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Expression of ecto-nucleoside triphosphate diphosphohydrolases-2 and -3 by the enteric nervous system impacts inflammation in experimental colitis and Crohn's disease

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Abstract

Background & Aims: Recent studies have suggested that the enteric nervous system can modulate gut immunity. Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) of the CD39 family regulate purinergic signaling by sequential phosphohydrolysis of extracellular ATP, a pro-inflammatory signaling molecule. Herein, we test the hypothesis that E-NTPDases modulate neuro-immune crosstalk in gut inflammation.

Methods: We determined expression patterns of NTPDase-2 and NTPDase-3 in murine and human colon. Experimental colitis was induced by dextran sodium sulfate (DSS) in mice deficient in NTPDase-2 or NTPDase-3. We compared plasma ADPase activity from Crohn's patients to healthy controls, and correlated levels of ADPase activity with Crohn's disease activity.

Results: NTPDase-2 and 3 were mainly expressed in cells of the enteric nervous system in both murine and human colon. When compared to wild type, DSS-induced colitis was exacerbated in NTPDase-2 null mice, as measured by both clinical disease activity and histology, while NTPDase-3 null mice merely developed more severe anemia. Colonic macrophages isolated from NTPDase-2 null mice displayed a more pro-inflammatory phenotype compared to wild type. Crohn's patients had decreased plasma ADPase activity when compared to healthy controls. The enzyme activity sensitive to an inhibitor against NTPDase-2 and NTPDase-3 showed the most striking difference and was inversely correlated with Crohn's disease activity.

Conclusions: NTPDase-2 and 3 are ecto-enzymes expressed in the enteric nervous system in both murine and human colon, and are protective against gut inflammation in experimental colitis and exhibit alterations in human Crohn's disease. These observations suggest that

purinergic signaling modulated by non-CD39 NTPDases governs neuro-immune interactions that are relevant in Crohn's disease.

Keywords: Crohn's disease, ectonucleotidase, CD39, NTPDase-2 (CD39L1), NTPDase-3 (CD39L3)

Introduction

Crohn's disease is a relapsing inflammatory condition that can be influenced by genetics, dysbiosis, mucosal barrier integrity and alterations in innate and adaptive immunity.[1, 2] Emerging evidence suggests that the enteric nervous system plays an important role in gut inflammation, and could be relevant in Crohn's disease.[3]

A recent study by Gabanyi and coworkers reported that enteric neurons can directly influence the phenotype of macrophages in the lamina propria and muscularis externa.[4] It has also been shown that glial cells in the gut can perpetuate the release of adenosine triphosphate (ATP) into the extracellular space, which mediates inflammation via purinergic signaling.[5] Extracellular ATP (eATP), a pro-inflammatory signaling molecule, is rapidly hydrolyzed via cell surface located ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) and 5-nucleotidase to ultimately produce adenosine which in turn has mostly anti-inflammatory properties.[6, 7] E-NTPDases therefore often mediate an anti-inflammatory response in immune regulation.

CD39, also known as NTPDase-1, the prototype of the E-NTPDase family, is expressed on immune cells and endothelial cells.[8] CD39/ENTPD1 deletion exacerbates colitis in an experimental murine model.[9, 10] Individuals with a single nucleotide polymorphism (SNP) variant associated with low levels of CD39 expression exhibit increased susceptibility to Crohn's disease in case-control studies.[9] Recent genome-wide association studies (GWAS) suggest strong associations between genotype variants of CD39 and immunophenotype of regulatory T cells.[11, 12] We have shown that CD39 can impact Crohn's disease in part via modulation of the number and function of Th17 cells.[13-15]

Within the gut, ATPase activity is predominantly localized in blood vessels, areas of the smooth muscle layers, and the enteric nervous system.[16] NTPDase-2 and NTPDase-3 are two

cell membrane located E-NTPDases that share significant structural homology and functional similarity to CD39.[7, 17] Both ectonucleotidases are known to be expressed in nerve tissues.[18, 19] In the gut, NTPDase-2 and NTPDase-3 expression have been described on cells of the enteric nervous system, both glial cells and neurons.[16] Nonetheless, any roles NTPDase-2 and NTPDase-3 might play in gut inflammation are unknown.

Herein, we test the hypothesis that purinergic signaling modulated by NTPDase-2 and NTPDase-3 may regulate intestinal neuro-immune interaction and impact gut inflammation. We further investigate the relevance of this mechanism to human Crohn's disease.

Methods and Reagents

Animals

All animal care and experiments were carried out under the guidelines and protocols approved by the animal care and use committee at BIDMC. We generated a global *Entpd2* (encoding NTPDase2) null mouse line, as described previously.[20] The strategy for generating global *Entpd3* (encoding NTPDase3) null mice is illustrated in **Supplemental Figure 1.** Male animals between 8-12 weeks of age were used in all experiments. Age-matched C57/B6 wild type mice were used as controls.

Induction and assessment of experimental colitis.

Age-matched *Entpd2* null, *Entpd3* null and wild type mice (n≥5 per group) were treated with 3% Dextran Sodium Sulfate (DSS) in drinking water that they received without restriction for 7 consecutive days. Control mice received standard drinking water. Every 24 hours body weights were measured and stools were examined. A disease activity score was calculated for each day using weight loss, degree of bleeding and stool consistency as detailed in **Supplemental Table 1**. After 7 days, mice were euthanized to retrieve colon tissue for histological analysis or cell isolation. Blood was sampled in 10% sodium citrate and hematocrit was measured from whole blood using microhematocrit capillary tubes (Fisherbrand, Pittsburgh, PA). Colons were opened longitudinally, cleaned and embedded in OCT freezing medium (Fisher Scientific, Waltham, MA). The tissues were immediately snap frozen in pre-cooled isopentane and stored at -80°C until use. The experiment was conducted three times, with n≥5 mice per group each time. Results from one representative experiment are shown.

