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Research Article

CD73 Is Critical for the Resolution of Murine Colonic Inflammation

Margaret S. Bynoe,[1] Adam T. Waickman,[1] Deeqa A. Mahamed,[1] Cynthia Mueller,[1] Jeffrey H. Mills,[1] and Agnieszka Czopik[2]

[1] *Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, C5 149 VMC, Ithaca, NY 14853, USA*
[2] *Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*

Correspondence should be addressed to Margaret S. Bynoe, msb76@cornell.edu

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CD73 is a glycosyl-phosphatidylinositol-(GPI-) linked membrane protein that catalyzes the extracellular dephosphorylation of adenosine monophosphate (AMP) to adenosine. Adenosine is a negative regulator of inflammation and prevents excessive cellular damage. We investigated the role of extracellular adenosine in the intestinal mucosa during the development of Dextran-Sulfate-Sodium-(DSS-)salt-induced colitis in mice that lack CD73 ($CD73^{-/-}$) and are unable to synthesize extracellular adenosine. We have found that, compared to wild-type (WT) mice, $CD73^{-/-}$ mice are highly susceptible to DSS-induced colitis. $CD73^{-/-}$ mice exhibit pronounced weight loss, slower weight recovery, an increase in gut permeability, a decrease in expression of tight junctional adhesion molecules, as well as unresolved inflammation following the removal of DSS. Moreover, colonic epithelia in $CD73^{-/-}$ mice exhibited increased TLR9 expression, high levels of IL-1β and TNF-α, and constitutive activation of NF-κB. We conclude that CD73 expression in the colon is critical for regulating the magnitude and the resolution of colonic immune responses.

1. Introduction

Many mechanisms are proposed to lead to chronic inflammation of the gut mucosa, which is exemplified by Crohn's disease (CD) and ulcerative colitis (UC), also known as inflammatory bowel diseases (IBDs) [1]. These include dysregulation of the innate immune response to enteric flora [2, 3], increased epithelial barrier permeability [4], and defective regulation of the adaptive immune response [5]. Several susceptibility genes carrying mutations have been found in patients suffering from IBD [1]. Mutations in the human leukocyte antigen genes are associated with both CD and UC [6], while mutations in the Nuclear Oligomerization Domain (NOD) 1/2 genes are associated with CD [1, 7].

The passage of soluble molecules across the epithelial barrier of the gastrointestinal tract is a tightly regulated process [8]. Nutrients must be absorbed into the bloodstream, while commensal bacteria normally found in the GI tract, and their associated antigens, must be kept isolated from intraepithelial lymphocytes to avoid abnormal and persistent immune responses that can cause inflammation or other types of trauma [8, 9]. The purine nucleoside adenosine, its receptors and ectoenzymes (CD39 and CD73) are emerging as critical players in the regulation of intestinal immune-mediated inflammation [10–12].

During inflammation and the initiation of primary immune responses, adenosine triphosphate (ATP) is released into the extracellular environment following cell damage [13]. Extracellular ATP, a damage-associated molecule and a potent activator of the immune response, increases the production of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL-12. ATP also enhances the potency of the oxidative stress response of activated macrophages [13]. The immune system has various mechanisms to resolve these inflammatory signals. One such mechanism is activated by the extracellular adenosine generated through the activities

of CD39 and CD73 [14]. CD39 catabolizes extracellular ATP to adenosine monophosphate (AMP) while CD73 (a 5′-ectonucleotidase) further converts AMP to adenosine [15]. Adenosine inhibits production of TNF-α, IL-1β, IL-6, IL-12, and IL-23 by monocytes and dendritic cells (DCs) and promotes the production of IL-10 [16]. Therefore, CD73-generated adenosine acts as a negative feedback signal to prevent uncontrolled inflammation that would otherwise cause collateral damage to healthy tissues.

CD39, CD73, and adenosine receptors are expressed on intestinal epithelial cells and various immune cell types [17–19]. CD39 and CD73 have recently been shown to be important for T regulatory cell function [20], and mice lacking these enzymes exhibit very severe IBD in trinitrobenzene sulfonate (TNBS) colitis model [10, 11]. Adenosine mediates its effects through four G protein-coupled receptors: A_1AR; A_{2A}AR; A_{2B}AR; A_3AR [14]. Adenosine receptors are expressed in the intestine of mice and humans**,** and signaling through these receptors is critical for regulation of the severity of intestinal inflammation following a wide range of insults [16]. For example, activation of the A_{2A} receptor has been shown to reduce intestinal inflammation in mice by decreasing leukocyte infiltration and inhibiting proinflammatory cytokines [21], while inhibition of adenosine kinase improves IBD pathology in DSS-induced colitis [22].

Because of the anti-inflammatory role of adenosine and its association with IBD, we investigated the role of extracellular (CD73-generated) adenosine in DSS-induced colitis in mice deficient in CD73. CD73$^{-/-}$ mice developed markedly more severe colitis and exhibit unresolved inflammation and produce high levels of the proinflammatory cytokines IL-1β and TNF-α compared to WT mice. Colonic epithelial cells from CD73$^{-/-}$ mice show increased TLR-9 and NF-κB activity and have decreased expression of several tight-junction-associated proteins when compared to WT mice, along with an associated increase in gastrointestinal permeability. From these findings, we hypothesize that CD73-generated adenosine is a key modulator in gut inflammation and IBD and mediates its effects through regulating inflammation and maintaining epithelial barrier integrity.

2. Materials and Methods

2.1. Mice. C57BL/6 WT and *rag*$^{-/-}$ mice were purchased from Jackson Laboratories. CD73$^{-/-}$ mice were obtained from Dr. Linda Thompson at the Oklahoma Medical Research Foundation [23]. Mice were bred and housed under specific pathogen-free conditions in the mouse facility at Cornell University. All procedures performed on mice were approved by the Cornell University IACUC. All mice used in these experiments were between 8 and 10 weeks old.

2.2. DSS-Induced Experimental Colitis. 3% Dextran Sodium Sulfate (DSS, MW = 36,000–50,000) was administered in drinking water to experimental animals [24]. Age-matched males and females WT and CD73$^{-/-}$ were used in these experiments. Weight loss was normally observed 4-5 days after the initiation of DSS treatment, and experiments were halted after animals lost ~20% of initial body weight.

2.3. Passively Induced IBD. CD4$^+$ cells were isolated from processed spleens and lymph nodes of naïve WT and CD73$^{-/-}$ mice by negative selection using magnetic beads (Invitrogen). CD4$^+$ cells were further subdivided into CD4$^+$CD45RB^{high}CD25$^-$ and CD4$^+$CD45RB^{low}CD25$^+$ populations using a BD-Biosciences FACS Aria system. Two million CD4$^+$CD45RB^{high} cells from either WT or CD73$^{-/-}$ mice were injected into *rag*$^{-/-}$ mice. Some mice additionally received 2×10^6 CD4$^+$CD45RB^{low}CD25$^+$ cells isolated from either WT or CD73$^{-/-}$ mice. Mice were weighed daily for 45 days to track disease progression.

2.4. Isolation of Primary Colonic Epithelial Cells. Whole colons were isolated, the lumen exposed by a longitudinal cut, washed with Ca^{++}Mg^{++} free HBSS + 2% FBS, cut into 1-2 mm^2 sections, and washed 2X with Ca^{++}Mg^{++} free HBSS + 2% FBS. The tissue was then incubated for 30 min at 37° C in Ca^{++}Mg^{++} free HBSS + 10% FBS + 1 mM EDTA + 1 mM DTT + penicillin/streptomycin. The digest was filtered through a 70 μm mesh and spun at 200 ×g for 10 min. Primary colonic epithelial cells include resident intraepithelial lymphocytes (iELs), as no Percoll gradient was used to separate this population.

