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CD39 and CD161 Modulate Th17 Responses in Crohn’s Disease

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CD39 (ENTPD1) is expressed by subsets of pathogenic human CD4+ T cells, such as Th17 cells. These Th17 cells are considered important in intestinal inflammation, such as seen in Crohn’s disease (CD). Recently, CD161 (NKR-P1A) was shown to be a phenotypic marker of human Th17 cells. In this study, we report that coexpression of CD161 and CD39 not only identifies these cells but also promotes Th17 generation. We note that human CD4+CD39+CD161+ T cells can be induced under stimulatory conditions that promote Th17 in vitro. Furthermore, CD4+CD39+CD161+ cells purified from blood and intestinal tissues, from both healthy controls and patients with CD, are of the Th17 phenotype and exhibit proinflammatory functions. CD39 is coexpressed with CD161, and this association augments acid sphingomyelinase (ASM) activity upon stimulation of CD4+ T cells. These pathways regulate mammalian target of rapamycin and STAT3 signaling to drive the Th17 phenotype. Inhibition of ASM activity by pharmacological blockers or knockdown of ASM abrogates STAT3 signaling, thereby limiting IL-17 production in CD4+ T cells obtained from both controls and patients with active CD. Increased levels of CD39+CD161+ CD4+ T cells in blood or lamina propria are noted in patients with CD, and levels directly correlate with clinical disease activity. Hence, coexpression of CD39 and CD161 by CD4+ T cells might serve as a biomarker to monitor Th17 responsiveness. Collectively, CD39 and CD161 modulate human Th17 responses in CD through alterations in purinergic nucleotide–mediated responses and ASM catalytic bioactivity, respectively. The Journal of Immunology, 2014, 193: 000–000.

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D4+ Th cells play an important role in adaptive immune responses. Upon Ag stimulation, CD4+ Th cells expand and differentiate into Th1, Th2, and regulatory T cells (Tregs) (1). A subset of IL-17–producing Th cells, termed Th17 cells, recently was established as a unique Th cell lineage (2). These cells appear to play critical roles in protective antibacterial responses, which, when perturbed, may contribute to the pathogenesis of inflammatory diseases and related disorders (3, 4). Indeed, inflammatory conditions, such as Crohn’s disease (CD), are characterized by predominant Th17 responses (5, 6).

CD4+ T cell differentiation into Th17 cells requires Ag-presentation stimulation by MHC complexes on dendritic cells, together with an appropriate proinflammatory cytokine milieu (e.g., IL-6, IL-1β, IL-23, and TGF-β). This process provides innate host defense against intracellular and extracellular pathogens and also promotes inflammatory responses, with persistence of adaptive immune responses (7, 8). Inhibiting Th17 responses was shown to mitigate the progression of inflammatory diseases (9, 10). Therefore, molecular mechanisms underpinning the modulation of human Th17 differentiation and development are a topic of great interest.

Murine naive CD4+ T cells can be differentiated into Th17 cells under certain circumstance in vitro (11), whereas in humans, memory CD4+ T cells appear to produce abundant IL-17 (12, 13). Recently, CD161 (also known as killer cell lectin-like receptor subfamily B member 1 or NKR-P1A), was found to be a relatively specific phenotypic marker of populations of these human Th17 cells (14, 15). These CD4+CD161+ T cells in the circulation and those within inflamed tissues of patients with CD are noted to possess IL-17–producing properties (15). Nevertheless, the frequency of CD4+CD161+ T cells in inflamed tissues of patients with CD is comparable to that seen in healthy donors or in non diseased tissues of these patients (15), indicating that CD161 alone might not be a pertinent marker defining human Th17 cells.

CD39/ENTPD1 is a cell surface–located prototypic member of the ectonucleoside triphosphate diphosphohydrolase family (16). Previously, we showed in the mouse that CD39 expression is noted on two subpopulations of CD4+ T cells. One subset also expresses CD73/ecto-5’-nucleotidase and largely consists of Tregs (17). The subset that does not express CD73 has memory phenotypic markers and secretes proinflammatory cytokines upon activation, typically representative of Th1, Th2, and Th17 effector subtypes (18). In humans, we note that CD39 expression by CD4+ T cells also distinguishes between regulatory memory T lymphocytes and other CD4+ T cell populations. The latter, seemingly pathogenic, cell populations have the capacity to produce proinflammatory cytokines inclusive of IL-17 (19).
Acid sphingomyelinase (ASM) is a hydrolase enzyme located at the plasma membrane, which plays an important role in mediating cell signaling by catalyzing sphingomyelin into ceramide (20). ASM was shown to promote LPS-induced proinflammatory cytokine release from macrophages. Furthermore, ASM inhibition protects mice against dextran sulfate sodium–induced colitis (21). However, it remains unclear whether ASM has the potential to modulate Th17 cell differentiation by specifically impacting ceramide-mediated cellular signaling pathways.

In this study, we observe substantial increases in IL-17–producing CD39+CD161+ CD4+ T cell subpopulations in both peripheral blood and lamina propria of patients with active CD. Coexpression of CD39 and CD161 modulates human CD4+ Th17 responses by interactions that are inclusive of links to ASM. Upon activation, this putative molecular association boosts ASM enzymatic activity, which enhances cellular ceramide production to further impact downstream signaling components via mammalian target of rapamycin (mTOR) and STAT3. These signaling pathways are crucial elements that control Th17 generation (22–24). Importantly, we also show that blockade of ASM bioactivity, with imipramine or by knockdown of ASM, limits CD4+ Th17 generation from T cells derived from healthy volunteers, as well as from patients with CD.