Immunohistochemistry

Formalin-fixed, paraffin-embedded human colon tissue or frozen murine colon tissue were cut into 6 µm sections. One slide from each block was stained by hematoxylin and eosin for morphological analysis. For immunohistochemistry, sections were fixed in acetone and blocked with 7% horse serum (Vector Labs, Burlingame, CA) for half an hour. The tissues were first incubated with primary antibodies overnight at 4 C°. A complete list of the primary and secondary antibodies used for immunohistochemistry is shown in **Supplemental Table 2**. After peroxidase and biotin activity blocking, sections were incubated with the biotinylated secondary antibody for one hour, continued with Avidin Biotin complex HRP and visualized with ImmPACT DAB (Vector Labs). All slides were mounted on Cytoseal, and examined and recorded on a Nikon microscope. For fluorescent double staining we used the respective fluorescent secondary antibodies, or streptavidin conjugated with Alexa Fluor 594 (Jackson ImmunoResearch, Wet Grove, PA). Sections were co stained with Hoechst and covered with polyvinyl alcohol mounting medium (Sigma-Aldrich) and examined on a Nikon MultiPhoton Fluorescent Microscope.

Isolation of colon macrophages and flow cytometry

Immune cells were isolated from freshly harvested colon tissue as described by Gabanyi *et al.* with modifications.[4] In short, colons were washed, briefly incubated with 1mM DTT and rinsed. The tissues were cut into small pieces and resuspended in digestion buffer (HBSS with Ca^{2+} and Mg^{2+}, 0.5mg/ml Collagenase IV (Worthington, Lakewood, NJ), 0.05mg/ml DNase I (Roche Diagnostics, Indianapolis, IN), 5% FBS, 1mM NaPyr, 25mM HEPES). After 40 minutes incubation at 37°C under constant shaking, tissues were mechanically homogenized, filtered through a 100 µm cell strainer and centrifuged. Cell pellets were then resuspended in 40%

percoll solution, layered over an 80% percoll solution (GE Healthcare, Uppsala, Sweden) and centrifuged for at 2,000 rpm for 20 minutes with no brake during deceleration. The interphase was collected and washed, then resuspended in PBS to test cell viability using the Zombie AcquaTM fixable viability kit (Biolegend, San Diego, CA). After washing, cells were resuspended in PBS with 2% fetal bovine serum and incubated with antibodies against CD45-Pacific Blue, CD11b-PE-Cy7, F4/80-APC, MHCII-APC-Cy7, or CD86-APC-Cy7, Ly6C-Alexa Fluor 700 (Biolegend) and CD39 (PE, eBioscience, San Diego, Ca) for 20 minutes, washed and then analyzed on a Gallios Flow Cytometer (Beckman Coulter, Danvers, MA).

Human plasma and tissue samples

Plasma samples from 14 healthy volunteers and 28 patients with Crohn's disease were used in the study. Crohn's disease patients had an established diagnosis based on history, endoscopic finding and pathology. Blood samples were obtained through standard phlebotomy, after which plasma were isolated and stored at -80°C prior to analysis. Patient history, Harvey-Bradshaw index (HBI) for Crohn's disease activity, routine labs were collected at the time of blood sampling.[21] The formalin-fixed colonic tissues from five patients who underwent ileocecectomy because of Crohn's disease were obtained. Cancer free colon tissues from five patients who underwent resection for colorectal cancer without history of inflammatory bowel disease were used as controls. The study has been approved by the institutional review board at Beth Israel Deaconess Medical Center (BIDMC).

ADPase activity assay

ADPase activity assay was performed with ^{14}C-ADP as substrate and analyzed by thin layer chromatography (TLC) as described previously.[22] We used three inhibitors, Ap5A, POM1

and POM6, to test for the primary enzymes responsible for the ADPase activity in the plasma. A schematic diagram for the choice of inhibitors is shown in **Supplemental Figure 2**. Each sample was tested in four conditions: without ADPase inhibitor to obtain total ADPase activity, and with Ap5A, POM1 or POM6 to obtain inhibitor specific activity. All reactions contain EHNA, an inhibitor for adenosine deaminase to block the conversion of adenosine to inosine. A detailed description of assay conditions and data analysis is provided in **Supplemental Methods**.

Statistical Analysis

We compared total and inhibitor specific ADPase activity in healthy controls and Crohn's patients by two tailed student t-test with unequal variance. Two tailed student t-test with equal variance was used to compare the total and inhibitor specific ADPase activities between inactive vs. active Crohn's disease characterized by HBI. The relationships between HBI and inhibitor specific ADPase activity were analyzed by linear regression. All statistical analyses were performed STATA/IC version 13.0 (Stata Corp, College Station, Texas).

Results

Expression of NTPDase-2 and 3 in murine intestine under normal condition and colitis

We first examined the expression of NTPDase-2 and 3 using frozen murine intestinal tissues. Both E-NTPDases are expressed predominantly in the enteric nervous system (**Figure 1A, B**). NTPDase-3 shows a strong expression on nerve / glial cells that penetrate the smooth muscle layers, whereas NTPDase-2 expression is scanty there. When co-stained using fluorescent microscopy, both enzymes demonstrated substantial overlap with glial fibrillary acidic protein (GFAP), a glial cell marker **(Figure 2A, B)**. Notably, the expression of NTPDase-2 appears to extend further into the submucosa and lamina propria, whereas the expression of NTPDase-3 was mostly limited to the muscularis externa **(Figure 2C)**.

After induction of experimental colitis by DSS treatment over 7 days, the overall expression pattern of NTPDases-2 and 3 remained largely unchanged (**Figure 1C, D**). NTPDase-2 appeared more prominent in the submucosa and lamina propria when compared to control mice, which might be an impression caused by the widening of the submucosa as a result of inflammation and edema. The integrity of the muscularis externa in the colon was largely maintained in DSS-treated mice **(Figure 1A, D)**. There were no apparent changes in NTPDase-2 or 3 expression in this layer.

Genetic deletion of NTPDase-2 exacerbates DSS-induced colitis in mice

To test the role of NTPDase-2 and 3 in gut inflammation, we compared the severity of DSS-induced colitis between mice deficient in NTPDase-2 (*Entpd2* null) or NTPDase-3 (*Entpd3* null) and wild type C57BL6 mice.

Under normal condition, both NTPDase-2 and 3 deficient mice developed normally, without clinical signs of spontaneous colitis or malabsorption. Both knockout strains had a similar baseline hematocrit compared to wild type (**Supplemental Figure 3A**). Intestinal histology showed normal morphology under baseline conditions, despite the absence of enzymes in the enteric nervous system **(Supplemental Figure 3B)**.

