/6 mice. Increased IDO activity and attenuated capacity for antigen presentation and production of inflammatory cytokines, observed in BALB/c DCs, was followed by a significantly lower number of inflammatory T helper 1 (Th1) and Th17 cells and a notably increased number of Tregs in the colons of DSS-treated BALB/c mice. DCs and Tregs were crucially important for the maintenance of mucosal healing since their depletion aggravated colitis. Mucosal healing, followed by an increase in kynurenine and intestinal Tregs, was re-established when BALB/c DCs were transferred into DC-depleted or Treg-depleted DSS-treated BALB/c mice. This phenomenon was completely abrogated by the IDO inhibitor. Significantly higher serum and fecal levels of kynurenine, accompanied by an increased presence of intestinal Tregs, were noticed in patients with UC with mucosal healing and negatively correlated with disease severity, fecal calprotectin, colon-infiltrating interferon γ and interleukin-17-producing cells, serum and fecal levels of inflammatory cytokines.

Conclusion: IDO-dependent expansion of endogenous Tregs should be further explored as a new approach for the induction and maintenance of mucosal healing in patients with UC.

Keywords: indolamine 2,3-dioxygenase, kynurenine, mucosal healing, T-regulatory cells, ulcerative colitis

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Introduction

Ulcerative colitis (UC) is a chronic, idiopathic and relapsing inflammatory disease of the gut limited to the mucosal layer of the colon and rectum.[1] Accordingly, mucosal healing, defined as the absence of friability, blood, erosions and ulcers in all visualized segments of the gut mucosa,[2] represents the ultimate therapeutic goal

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for the treatment of patients with UC[3] while the failure of mucosal healing is a major predictor of clinical recurrence.[4]

Currently, endoscopy is the best diagnostic approach to monitor mucosal status of patients with UC, but it should not be frequently performed because of the invasiveness and potential risk of disease exacerbation.[5] Additionally, one of the main clinical aims in UC treatment is continuous maintenance of mucosal healing in patients with UC.[6] Nowadays, mucosal healing can be maintained for 12 months with continued application of immunosuppressive drugs and biological agents, but aggravation of colon inflammation is inevitable when treatment stops.[3] Accordingly, new noninvasive markers and therapeutic approaches for monitoring and maintaining mucosal healing in patients with UC should be defined.

The importance of tryptophan and its metabolites for the progression of UC has been recently indicated by Nikolaus and colleagues[7] who noticed significantly lower serum levels of tryptophan in patients with UC than in healthy controls. Several recently published animal studies showed that administration of tryptophan or its metabolites led to the attenuation of experimental colitis, whereas removing tryptophan from the diet increases susceptibility to colitis.[8–10] In the inflammatory microenvironment of the gut, the kynurenin pathway comprises most of tryptophan metabolism.[11] In the presence of inflammatory cytokines, intestinal dendritic cells (DCs) produce indolamine 2,3-dioxygenase (IDO), a heme-containing enzyme that degrades tryptophan to kynurenine.[12] Gut DCs, in an IDO-dependent manner, promote the conversion of inflammatory T cells into immunosuppressive regulatory T cells (Tregs), enabling induction of immune tolerance in the gut.[12]

Dextran sodium sulphate (DSS)-induced colitis has been mostly used to elucidate the molecular and cellular pathways involved in the pathogenesis of UC and for the evaluation of new diagnostic and therapeutic approaches for UC treatment.[13] Interestingly, as it was recently demonstrated by Melgar and colleagues,[14] DSS induces a different degree of colon injury and inflammation when applied to C57BL/6 and BALB/c mice. The acute disease in C57BL/6 mice progresses to a severe, chronic colitis, while acute DSS-induced colitis in BALB/c mice is self limited and starts to resolve immediately after DSS removal.[14,15] It is supposed that strain-dependent differences in resolution of DSS-induced inflammation reflect the ability of these strains to mount different healing mechanisms.[16] Accordingly, the acute model of DSS-induced colitis in BALB/c mice is useful to study normal mucosal healing while that for DSS-injured C57BL/6 mice offers a promising animal model for the evaluation of pathological inflammatory changes observed in patients with chronic progressive UC.[14]

Here, we provide the first evidence that higher IDO activity in BALB/c DCs and the consequent IDO-dependent increase in the total number of colon-infiltrating Tregs are responsible for mucosal healing in DSS-treated BALB/c mice. Similarly, we observed higher serum and fecal levels of kynurenine and an increased presence of colon-infiltrating Tregs in patients with UC and mucosal healing. In contrast, patients with UC that is progressive had lower serum and fecal levels of kynurenine and a reduced number of gut infiltrated Tregs. Taken together, this experimental and clinical data suggest the importance of the IDO:Tregs axis for resolution of colon inflammation and strongly indicate the possible diagnostic and therapeutic potential of kynurenine for achieving and maintaining mucosal healing in patients with UC.

Material and methods

Study population

This study recruited a total of 65 patients with UC (38 male and 27 female) with a median age of 50 years (range 23–79). Additionally, 20 healthy subjects (11 male and 9 female) with a median age of 48 years (range 25–75), whose checkups were finished at the Clinical Center of Kragujevac as a routine item, were randomly chosen to represent the general population as healthy controls. Patients with UC were classified into two groups: mucosal healing (19 male and 20 female patients with a median age of 50 years) and chronic persistent disease (19 male and 7 female patients with a median age of 50 years) (Table 1). In each individual case, the diagnosis and assessment of the severity of UC was confirmed by the Mayo endoscopic subscore, histological and clinical scores and by measurement of C-reactive protein (CRP) and fecal calprotectin.[3,17–20] All endoscopies were

Table 1. Demographic and clinical characteristics of patients with UC.

Characteristic	Mucosal healing	Chronic persistent disease
Number of patients, *N* = 65 (%)	39 (60%)	26 (40%)
Sex (male/female), *N*	19/20	19/7
Age, median (range)	50.28 (23–71)	50.12 (26–79)
Disease location, *N* Proctitis/left sided distal UC/pancolitis	10/23/6	1/14/11
Hb, median (IQR)	134 (8)	109 (19)
Fe, median (IQR)	14.5 (6.10)	7.15 (5.53)
Ferritin, median (IQR)	239 (109)	44.5 (74.25)
Platelets, median (IQR)	392 (110)	480 (121.5)
UIBC, median (IQR)	44 (13)	32 (19)
TIBC, median (IQR)	52 (12)	56 (13.25)
C4, median (IQR)	0.21 (0.18)	0.35 (0.31)
C3, median (IQR)	1.3 (0.39)	1.75 (1.02)
CEA, median (IQR)	1.8 (1.95)	3.75 (2.54)
CA 19-9, median (IQR)	2.9 (2.4)	12.15 (16.50)

Fe, iron; Hb, hemoglobin; IQR, interquartile range; UC, ulcerative colitis; TIBC, transferrin and iron binding capacity; UIBC, unsaturated iron binding capacity.

performed by the same experienced endoscopist (NZ) and disease severity was independently determined by two gastroenterologists (MJ and NZ). Mucosal healing was defined when Mayo endoscopic subscore had been transitioned from a Mayo grade 2 or 3 to a Mayo grade 0 or 1, while chronic persistent disease was defined when the Mayo endoscopic subscore was 2 or 3.[3] The clinical score was determined by using the Truelove and Witts clinical activity index.[18] The histological score was determined by using Geboes grade.[19] Patients with previously diagnosed colorectal cancer, as well as patients with Crohn's disease, were excluded from the study. The study was conducted at the Center for Gastroenterology, Clinical Center of Kragujevac and Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia and was approved by the Ethics Committees of these institutions. The Principle of Good Clinical Practice and the Declaration of Helsinki were adhered to at all times. All patients gave their informed consent for blood and tissue analysis. Patients were under continuous medical supervision at the Clinical Center of Kragujevac.

Measurements of IDO activity, concentration of cytokines, fecal calprotectin and CRP levels in serum and fecal samples of patients with UC and healthy controls

Blood and fecal samples were prepared as previously described.[21] The serums and supernatant fluids were collected and stored at −80°C until they were measured by enzyme-linked immunosorbent assay (ELISA). IDO activity was determined by spectrophotometric assay for kynurenine in the serum and fecal samples of patients with UC and healthy controls.[22] Tumor necrosis factor (TNF)-α, interleukin (IL)-10, CXCL11 and IL-17, fecal calprotectin and CRP were measured in serum and fecal samples of patients with UC and healthy subjects by using commercially available ELISA tests, according to the manufacturer's instructions.[17]

Flow cytometry analysis of colon-infiltrating cells in patients with UC

The phenotype of colon-infiltrating cells was determined by flow cytometry. Immune cells were isolated from the colons of patients with UC, as previously described.[23] Biopsies were washed three times in calcium-magnesium-free Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich, St Louis, MO, USA). The specimens were incubated twice in calcium-magnesium-free HBSS with 1 mm ethylenediamine tetraacetic Acid (EDTA) for 10 min at 37°C under gentle shaking to remove intestinal epithelial cells. Specimens were washed with HBSS and incubated for 20–30 min in 2 ml Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Basel, Switzerland) with 1 mg/ml collagenase type I (336 U/ml) (Sigma-Aldrich), 0.1 mg/ml DNAse (Sigma-Aldrich) and 1 mg/ml hyaluronidase (Sigma-Aldrich) without fetal bovine serum (FBS) at 37°C. Cells were washed twice with phosphate-buffered saline (PBS) (Sigma-Aldrich) and finally submitted to Ficoll density gradient centrifugation for 20 min at 2000 rev/min (~690 g, without brake). The interphase was carefully removed and washed with PBS. Single-cell suspensions of colon-infiltrating cells were obtained and the cells were then washed twice with a buffer assigned for flow cytometry containing 0.05% NaN3, 0.1% bovine serum albumin (BSA), and 0.4% trisodium citrate dehydrate in PBS. About 1×10^6 cells per sample were incubated with antihuman CD4, CD56, IL-10, interferon (IFN)-γ and IL-17 antibodies conjugated with fluorescein isothiocyanate (FITC; BD Biosciences, Franklin Lakes, NJ, USA), phycoerythrin (PE; BD Biosciences), peridinin chlorophyll A protein (PerCP; BD Biosciences) or allophycocyanin (APC; BD Biosciences). For the intracellular staining, cells were previously stimulated with phorbol myristate acetate (PMA) and ionomycin for 4 h at 37°C with the addition of 1 μg/ml Golgi plug. Intracellular staining for Foxp3 was performed using the BD Bioscience fixation/permeabilization buffer kit. Flow cytometric analysis was conducted on a BD Biosciences FACSCalibur and analyzed by using the Flowing software analysis program.

