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ORIGINAL ARTICLE

Variable Impact of CD39 in Experimental Murine Colitis

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Abstract
Background Dysregulation of immune responses in inflammatory bowel diseases (IBD) results in intestinal inflammation and vascular injury while exacerbating systemic disease. CD39 is an ectonucleotidase, expressed by T regulatory cells and dendritic cells, that hydrolyzes extracellular nucleotides to modify those cellular immune responses implicated in IBD. Genetic polymorphisms of CD39 have been linked to Crohn's disease while gene deletion in mice exacerbates dextran sodium sulphate-induced colitis.
Aim The aim of this study was to test how global deletion of CD39 in mice impacts other models of experimental colitis.
Methods Colitis was induced in CD39-null and -wt mice, using trinitrobenzene sulfonic acid (TNBS, 125 mg/kg) administered intrarectally. Oxazolone colitis (1.5% oxazolone in 50% alcohol) was induced in comparable groups. Morphology, clinical and molecular parameters, and FACS analyses of lamina propria mononuclear cells (LPMC) were examined in CD39-null mice. CD39 expression was analyzed in human IBD biopsies.
Results Paradoxically, TNBS colitis in CD39-null mice was characterized by improved survival, favorable clinical scores, and decreased MPO activity, when compared to wt mice ($P < 0.05$). LPMC from TNBS colitis contained significantly increased amounts of T-cells ($CD3^+$ and $CD4^+$) and TNF-α mRNA expression were increased over those in CD39 null mice ($P < 0.05$). In contrast, oxazolone treated CD39-null and wt mice had comparable outcomes. In both ulcerative colitis and Crohn's disease, CD39 is present at high levels in intestinal tissue biopsies.
Conclusions TNBS colitis was attenuated in CD39-null mice whereas oxazolone-induced colitis was not impacted. Impaired adaptive cellular immune reactivity in the CD39-null environment appears protective in hapten-mediated Th1-type colitis. CD39 is expressed at high levels in clinical IBD tissues.

Keywords CD39 · NTPDase1 · P2-receptors · Inflammatory bowel disease (IBD) · Ulcerative colitis · Crohn's disease · Purinergic signaling · Murine colitis

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Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of inflammatory bowel disease (IBD), which are thought to develop as a consequence of

dysregulated immune responses and/or possible alterations in mucosal barrier function. CD is a granulomatous disease that may affect any portion of the gastrointestinal tract, whereas UC is an inflammatory disease limited to the colon. CD and UC are distinguished by distinct clinical appearances, by endoscopic investigation and by unique and distinctive histopathological features. Although the etiology of IBD remains largely unknown, these diseases are believed to be caused by abnormal mucosal immune cellular responses to specific antigens, including bacterial antigens contained in the bowel lumen [1]. Various genetic and environmental factors are also thought to influence both the initiation and progression of colitis [2, 3].

Inflammation causes cytokine-mediated activation and oxidant damage to cells [4] with consequent accumulation of extracellular nucleotides. Extracellular nucleotides regulate cell-cell communications via purinergic/pyrimidinergic P2-receptors (P2-R) [5, 6]. ATP, UDP, and others, acting at P2X7R and presumably also P2YR, trigger IL-1 and TNF-α release from activated macrophages and endothelium while promoting vascular remodelling [7–9].

In addition, commensal bacteria may be a major source of intestinal luminal ATP [10]. ATP that is derived from commensal bacteria is believed to activate $CD70^{high}CD11c^{low}$ cells in the lamina propria and generate IL-6, IL-23, and TGF-β [10]. Together this promotes T cell mediated colitis through enhanced Th17 differentiation.

The extracellular concentration of nucleotides is tightly regulated by ectoenzymes such as nucleoside triphosphate diphosphohydrolase-1 (CD39/NTPDase-1) [11]. CD39 is the dominant vascular and immune ectonucleotidase and hydrolyzes both pericellular ATP and ADP to AMP, which in turn is hydrolysed by CD73 to adenosine [11]. CD39 and other NTPDases interact functionally with multiple P2Y receptors [11] and have the capacity to terminate P2 receptor (P2-R) signaling, modulate receptor desensitization, alter specificities of the response or even generate signaling molecules (ADP) from precursors (ATP) [12].

CD39 was first described as an important vascular thromboregulatory factor inhibiting platelet aggregation [13], thrombosis [14], and altering leukocyte-endothelial interactions [15]. IBD is associated with vascular thrombosis and endarteritis [16]. Anemia and thrombocytosis are common secondary changes in inflammatory bowel disease (IBD). On the other hand, increased platelet function, fibrinolytic abnormalities, and hypercoagulation of patients with IBD predispose to thromboembolic events, and they may also contribute to the local microcirculatory alterations leading to IBD itself. In fact, dysregulation in purinergic signaling is associated with thromboembolic and microcirculatory abnormalities.

CD39 is also now known to play a significant regulatory role in immunity. The outcomes of CD39 deletion in irritant versus allergic contact dermatitis are quite different, reflecting differential effects on the regulation of inflammatory responses to environmental insults and upon dendritic cell with T cell communication in antigen presentation [17, 18]. Regulatory T cells (Treg) express CD39 and gene deletion is linked to impaired immune suppressive effects and autoimmune disease [19]. Moreover, CD39-null mice can be shown to display a predominant Th1 and Th17 phenotype with distinct upregulation of cellular expression of IFN-γ and IL-17 [19, 20].

IBD is a T cell mediated disorder with clinical features inclusive of vascular thrombosis and endarteritis [16]. Here we show that the deletion of CD39 differentially impacts two experimental murine models of colitis. Moreover, CD39 expression is altered in human colonic biopsies from patients with IBD.