We subjected age-matched wild type, *Entpd2* null and *Entpd3* null mice to seven days of treatment with 3% DSS. A clinical disease activity index, taking into account weight loss, rectal bleeding and stool consistency, was calculated every day for the duration of treatment **(Figure 3A).** Starting on day 5, *Entpd2* null mice showed a significantly higher disease activity index when compared to wild type (day 5: 8.6 ± 0.5 vs 3.2 ± 2.5, p = 0.002; day 6: 10.2 ± 0.8 vs 6.4 ± 3, p=0.03. day 7: 10.8 ± 0.4 vs 6.2 ± 1.8, p<0.001). The disease activity of *Entpd3* null mice was slightly higher on day 7 (8.6 ± 1.1 vs 6.2 ± 1.8, p=0.04), but otherwise similar to that of wild type mice. Individual scores for weight loss rectal bleeding and stool showed similar trends **(Supplemental Figure 4)**.

We also measured hematocrit after 7 days of DSS treatment as a more objective marker for the extent of colitis-induced blood loss. Both *Entpd2* null and *Entpd3* null mice had a significantly lower hematocrit when compared to wild type controls (14.0 ± 5.1% vs 16.8 ± 3.3% vs 21.8 ± 2.2%, p=0.01 and 0.02, respectively) **(Figure 3B)**.

In H&E stained histological sections, we observed an increase in mucosal destruction and infiltration of mononuclear cells in *Entpd2* null mice when compared to the wild type, while the histological changes in *Entpd3* null mice after DSS treatment were similar to wild type mice **(Figure 3C)**.

Genetic deletion of NTPDase-2 alters the macrophage phenotype in DSS-induced colitis

To test whether the more severe colitis seen in *Entpd2* null mice was a result from changes in the phenotype of intramural macrophages, we isolated macrophages from colon tissues after 7 days of colitis induction with DSS. Flow cytometric analysis of markers of macrophage activation showed a distinct profile in *Entpd2* null mice when compared to wild type mice (**Figure 4A-B**). These macrophages (live, CD45+ CD11b+ F4/80+ cells) had significantly higher expressions of MHCII (19 ± 5% vs 30 ± 3%, p=0.002), CD86 (33 ± 4% vs 25 ± 4%, p=0.01) and Ly6C (56 ± 5% vs 38 ± 3%, p<0.0001) as well as lower expression of CD39 (85 ± 2% vs 88 ± 1%, p=0.01), which is consistent with a more pro-inflammatory polarization. No significant changes in macrophage phenotype was observed in *Entpd3* null mice after DSS induction in comparison to wild type (data not shown).

Expression of NTPDase-2 and NTPDase-3 in human colon under normal condition and in Crohn's disease

To determine the relevance of our findings in human disease, we examined the expression of NTPDase-2 and 3 in human colon tissue. The expression of NTPDase-2 and 3 had similar features in the human colon as compared to mice. Both NTPDase-2 and NTPDase-3 are expressed predominantly in the enteric nervous system and some perivascular cells (**Figure 5A-D)**. While both ecto-enzymes can be observed in ganglia of the submucosal (Meissner's) plexus and myenteric (Auerbach's) plexus, nerve fibers within the smooth muscle layers as well as bigger nerve bundles stain markedly for NTPDase-3, and to a lesser extent for NTPDase-2 (**Figure 5B, D, arrows)**. NTPDase-2 expression was also noted in the subendothelial cells of most blood vessels, whereas NTPDase-3 expression was restricted to subendothelial cells of small venules.

Compared to controls, the overall expression of both NTPDase-2 and 3 showed similar patterns in Crohn's patients. However in Crohn's patients, the submucosal plexus appeared to be smaller compared to controls **(Figure 5E, G, arrowheads)**. Furthermore, the myenteric plexus in Crohn's patients were disintegrated into smaller fragments in diseased tissues, each of which was surrounded, and sometimes fully encircled by infiltrating mononuclear cells **(Figure 5F, H, arrows, Supplemental Figure 5E, F)**. In the absence of inflammation, macrophages (CD68+ cells) were present either within or adjacent to the myenteric plexus, whereas few lymphocytes (CD3+ cells) were noted there (**Supplemental Figure 5C, D**). In Crohn's disease, there is an increase in the number of both macrophages and lymphocytes, while macrophages are still the dominant immune cell population in the area surrounding the myenteric plexus (**Supplemental Figure 5G, H**).

Changes in ectonucleotidase activity in the plasma of patients with Crohn's disease

We have previously shown that cell membrane-localized E-NTPDases are present on microparticles circulating in the blood stream and contribute to the ectonucleotidase activity in the plasma.[22, 23] As microparticles are derived from cells, circulating ectonucleotidase activity may serve as a surrogate marker for cellular ecto-enzyme expression that cannot be easily sampled in humans. We compared ADPase activity in the plasma of both Crohn's patients and controls. The plasma of a total of 28 patients with Crohn's disease were collected for the study. Their background characteristics are summarized in **Table 1**. A control group was composed of 14 healthy volunteers without inflammatory bowel disease, with an average age of 51, and 64% females. The overall ADPase activity in Crohn's patients was 21.2 ± 1.7 U/L, significantly less than in controls (30.4 ± 4.9 U/L, $p < 0.0001$) (**Figure 6A, Supplemental Table 3)**.

We then measured the ADPase activity in the presence of three inhibitors: Ap5A (inhibits adenylate kinase 1), POM1 (inhibits CD39 and NTPDase-3) and POM6 (inhibits NTPDase-2 and 3) **(Figure 6B)**. The POM6-sensitive ectonucleotidase activity, defined by the difference between ADPase activity with and without POM6, was substantially lower among Crohn's patients (2.5 ± 1.2 U/L) when compared to healthy controls (13.0 ± 4.5 U/L, $p < 0.0001$) (**Figure 6C**, **Supplemental Table 3**). A similar, but smaller difference was seen in POM1 sensitive activity. No difference in Ap5A sensitive activities were noticed between Crohn's and control groups. As POM6 has a similar K_i for NTPDase-2 and -3, but does not inhibit CD39, non-CD39 E-NTPDases are likely the major contributor to the difference in ADPase activity between Crohn's patients and controls.

To further test the specificity of our findings, we investigated whether the plasma ADPase activity correlates with Crohn's disease activity. Patients with Harvey-Bradshaw index (HBI) values of 4 or higher had significantly lower levels of POM1 and POM6-sensitive ADPase activity than patients with an HBI of 3 or less (2.3 ± 1.0 vs. 3.2 ± 1.0 U/L, $p = 0.02$ and 1.9 ± 0.8 vs. 3.0 ± 1.2 U/L, $p = 0.005$ respectively) (**Figure 6D, Supplemental Table 4)**. Both POM1 and POM6 sensitive ADPase activities in the plasma demonstrate a near linear relationship with the HBI of Crohn's patients (**Supplemental Figure 6A, B**).

