

Indigo Naturalis Ameliorates Oxazolone-Induced Dermatitis but Aggravates Colitis by Changing the Composition of Gut Microflora

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Keywords

Indigo naturalis · Qing dai · Oxazolone · Dermatitis · Colitis · Herbal medicine · Microflora

Abstract

Background: Indigo naturalis (IND) is an herbal medicine that has been used as an anti-inflammatory agent to treat diseases including dermatitis and inflammatory bowel disease in China. However, the mechanism by which IND exerts its immunomodulatory effect is not well understood. **Methods:** A murine model of dermatitis and inflammatory bowel disease, both induced by oxazolone (OXA), was treated with IND. The severity of dermatitis was evaluated based on ear thickness measurements and histological scoring. The severity of colitis was evaluated by measuring body weight, histological scoring, and endoscopic scoring. The expression of inflammatory cytokines in ear and colon tissue was

evaluated using real-time PCR. 16S rRNA DNA sequencing of feces from OXA-induced colitis mice was performed before and after IND treatment. The effects of IND on OXA-induced colitis were also evaluated after depleting the gut flora with antibiotics to test whether alteration of the gut flora by IND influenced the course of intestinal inflammation in this model. **Results:** IND treatment ameliorated OXA dermatitis with a reduction in IL-4 and eosinophil recruitment. However, OXA colitis was significantly aggravated in spite of a reduction in intestinal IL-13, a pivotal cytokine in the induction of the colitis. It was found that IND dramatically altered the gut flora and IND no longer exacerbated colitis when colitis was induced after gut flora depletion. **Conclusions:** Our data suggest that IND could modify the inflammatory immune response in multiple ways, either directly (i.e., modification of the allergic immune cell activity) or indirectly (i.e., alteration of commensal compositions).

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Introduction

Indigo naturalis (IND), also known as Qing Dai, is an herbal medicine that is a blue powder made from extracts of plants such as *Strobilanthes cusia* and *Isatis tinctoria* [1]. It contains multiple ingredients including indirubin, indigo, and tryptanthrin [2]. IND has traditionally been used as an anti-inflammatory agent to treat a wide range of pathological conditions such as skin rash, dermatitis, venom detoxification, and upper airway inflammation [2–4]. IND is applied topically to the skin to treat psoriasis, and its efficacy has been demonstrated in several clinical reports [5, 6]. IND containing mixtures of herbs has also been used in hospitals in China for the treatment of inflammatory bowel disease (IBD). IBD is characterized by chronic inflammation of the alimentary tract, primarily manifested as ulcerative colitis (UC) and Crohn's disease. It is assumed that IBD is a multifactorial disease and genetic and environmental factors are important for its onset [7, 8]. As one of the mechanisms, it is widely recognized that deregulated immune responses toward gut microorganisms are involved. This idea is strongly supported by recent publications showing a significantly reduced diversity of gut flora in IBD patients [9] and modifications in the course of IBD by fecal microbial transplantation [10, 11]. Individuals with IBD possess a deregulated immune response, mainly skewed toward Th1 and Th2 in Crohn's disease and UC patients, respectively [12], and IND has mainly been used to treat UC. Despite the lack of English-language reports demonstrating clinical efficacy, the official indication of IND for UC in China and its reputation have caught the attention of Japanese gastroenterologists. Consequently, 2 recent clinical papers from Japan have documented a promising efficacy of IND for the treatment of UC [1, 13].

IND seems to be effective for the treatment of multiple diseases; however, the mechanism of its effect is still largely unknown. To investigate the efficacy of IND, we utilized 2 murine disease models of inflammation, i.e., dermatitis and colitis, both induced by oxazolone (OXA). Herein, we show that IND indeed ameliorated dermatitis through modification of inflammatory immune responses. However, surprisingly, IND aggravated this animal model of colitis. Our data suggest that IND may influence the course of colitis by changing the composition of the gut flora.

Materials and Methods

Animals

Male BALB/c mice were bought from Japan SLC (Fukuoka, Japan) and used for the OXA colitis model. Male C57BL/6J mice were bought from CLEA (Osaka, Japan) and used for the OXA dermatitis model. They were kept under specific pathogen-free conditions. Six- to 8-week-old mice were used in both the dermatitis and the colitis experiments. The protocols for this study were approved by the Animal Use and Care Committee of Kobe University (P130511, P130511-R1, and P130511-R2).

Induction of OXA Dermatitis and Treatment with IND

In order to presensitize mice, a 2×2 cm field of abdominal skin was shaved, and 100 μ L of 2% OXA (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich; St. Louis, MI, USA) in 100% ethanol was applied. Five days after presensitization, 100 μ L of 1% OXA in 100% ethanol was applied to the dorsal surface of the ear. This was repeated every other day for 2 or 3 applications until ear swelling was observed. From the day of dermatitis induction to sacrifice, IND was applied to the dorsal ear every day. For the assessment of dermatitis, the bifold ear thickness of the mice was measured every day. The mice were sacrificed for further evaluation within 72 h of the last OXA application. Experiments were conducted using IND (Seitain® and Seitai® from Seishinshoyakudo, Tokyo, Japan, and Tochimotoenkaido, Osaka, Japan, respectively).

