

# Ablation of IL-17A leads to severe colitis in IL-10-deficient mice: implications of myeloid-derived suppressor cells and NO production

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## Abstract

**IL-10 is an immune regulatory cytokine and its genetic defect leads to gastrointestinal inflammation in humans and mice. Moreover, the IL-23/T<sub>H</sub>17 axis is known to be involved in these inflammatory disorders. IL-17A, a representative cytokine produced by T<sub>H</sub>17 cells, has an important role for the pathological process of inflammatory diseases. However, the precise function of IL-17A in inflammatory bowel disease (IBD) remains controversial. In this study, we evaluated the effect of IL-17A on colitis in IL-10-deficient (*Il10*<sup>-/-</sup>) mice. Mice lacking both IL-10 and IL-17A (*Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup>) suffered from fatal wasting and manifested more severe colitis compared with *Il10*<sup>-/-</sup>*Il17a*<sup>+/-</sup> mice. Moreover, we found that CD11b<sup>+</sup>Gr-1<sup>+</sup> myeloid-derived suppressor cells (MDSCs) accumulated in the bone marrow, spleen and peripheral blood of *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice. These MDSCs highly expressed inducible nitric oxide synthase (iNOS) (*Nos2*) and suppressed the T-cell response *in vitro* in a NOS-dependent manner. In correlation with these effects, the concentration of nitric oxide was elevated in the serum of *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice. Surprisingly, the severe colitis observed in *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice was ameliorated in *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup>*Nos2*<sup>-/-</sup> mice. Our findings suggest that IL-17A plays suppressive roles against spontaneous colitis in *Il10*<sup>-/-</sup> mice in an iNOS-dependent manner and inhibits MDSC differentiation and/or proliferation.**

**Keywords:** colitis, IL-17A, MDSC, nitric oxide

## Introduction

IL-10 is an immunoregulatory cytokine, and genome-wide association studies (GWASs) showed its close relationship to human inflammatory bowel diseases (IBDs) (1–3). Moreover, homozygous loss-of-function mutations in IL-10 and IL-10 receptor genes manifest a neonatal onset of familial IBDs (4).

In concert with these human studies, IL-10 or IL-10 receptor-deficient mice spontaneously exhibit severe colitis (5). Since *Il10*<sup>-/-</sup>*Rag2*<sup>-/-</sup> mice fail to exhibit colitis (6) and T-cell-specific IL-10-deficient mice develop severe colitis comparable to that of *Il10*<sup>-/-</sup> mice (7), IL-10 derived from T cells is indispensable

for homeostasis of the gut mucosa. In addition, targeted disruption IL-23-specific subunit p19 gene, which abrogates development of  $T_H17$  cells but not  $T_H1$  cells, has been shown to rescue colitis of IL-10-deficient mice (8). This indicates the intimate involvement of  $T_H17$  cells in this model and accords with the finding that GWASs identify IL-23R as an IBD-susceptible gene (9). In the aggregate, IL-10-deficient mice could be regarded as a relevant model to study human IBD ontogeny, especially concerning the connection to the IL-23/ $T_H17$  axis. However, it still remains unclear which factor is important for the induction of colitis.

It has been reported that IL-17A plays crucial roles in several inflammatory conditions, such as IBD and cancer. For example, in colitis model mice, some reports have suggested that IL-17A promotes an inflammatory response (10–12), whereas other reports have shown that blockade of IL-17A by using neutralizing antibody exacerbates colitis (13). In cancer model mice, several reports have suggested that IL-17A promotes the progression of cancer (14, 15), whereas others have suggested that IL-17A has an antitumor effect (16, 17). Thus, the physiological roles of IL-17A under pathological conditions remain controversial.

Myeloid-derived suppressor cells (MDSCs) are myeloid-lineage cells which accumulate under pathological conditions such as cancer and inflammation, and these cells suppress the activation of T and natural killer (NK) cells (18, 19). Thus, it is thought that MDSCs promote cancer progression through the inhibition of immune surveillance and antitumor immunity. In colitis, the role of MDSCs is controversial. Several reports have shown that MDSCs are accumulated in colitis model mice, resulting in the amelioration of colitis (19, 20). On the other hand, Griseri *et al.* demonstrated that the neutralization of Granulocyte macrophage colony-stimulating factor (GM-CSF), which has a pivotal role for MDSC function and proliferation, attenuated colitis in mice (21). Currently, it remains unclear how MDSCs induced *in vivo* differentiate and accumulate and how they function in colitis.

Herein, to reveal the roles of IL-17A in chronic colitis, we introduced IL-17A-deficiency into the IL-10-deficient background mice. *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice exhibited more severe colitis and body weight loss compared with those of *Il10<sup>-/-</sup>* mice. We unexpectedly found that immunosuppressive myeloid-lineage cells, MDSCs, were dramatically increased in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. Moreover, we show that IL-17A-deficiency caused an enhanced IFN- $\gamma$  response followed by the up-regulation of inducible nitric oxide synthase (iNOS) (*Nos2*) in MDSCs. Finally, *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>Nos2<sup>-/-</sup>* mice failed to develop colitis. Taken together, these results suggest that IL-17A suppresses the colitis in *Il10<sup>-/-</sup>* mice, presumably through an iNOS-dependent pathway, and that the MDSCs–iNOS axis might exacerbate the pathology of *Il10<sup>-/-</sup>* mice.

## Methods

### Mice

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan) and used at 5–6 weeks of age. C57BL/6 *Il10<sup>-/-</sup>* and *Nos2<sup>-/-</sup>* mice were provided by the Jackson Laboratory. C57BL/6 *Il17a<sup>-/-</sup>* mice were the kind gift of Y. Iwakura (Tokyo

University of Science, Chiba, Japan). All animals were bred under specific-pathogen-free (SPF) conditions and were randomly assigned to cohousing irrespective of their genotype. All animal experimental procedures used in this study were performed in accordance with our institutional guidelines for animal experiments.

### Evaluation of colonic inflammation

The colonic weight/length ratio, which correlates well with the histological score and is recognized as an index of colonic edema, was calculated for the evaluation of colon inflammation. Briefly, the colon was excised and the fat and connective tissues were removed. After cutting longitudinally and washing out the fecal materials, the colon length and weight were measured. Independent experimental settings were subjected to histological analysis. Formalin-fixed, paraffin-embedded specimens derived from the middle to distal colons were sectioned transversely and stained with hematoxylin and eosin (HE) or with hematoxylin and periodic acid–Schiff (PAS).

### Flow cytometry analysis

Mononuclear cells from the peripheral blood (PB), bone marrow (BM) and spleen were obtained after lysing the red blood cells. A mesenteric lymph node (MLN) was smashed with a cell strainer, and then mononuclear cells were obtained. The small intestine and colon were cut into 1-cm long pieces and stirred for 20 min at 37°C in serum-free Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque, Kyoto, Japan) containing 5 mM ethylenediaminetetraacetic acid (EDTA) to dissociate epithelial and intra-epithelial cells. After washing with ice-cold phosphate buffered saline (PBS) three times, the remaining tissue was incubated for 50 min at 37°C with RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1 mg ml<sup>-1</sup> collagenase D (Roche, Basel, Schweiz) and 1 mg ml<sup>-1</sup> DNase I (Roche) with stirring. Mononuclear cells were further purified using a discontinuous Percoll gradient (40% and 75%) and subjected to flow cytometry. Before antigen staining, the cells were treated with antibodies to anti-CD16/32 (BioLegend, San Diego, CA, USA). The cells were stained with antibodies for 30 min at 4°C. The antibodies used were CD11b-PE-Cy7, Gr-1-FITC/PE, CD124 (IL-4R $\alpha$ )-PE, CD4-FITC, CD3 $\epsilon$ -PE-Cy7, CD19-allophycocyanin (APC) and CD11c-APC from eBioscience (San Diego, CA, USA), Ly-6G-APC, CD119 (IFN- $\gamma$ R1)-Biotin, CD284 (TLR4/MD2)-PE, H-2K<sup>b</sup>-FITC and CD8 $\alpha$ -APC-Cy7 from BioLegend, Ly-6C-APC-Cy7/V450, CD80-PE, CD86-PE, I-A<sup>b</sup>-PE and IFN- $\gamma$ -PE from BD Biosciences (San Jose, CA, USA) and F4/80-FITC from Serotec (Hercules, CA, USA). Streptavidin-FITC/PE were purchased from eBioscience. To remove dead cells, 7-AAD viability staining solution (eBioscience) was used. Flow cytometry analysis was performed using a MACS Quant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) or FACSCantoll (BD Biosciences).

### iNOS staining

For intracellular iNOS staining, a BD Cytofix/Cytoperm kit (BD Biosciences) was used according to the manufacturer's instructions. Briefly, the BM cells were stimulated with 2  $\mu$ g ml<sup>-1</sup>

of lipopolysaccharide (LPS; Sigma) for 24 h at 37°C, and BD GolgiPlug (BD Biosciences) was added for the last 6 h. The cells were harvested and subjected to dead-cell and surface staining, fixed and permeabilized with Cytotfix/Cytoperm solution, and stained for intracellular iNOS. Before antigen staining, the cells were stained with anti-CD16/32 (BioLegend). The cells were stained with antibodies for 30 min at 4°C. The antibodies used were CD11b-APC, CD45R (B220)-PE and iNOS-PE-Cy7 from eBioscience, Ly-6G-FITC and Ly-6C-APC-Cy7 from BioLegend and CD3ε-PerCP-Cy5.5 from BD Biosciences. To remove dead cells, a Zombie Aqua™ Fixable Viability Kit (BioLegend) was used. Flow cytometry analysis was performed using a FACSCanto II flow cytometer (BD Biosciences).