Discussion

Gut inflammation impacts the integrity and function of the enteric nervous system, as supported by both mechanistic investigations in animals and empirical observations of prolonged bowel dysfunction during recovery of flares of inflammatory bowel disease.[24] Emerging evidence suggests that neuronal modulation can in turn impact the intestinal immune system.[3, 4] However, the molecular mechanisms that underlie this neuro-immune crosstalk are not fully understood. It is also unclear whether such interactions are clinically relevant. Our study suggests that purinergic signaling may be a crucial pathway that regulates neuro-immune interaction in the gut. We found that ectonucleotidases as modulators of purinergic signaling impact gut inflammation in a murine model of colitis and appear to be relevant in human Crohn's disease.

Ectonucleotidases of the CD39 family catalyze the phosphohydrolysis of extracellular nucleotides, hence modulate purinergic signaling towards a more anti-inflammatory pathway.[17] CD39 is expressed on endothelial and immune cells and has been implicated in human Crohn's disease as well as experimental colitis.[9, 10] The protective effect of CD39 in colitis has been ascribed to its direct effect on the phenotype of T cells.[11, 14] However, it is not expressed on neurons or glial cells and cannot explain the finding of heightened ATPase activity in areas of the enteric nervous system.[16] Our present study thus focuses on NTPDase-2 and NTPDase-3, two ecto-enzymes of the CD39 family that are expressed primarily by cells of the enteric nervous system.

Extracellular ATP (eATP), a pro-inflammatory signaling molecule, activates ionotropic $P2X_7$ receptors in macrophages, dendritic cells and neutrophils, which in turn induce NLRP3 inflammasome assembly and the release of interleukin 1β and 18.[25] eATP has also been shown to mediate the communication between neurons and glia and thus contribute to the

maintenance of intestinal homeostasis and mucosal barrier.[26, 27] Glial cells can perpetuate the release of pro-inflammatory eATP in the setting of intestinal inflammation via the activation of $P2Y_1$ receptors, which in turn mediates neuronal cell death via $P2X_7$ receptors.[5] Furthermore, enteric glial cells have been shown to express pro-inflammatory cytokines such as interleukin-1β and interleukin-6.[28]

Here we observe that especially the loss of NTPDase-2, and to a lesser extent the loss of NTPDase-3, exacerbates gut inflammation in DSS-induced colitis. The increased severity of colitis in NTPDase-2 deficiency is associated with a change in intestinal macrophages toward a more pro-inflammatory phenotype, as defined by increased surface expression of MHCII, CD86, Ly6C and lower expression of CD39. Enteric neurons in the muscularis externa have been shown to mediate the polarization of tissue resident macrophages toward a tissue-protective phenotype.[4] It is plausible that NTPDase-2 in the enteric nervous system contributes to this immune-regulatory mechanism by decreasing the local eATP concentration and thus modulating inflammasome activation of intestinal macrophages and other immune cells. Consequently, the absence of NTPDase-2 results in a local accumulation of eATP that can lead to stronger pro-inflammatory macrophage activation through $P2X_7$ receptors as well as activation of glial cells via $P2Y_1$ receptors.[5, 25]

In humans, we found macrophages to be the dominant inflammatory cells surrounding the myenteric plexus under both normal and inflammatory states secondary to Crohn's disease, supporting the concept of macrophages as central players in intestinal neuro-immune crosstalk. In addition, we showed that plasma ADPase activity in humans corresponding to NTPDase-2 and 3 is lower among patients with Crohn's disease compared to controls, and inversely correlates with Crohn's disease activity, suggesting that alteration in ectonucleotidases may be associated with Crohn's disease. We have previously described the presence of E-NTPDases on the surface of circulating microparticles, which accounts for a significant proportion of plasma

ADPase activity.[22] Although it is not easy to confirm the origin of these microparticles, our data suggest that this quantifiable enzyme activity may serve as a surrogate marker of tissue ecto-enzyme activity, which is not easily accessible in humans, and can represent a useful biomarker for diagnosis and disease staging. We have previously shown that luminal extracellular vesicles impact function of epithelial cells and macrophages.[29] Whether the E-NTPDase-containing microparticles are functionally active needs to be explored.

In human plasma, both POM1- and POM6-sensitive ADPase activities inversely correlate with Crohn's disease activity. POM6, an inhibitor for NTPDase-2 and 3, shows a much larger impact on plasma ADPase activity than POM1, an inhibitor for CD39 and NTPDase-2.[30] This suggests that the substantial decrease in total ADPase activity in disease is caused, at least in part, by decrease of NTPDase-3, in addition to NTPDase-2. In our animal model, mice deficient in NTPDase-2 exhibit more severe experimental colitis. Interestingly, the impact of NTPDase-3 deletion is less dramatic, despite a broad overlap in the expression of these two related enzymes. This might be explained by slight differences in expression between NTPDase-2 and 3. In mice, NTPDase-3 is largely expressed in the muscularis externa, whereas NTPDase-2 expression extends to the mucosa and submucosa. The fact that DSS-induced colitis spares the muscularis externa may account for the stronger phenotype in *Entpd2* null mice. In contrast, the inflammation in Crohn's disease is transmural. In addition, NTPDase-2 has a significantly lower ADPase activity compared to NTPDase-3 which could lead to local accumulation of ADP and a relative lack of adenosine.[17] It is possible that the specific enzyme kinetics of NTPDase-2 and modified purinergic signaling mediated by eADP may contribute to the differences in experimental colitis.

Our study validates prior observations on the expression patterns of NTPDase-2 and 3 in murine enteric nervous system and confirms a similar expression in human intestine.[16] Lavoie and colleagues reported that in mice, NTPDase-3 is limited to neuronal cells in the myenteric

ganglia based on its co-immunostaining with PGP9.5.[16] Here, we observe a co-immunostaining of NTPDase-3 with glial cell marker GFAP, suggesting co-expression in enteric glial cells. NTPDase-3 is the predominant E-NTPDase in intramuscular fibers that are also positive for GFAP in both murine and human intestine. In comparison, the expression of NTPDase-2 is largely limited to areas of the submucosal and myenteric plexus of the enteric nervous system.