2.5. Flow Cytometry. Cells were isolated either from the spleen, mesenteric lymph nodes, colonic epithelium, or colonic patches of mice and processed into a single-cell suspension. These cells were subsequently stained with rat anti-mouse CD4, CD25 (BD Biosciences), CD73, and Foxp3 or hamster anti-mouse CD11c (eBioscience) at a 1 : 250 dilution in PBS. After incubating for 30 min at 4°C with the antibody cocktail, the cells were washed and resuspended in PBS. FoxP3 staining was performed using the eBioscience FoxP3-APC kit. Sample data were acquired using a FACScalibur with CellQuest software. Data files were analyzed using FlowJo software.

2.6. Histology. Hematoxylin and eosin (H&E) staining was performed by the Cornell University College of Veterinary Medicine Core Histology lab on formalin fixed tissue samples. Immunohistochemistry and immunofluorescence were performed on OCT embedding tissue snap-frozen in liquid nitrogen-chilled methylbutane and sectioned into 5 μm slices on a cryostat. Primary antibodies used were CD4, CD45, CD11c (hamster anti-mouse 1 : 30), and CD321 (JAM-A, rat anti mouse, 1 : 100). Secondary antibodies used were biotinylated goat anti-rat IgG and biotinylated goat anti-hamster IgG (1 : 100). For immunohistochemistry, slides were treated with streptavidin-HRP followed by AEC and counterstained with hematoxylin. Immunofluorescence staining for CD321 was performed by treating with a 1 : 200 dilution of streptavidin-AF488 for 90 minutes at 37°C and then fixed in 1% formalin for 10 minutes.

2.7. Clinical Score and Histological Analysis. Changes in body weight were assessed as a percent change in body weight compared to baseline. A scoring system (from 0–4) was used to determine the presence of occult or overt blood

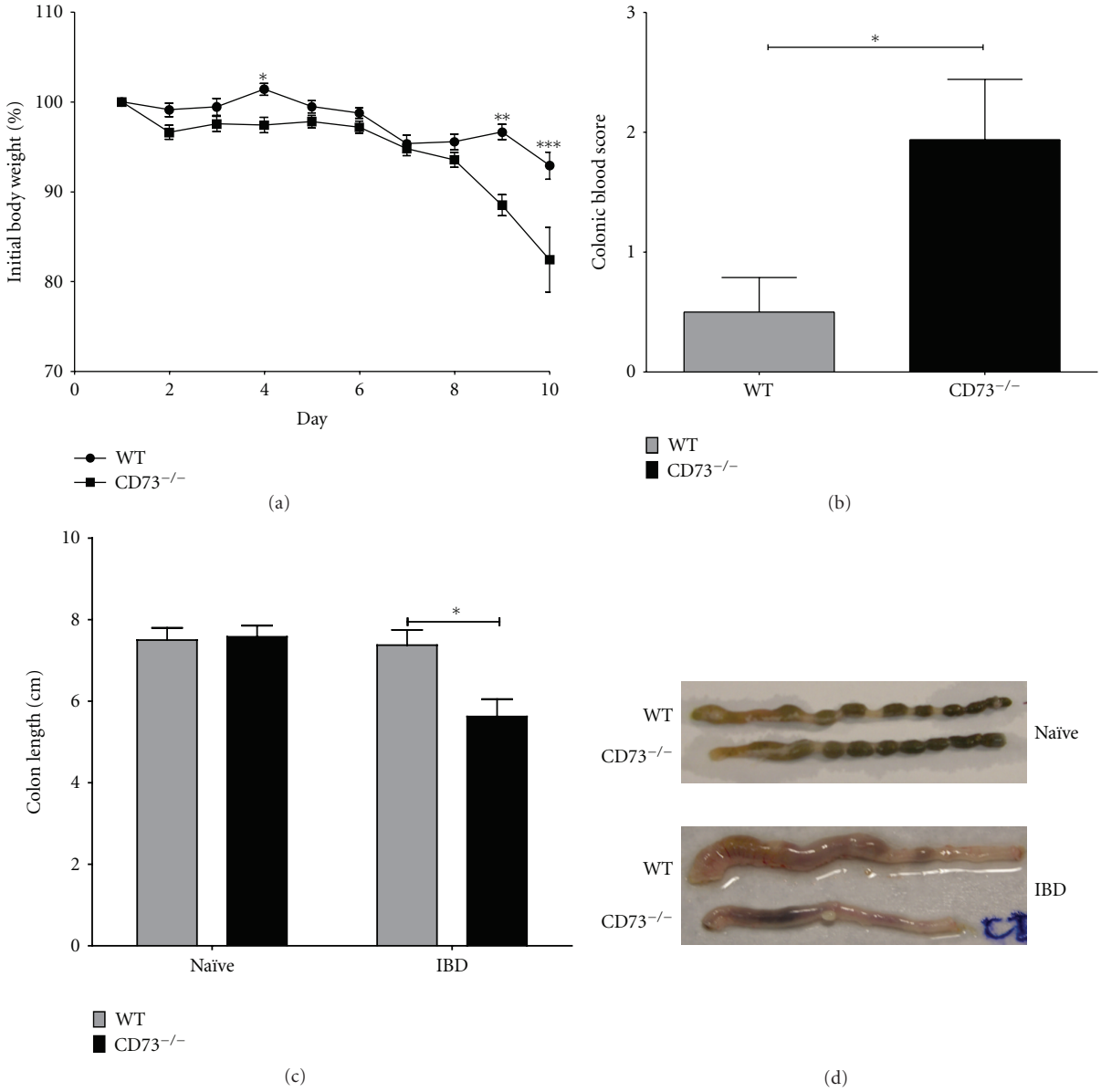

Figure 1: CD73$^{-/-}$ mice develop more severe clinical signs of IBD than WT mice. (a) WT (circles) and CD73$^{-/-}$ (squares) mice were given 3% DSS in drinking water and weighed daily for 10 days. Results are represented as % of initial weight (±SEM, summary of 5 experiments, $n = 26$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$). (b) Mean (±SEM, $n = 4$, $^{*}P < 0.05$, representative of 5 experiments) colonic blood score is displayed (0 = no blood in colon; 1 = blood in 25% of colon; 2 = blood in 50% of colon; 3 = blood in 75% of colon; 4 = blood in 100% of colon). (c) Mean (±SEM, $n = 4$, $^{*}P < 0.05$, representative of 5 experiments) colon length (cm) is displayed, along with (d) a representative photograph.

in the stool. Colonic inflammatory score was determined by the extent of inflammatory cell infiltrates (0–3) and tissue damage (0–3). Scoring was performed in a blinded manner. Cell infiltration and tissue damage were added and the combined "colitis severity score" ranged from 0 to 6 (description in legends to Figures 2, 3 and 4).

In Figure 2, (a) histology of colons from WT (top panels) and CD73$^{-/-}$ (bottom panels) mice treated with 3% DSS in drinking water. Left two panels show hematoxylin and eosin (H&E) stains displaying colonic epithelial crypt histopathology and appearance of granulomas (in CD73$^{-/-}$) mice. Right three panels show CD4^{+} cell (brown) infiltration in the colon. (b) Mean (±SEM, $^{****}P < 0.001$, $n = 8$) colonic inflammatory score is displayed. The scoring scale is comprised of two components: (i) inflammatory cell infiltrate (focus of inflammatory cells in the lamina propria = 1, confluence of inflammatory cells = 2, and transmural extension of the infiltrates = 3); (ii) tissue damage (discrete lymphoepithelial lesions = 1, mucosal erosion = 2 and extensive mucosal damage and extension through deeper structures

