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Induction of IDO-1 by Immunostimulatory DNA Limits Severity of Experimental Colitis

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The chronic inflammatory bowel diseases are characterized by aberrant innate and adaptive immune responses to commensal luminal bacteria. In both human inflammatory bowel disease and in experimental models of colitis, there is an increased expression of the enzyme IDO. IDO expression has the capacity to exert antimicrobial effects and dampen adaptive immune responses. In the murine trinitrobenzene sulfonic acid model of colitis, inhibition of this enzyme leads to worsened disease severity, suggesting that IDO acts as a natural break in limiting colitis. In this investigation, we show that induction of IDO-1 by a TLR-9 agonist, immunostimulatory (ISS) DNA, critically contributes to its colitis limiting capacities. ISS DNA induces intestinal expression of IDO-1 but not the recently described paralog enzyme IDO-2. This induction occurred in both epithelial cells and in subsets of CD11c+ and CD11b+ cells of the lamina propria, which also increase after ISS-oligodeoxynucleotide. Signaling required for intestinal IDO-1 induction involves IFN-dependent pathways, as IDO-1 was not induced in STAT-1 knockout mice. Using both the trinitrobenzene sulfonic acid and dextran sodium sulfate models of colitis, we show the importance of IDO-1 induction in limiting colitis severity. The clinical parameters and histological correlates of colitis in these models were improved by administration of the TLR-9 agonist; however, when the function of IDO is inhibited, the colitis limiting effects of ISS-oligodeoxynucleotide were abrogated. These findings support the possibility that targeted induction of IDO-1 is an approach deserving further investigation as a therapeutic strategy for diseases of intestinal inflammation. The Journal of Immunology, 2010, 184: 000–000.

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Abbreviations used in this paper: 1-mT, 1-methyl-tryptophan; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; ISS, immunostimulatory; ISS-ODN, immunostimulatory oligodeoxynucleotide; KO, knockout; M-ODN, mutated oligodeoxynucleotide; MLN, mesenteric lymph node; MPO, myeloperoxidase; TNBS, trinitrobenzene sulfonic acid; WT, wild-type.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10S16.00 via activation of GCN2 kinase (7). These changes include the inhibition of T cell replication, the induction of T cell apoptosis, and the support of regulatory T cell differentiation and suppressor function activation (8). Additionally, they create a hostile environment for invading microbes including numerous bacteria, viruses, and intracellular parasites (1). These important functions have led to evaluation of the role of IDO-1 in autoimmune and inflammatory conditions (9). The physiologic relevance of IDO-1 induction has already been demonstrated in models of pulmonary disease where upregulation of IDO-1 via TLR9 agonists was shown to inhibit pathogen growth and lessen asthma severity (10, 11).

Similar to the lungs, the luminal surface of the mammalian gastrointestinal tract continually interacts with foreign Ags, including microbial products from both commensal and pathogenic bacteria. In health, the mucosal immune system is geared toward tolerance and limits inflammatory responses to these Ags. In genetically predisposed individuals, when tolerance to commensal bacteria is lost, chronic inflammatory bowel disease (IBD) may develop. The in vivo mechanisms mediating immune tolerance is an area of active investigation and involves complex interactions between professional APCs, epithelial cells, and cells of the adaptive immune system. IDO-1 is a key molecule mediating tolerance by linking the innate and adaptive immune responses in other some systems (2). IBD may also play an important role in gut mucosal defense and act as a natural brake to intestinal inflammatory responses.

The gut is a site of high IDO-1 expression in the basal state, and its expression is increased in the setting of intestinal inflammation (12, 13). Biopsies from patients with an IBD have IDO-1 levels that are significantly increased compared with biopsies from healthy individuals. In IBD, the increased IDO-1 expression is apparent in both epithelial and mononuclear cells in areas of active disease, particularly near sites of ulceration (13–16). Similarly, in animal models of IBD, IDO-1 enzyme expression is
upregulated (13, 17). In the triminitrobenzene sulfonic acid (TNBS) colitis model, IDO expression and functionality is increased over baseline, particularly so in professional APCs of the lamina propria (17). Administration of a specific IDO inhibitor, 1-methyltryptophan (1-mT), significantly augmented the normal inflammatory response to TNBS, suggesting that IDO plays an important role in downregulating Th1 responses within the intestinal tract. These observations suggest that increasing IDO expression within the intestine may have the therapeutic capacity to abrogate colitis.

To address whether IDO upregulation lessens colitis severity, we examined the effect of a known IDO-inducing agent, ISS oligodeoxynucleotide (ISS-ODN), on two experimental models of colitis, TNBS and dextran sodium sulfate (DSS). In this study, we demonstrate that administration of an anticolonitic dose of ISS-ODN induces IFN-dependent expression of IDO-1, but not IDO-2, throughout the intestinal tract in both professional APCs of the lamina propria as well as intestinal epithelial cells. Furthermore, we show that IDO-1 induction is critical to the anticolonitic effects of ISS-ODN in both TNBS and DSS colitis in that coadministration of a specific IDO inhibitor negates the beneficial effects of this TLR9 agonist.