Although the expression of NTPDase-2 and 3 in the enteric nervous system suggests a link between neuronal signaling and inflammation, other possible mechanisms should be considered. Immunohistochemistry staining for both enzymes can also be observed in the perivasculature, as noted by Sévigny and colleagues.[16] However, we did not observe vascular inflammation in either *Entpd2* or *Entpd3* null animals. CD39, the E-NTPDase expressed on endothelial cells seems to be the dominant regulator of eATP in the vasculature. Furthermore, although the exacerbated colitis in *Entpd2* null mice suggests a role of this enzyme in the pathophysiology of colitis, the causal relationship between NTPDase-2 and 3 activities and Crohn's disease severity in humans needs to be validated. It is also possible that the lower ADPase activity in Crohn's disease is a reflection of damage to the enteric nervous system, as gut inflammation is known to cause plexitis and neuronal loss.[24]

In summary, we show that global deletion of NTPDase-2, and to a lesser extent NTPDase-3, exacerbates DSS-induced colitis in mice. In addition, we demonstrate a decrease in plasma ectonucleotidase activity among Crohn's patients along with alterations in the integrity of the enteric nervous system. Taken together, these observations support the concept of a neuro-immune crosstalk that appears to be, at least in part, mediated by purinergic signaling. Plasma ectonucleotidase activities may be useful biomarkers for inflammation or severity of disease. Further work is needed to fully understand the mechanism, including identification of cells and purinergic receptors involved in this pathway, and to explore the potential of

NTPDase-2 and NTPDase-3 as new diagnostic biomarkers and therapeutic targets in inflammatory bowel disease.

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Disclosure

The authors declare no financial interest in relation to this study.

TABLES

Table 1 Background characteristics of Crohn's disease patients

Background characteristics	
Age	
17-40	42.9%
41+	57.1%
Gender	
% female	42.9%
Duration (yr)	15 (5.5, 22)
Type of disease	
Inflammatory	28.6%
Stricturing	21.4%
Penetrating	50.0%
Location of Disease	
Ileal	32.1%
Colonic	25.0%
Ileocolonic	42.9%
Disease Activity	
HBI	3 (2, 5)
WBC ($\times 10^3$ /µl)	7 (5.7, 8.4)
CRP (ng/dLmg/L)	2.7 (1.2, 13.9)

FIGURE LEGENDS

Figure 1 NTPDase-2 and 3 expression in normal murine colon and experimental colitis

A. NTPDase-2 is expressed in the ganglia of the myenteric plexus (*), scarcely in fibers of the muscularis propria, and more strongly in thin elongated structures of the submucosa and lamina propria (arrows). B. NTPDase-3 expression is also localized in the ganglion cells, with an abundant expression in cells and fibers throughout the smooth muscle layer (arrow heads) and little expression in the submucosa and lamina propria.

Figure 2 Expression of NTPDase-2 and 3 in the murine enteric nervous system

A, B. Co-immunostaining of GFAP as a marker of glial cells with NTPDase-2 (A) and NTPDase-3 (B). C. Co-immunostaining of NTPDase-2 (in green) and NTPDase-3 (in red).

Figure 3 NTPDase-2 deficient mice develop exacerbated inflammation in DSS-induced colitis

A. Daily clinical disease activity index in DSS induced colitis. This index was calculated from individual scores for weight loss, bleeding and stool consistency as described in **Supplemental Table 1**. DSS induced colitis is most severe in *Entpd2* null mice when compared to wild type and *Entpd3* null mice (n=5 mice per group, this figure represents one out of 3 independent experiments that showed the same trends). B. Hematocrit was measured after 7 days of treatment with 3% DSS. C. Representative H&E stained tissue sections of mouse colon after 7 days of treatment with 3% DSS. Error bars show standard deviations. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 4 Colonic expression of NTPDase-2 and 3 in human enteric nervous system

Representative images of immunohistochemistry highlighting the expression of NTPDase-2 (A, B, E, F) and NTPDase-3 (C, D, G, H) in the human colon from controls (A-D) and Crohn's patients (E-F). Arrow heads: Submucous plexus, arrows: myenteric plexus.

Figure 5 NTPDase-2 deficiency mice exhibit pro-inflammatory macrophage phenotype in DSS-induced colitis

A. Gating strategy of macrophages after isolation from colon tissue. After gating of live, single cells (not shown), CD45+CD11b+F4/80+ cells were gated as shown and defined as macrophages (Gate "N"). Exemplary gating for MHCII positivity. B. Comparison of macrophage markers (in % of CD45+CD11b+F4/80+ cells as shown in A) between WT and Entpd2 null mice. Error bars show standard deviation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 6 Decreases in circulating ectonucleotidase activity in Crohn's disease

A. A comparison of total plasma ectonucleotidase activities. Samples from 28 Crohn's (CD) patients, 14 healthy controls were analyzed. B. The ectonucleotidase activity in the presence of inhibitors Ap5A (adenylate kinase 1), POM1 (CD39 and NTPDase 3) and POM6 (NTPDases2, 3). C. A comparison of POM1 and POM6 sensitive ectonucleotidase activities between Controls and Crohn's patients. D. A comparison of POM1 and POM6 sensitive ectonucleotidase activities between Crohn's patient with HBI 1-3 and HBI > 3. P values are calculated using Student's T test and labeled with asterisks: * $p < 0.05$ and ** $p < 0.0001$