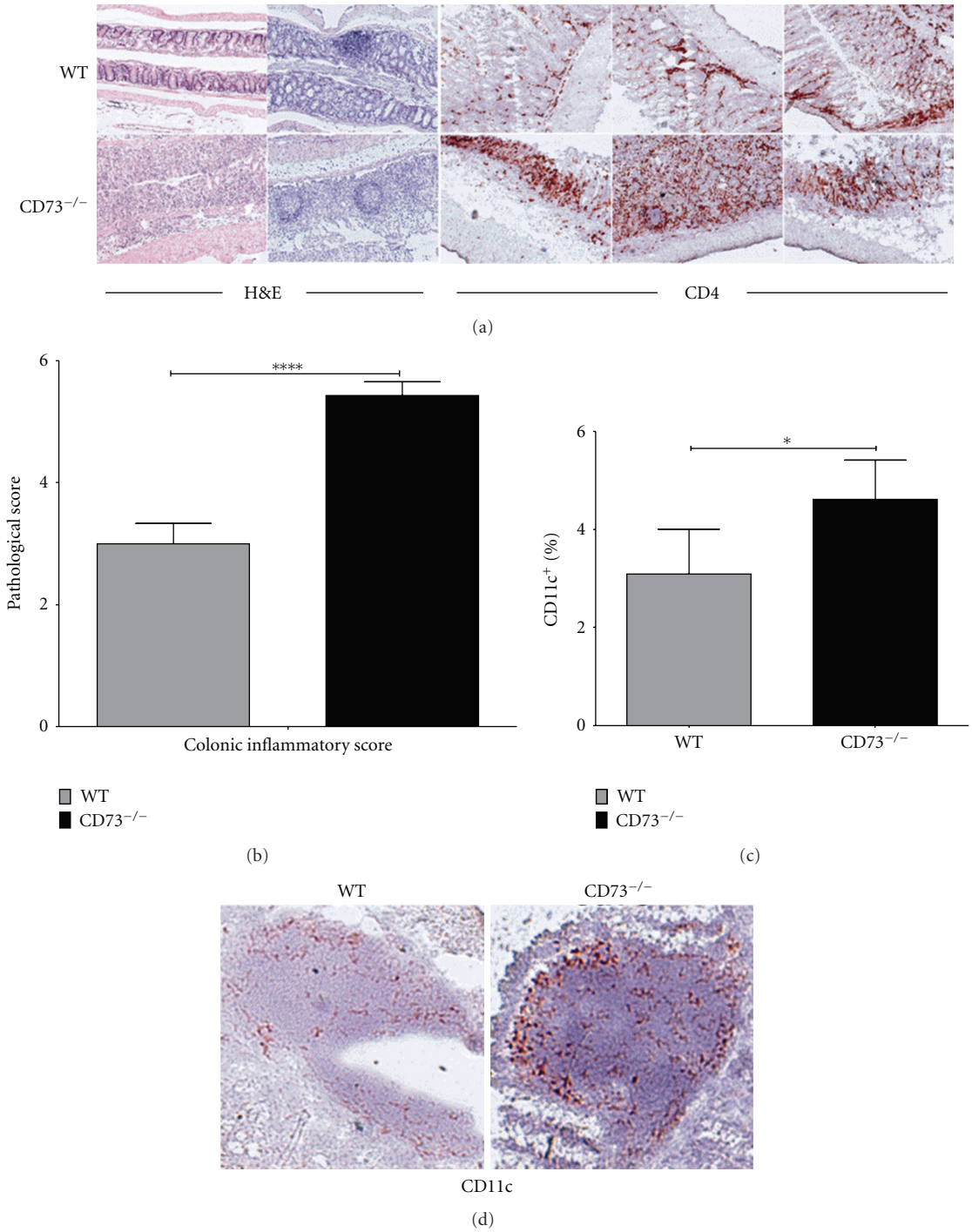

Figure 2: CD73$^{-/-}$ mice display a higher degree of infiltration in the colon compared to WT mice.

of the bowel wall = 3). The two scores (cell infiltration and cell damage) are added, and the combined histological colitis severity score ranges from 0 to 6. (c) Colonic patches from WT and CD73$^{-/-}$ mice treated with 3% DSS in drinking water were isolated and stained for CD11c and analyzed by FACS. Results are displayed as % of CD11c^{+} cells (data representative of 3 experiments). (d) Immunohistochemistry of colons from WT (left) and CD73$^{-/-}$ (right) mice treated with 3% DSS in drinking water. Sections show CD11c infiltration in the colons of mice. (e) IL-1β and TNF-α produced by lymphocytes from colonic patches of CD73$^{-/-}$ and WT mice after 24-hour incubation in medium without stimulation. Samples were analyzed by Bio-Plex instrument (from three different experiments, $n = 15$ mice, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, ND = not detected).

In Figure 3, (a) CD4^{+}CD45RB^{high} T cells from WT (circles) and CD73$^{-/-}$ (squares) were transferred into *rag*$^{-/-}$ mice, which were monitored for 45 days. Results

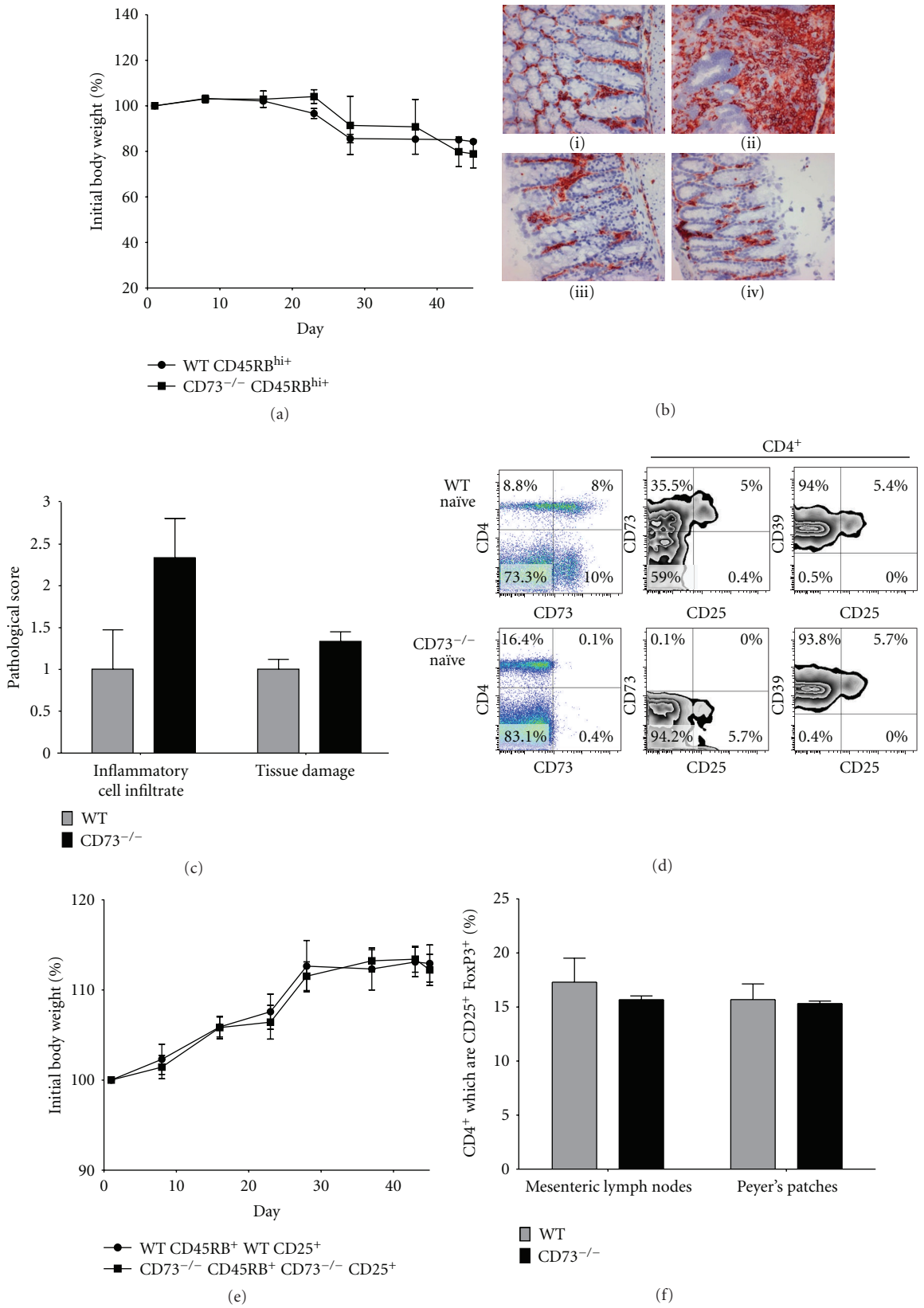

FIGURE 3: CD4+ T cells from CD73−/− mice transfer a similar degree of colitis compared to WT mice and show no defect in T_{reg} suppression of IBD.

