The Role of CD39 in Modulating Effector Immune Responses in Inflammatory Bowel Disease

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Glossary of Abbreviations

A2A: Adenosine A2A receptor
APC: Allophycocyanin fluorochrome
APC-Cy7: Allophycocyanin-cyanine 7
CCR6: Chemokine receptor 6
CD: Crohn’s disease
DSS: Dextran sodium sulfate
FITC: Fluorescein isothiocyanate fluorochrome
FOXP3: Forkhead box P3
IBD: Inflammatory bowel disease
iTreg: Induced regulatory T cells
LPMC: Lamina propria mononuclear cells
MFI: Mean fluorescence intensity
nTreg: Natural regulatory T cells
PB: Pacific blue fluorochrome
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate buffered saline
PE: Phycoerythrin fluorochrome
PE-Cy7: Phycoerythrin-cyanine 7 fluorochrome
RIPA buffer: Radioimmunoprecipitation assay buffer
SDS: Sodium dodecyl sulfate
SEM: Standard error of the mean
STAT: Signal Transducer and Activator of Transcription
supTh17: T helper type 17 cell with suppressor activity
Th17: T helper type 17
TLC: Thin layer chromatography
Treg: Regulatory T cell
UC: Ulcerative colitis
WT: Wild-type
Introduction

Inflammatory bowel disease (IBD) is composed of two major disorders, Crohn’s disease (CD) and ulcerative colitis (UC). While ulcerative colitis primarily impacts the colon and involves inflammation of the mucosal layer, Crohn’s disease can affect any portion of the gastrointestinal tract from the oral cavity to the anus. Crohn’s is also characterized by transmural inflammation. Inflammatory bowel disease predominantly affects individuals between 15 and 30 years of age (1). IBD manifests before 20 years of age in about one-fifth of patients with Crohn’s and one-tenth of ulcerative colitis patients (2). The predominant clinical symptoms seen in IBD patients include gastrointestinal symptoms, such as bloody diarrhea, tenesmus, and abdominal pain, as well as extraintestinal symptoms, such as aphthous stomatitis, erythema nodosum, pyoderma gangrenosum, uveitis, clubbing, jaundice, or hepatomegaly (3, 4). Although clinical features are quite similar among adults, the pediatric patient population may develop unique complications, such as growth failure and skeletal development (5, 6).

IBD is primarily diagnosed by a combination of laboratory tests, radiographic studies, endoscopy, and biopsies. Currently, the exact pathophysiological processes that lead to disease development are not thoroughly elucidated. Recently, there have been evolving data that demonstrate the impact that disordered regulatory T-cells (Treg) can play in excessive inflammation in the intestinal tract in genetically-susceptible individuals (7). Naive CD4\(^+\) T cells have been shown to differentiate into Th1, Th2, Th17 and Treg cells, upon recognition of cognate antigen in the context of associated environmental signals, as generated by cytokines or an inflammatory milieu. Immune cell plasticity in the gut may therefore be very relevant in IBD. Tregs were first described in 1970 and initially named as “suppressor T cells” (8). In the 1990s, a group of researchers showed that the CD4 pos (CD4\(^+\)) T cells expressing the \(\alpha\)-chain of the IL-2 receptor, CD25, were able to promote CD4 and CD8 T-cell mediated allograft unresponsiveness in cyclosporine-A treated mice (9). Subsequent studies have shown that CD4\(^+\) T cells expressing CD25 were essential for the maintenance of self-tolerance in mice (10-12). Since then, further research has demonstrated that CD4\(^+\)CD25\(^+\) cells in
humans were phenotypically similar to their murine counterparts, and that this population was able to suppress the proliferation of CD4\textsuperscript{pos}CD25\textsuperscript{neg} cells in vitro (13-15). In 2003, the transcription factor forkhead box P3 (Foxp3) was shown to control the generation and function of murine Tregs (16-18), and in later experiments, FOXP3 in humans was shown to be expressed predominantly by the CD4\textsuperscript{+} cells that expressed the highest level of CD25 (19). Moreover, Foxp3 regulates the transcription of genes for protein receptors on Treg cells, such as cytotoxic T lymphocyte antigen-4 (CTLA-4) (20). There are two subsets of Treg cells, natural Treg (nTreg) cells and induced Treg (iTreg) cells. While nTreg cells develop as a distinct lineage in the thymus, iTreg cells arise from peripheral naïve conventional T cells and can be generated in vitro (21). Both TGF-\textbeta1 and IL-2 are required for iTreg cell induction. TGF-\textbeta1 signaling promotes the binding of NFAT and Smad3 to the conserved non-coding sequence-1 (CNS1) enhancer and ultimately stimulates histone acetylation and Foxp3 induction (22).

The gastrointestinal tract is a major entry for microbial and dietary antigens. As a result, both beneficial commensal microflora and pathogens may reside in the gut. Thus, a significant regulatory mechanism within the gastrointestinal tract lies with Tregs, which can survey a diverse array of immune responses to maintain homeostasis and suppress inflammation. The transcription factor Foxp3 is critical in Treg development and stability (23). In the absence of Foxp3, inflammation increases, which supports the concept that Tregs are crucial for immune regulation and suppression of effector T cells.