Figure 1

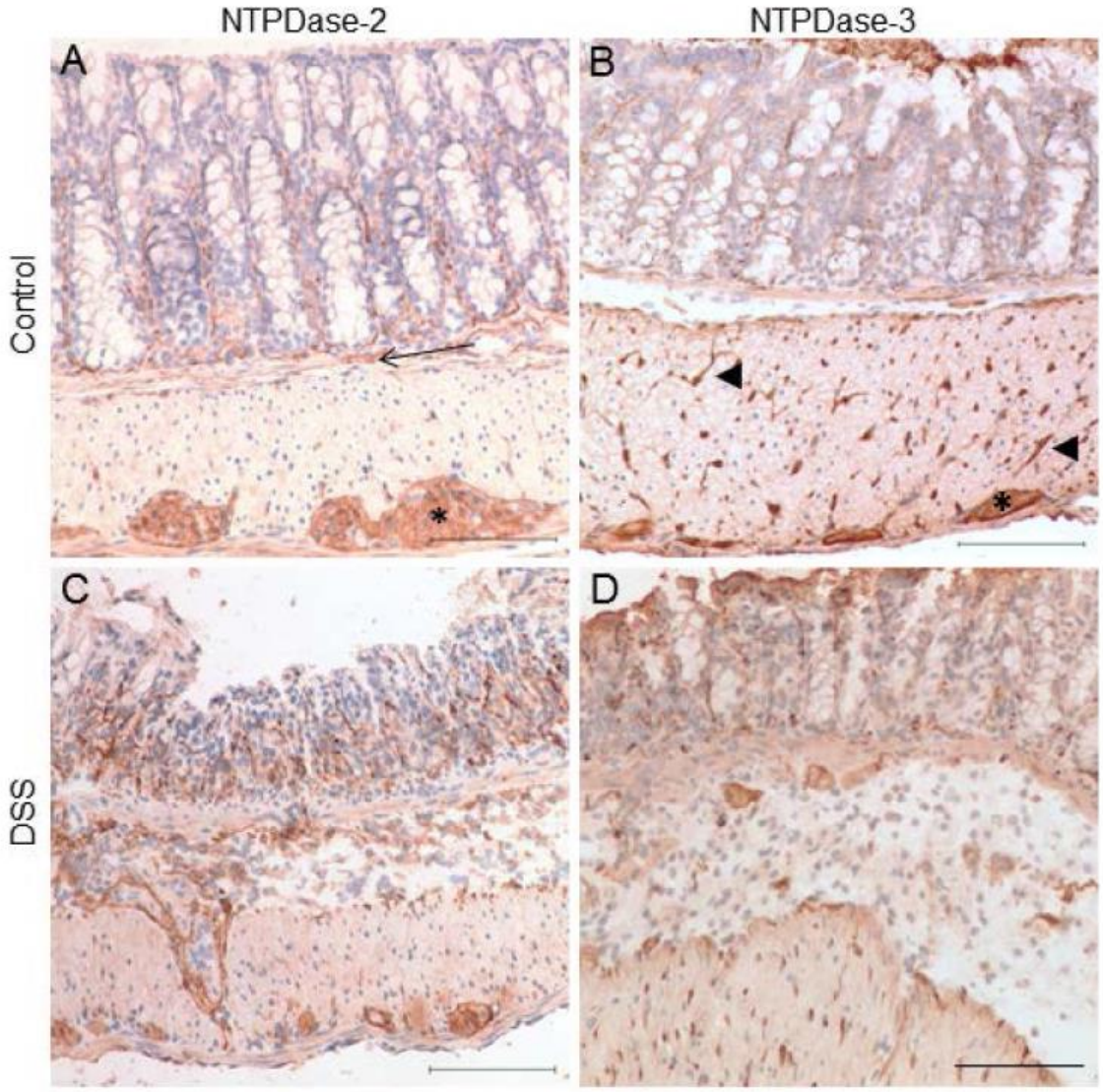


Figure 2

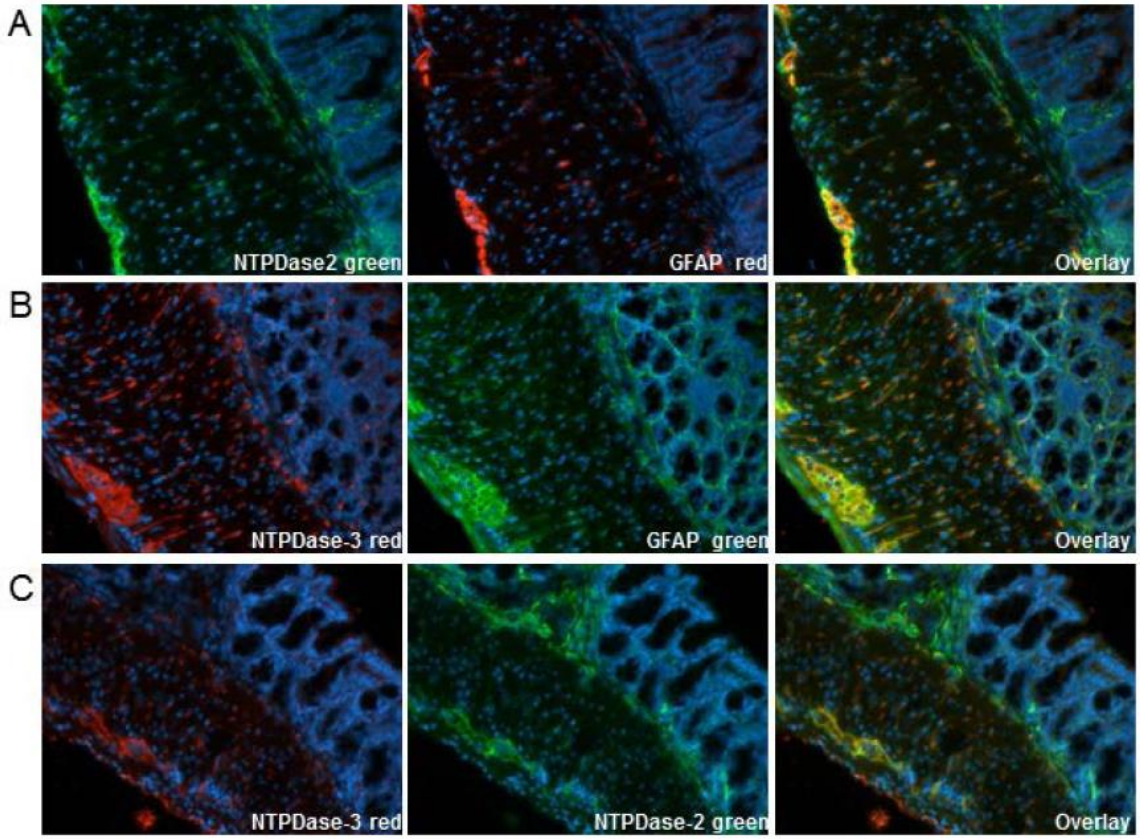

A
NTPDase2 green
GFAP red
Overlay
B
NTPDase-3 red
GFAP green
Overlay
C
NTPDase-3 red
NTPDase-2 green
Overlay

