Original Article

CD4⁺ Tissue-resident Memory T Cells Expand and Are a Major Source of Mucosal Tumour Necrosis Factor α in Active Crohn's Disease

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Abstract

Background and Aims: Tumour necrosis factor [TNF] α - and IL-17A-producing T cells are implicated in Crohn's disease [CD]. Tissue-resident memory T [T_{RM}] cells are tissue-restricted T cells that are regulated by PR zinc finger domain 1 [PRDM1], which has been implicated in pathogenic T_H17 cell responses. T_{RM} cells provide host defence but their role in CD is unknown. We thus examined CD4⁺T_{RM} cells in CD.

Methods: Colon samples were prospectively collected at endoscopy or surgery in CD and control subjects. Flow cytometry and ex vivo assays were performed to characterise CD4⁺T_{RM} cells.

Results: CD4⁺T_{RM} cells are the most abundant memory T cell population and are the major T cell source of mucosal TNF α in CD. CD4⁺T_{RM} cells are expanded in CD and more avidly produce IL-17A and TNF α relative to control cells. There was a unique population of TNF α ⁺IL-17A⁺ CD4⁺T_{RM} cells in CD which are largely absent in controls. PRDM1 was highly expressed by CD4⁺T_{RM} cells but not by other effector T cells. Suppression of *PRDM1* was associated with impaired induction of *IL17A* and *TNFA* by CD4⁺T_{RM} cells

Conclusions: CD4⁺T_{RM} cells are expanded in CD and are a major source of TNF α , suggesting that they are important in CD. PRDM1 is expressed by T_{RM} cells and may regulate their function. Collectively, this argues for prospective studies tracking CD4⁺T_{RM} cells over the disease course.

Key Words: T cells; tissue resident memory T cells; Crohn's disease; TNF; Th17 cells

1. Introduction

Crohn's disease [CD] is a chronic disorder characterised by segmental inflammation of the intestinal tract. CD4⁺ T cells are heavily implicated in the pathogenesis of CD.^{1,2} Furthermore, tumour

necrosis factor α [TNF α], a principal mediator of intestinal injury, is produced by CD4⁺ T cells and is directly associated with mucosal injury. Most contemporary and emerging biologics for CD either directly [anti-IL-23, anti- α 4 β 7] or partially [anti-TNFs] target

CD4⁺ T cells.^{3–6} Consistent with this, mucosal TNF α normalise with mucosal healing. However, despite new mechanisms of action, primary treatment failures remain common and rates of bowel resection have only marginally declined.⁷ Improving our understanding of T cell-mediated intestinal inflammation is therefore highly necessary to develop novel therapies.

Tissue resident-memory T cells [T_{RM}] are a recently described population of terminally differentiated memory T cells.^{8–10} T_{RM} cells are maintained in tissue and provide protective immunity.^{11–13} Consistent with their role in host defence, T_{RM} cells are enriched at mucocutaneous surfaces with high pathogen burden.^{11,12} The transcription factors PR domain zinc finger 1 [PRDM1] and homologue of BLIMP1 [HOBIT] are both required for the development of murine CD8⁺ T_{RM} cells.¹⁴ More broadly, T_{RM} cells across species and cell types exhibit overlap in expression of genes regulating cellular trafficking.^{15,16} In particular, nearly all T_{RM} cells express CD69 but have low expression of sphingosine-1-phosphate receptor 1 [S1PR1] and lymph node homing markers [CCR7, CD62L].¹⁷ Despite these similarities, however, there are important cell- and species-specific differences in T_{RM} cells.^{15,16,18–21}

T_{RM} cells are the most abundant memory T cells in the intestinal mucosa in healthy humans.^{9,10,22} In contrast to the consistently demonstrable role of T_{RM} cells in host defense, their role in immune-mediated conditions is not understood. Herein, we show that CD4⁺ T_{RM} cells are enriched in CD. In addition, CD4⁺ T_{RM} cells from CD patients more avidly produce TNF α and IL-17A compared with control CD4⁺ T_{RM} cells, and substantial fractions of T_H17 CD4⁺ T_{RM} cells also produce TNF α . Moreover, we show that CD4⁺ T_{RM} cells are a major T cell source of mucosal TNF α in CD. Finally, we demonstrate that PRDM1 is expressed more highly in T_{RM} cells compared with T_{EM} cells and that PRDM1 inhibition is associated with reduced expression of TNF α and IL-17A by CD4⁺ T_{RM} cells. Collectively, therefore, we show that this novel population of cells is enriched in CD and is a major source of mucosal TNF α .