Materials and Methods

Patients

Full thickness colonic tissue samples were obtained from patients undergoing surgery for refractory CD ($n = 65$) and colectomy for UC ($n = 49$). As controls, normal colonic biopsies from patients with benign and non-inflammatory colonic disease ($n = 46$) as well as nine colonic specimen from patients with acute sigmadiverticulitis ($n = 9$), that underwent sigma resection, were included in our studies. All patients were operated at the University Hospital in Heidelberg, Germany.

Studies of biopsies for CD39 expression were approved by the local Ethic Committee in Heidelberg, Germany. Written informed consent was obtained from all patients before the interventional procedure for this research investigation.

Animals

Pathogen-free C57BL6 CD39-null and matched wild type (wt) mice were studied in accordance with standard institutional animal welfare guidelines. The derivation and characterization of CD39-null mice has been described elsewhere [11]. Genotyping of the CD39 alleles was performed as previously described [11].

Animal care and experiments were carried out under the guidelines and protocols approved by the Animal Care and Use Committee, Beth Israel Deaconess Medical Center, Harvard University, Boston, USA.

Induction of Experimental Colitis

TNBS Colitis

Colitis was induced as previously described [21]. Briefly, CD39-null ($n = 11$) and wt mice ($n = 11$), 10–14 weeks of age, were anesthetized using Nembutal (50 mg/kg body weight) dissolved in saline 0.9%; then a 3.5 French polyurethane umbilical catheter (Utah Medical Products Inc., Midvale, UT, USA) attached on a 1-ml insulin syringe was inserted rectally and the tip was advanced to 4 cm. Trinitrobenzene sulfonic acid (TNBS) (125 mg/kg body weight TNBS in 100 µl 50% alcohol) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved and instilled into the colon through the catheter. After the instillation, mice were held vertically for 45 s. Control mice (CD39-null, $n = 4$; wt, $n = 5$) received 150 µl of 50% alcohol alone. All mice were sacrificed at day 4 after administration of TNBS.

Oxazolone Colitis

Oxazolone colitis was induced in CD39-null ($n = 13$) and wt mice ($n = 19$) as previously described [22]. 1.5% Oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 50% alcohol was injected per rectum (150 µl in a 50% alcohol mixture to 4 cm proximal the anal verge) via a 3.5 French polyurethane umbilical catheter (Utah Medical Products Inc.) attached on a 1-ml syringe. Mice were held in a vertical position for 45 s after injection. Control mice received an equal amount of 150 µl of 50% alcohol alone. All mice were sacrificed at day 4 after treatment.

During the course of TNBS and Oxazolone colitis, mice were weighed and examined daily for signs of diarrhoea and other distress symptoms (lethargy, periorbital exudates and piloerection).

Histological Assessment of Colitis

Mouse and human colonic specimens were snap-frozen [23] and stored at −80°C. Tissue sections of 4–5µm were stained with hematoxylin and eosin (H&E). Stained sections were examined for evidence of colitis as previously published [22], using as criteria the presence of lymphocytic infiltration, macrophages or polymorphonuclear cells, elongation and/or distortion of crypts, crypt abscesses, reduction in goblet cell number, ulceration, and edema formation of the colon wall.

Disease Monitoring and Scoring

Colons were removed and scored at day 4 after TNBS and oxazolone colitis, as previously described [24]. In brief, four criteria were scored: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild colon thickening; 2, moderate thickening; 3, extensive thickening); stool consistency, 0–3 (0, normal stool; 1, soft stool; 2, diarrhea, 3, bloody diarrhea) and ulcer formation, 0–3 (0, no ulcer; 1, mucosal ulcer; 2, bloody ulceration; 3, necrotic transmural ulcer). Tissue samples from the distal part of the colon were prepared and stained with H&E for histology by using standard techniques [24].

Colonic Myeloperoxidase (MPO) Activity

Colon samples were obtained from control and TNBS treated animals, and prepared as previously described [25]. The samples were thawed for MPO activity assay determination according to the *o*-dianisidine method as previously described [25]. In brief, tissue samples were thawed, weighed, suspended in 50 mM potassium phosphate buffer (Kp_i), pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide buffer (0.1 g/20 ml Kp_i), and homogenized. After sonication the samples were microcentrifuged. The reaction was started by mixing and incubating the supernatant in 50 mM Kp_i20 mg/ml *o*-dianisidine dihydrochloride and 20 mM hydrogen peroxide. The reaction was stopped by adding 2% sodium azide. The change of the absorbance was monitored at 460 nm for 10 min. MPO activity was expressed as the amount of enzyme necessary to produce a change in absorbance of 1.0 per minute per gram wet weight of colonic tissues.

Reverse-Transcription Polymerase Chain Reaction

Extracted mRNA from mouse colon was DNase treated and reverse transcribed using the SuperScript kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations using random hexamer primers for IFN-γ, IL-4, IL-10, IL-13, TNF-α and human CD39 (human biopsies) (Applied-Biosystems, Foster City, CA, USA). Quantitative PCR (Q-PCR) was performed using the 7700 Sequence Detector (Applied-Biosystems) and TaqMan technology as previously described [23]. Data were analyzed using the relative standard curve method, and expression levels were normalized to the expression of 18S ribosomal subunit as internal controls.

Serum Cytokines (IFN-γ and TNF-α)

Levels of IFN-γ in serum, isolated from wt and CD39-null mice at day 4 after induction of TNBS colitis, were determined using the MIF00-ELISA-kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol.

Cytokine concentrations of TNF-α were determined by commercially available specific ELISA assays, following manufacturer's recommendations (eBioscience, San Diego, CA, USA).

Western Blots

Cell lysates were prepared and (SDS)-polyacrylamide gel electrophoresis techniques employed to separate proteins exactly as previously described [23]. The membranes were probed with CD39 (C9F), CD39L1 (BZ3-4F), P2X7 (APR-0004) or P2Y2 (APR-010) (Alomone Labs Ltd., Jerusalem, Israel), or for control anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Amblion Inc., Woodward, TX, USA) primary antibodies. Appropriate secondary antibodies were used for detection, as previously described [23]. Equal gel loading was confirmed by analyses with GAPDH antibody.