Animals

For animal studies, 6–8-week-old male C57BL/6 and BALB/c mice were used. Mice were maintained in animal facilities of the Faculty of Medical Sciences, University of Kragujevac, Serbia. All animals received humane care and all experiments were approved by and conducted in accordance with the Guidelines of the Animal Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia. Mice were housed in a temperature-controlled environment with a 12 h light–dark cycle and were administered standard laboratory chow and DSS or water *ad libitum*.

Induction of experimental colitis

DSS (3%, molecular weight 40 kDa; TdB Consultancy, Uppsala, Sweden) was given to mice in place of normal drinking water for up to 5 days, followed by a recovery period of 7 days.[24] Control mice had access to DSS-free water.

Evaluation of DSS-induced colitis

Disease Activity Index (DAI) was used to assess the clinical signs of colitis.[25] Body weight measurements, analysis of stool consistency, and fecal occult blood tests were performed daily.[25]

For histological analysis, colons were removed from euthanized mice, rinsed with PBS, and cut longitudinally before being rolled into a 'Swiss roll'.[26] The length of each colon was measured starting from the end of the cecum to the rectum. The exact same areas (distal anorectal regions) were analyzed in all samples. The histology scores for each mouse were calculated as the sum of 'damage of epithelium' and 'infiltration' subscores. 'Damage of epithelium' subscore was scored as follows: 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas. 'Infiltration' subscore was scored as follows: 0, no infiltrate; 1, infiltrate around crypt basis; 2, infiltrate reaching to lamina muscularis mucosae; 3, extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema; 4, infiltration of the lamina submucosa.[27]

Pharmacological inhibition of IDO activity in vivo

For inhibition of IDO activity *in vivo*, BALB/c DSS-treated mice received IDO inhibitor, 1-methyltryptophan (1-MT, Sigma-Aldrich) dissolved in drinking water (2 mg/ml). DSS-treated BALB/c mice received 1-MT continuously during the first 5 days of the experiment (during DSS administration).[28]

Flow cytometry analysis of colon-infiltrating cells in DSS-treated mice

Isolation of immune cells from lamina propria of experimental animals was conducted as previously described.[29] Each colon was dissected away from the caecum. The colons were cut into pieces 3 cm long and then cut longitudinally, so that 3 × 3 cm flaps of colonic tissue were made. The flaps were placed in a 50 ml conical tube and washed three to five times with 30 ml cold HBSS, calcium and magnesium free. The pieces were incubated in 20 ml HBSS/EDTA for 30 min in a 37°C water bath. Each tube was shaken regularly during the incubation to ensure that epithelial cells were disrupted from the mucosa. The pieces were sedimented and the supernatant was decanted. The remaining EDTA were washed out with 40 ml HBSS, calcium and magnesium free. The fragments of colonic tissue were placed in a 10 cm Petri dish and cut into smaller pieces with a scalpel. The pieces were aspirated with a pipette, transferred to a fresh 50 ml conical tube and filled to 20 ml with DMEM supplemented with 10% FBS. Then, 1 ml of 4000 Mandl units/ml collagenase D (Sigma-Aldrich) and 200 µl of 1 mg/ml DNase (Sigma-Aldrich) were added to the tube and incubated for 1 h in a 37°C water bath. The supernatant was filtered through a 100 µm nylon cell strainer into a clean 50 ml conical tube. Cold HBSS, calcium and magnesium free, was added to 50 ml. Cells were pelleted by centrifuging 10 min at 450 g, at 4°C. The pellet was disrupted and cells were resuspended in 50 ml HBSS, calcium and magnesium free, and filtered through a 40 µm nylon cell strainer into a clean 50 ml conical tube. Cells were again pelleted by centrifuging for 10 min at 450 g, at 4°C. The pellet was disrupted and cells were resuspended in 20 ml of 30% Percoll. Afterwards, the cell suspension was layered over 25 ml of 70% Percoll in a 50 ml conical tube and centrifuged for 20 min at 1100 g, room temperature, with as low an acceleration rate as possible and with the brake off. Clumping of cells was prevented by the addition of 1 mM EDTA to the solution. Epithelial cells floated on the 30% Percoll layer, and immune cells were found between the 30% and 70% layers. Debris and dead cells were pelleted at the bottom of the conical tube. For flow cytometry analysis, about 1×10^6 cells per sample were incubated with antimouse CD45, CD4, CD11c and CD40, conjugated with FITC, PE, PerCP or APC. For the intracellular staining, cells were previously stimulated with PMA and ionomycin for 4 h at 37°C with the addition of 1 µg/ml Golgi plug. Following extracellular staining, cells were fixed, permeabilized and stained for IFN-γ, IL-4, IL-17, IL-12, IL-1β and IL-10, conjugated with FITC, PE, PerCP or APC. Intracellular staining for Foxp3 was performed using the BD Bioscience fixation/ permeabilization buffer kit, following the manufacturer's instructions. Flow cytometric analysis was conducted on a BD Biosciences FACSCalibur and analyzed by using the Flowing Software analysis program.

Determination of cytokines in serum samples of experimental animals

The commercial ELISA sets (R&D Systems, Minneapolis, MN, USA) were used to determine the concentration of IL-12, IL-1β and IL-10 in serum samples of control and DSS-treated animals.[25]

Isolation of DCs and analysis of their phenotype and function

DCs were isolated from spleens of DSS-treated C57BL/6 and BALBc mice 2 days after administration of DSS by magnetic cell sorting. Single-cell suspensions of mononuclear cells derived from the spleen were labelled with CD11c MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and positively selected using MACS Column and MACS Separator. Isolated DCs were stimulated with lipopolysaccharides (LPS, 10 ng/ml) for 48 h, harvested and analyzed by flow cytometry.[25]

Determination of IDO activity in DCs

Expression of IDO in DCs was determined by real-time polymerase chain reaction (PCR). The fold change of mRNA gene expression for IDO and β actin, used as a housekeeping gene (Invitrogen, Carlsbad, CA, USA), was calculated.[30] IDO activity was determined by spectrophotometric measurement of kynurenine in the supernatants of LPS (10 ng/ml) stimulated spleen DCs previously isolated from untreated or DSS-treated mice, 2 days after DSS administration.[22]

Depletion of DCs and adoptive transfer of DCs in DC-depleted DSS-treated mice

Since gut CD103+ DCs express IDO responsible for expansion of colon-infiltrating Tregs,[12] these DCs were selectively depleted in DSS-treated BALB/c mice by using immunotoxin-saporin capable of selectively depleting DCs (Advanced

Targeting Systems, San Diego, CA, USA).[31] Since this immunotoxin significantly reduces the frequencies and absolute numbers of DCs within 1 week of treatment,[31] DSS-treated BALB/c mice were, at day 5, injected with saporin (2 mg/kg, intraperitoneally) while clinical and histological manifestation of DC depletion were analyzed 1 week later.

IDO activity was inhibited in BALB/c DCs ($DCs^{1\text{-}MT}$) by culturing DCs, previously isolated from healthy BALB/c mice, in culture medium containing 1 mM 1-MT.[32] Immunotoxin-saporin used for depletion of DCs is cleared from the circulation by day 3 post injection.[31] Accordingly, for adoptive transfer experiments, DCs with normal or suppressed IDO activity ($DCs^{1\text{-}MT}$), previously isolated from healthy BALB/c mice, were transferred (intraperitoneally; 2×10^5 DCs/mouse) into DC-depleted DSS-treated BALB/c mice 72 h after saporin injection.

Depletion of Treg cells and adoptive transfer of DCs in Treg-depleted DSS-treated mice

For Treg depletion, mice were injected intraperitoneally with cyclophosphamide (CY, Galenika A.D., Belgrade, Serbia) at a dose of 10 mg/kg or anti-CD25 antibody (PC61 mAb, Sigma-Aldrich, Munich, Germany), at a dose of 250 mg/mouse, 3 days before DSS administration.[33,34]

For adoptive transfer experiments, isolated DCs (2×10^5 cells per mouse) were transferred intraperitoneally into CY+DSS-treated and anti-CD25-antibody-treated BALB/c mice at day 5.[35]

Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM). Results were analyzed by Student's *t* test, Pearson's or Spearman's correlation coefficient and SPSS 22.0 for Windows software (SPSS Inc., Chicago, IL, USA). The difference was considered significant when p was less than 0.05.

Results

Serum concentration of kynurenine reflected the strain-dependent difference in mucosal healing of DSS-treated C57Bl/6 and BALB/c mice

As previously reported by Melgar and colleagues,[14] a similar degree of DSS-induced colitis was noticed in C57BL/6 and BALB/c mice during the first 5 days of DSS treatment [Figure 1(Aa, Ba)]. However, at day 12, there was a striking difference in weight loss, clinical and histological manifestations of DSS colitis between C57BL/6 and BALB/c mice [Figure 1(Ab, Bb, D, E, Fb, Fd)], indicating faster mucosal healing in BALB/c mice. Accordingly, molecular and cellular mechanisms responsible for strain-dependent differences in mucosal healing 12 days after initial administration of DSS were analyzed.

There were significant differences in the clinical manifestation of disease [Figure 1(A–D)]. DSS-treated C57BL/6 mice developed severe colitis manifested by watery diarrhea, rectal bleeding and significant body weight loss (>5%) [Figure 1(Ab)]. The presence of blood in the feces was detected 1–2 days after the start of DSS treatment, whereas gross bleeding and diarrhea were initially observed from day 4 [Figure 1(Ca)]. Additionally, DSS-treated mice exhibited visible signs of illness, including a hunched back, raised fur and reduced mobility. Severe colitis observed in DSS-treated C57BL/6 mice was accompanied by noticeable decrease in colon length [Figure 1(D)]. DSS-treated BALB/c mice recovered from DSS-induced colitis faster than C57BL/6 mice [Figure 1(A–D)]. Bloody diarrhea, gross rectal bleeding [Figure 1(Cb)], hunched back, raised fur and reduced mobility were not observed in DSS-treated BALB/c mice at day 12. At the same time point, body weight loss [$p < 0.05$; Figure 1(Ab)], DAI [$p < 0.01$; Figure 1(Bb)] and colon shortening [$p < 0.05$; Figure 1(D)] were significantly lower in DSS-treated BALB/c animals compared with C57BL/6 mice.

