Deletion of cationic amino acid transporter 2 exacerbates dextran sulfate sodium colitis and leads to an IL-17-predominant T cell response

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Singh K, Coburn LA, Barry DP, Asim M, Scull BP, Allaman MM, Lewis ND, Washington MK, Rosen MJ, Williams CS, Chaturvedi R, Wilson KT. Deletion of cationic amino acid transporter 2 exacerbates dextran sulfate sodium colitis and leads to an IL-17-predominant T cell response. Am J Physiol Gastrointest Liver Physiol 305: G225–G240, 2013. First published May 23, 2013; doi:10.1152/ajpgi.00091.2013.—L-Arginine (L-Arg) is a semiessential amino acid that has altered availability in human ulcerative colitis (UC), a form of inflammatory bowel disease, and is beneficial in murine colitis induced by dextran sulfate sodium (DSS), a model with similarity to UC. We assessed the role of cationic amino acid transporter 2 (CAT2), the inducible transporter of L-Arg, in DSS colitis. Expression of CAT2 was upregulated in tissues from colitic mice and localized predominantly to colonic macrophages. CAT2-deficient (CAT2−/−) mice exposed to DSS exhibited worsening of survival, body weight loss, colon weight, and histological injury. These effects were associated with increased serum L-Arg and decreased tissue L-Arg uptake and inducible nitric oxide synthase protein expression. Clinical benefits of L-Arg supplementation in wild-type mice were lost in CAT2−/− mice. There was increased infiltration of macrophages, dendritic cells, granulocytes, and T cells in colitic CAT2−/− compared with wild-type mice. Cytokine profiling revealed increases in proinflammatory granulocyte colony-stimulating factor, macrophage inflammatory protein-1α, IL-15, and regulated and normal T cell-expressed and -secreted and a shift from an IFN-γ- to an IL-17-predominant T cell response, as well as an increase in IL-13, in tissues from colitic CAT2−/− mice. However, there were no increases in other T helper cell type 2 cytokines, nor was there a global increase in macrophage-derived proinflammatory cytokines. The increase in IL-17 derived from both CD4 and γδ T cells and was associated with colonic IL-6 expression. Thus CAT2 plays an important role in controlling inflammation and IL-17 activation in an injury model of colitis, and impaired L-Arg availability may contribute to UC pathogenesis.

arginine metabolism; macrophages; T helper cells; colitis; IL-17

Ulcerative colitis (UC) and Crohn’s disease (CD) constitute the two main forms of inflammatory bowel disease (IBD) (4, 5). These chronic diseases are characterized by frequent exacerbations that can severely compromise the quality of life of the ∼1.4 million affected people with IBD in the United States (4, 5). UC and CD can be differentiated histologically: in UC, inflammation is mucosal and restricted to the colon; in CD, inflammation can be transmural and can involve the entire gastrointestinal tract. Moreover, efforts have been made to characterize the pattern of cytokine responses in UC and CD, with the goal of targeting therapy. UC is generally considered to be a T helper (Th) cell type 2 (Th2)-type disease, and CD is considered to be a Th1-type disease, but this paradigm is overly simplistic, as the role for other cytokines, especially IL-17 made by Th17 cells, γδ T cells, and innate lymphoid cells has been emphasized in human IBD and in mouse models (22, 24, 30, 53, 54, 56, 62, 68). In addition, increased levels of IFN-γ, the prototype Th1 cytokine, have been observed in UC (47). A mainstay of therapy is the use of anti-TNF-α agents, since this appears to be a common pathway that is activated in both UC and CD. However, remission is achieved with such biological agents in only about half of the cases (58). Other biological therapies, including anti-IL-12/IL-23 (e.g., ustekinumab) (60), anti-α4-integrin (natalizumab) (25, 59), and anti-αβ7-integrin (vedolizumab) (51), are in use, but their long-term efficacy is still under investigation. Therefore, there is a compelling need to consider additional avenues for treatment.

Our laboratory has reported abnormalities in the availability of the amino acid L-Arg in UC (29). This is especially pertinent, because we have demonstrated that supplementation of L-Arg is beneficial (15) in mice with colitis induced by dextran sulfate sodium (DSS), a heparin-like polysaccharide that produces a disease with similarities to UC (15, 49), including weight loss, diarrhea, rectal bleeding, ulceration, loss of epithelium, and leukocyte infiltration (35, 36). A report that DSS can cause colitis in T cell-deficient mice (19) emphasizes the importance of the innate immune system. However, numerous studies, including those demonstrating reduced disease in IFN-γ−/− (31) and IL-17−/− (30) mice, implicate T cell responses in DSS colitis. The goal of the current study was to investigate the role of L-Arg transport in the DSS model of colitis in terms of clinical parameters and immune responses, including cytokine alterations.

In order for L-Arg to be fully utilized as a substrate, it needs to undergo uptake into cells. This is primarily dependent on the y+ transport system (33), which includes the cationic amino acid transporter (CAT) family of proteins (13, 33, 44). There are four types of CAT proteins. CAT1 is constitutively expressed and involved in basic metabolism. CAT2 is an inducible form, which includes the alternatively spliced isoforms CAT2A, a low-affinity transporter primarily in liver, and
CAT2, the high-affinity L-Arg transporter known to be abundant in macrophages (8). CAT3 is found in brain and thymus. The function of CAT4 is unknown (72).