RNA Isolation from the Skin and Real-Time PCR

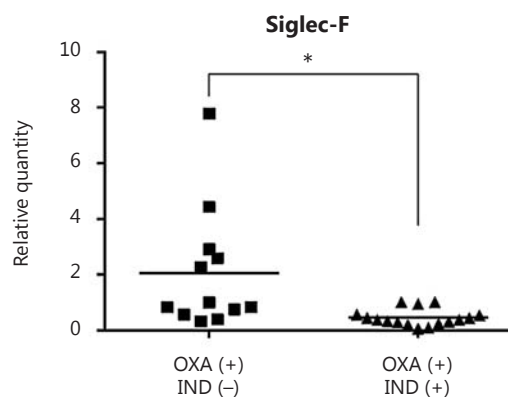
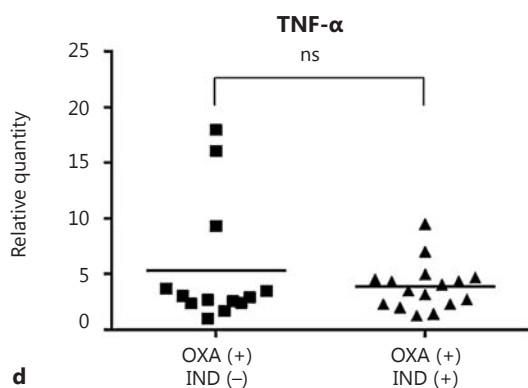
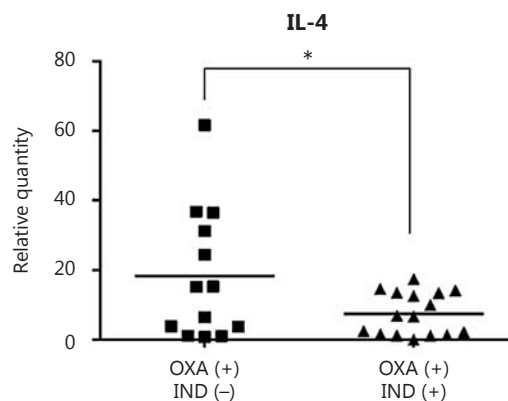
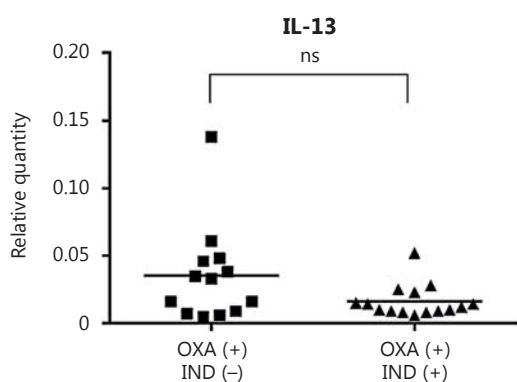
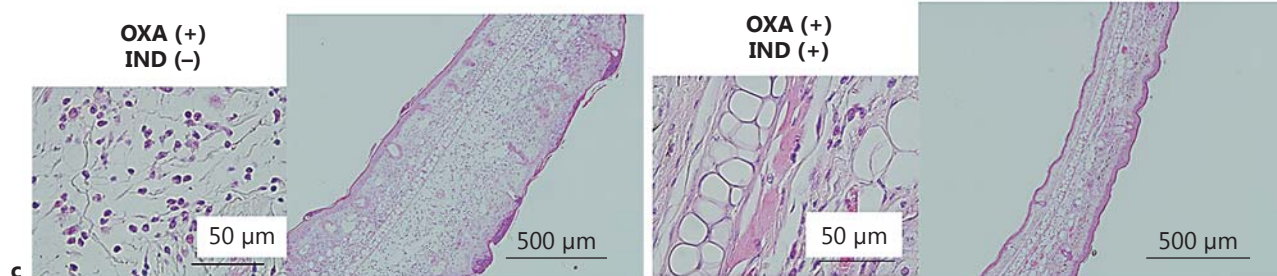
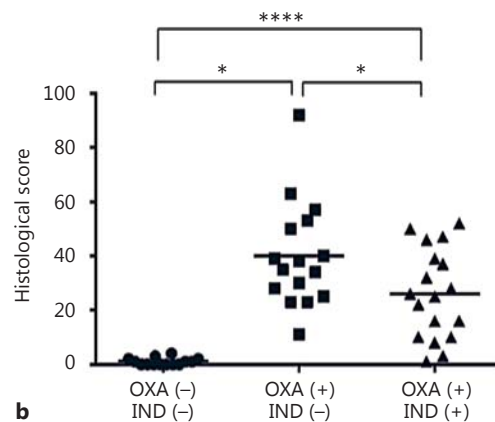
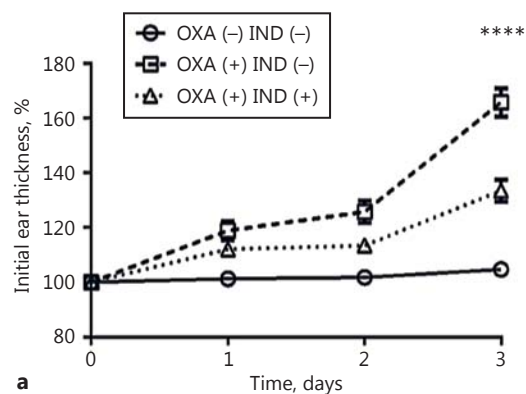
Skin tissue RNA from ear auricles was isolated using TRIzol® (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using a cDNA synthesis kit (Applied Biosystems; Foster City, CA, USA), and real-time PCR was carried out using primers and SYBR Green chemistry (Applied Biosystems) on an ABI 7500 real-time PCR system (Applied Biosystems). The relative quantity of target genes was normalized to hypoxanthine guanine phosphoribosyl transferase expression.

Histological Scoring of Skin Tissue

The ear auricle was fixed in Mildform® (Wako Pure Chemical Industries, Osaka, Japan) overnight and paraffin embedded, sectioned, and stained with hematoxylin/eosin. Four microscopic fields from each sample were randomly selected, and granulocytes were counted in each field ($\times 1,000$ HPF); the evaluation was done in a blind manner. Histological score equal to the total number of granulocytes counted in 4 fields were recorded.

Fig. 1. Indigo naturalis (IND) reduced the severity of oxazolone (OXA) dermatitis. **a** Bifold ear thickness was measured every day after the onset of dermatitis. OXA(–) IND(–), $n = 14$; OXA(+) IND(–), $n = 16$; and OXA(+) IND(+), $n = 18$. **b** Histological score was evaluated using H & E staining. OXA(–) IND(–), $n = 14$; OXA(+) IND(–), $n = 16$; and OXA(+) IND(+), $n = 18$. **c** Representative images of H & E staining. **d** Cytokine expression levels were evaluated by real-time PCR. OXA(+) IND(–), $n = 13$, and OXA(+) IND(+), $n = 16$. **a** The OXA(+) IND(–) and OXA(+) IND(+) groups were compared to calculate the p value. Data shown are the combined results of 3–4 repeated experiments. * $p < 0.05$, **** $p < 0.0001$.

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Induction of OXA Colitis and Treatment with IND

Mice were sensitized using OXA as described above in Induction of OXA Dermatitis and Treatment with IND. Five days after presensitization, 100 μ L of 0.5% OXA in 50% ethanol was administered intrarectally. From the day of colitis induction to sacrifice, the mice were fed a normal powdery chow containing 5% w/w IND. Body weight was measured every day. On days 0 and 3, mucosal damage to the colon was evaluated using murine endoscope. On day 4, the mice were sacrificed to harvest the tissue.

Assessment of Colitis with Murine Colonoscopy

The severity of the mucosal damage caused by OXA colitis was evaluated by murine colonoscopy under isoflurane anesthesia. The endoscopic score was evaluated based on mucosal granularity (0, none; 1, mild; 2, moderate; and 3, severe), vascular pattern (0, normal; 1, mildly decreased; 2, moderately decreased; and 3, absent), erosion/ulcer (0, normal; 1, erythema; 2, erosion; and 3, ulcer), and stool consistency (0, solid; 1, soft; 2, muddy; and 3, watery).