2. Materials and Methods

2.1. Patients

Subject selection criteria for CD patients are presented in [Box 1](#). Tissue specimens from the colon were collected by ileocolonoscopy or surgical resection. All tissue underwent histological review and

Box 1. Study criteria for Crohn's disease patients.

Inclusion criteria

Active Crohn's
Referred for ileo-caecal resection
Referred for endoscopy
Age >18 years
Age <80 years

Exclusion criteria

Unable to provide informed consent
Pregnant
Incarcerated
Active malignancy
Lymphocytic colitis
Collagenous colitis
Eosinophilic enteritis
Ischaemic colitis
Active gastrointestinal infection

was excluded if dysplasia or other exclusionary conditions were detected. Controls included subjects who were undergoing surgery for cancer, segmental colonic resection for diverticulitis or other benign indications, or endoscopy for colon cancer screening. All patients and controls provided informed consent and were prospectively recruited. This study was approved by the University of Michigan Institutional Review Board.

2.2. Tissue sampling sites and lymphocyte isolation

Endoscopically obtained tissue consisted of 2–4 mucosal biopsies with standard forceps from areas with endoscopic inflammatory activity in CD subjects; normal tissue from controls was collected from the transverse colon. We obtained tissue from regions with macroscopically inactive CD [$n = 8$ subjects]; this tissue was paired with active regions in a subset of CD patients [$n = 6$]. Gross and microscopically normal tissue was obtained from control subjects after review by clinical pathologists confirming they were free of inflammation or infection [disease-free margins]. Mononuclear cells were isolated using a modification of previously described methods [Supplementary methods, available as [Supplementary data](#) at ECCO-JCC online].²³

2.3. Flow cytometry

Freshly isolated cells were rested overnight in RPMI supplemented with glutamine, sodium pyruvate, 100 units/ml penicillin, 100 100 μ g/ml streptomycin, and 10% fetal bovine serum before stimulation with 1X stimulation cocktail which contains PMA/Iono [eBiosciences/Thermo Fischer, Waltham, MA] for 4–6 h with Brefeldin A. Flow cytometry was then performed on the LSR II [BD Biosciences, Franklin Lakes, NJ] or the FACS ARIA II [BD Biosciences] and the data were analysed with FlowJo [Ashland, OR]; see the Supplementary methods [available as [Supplementary data](#) at ECCO-JCC online] for the flow cytometry antibodies and clones.

2.4. Isolation of CD4⁺T_{RM} cells and ex vivo stimulation

The human CD4⁺ T cell memory effector cell isolation kit [Miltenyi Biotec, Bergisch Gladbach, Germany] was used to isolate CD4⁺ T_{RM} cells. Mononuclear cells were isolated from the colon with biotinylated antibodies [Supplementary methods, available as [Supplementary data](#) at ECCO-JCC online]. Magnetically labelled naïve and central memory CD4⁺ T cells, CD8⁺ T cells, non- $\alpha\beta$ ⁺ T cells, antigen-presenting cells, and B cells were positively selected, leaving a [negatively selected] cellular fraction heavily enriched for CD4⁺ T_{RM} cells. These cells were washed and then pre-treated for 24 h with butyrate at 1 μ M or bortezomib at 25 nM or were left in complete RPMI. For this stimulation, all cells were then stimulated with PMA/Iono [1X cell stimulation cocktail, eBiosciences] for 24 h with or without continued butyrate and bortezomib without brefeldin A. This stimulation protocol was distinct from that used for analytical flow cytometry as noted above. Cells were then harvested for quantitative reverse transcription polymerase chain reaction [qRT-PCR] and supernatant was collected for multiplex enzyme linked immunosorbent assay.