We suggest that CD39 and CD161 coexpression may serve as a reliable surface maker of Th17 cells in CD. We further note and describe novel and possibly linked elements of both extracellular nucleotide and sphingolipid homeostasis that are pivotal in the control of Th17 cells and which might be exploited therapeutically.

Materials and Methods

General reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. All cell culture media and reagents were from Life Technologies (Grand Island, NY), and all cytokines were from R&D Systems (Minneapolis, MN).

Abs

FACS studies were performed using FITC–, PE–, PE-Cy5–, PE-Cy7–, Pacific blue–, or allophycocyanin-conjugated anti-human Abs to CD4 (clone OKT4), CD3 (HIT3a), CD161 (HP-3G10), IL-17A (BL168), IFN-γ (AS.B1), IL-10 (JES3-19F1), and CCR6 (TG7) (all from BioLegend, San Diego, CA); CD39 (BU61) (Ancell, Bayport, MN); CD45RO (UCHL1) (eBioscience, San Diego, CA); IL-23R (218213) (R&D Systems); phospho-STAT3 (pS727) (49/p-Stat3) (BD Biosciences, Franklin Lakes, NJ); or phospho-mTOR (Ser2448) (#2971) (Cell Signaling Technology, Danvers, MA). Isotype-control Abs (IgG1) were from Ancell or Bio- 

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Abs used for Western blot included phospho-Akt (Ser473) (#9271), phospho-mTOR (Ser2448) (#2971), phospho-PKM2 (Tyr105) (#9272), phospho-AMPKα (Thr172) (#2535), phospho-AMPKβ1 (Ser177) (#4851), and phospho-ACC (Ser79) (#3261) (all from Cell Signaling Technology); ASM (SM201) (Cell Signaling Technology and R&D Systems); CD161 (D1X12) and CD4 (RPA-T4) (BD Biosciences); CD39 (BU61) (Ancell); or β-actin (AC-15, ab6276) (Abcam, Cambridge, MA).

Study populations

PBMCs were isolated from platelet-depleted blood (leukofilters) obtained from healthy blood donors (Blood Donor Center at Children’s Hospital, Boston, MA) or from patients with CD (active disease or in remission). A total of 49 patients with CD was recruited in the study (22 men and 27 women; age range, 24–71 y; ileocolonic or colonic disease). Among those patients, 11 had received or were still receiving anti-TNF treatment, 1 was being treated with oral steroids, and the other 37 patients were studied at either the initial presentation or while not on active immunomodulatory treatments (inclusive of anti-TNF, steroids, or thiopurines). All diagnoses were confirmed by clinical, radiologic, endoscopic, and histologic criteria. All human studies were conducted in accordance with the Declaration of Helsinki principles and were approved by the Beth Israel Deaconess Medical Center Institutional Review Committee (No. 2011-P-00022828). Written informed consent was obtained from all study participants.

Cell culture

Peripheral blood CD4+ T cells from healthy volunteers or patients with CD were isolated using a Human CD4+ T cell Enrichment Cocktail Kit (STEMCELL Technologies, Vancouver, BC, Canada) with slight modifications. Subtypes of CD3+CD4+ T cells were positively selected using a FACSaria cell sorter (BD Biosciences, San Jose, CA) with enrichment to >99% cell population. Cells were cultured in complete RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% nonessential amino acids, and 10% FCS.

Lamina propria mononuclear cells

Lamina propria mononuclear cells were isolated from freshly biopsied colonic mucosal tissues using a previously described method with minor modifications (25). Briefly, tissues were incubated in HBSS containing EDTA (0.75 mM) and DTT (1 mM) at 37°C to remove the epithelium, followed by incubation with digestion cocktails (RPMI 1640 medium containing collagenase IV [400 U/ml] and Dnase I [0.01 mg/ml]) at 37°C for 1–2 h. The digested tissues were filtered through a 100-µm cell strainer and centrifuged at 1500 rpm for 5 min. Cell pellets were resuspended in complete RPMI 1640 media and overlaid on a 40–100% Ficoll gradient (GE Healthcare Life Sciences, Pittsburgh, PA). After centrifugation, the interface (lamina propria mononuclear cells) was collected and washed once for subsequent experimentations.

Flow cytometric analysis

Flow cytometric analysis was performed as previously described (17) with slight modifications. The relevant fluorescent-labeled anti-CD4 or anti-CD3 Abs were used to set up the required compensations for flow cytometric analyses. For surface marker analyses, cells were stained with Abs diluted in PBS containing 0.1% BSA. For adenosine deaminase (ADA) expression assessment, cells were incubated with rabbit anti-human ADA Ab (Sigma-Aldrich; #AB001399), followed by fluorescein-conjugated anti-rabbit Ab (Santa Cruz Biotechnology). For intracellular staining, cells were treated for 5 h with PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (10 µg/ml). After surface staining, cells were permeabilized with 0.5% saponin in PBS for 20 min, followed by incubation with fluorescein-conjugated Abs. To determine intracellular phospho-mTOR levels, cells were incubated with phospho-mTOR (Ser2448) Ab after three washes with PBS containing 0.5% saponin, followed by fluorescein-conjugated anti-rabbit Ab. Fluorescein-labeled isotype IgG was used as a negative control in all cases. FACS data were acquired on a multicolor LSR II (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

In vitro T cell differentiation

Sorted CD4+ T cells were cultured in complete RPMI 1640 media. A total of 5 × 10^5 T cells was stimulated with anti-CD3/CD28 T-activation Dynabeads (Life Technologies) at a 1:1 cell/bead ratio. For Th17 polarization, cytokine cocktails including IL-6 (30 ng/ml), IL-1β (10 ng/ml), IL-23 (10 ng/ml), and TGF-β1 (1 ng/ml) were added to the cultures. Cell cultures supplemented with IL-12 (10 ng/ml), IL-4 (10 ng/ml), or IL-2 (10 ng/ml), together with TGF-β1 (10 ng/ml), were used for deviation toward Th1 cells, Th2 cells, or Tregs, respectively. For agonist or antagonist studies, chemical compounds or control vehicles were introduced to cell cultures at the beginning of stimulation.