RNA Isolation from the Colon and Real-Time PCR

Distal colonic tissue was used for RNA isolation. RNA isolation and real-time PCR were done as described above in RNA Isolation from the Skin and Real-Time PCR.

Histological Scoring of Colon Tissue

After the mice had been sacrificed, a 1-cm length of distal colon was removed and fixed in formalin. The tissue sample was then paraffin embedded, sectioned, and stained with hematoxylin/eosin. The histological score was evaluated based on epithelial hyperplasia (0, normal; 1, mild; 2, moderate; and 3, severe), loss of enterocytes (0, none; 1, mild; 2, moderate; and 3, severe), granulocyte infiltration in the lamina propria (0, none; 1, mild; 2, moderate; and 3, severe), and mononuclear cell infiltration in the lamina propria (0, none; 1, mild; 2, moderate; and 3, severe).

Bacterial DNA Sequencing and Bioinformatics

On days 0 and 4 of the colitis experiment, fresh feces were collected, immediately frozen, and stored at -80°C until use. The bacterial DNA content was extracted using a PowerFecalTM DNA isolation kit (MO BIO; Carlsbad, CA, USA). For 2-step PCR amplification, the bacterial V4 variable region of the 16S rRNA gene was amplified with primers containing Illumina adapter overhang nucleotide sequences (Illumina; San Diego, CA, USA), followed by amplification with barcoded primers [13]. The DNA concentration of each sample was measured using Qubit[®] and a dsDNA HS assay kit (Invitrogen; Carlsbad, CA, USA). DNA libraries adjusted to 6 pM were pooled with 20% 6 pM PhiX spike-in control (Illumina) and sequenced with the MiSeq[®] platform (Illumina). The raw data was converted to an FASTQ file and then demultiplexed using an MiSeq[®] reporter. Sequence data were quality filtered and analyzed using QIIMETM 8.1.0 open-source software [14]. Chimeric sequences were removed using USEARCH 6.1.544, and operational taxonomic unit clustering was performed using UCLUST at a similarity threshold of 97% [15]. The sequence reads were then searched against the Greengenes reference database (version 13.8) [16]. Principal coordinate analysis was performed by QIIMETM.

Depletion of Gut Microflora

Prior to OXA colitis induction, 200 μ L of a mixture of antibiotics consisting of vancomycin at 5 mg/mL (Sigma-Aldrich), metro-

nidazole at 10 mg/mL (Sigma-Aldrich), neomycin at 10 mg/mL (Sigma-Aldrich), and ampicillin at 10 mg/mL (Sigma-Aldrich) was administered by gavage every day for 7 days. The protocol of induction of OXA colitis was the same as described above.

Statistics

Statistical analysis was performed with an unpaired Student's *t* test using GraphPad Prism 6 (GraphPad Software; San Diego, CA, USA).

Results

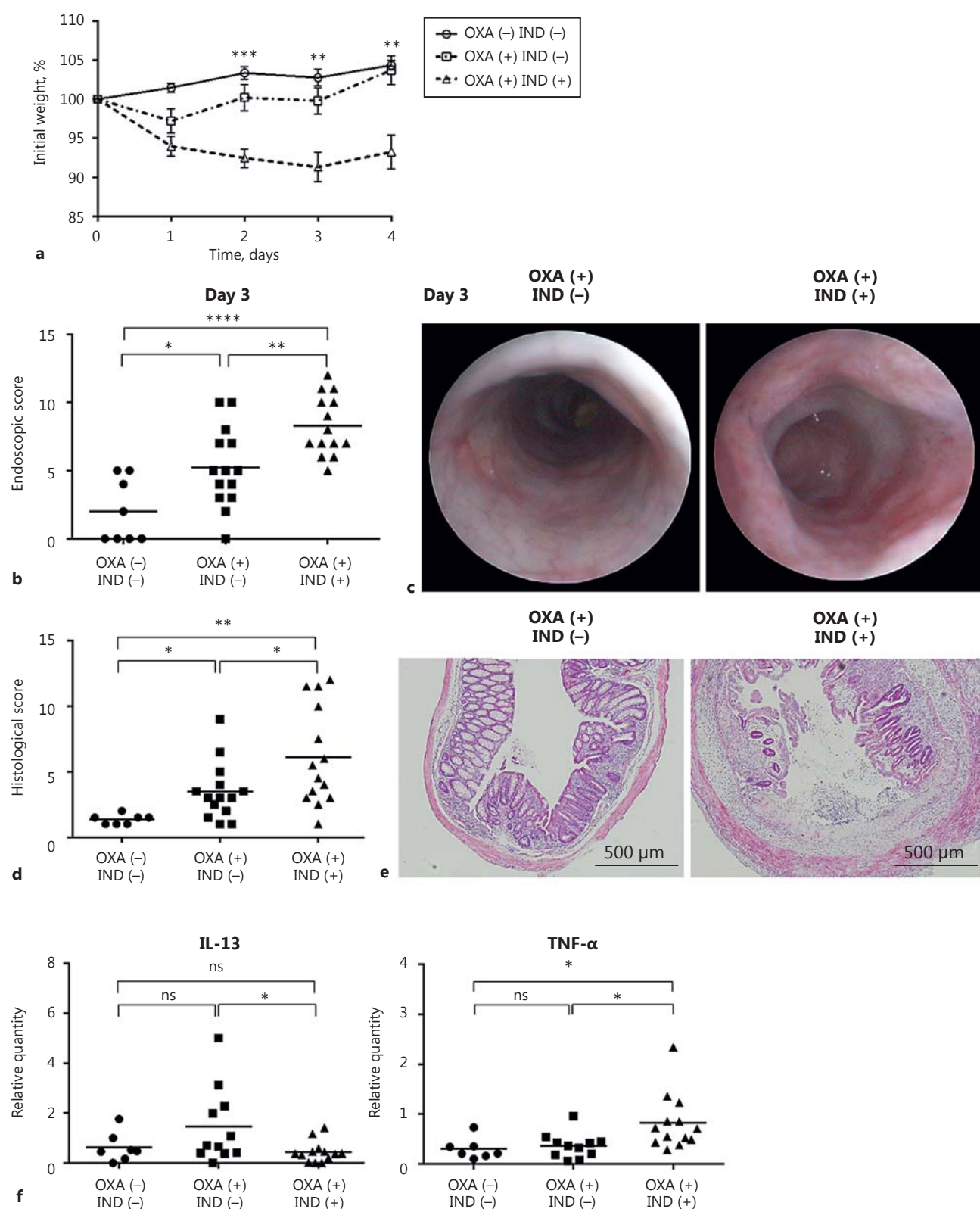
IND Reduced the Severity of OXA Dermatitis

IND has been traditionally used to treat skin rash, swelling, venom intoxication, and UC, all of which can be associated with allergy and/or a Th2 type response [17, 18]. Therefore, to investigate the effect of IND, we selected OXA to create animal models of inflammation. OXA is a haptenating agent that has been used to induce both allergic dermatitis [19, 20] and Th2 type colitis, with bowel wall edema and luminal exudates, that is more reminiscent of UC than of Crohn's disease [21].

