CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease

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CD39/ENTPD1 hydrolyzes proinflammatory nucleotides to generate adenosine. As purinergic mediators have been implicated in intestinal inflammation, we hypothesized that CD39 might protect against inflammatory bowel disease. We studied these possibilities in a mouse model of colitis using mice with global CD39 deletion. We then tested whether human genetic polymorphisms in the CD39 gene might influence susceptibility to Crohn's disease. We induced colitis in mice using Dextran Sodium Sulfate (DSS). Readouts included disease activity scores, histological evidence of injury, and markers of inflammatory activity. We used HapMap cell lines to find SNPs that tag for CD39 expression, and then compared the frequency of subjects with high vs. low CD39-expression genotypes in a case-control cohort for Crohn's disease. Mice null for CD39 were highly susceptible to DSS injury, with heterozygote mice showing an intermediate phenotype compared to wild type (WT). We identified a common SNP that tags CD39 mRNA expression levels in man. The SNP tagging low levels of CD39 expression was associated with increased susceptibility to Crohn's disease in a case-control cohort comprised of 1,748 Crohn's patients and 2,936 controls ($P = 0.005$ – 0.0006). Our data indicate that CD39 deficiency exacerbates murine colitis and suggest that CD39 polymorphisms are associated with inflammatory bowel disease in humans.

Crohn's disease | ENTPD1

Inflammatory bowel disease (IBD) results when the complex immune balance in the bowel environment is disrupted. Normally, the immune system suppresses inflammation in the presence of resident gut bacteria or food antigens but can respond rapidly to potentially pathogenic bacteria or viruses with vigorous inflammatory responses (1). In IBD, gut immunity is episodically activated in the absence of a clear “danger signal,” leading to a constellation of symptoms including abdominal pain, diarrhea, gastrointestinal bleeding, venous thrombotic complications, and often malnutrition (1). Strong genetic components contribute to an individual's susceptibility to IBD, particularly Crohn's disease (2).

CD39, also known as ENTPD1, is a vascular and immune cell ectonucleotidase that converts extracellular ATP and ADP to AMP. Since ATP is typically proinflammatory and AMP is rapidly converted by CD73 into the largely anti-inflammatory metabolite adenosine, CD39 tends to promote an anti-inflammatory and immune suppressive milieu. Within the immune system, CD39 is expressed on cells of both the innate and adaptive immune systems, including T cells (particularly T-regulatory cells) (3), B-cells (4), NK cells (5), NKT cells (6), dendritic cells (4), monocytes (5), and macrophages (7).

Studies in our laboratory indicate a particularly important role for CD39 and CD73 in the tandem generation of adenosine with subsequent activation of adenosine A2a receptors (3). Mice null for CD39 have heightened expression of proinflammatory me-

diators such as IFN gamma, IL-1 β , IL-6, and TNF (8). These mutant mice are also prone to increased injury in a wide range of acute, subacute, and chronic vascular inflammatory models including transplant rejection (9), ischemia-reperfusion (10), and diabetic microvascular disease (11).

Given the prominent role of CD39 in the response to inflammation and adaptive immunity, we asked whether CD39 might participate in regulating the complex immune balance that governs susceptibility to inflammatory bowel disease (IBD). We tested this hypothesis in mice null for CD39 by administering Dextran Sodium Sulfate (DSS), an experimental form of IBD, and characterized these mice clinically, histologically, and for markers of immune activation.

Recent advances in genetics and genomics, such as the HapMap and the advent of genome wide association studies, have generated a wealth of human genetic data. Using data from gene expression profiling studies and Crohn's case-control studies, we are able to identify human SNPs strongly associated with CD39 mRNA expression and have determined the effect of these SNPs on susceptibility to Crohn's disease.

Results

Initial Conditions. We studied adult wild-type (WT), heterozygous (hz) for CD39, and CD39-null (KO) C57BL6 mice at 16–22 weeks of age. Mice in the treatment groups for DSS (WT 37.9 ± 1.0 g vs. hz 34.9 ± 3.0 g vs. CD39-null 35.4 ± 1.2 g; $P > 0.05$, $n = 5$ for each group) had comparable body weights at initiation of the study.

Clinical Course of DSS Colitis. DSS treatment of C57BL6 mice resulted in acute colitis in all mice. We evaluated the clinical severity of the colitis using a disease activity index (DAI) (Fig. 1A), which incorporates weight loss, stool consistency, and GI bleeding (Fig. 1B). CD39-null mice had significantly worse colitis than WT mice starting on day 2 and continuing through day 7. Heterozygote mice were intermediate between WT and CD39-null at all time points, but did not differ significantly from either group at any time point (Fig. 1A).