#### Isolation of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells, CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells

Mononuclear cells from the BM and spleen were obtained after lysing the red blood cells. To enrich CD11b<sup>+</sup>Gr-1<sup>+</sup> cells, BM cells and splenocytes were treated with anti-Ly-6G-biotin antibodies or anti-CD11b-biotin antibodies and anti-biotin microbeads (Miltenyi Biotec), followed by positive selection using an autoMACS Pro Separator (Miltenyi Biotec). To enrich CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells, splenocytes were treated with anti-CD4 microbeads or anti-CD8 microbeads (Miltenyi Biotec), respectively, followed by positive selection. Purities of the enriched cells were confirmed to be higher than 90%.

#### Morphologic analysis

Cells were spun down on slides using a Cytospin cytocentrifuge (Thermo Fisher Scientific, Kanagawa, Japan). Diff-Quick staining was performed (Sysmex, Hyogo Japan) according to the manufacturer's recommended protocol. Images were obtained using a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

#### RNA extraction, reverse transcriptase reaction and real-time PCR

A part of spleen and colon was freeze-milled under liquid nitrogen into a fine powder using CoolMill (Toyobo, Osaka, Japan). Total RNA from the powder or cells was isolated with ISOGEN (Wako Pure Chemical Industries, Osaka, Japan) and complementary DNA (cDNA) was synthesized with High Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific). Real-time PCR was performed by SYBR Green-based gene detection analysis using a StepOnePlus™ system (Thermo Fisher Scientific). The sequences for the primers were as shown in [Supplementary Table 1](#). Data analysis was based on the  $\Delta\Delta Ct$  method, with normalization by a house-keeping gene (*Gapdh*). The data on the expression levels of each gene were normalized by that of *Il10*<sup>-/-</sup>/*Il17a*<sup>-/-</sup> mice, and the clustered heatmap was generated by the softwares Cluster 3.0 (Stanford University) and TreeView (Free Software Foundation).

#### MDSC suppression of T-cell proliferation

Splenocytes were obtained after lysing the red blood cells, and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Dojindo, Kumamoto, Japan).  $5 \times 10^5$  splenocytes and various amounts of MDSCs were co-cultured for 4 days

in a 96-well plate in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) with 10% FBS, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 μM non-essential amino acid (NEAA), 1 mM sodium pyruvate, 55 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U ml<sup>-1</sup> penicillin and 0.1 mg ml<sup>-1</sup> streptomycin. To induce T-cell proliferation, the cells were stimulated with 1 μg of anti-CD28 in a 96-well plate pre-coated with 1 μg ml<sup>-1</sup> of anti-CD3ε antibodies overnight at 4°C. T-cell proliferation was measured by CFSE dilution using a MACS Quant Analyzer (Miltenyi Biotec).

#### Intracellular cytokine staining

To remove dead cells, splenocytes were stained with a LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen, Carlsbad, CA, USA) and antibodies for surface molecules. Stained cells were stimulated with phorbol 12-myristate-13-acetate (PMA) (50 ng ml<sup>-1</sup>; Sigma)/Ionomycin (500 ng ml<sup>-1</sup>; Wako), and cultured in the presence of BD GolgiStop (1 μg ml<sup>-1</sup>; BD Biosciences) for 6 h at 37°C. These cells were fixed and permeabilized with Cytotfix/Cytoperm (BD Biosciences), followed by anti-IFN-γ staining.

#### Enzyme-linked immunosorbent assay

To enrich CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells, splenocytes were treated with MojoSort™ anti-CD4 Nanobeads or anti-CD8 Nanobeads (BioLegend), respectively, followed by positive selection. The purities of the enriched cells were confirmed to be higher than 90%. CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells were stimulated with PMA (50 ng ml<sup>-1</sup>; Sigma)/Ionomycin (500 ng ml<sup>-1</sup>; Wako) and cultured for 6 h at 37°C. The culture supernatants were collected, and IFN-γ concentrations were measured using an OptEIA™ Mouse IFN-γ ELISA Set (BD Biosciences) according to the manufacturer's protocol.

#### Quantification of nitric oxide

To remove proteins, serum samples were applied to an Amicon Ultra-0.5 centrifugal filter unit with an Ultracel-10 membrane and centrifuged for 20 min at 14 000 rpm. Nitric oxide (NO) concentrations were determined using an NO<sub>2</sub>/NO<sub>3</sub> Assay Kit-C II (Colorimetric) (Dojindo) according to the manufacturer's protocol.

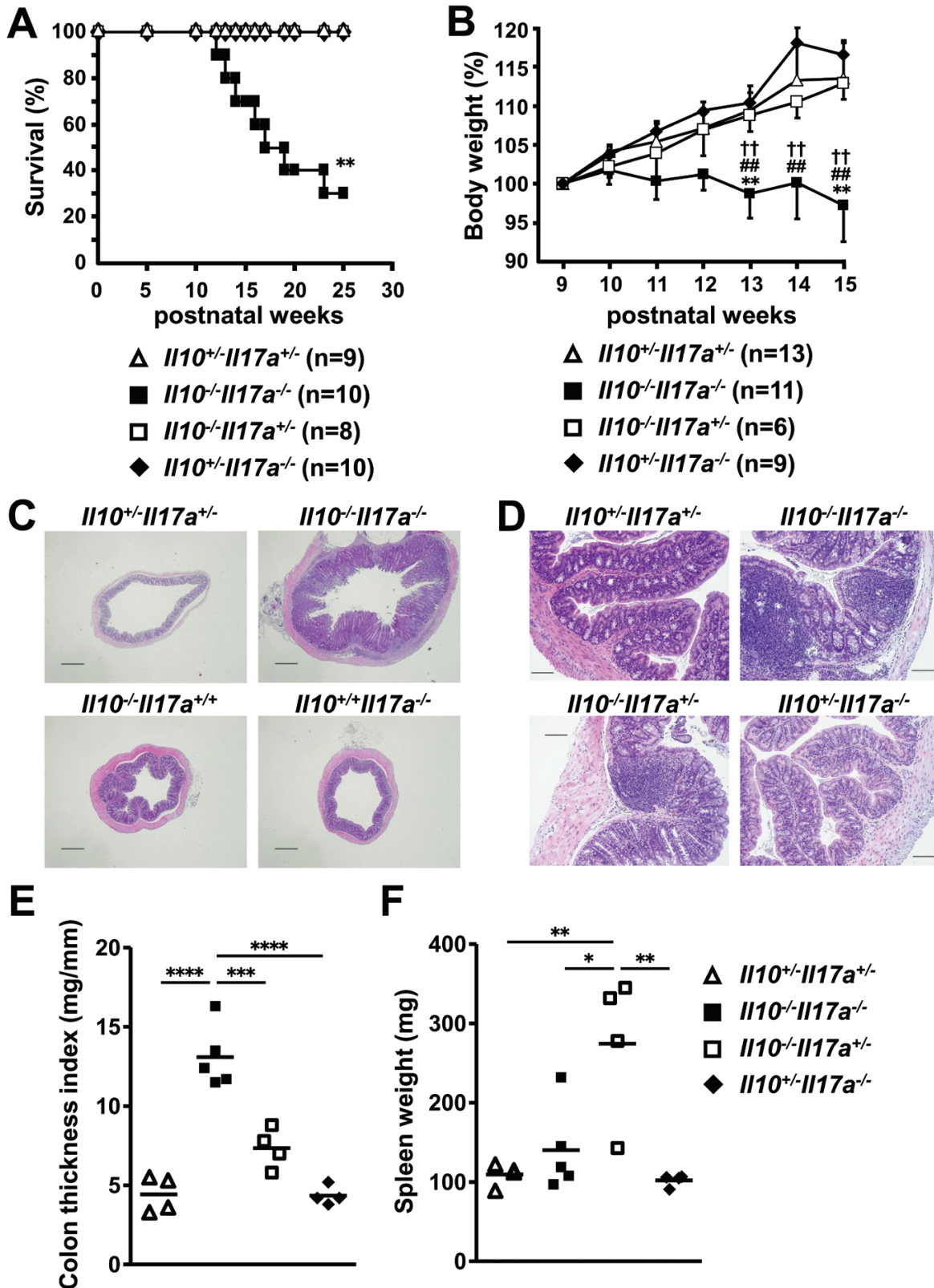
#### Statistics

All results are expressed as the mean ± standard error of the mean. Differences were analyzed for statistical significance by the Student's *t*-test, Mann-Whitney test or Kolmogorov-Smirnov test for comparison between two groups, one-way ANOVA for comparison among three or more groups, or log-rank test using Prism software (GraphPad Software Software, San Diego, CA, USA).

## Results

#### IL-17A suppressed spontaneous colitis in IL-10-deficient mice

To examine the effect of IL-17A on spontaneous colitis in *Il10*<sup>-/-</sup> mice, we generated *Il10*<sup>-/-</sup>/*Il17a*<sup>-/-</sup> mice. As shown in [Fig. 1\(A\)](#), the survival rate of *Il10*<sup>-/-</sup>/*Il17a*<sup>-/-</sup> mice reached 30% within 25 weeks after birth, and the body weight was significantly lower in *Il10*<sup>-/-</sup>/*Il17a*<sup>-/-</sup> mice than other mice after 13



**Fig. 1.** Survival, body weight changes, and symptoms of colitis of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. (A) Survival rates of *Il10<sup>+/-</sup>Il17a<sup>+/-</sup>*, *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>*, *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>* and *Il10<sup>+/-</sup>Il17a<sup>-/-</sup>* mice. \*\*Compared to other groups, log-rank test ( $P < 0.01$ ). (B) Body weight changes of each *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>*, *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>*, *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>* and *Il10<sup>+/-</sup>Il17a<sup>-/-</sup>* mice. Values represent the means  $\pm$  SEM ( $n = 6-13$ ). \*\*Compared to *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>*, ##compared to *Il10<sup>+/-</sup>Il17a<sup>-/-</sup>*, ††compared to *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>*, one-way ANOVA ( $P < 0.01$ ). (C and D) Representative histological images of HE-stained mid-colonic sections are shown. Bars indicate 500  $\mu$ m (C) and 100  $\mu$ m (D). (E) Colon length and weight of each mouse were measured and the length-to-weight ratio (colon thickness index) was calculated [\*\*\*\* $P < 0.0001$ , \*\*\*\* $P < 0.0001$  (one-way ANOVA)]. (F) Spleen weights were measured [\* $P < 0.05$ , \*\* $P < 0.01$  (one-way ANOVA)].

weeks of age (Fig. 1B). Histological analysis revealed that hyperplasia, cellular infiltration and severe ulceration were exacerbated in the colons of 12-week-old *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice (Fig. 1C and D). Additionally, the colon weight/length ratio (colon thickness index) was significantly increased (Fig. 1E). These results suggest that IL-17A is an important factor to limit or delay the onset of colitis in *Il10<sup>-/-</sup>* mice.