2.5. Quantitative PCR

CD4⁺ T_{RM} cells were suspended in RLT buffer [RNAeasy kit, Qiagen, Valencia, CA], RNA was extracted, and cDNA was synthesised as per kit instructions [iScript cDNA Synthesis, Bio-Rad, Hercules, CA]. qRT-PCR was performed as per kit instructions [SYBR Green,

Figure 1. CD4⁺T_{RM} cells are enriched in Crohn's disease [CD] and are the major mucosal T cell population. **A**] Study design. Patients with clinically active CD were prospectively recruited, and colonic samples were obtained at surgery or endoscopy. Colonic tissue samples from normal areas from controls were obtained either at surgery for non-inflammatory bowel disease indications or at colonoscopy for colorectal cancer screening. **B**] Gating strategy. Tissue-resident memory cells were identified as CD69⁺CCR7⁺ cells within the CD45RO⁺ antigen experienced memory T cell pool. **C**] CD4⁺T_{RM} cells are enriched in CD and are the major mucosal T cell population. Intestinal CD4⁺T cells were delineated according to the gating strategy in [B] in controls and patients with CD. Representative flow plots [left panels] and quantified data are presented [right panel]. **D**] The distribution of CD8⁺T_{RM} cells is similar between controls and CD. Intestinal CD8⁺T cells were identified and data are presented as in [C]. **E**] Total CD4⁺T_{RM} cells are increased in CD. CD4⁺T_{RM} cells [CD4⁺CD45RO⁺CD69⁺CCR7⁺] from control and CD surgical specimens were enumerated with flow cytometry and normalised to grams of tissue. **F**] CD4⁺T_{RM} cells outnumber CD8⁺T_{RM} cells in the intestinal mucosa in CD. CD4⁺T_{RM} cells. CD8⁺T_{RM} cells from endoscopic biopsy specimens from CD patients, from the epithelial and lamina propria compartments combined, were enumerated with flow cytometry and normalised to grams of tissue. Data for [C] and [D] comprise $n = 10$ controls and $n = 18$ Crohn's patients.; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by analysis of variance.

Table 1. Control and Crohn's disease patients' demographics.

	Controls	Crohn's disease	<i>p</i> -Value
Total [<i>n</i>]	15	23	
% female	50%	43%	0.74 ^a
Mean age [Standard deviation]	55 yrs [12]	42 yrs [18]	0.02 ^b
	Conditions [<i>n</i>]		
Screening colonoscopy	5		
Large polyp	2		
Adenocarcinoma	3		
Diverticulitis	1		
Liposarcoma	1		
Neuroendocrine tumour	1		
Lipoleiomyoma	1		
Adhesions	1		

Differences in age between groups are significant by the students t-test.

^aChi-square.

^bStudent's t test.

Table 2. Crohn's disease patients' disease features.

	Crohn's disease % [<i>n</i>]
Total [<i>n</i>]	23
Disease duration [years]	12 [8] ^a
Ileocolonic	83% [19]
Colonic	17% [4]
Previous medical therapies	
Steroids	70% [16]
Methotrexate	17% [4]
Imuran	43% [10]
Adalimumab	74% [17]
Infliximab	74% [17]
Certolizumab pegol	4% [1]
Vedolizumab	4% [1]

^aMean [standard deviation].

Bio-Rad] and were normalised to GAPDH. Some results were also normalised to baseline conditions, as indicated in the figure legends.

2.6. Statistics

All data were analysed on Prism [GraphPad, La Jolla, CA], using paired and unpaired Student's t test or analysis of variance [ANOVA], as indicated in the figure legends. Linear regression was used to determine the significance of correlations between clinical parameters and measured indices, as noted in the figure legends.

3. Results

3.1. CD4⁺T_{RM} cells are enriched in Crohn's disease and are the major mucosal T cell population

In order to examine the role of T_{RM} cells in CD, we designed a study to acquire colon tissue from patients with CD and controls [Figure 1A]. The demographics of included subjects are presented [Tables 1 and 2]. We used a standard gating scheme to distinguish naïve from memory T cells, based on expression of CD45RO.^{24–26} Within the CD45RO⁺ memory T cell pool, we used the coordinated expression of CD69 and CCR7 to classify T cells as T_{RM} [CD69⁺CCR7⁺], tissue-resident central memory [T_{RCM}; CD69⁺CCR7⁺], central memory [T_{CM}; CD69⁺CCR7⁺], or effector memory [T_{EM}; CD69⁺CCR7⁺] T cells [Figure 1B].^{9,10}