After sensitization by painting of OXA on the abdominal skin, allergic dermatitis was induced by direct painting of OXA on the ears. IND was applied to the ears 4 h after OXA painting to ensure that the OXA had dried, and changes in ear thickness were evaluated. IND significantly reduced ear thickening on day 2, and this had become more evident by day 3 (Fig. 1a). Ear tissue was also evaluated for histological score, and IND treatment reduced immune cell infiltration, leading to a significantly lower histological score (Fig. 1b, c). OXA dermatitis features increased levels of IL-4 [22], which is important in its immunopathology [23], and as expected IL-4 was downregulated in the ears of IND-treated mice (Fig. 1d). We noticed that OXA dermatitis ear tissue exhibited massive infiltration of cells with bright pink acidophilic cytoplasm. These were consistent in appearance with eosino-

Fig. 2. Indigo naturalis (IND) increased the severity of oxazolone (OXA) colitis. **a** Body weight changes. **b** Endoscopic scores. OXA(-) IND(-), *n* = 8; OXA(+) IND(-), *n* = 14; and OXA(+) IND(+), *n* = 14. **c** Representative endoscopic images. **d** Histological score was evaluated using H & E staining. OXA(-) IND(-), *n* = 7; OXA(+) IND(-), *n* = 14; and OXA(+) IND(+), *n* = 14. **e** Representative images of H & E staining. **f** Cytokine expression levels were evaluated by real-time PCR. OXA(-) IND(-), *n* = 7; OXA(+) IND(-), *n* = 11; and OXA(+) IND(+), *n* = 13. **a** OXA(+) IND(-) and OXA(+) IND(+) groups were compared to calculate the *p* value. Data shown are the combined results of 2 repeated experiments. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, **** *p* < 0.0001.

(For figure see next page.)



phils, which are known to infiltrate the skin in OXA dermatitis [24]. We measured the expression level of an eosinophil marker, i.e., Siglec-F, and confirmed that it was also significantly reduced in the ear tissue of IND-treated mice (Fig. 1d). These data indicate that IND has an anti-allergic effect by reducing the recruitment of immune cells and Th2-associated cytokine production.

IND Increased the Severity of OXA Colitis

Next, we investigated whether IND can also alleviate colitis. As mentioned before, IND is used to treat IBD, and especially UC, which is associated with mucosal expression of IL-4 and IL-13 [25, 26]. We used BALB/c mice because C57BL/6J mice are not susceptible to OXA colitis [27]. Feeding IND itself does not affect colonic tissue homeostasis (online suppl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000471923); however, to our surprise, IND-treated OXA colitic mice exhibited severe body weight loss. Endoscopic examination also revealed significantly more severe mucosal damage due to erosions and ulcers compared to the non-IND-treated mice (Fig. 2a–c). As the manufacturing process and/or the ingredients of Chinese herbals can vary slightly between companies [28], one could think that IND from different suppliers may lead to different results. However, this seems not to be the case because we repeated the experiments with IND from 2 different companies to collect the data and the phenotype was the same. Although it remains possible that the effect differs from product to product, our data at least suggest that it is not manufacturer dependent but rather it is a property of IND.

A histological evaluation supported those findings, with more severe epithelial depletion, edema, and inflammatory cell infiltration in the colonic tissue of IND-treated mice (Fig. 2d, e). We measured cytokine levels in the tissue by real-time PCR and found that IL-13, a pivotal cytokine in this model, was upregulated in the non-IND-treated colitic mice. However, IL-13 levels were significantly reduced in the IND-treated colitic mice despite aggravation of the mucosal damage (Fig. 2f). We also confirmed the known ability of IND to scavenge reactive oxygen species [1], which can also influence intestinal inflammation by electron spin resonance. Electron spin resonance results revealed a decreased spectrum of hydroxyl radical, superoxide radical, and singlet oxygen with IND treatment (online suppl. Fig. S2). These data indicate that a reduction of IL-13 or the reactive oxygen species scavenging capability of IND could improve OXA colitis.

To explain the discrepancy between phenotype and IL-13 level, the levels of other inflammatory cytokines

were investigated. IL-4, another cytokine reported to be associated with immunopathology in the OXA colitis model [21], was not upregulated in our setting (data not shown). However, we found that TNF- α levels were increased only in the IND-fed colitic mice (Fig. 2f).

Given these results, we wished to investigate the effects of IND in other models of colitis. We therefore treated dextran sulfate sodium (DSS)-induced colitis mice with dietary IND. However, we did not see any effect compared with non-IND-fed colitic mice in this model (online suppl. Fig. S3), suggesting that this was colitis model specific in our setting.