The difference in recovery from DSS-induced colitis between C57BL/6 and BALB/c mice was confirmed by the statistical difference in histological score [$p < 0.05$; Figure 1(E)]. Destruction of the entire epithelium, decreased number of goblet cells and loss of crypts, accompanied by severe submucosal edema and massive infiltration of inflammatory cells in the lamina propria and submucosa were observed in the colons of C57BL/6 DSS-treated mice [Figure 1(Fb)]. In contrast, microscopic examination of the distal colon sections of DSS-treated BALB/c mice revealed almost normal architecture of the colon, minimal changes in the surface epithelium and mild infiltration of inflammatory cells to the mucosa [Figure 1(Fd)].

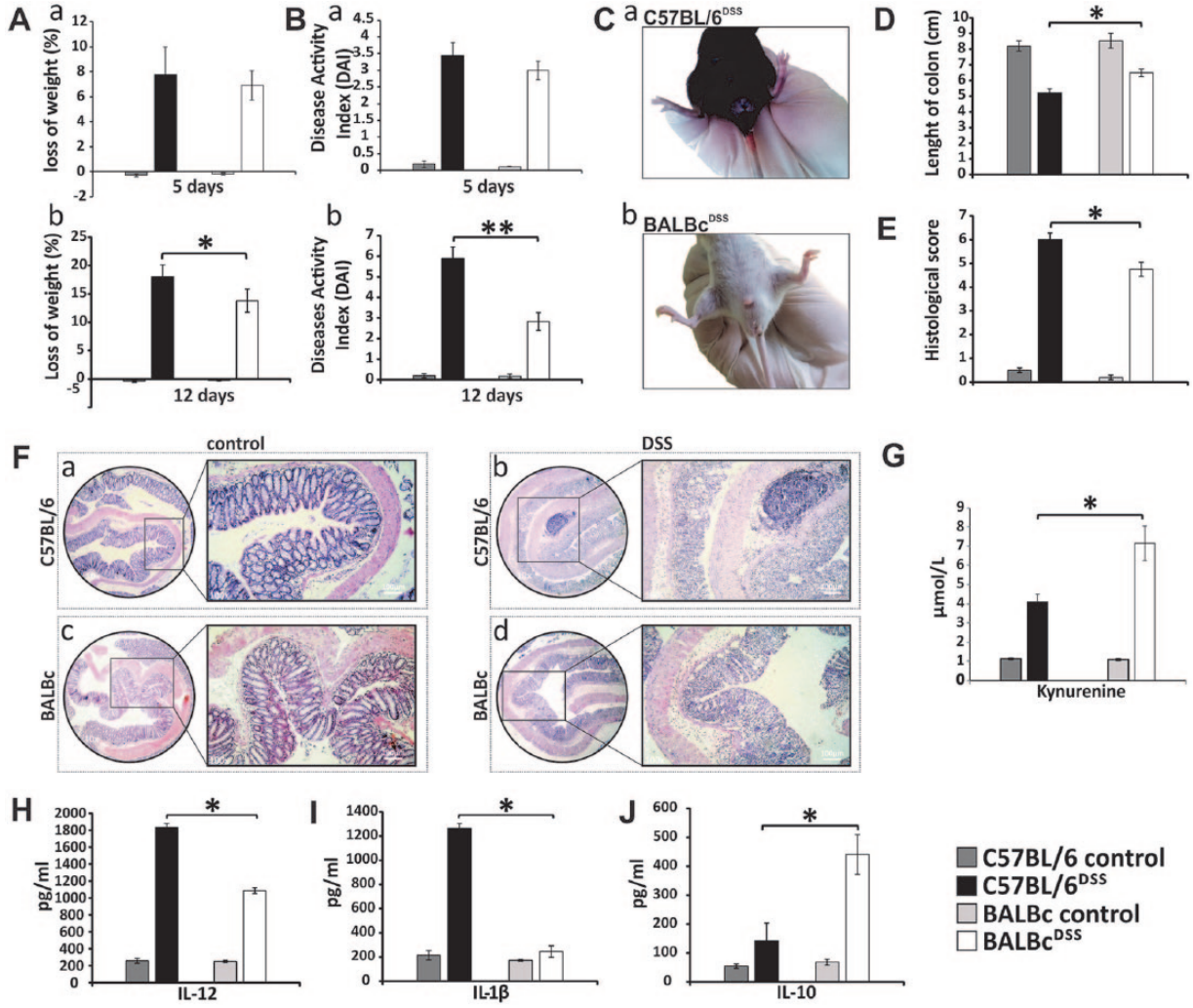

Figure 1. Serum concentration of kynurenine reflected the severity of dextran sodium sulphate (DSS)-induced colitis. Weight loss (A), Disease Activity Index (DAI) (B), rectal bleeding (C), length of colon (D) and histological score (E) revealed strain-specific difference in recovery from DSS at day 12. Destruction of the entire epithelium, decreased number of goblet cells and loss of crypts, accompanied by severe submucosal edema and massive infiltration of inflammatory cells in the lamina propria and submucosa were observed in the colons of C57BL/6 DSS-treated mice (Fb, 10×/100×). In contrast, distal colon sections of DSS-treated BALB/c mice revealed almost normal architecture of the colon, minimal changes in the surface epithelium and mild infiltration of inflammatory cells to the mucosa (Fd, 10×/100×). Significantly higher serum levels of kynurenine were noticed in DSS-treated BALB/c mice (G). Serum levels of inflammatory interleukin (IL)-12 (H) and IL-1β (I) were significantly lower ($p < 0.05$), while the concentration of anti-inflammatory IL-10 (J) was significantly higher in the sera of DSS-treated BALB/c mice compared with DSS-treated C57BL/6 mice. Data presented as mean ± standard error of the mean; n = 10/group. *$p < 0.05$, **$p < 0.01$.

Importantly, the serum concentration of kynurenine reflected the strain-dependent difference in mucosal healing. Significantly higher serum levels of kynurenine were noticed in DSS-treated BALB/c mice that recover faster from DSS-induced colon injury than C57Bl/6 mice [$p < 0.05$; Figure 1(G)].

To investigate whether a difference in serum concentration of kynurenine was accompanied by a different cytokine profile, concentrations of inflammatory cytokines (IL-12 and IL-1β) and immunosuppressive IL-10 were analyzed in the serum samples of DSS-treated C57BL/6 and BALB/c mice. Serum levels of inflammatory IL-12 and IL-1β were significantly lower [$p < 0.05$; Figure 1(H, I)], while the concentration of anti-inflammatory IL-10 was significantly higher in the sera of DSS-treated BALB/c mice compared with DSS-treated C57BL/6 mice

[$p < 0.05$; Figure 1(J)], indicating that increased serum levels of immunosuppressive kynurenine were accompanied by a notable decrease in inflammatory cytokines and a significant increase in anti-inflammatory IL-10.

Activated BALB/c DCs have higher capacity to express IDO and produce immunosuppressive kynurenine and IL-10 then activated C57BL/6 DCs

To delineate the cellular base of observed strain-dependent differences in mucosal healing, we first analyzed the effects of LPS on phenotype and function of DCs [Figure 2(A)], which are known to regulate colon inflammation in an IDO-dependent manner.[12] Significantly higher expression of IDO was noticed in LPS- and DSS-activated BALB/c DCs compared with similarly treated C57BL/6 DCs [$p < 0.05$; Figure 2(B)]. The difference in IDO expression was corroborated by measurement of kynurenine in supernatants of LPS- and DSS-activated BALB/c and C57BL/6 DCs. A significantly higher concentration of kynurenine was detected in the supernatants of activated BALB/c DCs then in activated C57BL/6 DCs [$p < 0.05$; Figure 2(C)], confirming the higher IDO activity in activated BALB/c DCs.

In line with these findings, compared with activated C57BL/6 DCs, a significantly higher percentage of LPS- and DSS-activated BALB/c DCs produced immunosuppressive IL-10 while a lower percentage of these cells produced inflammatory IL-12 and expressed CD40, a well known marker of DC activation[36] [$p < 0.05$; Figure 2(D)], suggesting that BALB/c DCs respond to specific (DSS) and general (LPS) stimuli mainly through the production of immunosuppressive factors. These findings were correlated with the cytokine production of gut-infiltrated DCs *in vivo* [Figure 2(E–G)]. A remarkably lower number of colon-infiltrating inflammatory (IL-12- and IL-1β-producing) DCs [$p < 0.05$; Figure 2(E, F)] and a significantly higher number of immunosuppressive IL-10-producing DCs [$p < 0.05$; Figure 2(G)] were noticed in the lamina propria of DSS-treated BALB/c mice compared with DSS-treated C57BL/6 mice, confirming the importance of strain-dependent differences for phenotype and function of colon-infiltrating DCs.

Recovery from DSS-induced colitis of BALB/c mice was a consequence of DC-driven polarization of T cells towards immunosuppressive Tregs

To determine whether colon-infiltrated DCs influenced the recovery from DSS-induced colitis by affecting polarization of CD4+ Th cells, the total number of IFN-γ, IL-4, IL-10 and IL-17 producing Th1, Th2 and Th17 cells in the colons of DSS-treated C57BL/6 and BALB/c mice was analyzed [Figure 2(H–M)]. The cellular makeup of the colons revealed a significantly lower number of CD45+CD4+ Th cells [$p < 0.01$; Figure 2(H)], IFN-γ-producing Th1 [$p < 0.05$; Figure 2(I)] and IL-17-producing Th17 cells [$p < 0.01$; Figure 2(J)] and a significantly higher number of IL-10-producing CD4+ T cells [$p < 0.01$; Figure 2(K)] in the lamina propria of DSS-treated BALB/c animals compared with DSS-injured C57BL/6 mice. There was no difference in the total number of colon-infiltrating IL-4-producing Th2 cells between experimental groups [Figure 2(L)]. Importantly, a remarkably higher presence of CD45+CD4+FoxP3+ Tregs, which are crucially involved in the suppression of DSS-induced inflammation,[37] was observed in the colons of DSS-treated BALB/c mice [$p < 0.05$; Figure 2(M)], suggesting that the strain-dependent recovery from DSS-induced colitis of BALB/c mice was a consequence of DC-driven polarization of T cells towards immunosuppressive Tregs.