Quantitative real-time PCR

Total RNA was extracted from cells using the RNeasy Kit (Qiagen, Valencia, CA) and retro-transcribed into cDNA using ABI Prism TaqMan reverse transcription reagents (cat. no. 204054; Applied Biosystems, Foster City, CA). Specific primers for quantitative real-time PCR (qRT-PCR) were obtained from Life Technologies; the sequences are shown in Supplemental Table I. Then, qRT-PCR was performed using a QuantFast SYBR Green PCR Kit (cat. no 204054; Qiagen) on a Stratagene Fast Real Time Machine (Mx3005P) (Agilent Technologies, Santa Clara, CA). Relative expression was calculated using the ΔΔCt algorithm and GAPDH as an internal control.

Confocal microscopy

Cells were stained with Abs to CD39 (FITC; Ancell) and CD161 (PE-Cy5; BioLegend) and viewed with a confocal microscope (ZEISS LSM 510 Meta; Carl Zeiss Microscopy, Thornwood, NY).
**Coimmunoprecipitation and immunoblotting**

Cells were washed with ice-cold PBS three times and lysed on ice in modified RIPA buffer (50 mM Tris-\(\text{HCl}\) [pH 7.4], 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl) supplemented with Complete Protease Inhibitor Cocktails (Roche Diagnostics, Indianapolis, IN) and Phosphatase Inhibitor Cocktails (Sigma-Aldrich). The lysates were spun at 10,000 \(\times g\) for 5 min at 4°C.

Protein concentrations were determined by Bio-Rad DC protein assay reagents (Bio-Rad, Hercules, CA).

For immunoprecipitation, 300 \(\mu\)g total lysates from each sample was incubated with mouse anti-human Ab to CD39 (BU61; Ancell), ASM (SMPD1, R&D Systems), or control IgG (BD Biosciences), with end-over-end rotation at 4°C for 5 h. A total of 30 \(\mu\)g protein G-Sepharose 4B (Sigma-Aldrich) was added to each mixture and incubated at 4°C overnight. The mixtures were centrifuged at 4°C for 5 min at 2500 rpm, washed five times with RIPA buffer, and eluted with 2× XT Sample Buffer (cat. no. 161-0791; Bio-Rad), followed by Western blot analysis.

For Western blotting, proteins were separated on 4–12% Criterion XT Bis-Tris SDS-PAGE gels (Bio-Rad) and transferred to a polyvinylidene difluoride membrane (cat. no. IPVH0010; EMD Millipore, Billerica, MA) by semidry electroblotting. The membranes were then probed with specific Abs against proteins of interest. Bands were visualized using HRP-conjugated goat anti-mouse, donkey anti-rabbit, or donkey anti-sheep IgG (Thermo Scientific, Rockford, IL) and SuperSignal West Femto Maximum Sensitivity Substrate reagents (cat. no. PI-34096; Thermo Scientific), according to the manufacturer’s instructions.

**Ectonucleotidase enzymatic activity analysis**

Cell-associated nucleoside triphosphate diphosphohydrolase activity was analyzed by thin layer chromatography (TLC), a technique fully established in the laboratory (26). Freshly sorted CD4+ T cells (2 \(\times 10^6\) and at other concentrations, as required) were incubated with 2 mCi/ml \([14C]\text{ADP}\) (GE Healthcare Life Sciences) in 10 mM Ca\(^{2+}\) and 5 mM Mg\(^{2+}\) for the indicated times. The mixtures were centrifuged at 4°C for 5 to 30 min. Then 5-\(\mu\)l aliquots were removed at each time point and analyzed for the presence of \([14C]\text{ADP}\) hydrolysis products by TLC. \([14C]\text{ADP}\), \([14C]\text{AMP}\), and \([14C]\)adenosine (ADO) incubated in PBS served as standards. The TLC glass plate was exposed in a storage phosphor cassette for 24 h, followed by visualization of phosphor release captured by a Strom Scanner. ADO uptake was inhibited by the use of the Ca\(^{2+}\) channel blocker diprydamole (10 \(\mu\)M).

**ASM activity assay**

Enzymatic activity of ASM of CD4+ T cells was measured, as previously reported, with minor modifications (27). Levels of derived ceramide, the lipid cleavage product of ASM, were used as an indicator for ASM bioactivity. Briefly, cells (1 \(\times 10^6\)) were processed in lysis buffer (50 mM sodium acetate [pH 5], 0.5% Nonidet P-40). A total of 10 \(\mu\)g cell lysates was incubated with 0.2 mg sphingomyelin (dissolved in a 1:1 mixture of chloroform and methanol) for 2 h and then loaded onto a TLC plate, followed by chromatographic separation using a solvent system of chloroform and methanol (3:2). Ceramide production was visualized in iodine vapor, identified using a standard run in parallel, and scanned by a scanner. Relative ceramide levels were evaluated by ImageJ software (National Institutes of Health).