Dynamic balances of Treg to Th17 cells in the intestine, alterations in bacterial flora and other environmental factors may regulate inflammatory responses both locally and systemically. Indeed, patients with Crohn's disease have decreased numbers of Treg in the blood and colon (7). While various T cell subsets orchestrate the host defense responses against pathogens, Th17 cells in particular have been shown to contribute to immunopathology in autoimmune diseases, such as in IBD (24). Consequently, it is theorized that massive lymphocyte infiltration of the gut lamina propria with T helper type 1 (Th1) and 17 (Th17) contributes to IBD (25-27).

Given that Tregs are also involved in the maintenance of immune homeostasis, Treg cellular defects have been observed in association with numerous other
autoimmune disorders, such as vascular thrombophilia, multiple sclerosis, rheumatoid arthritis, type 1 diabetes, and inflammatory bowel disease (28-30). Inflammation in the colon begins when dendritic cells on the mucosal epithelial layer of the lumen sense pathogenic bacteria and other antigens. Upon activation of MHC complexes by pathogens, local dendritic cells migrate to the lymph nodes to activate naïve T cells, which undergo differentiation into antigen-specific, pro-inflammatory effector T cells that fight infection in the lamina propria (12). Treg help prevent excess activation of pro-inflammatory T cells, such as Th1, Th2, and Th17 cells.

Through multiple signaling pathways, Tregs can exert immunosuppressive function. One of the mechanisms is thought to be due to the release of inhibitory cytokines, such as IL-10, TGF-β, and IL-35 (13). Another important pathway involves the Treg mediated generation of extracellular adenosine, which binds its receptor on the surface of pro-inflammatory T cells and decreases their development and proliferation (31). Two enzymes expressed constitutively on the surface of Treg cells are CD39 and CD73. The ectonucleotidase CD39 is expressed constitutively by murine Tregs and by a subset of human Tregs to catalyze the degradation of the pro-inflammatory molecule adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (32, 33). Subsequently, the ADP/AMP generated by ATP hydrolysis is degraded into adenosine by a second ecto-nucleotidase, CD73 on Tregs (31). Please see figure 1 on page 28 in the “Figures Section” for a schematic delineating CD39 and CD73.

The adenosine produced from the hydrolysis cascade initiated by CD39 has numerous anti-inflammatory properties and is also capable of binding the adenosine A\textsubscript{2A} receptor (ADORA\textsubscript{2A}) on effector T cells to block their activation and proliferation (34, 35). Previous studies have shown that Tregs in CD39 knock-out mice demonstrate a 50-60% reduction in their ability to suppress T cell proliferation when compared to those from wild-type mice (36). Furthermore, CD39 positive Tregs are also capable of suppressing IL-17 production (37). Another cell surface protein called CD26 has also been implicated in immune regulation, signal transduction, and apoptosis (33). Recently, research has demonstrated the importance of a group of proteins called the
Signal Transducer and Activator of Transcription (STAT) family which directs the differentiation of T helper cells, with specific STAT proteins promoting distinct effector subsets (38). For instance, Stat3 promotes the differentiation of Th17 (38) and can also modulate Th17 immunosuppressive activity by up regulating CD39 (39).

In a mouse model of chemically-induced acute colitis, CD39 knockout animals showed greater disease severity than controls that had colitis but expressed CD39. Moreover, the knockouts displayed elevated infiltration of leukocytes and colon wall thickening (40). Of note, a polymorphism associated with low levels of CD39 expression was significantly associated with Crohn’s patients, whereas healthy controls were enriched for the high CD39-expressing allele (p=0.0006) (41). Defective numbers of CD39+ Treg have also been reported in patients with multiple sclerosis and autoimmune hepatitis (42). Although the expression of CD39 by human Treg has been reported, the expression of CD39 by human Th17 cells has not been investigated yet.

T helper type 17 cells (Th17) constitute a subset of IL-17 producing effector T cells that develops separately from Th1 and Th2 cell lineages. Th17 cells have been shown to drive inflammatory and autoimmune conditions in both humans and mice and to be associated with intestinal inflammation, such as Crohn’s disease (31). Th17 cells are also critical in mediating protective antibacterial responses which, when perturbed, may contribute to the pathogenesis of inflammatory conditions (43). Inhibiting Th17 responses has been shown to mitigate the progression of inflammatory diseases (44).

When exposed to TGF-β in conjunction with IL-6 or IL-21 in mice and to IL-6, TGF-β, and IL-1β in humans, CD4+ T-cells can be differentiated into Th17 cells (45). Th17 cells can also develop into induced regulatory T cells or iTreg under the influence of TGF-β (46). Nonetheless, iTreg and Th17 cells may not be terminally differentiated and iTreg has shown phenotypic and functional plasticity.

Using genetic lineage tracing of Foxp3 Treg, Zhou and colleagues observed that a significant proportion of Foxp3+ cells undergo down-regulation and in some cases loss of Foxp3 expression is noted (47). These “ex-Foxp3” cells display an effector memory cell phenotype, produce pro-inflammatory cytokines and are numerically increased in experimental autoimmune diabetes (47). Moreover, exposure of Treg to IL-
6 can down-regulate both Foxp3 and IL-17 expression, suggesting that Treg may be transformed to Th17-like cells (48). In addition, it has been reported that Treg can further acquire effector properties, such as IFNγ production, when cultured in the presence of IL-12 (49). These Tregs show diminished suppressive activity that can be only partially reversed by blockade of IFNγ or IL-12 removal (49).

