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/Th17 axis is known to be involved in these inflammatory disorders. IL-17A, a representative cytokine produced by Th17 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−/−) mice. Mice lacking both IL-10 and IL-17A (Il10−/−Il17a−/−) suffered from fatal wasting and manifested more severe colitis compared with Il10−/−Il17a+/− mice. Moreover, we found that CD11b+Gr-1+ myeloid-derived suppressor cells (MDSCs) accumulated in the bone marrow, spleen and peripheral blood of Il10−/−Il17a−/− 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−/−Il17a−/− mice. Surprisingly, the severe colitis observed in Il10−/−Il17a−/− mice was ameliorated in Il10−/−Il17a−/−Nos2−/− mice. Our findings suggest that IL-17A plays suppressive roles against spontaneous colitis in Il10−/− 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−/−Rag2−/− mice fail to exhibit colitis (6) and T-cell-specific IL-10-deficient mice develop severe colitis comparable to that of Il10−/− 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/IL-23R 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. IL-17A−/− mice exhibited more severe colitis and body weight loss compared with those of IL-10−/− mice. Unexpectedly found that immunosuppressive myeloid-lineage cells, MDSCs, were dramatically increased in IL-17A−/− mice. Moreover, we show that IL-17A deficiency caused an enhanced IFN-γ response followed by the up-regulation of inducible nitric oxide synthase (iNOS) (Nos2) in MDSCs. Finally, IL-17A−/−/Nos2−/− mice failed to develop colitis. Taken together, these results suggest that IL-17A suppresses the colitis in IL-10−/− mice, presumably through an iNOS-dependent pathway, and that the MDSCs–iNOS axis might exacerbate the pathology of IL-10−/− mice.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan) and used at 5–6 weeks of age. C57BL/6 IL-10−/− and Nos2−/− mice were provided by the Jackson Laboratory. C57BL/6 IL17a−/− 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 cohoused 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−1 collagenase D (Roche, Basel, Schweiz) and 1 mg ml−1 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-4Rα)-PE, CD4-FITC, CD3e-PE-Cy7, CD19-allophycocyanin (APC) and CD11c-APC from eBioscience (San Diego, CA, USA), Ly-6G-APC, CD119 (IFN-γR1)-Biotin, CD284 (TLR4/MD2)-PE, H-2Kb-FITC and CD8α-APC-Cy7 from BioLegend, Ly-6C-APC-Cy7/V450, CD80-PE, CD86-PE, I-A^k-PE and IFN-γ-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 μg ml−1
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 Cytofix/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+Gr-1+ cells, CD4+ T cells and CD8+ T cells
Mononuclear cells from the BM and spleen were obtained after lysing the red blood cells. To enrich CD11b+Gr-1+ 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+ T cells or CD8+ 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 ∆∆Ct method, with normalization by a housekeeping gene (Gapdh). The data on the expression levels of each gene were normalized by that of Il10−/−Il17a−/− 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 × 10⁵ 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−1 penicillin and 0.1 mg ml−1 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−1 of anti-CD3e 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 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−1; Sigma)/Ionomycin (500 ng ml−1; Wako), and cultured in the presence of BD GolgiStop (1 μg ml−1; BD Biosciences) for 6 h at 37°C. These cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), followed by anti-IFN-γ staining.

Enzyme-linked immunosorbent assay
To enrich CD4+ T cells or CD8+ 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+ T cells or CD8+ T cells were stimulated with PMA (50 ng ml−1; Sigma)/Ionomycin (500 ng ml−1; 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 NO2/NO 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−/− mice, we generated Il10−/−Il17a−/− mice. As shown in Fig. 1(A), the survival rate of Il10−/−Il17a−/− mice reached 30% within 25 weeks after birth, and the body weight was significantly lower in Il10−/−Il17a−/− mice than other mice after 13
NO exacerbates the colitis of IL-10/IL-17A KO mice

Fig. 1. Survival, body weight changes, and symptoms of colitis of Il10−/−Il17a−/− mice. (A) Survival rates of Il10+/−Il17a+/−, Il10−/−Il17a−/−, Il10−/−Il17a+/− and Il10+/−Il17a−/− mice. **Compared to other groups, log-rank test (P < 0.01). (B) Body weight changes of each Il10+/−Il17a+/−, Il10−/−Il17a−/−, Il10−/−Il17a+/− and Il10+/−Il17a−/− mice. Values represent the means ± SEM (n = 6–13). **Compared to Il10−/−Il17a+/−, ##compared to Il10+/−Il17a−/−, ††compared to Il10+/−Il17a+/−, one-way ANOVA (P < 0.01). (C and D) Representative histological images of HE-stained mid-colonic sections are shown. Bars indicate 500 μm (C) and 100 μm (D). (E) Colon length and weight of each mouse were measured and the length-to-weight ratio (colon thickness index) was calculated [**P < 0.001, ****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−/−Il17a−/− 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−/− mice.