Utilization of L-Arg in macrophages and colonic epithelial cells (CECs) is an important response during gastrointestinal mucosal inflammation (8, 9, 15, 65). Briefly, L-Arg is the substrate for inducible nitric oxide (NO) synthase (iNOS), arginase I (Arg1), and arginase II (Arg2) enzymes. Expression of iNOS, Arg1, and Arg2 is upregulated in DSS colitis (15), indicating increased L-Arg metabolism in this model. In addition to a beneficial effect in DSS colitis, we have also demonstrated that L-Arg enhances CEC restitution and that this involves CAT2, but not CAT1 (65). However, CAT2 may also be important in regulating immune cell function in inflammation. We reported that generation of antimicrobial NO by macrophages exposed to the gastric pathogen Helicobacter pylori required CAT2 (8) and that CAT2-deficient (CAT2−/−) mice had alterations in innate and adaptive immune responses to H. pylori in vivo (3).

We now demonstrate that CAT2 expression in DSS colitis localizes to colonic macrophages and that CAT2 deletion is deleterious in this model, resulting in exacerbated clinical and immunological changes similar to those observed in UC. There was worsening of survival, body weight loss, colon weight, and histological injury in CAT2−/− compared with wild-type (WT) mice. DSS-stimulated colonic L-Arg uptake and the clinical benefit of L-Arg supplementation were attenuated in CAT2−/− mice. The exacerbation of colitis in CAT2−/− mice was associated with an increase in the number of myeloid cells and lymphocytes in the colon, an exaggerated chemokine response, and a shift in the T cell cytokine response from the Th1 cytokine IFN-γ to the Th17 cytokine IL-17. The IL-17 was derived from both CD4+ Th cells and γδ T cell. Together, these studies suggest that L-Arg uptake by CAT2 and L-Arg availability are important in the regulation of immune function in colitis.

MATERIALS AND METHODS

Animals. Male WT C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 6 wk of age, and CAT2−/− mice on a congenic C57BL/6 background (44) were provided by Lesley Ellies (University of California, San Diego). Age-matched male WT and mutant mice were used for experiments at 7–9 wk of age. All procedures using mice were reviewed and approved by the Institutional Animal Care and Use Committee of the Vanderbilt University Medical Center and the Research and Development Committee of the Veterans Affairs Tennessee Valley Healthcare System.

Induction of DSS colitis. DSS (mol wt 36,000–50,000; MP Bio, Solon, OH) was added to the drinking water as a 4% (wt/vol) solution for 1 wk after 6 days of DSS, as described elsewhere (15).

Survival and body weight measurement. For assessment of the effects of DSS treatment on mouse survival and changes in body weight, the animals were monitored daily over the course of colitis development. Any mice that lost 20% of initial body weight were euthanized, and the survival curves shown in RESULTS are based on this criterion.

Assessment of histological injury scores. By 10.220.32.246 on June 9, 2017 http://ajpgi.physiology.org/ Downloaded from

Western blot analysis. Frozen colon tissue samples were suspended in CellLytic MT lysis/extraction reagent (Sigma) containing the EDTA-free Protease Inhibitor Cocktail Set III and the Phosphatase Inhibitor Set I (EMD Chemicals, Billerica, MA) and disrupted by three 10-s pulses of sonication at 40 W with an ultrasonic processor (model GE 130PB, Hielscher, Ringwood, NJ). Protein was measured in cell lysates by the bicinchoninic acid (BCA) method, as described previously (15). The function of CAT4 is unknown (72).

The selection procedure was repeated three times, as described previously (9).

mRNA analysis. RNA from colon tissues was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA), as described previously (15, 64). One microgram of RNA was reverse-transcribed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Each PCR was performed with 2 μl of cDNA using iQ SYBR Green Supermix (Bio-Rad). Primers for IFN-α1 (18–19), IFNy (18–19), IL-12 (16–17), and IL-17A (16–17) were used as previously described. F0x3 primers were 5′-AGAGCCCT-CACAACACGCTA 3′ (forward) and 5′-CCAGATGTTGGTTGTTGATG 3′ (reverse); IL-13 primers were 5′-CCACAGCTTCCCTCGGAATC 3′ (forward) and 5′-CTCATGCTCCCGTGTGC 3′ (reverse); and IL-6 primers were 5′-AGTTGGCTTCTGGGAAGCTGA 3′ (forward) and 5′-TCCACGATTTCGCCAGAAC 3′ (reverse). RNA from F4/80+ and F4/80− cells and CECs was isolated using the PerfectPure RNA 96 Cell CS Kit (5 PRIME, Gaithersburg, MD). For these cells, RNA was reverse-transcribed as described above, and each PCR was performed with 5 μl of cDNA.

Western blot analysis. Frozen colon tissue samples were suspended in CellLytic MT lysis/extraction reagent (Sigma) containing the EDTA-free Protease Inhibitor Cocktail Set III and the Phosphatase Inhibitor Set I (EMD Chemicals, Billerica, MA) and disrupted by three 10-s pulses of sonication at 40 W with an ultrasonic processor (model GE 130PB, Hielscher, Ringwood, NJ). Protein was measured in cell lysates by the bicinchoninic acid (BCA) method, as described previously (9, 63, 64). For each mouse, 80 μg of protein were resolved per lane on 10% polyacrylamide gels (Bio-Rad) and transferred overnight onto polyvinylidene difluoride. The samples were blocked with 5% milk for 2 h, and iNOS and β-actin were detected by Western blotting, as described elsewhere (8, 9, 15, 65). iNOS (130 kDa) and β-actin (42 kDa) proteins were detected with the following
antibodies: rabbit polyclonal iNOS (1:2,000 dilution; BD Biosciences) and mouse polyclonal β-actin (1:10,000 dilution; Sigma) (8, 9, 15, 65).

τ-Arg uptake. The uptake studies were initiated by addition of 10 μl of [L-14C]Arg (specific activity 346 mCi/mmol) to freshly collected colon tissues placed in serum-free DMEM. After 5 min, tissues were washed three times with PBS and lysed with radioimmunnoassay precipitation buffer, and supernatants were collected. Tissue lysates were mixed with scintillation fluid, and the 14C content was determined in a scintillation counter. Protein was measured in tissue lysates by the BCA method. τ-Arg transport values were expressed as picomoles of [L-14C]Arg per minute per milligram of protein (8, 65).

