Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Role of Blood- and Tissue-Associated Inducible Nitric-Oxide Synthase in Colonic Inflammation

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There is evidence that inducible nitric-oxide synthase (iNOS)-derived NO contributes to the pathophysiology of intestinal inflammation. The aims of this study were to assess the role of iNOS in the development of dextran sodium sulfate (DSS)-induced colonic inflammation and to define the contribution of tissue-specific iNOS expression to this inflammatory response. Study groups included: 1) wild-type (WT) mice; 2) WT⇒WT bone marrow chimeras with normal iNOS function; 3) WT⇒iNOS^{-/-} chimeras (with functional blood cell iNOS, but iNOS-deficient tissue); 4) iNOS^{-/-}⇒WT chimeras (with iNOS-deficient blood cells, but normal tissue iNOS activity); and 5) iNOS-deficient mice. In WT mice and WT⇒WT chimeras, DSS-induced colonic inflammation was characterized by bloody diarrhea and a high disease activity index. However, WT⇒iNOS^{-/-} and iNOS^{-/-}⇒WT chimeras and iNOS^{-/-} mice exhibited an attenuated disease activity index, with parallel changes in histopathology. Colonic myeloperoxidase (MPO) was comparably elevated in DSS-treated WT mice (30.1 ± 1.7) and WT⇒WT chimeras (29.0 ± 1), whereas MPO was significantly reduced in iNOS^{-/-} mice and iNOS^{-/-}⇒WT chimeras (9.5 ± 1.7 and 15.6 ± 2.2, respectively). WT⇒iNOS^{-/-} chimeras exhibited the lowest MPO activity (3.7 ± 0.6). Our findings implicate both blood cell- and tissue-derived iNOS in DSS-induced colonic inflammation, with tissue-associated iNOS making a larger contribution to the recruitment of inflammatory cells. (*Am J Pathol* 2007, 170:490–496; DOI: 10.2353/ajpath.2007.060594)

It is well recognized that nitric oxide (NO) contributes not only to the normal functions of the gut but also to the pathophysiology of chronic inflammatory bowel diseases. Under physiological conditions, constitutive production of NO by nitric-oxide synthase (NOS) in endothelial cells elicits the relaxation of vascular smooth muscle cells and creates a nonthrombogenic environment in the vasculature^{1,2} while also preventing the accumulation of adherent leukocytes in postcapillary venules.² These anti-inflammatory properties of eNOS-derived NO differ from the primarily proinflammatory actions that have been described for NO derived from the inducible isoform of NO (iNOS). Whether the opposing actions of these two sources of NO relate to the differing amounts and/or cellular sources of the gaseous monoxide remains controversial¹; nonetheless, its involvement in the pathophysiology of most models of experimental inflammatory bowel disease has gained wide acceptance.

Nonselective (eg, L-NAME) and selective (1400W, L-NIL) inhibitors of iNOS have been studied in different models of intestinal inflammation.^{3–5} Although the results of these studies have been contradictory, the discrepant responses have been attributed to the model of inflammation used, with some of the more chronic models [eg, repeated application of dextran sodium sulfate (DSS) for 7 days] exhibiting a proinflammatory role for iNOS-derived NO.^{6,7} For example, we and others have shown that selective pharmacological inhibition of iNOS reduces the colonic inflammation and tissue injury induced by 7 days of DSS treatment.^{4,5} Likewise, we have shown that mice genetically deficient in iNOS show an attenuated colonic inflammatory response and disease activity in response to DSS treatment, compared with their wild-type (WT) counterparts.⁴

There is evidence that the cellular origin of NOS is one of the major factors that determines whether the NO-producing enzyme exerts a beneficial or detrimental ef-

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fect in models of tissue injury.^{6,8} iNOS is found in a variety of cells, including circulating blood cells of myeloid origin (eg, leukocytes) and resident cells (eg, endothelial cells). However, it remains unclear whether the protective effect of iNOS deficiency in DSS-induced intestinal inflammation reflects the involvement of iNOS associated with circulating blood cells, resident cells, or both. In an attempt to address this issue, we produced iNOS bone marrow chimeras that yielded either mutant mice with functional blood cell iNOS, but iNOS-deficient resident cells (WT $\Rightarrow$ iNOS^{-/-}), or mice with iNOS-deficient blood cells, but normal tissue iNOS activity (iNOS^{-/-} $\Rightarrow$ WT). A comparison of the inflammatory and tissue injury responses to DSS treatment in these mutants versus WT mice and iNOS^{-/-} mice has revealed that both blood cell- and resident cell-associated iNOS contribute to DSS-induced colonic inflammation, but the tissue-associated iNOS makes a larger contribution to the inflammatory response.