We measured the hematocrit in each group as another indicator of disease severity. There is no difference at baseline in

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Fig. 4. CD39 expression in 210 subjects across four populations. (A) CD39 mRNA expression in cell lines from subjects with Caucasian/European (CEU), Yoruba (YRI), Chinese (CHB), or Japanese (JPT) ancestry with each rs10748643 genotype. For Chinese subjects, AG and GG were combined because there were only two GG subjects.

examined CD39 mRNA expression in 210 HapMap subjects from four diverse populations to confirm our *in vitro* findings in a larger set of European subjects and to determine if this expression phenotype could be generalized to people of other ancestries. In Europeans, Africans, Chinese, and Japanese subjects, rs10748643 genotype correlates strongly with CD39 expression. GG is associated with much higher levels than AA (46–77% increase), with carriers of the AG genotype displaying intermediate expression levels (Fig. 4 and Tables 1 and 2). In Chinese subjects, the GG genotype was uncommon and AG and GG genotypes were pooled for analysis. For all populations combined, the G allele was associated with higher expression levels with a P value on the order of 10^{-22} using an additive, linear model (13). These results were confirmed in a second genome wide study, with similarly strong association (10^{-28}) (14).

Association of a SNP Tagging ENTPD1 Expression with Crohn's Disease. Next, we asked whether this SNP strongly correlated to CD39 expression might influence human susceptibility to colitis, as we saw in our mouse models. We obtained data from the Wellcome Trust Case Control Consortium study in Crohn's disease to test this hypothesis. At rs10748643, there was significant enrichment of the low CD39-expressing AA genotype in Crohn's disease cases, while controls were enriched for the higher CD39-expressing GG allele (Table 3). Using the Wellcome Trust Case Control Consortium (WTCCC) expanded control panel (all without IBD), we noted an increase in statistical significance in additive, genotypic, allelic, and homozygote vs. homozygote models (Table 3). The odds ratio for the A allele was 1.13 (1.04–1.23) or 1.14 (1.06–1.22) using the standard control or expanded control panel, respectively.

Table 1. HapMap cell lines from 4 ancestral populations

Genotype CD39	CEU	YRI	JPT	CHB
AA	9	10	20	26
AG	30	35	19	17
GG	21	15	6	2

Number of cell lines per genotype in each ancestral population.

Table 2. CD39 expression in HapMap cell lines

	Relative expression			
	CEU	YRI	JPT	CHB
AA	1.00	1.00	1.00	1.00
AG	1.34	1.13	1.21	1.20
GG	1.77	1.46	1.61	1.72

Relative CD39 expression for each population based on genotype at rs10748643.

Discussion

IBD may represent a tipping of the immune balance toward excessive inflammation in genetically susceptible individuals. Crohn's disease in particular has been associated with a skewing toward a Th1/IFN gamma-driven inflammatory response (1). Interestingly, CD39-null mice also exhibit a general tendency toward exaggerated Th1/IFN gamma responses (15).

In the selected mouse model (acute DSS exposure) that recapitulates important cardinal features of colitis in IBD such as diarrhea, weight loss, and gastrointestinal bleeding, we observed that CD39-null mice suffer more severe injury than WT mice. Although heterozygous mice did not differ from either group in our overall index (DAI) of IBD-activity, these mice did show a potential CD39 dose effect in limiting inflammation. DSS-colitis has traditionally been associated with features of both Crohn's disease and ulcerative colitis in rodents, with a bias toward the former.

In CD39-null mice, injury spanned the bowel wall, a typical and critically important feature of Crohn's disease that is not seen in classic ulcerative colitis (16). Examination of large bowel in the treated mouse groups revealed substantially more severe histological lesions in the null mice, with loss of crypts and a far more vigorous inflammatory response. Increased MPO activity in CD39-null mice provided further evidence of this heightened inflammatory response, emphasizing the role of infiltrating monocyte-macrophages in this disease process. Treatment of CD39-null mice with soluble apyrase could reverse weight loss from chronic DSS treatment, implicating the nucleotide hydrolyzing capacity of CD39 as the functional protective mechanism.

Immune system dysregulation is generally considered the major cause of IBD, but there is a growing appreciation for the role of the vasculature in modifying this response (17–19). CD39 is both the dominant immune cell and vascular ectonucleotidase, so its protective effect against DSS colitis in mice may be due to either of these two roles, or perhaps both. CD39 is important for T_{reg} cell function through generation of adenosine (3), and we would propose this function as the most likely factor in our model. However, recently others have shown that CD39 may specifically modify vascular regulation in murine colitis (20). We expect tissue specific deletion of CD39 in immune cells or vascular cells will answer this question definitively in the future.