To further validate P2 receptor expression (P2X7, P2Y2), primary antibodies against P2X7 and P2Y2 were blocked with the specific antigen, provided by the manufacturer (Alomone labs). Internal controls show complete negative blots (not shown) that signified signals detected were specific.

Immunohistochemistry

Immunohistochemistry for CD39 (C9F), CD4 (BD Pharmingen, San Diego, CA, USA) and F4/80 (Serotec, Oxford, UK) was performed exactly as previously described [23].

Isolation and Purification of Lamina Propria Mononuclear Cells (LPMC)

Lamina Propria Mononuclear Cells LPMC were isolated from freshly obtained colon samples as previously described by Boirivant et al., with some modifications [22]. Colonic specimens were dissected and digested in RPMI1640 containing collagenase type II (200 U/ml) (Invitrogen), dispase (1.04 U/ml) (Invitrogen) and DNase I (0.01 mg/ml) (Roche Diagnostics, Basel, Switzerland). After filtration, the remaining tissue debris and LPMC were resuspended in 30% Percoll-RPMI1640 mixture and layered on top of a 70% Percoll-RPMI 1640 gradient. After centrifugation the LPMC population accumulated at the 30–70% interface. This resultant cell population was further analyzed by flow cytometry.

Flow Cytometry

LPMC were washed with PBS, dissolved in MACS buffer (0.2% bovine serum albumin in 1x PBS) and then stained with various fluorochromes using standard methods provided by the manufacturer (eBioscience, San Diego, CA, USA and BD Pharmingen, San Jose, CA, USA). Antibodies used for surface-staining FITC-labelled anti-CD3, -CD4, were used (eBioscience). The anti-CD39 primary antibody (C9F) was labelled with a secondary anti-rabbit antibody (eBiocience) in a concentration of 0.04 μg/μl. Cells were incubated with FITC-labelled antibodies for 15 min at 4°C then washed for single staining. Surface staining was analysed by flow cytometry using a FACScan® (Becton Dickinson, Franklin Lakes, NJ, USA). Three independent experiments were performed and flow cytometric data were analysed using WinMDI2.8 (freeware) software. Each data point consisted of four mice treated with TNBS, Oxazolone or only 50% alcohol as internal control population.

Statistical Analysis

Data in this study are presented as means ± standard deviation (SD) of values (obtained from at least four mice per group and/or at least three independent in vitro experiments). All histology, Western blots and immunohistochemical images are representative of at least four mice per group. Statistical analysis of data was performed using the GraphPad PRISM4 software (GraphPad Software Inc., San Diego, CA, USA). Mean values of the experimental groups were compared using nonparametric testing (Mann-Whitney U test) for pair wise comparison and 1-way or 2-way ANOVA analysis for multiple comparisons. Values of $P < 0.05$ are considered statistically significant.

Results

CD39 Null Mice Are Protected from TNBS Colitis

CD39-null mice treated with TNBS exhibited lower pathological scores (Fig. 1a) ($P < 0.003$) and significantly lower myeloperoxidase (MPO) activity (Fig. 1b) ($P < 0.01$) than wild-type mice. Moreover, intestinal morphology was relatively well preserved in CD39-null mice, with transmural swelling evident. Leukocytic infiltration and ulcerations were only evident in wild type mice (Fig. 1d). Control mice treated with the vehicle only (50% alcohol), showed minor pathological changes (Fig. 1a, c).

At the initiation of treatment, the body weight of wild type and CD39-null mice did not differ (wt 25.6 ± 5.4 g vs. CD39-null 23.5 ± 5.7 g). Wild type mice lost 23% of their body weight over the four-day study compared to CD39 null mice, which lost 8% ($P < 0.004$) (Fig. 2a). Moreover, the mortality in wild type mice was significantly increased ($P < 0.05$) (Fig. 2c).