Materials and Methods

Animals

WT C57BL/6 mice, CD45 congenic B6.SJL-PTPRCPEP/BOY mice (which express CD45.1), and B6.129P2-NOS2 TM1 LAU/J (iNOS^{-/-}) mice on a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME).

Generation of Mice Chimeric for iNOS Expression

Three combinations of chimeric mice were used: WT $\Rightarrow$ WT, WT $\Rightarrow$ iNOS^{-/-}, and iNOS^{-/-} $\Rightarrow$ WT. The WT $\Rightarrow$ WT chimeras were WT animals that received bone marrow cells from CD45.1-expressing congenic WT mice. The iNOS function remained unchanged in the resulting WT $\Rightarrow$ WT chimeras. WT $\Rightarrow$ iNOS^{-/-} chimeras were produced by transplanting bone marrow from CD45.1-expressing congenic WT into iNOS^{-/-} mice, yielding mice with normal blood cell iNOS function, but an iNOS-deficient vessel wall. The iNOS^{-/-} $\Rightarrow$ WT chimeras were produced by transplanting bone marrow from an iNOS^{-/-} mouse into a congenic WT recipient, yielding mice with iNOS-deficient blood cells, but normal vascular wall enzyme activity. The use of CD45.1-expressing congenic WT mice facilitated verification of proper reconstitution in these chimeric mice because reconstitution was accompanied by a significant increase in the percentage of leukocytes expressing CD45.1, from 0 to 1% in regular WT (which express CD45.2) to >95% in the WT $\Rightarrow$ WT and WT $\Rightarrow$ iNOS^{-/-} chimeras. This was verified by fluorescence-activated cell sorting analysis by staining for CD45.1 and CD45.2 expression on splenocytes after the experiment.

Bone marrow transplantation was performed using CD45.2-expressing C57BL/6 WT and C57BL/6-iNOS^{-/-} mice as donors and/or recipients using a modified version of the method used by Tomita and colleagues.⁹ In

brief, bone marrow was isolated from sacrificed donor mice by flushing their femura and tibiae with HBSS. Large bone fragments were allowed to settle and the supernatant cell suspension was centrifuged. The pelleted bone marrow cells were resuspended in HBSS, counted, and finally resuspended in phosphate-buffered saline (PBS) for injection into irradiated recipient mice. To destroy the bone marrow cells of the recipient mice, they were irradiated with two doses of 500 to 525 rads, 3 hours apart, from a ¹³⁷Cs source. Thereafter, 200 μ l of PBS with $\sim 10^7$ donor bone marrow cells were injected into the femoral vein. To allow for full reconstitution of the bone marrow, the chimeric mice were maintained for 8 weeks on 12/12-hour light/dark cycles, in autoclaved boxes with filter top under specific pathogen-free conditions, and on standard laboratory chow and water ad libitum. For the first 2 weeks, 0.2% neomycin was added to the drinking water of chimeric mice. WT controls and iNOS^{-/-} mice were kept under the same conditions until the desired age (8 to 10 weeks) and/or weight (25 to 30 g).

Induction of Colitis

Colitis was induced by replacing normal drinking water with a 2.5% (w/v) solution of DSS (molecular mass, 40 kd; ICN Biomedicals, Inc., Aurora, OH) in Millipore water as previously described.

Assessment of Inflammation in DSS-Treated Mice

Daily clinical assessment of colonic inflammation included measurements of drinking volume and body weight, evaluation of stool consistency, and the presence of blood in the stools by a guaiac paper test (ColoScreen; Helena Laboratories, Beaumont, TX). A previously validated clinical disease activity index ranging from 0 to 4 was calculated using the following parameters: stool consistency, the presence or absence of fecal blood, and weight loss. Mice were sacrificed at day 7, blood was collected by cardiac puncture, and spleens were weighed. Colons were removed, and length and weight were measured before dividing the tissue for histology and evaluation of myeloperoxidase (MPO) activity. Blood samples were analyzed for white blood cells by manually counting cells on a hemocytometer (hemacytometer no. 0267110; Fisher Scientific Co., Pittsburgh, PA). Hematocrits were measured by centrifugation of heparinized microcapillary tubes (StatSpin Micro-Hct tubes, centrifuge, hematocrit rotor RH12, and Micro-Hct illuminated reader; all by StatSpin Technologies, Norwood, MA).