Like *Il10<sup>-/-</sup>* mice in a previous report (22), *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>* mice in the present study exhibited severe splenomegaly. On the other hand, surprisingly, *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice did not exhibit splenomegaly and their spleen weights were similar to those in IL-10-sufficient mice (Fig. 1F). Additionally, prominent inflammation was not observed in the thymus, pancreas, liver, heart, lung and kidney of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice (Supplementary Figure 1), suggesting that the impact of IL-17A-deficiency is restricted to the colon and accumulation of MDSCs. Therefore, it is most likely that either or both pathologies results in severe wasting leading to death. From these observations, it is speculated that there is a difference in inflammatory status, in terms of susceptibility to IL-17A-deficiency, between the systemic and mucosal compartment in *Il10<sup>-/-</sup>* mice.

#### *IL-17A-deficiency caused MDSC accumulation in IL-10-deficient mice*

We observed that the SSC<sup>high</sup> myeloid-lineage population was increased in the spleens of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. To characterize the myeloid-lineage cells in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice, we analyzed the surface marker expression on these cells in the BM, PB, spleen, MLN, small intestine and colon of 12- to 15-week-old *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. We found that the proportion and the numbers of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells were increased in the systemic compartments, such as BM and spleen (Fig. 2A). We also found that this proportion was increased in the mucosal compartments, except for the small intestinal lamina propria of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice (Fig. 2B). In *Il10<sup>-/-</sup>* mice, the proportions of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells were increased in the MLN and gut, suggesting that the increases of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells would be due to mild colitis (Fig. 1C–E). On the basis of the correlation between the severity of colitis and the proportion of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells (Figs 1C, D and 2B), we speculated that CD11b<sup>+</sup>Gr-1<sup>+</sup> cells were involved in the colitis. Further analysis revealed that most of the CD11b<sup>+</sup>Gr-1<sup>+</sup> subset accumulating in the BM, PB and spleen of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice showed the CD11b<sup>+</sup>Gr-1<sup>high</sup>Ly-6G<sup>+</sup>Ly-6C<sup>low</sup>F4/80<sup>low/-</sup> phenotype (Fig. 2C). This expression profile corresponded to polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), a subset of MDSCs (19). A morphological analysis revealed that each of these cells had a polymorphonucleus, and thus these cells corresponded to PMN-MDSCs (Fig. 2D). In addition, only CD11b<sup>+</sup>Gr-1<sup>high</sup>Ly-6G<sup>+</sup>Ly-6C<sup>low</sup>F4/80<sup>low/-</sup> PMN-MDSC-like cells in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice, but not CD11b<sup>+</sup>Gr-1<sup>mid</sup>Ly-6G<sup>-</sup>Ly-6C<sup>+</sup>F4/80<sup>+</sup> cells, exhibited an IL-4R $\alpha$ -positive and MHC class II-negative phenotype (Fig. 2E), which was similar to the phenotype of MDSCs in tumor-bearing mice (19). PMN-MDSC-like cells in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice also exhibited low-level expression of CD86, a co-stimulatory molecule, and MHC class I (Fig. 2E). These results suggest that the majority of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice showed PMN-MDSC-like surface marker expression patterns and morphology.

To clarify the mechanisms of MDSC accumulation in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice, we investigated the mRNA expression of several inflammatory molecules in the spleen and colon. We found that the mRNA expression levels of IL-17F (*Il17f*) and serum amyloid A1/2 (*SAA1/2*) both in the spleen and colon of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice were significantly higher than those in littermate *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>* mice (Fig. 2F). On the other hand, IL-22 (*Il22*), IL-1 $\beta$  (*Il1b*) and GM-CSF (*Csf2*) were expressed highly in some *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>* mice, and at lower levels in others, suggesting that these factors would not contribute to MDSC accumulation. Taken together, we speculate that IL-17F and SAA1/2 might coordinately enhance the MDSC accumulation in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice.

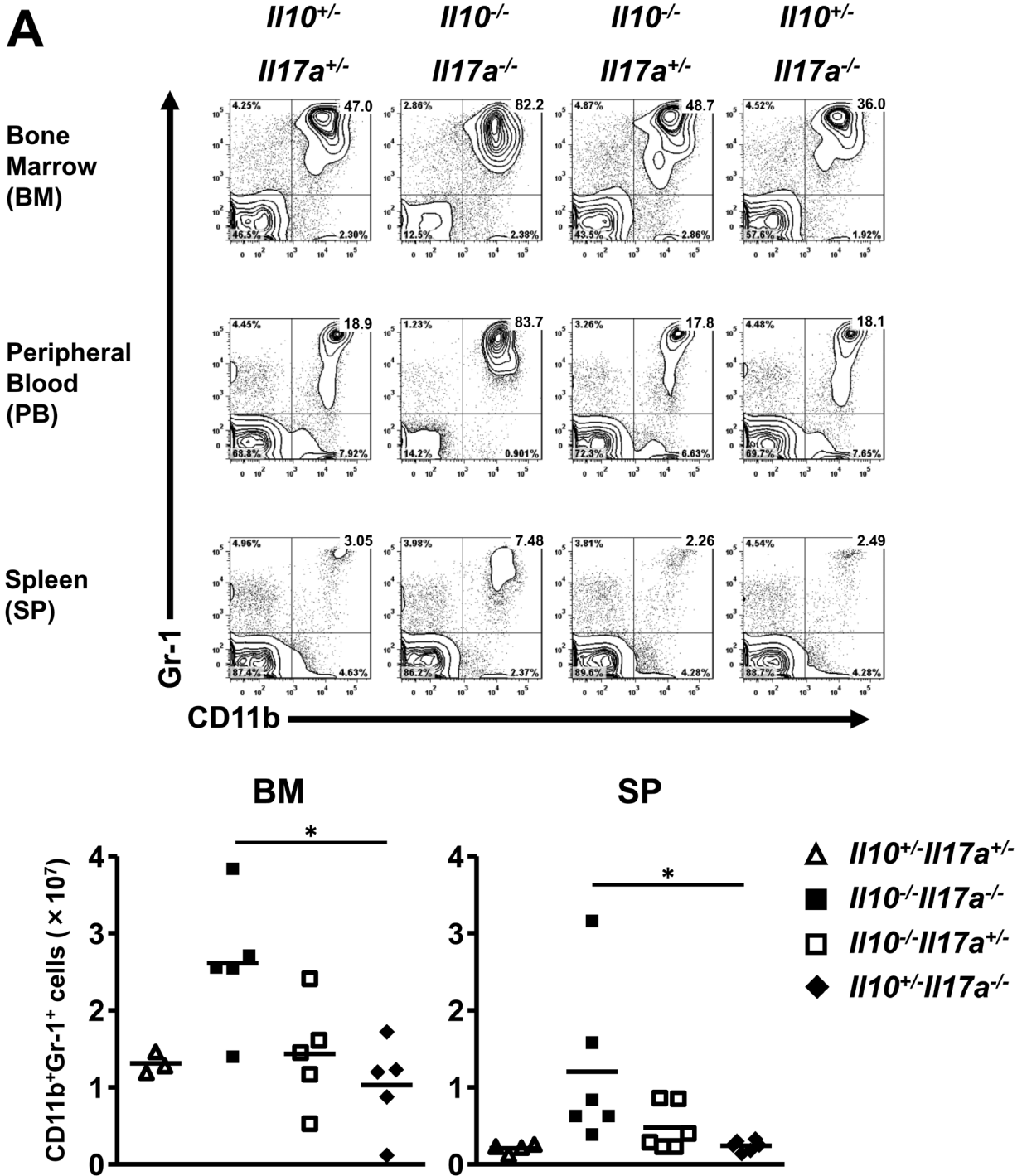
#### *Accumulating MDSCs highly expressed iNOS in Il10<sup>-/-</sup>Il17a<sup>-/-</sup> mice*

We next measured the mRNA expression levels of MDSC-related genes in CD11b<sup>+</sup>Gr-1<sup>+</sup> cells. Quantitative RT-PCR analysis revealed changes in the expression levels of MDSC-related genes, including suppressive molecules of MDSCs—that is, iNOS (*Nos2*), arginase (*Arg1*) and NADPH oxidase (*Cybb*). In particular, *Nos2* expression in CD11b<sup>+</sup>Gr-1<sup>+</sup> cells of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice was prominently higher than that in CD11b<sup>+</sup>Gr-1<sup>+</sup> cells of *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>* mice (Fig. 3A). On the other hand, in CD11b<sup>+</sup>Gr-1<sup>+</sup> cells of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice, the expression of *Arg1* and *Cybb* oxidase, one of the major reactive oxygen species (ROS) inducers, was lower than that in CD11b<sup>+</sup>Gr-1<sup>+</sup> cells of *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>* mice. Next, we checked iNOS expression among BM cells of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. We found that Ly-6C<sup>hi</sup>Ly-6G<sup>-</sup>CD11b<sup>+</sup> monocytes and the small population of Ly-6C<sup>low</sup>Ly-6G<sup>+</sup>CD11b<sup>+</sup> PMN-MDSCs expressed iNOS (Fig. 3B), suggesting that Ly-6C<sup>hi</sup>Ly-6G<sup>-</sup>CD11b<sup>+</sup> cells are M-MDSCs. On the other hand, T cells, B cells and CD11b<sup>-</sup> cells did not express iNOS. Thus, among BM cells, only M-MDSCs express iNOS. In addition, we examined the suppressive function of PMN-MDSC-like cells derived from *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice against T-cell responses. As shown in Fig. 3, PMN-MDSC-like cells suppressed CD4<sup>+</sup> T-cell proliferation. On the other hand, PMN-MDSC-like cells were less suppressive of CD8<sup>+</sup> T-cell proliferation. These results suggest that PMN-MDSC-like cells derived from *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice exhibit suppressive activity against T-cell proliferation. We observed a higher expression of *Nos2* in PMN-MDSC-like cells derived from *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. Since NO induces apoptosis and inhibits proliferation of T cells (23), we then hypothesized that PMN-MDSC-like cell-derived NO suppresses T-cell proliferation. In correlation with the higher *Nos2* expression, the suppressive activity of PMN-MDSC-like cells was canceled by a NOS inhibitor, N<sup>G</sup>-Monomethyl-L-arginine (L-NMMA) (Fig. 3C). These data suggested that the immune-suppressive activity of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice would largely depend on iNOS.