Serum amino acid analysis. Blood was collected from the mice at the time of euthanization via cardiac puncture, and serum was obtained in a scintillation counter. Protein was measured in tissue lysates obtained previously (15). Serum samples were provided to the Vanderbilt Hormone Assay Core for amino acid analysis via HPLC, as described elsewhere (15).

Immunofluorescent staining. For immunofluorescent staining, 5-μm sections from formalin-fixed paraffin-embedded Swiss-rolled tissues were deparaffinized, treated with citrate buffer for antigen retrieval, and blocked in 5% goat serum for 1 h at room temperature. Slides were incubated at 4°C for 16 h with primary antibodies to CAT2 (1:200 dilution) and F4/80 (1:50 dilution) (3, 11). For the isotype control for CAT2 and F4/80, slides were incubated with rabbit IgG and rat IgG2A, respectively, each at the same concentration, as the primary antibodies. Then the slides were incubated with FITC- and rhodamine-labeled secondary antibodies, each at 1:1,000 dilution, for CAT2 and F4/80, respectively, for 2 h at room temperature. Slides were washed, dried, mounted using mounting medium (Vector Laboratories, Burlingame, CA), and then visualized using a Nikon E800 fluorescence microscope and Spot RT Slider digital camera and Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI), as performed previously (10, 63).

Tissue cytokine and chemokine analysis. Colon tissues were lysed in CellLytic MT lysis extraction reagent (Sigma) with a mortar-and-pestle-type rotary homogenizer and used for Luminox assay, as described elsewhere (15, 55, 74). The 25-analyte MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (MCYTOMAG-70K-PMX, EMD Millipore, Billerica, MA) was used according to the manufacturer’s instructions, along with a FlexMAP 3D instrument (Luminex, Austin, TX) (55). Data were standardized to tissue protein concentration measured by the BCA method.

Immunophenotyping. Entire colons from untreated and DSS-treated mice were cut longitudinally, cleaned, weighed, cut into small pieces, and digested as described for the colonic macrophages. The resulting cell suspensions were analyzed by flow cytometry to identify infiltrating immune cells in the colon, as described elsewhere (55, 74); all antibodies were obtained from BD Biosciences. We detected expression of the granulocyte marker Gr1 with a phycoerythrin (PE)-tagged antibody, the macrophage marker F4/80 with an allophycocyanin-tagged antibody, the dendritic cell marker CD11c with an allophycocyanin-Cy7-tagged antibody, major histocompatibility complex (MHC) II with a Pacific Blue-tagged antibody, the T cell marker CD3 with a FITC-tagged antibody, and the Th cell marker CD4 with a peridinin-chlorophyll protein (PerCp)-Cy5.5-tagged antibody. For intracellular proteins, the cells suspensions were incubated with RPMI 1640 complete medium (containing 10 mM HEPES, 10 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS) plus GolgiPlug (BD Biosciences) for 4 h before staining. For IL-17, a PE-tagged antibody was used. The Mouse Regulatory T cell Staining Kit (eBioscience, San Diego, CA) was used for regulatory T cell staining; colonic immune cells were stained for CD4 with a FITC-tagged antibody, CD25 with a PE-tagged antibody, and Foxp3 with a Pacific Blue-tagged antibody, according to the manufacturer’s instructions. Single-cell suspensions from mesenteric lymph node (MLNs) of untreated and DSS-treated mice were cultured in RPMI complete medium, as described above, in round-bottom 96-well plates with 5 μg/ml plate-bound anti-CD3 and 1 μg of soluble anti-CD28. Cells were then stimulated with PMA (20 ng/ml) and ionomycin (1 μg/ml) in the presence of GolgiPlug for 4 h and stained for the γδ T cell receptor with a FITC-tagged antibody, CD4 cells with a PerCp-Cy5.5-tagged antibody, and IL-17 with a PE- or an Alexa Fluor 647-tagged antibody.

Statistical analysis. Values are means ± SE. Statistical analyses were performed with Prism version 5.0c (GraphPad Software, San Diego, CA). For comparisons between multiple groups, analysis of variance with the Student-Newman-Keuls post hoc multiple comparisons test was performed after transformation as appropriate. For comparisons between only two groups, Student’s t-test was performed.

RESULTS

CAT2 is upregulated in DSS-induced murine colitis. We previously reported that CAT2 expression was increased in DSS colitis tissues when assessed by real-time PCR and Western blotting (15). The aim of the present study was to assess the cellular source of CAT2 in WT mice treated with 4% DSS for 7 days. Compared with untreated control mice, there was a 6.2 ± 1.0 fold increase in CAT2 mRNA levels in tissues from DSS-treated mice, while CAT1 mRNA levels were not changed (Fig. 1A). CAT2 is known as an inducible τ-Arg transporter in macrophages, but we have also demonstrated that CAT2 can be upregulated in a mouse CEC line (65). We isolated colonic macrophages by positive selection with the macrophage marker F4/80 and found that these cells exhibited a 4.3 ± 1.3 fold increase in CAT2 mRNA levels, while there was a modest increase in F4/80− colon cells that was not significant. While CAT2 mRNA was detected in isolated CECs, there was no increase in expression levels in cells from mice treated with DSS (Fig. 1A). There was no increase in CAT1 mRNA expression in tissues, and CAT1 mRNA was not detected in any of the isolated cell fractions (Fig. 1A). We next examined protein levels of CAT2 and the macrophage marker F4/80 in situ by immunofluorescence and found that DSS treatment led to increased staining for both parameters (Fig. 1B). In the merged images, CAT2 localized predominantly to F4/80− cells (Fig. 1B). This was consistent with our mRNA results and indicates that CAT2 expression in DSS colitis derives primarily from colonic macrophages.