Histology

Histological examination was performed on samples of distal colon (~ 1 cm above the proximal rectum, defined by its passage under the pelvisternum), which were fixed in Zamboni's solution before embedding in paraffin and staining with hematoxylin and eosin. The histological ex-

amination was performed in a blinded manner using a previously validated scoring system.¹⁰ Three independent parameters were measured: severity of inflammation (0 to 3: none, slight, moderate, severe), depth of injury (0 to 3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0 to 4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement ($\times 1$, 0 to 25%; $\times 2$, 26 to 50%; $\times 3$, 51 to 75%; $\times 4$, 76 to 100%) and added to a sum. The maximum possible score is 40. In addition, the actual percentage of mucosal surface ulcerated was measured by computer-aided morphometric analysis.

Morphometric Analysis

The extent of ulcerated mucosa was determined by computerized morphometry that was performed on the more severely affected sections of each inflamed colon using a Metamorph image acquisition and processing software system (Universal Imaging Corp., West Chester, PA). Images of colon cross-sections were captured via an Eclipse E600 upright light microscope (Nikon Inc., Melville, NY) coupled to a SenSys CCD camera system (Roper Scientific Photometrics, Tucson, AZ), digitized using the Metamorph workstation, and displayed on a monitor. The ulcerated mucosal surface was defined by loss of mucosal epithelium and measured by tracing with the computer mouse. The total luminal perimeter of each colon cross-section was similarly determined. For each colon cross-section, the percentage of ulcerated mucosa was then calculated by dividing the length of ulcerated mucosa by the total luminal perimeter.

Colonic MPO Activity

Colon samples were obtained from either control or DSS-treated animals, rinsed with ice-cold PBS, blotted dry, and immediately frozen with liquid nitrogen. The samples were stored at -80°C until thawed for MPO activity determination using the *O*-dianisidine method, as previously described.^{11,12} Samples were thawed, weighed, suspended (10% w/v) in 50 mmol/L potassium phosphate buffer (Kp_i), pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide buffer (0.1 g/20 ml Kp_i), and homogenized. A 1-ml sample of the homogenate was sonicated using a Labsonic 2000 generator and a Braun-Sonic 2000U transducer with a 40T needle probe tip (B. Braun Biotech International, Allentown, PA) two times for 15 seconds (approximate average power output: 15 W with continuous operation). The sample was then microcentrifuged at $200 \times g$ for 10 minutes at 4°C . The reaction was started by mixing and incubating the supernatant (100 μl) at 20°C for 10 minutes with a solution composed of 2900 μl of 50 mmol/L Kp_i , 30 μl of 20 mg/ml *O*-dianisidine dihydrochloride, and 30 μl of 20 mmol/L hydrogen peroxide. The addition of 30 μl of 2% sodium azide stopped the reaction. The change in absorbance was read at 460 nm after 10 minutes using a spectrophotometer (Shi-

madzu UV-1201 series; Shimadzu Scientific Instruments Inc., Kyoto, Japan). MPO activity was expressed as the amount of enzyme necessary to produce a change in absorbance of 1.0 per minute per g of wet weight of tissue.

Experimental Protocol

Mice were treated with either normal drinking water or 2.5% DSS. During the time of induction of colitis, animals were weighed and stool specimens were examined on a daily basis. All animals were sacrificed on day 7. While under anesthesia, blood was collected for white cell count and hematocrit measurements. Thereafter, animals were euthanized by an overdose of anesthesia. The spleen was excised and weighed, the large bowel was removed completely, and after length measurement, cleaned of feces, dried, and weighed. A specimen was taken for histology, and the rest of the bowel was frozen at -80°C for subsequent measurement of MPO.