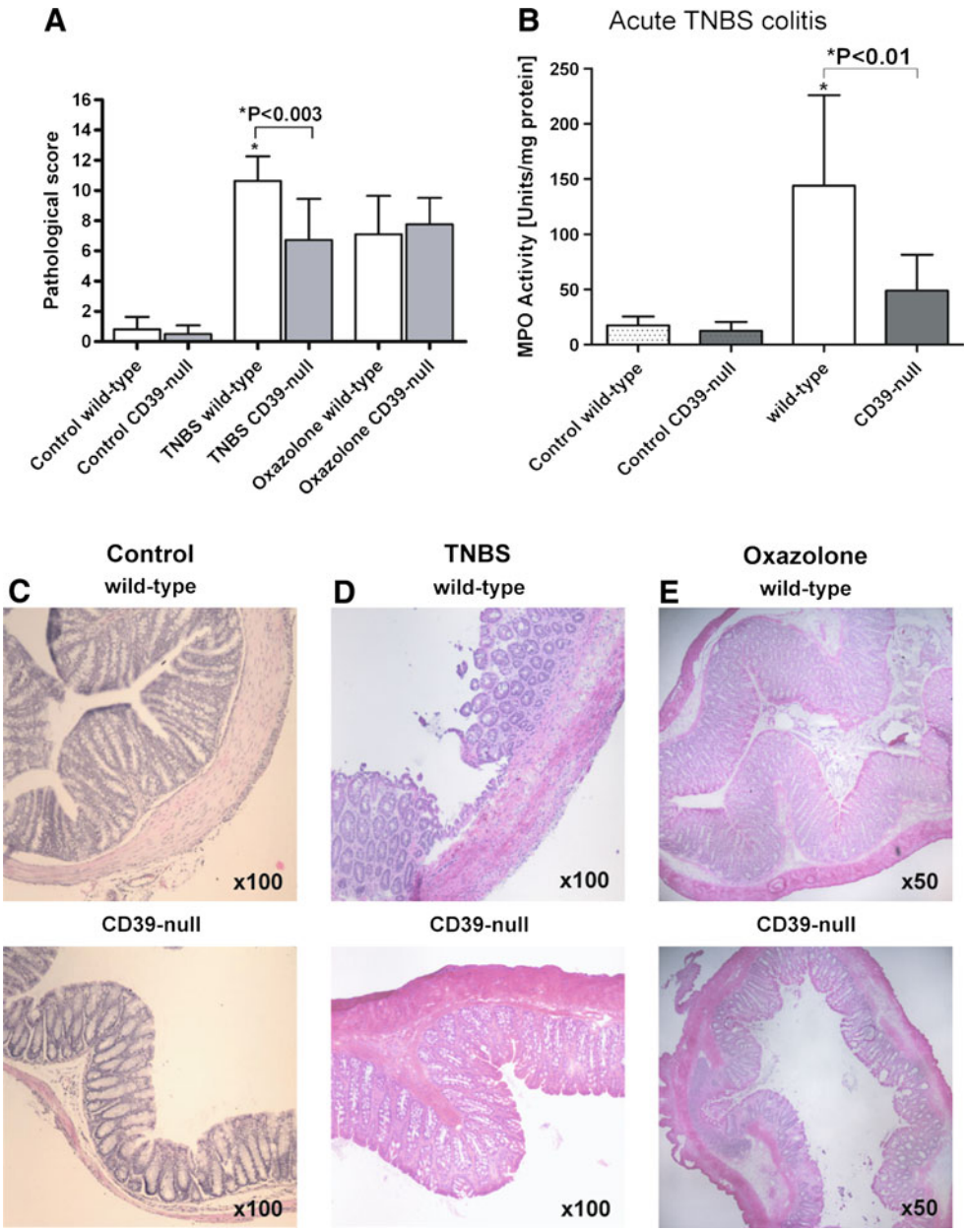

Fig. 1 CD39-null mice are protected in TNBS but not oxazolone colitis. Trinitrobenzene sulfonic acid (TNBS) (125 mg/kg) and 1.5% oxazolone were intrarectally introduced to cause colitis. **a** Pathological scores are significantly lower in TBNS colitis of CD39-null mice and indicate protection of CD39-null mice ($P < 0.003$). No significant pathological changes were observed when comparing wild-type and CD39-null mice. **b** Myeloperoxidase (MPO) activity was expressed as the amount of enzyme necessary to produce a change in absorbance of 1.0 per minute per gram wet weight of colonic tissues. According to pathological scores, wild-type mice are more severely affected in TNBS colitis ($P < 0.01$). **a**, **b**, **c** No differences in normal tissue morphology, pathological scores and MPO activity in wt and CD39-null control. **d** TNBS colitis is worse in wt mice compared to CD39-null, displayed by ulcerations and leucocyte infiltration of the colon wall, that was only seen in wt mice but not in CD39-null mice. **e** Oxazolone colitis revealed minor to moderate tissue changes in both wild-type and CD39-null mice based on (**a**) similar pathological scores

Oxazolone Colitis Is Not Affected by Deletion of CD39

Colitis was induced in both wild type and CD39-null mice following treatment with oxazolone. Pathological scores as well as the histomorphology of the induced colitis were comparable in wild type and CD39-null mice (Fig. 1a, e). Body weight was decreased by a comparable amount in both groups (Fig. 2b) and mortality was equivalent at about 50% in both groups at 4 days (Fig. 2d).

Serum Cytokine Levels Are Dysregulated in CD39 Null Mice

Levels of mRNA expression of inflammatory cytokines revealed a significant difference in TNF-α mRNA expression in TNBS treated wt mice compared to CD39-null mice ($P < 0.04$) (Table 1). IL-4, IL-10 and IL-13 were not significantly dysregulated in any kind of colitis, when contrasting wt and CD39-null mice (Table 1).

Systemic levels of IFN-γ were only detectable at low levels in wt and CD39-null mice, whereas TNF-α levels were increased in sera of CD39-null mice (wt 5.7 ± 7.9 pg/ml vs. CD39-null 36.0 ± 50.2 pg/ml; $P < 0.03$) (not shown).

Lamina Propria Mononuclear T Cells (LPMC) in TNBS Colitis

We analysed next the proportion of $CD3^+$, $CD4^+$ T cells, regulatory T cells (Treg) and natural killer cells (NKT)