Materials and Methods

ODNs

Oligonucleotides were purchased as reversed-phase HPLC purified, LPS-free, single-stranded phosphothioate oligodeoxynucleotides (Trilink, San Diego, CA). The ISS-ODNs and the control or mutated oligodeoxynucleotide (M-ODN) used were synthesized to match those found previously (17). For the recurrent TNBS model, mice initially received a 0.4 mg TNBS (Sigma-Aldrich, St. Louis, MO) in 35% ethanol via a flexible 3.5 Fr catheter inserted 4 cm proximal to the anus as described previously (17). For the recurrent TNBS model, mice initially received a 0.4 mg TNBS enema to reduce early deaths, followed by a 0.5-mg TNBS enema at day 7 similar to previously described techniques for chronic TNBS colitis (19, 20). In the DSS colitis model, a 2.0% aqueous solution of DSS (TdB Consultancy, Uppsala, Sweden) was passed through a 0.22-μm cellulose acetate filter then administered to mice for 7 d as drinking water. Ten micromers of the ISS DNA or the control-ODN was injected i.p. prior to each administration of TNBS or DSS. Pellets containing slow-release 1-mT at a rate of 0.9 mg/h (17). To assess morphological and histological differences and to obtain tissues for analysis, surviving mice were sacrificed at the times detailed with each result.

Morphological and histological analysis

The colon was removed from its mesentery to the pelvic brim by blunt dissection. The colon was opened longitudinally along the mesenteric attachment and pinned flat so that the mucosal surface could be examined. The entire length of the colon was pinned out, then in 10% formalin and transferred to 70% ethanol before being processed for embedding in paraffin. Five-micrometer serial sections were prepared and stained with H&E for histological grading. Methods for scoring TNBS-treated mice for morphology and histology have been described previously (17) and are based on the systems described, respectively, by Colon et al. (21) and Fuss et al. (22) for DSS-treated mice. A previously described 12-point scoring system was used that evaluated colonic epithelial damage (0–6 points) and inflammatory infiltrate separately assigned to the mucosa (0–3), submucosa (0–2), and muscular layer and serosa (0–1) (23). Scoring was performed in a blinded fashion independent by two of the authors (M.A.C. and E.E.B.).

Western blotting for IDO

Cell and tissue lysates were prepared from whole tissue or from the respective cell layers. Protein concentrations were determined using bicinechometric protein assay (Pierce Chemical Co., Rockford, IL). Samples were then subjected to electrophoresis on a 10% Tris-HCl ready gel (Bio-Rad, Hercules, CA) and transferred onto immobilon polyvinylidine difluoro membranes (Millipore, Bedford, MA). Blocking was with 5% nonfat dry milk in TBS. IDO-1 Abs were a polyclonal rabbit anti-mouse IDO Ab (BioLegend, San Diego, CA). The IDO-1 Abs were tested for specificity; none of them did not bind to recombinant IDO-2 protein. The mouse anti-IDO-2 Ab was used as described previously (4). Phospho-specific STAT1 (pY701) was from BD Biosciences (San Jose, CA). Ag detection was achieved using HRP-linked secondary Abs and the ECL system (Amersham Biosciences, Buckinghamshire, U.K.). The membranes were then stripped and reprobed for β-actin to confirm equal protein concentrations (Santa Cruz Bio¬technology, Santa Cruz, CA). Alternatively, a membrane from a simultaneously run gel with equal loading volumes was probed for β-actin.

Real-time PCR

Primers were designed for multiple genes using Primer Express Software (Applied Biosystems, Foster City, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). The following sequences were used: IDO-1 (5′-CGGACCTGAAGAGCAGGTCTC-3′), GAPDH (5′-TGACACCACACTGGCTTTC-3′). The PCR products were validated by melting point analysis. PCR was performed using the iScript One-Step RT-PCR kit (Bio-Rad) on a Bio-Rad iCycler (Applied Biosystems, Foster City, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). The ISS-ODNs and the control or mutated oligodeoxynucleotide (M-ODN) used were synthesized to match those found previously (17). The PCR products were validated by melting point analysis. PCR was performed using the iScript One-Step RT-PCR kit (Bio-Rad) on a Bio-Rad iCycler (Applied Biosystems, Foster City, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). The ISS-ODNs and the control or mutated oligodeoxynucleotide (M-ODN) used were synthesized to match those found previously (17). For the recurrent TNBS model, mice initially received a 0.4 mg TNBS (Sigma-Aldrich, St. Louis, MO) in 35% ethanol via a flexible 3.5 Fr catheter inserted 4 cm proximal to the anus as described previously (17). The TNBS colitis was homogenized in hexadecyltrimethyl ammonium bromide (0–2), and muscular layer and serosa (0–1) (23). Scoring was performed in a blinded fashion independent by two of the authors (M.A.C. and E.E.B.).