Like Il10−/− mice in a previous report (22), Il10−/−Il17a−/− mice in the present study exhibited severe splenomegaly. On the other hand, surprisingly, Il10−/−Il17a−/− 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−/−Il17a−/− 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−/− mice.

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

We observed that the SSC−/Lin− myeloid-lineage population was increased in the spleens of Il10−/−Il17a−/− mice. To characterize the myeloid-lineage cells in Il10−/−Il17a−/− 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−/−Il17a−/− mice. We found that the proportion and the numbers of CD11b+Gr-1+ 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−/−Il17a−/− mice (Fig. 2B). In Il10−/− mice, the proportions of CD11b+Gr-1+ cells were increased in the MLN and gut, suggesting that the increases of CD11b+Gr-1+ 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+Gr-1+ cells (Figs 1C, D and 2B), we speculated that CD11b+Gr-1+ cells were involved in the colitis. Further analysis revealed that most of the CD11b+Gr-1+ subset accumulating in the BM, PB and spleen of Il10−/−Il17a−/− mice showed the CD11b+Gr-1+Ly-6C+Ly-6G−F4/80low 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 these cells had a polymorphonuclear, and thus these cells corresponded to PMN-MDSCs (Fig. 2D). In addition, only CD11b+Gr-1+Ly-6C+Ly-6G−F4/80low PMN-MDSC-like cells in Il10−/−Il17a−/− mice, but not CD11b+Gr-1+Ly-6C+Ly-6G−F4/80− cells, exhibited an IL-4Rα-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−/−Il17a−/− mice also exhibited low-level expression of CD68, a co-stimulatory molecule, and MHC class I (Fig. 2E). These results suggest that the majority of CD11b+Gr-1+ cells in Il10−/−Il17a−/− mice showed PMN-MDSC-like surface marker expression patterns and morphology.

To clarify the mechanisms of MDSC accumulation in Il10−/−Il17a−/− 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−/−Il17a−/− mice were significantly higher than those in littermate Il10−/−Il17a−/− mice (Fig. 2F). On the other hand, IL-22 (Il22), IL-1β (Il1b) and GM-CSF (Csf2) were expressed highly in some Il10−/−Il17a−/− 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−/−Il17a−/− mice.

**Accumulating MDSCs highly expressed iNOS in Il10−/−Il17a−/− mice**

We next measured the mRNA expression levels of MDSC-related genes in CD11b+Gr-1+ cells. Quantitative RT–PCR analysis revealed changes in the expression levels of MDSC-related genes, including suppressive molecules of MDSCs—such as, iNOS (Nos2), arginase (Arg1) and NADPH oxidase (Cybb). In particular, Nos2 expression in CD11b+Gr-1+ cells of Il10−/−Il17a−/− mice was prominently higher than that in CD11b+Gr-1+ cells of Il10−/−Il17a−/− mice (Fig. 3A). On the other hand, in CD11b+Gr-1+ cells of Il10−/−Il17a−/− mice, the expression of Arg1 and Cybb oxidase, one of the major reactive oxygen species (ROS) inducers, was lower than that in CD11b+Gr-1+ cells of Il10−/−Il17a−/− mice. Next, we checked iNOS expression among BM cells of Il10−/−Il17a−/− mice. We found that Ly-6C+Ly-6G−CD11b+ monocytes and the small population of Ly-6C+Ly-6G−CD11b+ PMN-MDSCs expressed iNOS (Fig. 3B), suggesting that Ly-6C+Ly-6G−CD11b+ cells are M-MDSCs. On the other hand, T cells, B cells and CD11b− 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−/−Il17a−/− mice against T-cell responses. As shown in Fig. 3, PMN-MDSC-like cells suppressed CD4+ T-cell proliferation. On the other hand, PMN-MDSC-like cells were less suppressive of CD8+ T-cell proliferation. These results suggest that PMN-MDSC-like cells derived from Il10−/−Il17a−/− mice exhibit suppressive activity against T-cell proliferation. We observed a higher expression of Nos2 in PMN-MDSC-like cells derived from Il10−/−Il17a−/− mice than in PMN-MDSC-like cells derived from Il10−/−Il17a−/− 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, Nω-Monomethyl-L-arginine (L-NMMA) (Fig. 3C). These data suggested that the immune-suppressive activity of CD11b+Gr-1+ cells in Il10−/−Il17a−/− 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-γ production of T cells was enhanced in Il10−/−Il17a−/− mice**