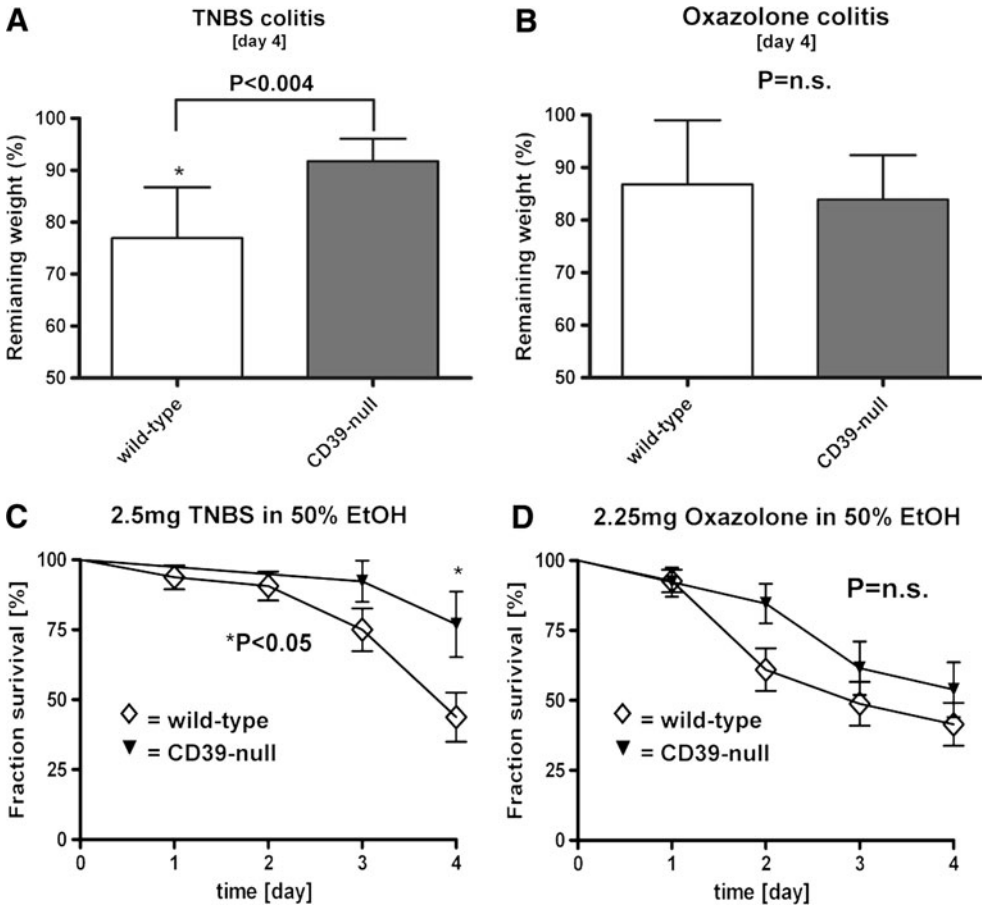

Fig. 2 TNBS and oxazolone colitis in wild-type and CD39-null mice. After induction of either TNBS or oxazolone colitis, mice were weighed and physically examined daily. Severely sick or distressed animals with signs of excessive weight loss, extreme lethargy and coarse respiratory movements were euthanized. Their status was included in the statistical analysis of fraction survival together with the animals that died unwitnessed throughout the experiment. **a** Body weights were significantly less in TNBS colitis of wt compared to CD39-null mice ($P < 0.004$). **b** Oxazolone colitis revealed no significant weight difference between wt and CD39-null mice. **c**, **d** The fraction survival of animals treated for TNBS and oxazolone colitis showed a significant drop in survival at day 4 for TNBS treated wt mice ($P < 0.05$). **d** No difference was noticed in fraction survival between wt and CD39-null mice treated with oxazolone at day 4

Table 1 Levels of mRNA Transcripts in TNBS and Oxazolone colitis

Tissue	mRNA	Control wild-type		Control CD39-null		TNBS wild-type		TNBS CD39-null		Oxa wild-type		Oxa CD29-null	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Colon	IFN-ag	0.48	0.24	1.03	0.42	2.88	4.40	1.62	2.36	0.74	0.92	5.26	8.09
	IL-4	0.157	0.076	0.505	0.318	0.732	0.486	0.460	0.197	0.400	0.450	0.464	0.721
	IL-10	3.96	1.44	8.42	1.44	8.44	4.33	12.97	6.61	4.25	2.93	6.38	4.50
	IL-13	1.49	1.33	1.15	0.59	1.41	0.95	10.44	9.21	0.84	0.91	7.27	11.30
	TNF-3a	93.4	27.49	118.1	46.39	481*	293*	241*	130	557	559	288	225

Mean values shown are standardized to 18 s mRNA levels

SD standard deviation, *TNBS* trinitrobenzene sulfonic acid, *Oxa* oxazolone

* $P < 0.04$

within the lamina propria. The populations of $CD3^+$ and $CD4^+$ T cells after induction of colitis (day 4) were significantly increased in wt compared to CD39-null LPMC ($CD3^+$: 75% vs. 59%; $P < 0.002$ and $CD4^+$: 28% vs. 15%; $P < 0.04$, respectively) (Fig. 3a, b). The proportions of T cells, especially regulatory T cells ($CD4^+$ and $CD25^+$) and natural killer T cells (NKT) ($CD3^+$ and $NK1.1^+$) were comparable in wild type and CD39-null mice (not shown). Staining for CD4 and F4/80 of colonic tissue sections of wt and CD39-null mice treated with TNBS revealed markedly increased CD4 and F4/80 cell accumulations in wt TNBS sections (Fig. 3c, d).

Expression of Ectonucleotidases and Purinergic Receptors in Murine Colitis

During the course of experimental colitis, CD39 levels increased in intestinal samples from TNBS colitis and were highly upregulated in oxazolone colitis at day 4 (Fig. 4). Whereas the related NTPDase CD39L1 is only moderately