The stimulation of naïve T-cells with TGF-β and IL-6 triggers IL-17 production, but it also induces the expression of IL-10, limiting the pathogenic potential of these cells (50). Indeed, additional studies have reported that IL-17+ T-cells can limit tissue damage during inflammation (39, 51). In experimental murine tumor settings, it has been demonstrated that CD39 and CD73 expressed by suppressor Th17 cells (supTh17) suppress tumor-specific immunity (52). However, there has been no investigation to date on whether comparable human supTh17 cells exist.

In this study, we aim to elucidate a population of human supTh17 cells with immune suppressive properties that display high levels of CD39 and Foxp3 in contrast to prototypic pathogenic Th17. We hypothesize that expression of CD39 in humans confers regulatory properties to effector Th17 cells, which limits their pathogenic potential. We theorize that circulating and lamina propria derived Th17 cells from patients with inflammatory bowel disease are defective in their CD39 expression and ability to acquire regulatory properties. Thus, inability of effector Th17 cells to suppress and respond to immune-modulation could represent a mechanism involved in tissue damage perpetuation.

Our new observations also provide mechanistic insights into the development of supTh17 as well as the role of CD39 in purinergic immunomodulation. The pathophysiological relevance of these cells is supported by the detection of decreased frequencies of CD39+ supTh17 cells in both peripheral blood and lamina propria of patients with Crohn’s disease.

**Part of the following work has been previously published in manuscript form (53).**

Roles of the student and colleagues:
Maria Huang (Student): Helped design, plan, and perform the experiments to generate
iTreg and supTh17 cells from CD4 cells, to determine the expression of CD39 and CD73 among the various cell types, to quantify the level of supTh17 from Crohn’s disease patients compared with healthy subjects. I also collaborated with Dr. Longhi to collect and analyze data to create figures 2-6. I helped write and edit the manuscript for publication, and I read more extensively on recent literature to incorporate new findings into this report. Only the methods section and some of the figures are adapted from the published manuscript. All of the other sections in this report were written independently by myself with feedback from Dr. Longhi and Dr. Robson.

Dr. Simon Robson is the Principal Investigator of the lab and suggested helpful background literature when I initially joined the lab. He also offered feedback on experimental design, data analysis, manuscript writing, and SIM report writing.

Dr. Serena Maria Longhi: Research fellow who taught me laboratory techniques for flow cytometry, purification of lamina propria mononuclear cells, and peripheral blood mononuclear cells. She also offered suggestions for improvement on my experimental design/techniques, data analysis, manuscript writing, and feedback on my SIM report.

Dr. Aiping Bai is a post-doctoral fellow who offered suggestions on experimental techniques. Dr. Yan Wu is the lab manager who provided assistance with troubleshooting on experimental protocols and offered feedback on data presented during lab meetings.

Drs. Adam Cheifetz and Francisco Quintana offered feedback on experimental design and suggestions for improving the published manuscript.

Methods

Subjects

Peripheral blood mononuclear cells (PBMCs) were isolated from platelet-depleted blood (leukofilters) obtained from 68 healthy blood donors at the Blood Donor Center of Boston Children’s Hospital. PBMCs were also obtained from 25 patients with Crohn’s disease who were recruited from the Gastroenterology Division at Beth Israel Deaconess Medical Center (BIDMC). Of these patients, 11 were studied during active disease (median Harvey Bradshaw Index, HBI: 8, range 2 to 25), while 14 were in
remission (median HBI: 0, range 0–12). The Harvey Bradshaw Index has been used to assess the degree of illness among patients with Crohn’s disease and has a 0.93 correlation coefficient with the Crohn’s Disease Activity Index (CDAI) (52). At the time of investigations, 11 patients were receiving infliximab, 2 were on steroids and 2 on immunomodulatory drugs. The study was approved by the BIDMC Institutional Review Committee. Written consent was obtained from all study participants.

Cell Purification

PBMCs were obtained by density gradient centrifugation on Ficoll-Paque (GE Healthcare, Uppsala, Sweden). Cell viability, determined by Trypan Blue exclusion, exceeded 98%. Lamina propria mononuclear cells (LPMCs) were isolated from freshly biopsied colonic tissue. The tissue was initially washed with phosphate buffered saline (PBS), cut into small sections, and incubated in calcium and magnesium-free Hanks’ Balanced Salt Solution containing 4 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol at 37°C for 15 min. Epithelia were removed by discarding the supernatants. This procedure was repeated three times. The tissue was then minced, re-suspended in RPMI 1640 medium containing 10% FCS, 400 U/ml collagenase D and 0.01 mg/ml DNase I, and then incubated at 37°C for 1.5 hours with pipetting every 30 min. The digested tissue was filtered and centrifuged at 600g for 7 min. Collected cells were pelleted, re-suspended in PBS 1% FCS and stained as indicated below.