DC-dependent expansion of Tregs and elevated serum levels of immunosuppressive kynurenine had a crucial role in the induction of mucosal healing in DSS-treated BALB/c mice

In order to confirm this hypothesis, DCs, isolated from healthy BALB/c mice, were transferred into DSS-treated BALB/c mice which were previously depleted of Tregs by CY or anti-CD25 antibody [Figure 3(A)]. Depletion of Tregs completely abrogated mucosal healing in DSS-treated BALB/c mice [Figure 3(B–D)], indicating the crucial importance of Tregs for the recovery from DSS-induced colon injury. CY as well as anti-CD25 antibody-treated BALB/c mice reacted to DSS challenge with severe colonic mucosal injury manifested by substantial, bloody diarrhea and considerable weight loss resulting in increased DAI [$p < 0.05$; Figure 3(B)]. Similar to that observed in DSS-susceptible C57BL/6 mice [Figure 3(Da)] notable mucosal damage and epithelial necrosis, focal crypt lesions, goblet cell loss

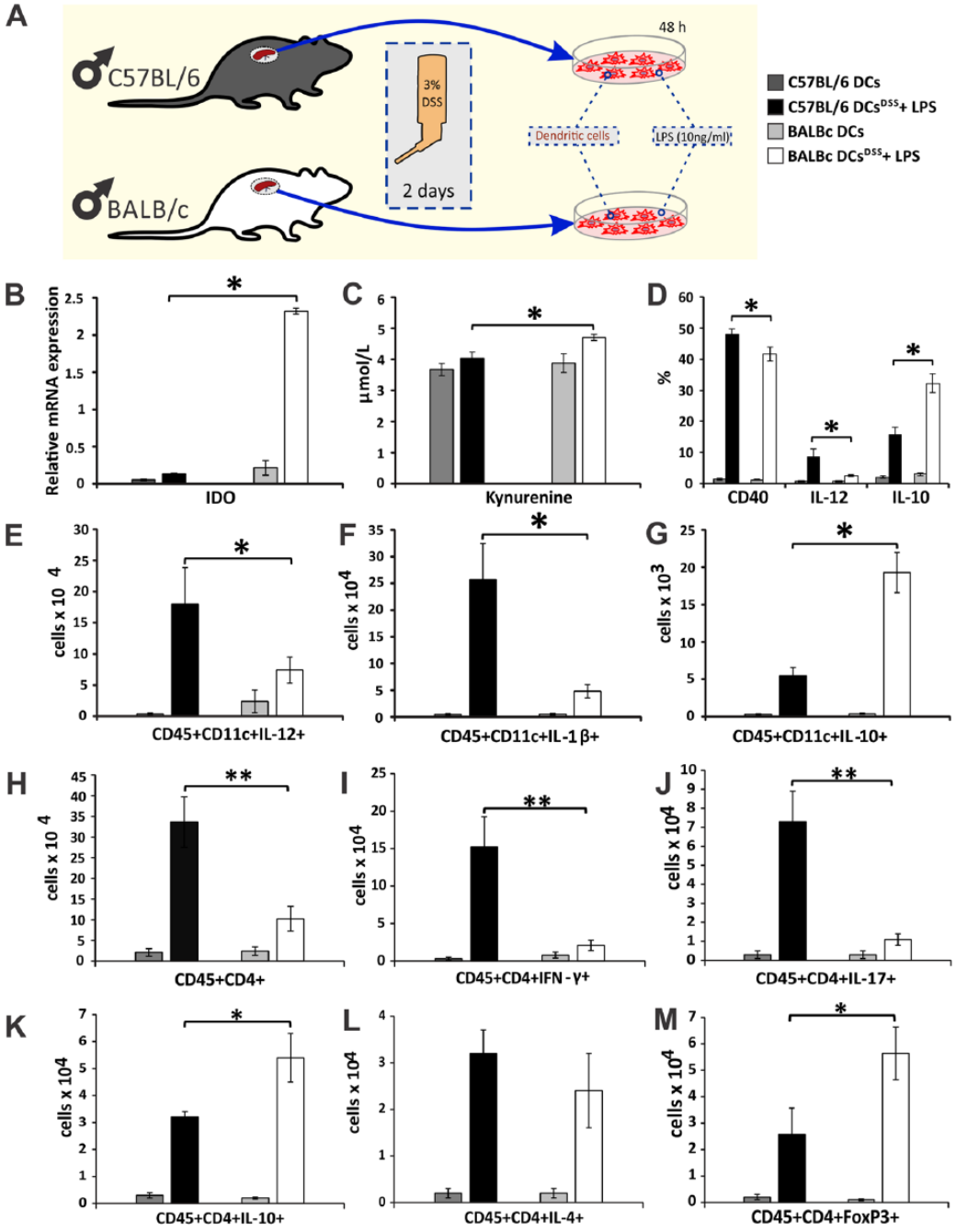

Figure 2. Activated BALB/c dendritic cells (DCs) have a higher capacity to express indolamine 2,3-dioxygenase (IDO) and produce immunosuppressive kynurenine and interleukin (IL)-10 then activated C57BL/6 DCs. DCs were isolated from the spleens of dextran sodium sulphate (DSS)-treated mice 2 days after administration of DSS and stimulated with lipopolysaccharide (LPS) for 48 h (A). Significantly higher expression of IDO was noticed in LPS- and DSS-activated BALB/c DCs compared with C57BL/6 DCs (B). A significantly higher concentration of kynurenine was detected in the supernatants of activated BALB/c DCs than in activated C57BL/6 DCs (C). A significantly higher percentage of LPS- and DSS-activated BALB/c DCs produced immunosuppressive IL-10 while a lower percentage of these cells produced inflammatory IL-12 and expressed CD40 (D). Remarkably, a lower number of IL-12-producing DCs (E) and IL-1β-producing DCs (F) and a significantly higher number of immunosuppressive IL-10-producing DCs (G) were noticed in the lamina propria of DSS-treated BALB/c mice compared with DSS-treated C57BL/6 mice. A significantly lower number of CD4+ T helper (Th) cells (H), interferon (IFN)-γ-producing Th1 cells (I), IL-17-producing Th17 cells (J) and a significantly higher number of IL-10-producing CD4+ T cells (K) and T-regulatory cells (Tregs) (M) were observed in the lamina propria of DSS-treated BALB/c animals compared with DSS-injured C57BL/6 mice. There was no significant difference in the total number of IL-4-producing CD4+ Th2 cells between experimental groups (L). Mean ± standard error of the mean; n = 10/group. *$p < 0.05$; **$p < 0.01$.

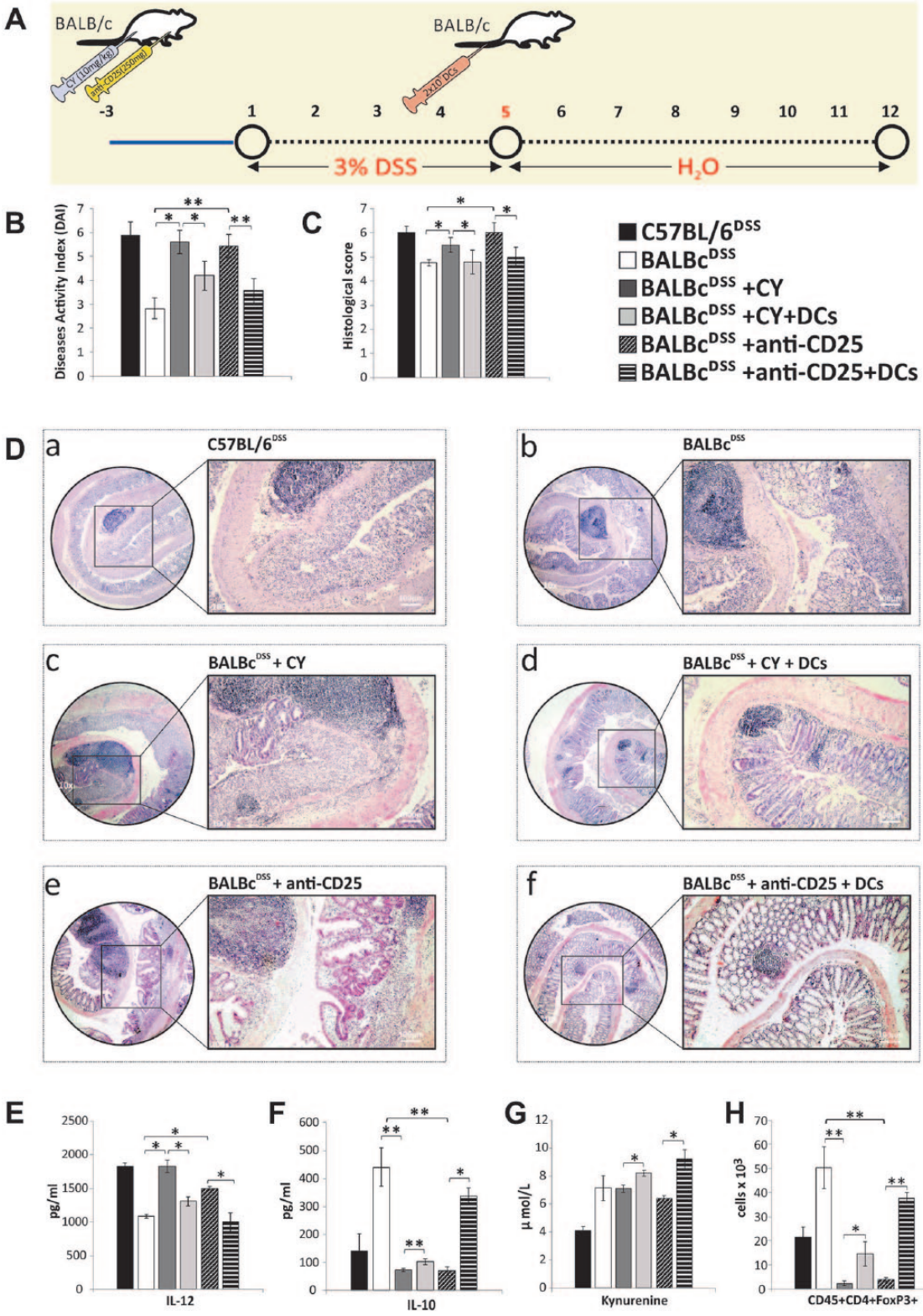

Figure 3. Dendritic cell (DC)-dependent expansion of T-regulatory cells (Tregs) and elevated serum levels of immunosuppressive kynurenine had a crucial role for mucosal healing of dextran sodium sulphate (DSS)-treated BALB/c mice. CY or anti-CD25 antibody were injected in BALB/c mice 3 days before DSS administration. DCs were transferred into CY+DSS-treated BALB/c mice at day 5 (A). CY-treated as well as anti-CD25-treated BALB/c mice reacted to DSS challenge with severe colonic mucosal injury manifested by substantial, bloody diarrhea and considerable weight loss, resulting in increased Disease Activity Index (DAI) (B).