CAT2−/− mice exhibit more severe DSS colitis. Because we have reported that τ-Arg supplementation is beneficial in DSS colitis and CAT2 is a major transporter of τ-Arg that is upregulated in this model, we determined the effect of CAT2 deletion in DSS colitis. WT and CAT2−/− mice were given 4% DSS in their drinking water for 7 days using our previously described model (64). Then DSS was discontinued and mice were monitored further for survival; profound mortality (Fig. 2A) on day 8 in the CAT2−/− mice precluded further observation of the mice. As shown in Fig. 2A, decreased survival in CAT2−/− mice started on day 7 (29.0% vs. 9.0% in WT) and worsened on day 8 (77.4% vs. 42.4%). Because of the substantial weight loss after starting DSS (Fig. 2B), mice of both genotypes began losing weight on day 5, but weight loss was significantly greater in CAT2−/− mice on days 5–7; weight as a percentage of initial weight for CAT2−/− compared with WT mice was as follows: 98.4 ± 3.0% vs. 102.1 ± 0.2%.
Fig. 1. Cationic amino acid transporter (CAT) 2 (CAT2) expression is upregulated and localizes to macrophages in dextran sulfate sodium (DSS)-induced murine colitis. Seven-week-old C57BL/6 mice were given water (control (Ctrl)), n = 3–5 or 4% DSS in their drinking water (n = 5–11) for 7 days, and colon tissues were harvested. Coloncic immune cells were isolated by enzymatic digestion, and F4/80 cells were obtained by positive selection, with the remaining cells considered F4/80− cells. Colonic epithelial cells (CECs) were isolated as described in MATERIALS AND METHODS. A: mRNA levels for CAT2 and CAT1 measured by real-time PCR in whole colon tissue (Tissue), F4/80+ colon cells, F4/80− colon cells, and CECs. ND, not detected. **P < 0.01 vs. Ctrl. B: tissues were obtained after 7 days of DSS treatment. Formalin-fixed, paraffin-embedded sections (5 μm) from Swiss-rolled tissues were stained for CAT2, which was detected with FITC-conjugated secondary antibody (green), and F4/80, which was detected with rhodamine-conjugated secondary antibody (red). Isotype control images are also shown. White rectangles enclose areas of the ×200 microscopic fields in DSS-treated mice that are shown at ×600 (DSS 600×) at right.

When assessed by real-time PCR, we again found a significant increase in CAT2 mRNA levels in WT mice that was eliminated in CAT2−/− mice (Fig. 3A). There was no induction of CAT1 mRNA expression in DSS colitis tissues in WT mice and no compensatory increase in CAT2−/− mice (Fig. 3A). Consistent with the induction of CAT2 expression, there was a marked increase in l-Arg uptake in WT tissues from mice treated with DSS, and this was significantly attenuated in colitic CAT2−/− mice (Fig. 3B). There was accumulation of L-Orn and L-Lys, as they also utilize CAT2 and, thus, are competitive inhibitors of L-Arg uptake. In mice exposed to DSS, L-Orn was not increased in WT mice but was increased in CAT2−/− mice (Fig. 3D); l-Lys increased in WT mice and further increased in CAT2−/− mice (Fig. 3E). These data suggest that deletion of CAT2 results in serum accumulation of

0.8 at day 5, 90.9 ± 4.5% vs. 98.8 ± 4.1% at day 6, and 86.7 ± 5.2% vs. 96.7 ± 3.8% at day 7 (Fig. 2B). When the animals were euthanized at day 7, we also measured the weight and length of the colon, as thickening and shortening of the colon indicate colitis severity. Colon weight-to-length ratio (Fig. 2C) was increased in WT mice treated with DSS compared with controls and was further increased by 20.9 ± 8.7% in CAT2−/− compared with WT mice. Histological sections revealed increased colonic inflammation and epithelial damage in CAT2−/− compared with WT mice (Fig. 2D). Using a comprehensive scoring system to quantify the degree of damage, we observed increased overall histological injury (Fig. 2E), including increases in scores for inflammation (Fig. 2F) and epithelial injury (Fig. 2G), in CAT2−/− mice. We also scored the histology from all the control mice shown in Fig. 2C, and there was no detectable inflammation or epithelial injury in untreated CAT2−/− or WT mice.

Effects of CAT2 deletion on L-Arg uptake and metabolism. L-Arg can be transported into cells by CAT1 or CAT2 and is metabolized by iNOS or competing arginase enzymes. We first sought to verify that CAT2 is absent in CAT2−/− colon tissues and that there was no compensatory upregulation of CAT1.
L-Arg under the conditions of colitis, but because there is also a concomitant increase in L-Orn and L-Lys, there is no increase in the arginine availability index (AAI; Fig. 3F), defined as [L-Arg]/([L-Orn] + [L-Lys]), as we described previously (15, 29). There was also a decrease in L-Arg uptake in untreated control CAT2−/− mice compared with WT mice (Fig. 3B), indicating that CAT2 plays a role in basal amino acid transport. Consistent with this finding, we detected a significant increase in serum L-Arg (Fig. 3C), L-Orn (Fig. 3D), and L-Lys (Fig. 3E) levels in CAT2−/− mice compared with WT control mice, and again there was no difference in the AAI in the control mice (Fig. 3F).