Data Analysis

Statistical analyses were performed with StatView 4.5 software (Abacus Concepts Inc., Berkeley, CA) using a one-way analysis of variance with Scheffé's (post hoc) test or the Kruskal-Wallis test where appropriate. All values are reported as means $\pm$ SEM. Statistical significance was set at $P < 0.05$.

Results

Clinical Course of DSS-Induced Colonic Inflammation

Treatment of WT mice and WT $\Rightarrow$ WT chimeras with DSS for 7 days was associated with significant and progressive increases in disease activity index (Figure 1), a gradual loss of body weight (Figure 2), rectal bleeding (Figure 3), and reduced stool consistency (Figure 4), with no differences noted between WT mice and WT $\Rightarrow$ WT chimeras. All of these DSS-induced responses were significantly attenuated in $\text{iNOS}^{-/-}$ mice. Attenuated responses were also noted in $\text{iNOS}^{-/-}\Rightarrow\text{WT}$ and WT $\Rightarrow\text{iNOS}^{-/-}$ chimeras, albeit these changes seemed to be less dramatic than those noted in the $\text{iNOS}^{-/-}$ mice. In fact, on day 6 there was a statistically significant difference between the $\text{iNOS}^{-/-}\Rightarrow\text{WT}$ and $\text{iNOS}^{-/-}$ groups for disease activity index and body weight loss. Drinking volume was similar in all groups, and no mortality was observed (data not shown).

DSS treatment of WT mice and WT $\Rightarrow$ WT chimeras resulted in a significant reduction in colon length, a significant increase of the weight-to-length ratio, and an increase in spleen weight. All these responses were significantly attenuated in the $\text{iNOS}^{-/-}$ mice and in both iNOS chimeras. DSS-induced leukocytosis and anemia were detected in WT and WT $\Rightarrow$ WT chimeras; however,

Figure 1. Clinical score (disease activity index) throughout 7 days of oral treatment with water in WT controls ($n = 8$) or 2.5% DSS in WT mice ($n = 7$), WT⇒WT, WT⇒iNOS^{-/-}, iNOS^{-/-}⇒WT bone marrow chimeric mice ($n = 8$ per group), and iNOS^{-/-} mice ($n = 7$). Mean ± SEM. For each time point, the letters indicate a significant difference ($P < 0.05$) versus the group indicated by the same letter. Starting on day 3, all groups were significantly different from water-treated WT.

these hematological changes were also abrogated in all iNOS-deficient and iNOS chimeric mice (Table 1).

DSS-Induced Neutrophil Infiltration

Compared with water-treated WT mice, DSS treatment produced an approximate 20-fold increase in colonic

Figure 2. Bodyweight in percent change from baseline = 100% throughout 7 days of oral treatment with water in WT controls ($n = 8$) or 2.5% DSS in WT mice ($n = 7$), WT⇒WT, WT⇒iNOS^{-/-}, iNOS^{-/-}⇒WT bone marrow chimeric mice ($n = 8$ /group), and iNOS^{-/-} mice ($n = 7$). Mean ± SEM. For each time point, the letters indicate a significant difference ($P < 0.05$) versus the group indicated by the same letter. Starting on day 4, all groups were significantly different from water-treated WT.

Figure 3. Rectal bleeding score throughout 7 days of oral treatment with water in WT controls ($n = 8$) or 2.5% DSS in WT mice ($n = 7$), WT⇒WT, WT⇒iNOS^{-/-}, iNOS^{-/-}⇒WT bone marrow chimeric mice ($n = 8$ per group), and iNOS^{-/-} mice ($n = 7$). Mean ± SEM. For each time point, the letters indicate a significant difference ($P < 0.05$) versus the group indicated by the same letter. Starting on day 5, all groups were significantly different from water-treated WT.

MPO activity in both WT mice and WT⇒WT chimeras. This index of neutrophil infiltration was reduced to a threefold increase in iNOS^{-/-} mice treated with DSS, with significant but less dramatic reductions also observed in the iNOS^{-/-}⇒WT (10-fold increase in MPO) and WT⇒iNOS^{-/-} (sevenfold increase in MPO) chimeras treated with DSS (Figure 5).