Immunofluorescence

Fresh-frozen intestinal sections were prepared by freezing tissues in Tissue-Tek OCT Compound (Miles, Elkhart, IN). Sections were cut 6 μm, allowed to warm to room temperature, fixed in a 1:1 mixture of methanol and acetone for 20 min, and then washed in water, followed by PBS. Endogenous peroxidases were quenched by incubation in 3% H2O2. An avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) was used and followed by blocking with 1% BSA in PBS with Mouse On Mouse (Vector Laboratories) when a murine primary Ab was used. Primary Abs used for IDO-1 staining was the rabbit anti-mouse IDO Ab (17). The IDO-2 Ab was the same as used for the Western blots. Other primary Abs included biotin anti-CD11c and anti-CD11b (eBioscience, San Diego, CA), mouse anti-IDO-2 (4), and mouse anticytokaterin 18 (Chemicon International, Temecula, CA). Secondary Abs bound to the described chromogens were from Jackson Immunoresearch Laboratories (West Grove, PA). VECTASTAIN DA-H was used for nuclear counterstaining, and sections were then subjected to a Zeiss Axioint 200 with an AxioCam MRm camera.

Determination of myeloperoxidase activity

Colon tissues were opened longitudinally and a lengthwise portion was taken from the anal verge to the ascending colon and weighed (~50 mg). This was homogenized in hexadecyltrimethyl ammonium bromide (0.5%)
in 50 mmol/L phosphate buffer (pH 7.0). The homogenate was sonicated for 10 s, frozen and thawed three times, and centrifuged for 15 min. An aliquot of the supernatant was used for determination of enzyme activity as described previously (18).

**Isolation of lamina propria mononuclear cells and epithelial cell layers**

The intestines were removed from mice, flushed with cold PBS, opened along the mesenteric border, and then washed again in cold PBS. The epithelial cell layer was removed by incubation in calcium- and magnesium-free HBSS containing 5 mM EDTA at 37˚C, then preserved for RNA or protein isolation. The lamina propria cell layer was isolated as described previously (24).

**Statistical analysis**

Morphological and histological data were assessed using Student t test. Survival data were assessed using a χ² test. Real-time PCR data and weights are expressed as mean ± SEM. Significance of fold increase in mRNA expression over control was assessed using a Student t test.

**Results**

**ISS-ODN induces IDO-1 but not IDO-2 throughout the intestinal tract**

Certain ISS-ODN sequences are protective in experimental colitis models, whereas M-ODNs, mutated versions of the ISS-ODNs, do not offer effective protection from colitis (18). In determining whether IDO-1 expression contributes to the anticolitic effect of ISS-ODN, we first sought to assess whether the same sequences, used at the same colitis preventing dosage, could induce intestinal IDO. ISS-ODN (10 μg), M-ODN (10 μg), or PBS was administered via i.p. injection, and tissues were harvested for RNA or protein. Time course experiments led to the choice of the 36-h time point for RNA and 72 h for protein. Small intestine and colon tissue from M-ODN–treated mice had no induction of IDO-1 protein over PBS-treated mice (Fig. 1A). Fold changes in IDO-1 mRNA also did not increase significantly (colon 1.10 ± 0.64, p = 0.89; small intestine 1.13 ± 0.15, p = 0.79). However, when compared with M-ODN treatment, ISS-ODN administration led to a significant increase in IDO-1 protein and mRNA in both the colon (average 10.7-fold increase) and in the small intestine (average 3.5-fold increase) (Fig. 1B,1C). Additionally, ISS DNA significantly increased IDO-1 in the mesenteric lymph nodes (1.9 ± 0.2), although to a lesser degree than in the intestinal tissue.

A paralog to the originally identified tryptophan catabolizing enzyme IDO (which is now referred to as IDO-1) was recently identified (4, 5). Metz et al. (4) had demonstrated that by Western blot the IDO-2 protein was slightly smaller than IDO-1. This raised the question of which intestinal IDO was upregulated by ISS-ODN. We first sought to determine the specificity of our IDO-1 Abs and found that neither of our IDO-1 Abs detected recombinant IDO-2 protein (data not shown). Having established the specificity of our reagents for IDO-1, we next sought to determine whether IDO-2 is

**FIGURE 1.** Intestinal tract IDO-1, but not IDO-2, is induced by ISS-ODN. Ten micrograms of either the ISS-ODN or the M-ODN was injected i.p., and the tissues were harvested at 72 h for protein. Compared with control (untreated mice), the administration of M-ODN does not lead to induction of IDO-1 in either the colon or the small intestine (A). However, the injection of ISS-ODN leads to a significant induction of IDO-1 mRNA and protein throughout the intestinal tract and mesenteric lymph nodes (MLNs) when compared with control mice injected with M-ODN (B, C). Analysis of the same samples did not show an increase in IDO-2 protein after ISS-ODN (D). All experiments consisted of at least four mice per group and were repeated at least twice. Two representative samples are shown in each Western blot for C. RT-PCR data are shown as fold change relative to control ± SEM. *p ≤ 0.05 shows statistical significance.