Taken together, these results suggested that IL-17A-deficiency resulted in the accumulation of PMN-MDSCs in mice with an IL-10-deficient background.

#### *IFN- $\gamma$ production of T cells was enhanced in Il10<sup>-/-</sup>Il17a<sup>-/-</sup> mice*

As shown above, PMN-MDSCs derived from *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice were less suppressive to CD8<sup>+</sup> T cells than CD4<sup>+</sup> T cells in



**Fig. 2.** Characterization of the MDSC-like subset in *IL10*<sup>-/-</sup>*IL17a*<sup>-/-</sup> mice. (A) CD11b and Gr-1 expression on mononuclear cells in bone marrow (BM), peripheral blood (PB) and spleen (SP) was analyzed by FACS (upper panels). The numbers of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in BM (left) and SP (right) are shown in lower graphs. Data are representative of at least five mice [*\*P* < 0.05 (one-way ANOVA)]. (B) CD11b and Gr-1 expression on mononuclear cells in mesenteric lymph node (MLN), small intestinal lamina propria (Si-LP) and colon lamina propria (C-LP) was analyzed by FACS. (C) Ly-6G/Ly-6C plots were gated on CD11b<sup>+</sup> cells among BM cells of *IL10*<sup>+/-</sup>*IL17a*<sup>+/-</sup> and *IL10*<sup>-/-</sup>*IL17a*<sup>-/-</sup> mice. Histograms show F4/80 expression on the cells gated as R1 and R2. Data are representative of two independent experiments. (D) The purified PMN-MDSC subset was subjected to Diff-Quick staining. (E) The expression of various surface markers was analyzed by FACS. Histograms show the indicated markers of *IL10*<sup>-/-</sup>*IL17a*<sup>+/-</sup> cells and *IL10*<sup>-/-</sup>*IL17a*<sup>-/-</sup> cells (dotted line and black line, respectively), and the isotype controls (shaded) represent cells gated as R1 and R2. Data are representative of two independent experiments. (F) Gene expression in the spleen and colon of *IL10*<sup>-/-</sup>*IL17a*<sup>-/-</sup> mice was measured by qRT-PCR. Data were normalized to the expression of the *Gapdh* housekeeping gene, and are shown relative to the expression levels in littermate *IL10*<sup>-/-</sup>*IL17a*<sup>+/-</sup> mice. Data were from *IL10*<sup>-/-</sup>*IL17a*<sup>-/-</sup> mice (*n* = 8) and *IL10*<sup>-/-</sup>*IL17a*<sup>+/-</sup> mice (*n* = 5) [*\*P* < 0.05, *\*\*P* < 0.01 (Kolmogorov–Smirnov test)]. *IL22* mRNA expression was from *IL10*<sup>-/-</sup>*IL17a*<sup>-/-</sup> mice (*n* = 5, 7) and *IL10*<sup>-/-</sup>*IL17a*<sup>+/-</sup> mice (*n* = 3, 4), since no expression was observed in some *IL10*<sup>-/-</sup>*IL17a*<sup>+/-</sup> mice.