Uptake of L-Arg has been shown to be required for iNOS protein translation in vitro (9, 39) and in vivo (8, 38, 39). We previously showed that iNOS expression is upregulated in DSS colitis tissues (15), so we also examined iNOS protein levels in colon tissues from untreated and DSS-treated CAT2−/− and WT mice. As shown in Fig. 3G, iNOS protein levels were undetectable in untreated WT and CAT2−/− mice and increased in DSS-treated WT mice, but in CAT2−/− mice, this expression was absent or very low. We previously reported that, in DSS colitis tissues, the arginase enzymes Arg1 and Arg2 are upregulated and the downstream metabolic enzymes that utilize L-Orn as substrate, ornithine decarboxylase and ---

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ornithine aminotransferase, are increased and decreased, respectively (15). When we assessed expression of Arg1, Arg2, ornithine decarboxylase, and ornithine aminotransferase, we found no differences in the colitis tissues of WT and CAT2/H11002/H11002 mice, nor did we find differences between the untreated control WT and CAT2/H11002/H11002 mice (data not shown).

Loss of beneficial effects of L-Arg supplementation in DSS colitis in CAT2/H11002/H11002 mice. Because we found defective uptake of L-Arg without an increase in AAI in CAT2/H11002/H11002 mice, we sought to determine if supplementation of L-Arg could overcome these defects and alter clinical effects in these mice. We used our established protocol of treating mice with 1% L-Arg in the drinking water for 4 days after induction of DSS colitis for 6 days, which we have shown to improve survival and body weight loss in WT mice (15). Consistent with our previous studies, there were no deaths among the WT mice treated with L-Arg during the course of the experiment (Fig. 4A), while DSS-treated WT mice not supplemented with L-Arg began dying at day 7 and only 37.5% of mice survived to the end of the experiment (Fig. 4A). Fewer CAT2/H11002/H11002 mice treated with...
We did not observe any survival benefit of L-Arg treatment in CAT2−/− mice receiving DSS (Fig. 5, A–C). Dendritic cells participate in antigen presentation, and MHCII expression is a marker of dendritic cell activation (74). MHCII levels in CD11c+ cells were modestly increased with DSS treatment in WT mice and significantly further increased in tissues from CAT2−/− mice (Fig. 5D). T cells are part of the adaptive immune response and can recognize antigens presented by dendritic cells and macrophages. We found no increase in CD3+ cells (total T cells) in DSS colitis tissues from WT mice but a marked increase in CD3+ cells in colitis tissues from CAT2−/− mice (Fig. 5E). Similarly, CD4+ cells (Th cells) were not increased in colitis tissues from WT mice but were significantly increased in CAT2−/− mice (Fig. 5F). Also, Gr1+, F4/80+, and CD11c+ cell numbers increased in control CAT2−/− compared with control WT mice (Fig. 5, A–C). However, there was no increase in MHCII levels, suggesting a lack of basal dendritic cell activation, nor was there an increase in CD3+ or CD3+CD4+ cells. Together, these data suggest some degree of basal dendritic cell infiltration in CAT2−/− mice, but there is not global immune cell activation, and this is subclinical, as there was no detectable histological inflammation or injury in these mice (data not shown).

Increased levels of immune-stimulating cytokines and chemokines in CAT2−/− mice. Because of the profound clinical worsening of disease and increased numbers of colonic immune cells in DSS-treated CAT2−/− compared with WT mice, we used Luminox analysis to conduct cytokine profiling of 25 cytokines and chemokines in colon tissues (Table 1). Granulocyte colony-stimulating factor (G-CSF), a cytokine that stimulates the production of granulocytes (7, 45, 66), was increased in DSS tissues from WT mice and further significantly increased in CAT2−/− mice (Fig. 6A). Similarly, macrophage inflammatory protein (MIP)-1α, a chemokine that is mainly produced by macrophages and also activates granulocytes, was increased in DSS colitis tissues from WT mice and further increased in CAT2−/− mice (Fig. 6B). IL-15 can be produced by activated macrophages and dendritic cells and signals through the IL-2 receptor to modulate innate and adaptive immune responses (21, 27, 41, 57); it was increased in tissues from WT mice but increased in CAT2−/− mice exposed to DSS (Fig. 6C). Regulated and normal T cell-expressed and -secreted (RANTES), a chemokine that attracts leukocytes, including T cells (61), was significantly increased in untreated control CAT2−/− compared with WT tissues (Fig. 6D). There was a modest increase in RANTES in colitis tissues from WT mice that was not significant but a marked increase in CAT2−/− colitis tissues (Fig. 6C). As shown in Table 1, the following proinflammatory chemokines were similarly increased above levels in untreated WT mice in DSS colitis tissues from WT or CAT2−/− mice: MIP-1β, MIP-2, monocyte chemotactic protein (MCP)-1, and keratinocyte-derived chemokine (KC, CXCL1); also the T cell-stimulatory cytokines IL-12p40 and IL-12p70 were not increased in either genotype. Furthermore, the innate immune response cytokines TNF-α, IL-1α, IL-1β, and IL-6, which are macrophage products, were actually increased less in CAT2−/− than WT mice (Table 1).
Alteration in cytokines related to Th1, Th2, and Th17 cells.

CAT2 has been shown to be an important regulator of Th1- and Th2-type immunity in the liver (70), and we previously reported effects in *H. pylori*-infected gastric mucosa (3), but this has not been investigated in the colon. Because we found increased CD4<sup>+</sup> cells in the colon of CAT2<sup>−/−</sup> mice exposed to DSS (Fig. 5F), we examined T cell cytokines in our Luminex-based profiling. There was an increase in the prototypic Th1 cytokine IFN-γ in WT tissues from DSS-treated compared with control mice, representing a 7.5 ± 1.9 fold increase; this level was reduced in CAT2<sup>−/−</sup> mice by 86.4 ± 13.3% (Fig. 7A). IFN-γ-induced protein (IP)-10, a cytokine secreted in response to IFN-γ, exhibited a similar pattern of a significant increase in WT mice treated with DSS that was attenuated in CAT2<sup>−/−</sup> mice (Fig. 7B). In contrast, IL-13 (Fig. 7C), a prototypic Th2 cytokine, showed a modest increase in DSS-treated WT mice that was not significant but a significant increase in tissues from DSS-treated CAT2<sup>−/−</sup> mice. IL-17 (Fig. 7D), which is the prototypic Th17 cytokine, was increased in DSS-treated WT mice compared with control mice and...
was significantly increased in CAT2−/− mice treated with DSS, but not in the other groups, as we observed at the protein level (Fig. 7F).