**Ab cross-linking experiment**

Mouse anti-human IgG of CD39 (4 \(\mu\)g/ml; Ancell) or CD161 (4 \(\mu\)g/ml, BD Biosciences) or both were incubated with goat anti-mouse IgG (4 \(\mu\)g/ml; BD Biosciences) at 37°C for 20 min. These cross-linked Ab complexes were preincubated with modified CD4+ T subsets (1 \(\times 10^6\) for the indicated times from 0 to 30 min). Reactions were stopped by suspending samples on dry ice, followed by subsequent analysis of ceramide levels. Mouse anti-human IgG (4 \(\mu\)g/ml) was used as control.

**ASM knockdown by short hairpin RNAs**

CD4+ T cells purified from healthy human blood were infected separately with lentiviral transduction particles (Life Technologies) containing an empty short hairpin RNA (shRNA) vector control (sh-C; pLKO.1-puro) or four human ASM shRNAs (sh-1: NM_000543.2-367s1c1, TRCN0000049014; sh-2: NM_000543.2-1054s1c1, TRCN0000049016; sh-3: NM_000543.2-1467s1c1, TRCN0000049015; sh-4: NM_000543.2-1225s1c1, TRCN0000049017), according to the manufacturer’s instructions. Recombinant lentiviral particles were produced by transient transfection of 293FT cells as described (28). CD4+ T cells were infected with lentiviral particles according to a previously described method, with minor modifications (29). Briefly, after stimulation with anti-CD3/CD28 Abs (both at 2 \(\mu\)g/ml) and human rIL-2 (1 ng/ml; R&D Systems) for 24 h, CD4+ T cells were infected by lentiviral particles by centrifugation at 2300 rpm for 1 h at room temperature in the presence of polybren (8 \(\mu\)g/ml). These cells were replenished with fresh media containing human rIL-2 (1 ng/ml; R&D Systems) for 48 h, followed by selection with puromycin (2.25 \(\mu\)g/ml) for an additional 5–10 d. The surviving cells were collected using the Dead Cell Removal Kit (Miltenyi Biotec, San Diego, CA) to deplete apoptotic cells, followed by examination of ASM protein expression using Western blot analysis.

**Statistical analysis**

Results in this study are expressed as the mean ± SEM of values. Differences between experimental groups were assessed by one-way ANOVA. The two-tailed Student t test was used to compare two groups, and the Tukey–Kramer multiple-comparison test was used to compare multiple groups. Correlations between subtype cell population and clinical activity was calculated by the Spearman test. Significance was defined as \(p < 0.05\).

**Results**

**CD39\(^{-}\)CD161\(^{+}\) CD4\(^{+}\) T cells exhibit molecular signatures of Th17 cells**

CD4+ T cells were isolated from peripheral blood of healthy volunteers and analyzed by FACS. These cells were grouped into four distinguishable subpopulations, based on the differential expression of CD39 and CD161 (Fig. 1A). To determine the exact nature of these subpopulations, we examined the expression of Th17 phenotypic markers by FACS and/or qRT-PCR analyses. CD39\(^{-}\)CD161\(^{+}\) cells exhibited robust expression of Th17 markers, such as chemokine receptors inclusive of CCR5, CCR6, and CXCR3, and cytokine receptors, including TGF\(_{\beta}1\), IL-23R, and IL-6R (30), compared with the other three subpopulations (Fig. 1B, 1C). Interestingly, the vast majority of CD39\(^{-}\)CD161\(^{+}\) cells appeared to be of the memory cell phenotype (CD45RO\(^{+}\)), with prototypic Th17 features (5, 31).

To determine whether dual expression of CD39/CD161 correlates with Th17 differentiation, we next directly stimulated quiescent peripheral blood CD4+ T cells with various cytokines and under different T cell–polarizing conditions for 18 h. We noted that the frequencies of CD39\(^{-}\)CD161\(^{+}\) populations increased markedly under these Th17-differentiation conditions (with or without IL-1\(\beta\) + IL-23), in contrast to parallel studies with conditions skewing differentiation toward Tregs (anti-CD3/28 + TGF-\(\beta\)1 + IL-2) (Fig. 1D) or Th1/2 cells (data not shown). Moreover, in the absence of Th17-skewing cytokines, the 72-h stimulation with anti-CD3/CD28 T-activation Dynabeads alone had minimal effects on the induction of IL-17 expression in any of the four CD4+ T cell subsets (data not shown). This induction of CD3 expression and Th17 cells was further linked to STAT3 signaling (23).

We then evaluated the proinflammatory potential and metabolic characteristics of CD4\(^{-}\)CD39\(^{-}\)CD161\(^{+}\) T cells. The four subpopulations were FACS sorted and then cultured under the same Th17-differentiation conditions. After 72 h, the CD4\(^{-}\)CD39\(^{-}\)CD161\(^{+}\) population produced substantial levels of intracellular IL-17 (Fig. 2A, Supplemental Fig. 1A). This activity was concordant with other typical aspects of Th17 cells (22–24, 30, 32, 33): robust gene expression of Th17 markers (IL-17, IL-22, and ROR\gamma), induction of glycolysis modulators (HIF1\(_{\alpha}\) and LDH1) (Fig. 2B), and enhanced signaling via Akt-mTOR and STAT3, together with other expected metabolic activation (PKM2 and AMPK-ACC) (34, 35) (Fig. 2C, 2D).