Figure 3. (Continued)

Similar to that observed in DSS-susceptible C57BL/6 mice (Da, 10×/100×), notable mucosal damage and epithelial necrosis, focal crypt lesions, goblet cell loss and inflammatory cell infiltration were noticed in the colons of CY+DSS-treated (Dc, 10×/100×) and anti-CD25+DSS-treated BALB/c mice (De, 10×/100×). These pathological changes in colon architecture were manifested by a significant increase in the histological score of CY+DSS-treated and anti-CD25+DSS-treated BALB/c mice (C). A significant increase in the serum levels of interleukin (IL)-12 (E), a decrease in the serum levels of IL-10 (F) and a significantly lower number of colon-infiltrating Tregs (H) were noticed in CY+DSS-treated and anti-CD25+DSS-treated BALB/c mice. Adaptive transfer of BALB/c DCs significantly attenuated DAI (B), histological score (C), colon injury and inflammation (Dd, Df), the serum levels of IL-12 (E) and significantly increased the serum concentrations of IL-10 (F) and kynurenine (G) as well as the total number of colon-infiltrating CD45+CD4+FoxP3+ Tregs (H) in CY+DSS-treated BALB/c mice that received DCs as well as in anti-CD25+DSS-treated BALB/c animals that were injected with DCs. Mean ± standard error of the mean; n = 10/group. *$p < 0.05$; **$p < 0.01$.

and inflammatory cell infiltration were noticed in the colons of CY+DSS-treated [Figure 3(Dc)] and anti-CD25+DSS-treated BALB/c mice [Figure 3(De)]. These pathological changes in colon architecture of Treg-depleted DSS-treated BALB/c mice were manifested by a significant increase in histological scores [$p < 0.05$; Figure 3(C)]. Additionally, a significant increase in the serum levels of IL-12 [$p < 0.01$; Figure 3(E)] and a decrease in the serum levels of IL-10 [$p < 0.01$; Figure 3(F)] were noticed in CY+DSS-treated and anti-CD25+DSS-treated BALB/c mice, indicating that augmented colon injuries in these animals were accompanied by enhanced systemic inflammatory responses.

Importantly, transfer of BALB/c DCs managed to significantly increase the serum levels of kynurenine in CY and anti-CD25+DSS-treated BALB/c mice [$p < 0.05$; Figure 3(G)] that resulted in a remarkably higher number of colon-infiltrated Tregs ($p < 0.05$; Figure 3H) and led to the attenuation of DSS-induced colitis in CY+DSS-treated and anti-CD25+DSS-treated BALB/c animals [$p < 0.05$; Figure 3(B–D)]. Compared with CY+DSS-treated and anti-CD25+DSS-treated BALB/c animals, clinically observed alleviation of intestinal inflammation [manifested by improved stool consistency, diminished fecal occult blood and reduced DAI; $p < 0.05$; Figure 3(B)] was verified by significantly decreased histological scores [$p < 0.05$; Figure 3(C)] that featured reduced numbers of mucosal erosions and ulcerations, lower hyperplasia and decreased colon infiltration of inflammatory cells in CY+DSS-treated BALB/c mice that received DCs [Figure 3(Dd)] as well as in anti-CD25+DSS-treated BALB/c mice that were injected with DCs [Figure 3(Df)]. Additionally, attenuated colon inflammation in these animals was accompanied by downregulated serum concentrations of inflammatory IL-12 [$p < 0.05$; Figure 3(E)] and upregulated serum levels of immunosuppressive IL-10 [$p < 0.01$; Figure 3(F)].

IDO inhibition completely abrogated Treg-dependent recovery from DSS-induced colitis in BALB/c mice

We next investigate whether IDO inhibition affects Treg-dependent recovery of BALB/c mice with DSS-induced colitis [Figure 4(A)]. As shown in Figure 4, 1-MT aggravated DSS-induced colon injury and inflammation in BALB/c mice. Loss of fecal consistency, bloody diarrhea and notable weight loss resulted in significantly increased DAI in 1-MT+DSS-treated BALB/c mice [$p < 0.05$; Figure 4(B)]. Consistent with the exacerbated clinical signs, greater severity in histological damage, evidenced by a widely disrupted tissue architecture, the disappearance of intestinal crypts and goblet cells, marked mucosal hypertrophy and massive infiltration of inflammatory cells was observed in 1-MT+DSS-treated BALB/c mice [Figure 4(Db)]. Significantly higher DAI [$p < 0.05$; Figure 4(B)] and histological score [$p < 0.05$; Figure 4(C)], accompanied by an increased serum level of IL-12 [$p < 0.05$; Figure 4(E)], a decreased serum level of IL-10 [$p < 0.01$; Figure 4(F)] and a significantly lower number of colon-infiltrating, IL-10-producing Tregs [$p < 0.05$; Figure 4(G)] were noticed in 1-MT+DSS-treated BALB/c mice compared with DSS-only treated animals. These findings strongly suggest the crucial importance of IDO activity in Treg-dependent recovery from DSS-induced colon injury and inflammation, and induction of mucosal healing in BALB/c mice.

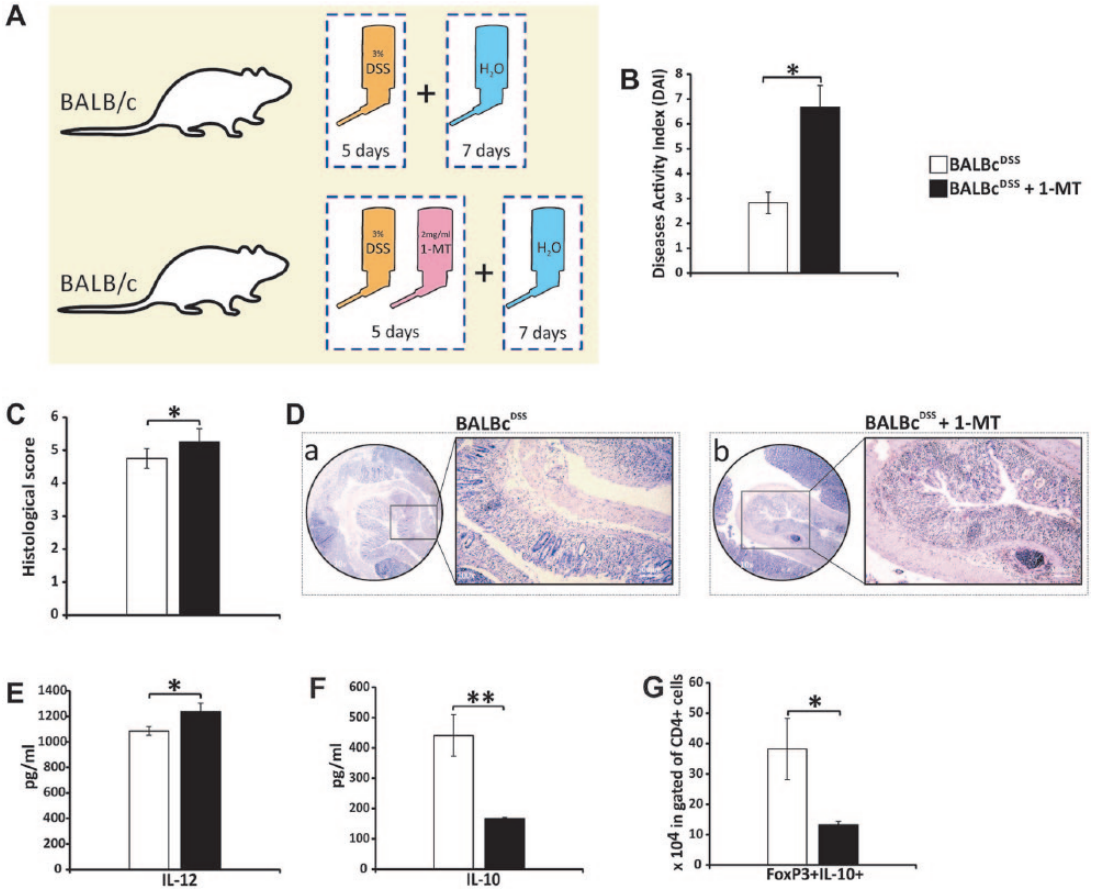

Figure 4. Indolamine 2,3-dioxygenase (IDO) inhibition completely abrogated T-regulatory cell (Treg)-dependent recovery from dextran sodium sulphate (DSS)-induced colitis in BALB/c mice. BALB/c DSS-treated mice continuously, during DSS administration, received 1-methyltryptophan (1-MT) dissolved in drinking water (A). A significantly increased Disease Activity Index (DAI) (B) and histological score (C) were observed in 1-MT+DSS-treated BALB/c mice compared with DSS-only treated animals. Representative images of H&E stained colon tissues demonstrate widely disrupted tissue architecture, the disappearance of intestinal crypts and goblet cells, marked mucosal hypertrophy and massive infiltration of inflammatory cells in 1-MT+DSS-treated BALB/c mice (Db, 10×/100×). An increased serum level of interleukin (IL)-12 (E), a decreased serum level of IL-10 (F) and a significantly lower number of colon-infiltrating, IL-10-producing Tregs (G) were noticed in 1-MT+DSS-treated BALB/c mice compared with DSS-only treated animals. Mean ± standard error of the mean; n = 10/group. *$p < 0.05$; **$p < 0.01$.

DCs, in IDO-dependent manner, promoted expansion of immunosuppressive Tregs enabling mucosal healing in DSS-treated BALB/c mice

To confirm that activity of IDO in DCs is mainly responsible for kynurenine-dependent expansion of immunosuppressive Tregs and consequent maintenance of mucosal healing in DSS-treated BALB/c mice, DCs with normal or suppressed IDO activity ($DCs^{1\text{-}MT}$) were transferred into DSS-treated BALB/c animals that were previously depleted of DCs (Figure 5). DSS-induced colitis in BALB/c mice was shown to be significantly more severe when DCs were selectively depleted, manifested by an increased DAI [Figure 5(A)] and histological score [Figure 5(B)]. Similar to that observed in DSS-treated C57BL/6 mice with progressive disease [Figure 5(Ca)], the abnormal structure of crypt glands, disrupted epithelium and severe edema accompanied by massive infiltration of inflammatory cells were noticed in the colonic mucosa of DC-depleted DSS-treated BALB/c mice [Figure 5(Cb)]. Exacerbated colitis observed in DC-depleted DSS-treated BALB/c mice was accompanied by a significant decrease in serum levels of kynurenine [$p < 0.05$; Figure 5(D)] and a significant reduction in the total number of colon-infiltrating Tregs [$p < 0.05$; Figure 5(E)], indicating the importance of DC-derived

kynurenine for the expansion of Tregs and consequent attenuation of DSS-induced colitis.