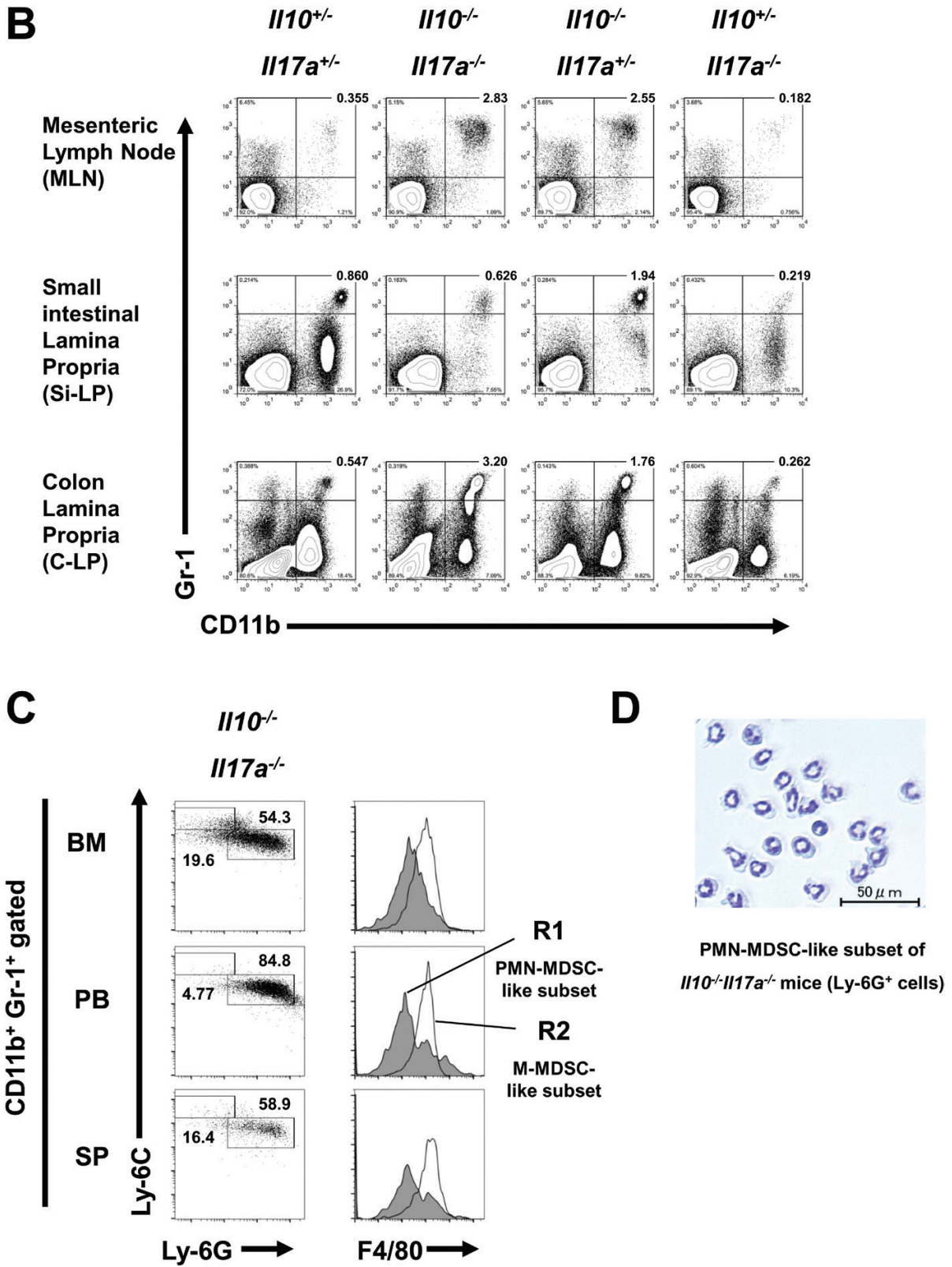


Fig. 2. Continued

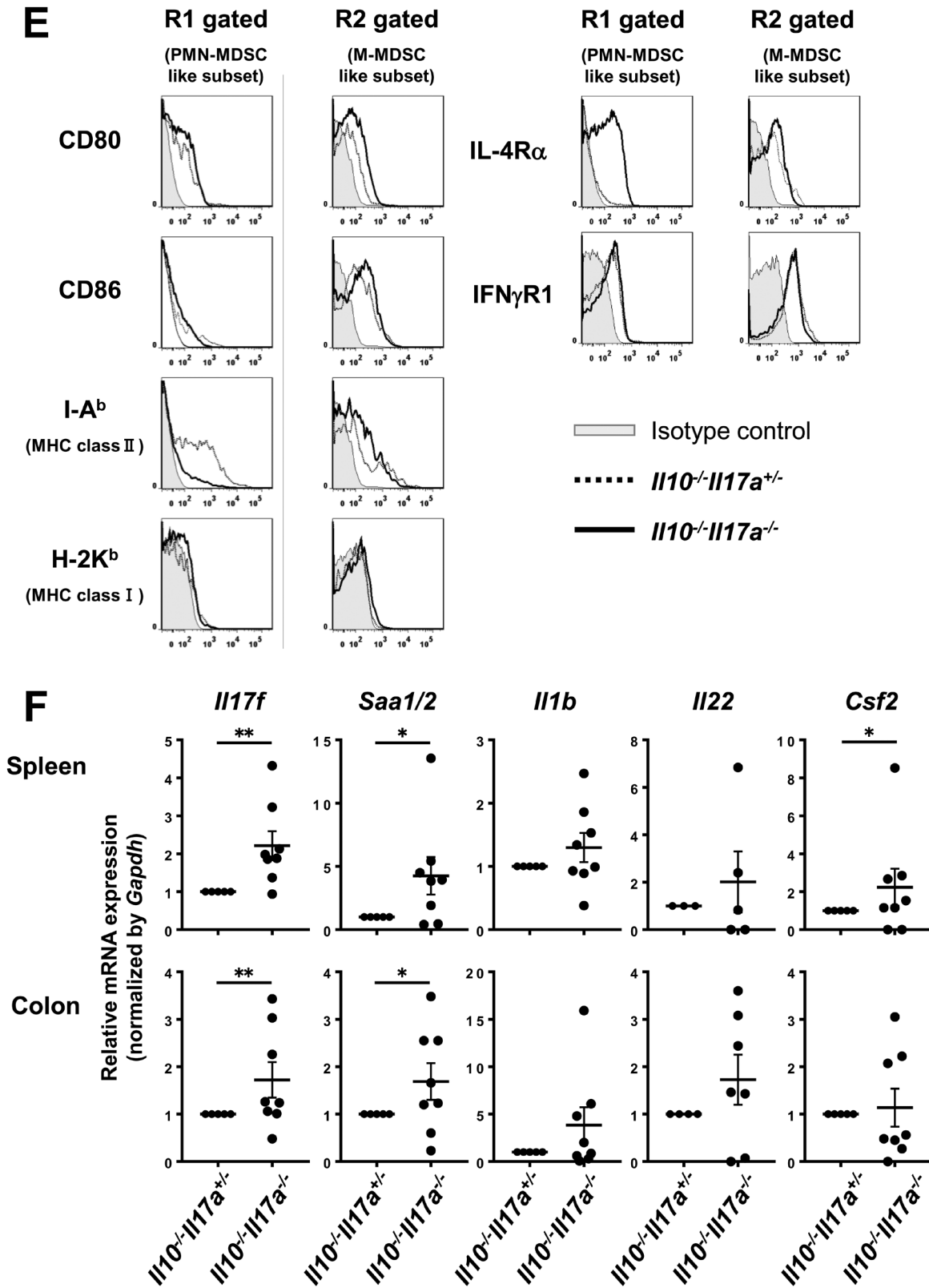
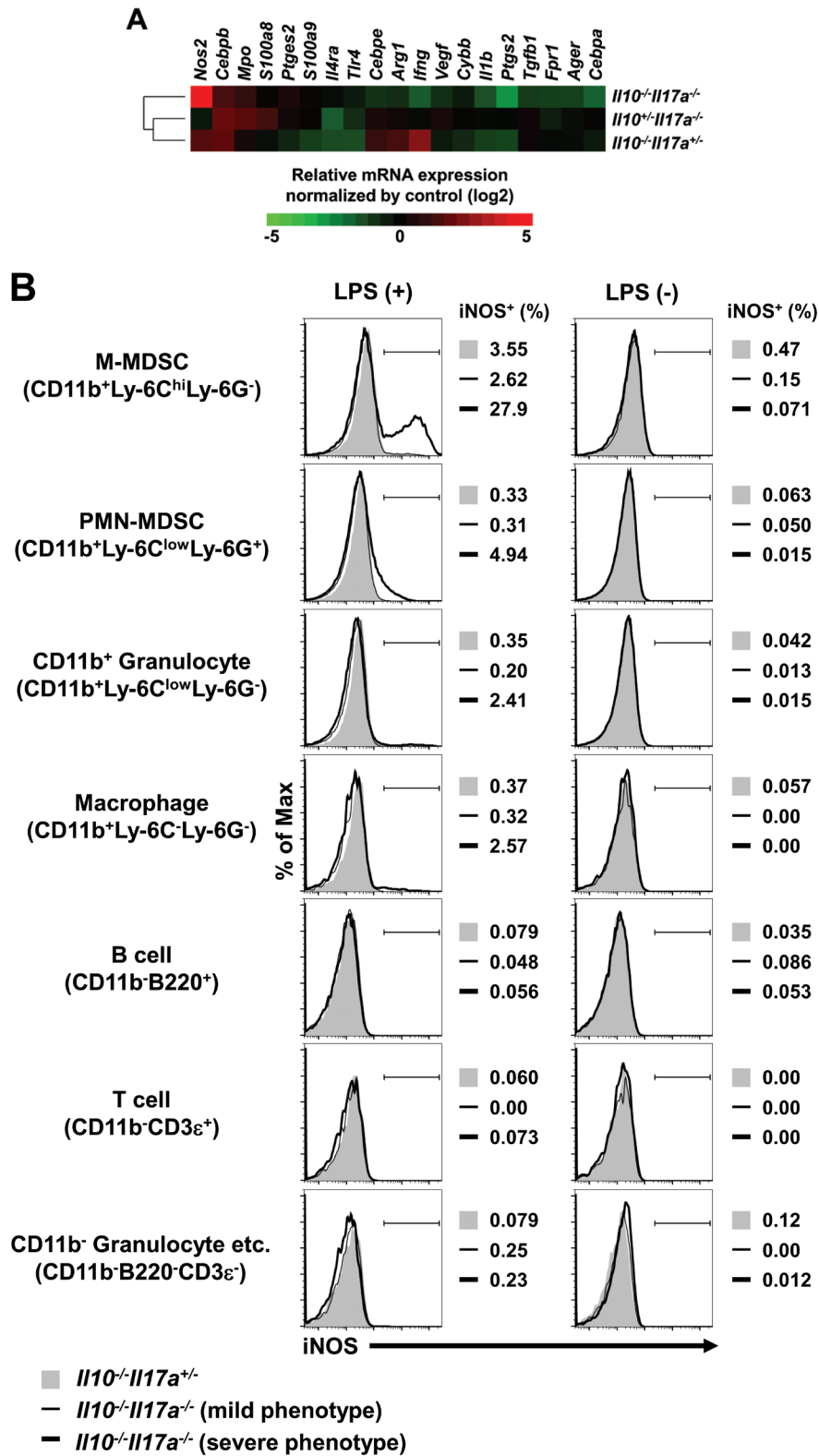


Fig. 2. Continued





**Fig. 3.** The PMN-MDSC-like subset in *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice has a capacity to suppress T-cell proliferation. (A) Gene expression of *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mouse-derived PMN-MDSCs in BM. mRNA levels of MDSC-related genes were measured by qRT-PCR. CD11b<sup>+</sup>Gr-1<sup>+</sup> cells were isolated from BM cells of *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice (12–15 weeks). Data were normalized to the expression of the *Gapdh* housekeeping gene and compared to mRNA levels in CD11b<sup>+</sup>Gr-1<sup>+</sup> cells derived from age-matched *Il10*<sup>-/-</sup>*Il17a*<sup>+/-</sup> mice. Data are representative of two independent experiments. (B) Expression of iNOS in various cells as indicated in BM of *Il10*<sup>-/-</sup>*Il17a*<sup>+/-</sup> mice (shaded) and *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice (line; mild phenotype one, bold line; severe phenotype one). Data are representative of two independent experiments. *Il10*<sup>-/-</sup>*Il17a*<sup>+/-</sup> (*n* = 3) and *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice (*n* = 4) were analyzed, and similar results were obtained. (C) Wild-type mice-derived splenocytes were stimulated with anti-CD3ε/CD28 monoclonal antibodies in the presence of a PMN-MDSC-like subset derived from *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice with or without L-NMMA, an NOS inhibitor (0.5 mM). After 4 days in culture, the percentage of proliferated T cells was calculated as described in the Methods. Data are representative of two independent experiments.

*vitro*.

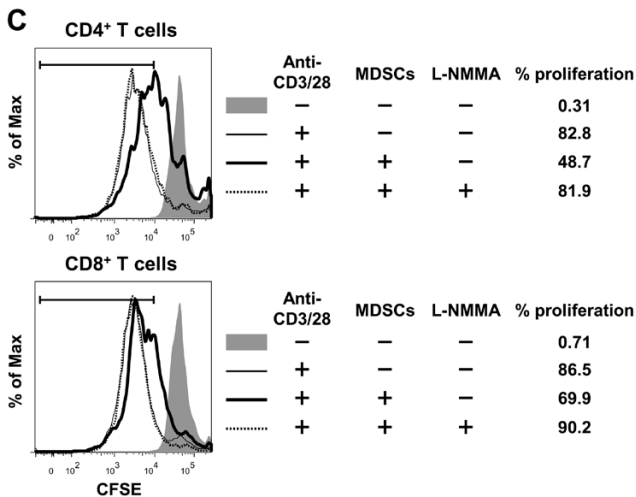


Fig 3. Continued

Next, to investigate whether this phenomenon is observed *in vivo*, we examined the IFN- $\gamma$  production of CD4<sup>+</sup> and CD8<sup>+</sup> T cells derived from the spleens of *Il10*<sup>-/-</sup>*Il17a*<sup>+/-</sup> and *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice. IFN- $\gamma$ -producing cells in CD8<sup>+</sup> T cells, but not CD4<sup>+</sup> T cells, of *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice increased (Fig. 4A and B). Moreover, we found enhanced IFN- $\gamma$  production from both CD4<sup>+</sup> and CD8<sup>+</sup> T cells of *Il10*<sup>-/-</sup>*Il17a*<sup>+/-</sup> mice compared to *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice (Fig. 4C). These data suggested that CD4<sup>+</sup> and CD8<sup>+</sup> T cells are activated in *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice. Given that IFN- $\gamma$  can alter the normal differentiation of myeloid cells (24, 25) and enhance *Nos2* expression (26), the IFN- $\gamma$  production of T cells in *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice would be expected to lead to MDSC accumulation.