As has been reported, most mucosal CD4⁺ T cells in controls were T_{RM} cells.^{9,10} Interestingly, controls also harboured substantial

fractions of CD4⁺ T_{RCM} cells but relatively few CD4⁺ T_{CM} and T_{EM} cells [Figure 1C]. As in controls, CD4⁺ T_{RM} cells were the major mucosal memory T cell population in the CD4⁺ T cell pool in CD. When compared with controls, the CD4⁺ T_{RM} and T_{EM} compartments were expanded in CD, consistent with a shift towards cytokine-producing effector populations in CD [Figure 1C, right panel]. The distribution of memory CD8⁺ T cells in controls differed slightly from their memory CD4⁺ T cell counterparts, as higher fractions of memory CD8⁺ T cells were in the T_{RM} compartment [Figure 1D]. Thus, although most memory CD8⁺ T cells were in the T_{RM} compartment in CD, there were no significant differences between controls and CD [Figure 1D, right panel]. The distribution of mucosal memory T cells from areas with quiescent CD was similar to that from areas from active CD, suggesting that these effects are due to disease state rather than mucosal location [Figure 1C, D; Supplementary Figure 1A–C, available as Supplementary data at ECCO-JCC online].

Consistent with the proportional shift towards CD4⁺ T_{RM} cells, the absolute number of mucosal CD4⁺ T_{RM} cells was also increased in CD relative to controls [Figure 1E]. We furthermore quantified CD4⁺ and CD8⁺ T_{RM} cells in CD patients in order to determine their relative absolute abundance. This demonstrated a preponderance of CD4⁺ T_{RM} cells over CD8⁺ T_{RM} cells in the mucosa in active CD [Figure 1F]. In addition, CD4⁺ T_{RM} cells in CD expressed the IL-15R, and the IL-7R but had variable expression of CD103, which is consistent with CD4⁺ T_{RM} cells [Supplementary Figure 1D, E, available as Supplementary data at ECCO-JCC online]. Interestingly, IL-15 is elevated in the intestine in CD, raising the possibility of enhanced IL-15R-dependent retention of CD4⁺ T_{RM} cells with disease.²⁷ Finally, we did not detect any difference in the distribution of CD4⁺ T_{RM} cells between biopsy and surgical samples in CD subjects [Supplementary Figure 1G, available as Supplementary data at ECCO-JCC online]. Collectively, these data demonstrate not only that CD4⁺ T_{RM} cells are enriched in CD compared with controls, but also that T_{RM} cells are the major mucosal T cell population in CD.

3.2. CD4⁺T_{RM} cells in active Crohn's avidly produce IL-17A and TNFα compared with cells from healthy tissue

It is reported that intestinal CD4⁺ and CD8⁺ T_{RM} cells from controls produced IL-2, TNFα, and interferon gamma [IFNγ], whereas IL-17 production is restricted to CD4⁺ T_{RM} cells.¹⁰ Murine models and genetic studies in humans have strongly implicated IL-17-producing

Figure 2. CD4⁺ T_{RM} cells in active Crohn's disease [CD] avidly produce IL-17A and TNF α compared with controls. **A**) CD4⁺ T_{RM} cells in CD exhibit increased IL-17A production relative to controls. CD4⁺ T_{RM} cells from colon of controls [$n = 10$] and CD patients [$n = 12$] were stimulated with PMA/ionomycin, and production of IL-17A was determined with flow cytometry [left panel] and quantified [right panel]. Differences between quiescent and active areas in the same subject are presented [bottom left panel]. **B**) CD4⁺ T_{RM} cells from CD patients express [B]. IL-22. CD4⁺ T_{RM} cells from CD patients were identified and stimulated as in [A] and [B] IL-22 expression was determined with flow cytometry. **C**) CD4⁺ T_{RM} cells from CD patients express *RORC*. Expression of *RORC* [left panel] and *TBET* [right panel] was determined on flow cytometry and sorted circulating CD4⁺ T_{EM} [CD4⁺CD45RO⁺CD69⁺CCR7⁺] and colonic T_{RM} cells [CD4⁺CD45RO⁺CD69⁺CCR7⁺] from control subjects and colonic CD4⁺ T_{RM} cells from Crohn's patients. Gene expression was normalised to *GAPDH* and is presented as fold induction relative to circulating CD4⁺ T_{EM} cells. **D**) Large fractions of T_{h17} CD4⁺ T_{RM} cells in CD produce TNF α . CD4⁺ T_{RM} cells were identified and stimulated as in [A], and the fraction of TNF α ⁺ cells within the IL-17A⁺ [T_{h17} CD4⁺ T_{RM} cells] and IL-17A⁻ subset of CD4⁺ T_{RM} cells was determined in controls [$n = 10$, left panels] and CD patients [$n = 12$, right panels]. **E**) Data in [C] from the [left panel] TNF α ⁺ T_{h17} CD4⁺ T_{RM} cells from CD patients and [right panel] the total cell number was calculated [per 1×10^6 total mononuclear cells]. **F**) Data from [C] for the TNF α ⁺ IL-17A⁺ CD4⁺ T_{RM} cell compartments are [left panel] quantified, and [right panel] the total number was calculated as in [D]. **G**) TNF α ⁺ T_{h17} CD4⁺ T_{RM} cells are expanded in CD. The ratio of the total number of TNF α ⁺ T_{h17} CD4⁺ T_{RM} cells to TNF α ⁺ IL-17A⁻ CD4⁺ T_{RM} cells is graphically presented [data are from Figure 2D, E]. Plots [B] and [C] are representative plots for $n = 3$ biological replicates; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ by analysis of variance [2A, 2D] or Student's t test.