Cell Sorting and Culture

CD4<sup>mem</sup> and CD4<sup>naive</sup> cells were sorted as CD4<sup>+</sup>CD45RO<sup>+</sup> and CD4<sup>+</sup>CD45RA<sup>+</sup> from PBMCs using a cell sorter (purity higher than 98%). Cells were cultured in complete RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acids and 10% fetal calf serum (FCS) and exposed for 3 days to Th17 polarizing conditions, i.e. IL-6 (50 ng/ml)+IL-1β (10 ng/ml)+TGF-β (3 ng/ml) (54-56) and anti-CD3/anti-CD28 T-cell
expander (bead/cell ratio: 1/50). Cells were then stimulated for 4 days in the presence of iTreg polarizing conditions comprised of high concentration IL-2 (300 U/ml) and T-cell expander (bead/cell ratio: 1/2) (57, 58) and then re-exposed to the same Th17 polarizing conditions as above for an additional 3 days. Cells obtained after exposure to Th17 and iTreg polarizing conditions are referred to as Th17 and iTreg; cells obtained after iTreg exposure to Th17 driving conditions are indicated as supTh17.

Flow Cytometry

Cell phenotype was assessed by 6-color flow cytometry following cell incubation with fluorochromes fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyamine 7 (PE-Cy7), Pacific blue (PB), allophycocyanin (APC) and allophycocyanin-cyamine 7 (APC-Cy7)-conjugated anti-human antibodies to: CD4, CD45RO, CD45RA, CD25, CD26, CD39, CD73, CCR6 and IL-23R. Frequency of FOXP3, RORC and Stat 3 positive cells was assessed by intracellular staining following cell fixation and permeabilization with Cytofix/Cytoperm and incubation with PB, APC and PE-conjugated anti-human FOXP3, RORC and Stat 3. Frequency of cytokine-producing cells was determined after exposure to phorbol 12-myristate 13-acetate (PMA, 10 ng/ml) and Ionomycin (500 ng/ml) for 60 minutes and to Brefeldin A (20 mg/ml) for an additional 5 hours. Staining was carried out using PE, PB, and APC-conjugated anti-human antibodies to IFNγ, IL-17A, IL-10, IL-2, and IL-22.

Cells were acquired on a BD LSRII flow cytometer and analyzed using BD FACSDiva software. 3–5x10^4 events were acquired for each sample. Positively stained cell populations were gated based on unstained, single stained and isotype control stained controls. Effect of adenosine on Th17, iTreg and supTh17 phenotype was assessed in parallel experiments. Adenosine was added at each stage of T cell development, i.e. 50 µM to memory CD4 cells at baseline; after 3 days when exposing cells to iTreg polarizing conditions; and after an additional 4 days when re-stimulating cells in the presence of Th17 skewing conditions. Controls consisted of cultures in the absence of adenosine.
In vitro Suppression Assay

The ability of Th17, iTreg and supTh17 to control target cell proliferation and effector cytokine production was evaluated following a 4-day co-culture with CD4 responder cells. Following 24 hours of incubation in cytokine and bead-free medium, Th17, iTreg and supTh17 were added at 1/8 ratio to autologous CD4 target cells (2.5x10^4 cells/well) previously exposed to IL-2 (30 U/ml) and T-cell expander (bead/cell ratio: 1:2) for 5 to 7 days. The 1:8 ratio was selected because in preliminary experiments where ratios of 1:16, 1:8, 1:4 and 1:2 were used, the 1:8 ratio exerted detectable regulatory function, which could putatively reflect pathophysiological proportions between suppressor and effector lymphocytes. Parallel cultures of CD4 responder cells and of Th17, iTreg and supTh17 on their own were performed under identical conditions. All experiments were performed in duplicates.

After 4 days, cultures were pulsed with 0.25 μCi/well ^3^H-thymidine and harvested 18 hours later using a cell harvester. Incorporated thymidine was measured by liquid scintillation spectroscopy. In preliminary experiments, inhibition of CD4 target cell proliferation in the absence and presence of suppressor cells was also analyzed using carboxy fluorescein succinimidyl ester (CFSE) staining. As CFSE- and ^3^H-thymidine-based assays gave comparable results, given the requirement for fewer cells, ^3^H-thymidine was used to measure proliferation in subsequent experiments. The ability of Th17, iTreg and supTh17 cells to control the production of IFNγ and IL-17 by target cells was determined by intracellular cytokine staining after 4-day co-culture as detailed above. The effect of adenosine on Th17, iTreg and supTh17 ability to suppress was tested in parallel experiments.

Ectonucleotidase Enzymatic Activity Analysis

Thin layer chromatography (TLC) was performed as previously described (36, 59). 3x10^5 Th17, iTreg and supTh17 were incubated with 2mCi/ml [C^14]ADP in 10 mM Ca^{2+} and 5 mM Mg^{2+}. Then, 5 μL aliquots, collected at 5, 10, 20, 40 and 60 minutes,
were analyzed for the presence of $[\text{C}^{14}]\text{ADP}$ hydrolysis products by TLC and applied onto silica gel matrix plates. $[\text{C}^{14}]\text{ADP}$ and the radiolabeled derivatives were separated using an appropriate solvent mixture as previously described (60).