Interestingly, all criteria used for clinical (weight loss, stool consistency and rectal bleeding) and histological evaluation of DSS-induced colitis (damage of epithelium, infiltration of inflammatory cells) were notably attenuated in DC-depleted DSS-treated BALB/c mice that received DCs, resulting in a significant decrease in DAI [$p < 0.01$; Figure 5(A)] and histological score [$p < 0.05$; Figure 5(B)]. Moreover, significantly higher serum levels of kynurenine [$p < 0.05$; Figure 5(D)] accompanied by a remarkable increase in the total number of colon-infiltrating Tregs [$p < 0.05$; Figure 5(E)] were noticed in DC-depleted DSS-treated BALB/c mice that received DCs.

Most importantly, suppression of IDO activity in DCs completely abrogated their capacity to promote expansion of immunosuppressive Tregs and to induce mucosal healing in DC-depleted DSS-treated BALB/c mice. Transfer of $DCs^{1\text{-}MT}$ neither attenuated colitis nor altered the total number of Tregs in the colons of DC-depleted DSS-treated BALB/c mice [Figure 5(E)], confirming that DCs, in an IDO-dependent manner, promoted the expansion of colon-infiltrating Tregs, enabling mucosal healing in DSS-treated BALB/c mice.

Increased serum and fecal levels of kynurenine are noticed in patients with UC and mucosal healing

To investigate the relevance of experimental findings for corresponding human pathology, we analyzed the concentration of kynurenine in serum and stool samples of healthy controls and patients with UC who were divided into two groups based on the presence or absence of mucosal healing (Table 1). The serum level of kynurenine was higher in patients with UC compared with healthy controls [Figure 6(A)]. Interestingly, significantly higher serum levels of kynurenine were observed in patients with UC and mucosal healing than in patients with UC and chronic persistent disease [$p < 0.05$; Figure 6(A)]. Clinical score which was significantly lower in patients with mucosal healing [$p < 0.01$; Figure 6(B)] negatively correlated with serum concentrations of kynurenine [$r = -0.418$, $p < 0.01$; Figure 6(C)]. These findings were in line with differences in histological score between these two groups of patients with UC [$p < 0.01$; Figure 6(D)]. Histological analysis revealed that higher serum levels of kynurenine were noticed in patients with UC and mucosal healing manifested by increased regeneration of epithelium and the disappearance of crypt abscesses in the colon [Figure 6(Ea)]. By contrast, lower serum levels of kynurenine were noticed in patients with UC and chronic persistent disease manifested by the presence of large abscesses and increased infiltration of inflammatory cells in the injured colons [Figure 6(Eb)].

In line with these results, serum levels of kynurenine negatively correlated ($r = -0.290$, $p < 0.05$) with endoscopic subscore, which was significantly lower in patients with UC and mucosal healing [$p < 0.01$; Figure 6(F)]. Lower serum levels of kynurenine were noticed in patients with UC and chronic persistent disease manifested by marked erythema, friability, erosions and absent vascular patterns on endoscopy [Figure 6(Gb)], while all endoscopic parameters were in remission [Figure 6(Ga)] in patients with UC and mucosal healing that had higher serum levels of kynurenine.

Additionally, a significantly higher concentration of kynurenine in the serum samples of patients with UC and mucosal healing corresponded with significantly lower serum levels of inflammatory mediators: CRP [$p < 0.01$; Figure 6(H)], TNF-α [$p < 0.05$; Figure 6(I)] and CXCL11 [$p < 0.05$; Figure 6(J)].

Similar to that observed in serum samples [Figure 6(A)], the concentration of kynurenine was higher in patients with UC compared with healthy controls [Figure 6(K)]. Importantly, the concentration of kynurenine in stool samples was significantly higher in patients with UC and mucosal healing [$p < 0.05$; Figure 6(K)] and it negatively correlated ($r = -0.335$, $p < 0.05$) with concentration of fecal calprotectin, which was significantly lower in patients with mucosal healing [$p < 0.01$; Figure 6(L)]. A significantly higher concentration of kynurenine in the stool samples of patients with UC and mucosal healing [Figure 6(K)] corresponded with significantly lower fecal levels of inflammatory mediators: TNF-α [$p < 0.05$; Figure 6(M)], IL-17 [$p < 0.05$; Figure 6(N)] and CXCL11 [$p < 0.05$; Figure 6(O)], indicating that both serum and fecal levels of kynurenine negatively correlated with colon injury and inflammation.

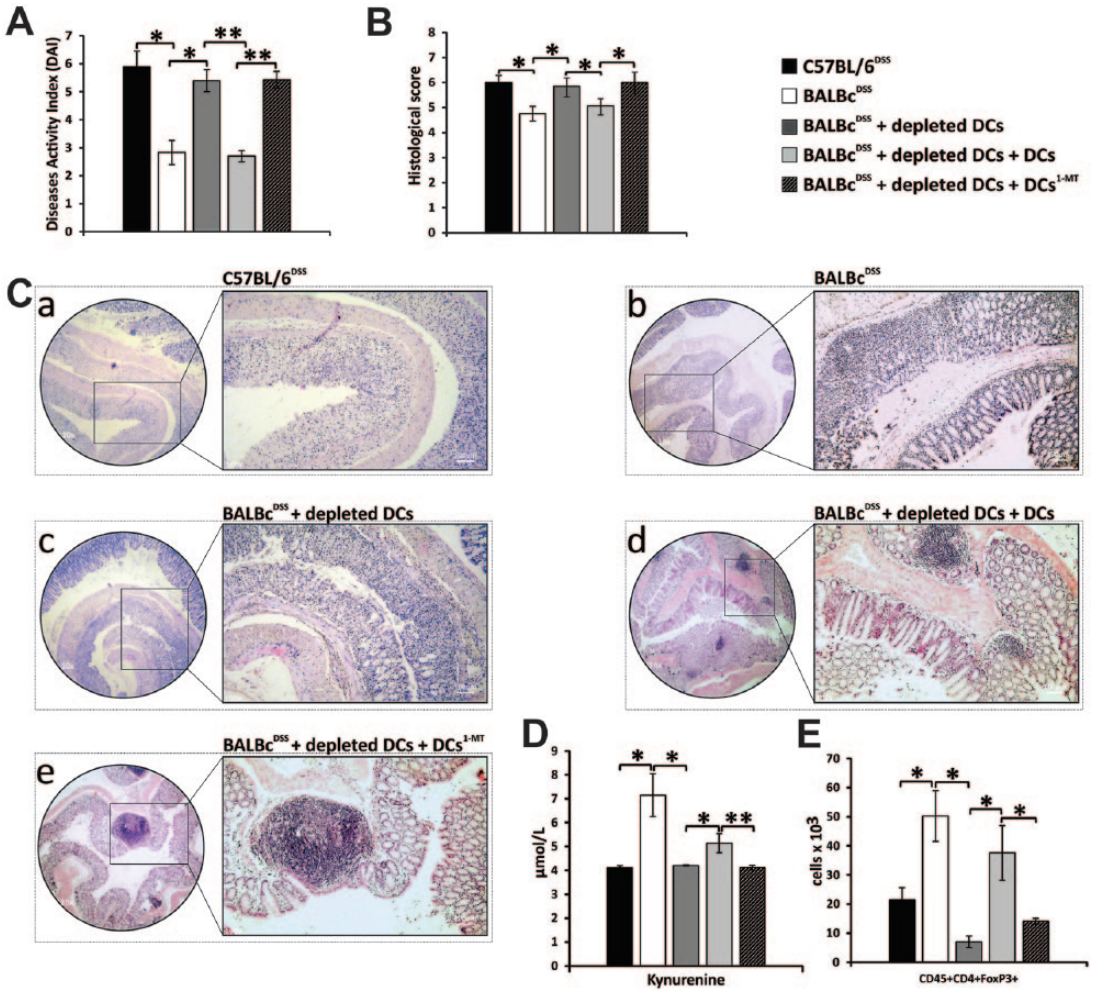

Figure 5. Dendritic cells (DCs), in an indolamine 2,3-dioxygenase (IDO)-dependent manner, promoted expansion of immunosuppressive T-regulatory cells (Tregs) enabling mucosal healing in dextran sodium sulphate (DSS)-treated BALB/c mice. A significantly increased Disease Activity Index (DAI) (A) and histological score (B) were observed in DC-depleted DSS-treated BALB/c mice compared with DSS-only treated BALB/c animals. Representative images of H&E stained colon tissues demonstrate the abnormal structure of crypt glands, disrupted epithelium and severe edema accompanied by massive infiltration of inflammatory cells in the samples of DC-depleted DSS-treated BALB/c mice (Cb), which were similar to those obtained from DSS-treated C57BL/6 mice with progressive disease (Ca). Depletion of DCs resulted in a significant decrease in the serum levels of kynurenine (D) and a significant reduction in the total number of colon-infiltrating Tregs (E). A remarkably lower DAI (A) and histological score (B), attenuated colon injury and inflammation (Cd), significantly higher serum levels of kynurenine (D) accompanied by a remarkable increase in the total number of colon-infiltrating Tregs (E) were noticed in DC-depleted DSS-treated BALB/c mice that received DCs. Transfer of $DCs^{1\text{-}MT}$ did not attenuate DAI (A), histological score (B), colon injury (Ce), serum level of kynurenine (D) and total number of colon-infiltrating Tregs in DC-depleted DSS-treated BALB/c mice. Mean ± standard error of the mean; n = 6/group. *$p < 0.05$; **$p < 0.01$. 1-MT, 1-methyltryptophan.