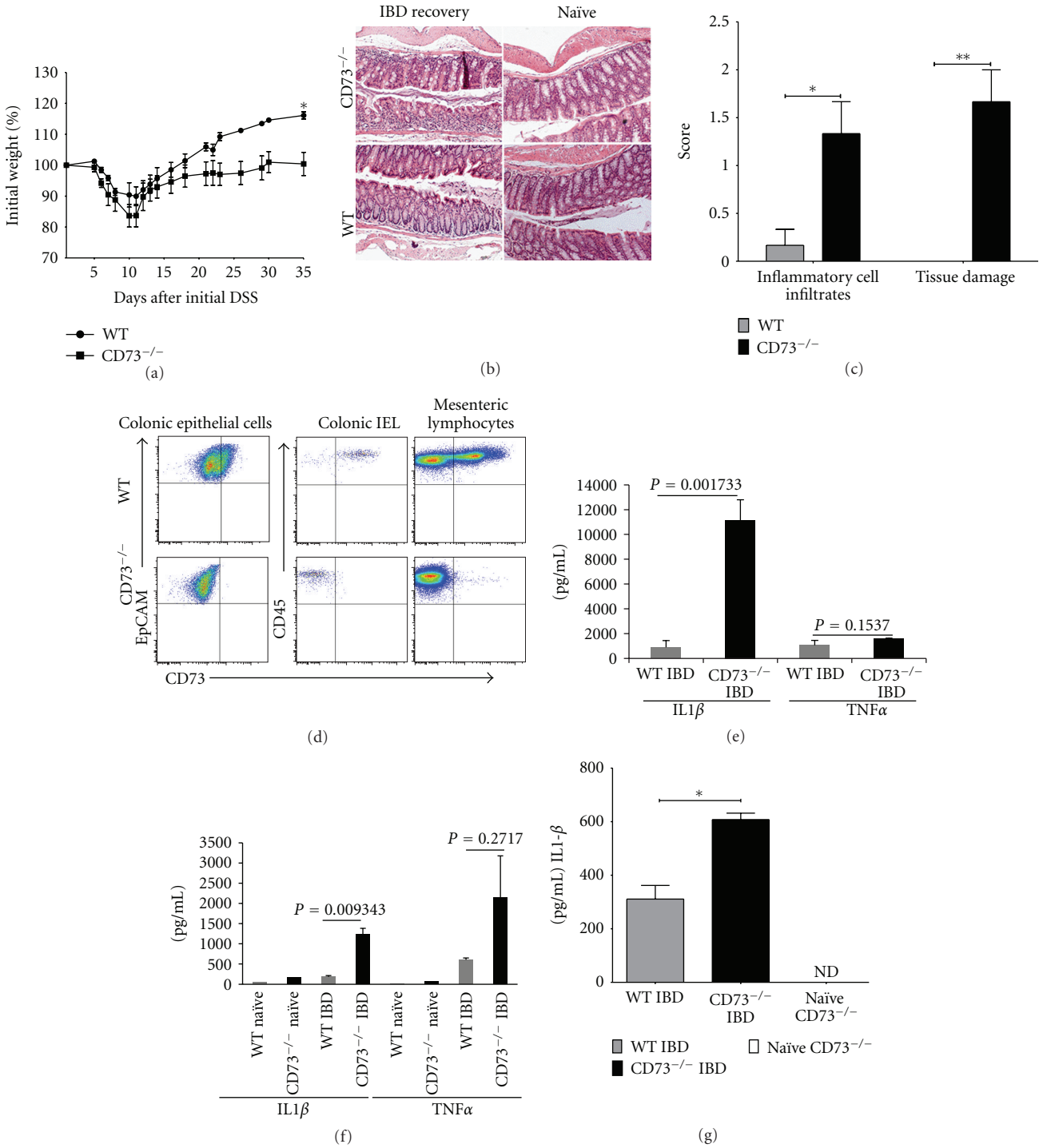

FIGURE 4: CD73$^{-/-}$ mice exhibit ongoing inflammation and slower recovery from IBD compared to WT mice. (a) WT (circles) and CD73$^{-/-}$ (squares) mice were given 3% DSS in drinking water and weighed daily for 35 days. DSS was removed at day 8 and replaced with drinking water. Results are represented as % of initial weight (±SEM, $^{*}P < 0.05$, one of two representative experiments). (b) H&E staining of colons from CD73$^{-/-}$ (top) and WT (bottom) mice taken on day 35 (left) compared to naïve (right) mice. (c) Mean (±SEM, $^{*}P < 0.05$, $^{**}P < 0.01$) colonic inflammatory score is displayed ($n = 5$ mice). The scoring scale is comprised of two components: (i) inflammatory cell infiltrate (focus of inflammatory cells in the lamina propria = 1, confluence of inflammatory cells = 2, and transmural extension of the infiltrates = 3); (ii) tissue damage (discrete lymphoepithelial lesions = 1, mucosal erosion = 2, and extensive mucosal damage and extension through deeper structures of the bowel wall = 3). (d) FACS analysis of CD73 expression on colonic epithelial cells from naïve WT (top left panel, 28%) and CD73$^{-/-}$ (bottom left panel, 0%) mice. Colonic epithelial cells identified with CD326/EpCam. Colonic IEL's identified with CD45 (middle panels, WT 87% CD73^{+}). Mesenteric lymph nodes shown as a positive control (right panels, WT 39% CD73^{+}). (e) IL-1β and TNFα production in colon preparation isolated from WT and CD73$^{-/-}$ mice on day three of DSS treatment and (f) day eight of DSS treatment. (g) Colons isolated from WT and CD73$^{-/-}$ mice on day 22 after removal of DSS were cultured (in medium alone) for 24 hours and supernatant analyzed by Bio-Plex for IL-1β. Results (mean ±SEM, $^{*}P < 0.05$, representative of one experiment, $n = 3$ mice per group) are presented as pg/mL IL-1β produced.

Table 1

Target gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
JAM-A	TCTCTTCACGTCTATGATCCTGG	TTTGATGGACTCGTTCTCGGG
Claudin 2	GTACCCTTTTAGGACTTCCTGC	CCCACCACAGAGATAATACAAGC
A Catenin	TCTCTACTGCCACCAGCTCAAC	AAGCCATCCCCTGTGACTTCT
GAPDH	CCCCAATGTGTCCGTCGTG	GCCTGCTTCACCACCTTCT
TLR1	GTCTCCCCACTTCATCCAGA	GCTTGTTCTTCTCTGTGGGC
TLR3	GGTCCCCAGCCTTCAAAGAC	ACGAAGAGGGCGGAAAGGT
TLR4	CAGGTGGAATTGTATCGCCT	CGAGGCTTTTCCATCCAATA
TLR5	CCACCGAAGACTGCGATGA	GTGACCGTGCACAGGATGAA
TLR9	ATG GTT CTC CGT CGA AGG ACT	GAG GCT TCA GCT CAC AGG G
PGK1	CTC CGC TTT CAT GTA GAG GAA G	GAC ATC TCC TAG TTT GGA CAG TG