In mice, several experimental models have been published showing involvement of purinergic signaling in IBD and toxin-induced colitis. The ultimate products of ectonucleotidases are nucleosides/adenosine that activate type 1 A2A adenosine receptors on immune and vascular cells, markedly attenuating intestinal inflammation in some animal models of IBD (21, 22). CD39 is not expressed on epithelial surfaces but rather has similar tissue distribution to the A2A receptor, and the similar response to colitis in CD39- and A2A-null mice suggests a functional interaction. A2A activation protects against colitis in these studies, associated with decreased production of inflammatory cytokines such as TNF- α and IFN gamma. Notably, these cytokines are upregulated in CD39-null mice (23). In contrast, conflicting recent reports show paradoxically that activation of another adenosine receptor, A2B, may be either pathogenic or

Histological Assessment of Colitis. Specimens were embedded and snap-frozen as previously described (37) and stored at -80°C . Four- to five-micrometer tissue sections were stained with hematoxylin and eosin. Stained sections were examined for evidence of colitis as previously published, (38) 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.

Colonic Myeloperoxidase (MPO) Activity. Colon samples were obtained from control and DSS treated animals, and prepared as previously described (39). The samples were thawed for MPO activity assay determination according to the o-dianisidine method as previously described (39). In brief, tissue samples were thawed, weighed, suspended in 50 mM potassium phosphate buffer (K_p), pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide buffer (0.1 g/20 mL K_p), and homogenized. After sonication the sample was microcentrifuged. The reaction was started by mixing and incubating the supernatant in 50 mM K_p, 20 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 min/g wet weight of colonic tissues.

Cell Culture. Lymphoblasts from HapMap participants of European (CEU) ancestry were purchased from Coriell Institute (www.coriell.org). Genotypes for each cell line were obtained from www.hapmap.org. Cells were grown in RPMI 1640 media with 2 mM L-glutamine, 15% FBS, penicillin-streptomycin mix, and 10 mM HEPES buffer. Cells were seeded at 200,000 per mL and harvested 24–48 h later in log-phase growth.

Real-Time PCR. mRNA was isolated using RNeasy (Qiagen) according to the manufacturer's instructions. mRNA (0.5 μg) was reversed transcribed to cDNA using the Taqman Reverse Transcription Kit (Applied Biosystems). Probe-primer sets for ENTPD1 and the 18S ribosomal subunit (loading control) were obtained from Applied Biosystems. Real-time PCR was performed with an Applied Biosystems 7900 system. ENTPD1 mRNA expression was normalized using 18S expression. mRNA was quantified twice from each cell line and averaged.

Bio-Informatic Assessment of ENTPD1 Expression. Expression data for 210 HapMap cell lines was downloaded from the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/humgen/genevar/>). The methods for both genotyping and expression profiling of these cell lines have been described in detail (13). ENTPD1 expression level for each genotype was calculated. We used log2-transformed data for tests of statistical significance.

Crohn's Disease Genetics. Genotypes at rs10748643 for both control and Crohn's patients in the Wellcome Trust Case Control Consortium (WTCCC) were downloaded from <http://www.wtccc.org.uk/>. Data were available for 1,748 Crohn's cases and 2,936 apparently healthy controls. We conducted a second analysis of the data using an expanded control panel (10,600 subjects) that included the healthy controls plus additional subjects from the Wellcome Trust case cohorts. We included hypertension, coronary artery disease, bipolar disorder, and type 2 diabetes cohorts that are not expected to have disease SNPs in common with Crohn's disease, but excluded subjects from autoimmune disease cohorts (type 1 diabetes and rheumatoid arthritis) that are likely to have disease SNPs that overlap with Crohn's disease (40).

Statistical Analysis. Statistical analysis was performed with Microsoft Excel or Graphpad Prism 4 software. For mouse data, we used ANOVA with Newman-Keuls posthoc test for parametric analyses between three or more groups and the Kruskal-Wallis test with Dunn's posthoc test for nonparametric analyses. For cell line data, we used unpaired Student t-test for comparison between groups. For Crohn's genetic data, we analyzed the data under a genotypic model (two-degree of freedom χ^2 test), an allelic model (one-degree of freedom χ^2 test), a homozygote vs. homozygote model (1-degree of freedom χ^2 test), and an additive model (one-degree of freedom χ^2 test for linear trend).

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