Increased serum and fecal levels of kynurenine were accompanied by increased presence of immunosuppressive Tregs in colons of patients with UC and mucosal healing

Similar to that observed in animal models, increased serum levels of kynurenine [Figure 6(A)] were accompanied by a significantly higher serum level of immunosuppressive IL-10 [$p < 0.05$; Figure 7(A)]. Additionally, there was a positive correlation between the fecal level of kynurenine [Figure 6(K)] and the fecal level of IL-10 ($r = 0.467$, $p < 0.01$), which was significantly higher in the stool samples of patients with UC and mucosal healing compared with patients with UC and persistent inflammation [$p < 0.05$; Figure 7(B)]. These findings corresponded with a remarkably increased presence of immunosuppressive cells [Tregs: $p < 0.01$; Figure 7(C);

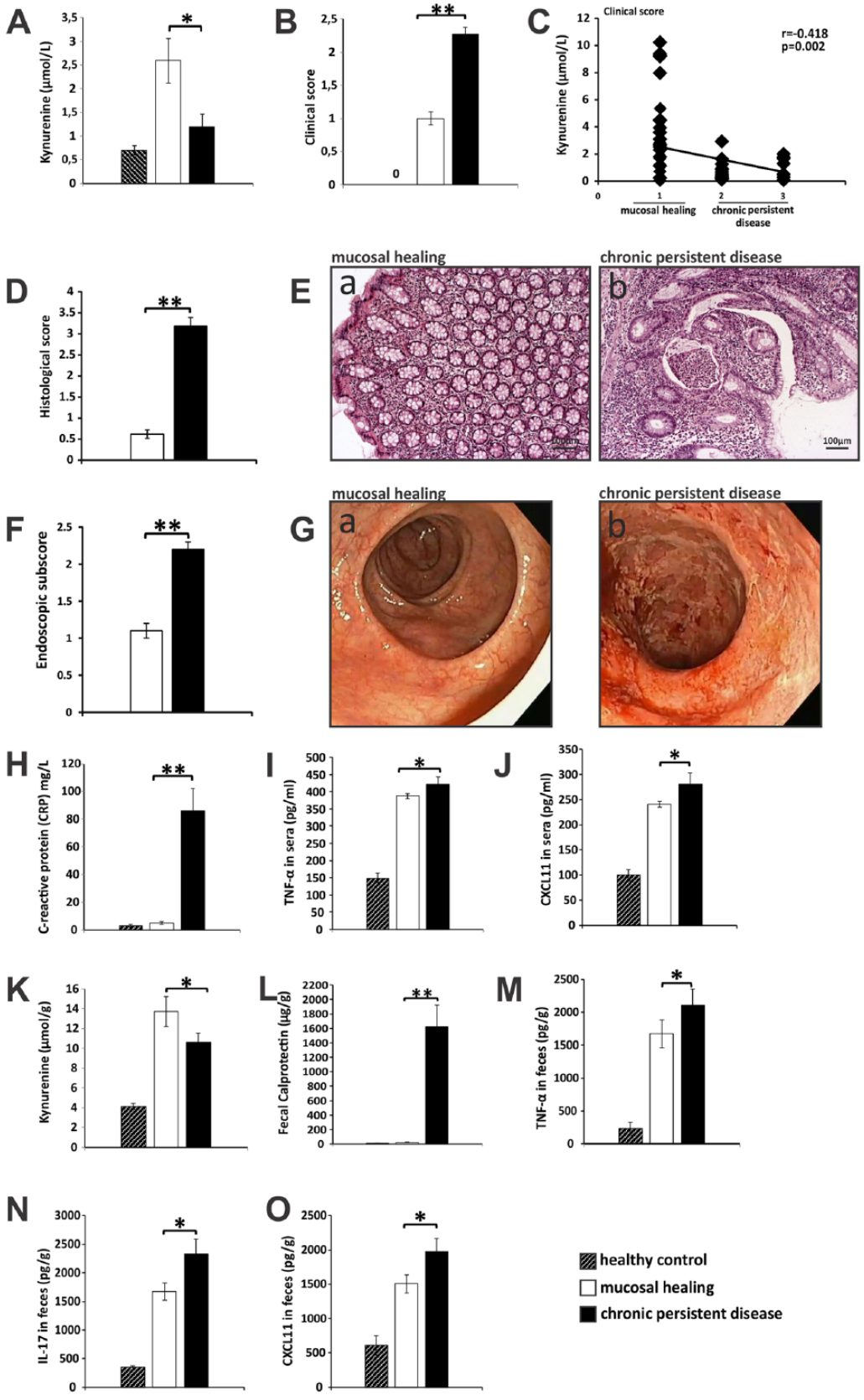

Figure 6. Increased serum and fecal levels of kynurenine are noticed in patients with ulcerative colitis (UC) and mucosal healing. The serum levels of kynurenine were significantly higher in patients with UC compared with healthy controls and were significantly

Figure 6. (Continued)

higher in patients with UC and mucosal healing compared with patients with UC and chronic persistent disease (A). Clinical score (B) and histological score (D) were significantly lower in patients with mucosal healing. Clinical score negatively correlated with serum concentrations of kynurenine (C). Mucosal healing was manifested by resolution of acute inflammation, regeneration of epithelium and disappearance of crypt abscesses in the colon (Ea, 100×). By contrast, chronic persistent disease was manifested by the presence of large abscesses and increased infiltration of inflammatory cells in the injured colons (Eb, 100×). The endoscopic subscore was significantly lower in patients with UC and mucosal healing (F). On endoscopy, chronic persistent disease was manifested by marked erythema, friability, erosions and absent vascular patterns (Gb), while all endoscopic parameters were in remission in patients with UC and mucosal healing (Ga). Significantly lower serum levels of inflammatory mediators, C-reactive protein (CRP) (H), tumor necrosis factor (TNF)-α (I) and CXCL11 (J) were noticed in patients with UC and mucosal healing. The concentration of kynurenine in stool samples was significantly higher in patients with UC compared with healthy controls (K). Also, the concentration of kynurenine in stool samples was significantly higher in patients with UC and mucosal healing compared with patients with UC and chronic persistent disease (K) and it corresponded with significantly lower stool levels of fecal calprotectin (L), TNF-α (M), interleukin (IL)-17 (N) and CXCL11 (O). Mean ± standard error of the mean; *$p < 0.05$; **$p < 0.01$.

IL-10-producing CD4+ T cells: $p < 0.05$; Figure 7(D); and IL-10-producing CD56+ natural killer (NK) cells: $p < 0.05$; Figure 7(E)] and with significantly reduced infiltration of inflammatory cells [IFN-γ-producing CD4+ T cells: $p < 0.05$; Figure 7(F); and IL-17-producing CD56+ NK cells: $p < 0.05$; Figure 7(G)] in the gut of patients with UC and mucosal healing compared with patients with UC and chronic persistent disease.

Discussion

In this paper, by using an experimental model and clinical data, we demonstrated that measurement of serum and fecal levels of kynurenine could be used as a new diagnostic approach that can complement endoscopy, CRP and fecal calprotectin for monitoring or predicting mucosal healing in patients with UC. Additionally, our results indicate the therapeutic potential of IDO-dependent expansion of endogenous Tregs as a possible new therapeutic approach for the induction of mucosal healing in patients with UC.

Diverse IDO activity manifested by different serum levels of kynurenine in DSS-treated BALB/c and C57BL/6 mice [Figure 1(G)], which were used as animal models for mucosal healing or chronic, persistent colitis,[14] indicated the importance of the IDO and kynurenine pathway for mucosal healing in UC. As shown in Figure 4, *in vivo* IDO inhibition completely abrogated mucosal healing in DSS-treated BALB/c mice. Opposite to our findings are the recently published results by Shon and colleagues,[38] who investigated acute DSS-induced colitis in IDO-deficient mice on a C57BL/6 background. They reported that genetic deletion of IDO protected against DSS-induced colitis. We believe that strain-dependent differences in DSS-induced colitis between C57BL/6 and BALB/c mice[14] might be responsible for the contrasting results obtained by us and Shon and colleagues. Additionally, Shon and coworkers investigated the impact of IDO deficiency on acute DSS-induced colitis, which is a T-cell-independent disease,[39] while we evaluated the effects of IDO inhibition 12 days after DSS administration when T cells play an important role in the pathogenesis of colitis.[40] Similar to our results, several experimental studies demonstrated that inhibition of IDO activity worsens colitis while induction of IDO expression limits disease progression,[12,41–43] indicating the importance of IDO activity in attenuation of colon inflammation. In line with these findings, we assume that striking differences in clinical and histological manifestations of DSS colitis in C57BL/6 mice with persistent disease and BALB/c mice with mucosal healing were consequences of IDO- and kynurenine-dependent effects on colon-infiltrating Tregs.

Similar to that observed in animal models, serum and fecal levels of kynurenine were increased in patients with UC and mucosal healing, and both negatively correlated with disease severity, serum levels of CRP and concentration of fecal calprotectin (Figure 6). These findings indicate that measurement of kynurenine in the serum and fecal samples of patients with UC could be used as a new diagnostic approach for the prediction or monitoring of mucosal healing in patients with UC.

Currently, measurement of fecal calprotectin is the most commonly used stool-based test for assessing