**FIGURE 2.** ISS-ODN administration leads to increased expression of IDO-1 in both lamina propria and epithelial cells layers. Ten micrograms of the ISS-ODN or the control M-ODN was injected i.p. into SJL/J mice, and the tissues were harvested at 36 h for RNA or 72 h for protein. The tissue was processed to isolate the epithelial cell layer (via calcium and magnesium-free HBSS + EDTA) and the lamina propria cell layer (via dispase/collagenase digestion). Induction of IDO-1 mRNA by ISS-ODN is shown in each cell layer from both the colon and small intestine (A). An increase in IDO-1 protein is confirmed on this western blot for two representative samples from isolated cell layers from the colon and small intestine (B). This experiment was repeated twice with two mice in each group.
also induced by ISS-ODN. RT-PCR using primers specific for IDO-2 did not detect any changes in mRNA expression after administration of ISS-ODN (Fig. 1B, gray bars). Finally, with high protein loading concentrations (50 μg/well) a distinct band for IDO-2 was identified in colonic and small intestinal tissues; however, the band was faint and showed no discernable difference in treatment verses control samples (Fig. 1D). Additionally, relative to our prior findings that IDO-1 is increased in TNBS colitis, we examined colonic tissue from mice with TNBS colitis and found no change in IDO-2 mRNA (average 0.8 ± 0.22-fold change versus control, \( p = \text{NS} \)) or IDO-2 protein expression over controls. Taken together, these findings suggest that only IDO-1 and not IDO-2 expression is up-regulated by ISS-ODN and TNBS colitis.

**Localization of intestinal IDO-1 expression after ISS-ODN**

Having confirmed the induction of IDO-1 by ISS-ODN in whole intestinal protein and mRNA extracts, we sought to determine which tissue layers and cell types demonstrated the increase in IDO-1 expression. Previous studies focused on the importance of IDO-1 expression in immune regulatory cells, specifically dendritic cell populations (2, 3). However, nonprofessional APCs have also been shown to express IDO-1 with physiologic relevance in other organ systems (10). After injection of ISS-ODN or M-ODN, whole colon and small intestine sections were flash-frozen or processed for isolation of the epithelial and lamina propria layers. RT-PCR and Western blotting showed that after ISS-ODN injection IDO-1 was upregulated in both lamina propria and the epithelial cell layers (Fig. 2A, 2B). In these experiments, the average induction of IDO-1 ranged from 5- to 14-fold over baseline within epithelial and lamina propria cell layers. The fold increases in IDO-1 induced by ISS-ODN were similar in the distal colon and terminal ileum. Moreover, the fold increases in IDO-1 were similar in the lamina propria and epithelial layers in both organs. The differential level of IDO expression between either the ileal or colonic layer types or between epithelial and lamina propria layers within the colon or small intestine was not statistically significant.

To further delineate where IDO-1 was expressed after ISS-ODN exposure, we used immunofluorescence to examine frozen sections of the colon and small intestine. Only faint IDO-1 staining was detected in the control-treated tissues (Fig. 3A, 3B, left image), whereas samples from ISS-ODN–treated mice demonstrated intense staining for IDO-1 (Fig. 3A, 3B, two right images). Cytokeratin-18 costaining (FITC) was used to assist in delineating the detection in colonic epithelial cells. In the colon, IDO-1 staining was strongest in the epithelial cells at the crypt base. In contrast, in the small intestine, staining was strongest in goblet cells and lamina propria cells of the villi. IDO-1 staining was also detected in select cells of small intestinal Peyer’s patches (Fig. 3B, far right image).

Given the previously identified physiologic importance of IDO’s induction in dendritic cells and macrophages, we costained for IDO-1 and Western blotting showed that after ISS-ODN injection IDO-1 was upregulated in both lamina propria and the epithelial cell layers (Fig. 2A, 2B). In these experiments, the average induction of IDO-1 ranged from 5- to 14-fold over baseline within epithelial and lamina propria cell layers. The fold increases in IDO-1 induced by ISS-ODN were similar in the distal colon and terminal ileum. Moreover, the fold increases in IDO-1 were similar in the lamina propria and epithelial layers in both organs. The differential level of IDO expression between either the ileal or colonic layer types or between epithelial and lamina propria layers within the colon or small intestine was not statistically significant.

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**FIGURE 3.** Cellular localization of IDO after ISS-ODN. Colon and small intestinal sections from control (M-ODN treated) mice shows faint staining for IDO-1 (red) (A, B; first image in each). Cytokeratin 18 (green) staining is used on colon sections to highlight structure. DAPI was used for nuclear staining. Seventy-two hours after ISS-ODN administration, IDO staining becomes markedly more positive in both epithelial cells (particularly at the base of the colonic crypts) as well as within the lamina propria and Peyer’s patches (A, B; images to right of arrow in each). Dual staining of frozen sections shows IDO-1 costaining in some but not all cells with CD11c (C) and CD11b (D), markers for dendritic cells, and macrophages, respectively. Staining for IDO-2 (red) was performed on the same control and ISS-ODN–treated tissues. Faintly positive cells were identified in the lamina propria with no difference identified between the treated and untreated tissues (E). Original magnifications ×400.
and CD11c or CD11b to determine whether ISS-ODNs were also inducing IDO-1 in these cell types (Fig. 3C, 3D). In the small intestine, there were numerous CD11c+ cells in the lamina propria cells of the villus. A few of these CD11c+ cells costained for IDO-1, suggesting that ISS-ODN induces IDO expression in a fraction of dendritic cells. There were also a small number of CD11b+ cells in the villus lamina propria. Some but not all of these CD11b+ cells also expressed IDO-1. After the administration of ISS-ODN, most of the IDO-1+ cells in the colon are epithelial cells at the base of the crypts, but there are also CD11b+ and CD11c+ cells that coexpress IDO-1. Moreover, we found that the administration of ISS-ODN is positively associated with a quantitative increase in both CD11b+ and CD11c+ cells (Fig. 4).