#### IL-17A enhanced the suppressive activity of GM-CSF-induced MDSCs *in vitro*

To determine how IL-17A prevents PMN-MDSC differentiation and proliferation, we examined the effect of exogenous IL-17A on *in vitro* MDSC differentiation and proliferation. Previous studies have indicated that short-term culture of BM-derived mononuclear cells upon stimulation of GM-CSF leads to the induction of MDSCs *in vitro* (27). As shown in Supplementary Figure 2A, recombinant IL-17A did not affect the proportion of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells which were differentiated *in vitro* from BM cells by GM-CSF. On the other hand, it was observed that IL-17A could enhance the suppressive activity of GM-CSF-induced MDSCs in a co-culture with splenocytes (Supplementary Figure 2B, MDSC:T = 1:8). In addition, we added IL-17A during the cultivation for *in vitro* MDSCs. The frequencies of iNOS<sup>+</sup> cells were slightly decreased or increased upon stimulation with IL-17A, suggesting that IL-17A would not directly regulate iNOS expression (Supplementary Figure 2C). Next, we could induce MDSCs from BM cells of *Il10*<sup>-/-</sup>*Il17a*<sup>+/-</sup> mouse and *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice (Supplementary Figure 2D), suggesting that endogenous IL-17A does not suppress MDSC differentiation *in vitro* and/or IL-17A is not produced during MDSC differentiation *in vitro*. We also added IL-17A into the culture, and we observed that exogenous

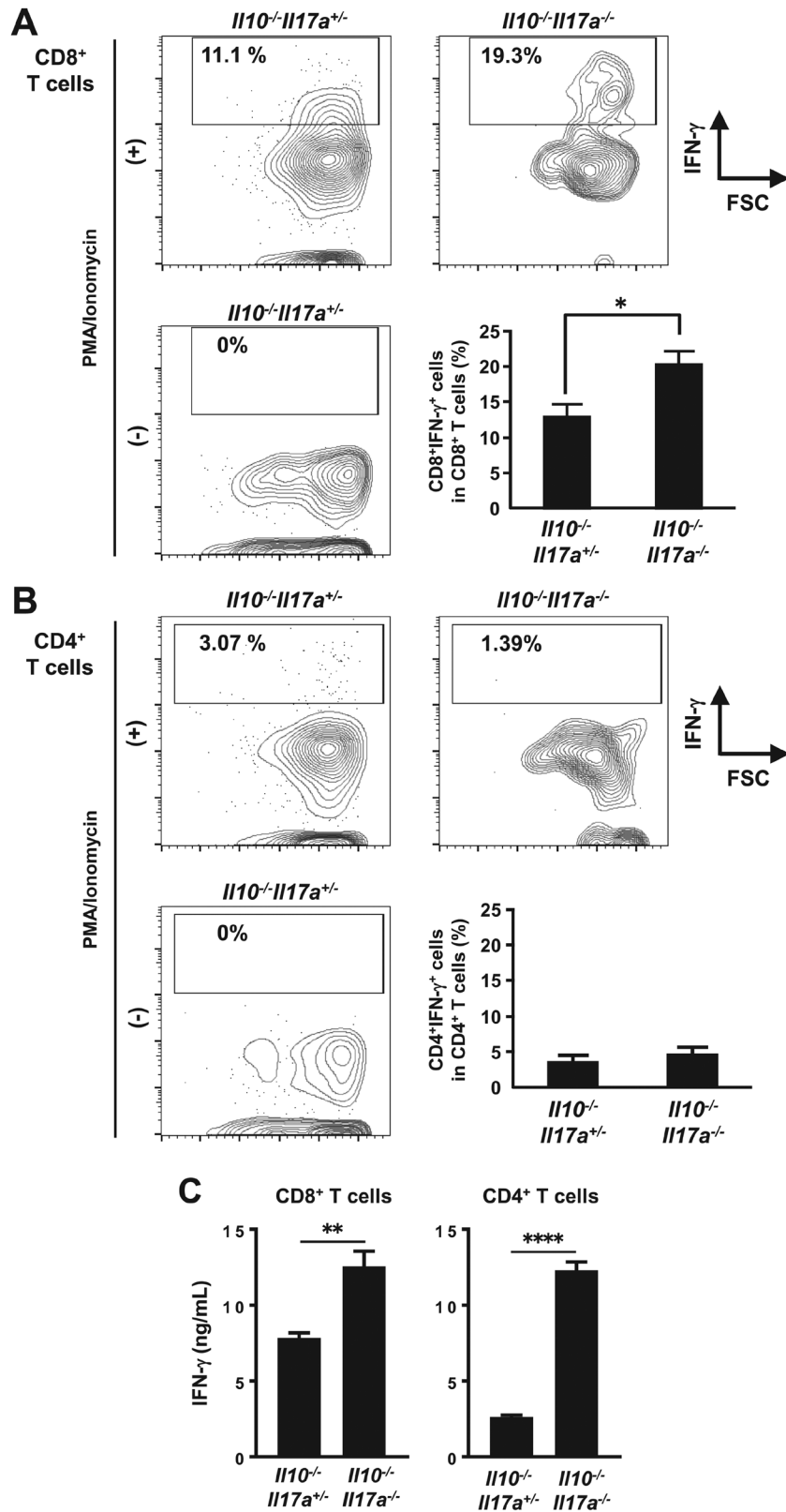
IL-17A did not alter the proportion of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells from BM cells of *Il10*<sup>-/-</sup>*Il17a*<sup>+/-</sup> mice and *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice. Thus, we concluded that IL-17A indirectly suppresses MDSC accumulation in an IL-10-deficient background. These findings suggested that IL-17A failed to directly prevent the differentiation, proliferation and/or suppressive activity of GM-CSF-induced MDSCs. In contrast, MDSC accumulation in *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice suggested the possibility that IL-17A directly prevented the differentiation, proliferation and/or suppressive activity of MDSCs. However, since this possibility was not supported by the data in Supplementary Figure 2, we concluded that IL-17A indirectly prevents the differentiation and/or proliferation of MDSCs *in vivo*.

#### Colitis of *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice was exacerbated by iNOS

Given the accumulation of iNOS-expressing MDSCs (Fig. 2F and G), we next measured the NO concentration in the serum of *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice. The serum NO concentration in *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice was prominently higher compared with that in other mice (Fig. 5). It is speculated that NO produced from MDSCs exacerbates colitis and promotes the death of mice via IL-10-deficiency. Therefore, in order to identify the role of NO, we introduced iNOS-deficiency into *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice. Although *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup>*Nos2*<sup>+/-</sup> mice exhibited ulceration, hyperplasia, massive lymphocyte infiltration and higher colon thickness index, *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup>*Nos2*<sup>-/-</sup> mice did not (Fig. 6A–D). As shown in Fig. 6(E), a loss of mucus-secreting cells, including goblet cells, was observed in *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup>*Nos2*<sup>+/-</sup> mice, but not in *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup>*Nos2*<sup>-/-</sup> mice. These results suggest that NO exacerbates the colitis of *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice.

## Discussion

Accumulating evidence has revealed the roles of IL-17A under several pathological conditions (13, 15). In particular, many researchers have reported that IL-17A-producing T helper (T<sub>H</sub>17) cells exacerbate inflammatory responses (10, 28, 29). In contrast, O'Connor *et al.* revealed that adoptive transfer of CD45RB<sup>high</sup> T cells derived from IL-17A-deficient mice resulted in more severe colitis in immune-deficient mice because of the aberrant deviation to T<sub>H</sub>1 cells (30). Therefore, the question of whether IL-17A and T<sub>H</sub>17 promote or suppress inflammatory responses still remains controversial and needs to be evaluated in various experimental settings, especially colitis models. Our present study demonstrated that IL-17A-deficiency exacerbates the pathology of the colitis and induces a lethal wasting syndrome, cachexia, on an IL-10-deficiency background, in concert with the systemic accumulation of MDSCs and NO production. Furthermore, the severe colitis in *Il10*<sup>-/-</sup>*Il17a*<sup>-/-</sup> mice was completely ameliorated by iNOS-deficiency. It has been reported that treatment of Crohn's disease patients with secukinumab, a human anti-IL-17A monoclonal antibody, and also AMG827, a human anti-IL-17 receptor antibody, unexpectedly deteriorated disease activity scores via unknown mechanisms (31–33). This clearly shows that IL-17A plays a different role in Crohn's disease than in psoriasis, for which targeting IL-17A or IL-17 receptor by therapeutic antibodies has been proved



**Fig. 4.** IFN- $\gamma$  production of T cells was enhanced in the spleens of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. IFN- $\gamma$  secretion in CD8<sup>+</sup> T cells (A) and CD4<sup>+</sup> T cells (B) derived from the spleens of *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>* and *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice were analyzed by intracellular cytokine staining as described in the Methods. Representative plots are shown. Values represent the means  $\pm$  SEM (*Il10<sup>-/-</sup>Il17a<sup>+/-</sup>*;  $n = 3$ , and *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>*;  $n = 5$ ). \* $P < 0.05$ , Student's  $t$ -test. (C) IFN- $\gamma$  production of CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells derived from the spleens of *Il10<sup>-/-</sup>Il17a<sup>+/-</sup>* ( $n = 2$ ) and *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* ( $n = 3$ ) mice was measured by ELISA. Data are representative of two independent experiments. Values represent the means  $\pm$  SD in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , Student's  $t$ -test.



**Fig. 5.** The serum NO level was elevated in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. Serum samples were collected from the mice and NO concentrations were determined as described in the Methods. Bars indicate averages. **\*\*** $P < 0.01$ , compared to other groups (one-way ANOVA).