Figure 4. Stool score throughout 7 days of oral treatment with water in WT controls ($n = 8$) or 2.5% DSS in WT mice ($n = 7$), WT⇒WT, WT⇒iNOS^{-/-}, iNOS^{-/-}⇒WT bone marrow chimeric mice ($n = 8$ per group), and iNOS^{-/-} mice ($n = 7$). Mean ± SEM. For each time point, the letters indicate a significant difference ($P < 0.05$) versus the group indicated by the same letter.

Table 1. Colon, Spleen, and Hematological Changes after 7 Days of Oral Treatment with Water in Controls ($n = 8$) or 2.5% Dextran Sodium Sulfate (DSS) in WT Mice ($n = 7$), WT $\Rightarrow$ WT, iNOS $^{-/-}$ $\Rightarrow$ WT, WT $\Rightarrow$ iNOS $^{-/-}$ Bone Marrow Reconstituted Groups ($n = 8$ /group), and iNOS $^{-/-}$ Mice ($n = 7$)

	Colon length (cm)	Colon weight/length (mg/cm)	Spleen weight (% body weight)	White blood cells (WBC) ($\times 10^{-3}/\mu\text{l}$)	Hematocrit (HTC) (%)
Water-treated control mice	7.4 $\pm$ 0.1	27.2 $\pm$ 0.7	0.23 $\pm$ 0.07	8.6 $\pm$ 0.3	50.3 $\pm$ 1.3
(a) DSS-treated WT	4.9 $\pm$ 0.2*	43.8 $\pm$ 2.4*	0.49 $\pm$ 0.02*	12.0 $\pm$ 0.5*	31.3 $\pm$ 0.9*
(b) DSS-treated WT $\Rightarrow$ WT mice	5.1 $\pm$ 0.1*	38.3 $\pm$ 1.6*	0.40 $\pm$ 0.02*	10.2 $\pm$ 0.3*	27.4 $\pm$ 2.0*
(c) DSS-treated iNOS $^{-/-}$ $\Rightarrow$ WT mice	6.3 $\pm$ 0.1* ††	27.9 $\pm$ 1.0 ††	0.32 $\pm$ 0.01* †	7.1 $\pm$ 0.4* ††	39.8 $\pm$ 1.7* †
(d) DSS-treated WT $\Rightarrow$ iNOS $^{-/-}$ mice	6.0 $\pm$ 0.2* ††	30.0 $\pm$ 1.1 ††	0.36 $\pm$ 0.02* †	6.7 $\pm$ 0.6* ††	35.3 $\pm$ 2.2*
(e) DSS-treated iNOS $^{-/-}$ mice	6.1 $\pm$ 0.1* ††	32.1 $\pm$ 0.9 †	0.35 $\pm$ 0.02* †	7.8 $\pm$ 0.5 ††	41.1 $\pm$ 1.5* ††

Mean $\pm$ SEM.

* $P < 0.05$ versus water-treated controls.

$^{\dagger}P < 0.05$ versus DSS-treated wild-type (WT) mice.

$^{\dagger\dagger}P < 0.05$ versus DSS-treated WT $\Rightarrow$ WT mice.

Histological Changes after DSS Treatment

Blinded histological injury scoring was quantified in the distal colon from WT, WT $\Rightarrow$ WT chimeras, iNOS $^{-/-}$, and in both iNOS $^{-/-}$ $\Rightarrow$ WT and WT $\Rightarrow$ iNOS $^{-/-}$ chimeras after 7 days of treatment with 2.5% DSS. The colonic inflammation induced by DSS treatment in WT and WT $\Rightarrow$ WT chimeras was mostly confined to the submucosa with loss of goblet cells, crypt damage, mucosal ulceration, and submucosal edema. iNOS $^{-/-}$ mice consistently showed an

attenuated injury response to DSS, with all elements of the injury reduced (total score, depth, crypt damage, and severity). A qualitatively and quantitatively similar pattern was noted in the DSS-treated WT $\Rightarrow$ iNOS $^{-/-}$ chimeras; however, the histological injury indices were attenuated to a lesser degree in the iNOS $^{-/-}$ $\Rightarrow$ WT chimeras, with injury severity and depth measurements showing no significant reduction compared with DSS-treated WT or WT $\Rightarrow$ WT chimera groups (Figure 6). A similar pattern of protection in the DSS-treated mutant mice was noted using ulcerated mucosal area as a measure of tissue injury (Figure 7).