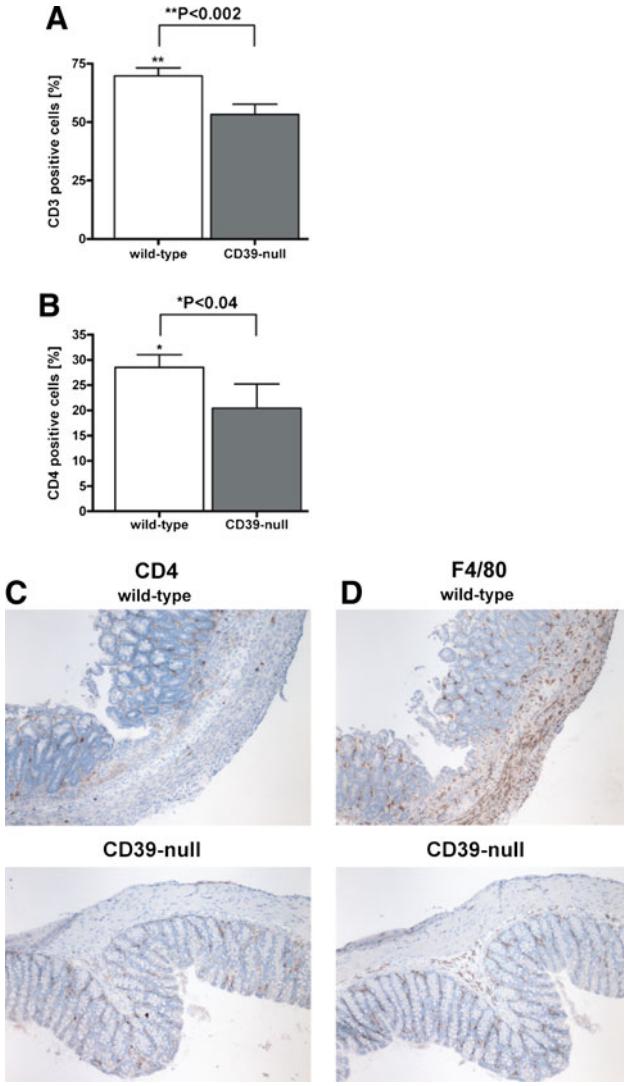

Fig. 3 Subpopulations of T cells (CD4- and F4/80 positive cells) are highly abundant in TNBS colitis of wild-type but not in CD39-null mice. Analyzing T cells in TNBS colitis of wt and CD39-null mice, we demonstrated (**a**, **b**) significant predominance of CD3 ($P < 0.002$) and CD4 ($P < 0.04$) positive immune cells in wt compared to CD39-null environment. Further analysis of regulatory T cells (Treg, $CD4^+$ and $CD25^+$) and natural killer cells (NKT, $CD3^+$ and $NK1.1^+$) did not indicate significant differences between wt and CD39-null immune cells (not shown). **c**, **d** In situ localization of $CD4^+$ and $F4/80^+$ positive cells revealed a more predominant and numerous accumulation of T cells and macrophages in wt than CD39-null mice. **d** Of note, F4/80 positive cells were predominantly present in the muscular layer of wt TNBS colitis but mainly in the sub mucosa and intervillous spaces in CD39-null colitis

expressed in TNBS colitis, this ectoenzyme is present at high levels in oxazolone colitis of CD39-null animals at day 4 (Fig. 4).

At the protein level, P2X7 was weakly expressed in normal colon, TNBS and oxazolone colitis and no significant difference in expression was found between wt and CD39-null mice. Interestingly the protein levels of P2Y2

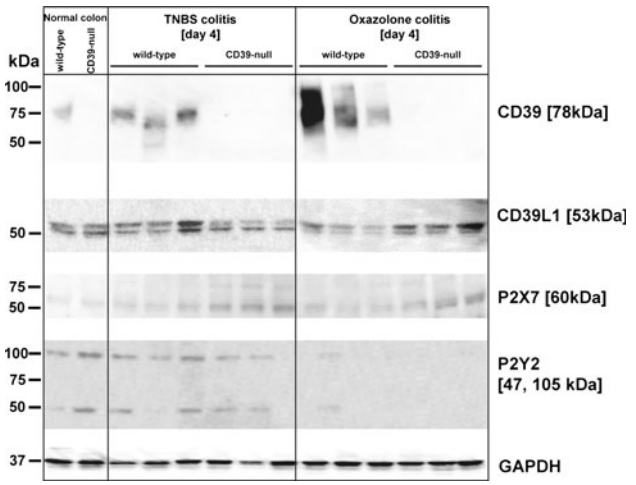

Fig. 4 CD39 and P2Y2 protein expression is dysregulated in murine colitis. Protein lysates of TNBS- and oxazolone colitis samples were prepared and (SDS)-polyacrylamide gel electrophoresis techniques (Western Blot) employed to separate proteins. Membranes were probed with specific antibodies for CD39, CD39L1, P2X7 and P2Y2. CD39 is upregulated in wild-type TNBS and oxazolone colitis. CD39-null mice show significant upregulation of CD39L1 in oxazolone colitis. P2X7 is only faintly expressed in normal colon and only minimal increased expression of P2X7 in TNBS and oxazolone colitis. P2Y2 expression was suppressed in wt and CD39-null mice in oxazolone colitis

were almost completely lost in oxazolone colitis, whereas P2Y2 expression in TNBS colitis was comparable to that in normal colon (Fig. 4).

CD39 Is Highly Upregulated in Human Biopsies of Crohn's Disease (CD) and Ulcerative Colitis (CU)

At the mRNA level, human CD39 was noted to be upregulated in colitis from CD and UC, when compared to normal colon (Fig. 5a). We investigated human surgical specimens obtained from highly inflamed colon but also regions where macroscopically the colitis was absent or only marginally detectable (inactive biopsies). Patients with inactive CD (416 ± 156 number of CD39 mRNA copies) expressed significantly more CD39 mRNA compared to inactive UC (291 ± 108 numbers of CD39 mRNA copies) ($P < 0.01$) (Fig. 5a).

Investigating CD and UC at sites of severe inflammation in specimens of patients with acute sigmoid diverticulitis, there was significant overexpression of CD39 in both diseases (albeit no significant difference amongst CD and UC samples).

In normal colon biopsies from controls, CD39 was expressed in endothelial cells between the colonic crypts and in mononuclear cells (Fig. 5b). In Crohn's disease, CD39 localized to endothelial cells (Fig. 5c, black arrowhead) but also to stromal cells and infiltrating immune cells