(5 mice/group, mean ± SEM, representative of 3 experiments) are displayed as % of initial weight. (b) CD45 staining of frozen colons from $rag^{-/-}$ mice 45 days after T cell transfer: (i) WT CD45RB$^+$ donor, (ii) CD73$^{-/-}$ CD45RB$^+$ donor, (iii) WT CD45RB$^+$ and WT CD25$^+$ donors, and (iv) CD73$^{-/-}$ CD45RB$^+$ and CD73$^{-/-}$ CD25$^+$ donors. (c) Colonic inflammatory score is displayed with separate scores for inflammatory cell infiltrate (focus of inflammatory cells in the lamina propria = 1, confluence of inflammatory cells = 2 and transmural extension of the infiltrates = 3), and tissue damage (discrete lymphoepithelial lesions = 1, mucosal erosion = 2, and extensive mucosal damage and extension through deeper structures of the bowel wall = 3) ($n = 6$ mice, inflammatory cell infiltrate, ±SEM, $P = 0.05$, tissue damage, $P = 0.4$). (D) CD25, CD73, and CD39 expression on CD4$^+$ T cells from mesenteric lymph nodes and colonic patches of naïve WT and CD73$^{-/-}$ mice. Left-hand panels show distribution of CD73 on CD4$^+$ cells. Middle panels show distribution of iELs, and right panels are gated on CD4$^+$ cells and show distribution of CD25/CD73 and CD25/CD39 expression. (e) CD4$^+$CD45RB^{high} and CD4$^+$CD25$^+$ (CD45RB^{lo}) T cells from WT (circles) or CD73$^{-/-}$ (squares) were transferred into $rag^{-/-}$ mice. Results (mean ± SEM, representative of 3 experiments) are displayed as % of initial weight. (f) FoxP3 expression in colonic patches and mesenteric lymph nodes from CD73$^{-/-}$ versus WT mice (representative of two experiments).

2.8. Cytokine Analysis. For Figure 2, colonic patches were cultured overnight at 37°C in Bruff's media. For Figure 4, whole colons were isolated from DSS-treated and naïve CD73$^{-/-}$ and WT animals and cultured overnight at 37°C in Bruff's media. The culture supernatant was isolated and frozen at −80°C. Analysis of cytokine levels was performed using a Bio-Plex system.

2.9. Real-Time PCR. All data was generated using either the Thermo Scientific SYBR Green kit and the ABI 7500 Real-Time PCR machine or the Kapa SYBR FAST qPCR kit and the Bio-Rad C1000 Real-Time PCR machine. Expression of all genes was standardized to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and relativised to WT expression of the gene in question or was compared to PGK1 (housekeeping gene) expression. Primers, whose sequences are shown in Table 1, were obtained from IDT.

2.10. Gastrointestinal Permeability. Briefly, mice were fasted for 3 hrs and were rectally administered 200 μL of 10 mg/mL (0.1 mg/g body weight) 10 kDa FITC-dextran (Invitrogen) in PBS [25]. After 1 hour, mice were bled via their submandibular vein, the whole blood spun at 2,000 ×g for 5 min, and the serum isolated. This isolation yielded approximately 150 μL of serum per animal. Serum fluorescence was measured on a BioTek (Winooski, VT) Synergy 4 analyzer. FITC-Dextran concentration was calculated from standard curves generated by serially diluting FITC-Dextran.

2.11. Western Blotting. Western blot analysis as described in [26] was performed on colonic epithelial cells of CD73$^{-/-}$ and WT mice. Briefly, cells (2×10^6) were lysed in reducing SDS sample buffer followed by centrifugation at 18,000 g for 5 min. Samples were boiled for 10 minutes after which equal amounts of total cell lysates were separated by 10% SDS-PAGE and electrophoresed onto PVDF (Millipore, Bedford, MA). Samples were incubated with Ab specific for dual-phosphorylated (active) NF-κB P65 subunit used at the concentrations recommended by the manufacturer (Cell Signaling Technology, Beverly, MA) in 5% BSA in Tris buffered saline, 0.1% Tween20 overnight at 4°C.

3. Results

3.1. CD73$^{-/-}$ Mice Develop More Severe IBD Than WT Mice. Since CD73 catalyzes the formation of extracellular immunosuppressive adenosine from damaged cells during inflammation and is highly expressed on colon tissue [16–19], we investigated the impact of a lack of CD73 in the intestinal mucosa. To do this, we induced IBD in CD73$^{-/-}$ and WT mice by oral treatment with 3% DSS in drinking water. DSS-induced colitis resulted in significant weight loss, epithelial damage, and robust colonic inflammation (Figure 1). Daily monitoring of IBD revealed that CD73$^{-/-}$ mice developed markedly more severe clinical signs of IBD, including diarrhea and dehydration (data not shown), when compared to WT mice (Figure 1). Frequently, disease had to be aborted before the duration of the 10

day protocol because greater than 50% of CD73$^{-/-}$ mice exhibited substantial weight loss and severe rectal bleeding. This was accompanied by more pronounced weight loss (Figure 1(a)), higher colonic blood scores (Figure 1(b)), and shorter colon length (Figure 1(c)).

Histological analyses of the colons of DSS-treated CD73$^{-/-}$ mice revealed extensive destruction of colonic epithelial architecture, including epithelial crypt destruction, massive immune cell infiltration into the lamina propria, and the appearance of granulomas (in 30% of mice), and an overall increase in colon inflammatory score compared to WT mice (Figures 2(a) and 2(b)). These immune cell infiltrates consist of CD45$^+$ cells (a general marker for leukocytes, data not shown) with a substantial number being CD4$^+$ T cells (Figure 2(a)). FACS analysis of colonic patches (lymphoid aggregates from colon) also revealed a higher percentage of CD11c$^+$ dendritic cells in CD73$^{-/-}$ mice compared to WT mice (Figures 2(c) and 2(d)).

We next determined the cytokine profile of infiltrating leukocytes isolated from the colon of DSS-treated CD73$^{-/-}$ and WT mice. Cells from colonic patches of CD73$^{-/-}$ mice produced higher levels of IL-1β compared to those from WT mice (data not shown). There was no significant difference in TNF-α, IFN-γ, IL-17, IL-4, or IL-10 levels between WT and CD73$^{-/-}$ mice (data not shown). The increase in IL-1β suggests that infiltrating inflammatory cells into the colon are capable of contributing to the severe IBD in CD73$^{-/-}$ mice.

3.2. CD45RB^{high}CD4$^+$ Cells from CD73$^{-/-}$ Mice Transfer Similar Degree of Colitis as WT Mice. Due to the immunoregulatory effects of localized CD73-driven adenosine production, we next determined if the severe IBD phenotype observed in DSS-treated CD73$^{-/-}$ mice was due to a more proinflammatory nature of the CD4$^+$ T-cell population. To address this question, we induced IBD by transferring CD4$^+$CD45RB^{high} T cells into *rag*$^{-/-}$ recipients [27]. IBD induced by this method involves differential activation of T_H-1 cells and can be inhibited by cotransfer of CD4$^+$CD45RB^{low} (consisting of CD25$^+$ T regulatory cells) cells into *rag*$^{-/-}$ recipients [28]. CD4$^+$CD45RB^{high} cells were isolated from spleens of naïve WT or CD73$^{-/-}$ mice and adoptively transferred into *rag*$^{-/-}$ recipients. Recipients of CD73$^{-/-}$ CD4$^+$CD45RB^{high} T cells exhibited a similar weight loss pattern to those recipients of WT CD4$^+$CD45RB^{high} T cells (Figure 3(a)). Histological analysis of colons revealed *rag*$^{-/-}$ mice that received CD4$^+$CD45RB^{high} cells from CD73$^{-/-}$ mice had significantly more immune cell infiltration in the lamina propria compared to recipients of CD4$^+$CD45RB^{high} cells from WT mice (Figures 3(b) and 3(c)). However, the resultant colonic epithelial damage compared between recipients of WT and CD73$^{-/-}$ CD4$^+$CD45RB^{high} donor cells is not statistically significant (Figures 3(c) and see S1 in supplementary material available online at doi:101155/2012/260983). This suggests that the inflammatory immune cells infiltrating the large intestine is not the sole cause of the very severe IBD observed in CD73$^{-/-}$ mice.