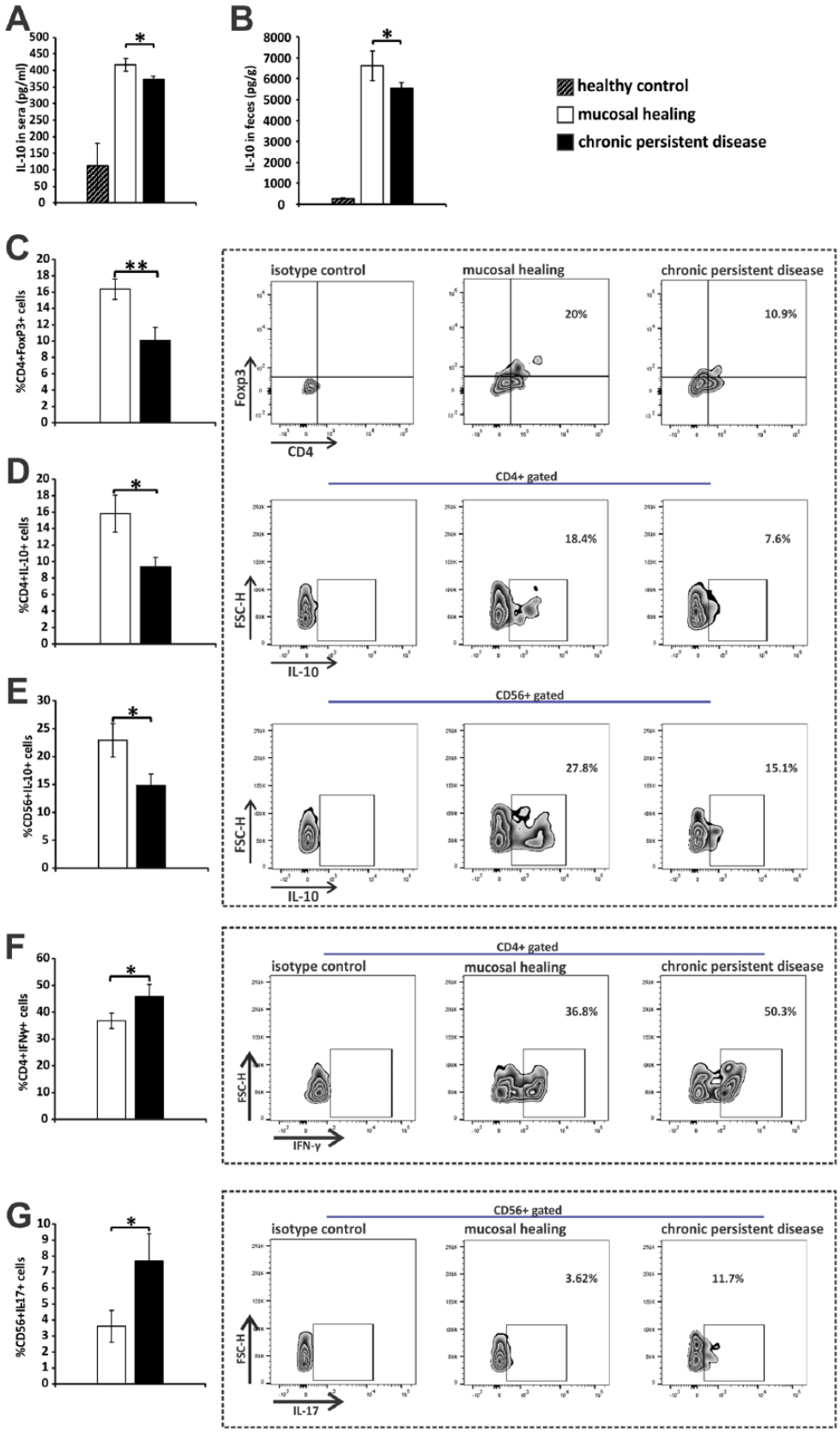

Figure 7. Increased serum and fecal levels of immunosuppressive kynurenine and interleukin (IL)-10 were accompanied by increased presence of T-regulatory cells (Tregs) in the colons of patients with UC and mucosal

Figure 7. (Continued)

healing. Increased serum (A) and fecal levels of IL-10 (B), significantly higher percentage of immunosuppressive Tregs (C), IL-10-producing CD4+ T cells (D) and IL-10-producing CD56+ natural killer (NK) cells (E) and a notably lower percentage of inflammatory interferon (IFN)-γ-producing CD4+ T cells (F) and IL-17-producing CD56+ NK cells (G) were noticed in patients with UC and mucosal healing compared with patients with UC and persistent disease. Representative dot plots are shown. Mean ± standard error of the mean; *$p < 0.05$; **$p < 0.01$. FSC-H, forward scatter height.

progression of UC.[44] Reduction in the concentration of fecal calprotectin represents the most reliable predictor of mucosal healing in patients with UC.[45] Nevertheless, the fecal calprotectin test lacks a validated cutoff, optimal specificity and accuracy, indicating the need for other stool-based biomarkers to complement fecal calprotectin in monitoring mucosal healing.[44] Correlation between increased fecal levels of kynurenine [Figure 6(K)] and decreased concentration of fecal calprotectin [Figure 6(L)] noticed in patients with UC and mucosal healing indicates that measurement of kynurenine in the fecal samples of patients with UC could become a useful diagnostic tool that can complement fecal calprotectin in monitoring or predicting mucosal healing.

In the inflammatory microenvironment of the gut, the balance between pro- and anti-inflammatory cytokines regulates progression of UC towards mucosal healing or chronic inflammation.[46] Upregulation of DC-derived inflammatory cytokines (IL-1β and IL-12) in injured colons and their elevated concentration in serum samples follow the development of chronic colitis in C57BL/6 mice, while increased serum levels of immunosuppressive IL-10 is accompanied by restoration of the mucosal barrier in BALB/c mice.[14] In line with these findings, BALB/c DCs, compared with C57BL/6 DCs, had reduced capacity for antigen presentation [Figure 2(D)] and respond to specific (DSS) and general (LPS) stimuli, mainly by producing immunosuppressive kynurenine and IL-10 [Figure 2(D–G)]. Accordingly, mucosal healing noticed in BALB/c mice was accompanied by increased serum levels of kynurenine [Figure 1(G)], elevated serum levels of IL-10 [Figure 1(J)], an increased number of colon-infiltrating, IL-10-producing DCs [Figure 2(G)], decreased serum levels of IL-1β and IL-12 [Figure 2(D)] and a decreased number of IL-1β and IL-12-producing DCs in the gut [Figure 2(E, F)], indicating a negative correlation between the serum levels of kynurenine and inflammatory cytokines in DSS-treated mice.

Similar to that observed in DSS-induced colitis, an increased concentration of kynurenine in the serum and fecal samples of patients with UC and mucosal healing was accompanied by decreased serum and fecal levels of inflammatory cytokines and elevation of anti-inflammatory IL-10 (Figure 6). In human pathology, among inflammatory cytokines, TNF-α and IL-17 are the most important for progression of UC towards chronic inflammation.[1,47] Reduction of serum levels of TNF-α predicts long-term remission of UC[48] and the use of anti-TNF-α biological agents represents the most effective therapeutic approach for inducing mucosal healing in patients with UC.[49] Accordingly, downregulation of serum and fecal levels of TNF-α, followed by decreased levels of IL-17, are associated with achievement of mucosal healing in patients with UC who receive anti-TNF-α biologics.[50] Additionally, elevated serum and fecal levels of TNF-α and IL-17 in patients with UC are always accompanied by increased production of CXCL11, which is important for recruitment of activated IFN-γ- and IL-17-producing T lymphocytes and NK cells in the inflamed gut.[51,52] Through the production of IFN-γ, gut-infiltrated T cells induce activation of IDO, resulting in the conversion of tryptophan in kynurenine that, in turn, increases expression of the IL-10 receptor on intestinal epithelial cells, enabling IL-10-dependent mucosal healing[53] mediated by IL-10-producing T cells which rapidly migrate to the injured colons where they attenuate Th1- and Th17-driven inflammation.[54] Accordingly, induction of mucosal healing is manifested by downregulation of CXCL11, reduced influx of inflammatory cells and increased presence of IL-10-producing anti-inflammatory T and NK cells in the injured colons.[52] In line with these findings, increased serum and fecal levels of kynurenine, observed in patients with UC and mucosal healing [Figure 6(A, K)], correlated with elevated serum and fecal levels of immunosuppressive IL-10 [Figure 7(A, B)] and were accompanied by downregulated serum and fecal levels of CXCL11 [Figure 6(J, O)], reduced

presence of colon-infiltrating IFN-γ-producing T lymphocytes [Figure 7(F)] and IL-17-producing NK cells [Figure 7(G)] and increased presence of immunosuppressive IL-10-producing T lymphocytes, NK cells and Tregs [Figure 7(C–E)].

Tregs accumulate in the injured colons of DSS-treated mice and are present at increased density in the colon samples of patients with UC, where, through the production of immunosuppressive IL-10, they suppress IFN-γ-producing Th1 and IL-17-producing Th17 cells, resolving ongoing inflammation.[54] Here, we have demonstrated that Tregs are crucially important for the maintenance of mucosal healing and recovery of BALB/c mice from DSS-induced colitis, since their depletion led to the significant aggravation of colitis (Figure 3). Importantly, adoptive transfer of BALB/c DCs managed to increase the serum levels of kynurenine [Figure 3(G)] in Treg-depleted DSS-treated BALB/c mice which was accompanied by the generation and enhanced presence of Tregs in the colons of CY+DSS- and anti-CD25+DSS-treated mice [Figure 3(H)], resulting in the attenuation of colon inflammation [Figure 3(B–D)]. These results strongly indicate that DCs promoted expansion of colon-infiltrating Tregs in DSS-treated BALB/c animals and induced mucosal healing. It is well known that kynurenine, the product of DC-derived IDO, promotes the expression of Treg lineage-defining transcription factor FoxP3 in CD4+ T cells and enables generation of Tregs.[12] Indeed, increased IDO activity, noticed in BALB/c DCs [Figure 2(B, C)], was followed by significantly lower numbers of Th1 and Th17 cells [Figure 2(I, J)] and a notably increased number of Tregs in the colons of DSS-treated BALB/c mice [Figure 2(M)]. This indicated that, in the injured gut, DCs in an IDO-dependent manner promote conversion of inflammatory T cells in immunosuppressive Tregs, enabling mucosal healing. Although macrophages (particularly alternatively activated) and B cells may express enhanced IDO activity,[55] the highest expression of IDO is, in the inflamed gut, noticed in colon-infiltrating tolerogenic DCs.[12] In line with these findings, depletion of DCs completely abrogated Treg-dependent mucosal healing in DSS-treated BALB/c mice (Figure 5). Attenuation of DSS-induced colitis in DC-depleted BALB/c mice was observed when these animals received DCs. Transfer of DCs was accompanied by a significant increase in the serum levels of kynurenine [Figure 5(D)] followed by a significantly higher presence of colon-infiltrating Tregs in DC-depleted DSS-treated BALB/c mice [Figure 5(E)]. Most importantly, suppression of IDO activity in DCs completely abrogated their capacity to promote expansion of immunosuppressive Tregs and to induce mucosal healing in DC-depleted DSS-treated BALB/c mice (Figure 5), confirming the crucial importance of DC-derived IDO for expansion of Tregs and the consequent induction and maintenance of mucosal healing.

Interestingly, several studies have shown that immunosuppressive Tregs are present in significantly higher numbers in the intestines of patients with UC than in healthy subjects.[56–58] Our findings are in line with these results [Figure 6(A)], as are data obtained by Nikolaus and colleagues,[7] who noticed elevated serum levels of kynurenine in patients with UC compared with healthy controls. It is well known that immunosuppressive Tregs represent a great fraction of lamina propria CD4+ T cells in the colons of patients with UC and that their number is increased during the remission of disease.[57] In line with these findings were significantly increased serum and fecal levels of kynurenine [Figure 6(A, K)], followed by an increased presence of colon-infiltrating Tregs [Figure 7(C)] that we noticed in patients with UC and mucosal healing compared with patients with UC and chronic persistent disease. This confirms the importance of the IDO/kynurenine pathway for the expansion of endogenous Tregs during mucosal healing. Since the clinical application of Tregs in patients with UC is not easy to perform given their rarity in peripheral blood,[58] IDO-dependent expansion of endogenous Tregs should be further explored as a potentially new approach for the induction and maintenance of mucosal healing in patients with UC. Since colon epithelial cells exhibit an IDO-activity-dependent proliferation phenotype, careful monitoring will be important in clinical trials which are going to investigate the therapeutic potential of agents that will induce IDO expression in patients with UC.[59]

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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