Figure 5. MPO activity in colonic tissue of water-treated controls ($n = 8$) or after 7 days of treatment with water in WT controls ($n = 8$) or 2.5% DSS in WT mice ($n = 7$), WT $\Rightarrow$ WT, WT $\Rightarrow$ iNOS $^{-/-}$, iNOS $^{-/-}$ $\Rightarrow$ WT reconstituted mice ($n = 8$ per group), and iNOS $^{-/-}$ mice ($n = 7$). Tissue concentrations were measured by the *O*-dianisidine method described in Materials and Methods. The change in absorbance was read at 460 nm in a spectrophotometer. MPO activity was expressed as the amount of enzyme necessary to produce a change in absorbance of 1.0 per minute per g of wet weight of tissue. Mean $\pm$ SEM. # $P < 0.05$ versus water-treated controls, * $P < 0.05$ versus DSS-treated WT and WT $\Rightarrow$ WT, and $^{\dagger}P < 0.05$ versus DSS-treated iNOS $^{-/-}$ $\Rightarrow$ WT mice.

Figure 6. Blinded histological assessment of colitis after oral treatment with 2.5% DSS for 7 days in WT mice ($n = 7$), WT $\Rightarrow$ WT, WT $\Rightarrow$ iNOS $^{-/-}$, and iNOS $^{-/-}$ $\Rightarrow$ WT bone marrow chimeric mice ($n = 8$ per group), and iNOS $^{-/-}$ mice ($n = 7$), based on validated scoring system described in Materials and Methods. Total histological score is shown as the sum of its components: severity, depth, and crypt damage. Mean $\pm$ SEM. * $P < 0.05$ versus DSS-treated WT and WT $\Rightarrow$ WT.

Figure 7. Blinded morphometric assessment of colitis after oral treatment with 2.5% DSS for 7 days in WT mice ($n = 7$), WT⇒WT, WT⇒iNOS^{-/-}, iNOS^{-/-}⇒WT bone marrow chimeric mice ($n = 8$ per group), and iNOS^{-/-} mice ($n = 7$), based on morphometric measurements described in Materials and Methods. Showing percentage of surface ulcerated. Mean ± SEM. * $P < 0.05$ versus WT, and * $P < 0.05$ versus WT⇒WT.

Discussion

Although the mechanisms underlying the inflammation and tissue injury in inflammatory bowel diseases remain poorly defined, there is a large amount of evidence that implicates NO in this disease process.^{1,6} A major source of NO in the inflamed intestine is iNOS, an isoform of the NO-producing enzyme that can produce large sustained fluxes of this gaseous monoxide. Based on research dealing with the biological actions of NO throughout the past 3 decades, it is widely accepted that the cellular origin and amounts of NO produced during inflammation determine the quality and magnitude of the responses elicited by NO, with small amounts of NO generated by endothelial NOS exerting an anti-inflammatory response whereas large fluxes of macrophage-derived NO mediate a proinflammatory response.¹ Previously published findings in animal models of gut inflammation tend to support this view inasmuch as *in vivo* and *in vitro* experiments using pharmacological antagonists or inhibitors and genetic manipulation to ablate the effects of iNOS^{4,5} have shown significant protection against the inflammatory and tissue injury-inducing effects of different inflammatory stimuli, including DSS and immunological models.^{4,7,13} However, not all experimental models of intestinal inflammation show similar results. A number of studies show beneficial effects of NO in experimental inflammatory bowel disease, as evidenced by worsening with NOS inhibitors or improvement with NO donors.^{14,15} Furthermore, genetic ablation of iNOS is not beneficial in the interleukin 10 knockout model of colitis.¹⁶ Although there is no single animal model that truly mimics human inflammatory bowel disease, each of these models can be used to address different aspects of disease pathogenesis. Here, we chose to further evaluate the role of

iNOS using a model (DSS) in which we have previously demonstrated protection by ablation of iNOS.⁴