Recent studies have indicated that CD73 and CD39 are important for immune suppression by T regulatory cells [20]. We therefore asked whether CD4$^+$ T regulatory (T_{reg}) cells from CD73$^{-/-}$ mice were defective in their ability to suppress IBD. In naïve WT mice, fifty percent of CD4$^+$ T cells express CD73 (Figure 3(d)) and all CD4$^+$CD25$^+$ cells express both CD73 and CD39 (Figure 3(d)). CD4$^+$CD45RB^{high} and CD4$^+$CD25$^+$ (CD45RB^{lo}) cells from spleens of naïve WT mice were cotransferred into *rag*$^{-/-}$ recipients. Similarly, naïve CD4$^+$CD45RB^{high} and CD4$^+$CD25$^+$ (CD45RB^{lo}) cells from CD73$^{-/-}$ mice were cotransferred into *rag*$^{-/-}$ recipients. *Rag*$^{-/-}$ mice that received CD73$^{-/-}$ CD4$^+$CD45RB^{high} and CD4$^+$CD25$^+$ cells developed no clinical signs of IBD and showed a similar weight-gain pattern to *rag*$^{-/-}$ mice receiving WT CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{lo}CD25$^+$ cells (that also showed no signs of IBD) (Figure 3(e)). Further, similar to WT mice, CD73$^{-/-}$ mice harbor similar frequency of CD4$^+$ FoxP3$^+$ T cells in colonic patches (lymphoid follicles) and mesenteric lymph nodes compared to those of WT mice (Figure 3(f)) (also in peripheral lymphoid organs [29]). These results indicate that neither the numbers nor the function of CD4$^+$Treg cells from CD73$^{-/-}$ mice is significantly altered and thus is not the only contributing factor in the severe IBD in CD73$^{-/-}$ mice. From these studies we conclude that a lack of CD73 expression on CD4$^+$$T_{reg}$ cells did not majorly hamper their ability to suppress IBD.

3.3. CD73$^{-/-}$ Mice Exhibit Ongoing Inflammation and Slower Recovery Than WT Mice. Under normal conditions, WT mice can recover from colitis once DSS treatment is removed [24]. To determine whether CD73$^{-/-}$ mice can recover from DSS-induced colitis we replaced DSS with drinking water on day eight in both WT and CD73$^{-/-}$ mice and monitored their body weight as an indication of disease alteration. CD73$^{-/-}$ mice recovered at a slow rate following the removal of DSS from their drinking water, regaining their initial body weight 22 days after DSS removal (Figure 4(a)). This was in sharp contrast to WT mice that regained their initial body weight seven days after the removal of DSS and gained an additional 20% of their initial body weight by 22 days after T DSS removal (Figure 4(a)). Examination of colon pathology of WT mice after day 22 showed full recovery: colonic architecture showed little to no cell infiltration, no tissue damage, and normal looking epithelial crypts and goblet cells (Figures 4(b) and 4(c)). While CD73$^{-/-}$ mice (day 22) exhibited significantly reduced colonic pathology compared to that observed during DSS treatment, they still show modest cell infiltration, some epithelial hyperplasia, and noticeable crypt destruction (Figures 4(b) and 4(c)), indicating prolonged or ongoing inflammation illustrated by the colonic inflammatory score (Figure 4(c)).

3.4. Increased IL-1β and TNF-α Production in Colonic Epithelium of CD73$^{-/-}$ Compared to WT Mice. While inflammatory immune cells lacking CD73 play an important role in IBD pathogenesis in CD73$^{-/-}$ mice (Figure 3(b)), it is apparent that they are not the only contributing factor that result in the very severe IBD. We next focused our attention on the colonic epithelium that is comprised of a single layer of intestinal epithelial cells that forms a physical as well as

a chemical barrier between the host and the microbial community [30]. FACS analysis of colonic epithelial cells from naïve WT mice, double stained with an epithelial marker plus CD73, showed that about 40% of epithelial cells expressed CD73 (including intraepithelial lymphocytes (iELs) which make up about 10% of $CD73^+$ cells) (Figure 4(d)).

We next focused on the colon in $CD73^{-/-}$ mice. We reasoned that the lack of CD73 expression on the colonic epithelium must contribute significantly to the severe IBD, since the immune cells lacking CD73 cannot recapitulate the severe colitis in $CD73^{-/-}$ mice. The intestinal epithelium closely interacts with underlying mucosal immune cells to control the immune response [2, 31]. The intestinal epithelium and its products determine whether the underlying immune status is suppressive or inflammatory. Damage or activation of the epithelial barrier results in production of inflammatory mediators by immune cells and epithelial cells [8]. We hypothesized that the lack of CD73 expression on colonic epithelial cells might contribute to the heightened proinflammatory conditions in IBD in $CD73^{-/-}$ mice. We isolated colons of WT and $CD73^{-/-}$ mice over the course of DSS treatment, including the period after DSS removal, cultured them in media overnight, and analyzed supernatants for cytokines by ELISA. We focused specifically on IL-1β and TNF-α, as these cytokines are implicated in colitis both in mice and humans [32]. Further, extracellular adenosine is a potent regulator of proinflammatory cytokines including IL-1β and TNF-α [14]. Colons from $CD73^{-/-}$ mice produced dramatically higher levels of IL-1β (more than 10-fold) and with slight increase in TNF-α on day 3 of DSS treatment compared to WT (Figure 4(e)). By day 8 of DSS treatment, $CD73^{-/-}$ mice consistently produced significantly higher levels of these cytokines compared to WT mice (Figure 4(f)). Furthermore, supernatants from colons isolated after DSS removal (day 22) show significantly higher levels of IL-1β (similar to day 8 of DSS, but not TNF-α) observed in $CD73^{-/-}$ mice (Figure 4(g)). IL-1β and TNF-α are potent proinflammatory factors that stimulate proliferation, apoptosis, phagocytosis, and transmigration of cells across epithelial and endothelial barriers as well as activate innate receptors on innate immune and epithelial cells [33]. The increased production of IL-1β and TNF-α is tightly correlated with IBD severity and tissue destruction in animals and human patients [1] and is consistent with the severe colitis and apparent lack of resolution of the inflammatory process in $CD73^{-/-}$ mice.

3.5. Increased Activation of NF-κB in $CD73^{-/-}$ Mice Compared to WT Mice. IL-1β induces the phosphorylation of IκB resulting in its degradation and the activation of NF-κB [34]. The constitutive activation of NF-κB is associated with aberrant expression of proinflammatory genes induced by cytokines such as IL-1β and is linked to the pathogenesis of IBD in both human and mice [35]. Adenosine is a potent regulator of IL-1β, and IL-1β can increase the expression of the A_{2A} adenosine receptor [36]. Further, adenosine is a negative regulator of NF-κB activation in human intestinal epithelial cells [36]. Thus, we hypothesize that constitutive production of IL-1β in $CD73^{-/-}$ mouse colonic epithelia (including intraepithelial lymphocytes) even after DSS removal may cause constitutive activation of NF-κB. To investigate this, we isolated colonic epithelial cells from WT and $CD73^{-/-}$ mice over the course of IBD and tested them for presence of NF-κB activity. Figure S2 shows that, at various disease intervals, NF-κB p65 protein is augmented in colonic epithelial cells in $CD73^{-/-}$ compared to WT mice. These findings suggest that the lack of extracellular adenosine may result in prolonged NF-κB activity after the inflammatory trigger (DSS) is removed. This inflammatory program, through constitutive production of IL-1β, can promote a proinflammatory feedback loop and a vicious inflammatory cycle.