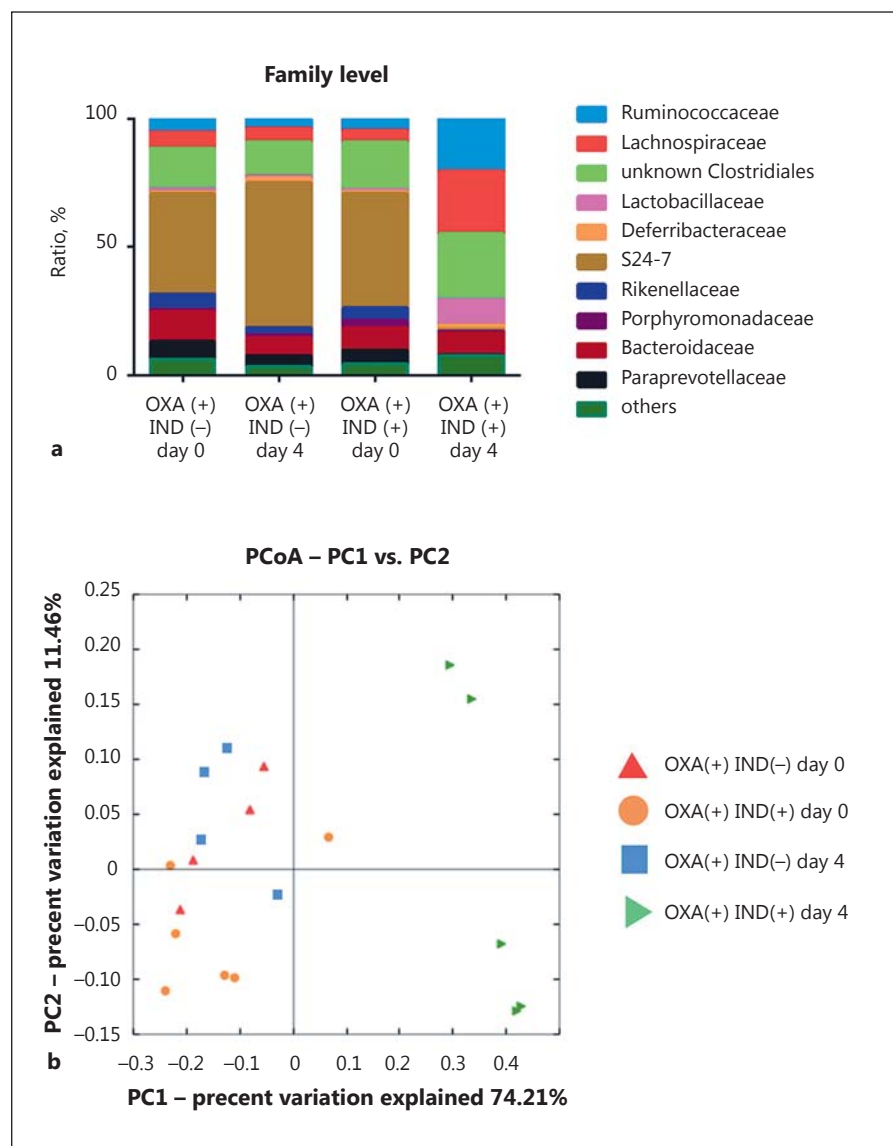
IND Changed the Composition of Gut Microflora

It is well known that the severity of inflammation in murine colitis models can vary greatly from facility to facility, which is partly because of the difference in gut flora between mice colonies. Indeed, the composition and existence of commensal bacteria greatly influence the course of intestinal inflammation. For example, IL-2 knockout mice exhibit a delayed onset of disease [29], and IL-10 knockout mice do not develop colitis when reared in germ-free conditions [30, 31]. Furthermore, even when using the same genetically manipulated mice, similar colitis experiments often yield different results [32, 33]. These facts suggested to us that IND administration may change the composition of gut flora and thus alter the course of OXA colitis. When commensal bacteria in mice feces were subjected to 16S rRNA gene sequencing, we found that the bacterial composition was dramatically altered in the IND-treated colitic group (Fig. 3a, b). Principal coordinate analysis revealed that PC1 separated the IND-treated colitic group from other groups (Fig. 3b). Before IND treatment, approximately 50–70% of the gut flora were of the *Bacteroidetes* phylum, mainly containing S24-7, which significantly decreased after treatment with IND. Meanwhile, the *Firmicutes* phylum, containing *Lachnospiraceae*, *Ruminococaceae*, and *Lactobacillaceae*, was relatively increased by IND.

Depletion of Gut Microflora by Antibiotic Treatment Blocked the Aggravation of OXA Colitis in IND-Fed Mice

To test whether the change in the composition of gut bacteria was the reason for OXA colitis aggravation, we treated mice with a combination of antibiotics prior to induction of OXA colitis and IND treatment. As expected, the exacerbation of colitis in the IND-treated group was no longer observed (Fig. 4a–e); an increase in TNF- α in the IND-treated group was also not detected (Fig. 4f).

Fig. 3. Indigo naturalis (IND) changed the composition of gut microflora. DNA was isolated from stool and 16S rRNA of gut flora was analyzed with a next-generation sequencer. **a** Taxa distribution of the average microbial composition. **b** Principal coordinate analysis plot based on weighted UniFrac distances of operational taxonomic units (POTU) created by QIIME™. Each symbol corresponds to a single fecal sample. Day 0 of oxazolone (OXA)(+) IND(-), $n = 4$; day 0 of OXA(+) IND(+), $n = 6$; day 4 of OXA(+) IND(-), $n = 4$; and day 4 of OXA(+) IND(+), $n = 5$.