As shown above, PMN-MDSCs derived from Il10−/−Il17a−/− mice were less suppressive to CD8+ T cells than CD4+ T cells in...
Fig. 2. Characterization of the MDSC-like subset in Il10−/−Il17a+/− 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+Gr-1+ 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+ cells among BM cells of Il10+/−Il17a−/− and Il10−/−Il17a−/− 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−/−Il17a+/− cells and Il10−/−Il17a−/− 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−/−Il17a−/− 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−/−Il17a+/− mice. Data were from Il10−/−Il17a−/− mice (n = 8) and Il10+/−Il17a−/− mice (n = 5) [*P < 0.05, **P < 0.01 (Kolmogorov–Smirnov test)]. Il22 mRNA expression was from Il10−/−Il17a−/− mice (n = 5, 7) and Il10+/−Il17a−/− mice (n = 3, 4), since no expression was observed in some Il10−/−Il17a−/− mice.
NO exacerbates the colitis of IL-10/IL-17A KO mice

Fig. 2. Continued
NO exacerbates the colitis of IL-10/IL-17A KO mice

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Fig. 3. The PMN-MDSC-like subset in \( I{\textit{I}}^{10-/-}I{\textit{I}}^{17a-/-} \) mice has a capacity to suppress T-cell proliferation. (A) Gene expression of \( I{\textit{I}}^{10-/-}I{\textit{I}}^{17a-/-} \) mouse-derived PMN-MDSCs in BM. mRNA levels of MDSC-related genes were measured by qRT–PCR. CD11b\(^+\)Gr-1\(^-\) cells were isolated from BM cells of \( I{\textit{I}}^{10-/-}I{\textit{I}}^{17a-/-} \) mice (12–15 weeks). Data were normalized to the expression of the \( Gapdh \) housekeeping gene and compared to mRNA levels in CD11b\(^+\)Gr-1\(^-\) cells derived from age-matched \( I{\textit{I}}^{10+/-}I{\textit{I}}^{17a+/-} \) mice. Data are representative of two independent experiments. (B) Expression of iNOS in various cells as indicated in BM of \( I{\textit{I}}^{10-/-}I{\textit{I}}^{17a-/-} \) mice (shaded) and \( I{\textit{I}}^{10-/-}I{\textit{I}}^{17a-/-} \) mice (line; mild phenotype one, bold line; severe phenotype one). Data are representative of two independent experiments. \( I{\textit{I}}^{10-/-}I{\textit{I}}^{17a+/-} (n=3) \) and \( I{\textit{I}}^{10-/-}I{\textit{I}}^{17a-/-} (n=4) \) were analyzed, and similar results were obtained. (C) Wild-type mice-derived splenocytes were stimulated with anti-CD3\(\epsilon\)/CD28 monoclonal antibodies in the presence of a PMN-MDSC-like subset derived from \( I{\textit{I}}^{10-/-}I{\textit{I}}^{17a-/-} \) 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.
Next, to investigate whether this phenomenon is observed in vivo, we examined the IFN-γ production of CD4+ and CD8+ T cells derived from the spleens of Il10−/−Il17a−/− and Il10−/−Il17a+/− mice. IFN-γ-producing cells in CD8+ T cells, but not CD4+ T cells, of Il10−/−Il17a−/− mice increased (Fig. 4A and B). Moreover, we found enhanced IFN-γ production from both CD4+ and CD8+ T cells of Il10−/−Il17a−/− mice compared to Il10−/−Il17a+/− mice (Fig. 4C). These data suggested that CD4+ and CD8+ T cells are activated in Il10−/−Il17a−/− mice. Given that IFN-γ can alter the normal differentiation of myeloid cells (24, 25) and enhance Nos2 expression (26), the IFN-γ production of T cells in Il10−/−Il17a−/− 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, recombiant IL-17A did not affect the proportion of CD11b+Gr-1+ 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+ 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−/−Il17a−/− mouse and Il10−/−Il17a+/− 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+Gr-1+ cells from BM cells of Il10−/−Il17a−/− mice and Il10−/−Il17a+/− 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−/−Il17a−/− 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−/−Il17a−/− 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−/−Il17a−/− mice. The serum NO concentration in Il10−/−Il17a−/− 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−/−Il17a−/− mice. Although Il10−/−Il17a−/−Nos2−/− mice exhibited ulceration, hyperplasia, massive lymphocyte infiltration and higher colon thickness index, Il10−/−Il17a−/−Nos2−/− 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−/−Il17a−/−Nos2−/− mice. These results suggest that NO exacerbates the colitis of Il10−/−Il17a−/− 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 (Th) cells exacerbate inflammatory responses (10, 28, 29). In contrast, O’Connor et al. revealed that adoptive transfer of CD45RB+CD8+ T cells derived from IL-17A-deficient mice resulted in more severe colitis in immune-deficient