CD4⁺ T cells [T_h17] in the pathogenesis of CD.^{1,28,29} Thus, we first focused on IL-17A production by CD4⁺ T_{RM} cells, given this link.

Very few CD4⁺ T_{RM} cells in controls were IL-17A⁺. In contrast, CD4⁺ T_{RM} cells in quiescent and active CD had a proclivity for IL-17A production [Figure 2A] and many cells were IL-22⁺ which is associated with T_h17 cells via the aryl hydrocarbon receptor [Figure 2B]. Consistent with a IL-17A production and a T_h17 signature, CD4⁺ T_{RM} cells from CD patients expressed the T_h17 master transcription factor RORC highly relative to circulating T_{EM} cells from control subjects, but not *TBET* [Figure 2C].

Anti-IL-23 agents are effective for CD, but anti-IL-17 agents are not. This discrepancy suggests that T_h17 cells are pathogenic independently of IL-17.^{30–32} TNF α , however, is irrefutably pathogenic in CD.⁵ We considered the possibility that CD4⁺ T_{RM} cells also produced TNF α . In order to investigate this, we assessed TNF α production by the IL-17A⁺ fraction of CD4⁺ T_{RM} cells [T_h17 CD4⁺ T_{RM}], given the link between T_h17 cells and CD. We then assessed TNF α production in the IL-17A⁺ CD4⁺ T_{RM} fraction and finally by the total CD4⁺ T_{RM} pool, given the link between TNF α and CD. There were relatively few T_h17 CD4⁺ T_{RM} cells in controls but many were TNF α ⁺ [Figure 2D]. Not only were there higher fractions of T_h17 CD4⁺ T_{RM} cells in quiescent and active CD, but also greater proportions were TNF α ⁺ [Figure 2D, top right panels, 2E, left panel]. There were, however, no differences between quiescent and active CD [Supplementary Figure 2A, available as Supplementary data at ECCO-JCC online]. Thus, the total number of TNF α ⁺ T_h17 CD4⁺ T_{RM} cells was substantially increased in active CD relative to controls [Figure 2E, right panel]. These findings were reflected in the IL-17⁺ CD4⁺ T_{RM} compartment as well [Figure 2D, bottom right panels, 2F]. Although most TNF α ⁺ CD4⁺ T_{RM} cells were in the IL-17A⁺ compartment, there was a relative expansion of the TNF α ⁺ IL-17A⁺ double-positive CD4⁺ T_{RM} subset in CD which was largely absent in controls [Figure 2E, right panel, G]. Finally, consistent with Figure 2D–F, significantly higher fractions of total CD4⁺ T_{RM} cells from quiescent and active CD were TNF α ⁺ compared with controls [Supplementary Figure 2B, available as Supplementary data at ECCO-JCC online]. Collectively, therefore, larger fractions of CD4⁺ T_{RM} cells in CD were TNF α ⁺IL-17A⁺ [double-positive] and TNF α ⁺ [single-positive] compared with control cells. These findings show that CD4⁺ T_{RM} cells in CD exhibit an activated profile compared with controls, and raises the intriguing possibility that T_h17 CD4⁺ T_{RM} cells may have pathogenic potential via TNF α production.