Furthermore, blocking glycolysis (by 2-DG), IL-17A, and LDH1) (Fig. 2B), and enhanced signaling via Akt-mTOR and STAT3, together with other expected metabolic activation (PKM2 and AMPK-ACC) (34, 35) (Fig. 2C, 2D).
Supplemental Fig. 1B). These data suggest that CD39+CD161+ Th17 cells are preferentially active metabolically with respect to glycolytic pathways (32, 33). Taken together, the data clearly show that coexpression of CD39 and CD161 defines a CD4+ T cell population that is more highly enriched in Th17 cells and has distinct phenotypic features.

CD39 and CD161 coexpression denotes Th17 populations in CD

To further investigate the physiological relevance of CD39 and CD161 coexpression in CD, we next contrasted patterns of CD39 and CD161 expression in CD4+ T cells isolated from peripheral blood and lamina propria of healthy volunteers and from patients with CD.

We noted that levels of CD4+ T cells expressing CD39+, CD161+, or the double-positive label of CD39+CD161+ in blood or lamina propria were consistently higher in patients with active CD compared with healthy controls (Fig. 3A, 3B). Concurrently, levels of CD39+ CD4+ and/or CD39+CD161+ CD4+ T cells in patients with active CD were significantly higher than those in patients with quiescent disease. In contrast, levels of CD4+ CD161+ T cells without the additional CD39 expression marker in these lamina propria cells did not allow differentiation between patients with active and inactive CD (Fig. 3B).
Given the above findings, we next evaluated relationships between CD4+ T cell expression of CD39/CD161 and clinical disease activity scores. The varied levels of CD39+ and CD39+CD161+ CD4+ T cells in peripheral blood correlated positively with disease activity using the Harvey Bradshaw Index (36) (Supplemental Fig. 2A). In contrast, levels of lamina propria CD161+CD4+ T cells, not considering levels of CD39 expression, did not show any direct statistical correlation with disease activity (Supplemental Fig. 2A).

It was noted previously that lamina propria CD4+ T cells from patients with active CD express higher levels of intracellular IL-17.
**FIGURE 3.** CD4+CD39+ CD161+ T cells are indicative of Th17 responses in CD. (A and B) CD4+ T cells were isolated from healthy volunteers and patients with active or inactive CD using peripheral blood (n = 36, 16, or 12, respectively) or lamina propria (n = 17, 15, or 6, respectively) cells. Representative FACS analysis of subpopulations sorted by CD39 and CD161 (A) are expressed as a percentage of total CD4+ T cells (B). (C-E) CD4+ T cells were purified from lamina propria of patients with active CD (n = 8) and treated with PMA (50 nM) and ionomycin (500 nM) for 5 h. This was followed by flow cytometry analysis of intracellular IL-17 and IFN-γ (C and D) and CD39 and CD161 expression patterns in gated IL-17+CD4+ T cells (E). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
and IFN-γ (37, 38). In this study, we demonstrated that lamina propria CD4+CD39+CD161+ T cells in patients with active inflammation also exhibited substantial levels of intracellular IL-17 and IFN-γ (Fig. 3C, 3D), in association with high levels of mTOR phosphorylation (Supplemental Fig. 2B, 2C) and STAT3 (data not shown).

We also noted that most IL-17+ CD4+ T cells express both CD39 and CD161 (Fig. 3E). Therefore, dual expression of high levels of CD39 and CD161 by CD4+ T cells appears indicative of the Th17 phenotype within both the gut and the peripheral blood of patients with CD.

**CD39 associates with CD161 on CD4+ T cells**

Next, we investigated how dual expression of CD39 and CD161 might impact Th17 cell phenotype and functions. Because both molecules are cell surface markers, we first examined their localization on CD4+ T cell membranes. We noted that CD39 potentially colocalized with CD161 in CD4+ T cells, as shown by confocal imaging and coimmunoprecipitation studies (Fig. 4A, 4B). Such associations were noted regardless of the state of cell activation (resting or after activation with anti-CD3/CD28 beads).

In addition to CD39, differential expression and activity of other purinergic ecto-enzymes in sorted CD4+ T cell subsets were examined. Our specific focus was on the ecto-enzymes CD73 [ecto-5'-nucleotidase, which catalyzes the hydrolysis of AMP to ADO (39)] and ADA [which catalyzes deamination of ADO to form inosine (40)].

The two CD39+ subpopulations exhibit substantial CD39 activities, whereas CD39+CD161+ cells contained higher ADA expression and possible specific activities (inferred by higher inosine levels) (Fig. 4C–E). Hence, ADO is readily cleaved by ADA on Th17 cells to form inosine (Fig. 4C), which has no effect on human Th17 generation (data not shown). These functional data were concordant with the protein expression levels of these molecules, in that CD39+CD161+ cells were shown to express high levels of ADA (Fig. 4D, 4E).

We previously showed that extracellular purine metabolism, with ADO generation as modulated by CD39 and CD73, impacts murine adaptive immune responses (16, 17). We then tested the relevance of this system in human CD4+ Th17 cells. In contrast to mouse cells (16, 17, 23), purines (i.e., ATP, AMP, or ADO) exogenously added to CD39+CD161+ cells had no substantial impact on human Th17 generation (Fig. 4F). These data infer that rapid scavenging of ATP and ADO by the high levels of CD39 and ADA expressed by CD4+CD39+CD161+ cells results in this particular Th17 phenotype. These cells are refractory to both ATP (because of CD39 activity) and ADO (because of ADA associations, which rapidly deaminates this nucleoside).