Immunofluorescence for IDO-2 in tissue from untreated mice showed very faint staining in lamina propria cells of the colon without significant epithelial cell staining (Fig. 3E, left image). Staining for IDO-2 did not appear to be increased after ISS-ODN treatment as it had with IDO-1 (Fig. 3E, middle and right images). Thus, the immunofluorescence staining supports our findings that IDO-1 expression is upregulated by ISS-ODN in both intestinal lamina propria mononuclear cells and epithelial cells.

Gut IDO-1 expression after ISS-ODN is associated with IFN induction and STAT1-dependent signaling

IDO-1 expression has been shown to be increased by cytokines, including TNF-α and IL-1, as well as TLR ligands, including LPS (2). Both type I and type II IFNs also induce IDO-1 (2, 3, 10). Similarly, both type I and type II IFNs have been implicated as mediators of the physiological effects of TLR-9 agonists (3, 10, 23, 26). STAT1 is a downstream signaling molecule for both type I and type II IFNs (27, 28). We evaluated cytokine expression by mRNA at 12 and 72 h after ISS-ODN and found TNF-α, IFN-α, and IFN-γ all to be significantly increased (Fig. 5). After 72 h of dosing ISS-ODN twice to maximize detection, IDO-1 expression was high at 35-fold over untreated mice. Then to determine whether, within the intestinal tract, ISS-ODN’s upregulation of IDO was dependent on IFN signaling, we used STAT1 knockout (KO) mice. These STAT1 KO mice were compared with background-matched C57BL/6 wild-type (WT) controls. After ISS-ODN, IDO-1 expression was increased in both the colon and small intestine of the WT but not the STAT1 KO mice (Fig. 6A). RT-PCR confirmed these findings (data not shown). Using the intestinal samples from the WT mice, we also used Western blotting to confirm that ISS-ODN administration leads to activation of STAT-1 through phosphorylation (Fig. 6B).

Clinical and histological benefits offered by ISS-ODNs in TNBS colitis are abrogated by concurrent IDO inhibition

Having demonstrated that doses of ISS-ODN that prevent colitis also significantly increase intestinal IDO-1, a known tolerance promoting enzyme, we next wanted to determine whether IDO-1 upregulation contributed to the beneficial effects of ISS-ODN on TNBS colitis. To address this question, we concurrently administered ISS-ODN and 1-mT, a specific IDO inhibitor (10, 17, 25, 29–31), and then followed clinical and pathology parameters known to be altered in TNBS colitis (17, 29).

Mice were given ISS-ODN, and subsequently, a slow release tablet of 1-mT was inserted under their dorsal skin at the time of TNBS enema. All mice lost weight by day 1, but by day 2, the mice receiving ISS-ODN along with either the TNBS or ethanol (control) enema began regaining weight (Fig. 7A). The mice receiving TNBS alone or in combination with 1-mT, with or without concurrent ISS-ODN, all continued to lose weight. At day 5, weight loss in the ISS-ODN–treated group was significantly less than in the TNBS alone group (Table I). However, weight loss in mice receiving the ISS-ODN along with the IDO inhibitor was significantly greater than mice in the ISS-ODN/TNBS group but not statistically different from the group receiving TNBS alone. Similarly, survival experiments performed using a higher-dose TNBS enema confirmed that ISS-ODN lessened mortality, and this survival benefit was negated by concurrent IDO inhibition (Fig. 7B).

Colons were harvested from the mice after day 5 of the weight loss experiments and were compared for histology and morphology. Mirroring the weight loss and mortality results, mice who
received ISS-ODN prior to a single TNBS enema had lower histological and morphologic scores; however, this benefit was lost in the mice who received the 1-mT pellet for concurrent IDO inhibition (Fig. 7C, Table II). TNBS colitis has most frequently been used as an acute colitis model in which mice received only one TNBS enema (18, 29). However, use of a second TNBS administration increases T cell-mediated disease, whereas repeated dosing (up to eight times) leads to chronic inflammation with IL-13 triggered tissue fibrosis (19, 20). Because a key function of IDO-1 is to downregulate T cell responses, we compared the same groups previously tested when TNBS enema was administered twice. The ISS-ODN was administered on days 0 and 7, whereas the 1-mT pellet was administered on the day before the second enema performed on day 7. The results were similar to the single TNBS administration where ISS-ODN reduced colitis severity, but the effect was abrogated by inhibition of IDO-1 with 1-mT (Table III). There was no significant differences in final weights between groups; however, colon lengths were significantly longer in the TNBS plus ISS-ODN groups than the TNBS alone or with co-administration of ISS-ODN and 1-mT or 1-mT alone (9.5 versus 8.9 and 9.0 cm, respectively; \( p < 0.01 \)). These findings suggest that the beneficial effects of ISS-ODN in both single and recurrent TNBS colitis involved an IDO-1–dependent mechanism.