We also investigated the effect of CAT2 deletion on regulatory T (Treg) cells. Expression of Foxp3, a transcription factor involved with Treg cell development, is a marker for Treg cell infiltration. We found increased Foxp3 mRNA levels in WT mice treated with DSS, but these levels were increased to a similar level in colitic CAT2−/− mice (Fig. 8B). We also detected an increase in Foxp3 mRNA expression in untreated CAT2−/− mice. When we quantified the percentage of CD4+CD25+Foxp3+ cells in colon tissues by flow cytometry, we found a pattern that was the same as that for the Foxp3 mRNA levels, i.e., a similar increase in colitic WT and CAT2−/− mice and an increase in control CAT2−/− mice (data not shown).

Effect of CAT2 deletion on IL-17+CD4+ and IL-17+γδ T cells. Because the shift to an increase in IL-17 was more marked than to an increase in IL-13 and because IL-17 has been strongly linked to the pathogenesis of IBD and can be produced during colitis by T cell subsets that include Th17 cells and γδ T cells (22, 28, 53, 54, 56), we investigated this further. We analyzed CD4+ cells for IL-17 expression by flow cytometry (Fig. 9) and found a significant increase in IL-17-expressing CD4+ cells in the colon in WT mice treated with DSS and a modest further increase in CAT2−/− mice treated with DSS (Fig. 9, A and B). In MLN cells, the IL-17-expressing CD4+ cells were not increased in WT mice treated with DSS significantly further increased in CAT2−/− mice treated with DSS. Consistent with these findings, there was a marked increase in the ratios of IL-13 to IFN-γ (Fig. 7E) and IL-17 to IFN-γ (Fig. 7F) in the colon tissues of DSS-treated CAT2−/− mice. Together, these ratios suggest that, with CAT2 deletion, there is a strong shift from a Th1- to a Th2- and Th17-skewed immune response in DSS colitis. Notably, there was no change in IFN-γ, IL-13, or IL-17 levels in control CAT2−/− compared with control WT mice, suggesting that these changes are unmasked only under conditions of stress in the colon. The Th2 cytokines IL-4, IL-5, and IL-10 did not follow the pattern of IL-13, as they were not upregulated in tissues from DSS-treated CAT2−/− mice (Table 1).

Altered cytokine mRNA levels related to Th1, Th2, and Th17 cytokines. Next we sought to determine if the differences in protein levels could be due to altered mRNA expression. As in the protein studies, there was an increase in IFN-γ (Fig. 8A) and IL-17A (Fig. 8D) mRNA expression in WT mice treated with DSS and a decrease in IFN-γ (Fig. 8A) and a further increase in IL-17A (Fig. 8D) levels in colitic CAT2−/− mice. There was a similar degree of increased IL-13 expression in CAT2−/− and WT mice treated with DSS (Fig. 8C). However, the ratio of IL-13 to IFN-γ mRNA was not increased in WT mice treated with DSS but was significantly increased in the colitic CAT2−/− mice (Fig. 8E), similar to the protein data (Fig. 7E). Also the ratio of IL-17 to IFN-γ mRNA (Fig. 8F)
but were significantly increased in colitic CAT2−/− mice compared with colitic or control WT mice (Fig. 9, C and D). We investigated γδ T cell receptor cells, a type of intraepithelial T lymphocyte (67), because they have been shown to have a protective role in DSS colitis (12) but can also be a source of IL-17 and recruit neutrophils (17, 22, 28). We found, in WT mice treated with DSS, no increase in IL-17-expressing γδ T cells above control levels in either the colon (Fig. 10, A and B) or MLN (Fig. 10, C and D). In contrast, in both locations, there was a significant increase in IL-17-expressing γδ T cells from colitic CAT2−/− mice (Fig. 10). Taken together, these data suggest that increased IL-17 expression in CAT2−/− mice derives from CD4+ and γδ T cells and that both cell types may contribute to the exacerbation of colitis in CAT2−/− mice.

**Effect of CAT2 deletion on IL-23 and IL-6 mRNA levels.**

Because we have found that IL-17 is increased in DSS-treated CAT2−/− mice and because IL-23 and IL-6 mRNA levels in innate immune cells, we assessed IL-23 and IL-6 mRNA expression in tissues after 4 days of DSS, the time at which weight loss begins to occur in our model. As shown in Fig. 11A, IL-23 mRNA levels were increased in WT mice treated with DSS; in the CAT2−/− mice treated with DSS there was a modest, but not significantly different, further increase. There was no increase in IL-6 mRNA levels in WT mice treated with DSS, whereas the colitic CAT2−/− tissues exhibited a significant, 4.9 ± 0.6 fold, increase (Fig. 11B).