**Statistical Analysis**

Results are expressed as mean +/- SEM (obtained from at least 5 subjects per group and from at least 3 independent in vitro experiments). Smirnov goodness of fit test was performed to test the normality of variable distribution. Paired and unpaired Student’s t-test were used for comparing normally distributed data; Wilcoxon’s rank sum test and Mann Whitney test were used for non-normally distributed data. ANOVA repeated measures or one-way ANOVA, followed by Tukey’s multiple comparisons test, was used to compare the means of multiple samples. For all comparisons a P value $\leq 0.05$ was considered significant. Statistical analysis was performed using SPSS version 19.0.

**Results**

**supTh17 Cells are Phenotypically Different from Prototypic Th17 Cells and Exert Regulatory Function**

To assess whether human Th17 cells could acquire regulatory functions, we activated CD4$^+$CD45RO$^+$ memory (CD4$^{\text{mem}}$) and CD4$^+$CD45RA$^+$ naïve (CD4$^{\text{naïve}}$) T-cells under Th17 polarizing conditions by exposing them to cytokines IL-6, IL-1β, and TGF-β for 3 days, as well as low-dose anti-CD3 and anti-CD28, which have previously been shown to lead to the differentiation of IL-17 producing cells in humans (54-56, 61). Then, we exposed these cells to iTreg polarizing conditions with a high concentration of IL-2 and anti-CD3/anti-CD28 for 4 days. These conditions have been shown to be effective at inducing high numbers of iTreg (57, 61). Finally, to assess the stability of the polarized T-cells, we re-activated them in the presence of Th17 driving conditions as detailed earlier.

Our results showed that iTreg obtained from CD4$^{\text{mem}}$-derived Th17 cells
demonstrated persistent and stable suppressor activity following re-activation in the setting of Th17 polarizing conditions (Figure 2). (Please see page 28 for figure 2.) In contrast, iTReg obtained from CD4 naïve-derived Th17 cells lost most of their suppressive ability once re-activated in the presence of Th17 polarizing conditions (Figure 2). Therefore, we focused subsequent studies on iTReg derived from CD4 mem cells.

Figure 3 illustrates the phenotype of CD4 mem cells at baseline, after 3 days of exposure to Th17 polarizing conditions, after 4 days of exposure to iTReg polarizing conditions, and then after 3 days of re-exposure to Th17 driving cytokines. At baseline, CD4 mem cells showed low frequencies of IL-17-producing, CD25+ and FOXP3+ lymphocytes (Figure 3A). After 3 days of exposure to Th17 polarizing cytokines IL-6, IL-1β and TGF-β, CD4 mem cells contained higher frequencies of IL-17-producing cells, while they still maintained low frequencies of CD25+ and FOXP3+ lymphocytes (Figure 3A). (Please see page 29 for figure 3.)

Cells that were obtained following Th17 exposure to iTReg polarizing conditions showed a decrease in the number of IL-17+ lymphocytes and an increase in the frequency of CD25+ and FOXP3+ cells (Figure 3A). Moreover, these cells displayed minimal proportions of effector cytokines, such as IFNγ or IL-2. After re-exposing the iTReg cells to Th17 polarizing conditions, phenotypic changes that were seen included increases in the number of cells producing IL-17, decreases in CD25+ and FOXP3+ lymphocytes that were similar to iTReg although higher than prototypic Th17 cells (Figure 3A). Also in contrast to prototypic Th17, these supTh17 cells displayed higher expression of RORC, higher numbers of IL-22+ lymphocytes and similar proportions of cells positive for chemokine receptor 6 (CCR6) and IL-23 receptor (IL-23R) (Figure 3B).

When considering suppressive functions (Figure 4A), we noted that supTh17 controlled CD4 target cell proliferation in a manner comparable to iTReg, and did so more effectively than prototypic Th17 cells. In terms of suppressing pro-inflammatory cytokine production (Figures 4B), supTh17 effectively controlled IL-17 and IFNγ
cytokine production by CD4 effector cells. In contrast, although iTreg effectively inhibited production of IL-17, it exerted only weak control over CD4 T-cell IFNγ production. (Please see page 30 for figure 4.)

These results demonstrated that supTh17 can be obtained following exposure of CD4<sup>mem</sup>-derived iTreg to Th17 polarizing conditions. In contrast to prototypic Th17, these cells contain higher frequencies of IL-17 producing and FOXP3<sup>+</sup> lymphocytes and furthermore display effective and stable suppressive function.

**supTh17 Cells Express CD39 and CD73 to Facilitate ATP Hydrolysis into Adenosine**

Because of supTh17 cells’ regulatory properties and the putative role of CD39 in immunoregulation (36, 37), we examined the expression of CD39 in supTh17 and compared it with that of CD4<sup>mem</sup> cells at baseline, Th17 and iTreg. As demonstrated in Figures 5A and 5B, supTh17 contained the highest numbers of CD39<sup>+</sup> cells as well as the CD39 mean fluorescence intensity (MFI), in contrast to prototypic Th17 cells that displayed lower frequencies of CD39<sup>+</sup> lymphocytes and lower CD39 MFI. (Please see page 31 for figure 5.)