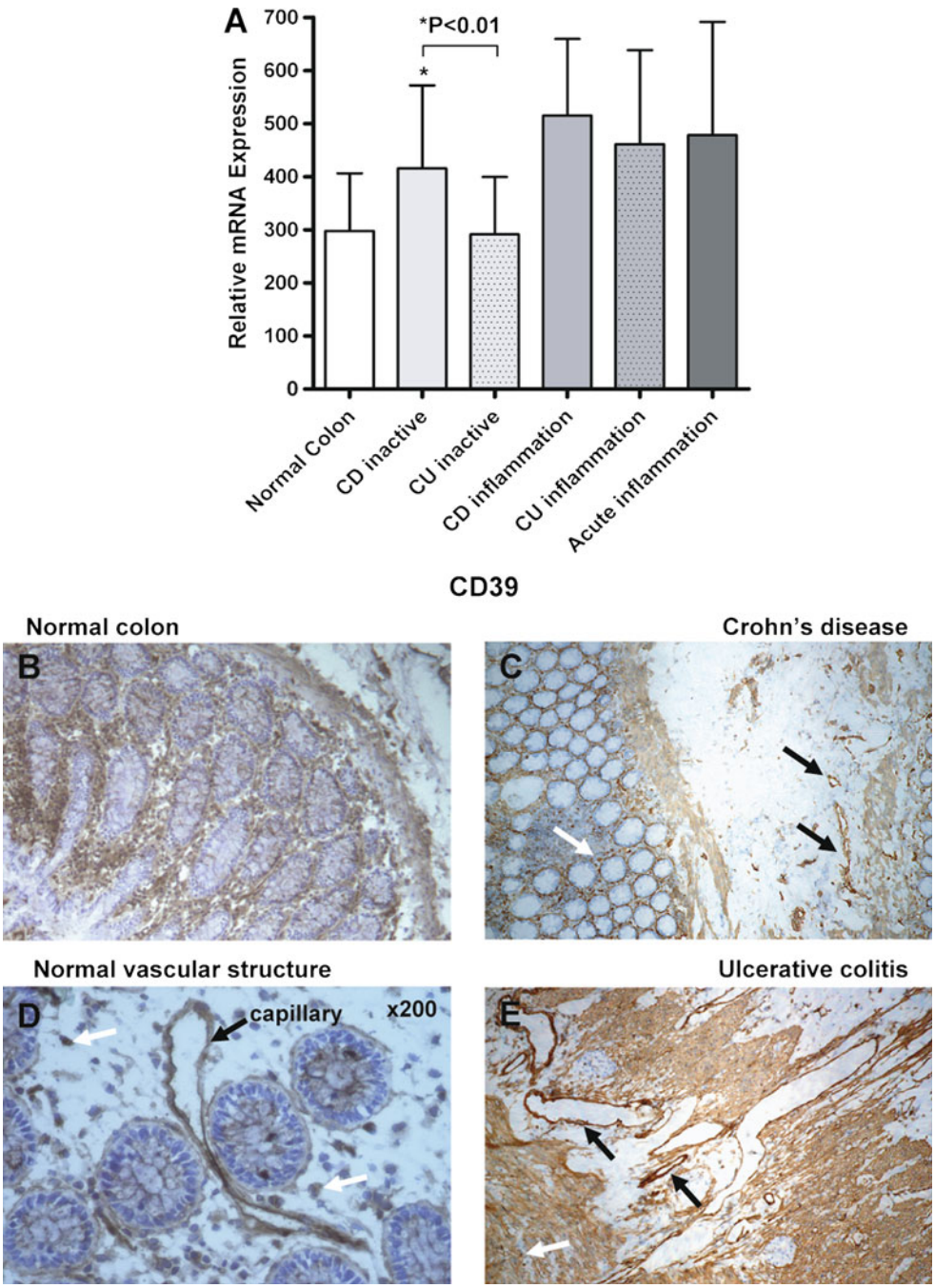

Fig. 5 CD39 is overexpressed in inactive human Crohn's disease (CD) and localized to capillaries, stromal- and mononuclear cells in normal and inflamed human colon. **a** Quantification of CD39 mRNA expression revealed significant increased levels of CD39 mRNA in inactive Crohn's disease (CD) compared to inactive ulcerative colitis (CU). Acute bowel inflammation, as well as acute inflammation of CD and CU, displayed comparable expression levels of CD39 mRNA in human IBD. Immunohistochemical analysis for CD39 revealed positivity for CD39 to stromal cells, smooth muscle cells, vascular endothelial cells, mononuclear cells and immune cells (**b**–**e**) for normal colon. In Crohn's disease and ulcerative colitis tissue architecture was vastly disrupted and CD39 was mainly localized to blood vessels, endothelium (*black arrowhead*) and connective tissue/stromal cells as well as mononuclear cells (*white arrowhead*)

(Fig. 5c, white arrowhead). In ulcerative colitis, CD39 is localized to vascular endothelium; the stromal cells/interstitial areas also stained for CD39 (Fig. 5e, white arrowhead). No CD39 was detected in control or diseased colonic epithelium.

Discussion

CD39 is a dominant vascular and immune ectonucleotidase [19, 20] localized to T regulatory and dendritic cells which mediates immune suppression and cellular responses through the generation of adenosine from extracellular nucleotides [17, 19]. The deletion of CD39 in C57BL/6 mice is associated with a Th1 bias [19, 20] as evidenced by excessive IFN-γ production. Recent studies have suggested an important role for purinergic signaling in inflammatory disorders [7, 19, 20, 26–28]. Our data here and recently published [29] suggest that CD39 may be also implicated in IBD.

CD39-null mice developed minimal disease and had more favorable outcomes after exposure to the hapten-Th1-mediated TNBS colitis model. This may be in keeping with the

dendritic cell phenotype that results in attenuated delayed type of hypersensitivity (DTH) and inflammation seen in skin following exposure to haptens in CD39-null mice [17].

Interestingly, oxazolone, a Th2 related form of colitis, caused moderate colitis in both wild type and CD39-null mice. The initial haptenic effect of TNBS does not occur in the oxazolone colitis, potentially explaining the comparable clinical manifestations in wild type and CD39-null mice in this alternative DTH model [30]. These findings illustrate the differential nature and responses of mice in these two models of colitis as modulated by the expression of CD39.