**DISCUSSION**

We previously found increased levels of the l-Arg transporter CAT2 in the DSS murine model of colitis and showed that l-Arg supplementation is beneficial as a treatment in this model (15). In the current report, we show that CAT2−/− mice exhibit profound exacerbation of DSS-induced colitis, with worsening of survival, body weight loss, and histological inflammation and epithelial injury. These changes were associated with increased IL-17 expression in colonic tissues.
CAT2 deletion worsens DSS colitis

associated with defective l-Arg uptake and accumulation of serum l-Arg without an increase in the AAI. The increased l-Arg in the serum of WT mice is most likely due to metabolic stress, which could include increased conversion of other amino acids, such as citrulline, to l-Arg, and the increased serum l-Arg in the CAT2−/− mice is most likely due to defective tissue utilization of l-Arg. These findings are consistent with our results in human UC patients, in whom we have reported increased serum l-Arg levels without an increase in the AAI (29). In addition, in a prospective study of 137 UC patients and 40 control subjects, we have found that CAT2 mRNA expression is decreased in UC tissues in a histological disease activity-specific manner: tissue AAI was inversely correlated with the disease activity index, and in patients with left-sided colitis, l-Arg levels and l-Arg uptake were decreased in involved compared with uninvolved colon tissues (unpublished data). While DSS as a model of UC has significant limitations, it is considered to be primarily a chemically induced injury model, our findings that CAT2 is downregulated in human UC suggest that the CAT2−/− DSS model has more similarity to UC than does DSS colitis in WT mice.

Additionally, we show that the increase in iNOS in colon tissues from WT mice treated with DSS no longer occurred in CAT2−/− mice. In considering the importance of this finding, we previously showed, in iNOS−/− mice, that DSS colitis worsened (15) but that benefits of l-Arg supplementation were lost (15), suggesting that when iNOS substrate levels are enhanced, NO can be beneficial in the DSS model. Similarly, a recent study showed that an arginase inhibitor improved clinical and histological features in DSS colitis, and this effect was attributed to enhanced NO production (1). We now show that CAT2−/− mice similarly lose the benefits of l-Arg supplementation in DSS colitis, indicating that the loss of iNOS in CAT2−/− mice could be involved in the exacerbation of colitis.

CAT2 is established as the main l-Arg transporter in macrophages and is essential for iNOS translation (9) and NO production (9, 14). In addition, increased arginase activity has been shown in CAT2−/− bone marrow-derived macrophages exposed to the alternative activation stimuli IL-4 and IL-13 (46). In the current study, we show that the increase in mRNA expression for CAT2 in DSS colitis derives mainly from F4/80+ colonic macrophages, rather than from the residual F4/80− cell population or isolated CECs. CAT2 mRNA expression was indeed detectable, but not upregulated, in CECs from WT colitic mice compared with control tissues. In addition, we previously demonstrated that CAT2 is expressed in CECs in vitro (65). However, our immunofluorescence data indicate that the CAT2 expression is primarily derived from colonic macrophages. Because we have shown that CAT2 can play an important role in epithelial restitution, the high level of expression in colonic macrophages compared with CECs in DSS colitis suggests that macrophages may act to deplete...
Fig. 10. IL-17 in γδ T cells from colon and MLNs. Colonic immune cells and MLN cells were isolated, stained, and analyzed by flow cytometry. A: percentage of IL-17+ cells within the CD4+ cell population in the colon. B: representative flow cytometry zebra plots (density of cells positive for a specific antigen). Left: percentage of CD4+ cells within the total colonic immune cell fraction. Right: percentage of IL-17+ cells within the CD4+ cells. C: percentage of IL-17+ cells within the CD4+ cell population in MLNs. D: zebra plots. Left: percentage of CD4+ cells within the MLN cell population. Right: percentage of IL-17+ cells within the CD4+ cells. **P < 0.01 vs. WT Ctrl; §§§P < 0.001 vs. WT DSS.

1-Arg substrate availability for the relatively low level of CAT2 or for other 1-Arg transporters in CECs. Thus deletion of CAT2 could affect CECs, including barrier function and inflammatory chemokine responses. These events may lead to increased exposure of colonic mucosal immune cells to luminal bacteria.

There was an increase in F4/80 staining in DSS colitis tissues, indicating an increase in macrophage infiltration; moreover, the finding that F4/80 and CAT2 staining strongly colocalized suggests that CAT2 expression could represent a marker of activated macrophages in DSS colitis. Consistent with this concept, we showed increased levels of proinflammatory G-CSF, MIP-1α, IL-15, and RANTES in the colonic mucosa of CAT2−/− compared with WT mice treated with DSS; these cytokines/chemokines can be made by macrophages (2, 18, 42, 43, 69), indicative of innate immune activation. Importantly, IL-15−/− mice have been shown to have attenuated colitis in the DSS model (75), which is consistent with our findings of increased IL-15 in CAT2−/− mice that exhibit increased colitis with DSS. However, in our Luminox profiling, multiple proinflammatory chemokines and monocyte/macrophage-derived cytokines noted in Table 1 were not increased in CAT2−/− mice compared with WT mice exposed to DSS and, in some cases, were even decreased in the CAT2−/− mice (TNF-α, IL-1α, IL-1β, and IL-6). Thus there may not be a global gain or loss of innate immune function in CAT2−/− macrophages. We also found an increase in dendritic cell activation in CAT2−/− colitis tissues, which may act to compensate for limitations in macrophage function by also acting as antigen-presenting cells. There was also an increase in granulocyte infiltration in the colitis tissues of CAT2−/− compared with WT mice, indicating that MIP-1α, which is known to recruit neutrophils (2, 42, 69), may be sufficient, although the neutrophil chemotactic proteins KC, MIP-1β, and MIP-2 were not increased. This suggests redundancy in the molecules that can enhance granulocyte infiltration in the DSS model. Also the increase in colonic granulocytes is consistent with the increase in G-CSF, which acts to stimulate the proliferation and function of neutrophils (7, 45, 66).