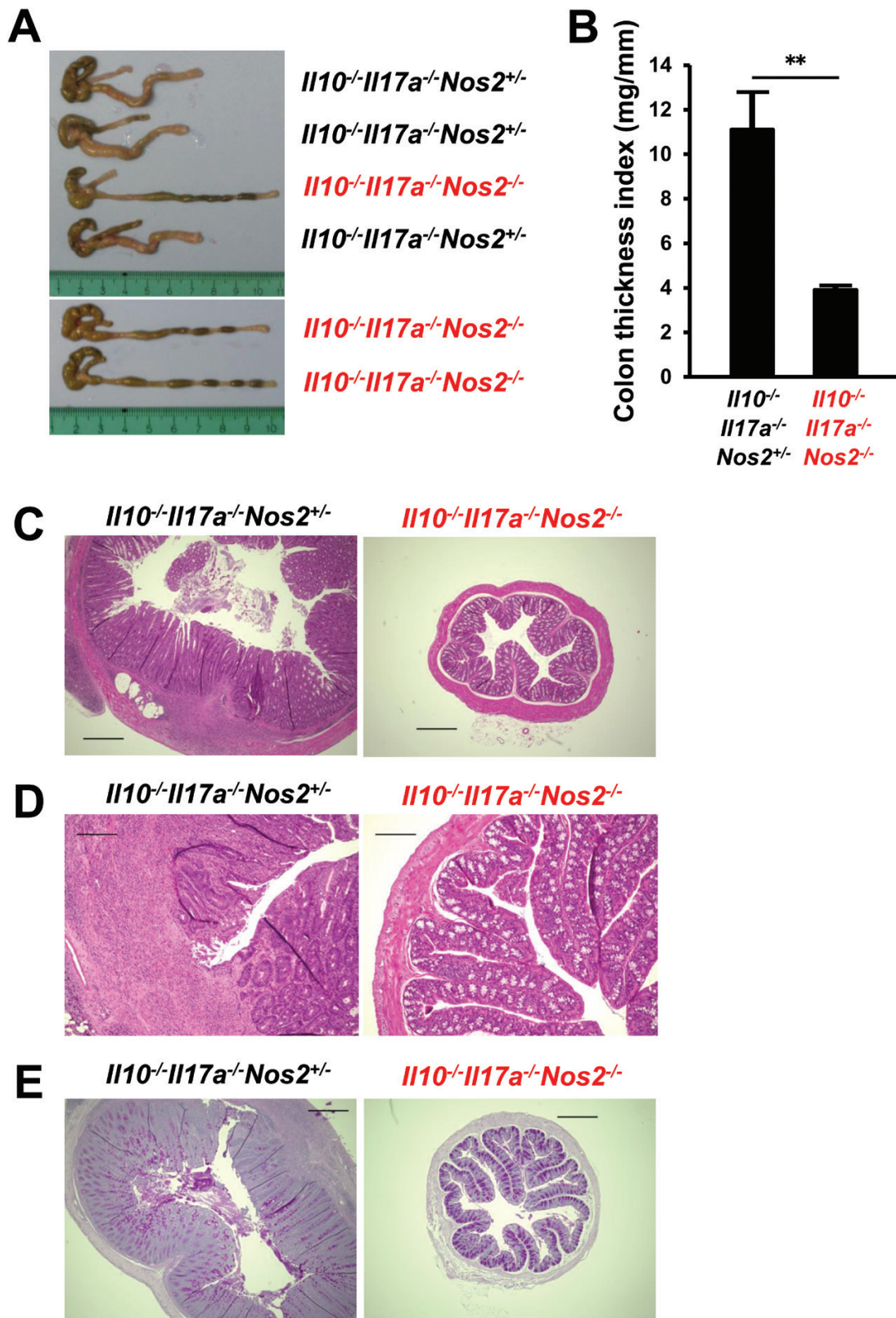
effective. In this study, we genetically confirmed this unique property—i.e. the adverse effect of IL-17A on the pathogenesis of a representative mouse model of Crohn's disease. We also clarified the involvement of iNOS in this process. Thus, this study paves the way to a better understanding of the molecular pathology of Crohn's disease and to the development of novel therapeutics for its treatment.

Recently, IL-17A has been shown to regulate the induction and the suppressive activity of MDSCs in tumor-bearing mice (15, 34). In line with this finding, it was observed that IL-17A enhanced the suppressive activity, but not the differentiation or proliferation, of MDSCs *in vitro* under our experimental conditions (Supplementary Figure 2). From this observation, we reason that massive accumulation of MDSCs in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice was provoked via an indirect effect of IL-17A. The qRT-PCR experiments indicated the up-regulation of *Il17f* and *SAA1/2* expression. Although there has been no report about the relationship between MDSCs and IL-17F, it was reported that the number of MDSCs in IL-17R $\alpha$ -deficient mice, which lack both IL-17A and IL-17F signaling, was lower than that in wild-type mice (15), suggesting the possibility that IL-17F induces MDSCs. In addition, it has been reported that SAA induces MDSCs (35), and enhances the survival of MDSCs under inflammatory conditions (36). Moreover, SAA has been shown to induce iNOS expression and NO production in macrophages (37), suggesting that SAA might be an inducer of iNOS in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* MDSCs. Since SAA also induces T<sub>H</sub>17-related inflammatory cytokines, such as IL-17A, IL-17F and IL-22 (38), it is possible that SAA induces IL-17F expression in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. Taken together, these results suggest that IL-17F and/or SAA could enhance the accumulation of MDSCs in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice showed an immunosuppressive activity. In *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice, only the PMN-MDSC subset, which is a major population among CD11b<sup>+</sup>Gr-1<sup>+</sup> cells, showed an IL-4R $\alpha$ -positive and MHC class II-negative phenotype, which is related to the suppressor activity of MDSCs (19). These findings suggest that MDSC accumulation is induced by a certain inflammatory signal which is negatively regulated by IL-17A.

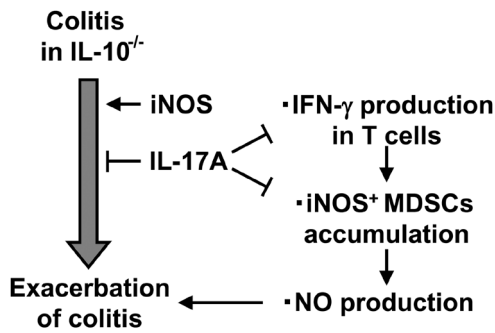
Several inflammatory signals enhance emergency myelopoiesis (39, 40). In this study, we found that MDSCs in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice highly expressed *Nos2* (Fig. 2E). It has been reported that IFN- $\gamma$  enhanced *Nos2* expression and that IFN- $\gamma$  promoted myelopoiesis (24–26). These

observations suggested that IFN- $\gamma$  might be important for the induction and/or suppressor activity of MDSCs. Further, we found that the frequencies of IFN- $\gamma$ -producing T cells were increased in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. A previous report showed that STAT3-deficiency leads to IFN- $\gamma$  up-regulation in CD4<sup>+</sup> T cells, suggesting that STAT3 suppresses IFN- $\gamma$  expression (41). On the other hand, IL-17A could induce the production of IL-6, a STAT3 activator, from fibroblasts (42). In line with this, we speculate that the IL-6 produced by IL-17A-stimulated fibroblasts suppresses IFN- $\gamma$  expression via STAT3 activation even in CD8<sup>+</sup> T cells at the transcriptional level. Thus, we speculated that the IFN- $\gamma$  produced by T cells would up-regulate *Nos2* expression in MDSCs of *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. We found that, in correlation with the *Nos2* expression levels, the serum NO levels were strikingly higher in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice than in other mice (Fig. 5) and MDSCs expressed iNOS (Fig. 3B), suggesting that MDSCs are a source of NO. Although NO has several biological effects, its role in the pathogenesis of colitis or other types of inflammation is still controversial (43). Specifically, it has been reported that iNOS-deficiency has no effect on the pathology of colitis induced by IL-10-deficiency (44). In the present study, we found that *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>Nos2<sup>-/-</sup>* mice failed to exhibit prominent colitis (Fig. 6), indicating that NO exacerbates the colitis in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice. Although the serum NO levels in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice were higher than those in *Il10<sup>-/-</sup>* mice under our experimental conditions, a local high concentration of NO or the source of NO production, e.g. epithelial cells, might have an important role in the induction of colitis. It has been reported that the level of NO is elevated in IBD patients in the active phase compared with in those in the inactive phase (45, 46). Accumulating evidence shows that the gut microbiota is important for the homeostasis of health. Recently, it was revealed that host-derived NO alters the composition of gut microbiota (47, 48). On the basis of the finding that T<sub>H</sub>17 cells protect against pathogens in gut, the severe colitis in *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice may be caused by NO-induced disruption of the composition of gut microbiota.

IL-10 is an immunoregulatory cytokine, and the lack of IL-10 causes severe colitis (5). However, young *Il10<sup>-/-</sup>* mice are healthy, and the molecular mechanism underlying their unexpected health is unresolved. In this study, we found that *Il10<sup>-/-</sup>Il17a<sup>-/-</sup>* mice exhibited much more severe colitis than *Il10<sup>-/-</sup>* mice, suggesting that IL-17A plays an immunoregulatory role in the pathogenesis of colitis in an IL-10-deficient genetic background. In young mice, it is suggested that IL-17A indirectly maintains the homeostasis of myelopoiesis, probably by suppressing the IFN- $\gamma$  production from CD8<sup>+</sup> T cells. Since the arising abnormal myeloid cells, PMN-MDSCs, are less suppressive against CD8<sup>+</sup> T cells (Fig. 3C), IFN- $\gamma$  production from CD8<sup>+</sup> T cells could be sustained. This IFN- $\gamma$  production would enhance iNOS expression in MDSCs, leading to the elevation of serum NO levels. The overproduction of NO would exacerbate the colitis elicited by IL-10-deficiency, although the precise molecular mechanisms remain to be clarified (Fig. 7). Taken together, these results reveal the suppressive function of IL-17A under a pathological condition—namely, the colitis induced by



**Fig. 6.** Inflammatory symptoms of the colon were attenuated by iNOS-deficiency in *IL10<sup>-/-</sup>IL17a<sup>-/-</sup>* mice. (A) Representative macroscopic images of colons are shown. (B) Colon length and weight were measured and the colon thickness index of *IL10<sup>-/-</sup>IL17a<sup>-/-</sup>Nos2<sup>+/-</sup>* mice ( $n = 5$ ) and *IL10<sup>-/-</sup>IL17a<sup>-/-</sup>Nos2<sup>-/-</sup>* mice ( $n = 6$ ) were calculated. Data were analyzed by Mann-Whitney test (\*\* $P < 0.01$ ). (C–E) Representative histological images of (C and D) HE- or (E) PAS-stained mid-colonic sections are shown. Bars indicate 500  $\mu\text{m}$  (C and E) and 200  $\mu\text{m}$  (D).