**CD39–CD161 association involves ASM activation and impacts STAT3/mTOR signaling in vitro**

Human CD161 was shown to interact with, and then activate, ASM to generate ceramide, a second messenger with prominent roles in NK cell biology (41). In this study, we evaluated the role of ASM in human Th17 development. Production of ceramide, a lipid cleavage product of ASM and an indicator of bioactivity, was dramatically elevated by 2 h and reached a plateau after 24 h under Th17-polarization conditions (data not shown).

This change was noted to occur with increased activation of Akt-mTOR and STAT3 signaling. Conversely, inhibition of ASM activity in CD4+ T cells by imipramine substantially blocked ceramide generation and concurrently inhibited Akt-mTOR and STAT3 activation (Fig. 5A), which differentially limited IL-17+ cell induction (Fig. 5B). The results suggest that ASM bioactivity might be an integral component, at least in part, of Th17 generation.

We then examined the properties of possible CD39/CD161 colocalization, as might concern ASM activation. By performing coimmunoprecipitation, we also noted that ASM associates with CD39 and CD161 in human CD4+ T cells (data not shown), and in CD4+CD39+CD161+ T cells. ASM has the potential to interact in the same manner with both CD39 and CD161 in quiescent cells, as well as in cells stimulated to drive Th17 differentiation (Fig. 5C).

Next, we detailed profiles of ASM enzymatic activity among the four CD4+ T cell subpopulations. At 48 h post-Th17 differentiation, robust ceramide generation was observed only in CD4+ CD39+CD161+ cells (Fig. 5D). No differences in ASM protein levels were noted among the subsets (data not shown).

We then evaluated the impact of manipulating cells, by cross-linking Abs to CD39 and/or CD161, on ASM activity in the four freshly isolated CD4+ T subsets. Ceramide production indicating ASM bioactivity was stimulated by TNF (27) or by these cross-linked Abs to CD161 (41). These processes appeared regulated albeit shown to be transient. We measured ceramide production at various time points after the addition of cross-linked Abs. Peak ASM activity was observed at or before 5 min post-stimulation. As shown in Fig. 5E, cross-linking Abs to either CD39 or CD161 appeared to augment ASM activity in CD39+CD161+ cells, reaching the highest level at 5 min, but not in CD39–CD161− cells (data not shown). Additionally, additive effects were seen when both Abs were applied (Fig. 5E).

Lastly, blocking ASM activity (by imipramine) or mTOR signaling (by rapamycin) markedly decreased Th17 generation of CD4+CD39+CD161+ T cells, in contrast to cells treated with vehicle only (Fig. 5F).

Because of the pivotal role of ASM in Th17 generation, as suggested above with the use of the pharmacological inhibitor imipramine, we next tested shRNA treatment to “knockdown” ASM levels in healthy blood CD4+ T cells (Fig. 6A). In these studies, two shRNAs, termed sh-1 and sh-3, were used to assess the effect of direct downregulation of ASM on downstream STAT3 signals and Th17 generation.

**Knockdown of ASM or ASM inhibition abrogates Th17 generation**

Because of the pivotal role of ASM in Th17 generation, as suggested above with the use of the pharmacological inhibitor imipramine, we next tested shRNA treatment to “knockdown” ASM levels in healthy blood CD4+ T cells (Fig. 6A). In these studies, two shRNAs, termed sh-1 and sh-3, were used to assess the effect of direct downregulation of ASM on downstream STAT3 signals and Th17 generation.

Compared with sh-C, knockdown of ASM by sh-1 or sh-3 significantly inhibited ASM bioactivity, as determined by ceramide production (Fig. 6B), STAT3 signaling (Fig. 6C), and decreased IL-17 levels (Fig. 6D). These data further suggest ASM links to STAT3 signaling that might be associated with Th17 generation.

Next, CD4+ T cells from lamina propria of patients with active CD were treated with imipramine, followed by evaluation of all aspects of Th17 differentiation, as above. Ceramide production was substantially abrogated by imipramine (Fig. 7A), concurrently with decreased activation of Akt-mTOR and STAT3 signaling (data not shown) and diminished levels of intracellular IL-17 and IFN-γ (Fig. 7B, 7C). These data infer that blockade of ASM bioactivity might have therapeutic potential in Th17-dominant inflammatory states, such as in CD.
Discussion

Th17 cells are novel and important immunomodulatory cells in the mammalian immune system. These proinflammatory lymphoid cells are crucial in mediating host defense against both intracellular and extracellular pathogens. Moreover, Th17 cells were implicated in the pathogenesis of CD and other inflammatory conditions (5, 6). We (10) and other investigators (42, 43) showed that the likely inhibition of Th17 responses can lead to amelioration of inflammation in experimental models of intestinal disease and other inflammatory disorders. Hence, more detailed molecular insights into Th17 generation are of paramount importance in developing new therapies to supplement the current clinical approaches in CD.

Specifically identifying Th17 cells in patients has remained problematic, which is due, in part, to the lack of reliable surface
markers. Recently, CD161 was identified as a relatively specific surface marker for human Th17 cells, with application specifically to CD (14, 15). However, CD161 expression is also associated with NK cells and, as we demonstrate in this study, CD161 alone is insufficient for depicting and characterizing these Th17 cells and associated responses.