3.6. Increased Intestinal Inflammation Is Associated with Increased Intestinal Permeability and Decreased Expression of Tight-Junction Associated Proteins in the Colons of $CD73^{-/-}$ Mice. In IBD patients and animal models of IBD, increased inflammation strongly correlates with an increase in intestinal permeability and vice versa [37, 38]. To determine if intestinal barrier permeability is altered, we examined the barrier integrity in $CD73^{-/-}$ and WT mice by treating mice rectally with FITC-Dextran and measuring its concentration in sera one hour later. As shown in Figure 5(a), $CD73^{-/-}$ mice exhibit significant increase in gastrointestinal permeability compared to WT. The protective barrier function of the intestinal epithelium is regulated by intercellular tight junctions that seal the space between epithelial cells [31]. Tight junction molecules are comprised of occludins, claudins, and JAM proteins [31]. Further, JAM-A/JAM-1 has been shown to be critical in the maintenance of epithelial barrier permeability, as $JAM\text{-}A^{-/-}$ mice have increased leukocyte infiltration and increased mucosal barrier permeability to macromolecules in the intestinal mucosa [39]. We analyzed mRNA transcript levels of several junction molecules that are associated with tight-junction stability to determine if the loss of these factors could be responsible for the destruction of the colonic architecture observed in DSS-treated $CD73^{-/-}$ mice. We observed a significant decrease in the mRNA levels of JAM-A, α-Catenin, and Claudin 2 in the colons of $CD73^{-/-}$ mice compared to WT mice (Figure 5(b)). Furthermore, immunofluorescence staining with anti-JAM-A antibody shows JAM-A protein expression is decreased at the borders of epithelial cells in naïve $CD73^{-/-}$ mice, while expression is localized to the plasma membrane in naïve WT mice (Figure 5(c)). These findings further demonstrate that extracellular adenosine is important in regulation and maintenance of intestinal barrier integrity.

3.7. TLR9 Is Upregulated in Colons of $CD73^{-/-}$ Mice during IBD. To further evaluate the status of the colonic epithelia and their ensuing inflammatory state, we next focused on Toll-like receptors (TLRs) [40]. We hypothesize that the ongoing inflammation in $CD73^{-/-}$ mice is due to a breakdown of the intestinal epithelial barrier that leads to leakage of luminal antigens into the lamina propria and activation of innate receptors. Intestinal epithelial cells express a spectrum of Toll-like receptors (TLRs), recognize and respond to microbial products (MAMP/PAMPS), and

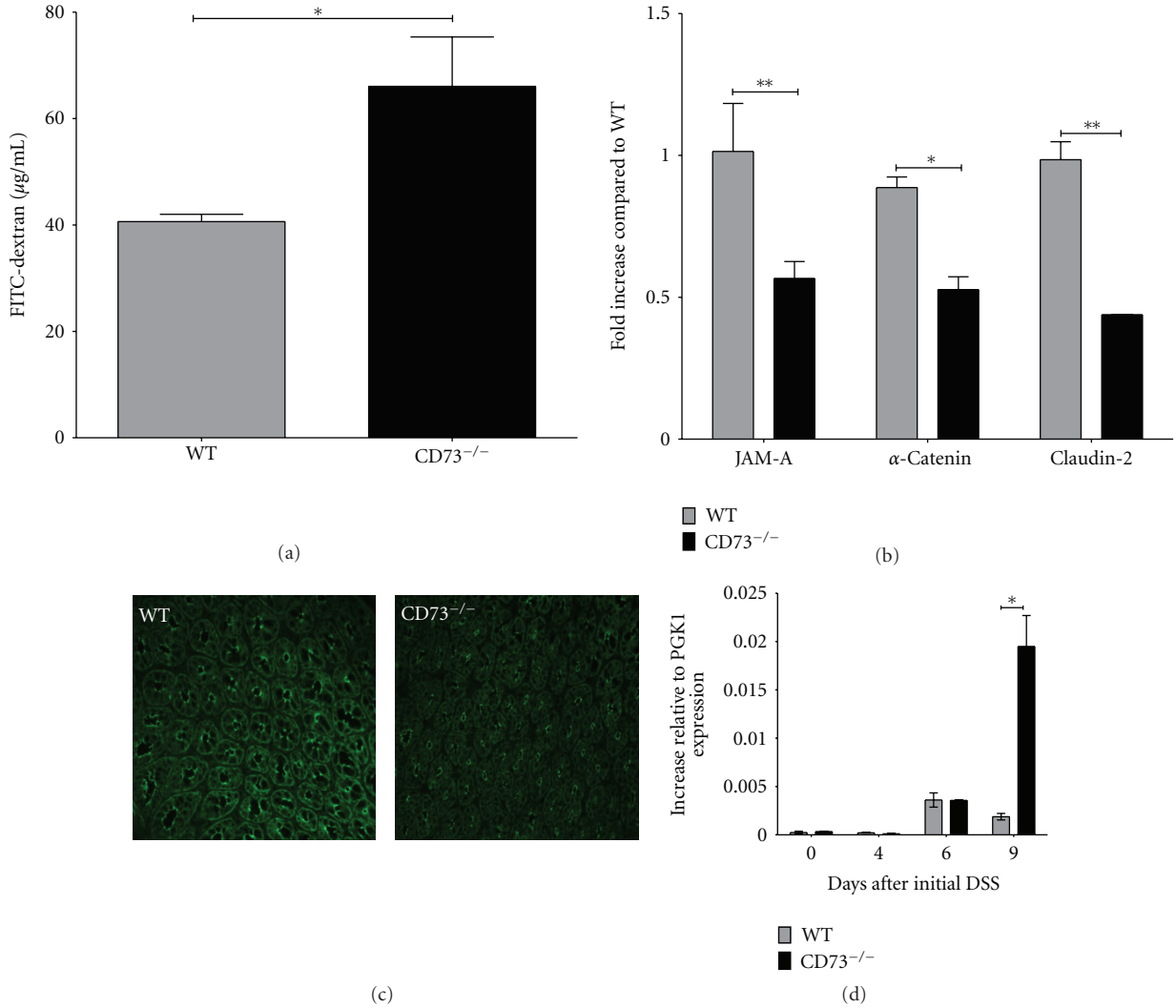

Figure 5: Upregulation of TLR9 in CD73$^{-/-}$ decreased expression of tight-junction associated proteins observed in the colons of CD73$^{-/-}$ mice compared to WT mice. (a) Colonic permeability was determined in WT and CD73$^{-/-}$ mice. Serum concentrations of FITC-Dextran (MW 10,000) in WT (gray bar) and CD73$^{-/-}$ (black bar) mice were obtained following rectal administration of 10 KDa FITC-Dextran (0.5 mg/Kg) and measuring FITC-Dextran in the blood 1 hour later. Results are expressed as mean ±SEM, $n = 5$, $^{*}P < 0.05$). Results are expressed as (mean ±SEM, $n > 3$, $^{*}P < 0.05$) relative fluorescence compared to WT. (b) Colons of WT (gray bars) and CD73$^{-/-}$ (black bars) were analyzed for mRNA expression of the adhesion molecules JAM-A, αCatenin, and Claudin-2. Results are expressed as (mean ±SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$) fold increase in CD73$^{-/-}$ compared to WT. (c) Fluorescent microscopy of colons from naïve WT (left panel) and CD73$^{-/-}$ (right panel) mice stained for JAM-A (green). (d) Colons were isolated from CD73$^{-/-}$ or WT mice over the course of IBD and analyzed for TLR9 mRNA expression by real-time PCR. Results are presented as TLR9 expression level relative to the housekeeping gene PGK1. (Representative of 3 experiments.)

can trigger inflammatory responses by mucosal immune cells [41]. To determine if the lack of CD73 expression on the colon results in increased expression of TLRs, we analyzed colons of IBD WT and CD73$^{-/-}$ mice by quantitative PCR analysis for mRNA expression. WT mice expressed background mRNA levels of a few TLRs, while other TLRs are not detected (Figure S3). In stark contrast, CD73$^{-/-}$ mice express more than 10-fold higher levels of TLR9 and just above background levels of TLR 3 and 5 (Figure S3). To determine the onset of TLR9 expression over the course of DSS treatment, we analyzed colons at different time points during IBD. We observed consistent increase in TLR9 expression in the latter stages of IBD (Figure 5(d)). These findings strongly support the idea of a more activated and proinflammatory state of the colonic epithelium in CD73$^{-/-}$ mice compared to WT mice.