**IDO-1 induction by ISS-ODN is critical to limiting severity of DSS colitis**

ISS-ODN has also been shown to be effective in limiting disease severity in non-T cell-mediated DSS colitis model (18, 23). Recognizing that IDO-1’s most examined immunologic function is its ability to downregulate T cell responses, we sought examine the possibility that induction of IDO-1 could also be critical to the ISS-ODN’s effects in the DSS model. A similarly designed experiment to the TNBS model evaluated DSS colitis at 7 d with ISS-ODN being tested with or without IDO-1 inhibition (Fig. 8). ISS-ODN administration lessened DSS disease severity as evidenced by lower myeloperoxidase activity, disease activity indexes, and histology scores (Fig. 8A, 8B, 8D). Regenerative crypts and epithelial proliferation were also more apparent in these mice as evidenced on H&E and Ki-67 staining (Fig. 8C, 8E). Administration of the IDO inhibitor 1-mT alone did not significantly worsen any of these measured parameters compared with DSS alone. However, 1-mT blockade of IDO significantly abrogated

**FIGURE 6.** ISS-ODN induces IDO-1 in a STAT-1–dependent manner. Age-matched WT C57BL/6 mice and STAT-1 KO mice on a C57BL/6 background were given 10 \( \mu \)g ISS-ODN or control M-ODN. IDO-1 expression increases in both the small intestine and colon of WT C57B/6 mice but not in the STAT1 KO mice (A). Two representative mice are shown for each condition. In the WT mice, activation of STAT-1 signaling by phosphorylation is shown on this Western blot analysis (B).

**FIGURE 7.** The clinical, histological, and morphologic benefits offered by ISS-ODN prior to TNBS colitis are abrogated by concurrent IDO inhibition. ISS-ODN was offered at 10 \( \mu \)g i.p. prior to TNBS or ethanol enema. The 1-mT slow release pellet was inserted concurrently with the TNBS enema; \( n = 10 \) in each group. Relative weight changes from baseline weight are graphed as mean change ± SEM (A). TNBS enema was at 0.5 mg per mouse. Survival curve of mice with the same treatment groups is shown (B), where TNBS was administered at 0.7 mg/mouse. Distal colon sections were harvested 5 d after treatment and representative sections are shown (H&E staining, original magnification \( \times 100 \)) (C).
the beneficial effects of ISS-ODN on all of these parameters. The results from this second model of colitis strengthen the case for IDO-1 induction by ISS-ODN being critical for its anticolitic properties. Furthermore, the efficacy of IDO-1 induction in the acute DSS model suggests that this enzyme’s colitis preventing properties are not limited to its ability to dampen T cell responses.

**Discussion**

In the mammalian gastrointestinal tract, the innate immune system has a key function to recognize and determine appropriate responses to luminal Ags and microbial products (32). Some of the genes encoding for proteins that regulate the innate immune response pathways are susceptibility genes for the development of the chronic IBDs (33). TLRs, a class of transmembrane pattern recognition receptors, are an important part of the innate immune system and are widely expressed by various cell types in the gastrointestinal mucosa (32). Upon activation, TLRs initiate signaling to induce antimicrobial effector pathways as well as to control adaptive immune responses. IDO, an enzyme that regulates tryptophan catabolism, is a downstream mediator of TLR signaling known to exhibit both antimicrobial effects and effects on the adaptive immune response (1–3, 44). The overall decrease in mortality after administration of 1-mT suggests that the beneficial effects were mediated through the induction of IDO-1. The effect of 1-mT administration alone also worsened disease severity, although less dramatically than previously observed (17), a finding that may be explained by variability in TNBS stock or mouse vendor (29, 42). All experiments in this study were performed with age/sex-matched mouse groups from the same vendor.

TNBS colitis is commonly evaluated within 7 d after the first rectal dose (29). A second dose of TNBS still results in a Th-1 response; however, dosing greater than three times leads to an IL-13–triggered TGF-β–dependent tissue fibrosis with a concurrent decline in Th1 cytokines (19, 43). In recognition of IDO’s role in dampening T cell responses, we also evaluated ISS-ODN in a repeat model of TNBS. The results were similar, although less dramatic, than with the single administration, a finding that may be explained by the reduced TNBS dose so as to avoid excessive mortality. IDO-1 expression by CD11b+ and CD11c+ APCs is associated with inhibition of effector T cell activation and downregulation of the immune response (2, 3, 44). The overall decrease in severity of this Th-1–mediated colitis after administration of ISS-ODN, an agent that induces IDO-1 in dendritic cells, fits with this model.

**Table I.** *Day 5 weight and mortality comparison*

<table>
<thead>
<tr>
<th>Mortality</th>
<th>p Value</th>
<th>% Δ in Weight</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISS-ODN + EtOH enema</td>
<td>0/10</td>
<td>+3 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>TNBS</td>
<td>5/10</td>
<td>0.02 versus EtOH</td>
<td>−22 ± 1.7</td>
</tr>
<tr>
<td>TNBS + 1-mT</td>
<td>6/10</td>
<td>N.S. versus TNBS</td>
<td>−23 ± 2.4</td>
</tr>
<tr>
<td>TNBS + ISS-ODN</td>
<td>1/10</td>
<td>0.07 versus TNBS</td>
<td>−3 ± 1.7</td>
</tr>
<tr>
<td>TNBS + ISS-ODN + 1-mT</td>
<td>5/10</td>
<td>0.07 versus TNBS + ISS-ODN; N.S. versus TNBS</td>
<td>−20 ± 3.9</td>
</tr>
</tbody>
</table>

Ten mice per group. This table numerically summarizes day 5 of the data presented in Fig. 5 and provides statistical analysis using the Fisher’s exact test for mortality and Student t test for weight loss.