The data from the present study on DSS-treated iNOS^{-/-} mice are consistent with a major role for this enzyme in intestinal inflammation. In addition to the improved clinical outcome in the iNOS^{-/-} and iNOS chimeric groups, leukocyte-endothelial cell adhesion was significantly attenuated. This may result from an influence of NO on MAdCAM-1 expression in the inflamed gut. iNOS deficiency has been shown to down-regulate intestinal MAdCAM-1 expression after DSS challenge, with anti-MAdCAM-1 antibodies showing protection in the same experimental model.^{7,17} Furthermore, MAdCAM seems to play a major role in the recruitment of T lymphocytes into postcapillary venules of the inflamed mouse colon.¹⁸

An important unresolved issue in the literature dealing with the contribution of iNOS to intestinal inflammation is cellular localization of this NO-producing enzyme. Multiple cell types located within the blood (eg, leukocytes) and in vascular wall/extravascular (eg, endothelial cells, resident macrophages) compartments are capable of generating NO from iNOS. A major objective of this study was to determine whether the iNOS-associated cells that contribute to gut inflammation reside in the blood and/or vascular wall/extravascular compartments. Using chimeras generated from the transplantation of bone marrow between WT and iNOS^{-/-} mice, we produced mutants that were selectively deficient in iNOS either in the blood or in vascular wall/extravascular compartments. These mutant mice, which were treated with DSS, reveal a role for both blood cell-derived and vascular/extravascular iNOS in mediating the neutrophil infiltration and tissue injury associated with gut inflammation. In addition, our findings with the chimeras suggest that blood cell-associated iNOS is quantitatively less important than vascular wall/extravascular iNOS in mediating DSS-induced gut inflammation.

A large number of circulating blood cells are capable of producing NO via iNOS after an inflammatory stimulus. Neutrophils, monocytes, macrophages, NK cells, and tumor cells of T- and B-cell origin can express iNOS and may be able to produce large ($\mu\text{mol/L}$) amounts of NO for a sustained period of time.^{2,19} Anti-microbial, anti-viral, and anti-tumor effects of iNOS-derived NO have been described *in vitro*,¹⁹ and leukocyte-associated iNOS has been implicated in the pathogenesis of numerous inflammatory disorders, including asthma, arthritis, and colitis.²⁰ The consequences of iNOS-dependent NO production from circulating cells include inhibition of T- and B-cell proliferation, regulation of inflammatory signaling cascades and transcription factors, and the subsequent regulation of leukocyte recruitment.² The protective effect against DSS-induced colonic inflammation noted in our iNOS^{-/-}⇒WT chimeras whose blood cells were deficient in iNOS indicate that NO produced by blood cell-associated iNOS plays an important role in mediating the inflammatory and tissue injury responses in this model. This finding is also consistent with results from other models of inflammation, wherein NO derived from leukocyte-associated iNOS exerts significant detrimental effects.⁸

Our observation that iNOS^{-/-}→WT chimeras exhibit less protection against DSS-induced colonic inflammation compared with iNOS^{-/-} mice suggests that a source of iNOS other than myeloid cells also contributes to the DSS-induced inflammatory responses. Intestinal epithelial cells, endothelial cells, vascular smooth muscle cells, mast cells, and fibroblasts are cells located in the vascular wall or extravascular compartment that can express iNOS in response to certain stimuli.^{2,20} Cells in this compartment have traditionally been regarded as the quantitatively more important source of iNOS during inflammation. A previous report on endotoxin-induced inflammation provides evidence that resident colonic cells are a more important source of iNOS than circulating cells.²¹ Resident macrophages after irradiation and subsequent bone marrow transplantation at the time point of our experiments seem to derive from the donor bone marrow.²² Therefore, in our model, the resident macrophages in the iNOS^{-/-}→WT should be incapable of iNOS production, whereas all other cells would be able to produce levels sufficient to mediate the DSS effects. In contrast, in the WT→iNOS^{-/-} group, the absence of iNOS in all resident cells except macrophages seems to be protective, suggesting that the macrophages are not the major source of tissue-derived iNOS in this model. The results of the present study are consistent with this view and suggest that iNOS associated with resident colonic cells are quantitatively more important than circulating blood cells in mediating the colonic inflammation and tissue injury induced by DSS in mice.

In conclusion, our findings indicate that both blood cell-associated and vascular wall/extravascular cell-associated iNOS contributes to the inflammation and tissue injury induced by DSS. These results suggest that drugs designed to target iNOS for treatment of colonic inflammation must have ready access to both the intravascular and extravascular compartments to exert their full potential as therapeutic agents.

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