3.3. CD4⁺ T_{RM} cells are the major source of TNF α in the mucosa in Crohn's disease

Activated antigen-presenting cells [APCs], stromal cells, and T cells all produce TNF α in the intestinal mucosa in CD.^{33–35} Within the CD4⁺ T cell compartment, CD4⁺ T_{EM} cells [CD4⁺CD45RO⁺CCR7⁺CD69⁺] are thought to be the major source of TNF α in CD. We found that T_{RM} cells are abundant and avidly produce TNF α , but this does preclude the possibility that non-T_{RM} cells contribute to total mucosal TNF α . In order to examine this more directly, we used a gating strategy centred on identifying all TNF α ⁺ T cells after stimulation with phorbol 12-myristate 12-acetate [PMA] and ionomycin [I] [Figure 3A].

Consistent with reported data, significantly higher fractions of live cells were TNF α ⁺ in the colonic mucosa in CD patients than in controls [Figure 3B].³ In controls, gating on the TNF α ⁺ population and discriminating by CD3, CD4 and CD45RO demonstrated that CD4⁺ cells comprised ~75% of all TNF α ⁺ T cells, whereas CD8⁺ T cells accounted for ~10% with non-T cells, accounting for the

remainder. Nearly all of these cells were CD45RO⁺ [Supplementary Figure 3A, B, available as Supplementary data at ECCO-JCC online]. Within the TNF α ⁺CD4⁺CD45RO⁺ compartment, T_{RM} cells were the major mucosal source of TNF α . However, T_{RCM} cells were also major contributors, whereas T_{EM} cells only provided a marginal contribution [Figure 3C].

There was a relative expansion of the TNF α ⁺ CD8⁺ T cell pool in CD compared with controls. However, as in controls, most TNF α ⁺ T cells were in the CD4⁺CD45RO⁺ pool [Supplementary Figure 3C, D, available as Supplementary data at ECCO-JCC online]. Within the CD4⁺CD45RO⁺ pool, CD4⁺ T_{RM} cells were the major source of mucosal TNF α in active CD [Figure 3D]. This finding was unexpected, since it had been thought that T_{EM} cells were the major T cell source of mucosal TNF α in CD. Compared with controls, higher fractions of TNF α ⁺ T cells in CD patients were in the T_{RM} and T_{EM} compartments [Figure 3E]. This difference is consistent with a shift toward effector phenotypes in CD [T_{RM} and T_{EM}] at the expense of resting memory phenotypes in controls [T_{RCM}] [Figure 3C, D, right panels]. These data collectively demonstrate that CD4⁺ T_{RM} cells are a major T cell source of TNF α in the intestinal mucosa in CD.

3.4. CD4⁺ T_{RM} cells express the transcription factor PRDM1

The transcription factors PRDM1, HOBIT, and T-bet are thought to direct the CD8⁺ T_{RM} programme, but there is comparatively less is known about CD4⁺ T_{RM} cell regulation.^{14,15,36} T_{RM} cells in healthy humans do not express HOBIT, suggesting species-specific differences in T_{RM} biology.¹⁶

PRDM1 is expressed by intestinal CD4⁺ T_{RM} cells [Figure 4A, left panel]. Mucosal and circulating antigen-experienced cells in CD patients are reactive to enteric flora.³⁷ Therefore, in order to determine whether PRDM1 expression was restricted to T_{RM} cells or was a feature of mucosal localisation or antigen-experienced T cells, we also determined the expression of PRDM1 in intestinal and circulating T_{EM} cells. However, neither intestinal nor circulating T_{EM} cells exhibited high expression of PRDM1 [Figure 4A, B, left panel]. Moreover, because there were substantially more T_{RM} than T_{EM} cells in the mucosa, there was a large preponderance of PRDM1⁺ CD4⁺ T_{RM} cells relative to PRDM1⁺ CD4⁺ T_{EM} cells [Figure 4B, right panel].