Figure 3

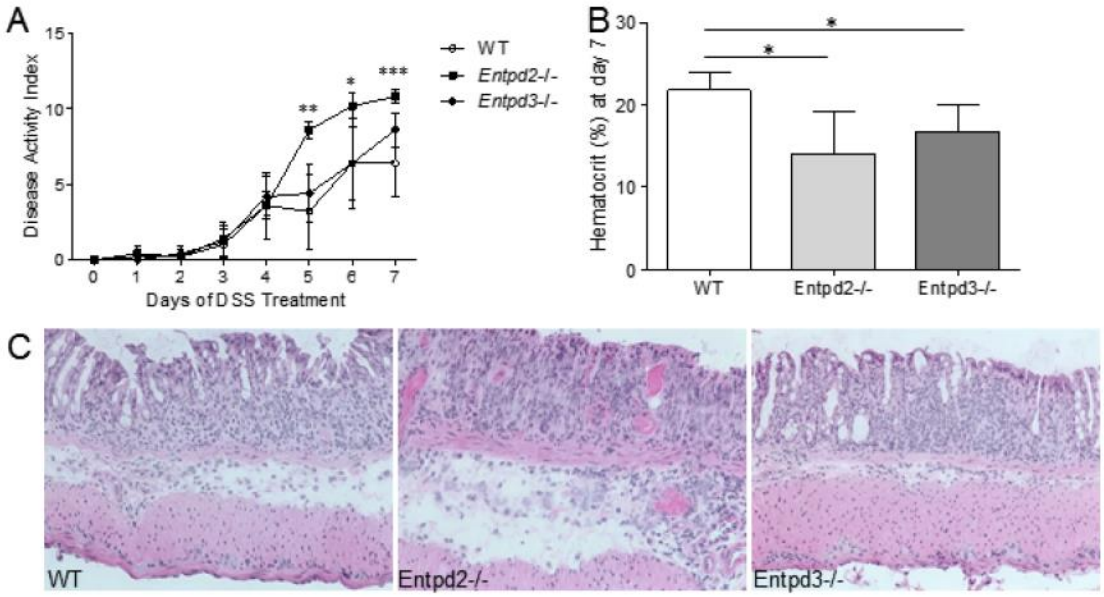

A
Disease Activity Index
Days of DSS Treatment
WT
Entpd2-/-
Entpd3-/-
**
*
***
B
Hematocrit (%) at day 7
WT
Entpd2-/-
Entpd3-/-
C
WT
Entpd2-/-
Entpd3-/-

Figure 4

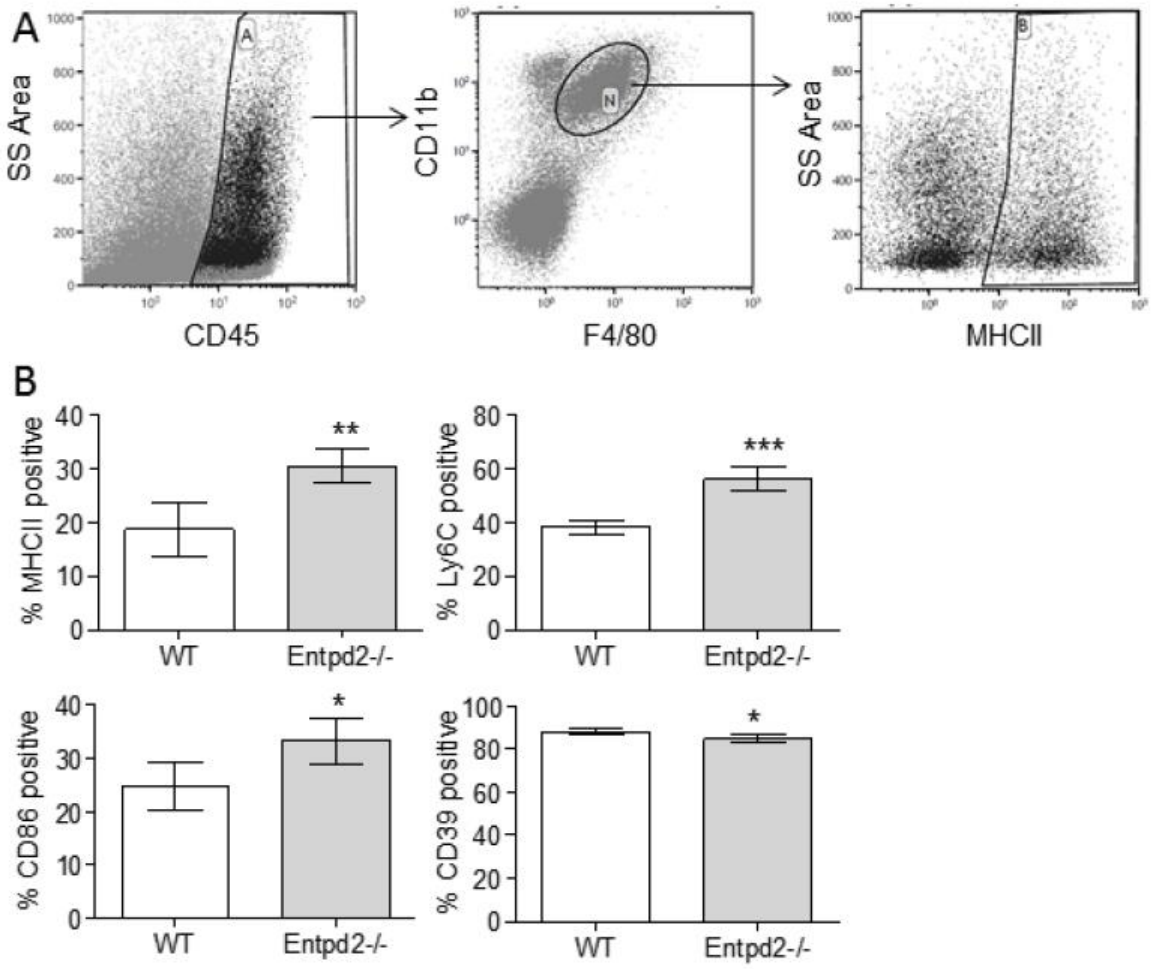

A
SS Area
CD45
CD11b
F4/80
SS Area
MHCII
B
% MHCII positive
% Ly6C positive
% CD86 positive
% CD39 positive
WT
Entpd2-/-
**
***
*
*