Next, we evaluated the phenotypic properties of CD39<sup>+</sup> cells within supTh17 and compared them with those of CD39<sup>+</sup> cells within CD4<sup>mem</sup> at baseline, Th17 and iTreg (Figure 5C). Interestingly, supTh17 cells contained proportions of cells positive for CD73 comparable to iTreg and higher than Th17 cells and CD4<sup>mem</sup> cells at baseline (Figure 5C). CD73 is the ectonucleotidase working in conjunction with CD39 to generate adenosine from ATP. The concomitant expression of CD39 and CD73 by supTh17 and iTreg suggests that both of these cells could be capable of hydrolyzing the pro-inflammatory ATP molecule into adenosine, which has immune suppressive properties (36). To verify this, we assessed the cell ectoenzymatic activity with thin layer chromatography (TLC) following cell incubation with [C<sup>14</sup>] radiolabeled ADP.

As portrayed in Figure 5D, supTh17 and iTreg both generated adenosine that supTh17 cells further effectively degraded into inosine through deamination. Inosine is a
metabolite that has also been shown to exert immune-suppressive modulatory effect in mouse models (62). In contrast, prototypic Th17 cells could only hydrolyze ADP into AMP but was incapable of generating extracellular adenosine, in accordance with their low levels of CD39 and CD73 expression. Thus, the TLC results verified that the concomitant CD39 and CD73 expression enabled supTh17 to generate adenosine, which is then effectively degraded into inosine.

**supTh17 in Healthy Subjects and Patients with Crohn’s Disease**

To evaluate the clinical significance of supTh17, we determined the frequency of CD4^+IL-17^+ and supTh17 in peripheral blood mononuclear cells (PBMCs) and lamina propria mononuclear cells (LPMCs) from healthy subjects and patients with Crohn’s disease. The supTh17 were identified by first gating CD4^+CD45RO^+ cells within PBMCs or LPMCs and then by determining the proportion of CD39^+IL-17^+ and FOXP3^+ cells within this population.

Although the proportion of CD4^+IL-17^+ in PBMCs cells was similar among both healthy subjects and patients, the proportion of CD4^+IL-17^+ lymphocytes obtained from the lamina propria was higher in Crohn’s patients than that in healthy subjects (Figure 6A). In patients, the frequency of CD4^+IL-17^+ cells was significantly higher in the lamina propria compared to the circulation (Figure 6A). Subsequently, we determined the frequency of supTh17 in both PBMCs and LPMCs and found that Crohn’s patients had decreased levels of supTh17 cells within PBMC and LPMC populations when compared to healthy subjects (Figure 6B). In both groups, supTh17 were increased in the lamina propria compared to the circulation (Figure 6B). (Please see page 32 for figure 6.)

Because the transcription factor Stat3 has previously been found to modulate Th17 immunosuppressive activity through up regulation of CD39 (39), we compared the level of Stat3 expression between healthy subjects and Crohn’s patients. The results showed that circulating supTh17 cells from Crohn’s patients displayed a higher proportion of cells positive for Stat3 than did the healthy subjects’ peripheral cells (Figure 6C). Interestingly, among both population groups, supTh17 from the lamina
propria contained higher proportions of lymphocytes positive for Stat 3 than did peripheral blood (Figure 6C).

Next, we examined the proportion of the cytokines TNF-α and IL-2 in supTh17 cells, as both cytokines have previously been found to be pro-inflammatory proteins that are decreased in populations of Th17 cells (63). The flow cytometry profile results show that supTh17 from Crohn’s disease patients had higher frequencies of TNF-α⁺ and IL-2⁺ cells than the respective supTh17 cells from the peripheral circulation of healthy subjects (Figure 6D). Furthermore, in both groups, supTh17 in the lamina propria contained higher proportions of TNF-α⁺ and IL-2⁺ cells than did their counterparts in peripheral circulation (Figure 6D). These results suggest that supTh17 are highly represented in the lamina propria and that the frequencies of these cells are lower in the periphery in Crohn’s disease. Additionally, these cells also express increased levels of pro-inflammatory cytokines.

Discussion

By exposing iTreg cells to Th17 polarizing conditions in vitro, we have been able to derive a population of human supTh17 cells. Interestingly, these supTh17 cells display overlapping features of both the effector Th17 and regulatory iTreg cells. More specifically, the phenotypic features characteristic of effector cells include production of IL-17, expression of CCR6, IL-22, and IL-23R, while the regulatory functions are reflected in its ability to inhibit CD4 cell proliferation and production of IFNy and IL-17. It is possible that the supTh17 cells are representative of a late stage in Th17 differentiation, in which the effector potential of prototypic Th17 cells is attenuated or might consist of cell subsets with overlapping regulatory and effector features.

By studying supTh17 cells we can provide a mechanism through which effector cells can be down-modulated with consequent loss of their pathogenic potential. This is relevant to Th17 cell biology and provides a novel mechanism for understanding the pathogenesis of inflammatory and autoimmune conditions, such as IBD.

Using the in vitro system in this project allowed us to observe changes in T-cell
phenotype and function upon stimulation in the presence of Th17 and iTreg polarizing conditions. The differentiation of CD4 cells into Th17 or iTreg following cell culture in vitro can facilitate better understanding of the modulation of antigen-primed CD4 memory cells during various disease states.