We note in this article that dual expression of CD39 and CD161 more clearly defines Th17 lineages of human CD4+ T cells. Furthermore, we also show that blood and lamina propria levels of IL-17 and IFN-γ (72 h) were evaluated by TLC (A) and FACS (B), respectively. (C) CD39+CD161+ and CD39−CD161− CD4+ T cells were stimulated with Th17-driving conditions for 12 h or were left unstimulated (0 h). Cell lysates were used for coimmunoprecipitation against anti-ASM Ab, followed by Western blot using the probes indicated. TLC analysis of ceramide production by 48-h Th17-deviated CD4+ T cell subsets (D) or CD4+CD39+CD161+ T cells stimulated with cross-linked anti-CD39 Ab (4 μg/ml), anti-CD161 Ab (4 μg/ml), or both for different times (E). Mouse anti-human IgG (4 μg/ml) served as control. (F) Effects of imipramine or rapamycin on IL-17 and IFN-γ production by CD4+CD39+CD161+ T cells at 72 h post-Th17 polarization. Data are mean ± SEM of three or four independent experiments. *p < 0.05 versus the other three groups.

Extracellular purinergic products, including ATP and ADO, were reported to impact the differentiation and function of murine CD4+ T cells, as highlighted by the contribution of ADO in mediating the suppressive function of Tregs (17, 44) and the instructive role of ATP in Th17 cell differentiation (44). CD39 is the major ectonucleotidase expressed by regulatory type immune cells, and it sequentially hydrolyzes extracellular nucleotides, such as ATP and ADP, which are involved in immune purinergic signaling, to AMP. This AMP is degraded to ADO via catalytic activity of CD73, and the nucleoside product is further converted to inosine by ADA (16).

Interestingly, one of our recent studies showed CD73 to be a phenotypic marker of an effector memory Th17 cell subset in...
CD (45). In this study, we note that human CD39⁺CD161⁺ CD4⁺ T cells express high levels of ecto-NTPDase as well and that extracellular ADO is extremely short lived, as a result of higher rates of ADA-mediated catabolism. Supplementing purine products ATP, AMP, or ADO did not affect expansion of CD39⁺ CD161⁺ CD4⁺ Th17 cells in these models, given the rapid

FIGURE 6. Knockdown of ASM blocks STAT3 signals and Th17 generation. (A) Western blotting of ASM expression in healthy blood CD4⁺ T cells after inhibition of ASM using lentiviral shRNAs. Sh-C and ASM knockdown (sh-1 and sh-3) healthy peripheral blood CD4⁺ T cells were stimulated with anti-CD3/CD28 beads under the defined Th17 differentiation conditions, followed by representative TLC analyses of ceramide at 2 h (B), flow cytometry analysis of intracellular p-STAT3 at 3 h (C), and intracellular IL-17 and IFN-γ expression at 96 h (D). Data are mean ± SEM of three independent experiments. *p < 0.05 versus the sh-C group.

FIGURE 7. ASM inhibition abrogates in vitro generation of Th17 cells using lamina propria CD4⁺ T cells purified from patients with CD. Lamina propria CD4⁺ T cells isolated from patients with active disease were stimulated with anti-CD3/CD28 activation beads in the presence of vehicle or imipramine (20 µM), followed by TLC analysis of ceramide levels at 2 h (n = 3) (A) and flow cytometry determination of intracellular IL-17 and IFN-γ expression at 5 h (n = 6) (B and C). *p < 0.05, **p < 0.01 versus vehicle.
cleavage of both extracellular nucleotides and ADO. Moreover, blocking or activating purinergic receptors, including type 1 (P1) and type 2 (P2), did not markedly promote or block Th17 expansion of CD39+CD161+ CD4+ T cells, suggesting a degree of resistance in these Th17 cells to such mediators.

However, in the current study we identified another aspect of purinergic signaling, wherein CD39 might play a mechanistic role in shaping human Th17 responses by influencing any of the putative associations between CD161 and ASM. ASM, the hydrolase-type enzyme converting sphingomyelin to ceramide (41), appears to be one of the key lipid cellular signals regulating cellular differentiation, proliferation, and apoptosis (46, 47). CD161 was shown to modulate NK cellular functions via ASM–ceramide-mediated signaling (41). We showed previously that CD39 may directly interact with RanBPM (48), a scaffolding protein linked to GTPases and c-Met signaling (49). These novel molecular associations between CD39 and CD161 might also link ASM-dependent mTOR/STAT3-signaling transduction to the control of Th17 cell responses in both normal and pathological conditions.

We further demonstrate that, in CD4+ T cells, ASM enzymatic activity (as indicated by ceramide production) mediates intracellular signaling inclusive of mTOR and STAT3, key putative elements controlling Th17 generation (22–24). Upon stimulation, in conjunction with CD161, CD39 is associated with ASM bioactivity to a greater extent in human CD4+ T cells. Furthermore, augmented ASM activity might govern the extent of human Th17 generation through modulating mTOR/STAT3 signals, whereas inhibition or knockdown of ASM abrogates mTOR/STAT3 signaling and Th17 cell generation.

A recent study showed that mTOR signaling mediates glycolysis during Th17 differentiation in an HIF1α-dependent manner (32). We affirm that the chief signaling transduction components controlling Th17 generation of CD39+ CD161+ CD4 T cells in our studies are ultimately altered to meet the needs of Th17 polarization. Such fundamental cellular aspects/functions of CD39+CD161+ Th17 cells appear to be regulated by ASM.