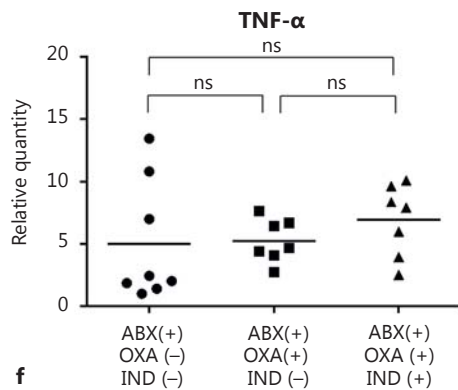
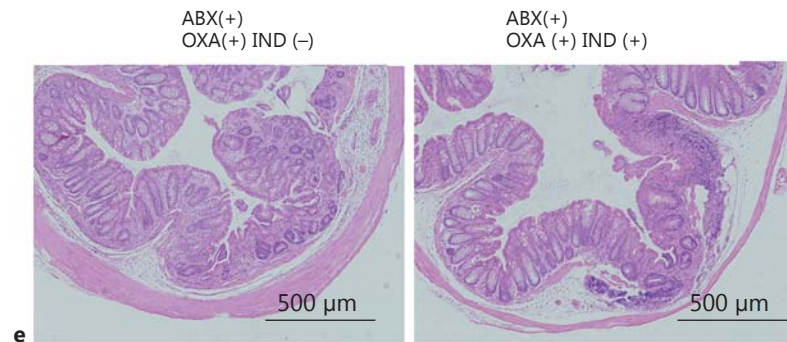
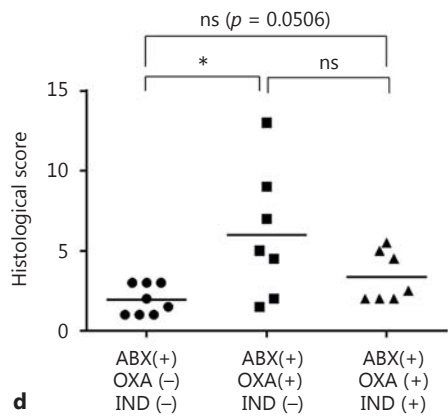
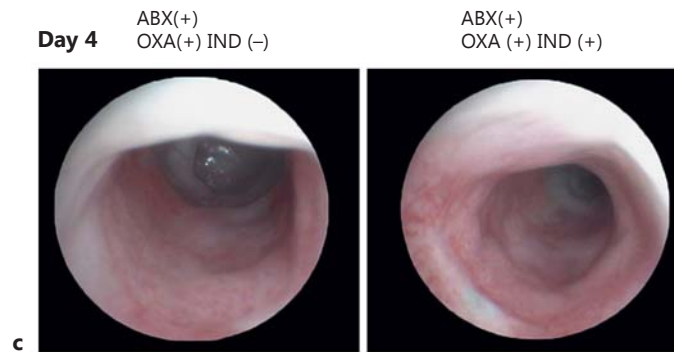
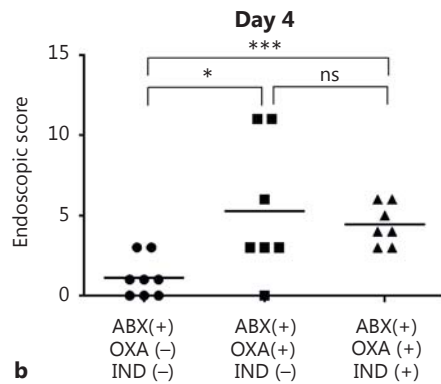
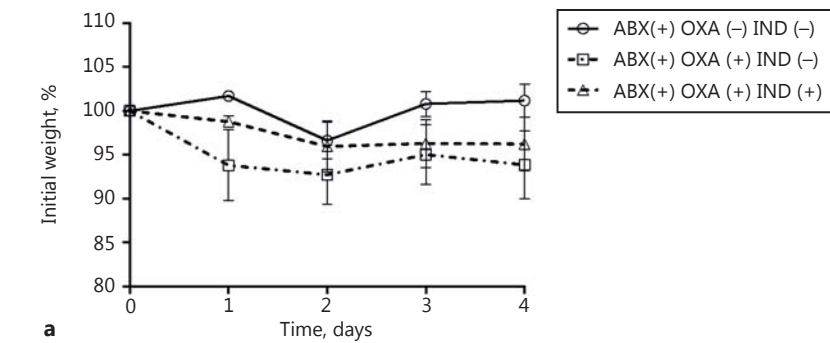
Taken together, our data suggest that IND could reduce IL-13 expression in the colon of OXA colitis mice in the presence of gut flora. However, due to IND-induced alterations in gut commensal composition, this effect may have been overwhelmed and colitis was phenotypically aggravated in these mice.

Discussion

Chinese herbal medicines have been widely used as folk remedies, and they are employed clinically in many countries including China, Japan, South Korea, the USA,

Russia, and many European countries [34]. However, even though their effect is empirically obvious, inadequate scientific evidence from basic research often makes doctors skeptical and hesitant to use them. It is also possible that many effective and useful Chinese herbals are used only in alternative medicine simply because of a lack of research-based evidence. Clearly, studies providing solid scientific data on the use of these herbal medicines would be beneficial in promoting their use in more medical facilities.

The results of the present study suggest that IND dampens allergic responses by reducing eosinophil recruitment and downregulating IL-4. IND is a water-in-



(For legend see next page.)

soluble powder and it is therefore easy to apply on the skin. It has been claimed that IND is an effective treatment for a range of skin conditions including rashes, bug bites, and snake bites. Therefore, our results seem to be compatible with their empirical use, and our data suggest one of the mechanisms of IND immune modulation.

On the other hand, the results of the colitis trial are enigmatic. IND clearly exacerbates OXA colitis; however, it reduces IL-13 expression. This result conflicts with the previously demonstrated fact that OXA colitis is mediated by IL-13 [35]. It indicates that other factors can contribute to the aggravation of OXA colitis, and our data suggest that it is a change in gut flora. It is now widely accepted that changes in gut flora, due to antibiotics, for example, can greatly influence the severity of murine experimental colitis such as in DSS and IL-10-deficient colitis models [30, 36]. No previous studies have determined whether the severity of OXA colitis could be influenced by alteration of the gut flora, but it should be possible. Indeed, commensal-dependent aggravation of OXA colitis by IND in this study supports that idea. Considering that OXA acts as a hapten to induce inflammation, one hypothesis to explain the aggravation is that IND itself contributes to the efficient OXA haptenization of commensal-derived products. This could then lead to severe tissue damage and commensal invasion, thus inducing TNF- α . Another could be that some of the commensal populations increased by IND, perhaps among the *Firmicutes* phylum, could simply induce more severe inflammation when there is invasion into colonic lamina propria than the original gut commensal composition. However, further experiments are required to investigate these theories.

We selected an OXA colitis model to investigate whether IND modifies Th2 type colitis because IND has been shown to be effective in the treatment of UC patients. However, it is important to bear in mind that a mouse model of colitis does not exactly mimic the immunopathology of human disease. OXA colitis was worsened by IND in this study, but it should not be assumed that IND would also worsen UC. Our data indicate that

IND has the effect of reducing Th2 type cytokines such as IL-4 and IL-13, which suggests that it may indeed help to alleviate inflammation involving these cytokines. Increased levels of IL-13 have been proven to exist in the intestines of UC patients [26, 37]. Therefore, the amelioration of UC symptoms by IND reported by 2 clinical studies [1, 13] may be by this mechanism. Furthermore, we have also confirmed that IND reduces free radicals (online suppl. Fig. S2). This is also the effect of 5-aminosalicylic acid, the main medication used to treat UC patients. This result agrees with that of a previous study [1, 38], and it is highly possible that IND as a reactive oxygen species scavenger plays an important role in reducing disease activity in UC patients. Another important finding is that IND can modify the gut flora. Modification of UC disease activity by fecal microbial transplantation [10, 11] and probiotics [39, 40] indicates the involvement of commensals in UC pathology. Our data suggest that IND has the potential to alter the gut flora to influence the disease activity of UC for better or worse.

As mentioned above, we did not observe any effect on DSS colitis despite previous papers describing alleviation of DSS colitis by IND [41, 42]. The dose used was similar [41], but the preparation of IND used was different from ours, and this may have led to discrepancies in the results. Additionally, differences in gut flora among animal facilities may also have affected the outcomes.