CAT2−/− colitic mice had increased levels of RANTES, which is a marker of resting, as well as activated, T cells. Consistent with this finding, we detected increased numbers of T cells in the colitis tissues from the CAT2−/− mice, as well as increased Th cells, compared with WT mice. A significant finding from our study was an upregulation of colonic IFN-γ protein and mRNA expression in colitis tissues from DSS-treated WT mice compared with untreated mice. However, this may not have a role in driving the inflammation, because in CAT2−/− mice with more colitis, the IFN-γ levels were actually attenuated. Instead, the levels of IL-17 protein and mRNA were markedly potentiated in the colitis tissues from the CAT2−/− mice, and consistent with the shift to this T cell...
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Fig. 11. Effect of CAT2 deletion on levels of IL-23 (A) and IL-6 (B) mRNA expression. RNA from colon tissues of control mice and mice treated with DSS for 4 days were analyzed by real-time PCR. *P < 0.05, **P < 0.01 vs. WT Ctrl; §P < 0.05 vs. WT DSS.

cytokine, the IL-17-to-IFN-γ ratio was not increased in WT mice but was markedly increased in CAT2−/− mice. Similarly, the IL-13-to-IFN-γ ratio was also increased in colitic CAT2−/− mice. Prior studies showed that DSS colitis still occurs in C.B17 severe combined immunodeficient mice on a background similar to BALB/c (19) and in Rag1−/− mice on a background similar to C57BL/6 (34). However, our data suggest that T cell-derived IL-17 and IL-13 are important in the pathogenesis of DSS colitis in CAT2−/− mice, especially since both of these cytokines have been implicated in human UC (22, 23, 67). Other Th2 cytokines did not follow the pattern of IL-13, again indicating that the effect of CAT2 deletion is not global.

We focused on the marked difference in IL-17 between the WT and CAT2−/− mice, since the difference in IL-13 was more marked between CAT2−/− and WT mice. Also the area of regulation of IL-17 in colitis is a rapidly evolving field and is a downstream target of new therapies in IBD. Our data implicating IL-17 as a causal factor in the exacerbation of colitis in CAT2−/− mice is consistent with reports that IL-17A−/− mice exhibit reduced severity of colitis in the DSS model (30). A conflicting report shows that monoclonal anti-IL-17 worsened DSS colitis (48), but these latter experiments were done in BALB/c mice, while the IL-17A knockout studies were conducted in C57BL/6 mice, as we used in the current study.

Furthermore, we show that the increased IL-17 in the DSS-treated CAT2−/− mice can derive from CD4+ cells and γδ T cells. In DSS colitis, several studies have indicated that γδ T cells can be protective in DSS colitis, as knockout mice (12, 71) and γδ T cell depletion models (37) showed worsening of DSS colitis. However, in our studies, in the specific case of CAT2 deletion, the γδ T cells in the colon and MLN show activation of IL-17 expression, suggesting that they have now become proinflammatory. This finding suggests that alterations in 1-Arg availability may modulate γδ T cell activation status. Because γδ T cells have been implicated in a variety of aspects of innate immunity (6, 32, 67), studies of the effects of CAT2 deletion on γδ T cells in other models of innate immune activation are a future area for investigation. A question raised by the increase in IL-17 expressing CD4+ cells in the colon and MLN indicative of increased Th17 cells is the role of innate immune cell cytokines in this process. Our data show that while IL-23 mRNA levels are increased in colon tissues but are not further increased in CAT2−/− mice, IL-6, which is also required for Th17 differentiation (17, 40), is specifically upregulated only in CAT2−/− mice. These data suggest that the increase in Th17 cells in DSS colitis that occurs with CAT2 deletion may be due, at least in part, to the increase in IL-6 in colonic innate immune cells, such as macrophages.

While CAT2 deletion resulted in more inflammation in DSS colitis, this is not necessarily a nonspecific phenomenon. We have reported that, in CAT2−/− mice infected with the gastric pathogen H. pylori, there was actually less gastritis, which was associated with ineffective immunity, since there was a higher level of H. pylori colonization in these mice than in WT mice (3). In contrast to our findings in DSS-treated mice, H. pylori-infected CAT2−/− mice exhibited fewer gastric macrophages, fewer MHCII+ dendritic cells, and less G-CSF and IL-17 (3). However, there was a decrease in IP-10 and an increase in IL-13 (3) similar to the current study. In addition, our laboratory has extensive experience with the Citrobacter rodentium murine model of colitis, in which there is Th1 and Th17 activation (26, 50, 64, 74). We have found that CAT2 mRNA expression is upregulated in C. rodentium colitis and that, in contrast to the findings in the DSS model, CAT2−/− mice in this model exhibit less clinical evidence of colitis, less colonic immune cell infiltration, and less Th1 and Th17 response, without induction of Th2 cytokines (unpublished data). One potential explanation for the different effect of CAT2 deletion in the two colitis models may be that, in the C. rodentium colitis tissues, the CAT2 expression derives mainly from CECs, rather than from the colonic macrophages, as in DSS-induced colitis; consistent with this finding, we have observed decreased expression of talin, a host protein to which C. rodentium binds, in infected tissues from CAT2−/− mice (unpublished data). We have also found that CAT2 mRNA expression is upregulated in colon tissues in the oxazolone colitis model, which is hapten-induced, and in the CD45RBmab-to-Rag2−/− adoptive transfer colitis model; both of these models could be useful for future studies.

Taken together with our current study, these findings indicate that CAT2 deletion does not cause a global shift in macrophage polarization and that the role of CAT2 in mucosal macrophages in the gastrointestinal tract is likely to be model/
stimulus-specific. Our finding that CAT2 levels are decreased in human UC suggests that altered macrophage function may be important in UC pathogenesis. Furthermore, combination amino acid therapy with L-Arg and its downstream metabolites L-Orn and L-Pro could be beneficial in UC to counteract impaired L-Arg uptake.

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AUTHOR CONTRIBUTIONS


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