Figure 5

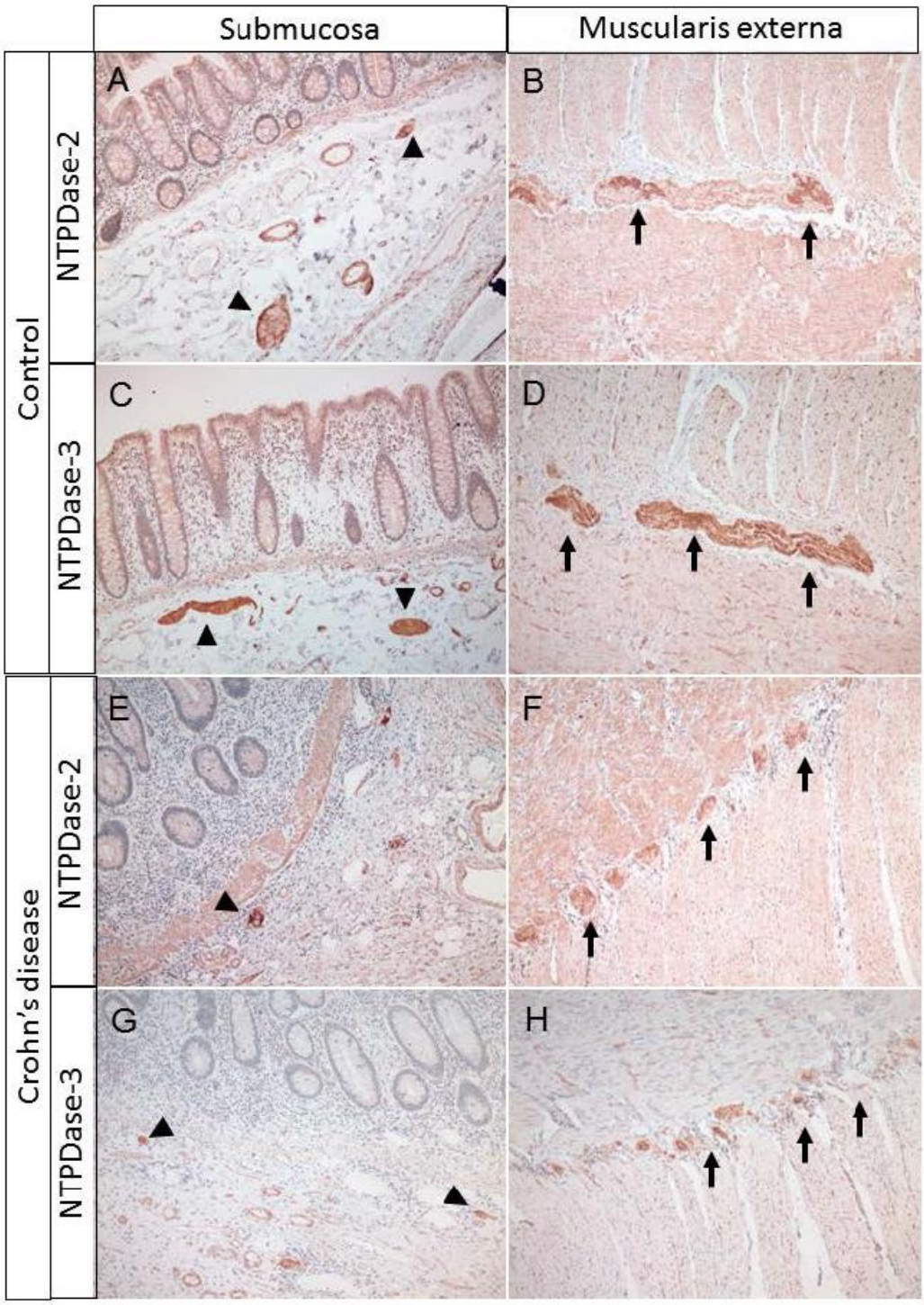
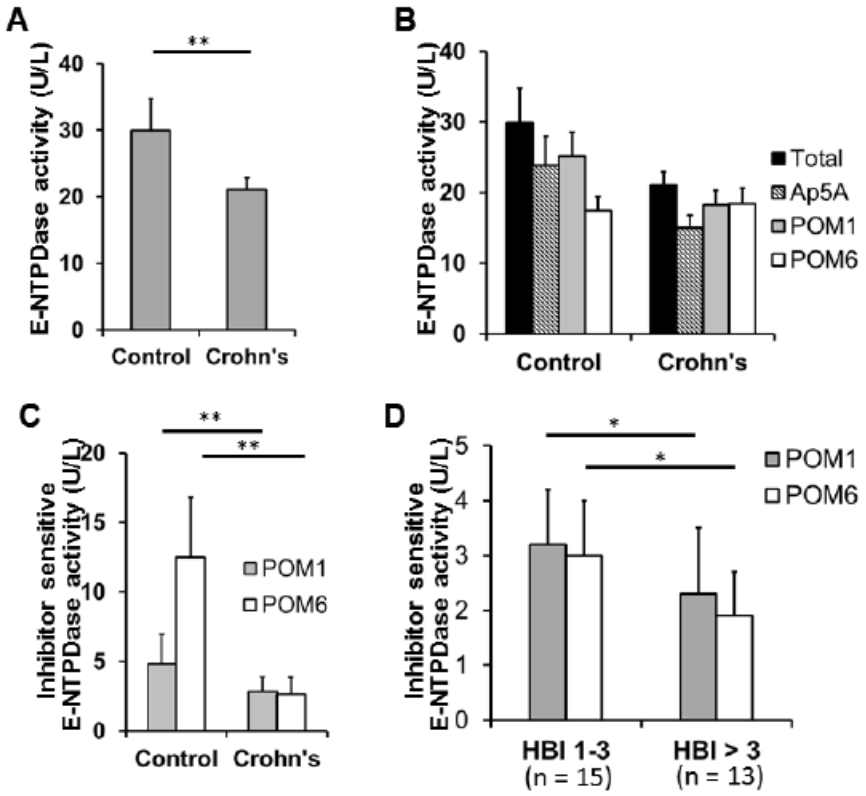

Submucosa
Muscularis externa
Control
NTPDase-2
NTPDase-3
Crohn's disease
NTPDase-2
NTPDase-3
A
B
C
D
E
F
G
H

Figure 6


A
B
C
D
E-NTPDase activity (U/L)
Inhibitor sensitive E-NTPDase activity (U/L)
Control
Crohn's
Total
Ap5A
POM1
POM6
HBI 1-3
(n = 15)
HBI > 3
(n = 13)