mice because of the aberrant deviation to Th1 cells (30). Therefore, the question of whether IL-17A and Th17 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−/−Il17a−/− mice was completely ameliorated by iNOS-deficiency. It has been reported that treatment of Crohn’s disease patients with secukinumab, an 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-γ production of T cells was enhanced in the spleens of II10−/−II17a−/− mice. IFN-γ secretion in CD8+ T cells (A) and CD4+ T cells (B) derived from the spleens of II10−/−II17a+/− and II10−/−II17a−/− mice were analyzed by intracellular cytokine staining as described in the Methods. Representative plots are shown. Values represent the means ± SEM (II10−/−II17a−/−; n = 3, and II10−/−II17a−/−; n = 5). *P < 0.05, Student’s t-test. (C) IFN-γ production of CD8+ T cells and CD4+ T cells derived from the spleens of II10−/−II17a−/− (n = 2) and II10−/−II17a−/− (n = 3) mice was measured by ELISA. Data are representative of two independent experiments. Values represent the means ± SD in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Student’s t-test.
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 IL-17A−/− 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-17A-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−/−Il17a−/− MDSCs. Since SAA also induces T17-related inflammatory cytokines, such as IL-17A, IL-17F and IL-22 (38), it is possible that SAA induces IL-17F expression in IL10−/−Il17a−/− mice. Taken together, these results suggest that IL-17F and/or SAA could enhance the accumulation of MDSCs in IL10−/−Il17a−/− mice. CD11b+Gr-1+ cells in IL10−/−Il17a−/− mice showed an immunosuppressive activity. In IL10−/−Il17a−/− mice, only the PMN-MDSC subset, which is a major population among CD11b+Gr-1+ cells, showed an IL-4Rα-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−/−Il17a−/− mice highly expressed Nos2 (Fig. 2E). It has been reported that IFN-γ enhanced Nos2 expression and that IFN-γ promoted myelopoiesis (24–26). These observations suggested that IFN-γ might be important for the induction and/or suppressor activity of MDSCs. Further, we found that the frequencies of IFN-γ-producing T cells were increased in IL10−/−Il17a−/− mice. A previous report showed that STAT3-deficiency leads to IFN-γ up-regulation in CD4+ T cells, suggesting that STAT3 suppresses IFN-γ 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-γ expression via STAT3 activation even in CD8+ T cells at the transcriptional level. Thus, we speculated that the IFN-γ produced by T cells would up-regulate Nos2 expression in MDSCs of IL10−/−Il17a−/− mice. We found that, in correlation with the Nos2 expression levels, the serum NO levels were strikingly higher in IL10−/−Il17a−/− 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−/−Il17a−/−Nos2−/− mice failed to exhibit prominent colitis (Fig. 6), indicating that NO exacerbates the colitis in IL10−/−Il17a−/− mice. Although the serum NO levels in IL10−/−Il17a−/− mice were higher than those in IL10−/− 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 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 T17 cells protect against pathogens in gut, the severe colitis in IL10−/−Il17a−/− 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−/− mice are healthy, and the molecular mechanism underlying their unexpected health is unresolved. In this study, we found that IL10−/−Il17a−/− mice exhibited much more severe colitis than IL10−/− 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-γ production from CD8+ T cells. Since the arising abnormal myeloid cells, PMN-MDSCs, are less suppressive against CD8+ T cells (Fig. 3C), IFN-γ production from CD8+ T cells could be sustained. This IFN-γ 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 \( \text{II}^{10^{-}}\text{II}^{17a^{-}} \) mice. (A) Representative macroscopic images of colons are shown. (B) Colon length and weight were measured and the colon thickness index of \( \text{II}^{10^{-}}\text{II}^{17a^{-}}\text{Nos}^{2^{-}} \) mice (n = 5) and \( \text{II}^{10^{-}}\text{II}^{17a^{-}}\text{Nos}^{2^{-}} \) 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 \( \mu \)m (C and E) and 200 \( \mu \)m (D).
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Fig. 7. Scheme of the suppressive role of IL-17A in the colitis elicited by IL-10-deficiency. In the presence of IL-17A, IL-10-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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