4. Discussion

The goal of this study was to determine the role of extracellular adenosine in the regulation of intestinal inflammation. We demonstrate that mice lacking CD73 (and thus unable to adequately synthesize extracellular adenosine) exhibit severe

colitis induced by DSS treatment. This finding is consistent with previous studies that showed CD73$^{-/-}$ mice presented a more severe IBD induced by trinitrobenzene sulfonate (TNBS) administration than their WT counterparts [11]. In this study, CD73$^{-/-}$ mice were found deficient in INF-α, which may be important for regulating colonic inflammation. Here, we demonstrate that CD73$^{-/-}$ mice are unable to resolve inflammation even after the removal of DSS. Compared to WT mice, colonic epithelia from CD73$^{-/-}$ mice produced consistently high levels of IL-1β and TNF-α that correlate with increased NF-κB and TLR9 expression and resultant gastrointestinal permeability. In this study, we demonstrate that heightened inflammation, induced by aggressive inflammatory immune cells, activated inflammatory colonic epithelial cells and increased intestinal barrier permeability result in severe unresolved DSS-induced colitis in mice lacking CD73. Similar outcomes from previous studies demonstrate that CD73$^{-/-}$ mice presented more severe IBD, while these two studies use different approaches.

CD73$^{-/-}$ mice exhibit massive immune cell infiltration into the colon. The infiltrating cells are comprised primarily of CD4^{+} T cells and dendritic cells. These CD4^{+} T cells in CD73$^{-/-}$ mice produced high levels of the proinflammatory cytokines IL-1β and TNF-α, which are known to play a major role in the pathogenesis of IBD in humans and mice. However, adoptive transfer of naïve CD4^{+}CD45RB^{high} (inducer) T cells from CD73$^{-/-}$ mice into naive *rag*$^{-/-}$*cd73*$^{+/+}$ recipients shows that while these mice developed severe colitis, the extent of the tissue damage was not dramatically different when compared to recipients that received CD4^{+}CD45RB^{high} T cells from WT mice. These findings suggest that the infiltrating immune cells are not the only cause of the severe IBD in CD73$^{-/-}$ mice. Furthermore, cotransfer of regulatory T cells from either WT or CD73$^{-/-}$ mice prevented weight loss and intestinal pathology to an equal degree. Thus, CD4+Treg cells lacking CD73 are not defective in their ability to suppress IBD and therefore are not a major contributor to the severe IBD in CD73$^{-/-}$ mice. From these observations, we determined that, while the lack of CD73 expression on immune cells contributed to the pathogenesis of IBD, this was not the major cause of the extensive pathology and ongoing inflammation observed in CD73$^{-/-}$ mice with colitis. We propose therefore that lack of CD73 expression on the colonic epithelium itself plays a critical role in IBD in CD73$^{-/-}$ mice.

CD73$^{-/-}$ mice also exhibited a higher frequency of CD11c^{+} DCs infiltration in the colon. Mucosal DCs located in the epithelial dome of the colonic patches or in colonic lymphoid follicles are known to be involved in endocytosis and presentation of luminal antigens [42, 43]. The colonic epithelium is critical for maintenance of immune homeostasis in the colonic environment [44–46]. It does this in part by keeping mucosal DCs in a quiescent state [44–46]. The location and increase in these DCs suggest that they may be involved in presentation of luminal antigens and propagation of colonic inflammatory immune responses, driven by the activated inflammatory colonic epithelial cells in CD73$^{-/-}$ mice. It is also possible that CD73 expression on the intestinal vasculature could play a role in regulating intestinal inflammation. Future studies would be able to address these questions.

The intestinal epithelium plays a critical role in maintaining the homeostasis of the intestinal environment [8]. It does this in multiple ways including regulation of TLRs, NF-κB, innate and adaptive immune responses, as well as by providing a physical barrier comprised of junction molecules that separate the luminal contents from the underlying lamina propria [8]. Whole colons from DSS-treated CD73$^{-/-}$ mice produce high levels of TNF-α and IL-1β over the course of DSS treatment. IL-1β was detectable in colonic epithelial preparation in non-DSS-treated CD73$^{-/-}$ mice and remains elevated even after DSS treatment is aborted. Both IL-1β and TNF-α are well-known contributors to the pathogenesis of colitis in mice and humans [32]. Consistent with this ongoing inflammatory program, constitutively high levels of the transcription factor NF-κB, which is a critical mediator in inflammatory and remodeling responses, are also observed. Both IL-1β and TNF-α are potent activators of NF-κB. NF-κB is a key player in the cycle of inflammation in CD and is found constitutively active in colonic epithelial cells in IBD [47]. Adenosine is a negative regulator of NF-κB in human intestinal epithelial cells [36, 47]. The increase in IL-1β levels during and after DSS treatment correlates with active NF-κB protein and ongoing inflammation and pathology in colons of CD73$^{-/-}$ mice. Although IL-1β is observed in non-DSS-treated CD73$^{-/-}$ mice, albeit lower than is seen in DSS treatment, these mice do not display any signs of colon pathology. Therefore, we hypothesize that an inflammatory trigger (DSS) in the absence of extracellular adenosine in CD73$^{-/-}$ mice results in elevated IL-1β that is unresolved even after the trigger is removed. This potentially can contribute to the severe and unresolved inflammation in CD73$^{-/-}$ but not in WT mice.

Intestinal epithelial cells express a spectrum of Toll-like receptors (TLRs), recognize and respond to microbial products (MAMP/PAMPS), and can trigger inflammatory responses by mucosal immune cells. Studies have shown that exposure of the apical side of a polarized epithelial barrier to the TLR9 ligand, CpG DNA, results in a decreased ability to produce inflammatory cytokines, such as IL-6 and IL-8 [41, 48]. However, basolateral stimulation of the epithelial barrier with the same agent resulted in a potent proinflammatory response [48]. Colons of CD73$^{-/-}$ mice with colitis exhibited increased TLR9 expression that is consistent with the heightened and unresolved inflammation in CD73$^{-/-}$ mice but not in WT mice with colitis. Increased TLR9 expression, together with the increase in proinflammatory cytokines such as TNF-α and constitutive NF-κB activity, is indicative of an activated intestinal epithelium. We hypothesize that the ongoing inflammation in CD73$^{-/-}$ mice could potentially be due to a breakdown of the intestinal epithelial barrier that leads to leakage of luminal antigens (PAMPS/MAMPS) into the lamina propria and activation of innate receptors like TLR9. Altered TLR expression is commonly associated with chronic inflammatory diseases including IBD. An inflammatory reaction is only required when an antigen is able to bypass

the protective epithelial barrier. Consistent with the idea of a severely compromised barrier, $CD73^{-/-}$ mice express significantly lower levels of JAM-A protein and decreased levels of JAM-4 and α-catenin transcripts. The increased gastrointestinal permeability in $CD73^{-/-}$ mice would allow easy passage of commensal bacteria or their ligands across the epithelial barrier, resulting in stimulation of basolateral TLRs and production of proinflammatory cytokines. These findings strongly indicate that the lack of CD73 expression on colonic epithelial cells plays a critical role in the ongoing inflammation in $CD73^{-/-}$ mice. This leads us to conclude that extracellular adenosine function in the colon is critical for regulation of colonic immune responses and for the resolution of the inflammatory program and establishment of intestinal homeostasis.

In summary, our data show that the lack of CD73 expression on effector immune cells and on colonic epithelium causes severe IBD in $CD73^{-/-}$ mice. While effector immune cells in $CD73^{-/-}$ mice contribute to IBD pathogenesis, it appears that the greater degree of inflammation and colonic tissue damage is caused by a lack of CD73 expression on the colonic epithelium. This results in breakdown of the epithelial barrier, possibly leading to infiltration of luminal antigens and subsequent presentation of microbial products and activation of immune responses by innate and adaptive immune cells. This perpetuates a vicious cycle of unresolved inflammation due to the lack of CD73-generated adenosine. We conclude therefore that CD73 expression on colonic immune cells is important for immune regulation whereas CD73 expression on colonic tissue is critical for regulating the magnitude and the resolution of the mucosal immune response. These studies provide deeper insights into adenosine's role in mucosal immunity and demonstrate the potential for future development of adenosinergic therapy in treatment of colitis.

Authors' Contribution

A. T. Waickman and D. A. Mohamed contributed equally to this work.

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