**Fig. 7.** Scheme of the suppressive role of IL-17A in the colitis elicited by IL-10-deficiency. In the presence of IL-17A, iNOS-deficiency fails to ameliorate the colitis elicited by IL-10-deficiency. IL-17A suppresses the exacerbation of the colitis, probably through the inhibition of NO production from MDSCs.

IL-10-deficiency. Our present study thus provides novel insight into the context-dependent role of IL-17A.

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*Conflicts of interest statement:* the authors declared no conflicts of interest.

### References

- Glocker, E. O., Kotlarz, D., Boztug, K. *et al.* 2009. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N. Engl. J. Med.* 361:2033.
- Franke, A., Balschun, T., Karlsen, T. H. *et al.*; IBSEN Study Group. 2008. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat. Genet.* 40:1319.
- Lees, C. W., Barrett, J. C., Parkes, M. and Satsangi, J. 2011. New IBD genetics: common pathways with other diseases. *Gut* 60:1739.
- Glocker, E. O., Kotlarz, D., Klein, C., Shah, N. and Grimbacher, B. 2011. IL-10 and IL-10 receptor defects in humans. *Ann. N. Y. Acad. Sci.* 1246:102.
- Kühn, R., Löhler, J., Rennick, D., Rajewsky, K. and Müller, W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75:263.
- Chinen, T., Komai, K., Muto, G. *et al.* 2011. Prostaglandin E2 and SOCS1 have a role in intestinal immune tolerance. *Nat. Commun.* 2:190.
- Roers, A., Siewe, L., Strittmatter, E. *et al.* 2004. T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. *J. Exp. Med.* 200:1289.
- Yen, D., Cheung, J., Scheerens, H. *et al.* 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J. Clin. Invest.* 116:1310.
- Duerr, R. H., Taylor, K. D., Brant, S. R. *et al.* 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 314:1461.
- Ito, R., Kita, M., Shin-Ya, M. *et al.* 2008. Involvement of IL-17A in the pathogenesis of DSS-induced colitis in mice. *Biochem. Biophys. Res. Commun.* 377:12.
- Zhang, Z., Zheng, M., Bindas, J., Schwarzenberger, P. and Kolls, J. K. 2006. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflamm. Bowel Dis.* 12:382.
- Kayama, H. and Takeda, K. 2012. Regulation of intestinal homeostasis by innate and adaptive immunity. *Int. Immunol.* 24:673.
- Ogawa, A., Andoh, A., Araki, Y., Bamba, T. and Fujiyama, Y. 2004. Neutralization of interleukin-17 aggravates dextran sulfate sodium-induced colitis in mice. *Clin. Immunol.* 110:55.
- Wang, L., Yi, T., Kortylewski, M., Pardoll, D. M., Zeng, D. and Yu, H. 2009. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *J. Exp. Med.* 206:1457.
- He, D., Li, H., Yusuf, N. *et al.* 2010. IL-17 promotes tumor development through the induction of tumor promoting micro-environments at tumor sites and myeloid-derived suppressor cells. *J. Immunol.* 184:2281.
- Kryczek, I., Wei, S., Szeliga, W., Vatan, L. and Zou, W. 2009. Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood* 114:357.
- Marshall, N. A., Galvin, K. C., Corcoran, A. M., Boon, L., Higgs, R. and Mills, K. H. 2012. Immunotherapy with PI3K inhibitor and Toll-like receptor agonist induces IFN- $\gamma$ +IL-17+ polyfunctional T cells that mediate rejection of murine tumors. *Cancer Res.* 72:581.
- Kumar, V., Patel, S., Tcyganov, E. and Gabrilovich, D. I. 2016. The nature of myeloid-derived suppressor cells in the tumor micro-environment. *Trends Immunol.* 37:208.
- Gabrilovich, D. I. and Nagaraj, S. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* 9:162.
- Haverkamp, J. M., Crist, S. A., Elzey, B. D., Cimen, C. and Ratliff, T. L. 2011. In vivo suppressive function of myeloid-derived suppressor cells is limited to the inflammatory site. *Eur. J. Immunol.* 41:749.
- Griseri, T., Arnold, I. C., Pearson, C. *et al.* 2015. Granulocyte macrophage colony-stimulating factor-activated eosinophils promote interleukin-23 driven chronic colitis. *Immunity* 43:187.
- Rennick, D. M., Fort, M. M. and Davidson, N. J. 1997. Studies with IL-10-/- mice: an overview. *J. Leukoc. Biol.* 61:389.
- Bronte, V. and Zanovello, P. 2005. Regulation of immune responses by L-arginine metabolism. *Nat. Rev. Immunol.* 5:641.
- Baldrige, M. T., King, K. Y., Boles, N. C., Weksberg, D. C. and Goodell, M. A. 2010. Quiescent haematopoietic stem cells are activated by IFN- $\gamma$  in response to chronic infection. *Nature* 465:793.
- Medina-Echeverez, J., Haile, L. A., Zhao, F. *et al.* 2014. IFN- $\gamma$  regulates survival and function of tumor-induced CD11b+ Gr-1high myeloid derived suppressor cells by modulating the anti-apoptotic molecule Bcl2a1. *Eur. J. Immunol.* 44:2457.
- Xiong, H., Zhu, C., Li, H. *et al.* 2003. Complex formation of the interferon (IFN) consensus sequence-binding protein with IRF-1 is essential for murine macrophage IFN- $\gamma$ -induced iNOS gene expression. *J. Biol. Chem.* 278:2271.
- Marigo, I., Bosio, E., Solito, S. *et al.* 2010. Tumor-induced tolerance and immune suppression depend on the C/EBP $\beta$  transcription factor. *Immunity* 32:790.
- Sujino, T., Kanai, T., Ono, Y. *et al.* 2011. Regulatory T cells suppress development of colitis, blocking differentiation of T-helper 17 into alternative T-helper 1 cells. *Gastroenterology* 141:1014.
- Leppkes, M., Becker, C., Ivanov, I. I. *et al.* 2009. ROR $\gamma$ -expressing Th17 cells induce murine chronic intestinal inflammation via redundant effects of IL-17A and IL-17F. *Gastroenterology* 136:257.
- O'Connor, W., Jr, Kamanaka, M., Booth, C. J. *et al.* 2009. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nat. Immunol.* 10:603.
- Hueber, W., Sands, B. E., Lewitzky, S. *et al.* 2012. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* 61:1693.

- 32 Targan, S. R., Feagan, B., Vermeire, S. *et al.* 2016. A randomized, double-blind, placebo-controlled phase 2 study of brodalumab in patients with moderate-to-severe Crohn's disease. *Am. J. Gastroenterol.* 111:1599.
- 33 Colombel, J. F., Sendid, B., Jouault, T. and Poulain, D. 2013. Secukinumab failure in Crohn's disease: the yeast connection? *Gut* 62:800.
- 34 Zhuang, Y., Peng, L. S., Zhao, Y. L. *et al.* 2012. CD8(+) T cells that produce interleukin-17 regulate myeloid-derived suppressor cells and are associated with survival time of patients with gastric cancer. *Gastroenterology* 143:951.
- 35 De Santo, C., Arscott, R., Booth, S. *et al.* 2010. Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. *Nat. Immunol.* 11:1039.
- 36 Sander, L. E., Sackett, S. D., Dierssen, U. *et al.* 2010. Hepatic acute-phase proteins control innate immune responses during infection by promoting myeloid-derived suppressor cell function. *J. Exp. Med.* 207:1453.
- 37 Sandri, S., Rodriguez, D., Gomes, E., Monteiro, H. P., Russo, M. and Campa, A. 2008. Is serum amyloid A an endogenous TLR4 agonist? *J. Leukoc. Biol.* 83:1174.
- 38 Ivanov, I. I., Atarashi, K., Manel, N. *et al.* 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139:485.
- 39 Takizawa, H., Boettcher, S. and Manz, M. G. 2012. Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood* 119:2991.
- 40 Manz, M. G. and Boettcher, S. 2014. Emergency granulopoiesis. *Nat. Rev. Immunol.* 14:302.
- 41 Zhou, L., Ivanov, I. I., Spolski, R. *et al.* 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat. Immunol.* 8:967.
- 42 Murakami, M. and Hirano, T. 2011. A four-step model for the IL-6 amplifier, a regulator of chronic inflammations in tissue-specific MHC class II-associated autoimmune diseases. *Front. Immunol.* 2:22.
- 43 Koliós, G., Valatas, V. and Ward, S. G. 2004. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology* 113:427.
- 44 McCafferty, D. M., Sihota, E., Muscara, M., Wallace, J. L., Sharkey, K. A. and Kubes, P. 2000. Spontaneously developing chronic colitis in IL-10/iNOS double-deficient mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 279:G90.
- 45 Cross, R. K. and Wilson, K. T. 2003. Nitric oxide in inflammatory bowel disease. *Inflamm. Bowel Dis.* 9:179.
- 46 Avdagić, N., Zaćiragić, A., Babić, N. *et al.* 2013. Nitric oxide as a potential biomarker in inflammatory bowel disease. *Bosn. J. Basic Med. Sci.* 13:5.
- 47 Faber, F., Tran, L., Byndloss, M. X. *et al.* 2016. Host-mediated sugar oxidation promotes post-antibiotic pathogen expansion. *Nature* 534:697.
- 48 Winter, S. E., Winter, M. G., Xavier, M. N. *et al.* 2013. Host-derived nitrate boosts growth of *E. coli* in the inflamed gut. *Science* 339:708.