Day 5 after single TNBS enema to SJL/J mice.

**Table II.** *Histology and morphology scores for acute phase after TNBS enema*

<table>
<thead>
<tr>
<th>Histology</th>
<th>p Value</th>
<th>Morphology</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNBS</td>
<td>5.5 ± 0.4</td>
<td>3.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>TNBS + ISS-ODN</td>
<td>2.9 ± 0.5</td>
<td>0.001 versus TNBS</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>TNBS + 1-mT</td>
<td>6.5 ± 0.4</td>
<td>0.038 versus TNBS</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>TNBS + ISS-ODN + 1-mT</td>
<td>5.2 ± 0.5</td>
<td>0.004 versus TNBS + ISS-ODN; 0.62 versus TNBS</td>
<td>3.3 ± 0.3</td>
</tr>
</tbody>
</table>
ISS-ODN induced IDO-1 expression in colonic epithelial cells, which was greatest at the crypt base. Although it is not clear why IDO-1 was selectively expressed in these epithelial cells, it is possible that TLR9 is selectively expressed in these epithelial cell populations or that IFN signaling is differentially regulated in these cells compared with other epithelial cells. It is also possible that a paracrine, rather than an epithelial cell autonomous mechanism, mediates the induction of IDO-1. The ISS-ODN–mediated induction of IDO-1 in colonic epithelial cells raised the question of whether the induction of IDO-1 expression in epithelial cells might also affect intestinal inflammation. To address this issue, i.p. ISS-ODN was given prior to oral DSS. The colitis induced by DSS is T cell independent and is thought to be a product of epithelial injury induced by DSS (45, 46). In DSS colitis, IDO-1 has been reported to be upregulated in colonic epithelial cells (47), particularly in those at the crypt base (48). ISS-ODN lessens the severity of DSS colitis, and similarly to the TNBS model, this effect is blocked by 1-mT. These results raise the possibility that the induction of epithelial IDO-1 protects the animal from DSS-induced colitis. It is possible that the protective effects of IDO-1 induction in the DSS model may be mediated indirectly by IDO-1 induced in lamina propria mononuclear cells; however, the induction of IDO-1 in colonic crypt epithelial cells in the area of greatest epithelial proliferation suggests that the epithelial IDO may be involved in the protective effects of ISS-ODN in the DSS colitis model.

Taken together, the results from the DSS and TNBS models suggest that IDO-1 in experimental colitis has multiple roles. The induction of IDO-1 in epithelial cells at the colonic crypt base may limit the initial acute changes associated with epithelial disruption by enabling epithelial proliferation through limiting bacterial invasion and perhaps direct proproliferative effects. The DSS and acute stages of the TNBS model best demonstrate this. Expression of IDO-1 within APCs of the lamina propria may then limit the progression of adaptive immune responses driving the subsequent perpetuation of colitis.

Type I IFNs play a key role in mediating the protective effects of ISS-ODN in DSS colitis (23). In WT mice, ISS DNA leads to...

| Table III. Histology and morphology scores after recurrent TNBS treatment |
|---------------------------|-------------------|-----------------|-------------------|
|                          | Histology | p Value | Morphology | p Value |
| TNBS                     | 3.8 ± 0.51 |        | 2.3 ± 0.19 |
| TNBS + ISS-ODN           | 2.7 ± 0.39 | 0.16 versus TNBS | 1.4 ± 0.29 | 0.041 versus TNBS |
| TNBS + 1-mT              | 4.9 ± 0.55 | 0.048 versus TNBS | 2.6 ± 0.22 | 0.33 versus TNBS |
| TNBS + ISS-ODN + 1-mT    | 4.4 ± 0.32 | 0.018 versus TNBS; 0.44 versus TNBS; 2.3 ± 0.15 | 0.046 versus TNBS; 0.44 versus TNBS; 0.81 versus TNBS |

Day 13 after first TNBS enema and day 6 after second TNBS enema to BALB/c mice. Five mice per group performed twice.