In conclusion, our results show that IND effectively reduced skin inflammation by reducing eosinophil recruitment and a lower IL-4 production. However, it aggravated colitis by changing the components of the gut flora regardless of the IL-13 reduction. This suggests that IND has the ability to suppress allergic responses to reduce inflammation. Nonetheless, our results also demonstrated the potential to alter the gut flora to affect the course of intestinal inflammation. IND may be effective in treating human disease; however, our data suggest that careful gathering of basic data on possible adverse events is warranted.

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Fig. 4. Depletion of microflora blocked the indigo naturalis (IND)-induced aggravation of oxazolone (OXA) colitis. **a** Body weight changes. **b** Endoscopic scores. **c** Representative endoscopic images. **d** Histopathological scoring. **e** Representative images of H & E staining. **f** The TNF- α expression level was evaluated by real-time PCR. OXA(-) IND(-), $n = 8$; OXA(+) IND(-), $n = 7$; and OXA(+) IND(+), $n = 7$. Data shown are the combined results of 2 repeated experiments. * $p < 0.05$, *** $p < 0.001$.

Disclosure Statement

The authors have no conflicting financial interests.

Author Contributions

S.A. and N.H. conceived and designed the experiments. S.A., N.H., J.I., E.Y., T.O., Toshihiro T., Y.M., and S.N. performed the experiments. S.A., N.H., R.D., Toshihiro T., and Y.M. analyzed the data. S.A., N.H., J.I., E.Y., T.O., R.D., Z.W., Y.K., Toshihiro T., Y.M., H.Y., D.W., M.O., Toshihito T., S.N., M.Y., and T.A. wrote this paper.

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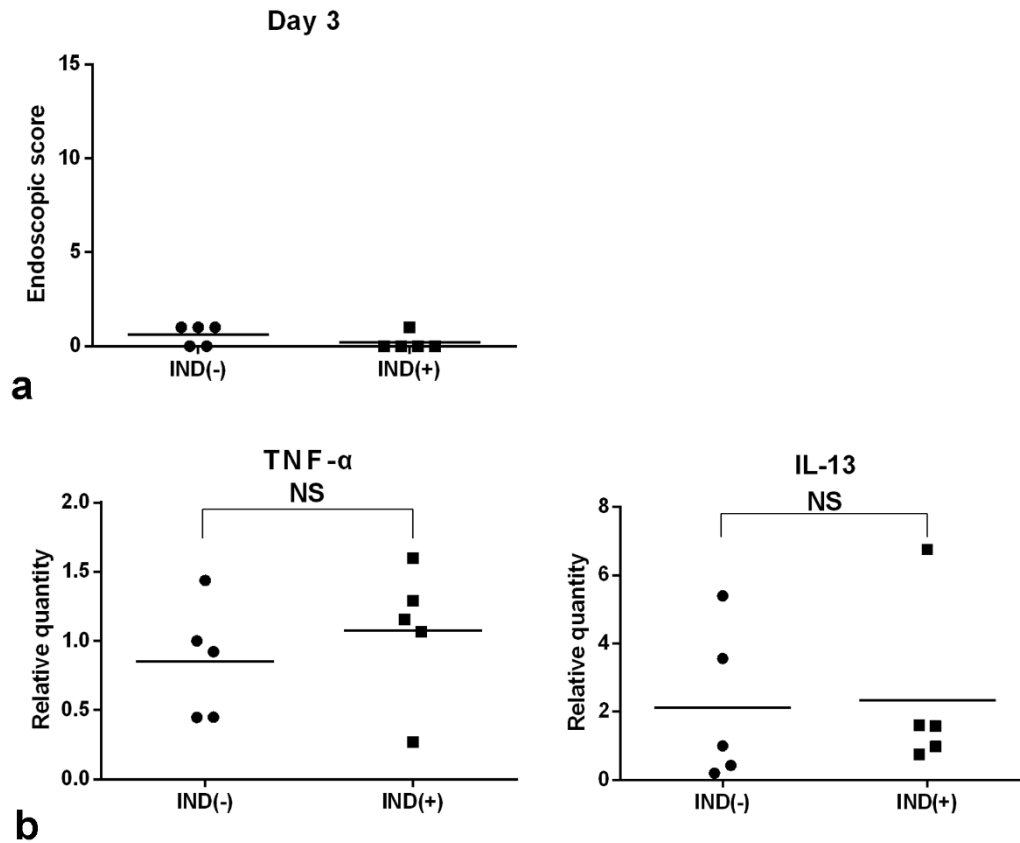


Fig S1. IND did not induce colitis by itself

Mice were fed a diet containing 5 % IND. (a) Endoscopic score was recorded. (b) Cytokine expression levels were evaluated by real-time PCR. IND(-) n = 5, IND(+) n = 5.