Finally, imipramine, in addition to other known functions, was recently shown to inhibit ASM bioactivity and, hence, to block ceramide generation (51). Such pharmacological activity limits inflammatory responses in an experimental colitis model (52). In this study, we demonstrate that imipramine treatment blocks both mTOR and STAT3 signals when CD4+ T cells from controls and from patients with active CD are studied. This salutary effect is concurrent with ASM blockade and impaired Th17 generation. Furthermore, in line with recent observations in mice, Th17 function is tightly controlled by STAT3-regulated ectonucleotidase expression (23). We also note that blockade of ASM-mTOR/STAT3 signals inhibits human Th17 cell expression of CD39. This inhibitory effect, secondary to limited activation of ASM, may eventually limit Th17 generation.

CD39 and CD161 coexpression and functional data taken together suggest associations between purinergic signaling and sphingolipid/ceramide metabolism. We also demonstrate putative molecular associations between CD161 and ASM-dependent intracellular signaling transduction that appear crucial in promoting human Th17 responses in both health and disease. Our findings further improve the understanding of the molecular control of Th17 responses, provide possible new alternatives to current clinical approaches to suppress Th17 responsiveness, and may help to direct novel strategies for the diagnosis, prevention, and treatment of inflammation.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Fig. 1. STAT3 and AMPK signaling controls the Th17 phenotype of CD4⁺CD39⁺CD161⁺ cells. (A) Frequency of four healthy peripheral blood CD4⁺ T cell subsets expressing IL-17 or IFNγ post 72 hr. Th17-polarization (n=16). (B) Effects of WP1066 (STAT3 inhibitor), AICAR (AMPK activator), or compound C (AMPK inhibitor) on IL-17 and IFNγ production by CD4⁺CD39⁺CD161⁺ T cells at 72 hr. after Th17 differentiation. ***P < 0.001, ns, not significant, vs. other three groups.
Supplemental Fig. 2. CD39 and CD161 are valuable makers for tracking degree of illness in Crohn’s disease (CD) patients. (A) Correlation between disease activity of CD (HBI) and percentage of CD4⁺ T cell subsets in peripheral blood (PB) (n = 28) or lamina propria (LP) (n = 19) of patients with active CD. (B, C) Levels of phosphorylated mTOR in four CD4⁺ T cell subsets from lamina propria of active CD patients (n=3), the shaded graph showed staining with isotype control antibody. *P < 0.05 vs. other three groups.
**Supplemental Fig. 3. Inhibition of ASM, mTOR or STAT3 blocks CD39 expression on Th17 cells.** CD4⁺ T cells from healthy peripheral blood were cultured under Th17 differentiation condition for 72 hr, in the presence of vehicle, imipramine, rapamycin, or WP1066. The cells before culture and treatment were set up as the basal Control. Representative FACS analyses of CD39 expression were shown (n = 5). The shaded graph showed staining with isotype control antibody.
Supplemental Table 1. qRT-PCR primers.

<table>
<thead>
<tr>
<th>Transcript</th>
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| CCR5       | Forward 5'-AATGAGAAGAAGAGGCACAGGGCT-3'  
             | Reverse 5'-TCTCTGTACCTGCATAGCTTGGT-3' |
| CCR6       | Forward 5'-TGTCACAGAAGTCTGGCTTTCT-3'  
             | Reverse 5'-ACTTCCTTTCACACACCACAGGT-3' |
| CXCR3      | Forward 5'-ACATAGTTCTGCGACCAGCTCTT-3'  
             | Reverse 5'-AAGATGAAGTCTGGAGGGCGAAG-3' |
| TGFR1      | Forward 5'-TGTTCTATATCTGCGACACCCGCA-3'  
             | Reverse 5'-TGAGCCAGAAGCTGAGTCTGAT-3' |
| IL-23R     | Forward 5'-ACTGCAGTCTGCTAGTCCCACAC-3'  
             | Reverse 5'-ATTCCAGGCTGACTGCTGAT-3' |
| IL-6R      | Forward 5'-AGCCTCCCCAGTGCAAGATTCTTCT-3'  
             | Reverse 5'-TGCATGCTTGTCTTTGCTCT-3' |
| IL-17      | Forward 5'-AGGCCATAGTGAGGAGAGCTCCAC-3'  
             | Reverse 5'-ATTCCAAAGGTGAGGTGATCGAT-3' |
| RORγ       | Forward 5'-ACCAGCTCTTTTGGAAGTGAGGAA-3'  
             | Reverse 5'-TCCAGAAGCTGGAAGCTGAG-3' |
| IL-22      | Forward 5'-TGCTGATATTGACAGGCTGCAAC-3'  
             | Reverse 5'-TGGAGCTGTGAGCTGACGAGT-3' |
| HIF1α      | Forward 5'-TTGGCGAGCAACAGCAGCAACTG-3'  
             | Reverse 5'-TTGAGTGCAGGTCAGCAGTAC-3' |
| LDH1       | Forward 5'-TGGTCAGCTGCTAGAAGCTTGAT-3'  
             | Reverse 5'-TTGCAACCCGCTTCAATAACACGG-3' |
| Glu1       | Forward 5'-ATCGTGCCCATTTGGCTTCTTGTG-3'  
             | Reverse 5'-CTGGAAGCAGCATGCCCAATGAA-3' |
| GAPDH      | Forward 5'-CCTGCACCACACACTGCTTTA-3'  
             | Reverse 5'-CATGAGTCTTCCACGATACCA-3' |

The primer sequences used in the experiments.