Important efforts have been made to more clearly dissect the immunological phenotypes and significance of murine models of colitis in order to relate these with clinical aspects of IBD [1, 22, 31]. The TNBS model exhibited clinicopathological findings that relate more to CD, whereas oxazolone colitis is more commonly associated with an essentially UC-like phenotype [22]. Our study indicates the importance and regulatory capacity of CD39 and the purinergic system in these different and distinct models of murine IBD. Extracellular nucleotides, especially ATP, are not only derived from dead epithelial cells but are also derived in high concentrations from commensal bacteria in the gut. ATP activates a subset of lamina propria cells, that are $CD70^{high}CD11c^{low}$, leading to a differentiation of Th17 cells [10]. The stimulation of these lamina propria cells with ATP, release Th17 related cytokines, such as IL-6, IL-23 and TGF-β. While initiating this cascade it is plausible that the purinergic system, with P2-receptors, might be involved in the regulation of the differentiation of Th17 cells.

Further studies show opposing outcomes of CD39 deficiency in irritant vs. allergic contact dermatitis, reflecting its diverse roles in regulating extracellular nucleotide-mediated signaling in inflammatory responses to environmental insults and dendritic cell and T cell communication in antigen presentation [17]. These types of acute immune responses are most probably only marginally affected by T cell interactions and therefore are exacerbated in CD39-null mice. The haptenic form of Th1 colitis (TNBS model) is dependent on a delayed type of immune response, where dendritic cells and T cells come into play and cell-interactions are thought to be a key factor in mediating disease. Dysfunctional dendritic cell interactions with T cells could inhibit the development of hapten-mediated type of colitis [17, 19].

In contrast, the oxazolone colitis model is a Th2 mediated form of colitis that caused comparable clinicopathological phenotypes in wild type and CD39-null mice. No distinct differences could be observed in terms of pathological score, weight loss and mortality. In contrast to some inflammatory models, CD39 dependent signaling did not significantly influence the course of this Th2 modulated state. This difference could be explained by the essential immunological phenotype in CD39-null mice that is driven by a Th1 deviation, with a simultaneous competent Th2 system cascade, and consecutively leading to an exacerbation in haptenic Th1 colitis models (e.g. TNBS colitis).

At sites of inflammation, IFN-γ is highly expressed in CD39-null colitis tissues, which is in keeping with previous studies [19]. Concentrations of IFN-γ in tissues of CD39-null mice were higher in TNBS colitis, as compared to wild type mice (not shown). CD39 is localized not only to endothelial and immune cells, but also to other resident cells, e.g. macrophages [7] and stromal cells [20] in TNBS colitis. Investigating infiltrating lamina propria mononuclear cells (LPMC) by FACS analyses, we noted significantly decreased numbers of $CD3^+$ and $CD4^+$ cells in the colonic wall of TNBS treated CD39-null vs. wild type mice. This is in accordance with the minimal disease activity in TNBS treated CD39-null mice. TNF-α represents a proinflammatory cytokine that is overexpressed in IBD. The limited expression of TNF-α at the protein and mRNA levels in TNBS treated CD39-null mice correlated with only minimal inflammatory effects on CD39-null mice.

F4/80 positive cells, such as macrophages, are predominant and numerous in wild-type TNBS colitis. Deficient macrophage migration has been described [17] in the CD39-null environment and could at least partially explain the decreased MPO production in TNBS colitis.

This cellular migration deficit might explain differences of severity in TNBS colitis between wild-type and CD39-null mice. Furthermore, the regulatory compensation in the purinergic cascade could explain why oxazolone colitis seems to affect wild-type and CD39-null mice in an equivalent manner: CD39L1 is markedly increased in the colon of oxazolone treated CD39-null mice. This overexpression of CD39L1 in CD39-null mice, treated with oxazolone, compared to those affected with TNBS colitis, could compensate for the lack of CD39 and as a result, these mice do not differ from their wild-type counterparts. Interestingly, in TNBS colitis, CD39L1 expression is higher in wild-type when compared to CD39-null mice.

The published data detailing purinergic signaling and colitis do not address or explain the role of T–dendritic cell communication in antigen-/hapten presentation and further studies are needed. Furthermore, an important aspect in CD39 signaling is the critical role of A2A adenosine receptors in the T-cell mediated regulation of colitis [32]. A2A adenosine receptors seem to interact with T cell-mediated types of colitis and are critical in the immune regulation of murine colitis. Furthermore, A2B adenosine receptor (A2BR) is the predominant adenosine receptor expressed in the colonic epithelia [33]. Interestingly, the colonic inflammation induced by DSS and TNBS colitis as

well as S typhimurium was attenuated in A2BR-null mice and neutrophil chemotaxis in response to exogenous IL-8 preserved [33]. Therefore, a proinflammatory role for A2BR in colitis is proposed [29, 33].

On the other hand, profiling studies revealed that murine intestinal epithelial cells express predominantly A2BR and to a lesser extent A2AR [34]. Frick et al. [34] demonstrated that the severity of colitis was increased in A2BR-null mice relative to A2BR(+/+) mice. Furthermore, the enteral administration of selective A2BR inhibitor (PSB1115) resulted in a similar increase in severity of DSS colitis. The cytokine profiling revealed deficiencies in IL-10 in A2BR-null mice in relation to control mice. These data indicate a central regulatory role for A2BR modulated IL-10 in the acute phase of DSS colitis. Thus, the A2BR is considered a potential protective molecule expressed on the intestinal epithelial cells [34].

Our studies might have implications for human IBD. In this series of experiments in human IBD samples, we found high expression levels of CD39 mRNA in inactive CD but not in UC, where expression levels of CD39 were at control levels. The clinical relevance of this is yet to be determined. Interestingly, in states of clinically apparent acute inflammation (e.g. sigmoid diverticulitis), there are no differences in CD39 mRNA expression levels.

In this context, further studies have to clarify in what way the described effects in murine colitis may be of potential therapeutic use in controlling or at least influencing IBD by purinergic mediators. Other avenues of investigation include the genetic screening of patients with IBD in terms of CD39 expression to correlate, for example, the severity of disease with the expression levels of CD39 or related purinergic elements.

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