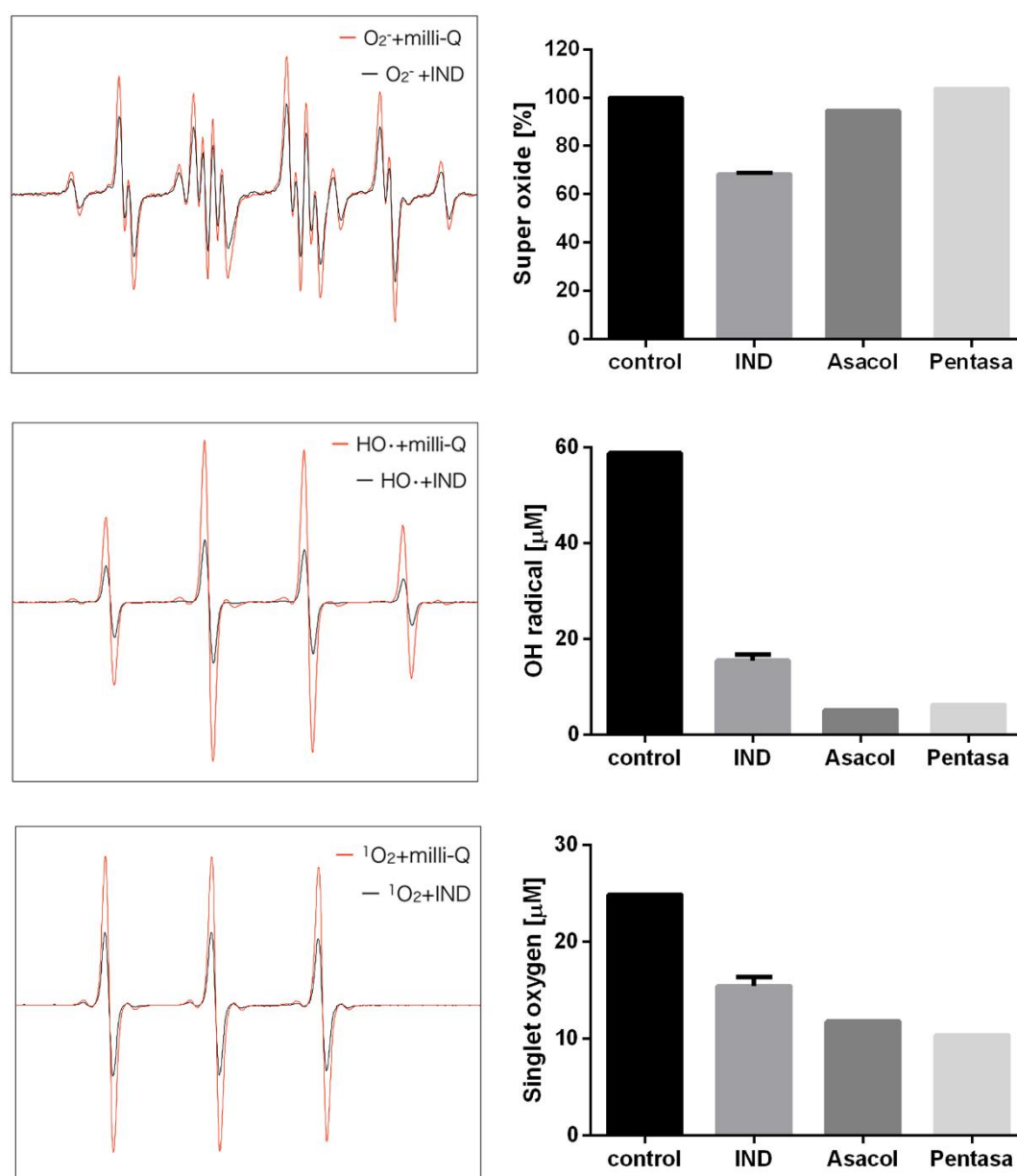


Fig S2. IND has the ability to scavenge ROS

The ability to scavenge ROS was measured using an electron spin resonance (ESR) spectrometer. The figures on the left represent the ESR spectrum from which the ROS level was calculated, which is represented by the figures on the right. The spin-trapping agents 2,2,5,5-tetramethyl-3-pyrroline-3-carboxamide (TPC) [Sigma Aldrich] as $^1\text{O}_2$ detector, and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) [LaboTec Co., Ltd.; Tokyo, Japan] as OH radical and $\text{O}_2^{\cdot -}$ detector, were used. They were dissolved in phosphate buffered saline, and the concentration of TPC was fixed at 100 mM for OH radicals, and DMPO was fixed at 5 mM and 445 mM for O_2 . As for ESR setup, 9.425 GHz microwave frequency, 2 min sweep time, 100 kHz modulation frequency, 335.5 ± 5 mT magnetic field,

and 0.1 s time constant were used. For OH radical and $^1\text{O}_2$ measurements, 0.1 mT modulation width was set. O_2^- measurement is fixed at 0.07 mT modulation width.

Indigo naturalis (IND) was dissolved to 2500 $\mu\text{g/mL}$ in ultra-pure water. For OH radical and $^1\text{O}_2$ scavenging ability measurement, 100 μL IND solution and 100 μL TPC and DMPO solution were mixed. For O_2^- scavenging ability, 50 μL IND solution and 150 μL DMPO solution were mixed. As a positive control, 2500 $\mu\text{g/mL}$ Asacol or Pentasa was mixed with DMPO as for OH radical and O_2^- scavenging measurement, and 125 $\mu\text{g/mL}$ Asacol or Pentasa was mixed with TPC as for $^1\text{O}_2$ scavenging ability measurement. As a negative control, only DMPO or TPC was used.

To measure $^1\text{O}_2$ scavenging ability, 100 μM methylene blue was prepared in the IND with TPC solution, and a light source [Pencure 2000, J. MORITA MFG. CORP.; Kyoto, Japan] was used for 20 s. To measure OH radical scavenging ability, 10 mM hydrogen peroxide was prepared in the IND with DMPO solution, and UV light [Compact UV lamp UVGL-25, Funakoshi Co., Ltd.; Tokyo, Japan] was applied for 1 min. To measure O_2^- scavenging ability, the concentration of HPX and XOD was fixed at 0.5 mM and 0.1 U/mL, respectively, in the IND with DMPO solutions with 25 % added DMSO.

200 μL of solution was examined by X-band ESR spectrometer [JES-FA-100, JEOL; Tokyo, Japan], and OH radical and $^1\text{O}_2$ were calibrated with spin adducts of 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) [Tokyo Chemical Industry Co., Ltd.; Tokyo, Japan] with known amounts of radical. In the case of O_2^- spin adducts, the scavenging rate was compared.

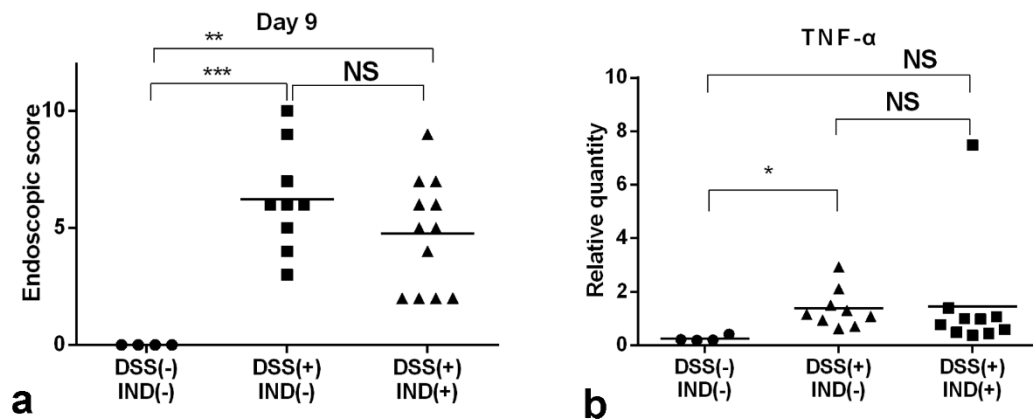


Fig S3. IND did not influence the severity of DSS colitis

Mice were treated with 2.5 % DSS (dextran sulfate sodium) in drinking water for 5 days. Mice in the IND(+) group were fed with IND every day. At day 9, evaluation of endoscopic score was performed. At day 10 mice were sacrificed for further analysis. (a) Endoscopic score. (b) Expression level of TNF- α was evaluated by real-time PCR. (DSS(-)IND(-) n = 4, DSS(+)IND(-) n = 9, DSS(+)IND(+) n =12).