Figure 8. IDO-1 induction is key to ISS-ODN’s protection against DSS colitis. Eight-week-old C57B/6 mice were offered 2.0% DSS in drinking water. On the day DSS was started, mice received 10 μg ISS-ODN and/or 1-mT as listed; n = 5 mice per group and the experiment was repeated three times with averaged composite values being shown in the figure. On day 7, disease activity index scores were compiled, and the colons were subsequently harvested to test myeloperoxidase activity and fixed for histology evaluation of the distal rectum and proximal descending colon. The myeloperoxidase (MPO) activity (A), disease activity index (B), and colitis severity (C, D) were all significantly lower in the mice treated with ISS-ODN. No difference was found if 1-mT was administered alone. However, the addition of 1-mT eliminated the protective effect of each parameter, which was improved by ISS-ODN. Representative histology sections (paired images, original magnification ×40) are shown in the bottom panel with the arrow marking the anorectal junction. Regenerative crypts (arrowhead [C]) with proliferating epithelial cells by Ki-67 staining (E) were more prevalent in the distal rectum of ISS-ODN–treated mice. Similarly, this effect was lost with 1-mT coadministration. (Histology p values: a = 0.039 and b = 0.001 versus DSS controls; c = 0.003 and d = 0.004 versus DSS + ISS-ODN–treated groups.). MPO, myeloperoxidase.
phosphorylation of intestinal STAT1, a member of the signal transducers, and activators of transcription family activated by IFN binding (Fig. 6B). Mice lacking STAT1 signaling did not upregulate IDO-1 after ISS-ODN exposure. This complements the previous observation that STAT1 mice developed a more severe form of TNBS colitis than WT mice (17). Although signaling of ISS-ODN through IFN-α/β may be critical to the preventive effects in the DSS model, there may be other beneficial effects mediated through IFN-γ, which is a more potent stimulus for IDO induction (49). Both type I (α/β) and type II (γ) IFNs have been shown to signal through STAT1 and can lead to increased levels of IDO-1 (27, 28).

Administration of ISS-ODN results in an increase in the number of CD11b and CD11c cells as assessed by immunohistochemistry. The CD11b+ cells were in the lamina propria surrounding the crypts, whereas the CD11c+ cells were also in the lamina propria but more associated with the surface epithelium. ISS-ODN also results in an ~5-fold increase in CD11b mRNA and a 2-fold increase in CD11c mRNA as assessed by quantitative RT-PCR. The increases in CD11b and CD11c may represent a combination of increased cell expression of CD11b and CD11c, the proliferation of CD11b and CD11c+ cells, and/or the infiltration of monocytes that differentiate into CD11b and CD11c+ cells. The increase in IDO-1 expression is significantly higher than each, at ~35-fold, suggesting that the increase in CD11b and CD11c+ cells is not alone responsible for the increased IDO-1 levels.

Administration of ISS-ODN resulted in increased cytokine production as assessed by quantitative RT-PCR of mRNA from the distal colon. Two doses of i.p. ISS-ODN resulted in a >5-fold increase in TNF-α and a ~4-fold increase in IFN-γ, cytokines associated with Th1 responses. ISS-ODN also induced an ~2-fold increase in IFN-α. The increases in IFN-γ and IFN-α are likely to be involved in the STAT1-dependent induction of IDO-1 by ISS-ODN. Our results show that among cytokines and enzymes that favor tolerance, that the fold changes in IL-10 and TGFB were more modest than IDO-1. Increases in intestinal IL-10 as well as IL-6, IL-12, and cyclooxygenase-2 at alternate time points after ISS-ODN administration have also been reported, although these experiments found the PG pathway not to be an important mediator of ISS-ODN’s anti-inflammatory properties (18, 23).

In addition to assessing the role of IDO-1 in intestinal inflammation, we also studied the expression of IDO-2. This is the first report on IDO-2 in the gastrointestinal tract and among the first to distinguish between and describe the individualized physiologic effects of the two IDO enzymes (4, 50, 51). IDO-1 is the more widely expressed and more thoroughly investigational of the two enzymes, both of which function as the initial and rate-limiting steps in tryptophan metabolism. Roles for IDO-1 have been found in autoimmune, allergic, malignant, and infectious diseases (1, 9, 10, 52). IDO-2 has only recently been described and seems to be highly expressed in the kidney, epididymis, and liver, but it has also been found to be expressed in dendritic cells and tumors, including those of the gastrointestinal tract (4–6, 50, 51). The functional significance of IDO-2 is still being investigated. IDO-1 and IDO-2 are differentially regulated, whereas IDO-2 was detected at low levels of the colon and intestine, its expression was not increased by ISS-ODN. This finding supports previous reports that IDO-2 expression is less responsive to IFN-γ than is IDO-1 (4, 5). The activity of IDO-2 can be suppressed by 1-mT; in light of our prior studies, we cannot exclude the possibility that IDO-2 activity may be contributing to the natural brake tryptophan metabolism puts on intestinal inflammation (17). However, given its significantly greater expression and activity level, we propose that IDO-1 rather than IDO-2 exerts the greater impact on control of colitis.

IDO-1 expression is increased in animal models of colitis and human IBD. Increased IDO-1 expression is a component of the innate immune system’s effort to control microbial invasion and dampen or modulate the adaptive immune response. IDO-1 activity promotes depletion of the rarest of essential amino acids, tryptophan, in the microenvironment and leads to the generation of toxic and immunomodulating kynurenine metabolites. These effects have been shown to result in inhibition of T cell replication, induction of T cell apoptosis, differentiation, and suppressor function.

The studies described in this study show that a TLRR agonist induces IDO-1 and that this induction is critical in its ability to decrease severity of both a T cell-dependent and T cell-independent model of colitis. Furthermore, in addition to IDO-1 expression by professional APCs, these studies raise the possibility that IDO-1 induction in epithelial cells may also contribute to limiting colitis severity. Taken together, these findings suggest possibility that pharmacologic upregulation of IDO-1 may be an approach deserving further investigation for the induction and maintenance of remission in human IBD.

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Disclosures

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References