As demonstrated in previous research, the ectonucleotidase CD39 seems to play an important role in immunoregulation, especially in mediating suppressive function of iTreg. Consequently, we were interested in investigating whether supTh17 expressed CD39. When compared to the prototypic Th17 cells, supTh17 not only display high levels of CD39, but they also co-express the ectonucleotidase CD73, which is involved in metabolizing AMP to adenosine. This is clinically important because extracellular adenosine and adenosine A2A purine receptors have previously been identified as both anti-inflammatory signals and sensors of excessive inflammatory tissue damage (64). In contrast to the prototypic Th17, the supTh17 cells could generate adenosine similarly to iTreg. Experimental data indicate that the effective degradation of adenosine into inosine displayed by supTh17 relies on the co-expression of both ADA and CD26 (53). Moreover, because of the concomitant expression of adenosine deaminase and CD26 by supTh17, the extracellular adenosine that is generated undergoes further degradation to inosine, unlike that produced by prototypic Th17 (53). Inosine can also bind to adenosine receptors and exert immunomodulatory effects (65). For instance, recent murine studies have shown that inosine administration to immune-stimulated mouse peritoneal macrophages can reduce the production of several pro-inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin 1 (IL-1) and macrophage inflammatory protein α (MIP-1α) (62).

Interestingly, supTh17 cells seemed resistant to the effect of exogenous adenosine, as these cells neither underwent CD39 upregulation nor exhibited amelioration of suppressive function (53). It is plausible, then, that adenosine resistance in supTh17 is due to low levels of A2A adenosine receptor and higher levels of adenosine catalysis, which is enabled by ADA and CD26 co-expression. A prior study has shown that the A2A adenosine receptor mediates anti-inflammatory effects, as lymphocytes from A2A receptor mice exhibit higher rates of cell proliferation and
produce high IFNγ levels upon stimulation (66).

Though supTh17 are resistant to adenosinergic modulation and are not conventional suppressors, they still exert homeostatic properties by generating adenosine. These dual functions suggest that supTh17 could undertake both regulatory and pro-inflammatory roles based on the immunological context, which differentiates them from iTreg. For instance, supTh17 exerts suppressive regulatory function by generating adenosine. On the other hand, supTh17 can also be pro-inflammatory because of its low A2A level and thus maintain intrinsic resistance to the immune suppressive function of adenosine.

**Limitations**

Even though Crohn’s disease shares similarities with ulcerative colitis in terms of the inflammatory pathogenesis involving the dynamic balance between cytokines, intestinal microflora, and possible environmental factors, these two subtypes of IBD also display clinical differences, such as the transmural inflammation of Crohn’s disease as opposed to the mucosal and submucosal involvement in ulcerative colitis. Additionally, patients with Crohn’s disease often experience strictures or fissures that are not as prevalent among ulcerative colitis patients. Thus, since this study only contains peripheral blood mononuclear cells and lamina propria mononuclear cells from healthy individuals and Crohn’s patients, the results may not be generalizable for all IBD patients, as variations could exist for ulcerative colitis patients.

Furthermore, all of the experiments in this study were in vitro, and it is difficult to ensure that the Th17 cells traced over time can convert into iTreg and then supTh17 upon sequential exposure to Th17 and iTreg polarizing conditions in an in vivo model. Thus, incorporating in vivo data by using murine models may help elucidate the findings in a more clinically directed context. Please see a discussion of future in vivo studies in the section, “Suggestions for Future Work.”
Conclusion

The clinical relevance of this study is also important, as it demonstrates that supTh17 can be found in both the peripheral circulation and lamina propria of healthy subjects and Crohn’s disease patients. However, the results show that supTh17 cells preferentially home to the intestine, since a higher percentage of these cells are found in the lamina propria. Moreover, a higher percentage of supTh17 cells express the transcription factor Stat-3 in the lamina propria, which suggests that Stat-3 may play a CD39 expression and supTh17 induction in the colon (39). These results indicate that Th17 cells may undergo regulation in the colon. A previous study has shown that pathogenic Th17 cells undergo regulation in the intestine where they acquire functional and phenotypic properties similar to Tregs (63). However, in this previous study, the authors did not test whether Th17 cells that have acquired regulatory properties in the intestine also express the CD39 ectonucleotidase.

More strikingly, in patients with Crohn’s disease, the number of supTh17 cells is significantly decreased, which could consequently lead to exacerbation of disease given the impaired ability of effector Th17 to undergo regulation. Prior research has shown that numerically defective and dysfunctional Treg along with impaired immunoregulation in the context of increased numbers of effector Th17 cells (67). When compared with those in healthy controls, the supTh17 from Crohn’s disease patients also seemed to contain higher frequencies of TNF-α and IL-2, pro-inflammatory cytokines that could be also involved in the tissue damage. This suggests that the supTh17 in Crohn’s patients may have a more inflammatory phenotype.

Our results have demonstrated that when iTreg cells are exposed to Th17 driving conditions in vitro, supTh17 cells can develop. These supTh17 cells express high levels of CD39, which distinguishes them from effector pathogenic Th17 cells. The immune suppressive function of these cells could help ameliorate inflammatory autoimmune ailments, such as Crohn’s disease and ulcerative colitis. High levels of CD39 expression distinguish the supTh17 immune suppressive cells from effector pathogenic Th17 cells. We propose that these fundamental alterations in purinergic signaling might control tissue damage while limiting cellular pathogenicity in local and systemic inflammatory
illnesses, such as in Crohn’s disease. Promoting the local expansion of supTh17 cells and the maintenance of these should boost local immune suppressive activities and augment diminished Treg functionality, as previously noted in IBD (68). Indeed, these studies and development of modalities to boost CD39 expression have implications for the development of innovative therapeutic strategies to treat or cure Crohn’s disease.

Suggestions for Future Work

To investigate the expression of CD39 in vivo would constitute a major trajectory for future research. Given that CD39 plays a crucial role in suppressing inflammation and tissue injury by hydrolyzing extracellular ATP, it would be interesting to use animal models in which CD39 expression has been knocked out to determine the proportions of various T cell populations and cytokine production.

Recently, for instance, a study has transplanted livers from mice null for CD39 (CD39KO) as well as those from wild-type (WT) mice into recipients, and the results showed a significant reduction in the frequency of Tregs in CD39KO recipients than in WT (69). Also, WT liver allografts survived more than 100 days, whereas no CD39KO recipients survived beyond 40 days (69). Moreover, soluble CD39 administration prolonged the survival of CD39KO liver allograft, which further supports that CD39 can help modulate inflammation and suppress transplant rejection in vivo (69).

In future studies, colitis can be induced chemically by injecting mice with dextran sodium sulfate (DSS), which is a negatively charged sulfated polysaccharide that can result in damage to the epithelial monolayer lining the colon to allow the dissemination of pro-inflammatory intestinal contents into underlying tissue (70). DSS has been used in some studies to mimic the clinical and histological features of ulcerative colitis (70). Future experiments can use CD39KO mice with DSS-induced colitis to determine the level of supTh17 cells and pro-inflammatory cytokines, such as TNF-α and IL-2. The studies could also evaluate the functional properties of Th17 cells from both WT and CD39KO mice. Additionally, isolation of Th17 cells from WT and CD39 KO could be attempted with the aim of injecting these cells in adoptive transfer experiments and
subsequently exploring their pathogenic or immune-modulatory potential in vivo (e.g. in the DSS colitis model).

Furthermore, various stages of acute, chronic, and relapsing intestinal inflammation can be achieved by modifying the concentration of DSS and the frequency of administration. Ultimately, the goal will be directed toward understanding whether delivering CD39 or elevating endogenous levels of CD39 in IBD patients can increase the local pool of extracellular adenosine to have an immunosuppressive effect on pathogenic T cell populations.

References

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Figures

Figure 1. CD39 and CD73 are integral functional components of T regulatory cells.

Figure 2. Suppressor ability of iTreg derived from CD4\(^+\)CD45RO\(^+\) memory (CD4\(^{mem}\)) and CD4\(^+\)CD45RA\(^+\) naïve (CD4\(^{naive}\)) cells. Mean percentage (+SEM) suppression of CD4 effectors by CD4\(^{mem}\) and CD4\(^{naive}\) iTregs were measured before
and after exposure to IL-6, IL-1β, and TGF-β. CD4<sup>mem</sup> iTreg maintained their suppressor ability after exposure to Th17 driving conditions. *P<0.05

**Figure 3. Phenotypic properties displayed by supTh17 cells.** A) Representative flow cytometry plots of CD4 (X axis) and IL-17, CD25 and FOXP3 (Y axis) fluorescence. B) Representative histogram depicting RORC fluorescence in CD4<sup>mem</sup> at baseline, Th17 and supTh17; representative flow cytometry plots of CD4 (X axis) and CCR6, IL-23R and IL-22 (Y axis) fluorescence.
Figure 4. supTh17 cells demonstrate suppressive ability. A) Mean (+SEM) percentage inhibition of CD4 effector cell proliferation by Th17, iTreg and supTh17 cells. B) Mean (+SEM) percentage inhibition of CD4 effector cell IL-17 and IFNγ production by Th17, iTreg and supTh17 cells. When compared with prototypic Th17, supTh17 demonstrated more effective suppression of CD4 cell proliferation and pro-inflammatory cytokine production. *P<0.05; **P<0.01.
Figure 5. Expression of CD39 and CD73 ectonucleotidases and associated ectoenzymatic activity. Mean (+SEM) frequency of (A) CD39$^+$ cells, (B) CD39 mean fluorescence intensity (MFI) and of (C) CD39$^+$CD73$^+$ cells within CD4$^{\mathrm{mem}}$ at baseline and within Th17, iT-reg and supTh17. *P<0.05; **P<0.01; ***P<0.001. (D) CD39 ADPase enzymatic activity was evaluated by TLC following incubation of Th17, iTreg and supTh17 with $^{[14]}$C radiolabeled ADP substrates.
Figure 6. Demonstration of supTh17 cells in healthy subjects and associated decreases in Crohn’s disease. Flow cytometry was used to assess the frequency of CD4$^+$IL-17$^+$ and supTh17 in PBMCs and LPMCs. Mean (+SEM) frequency of (A) CD4$^+$IL-17$^+$ and of (B) supTh17 cells in the peripheral circulation and in the lamina propria. Mean (+SEM) frequency of supTh17 cells positive for (C) Stat-3 and for (D) TNF-α and IL-2 in the circulation and in the lamina propria. *P<0.05; **P<0.01; ***P<0.001.