As PRDM1 was more highly expressed on CD4⁺ T_{RM} cells relative to their T_{EM} counterparts, we sought to determine the functional consequences of PRDM1 in CD4⁺ T_{RM} cells. Purified CD4⁺ T_{RM} cells from CD patients were stimulated with PMA/I and the PRDM1 inhibitors butyrate and bortezomib. Butyrate suppresses *PRDM1* via micro-RNAs.³⁸ Bortezomib suppresses transcription of *PRDM1* via nuclear factor kappa β .³⁹ Stimulation led to robust induction of *PRDM1* in CD4⁺ T_{RM} cells relative to unstimulated cells. However, butyrate and bortezomib inhibited the induction of *PRDM1* [Figure 4C]. Furthermore, the inhibition of *PRDM1* by butyrate and bortezomib was associated with marked reductions in *IL17A* and *TNF*, and more broadly in the production of several inflammatory cytokines and IL-2 [Figure 4D]. There were no differences in cell viability with butyrate and bortezomib, suggesting that the observed differences were not due to increased cell death with inhibitor co-culture [Figure 4E]. Finally, CD4⁺ T_{RM} cells from control subjects, which also express PRDM1, reacted similarly to butyrate and bortezomib [Supplementary Figure 4A, B, available as Supplementary data at ECCO-JCC online]. Collectively, these data show that PRDM1 is more highly expressed by T_{RM} compared with T_{EM} cells, and raises the possibility that PRDM1 may regulate CD4⁺ T_{RM} function.^{40,41}

Figure 3. CD4⁺ T_{RM} cells are a major source of T cell-produced TNF α in the mucosa in active Crohn's disease [CD]. **A**) Experimental scheme and gating strategy. Epithelial and lamina propria fractions of mononuclear cells were processed from colonic tissue samples and stimulated with PMA/ionomycin. **B**) Higher fractions of stimulated cells are TNF α ⁺ in CD relative to controls. Cells were processed and stimulated as in **A**), and live TNF α ⁺ cells in controls and CD patients were identified by flow cytometry [left panels], and quantified [right panel]. **C**), **D**) CD4⁺ T_{RM} cells are a major source of T cell TNF α in controls and CD patients. T_{RM} cells within the TNF α ⁺ CD3⁺ CD4⁺ CD45RO⁺ fraction were determined by coordinated expression of CD69 and CCR7 in **C**) controls and **D**) CD patients by flow cytometry [left panels], and quantified [right panels]. **E**) Mucosal memory CD4⁺ T cells in CD are shifted toward effector phenotypes. CD4⁺ T_{RM} [left panel] and T_{EM} fractions [right panel] from **C**) and **D**) were compared between controls and CD patients. Data for **B**), **C**), and **D**) comprise $n = 5$ –10 controls and $n = 13$ CD patients; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by the unpaired **B**, **E**) and paired **C**, **D**) Student's t test.

4. Discussion

It was thought that mucosal T cells consisted of naïve and effector [T_{EM}] and central memory [T_{CM}] subsets, and that T_{EM} cells were the

major T cell contributors to mucosal TNF α .^{24,25,42} However, the discovery of T_{RM} cells has prompted a reassessment of mucosal T cells in CD. T_{RM} cells are tissue-restricted and therefore cannot be identified

Figure 4. The transcription factor PRDM1 is selectively expressed by CD4⁺ T_{RM} cells compared with colonic or circulating T_{EM} cells. A) PRDM1 is selectively expressed by CD4⁺ T_{RM} cells. CD4⁺ T_{RM} and colonic and circulating T_{EM} cells were identified in colonic tissue specimens or from peripheral blood mononuclear cells of Crohn's disease [CD] patients by flow cytometry as CD69⁺ or CD69⁻ fractions within the CD4⁺CD45RO⁺CCR7⁺ compartment. B) Data from [A] are quantified [left panel] and [right panel] the number of PRDM1⁺ cells in each compartment is enumerated [per 1 × 10⁶ total mononuclear cells]. C) PRDM1 inhibition is associated with reduced inflammatory cytokine production by CD4⁺ T_{RM} cells. CD4⁺ T_{RM} cells were MACS purified, stimulated with PMA/ionomycin, and co-cultured with the PRDM1 inhibitors butyrate and bortezomib. RNA was extracted and quantitative reverse transcription polymerase chain reaction [qRT-PCR] was performed for the indicated genes. Values were normalised to GAPDH and presented as fold induction relative to the unstimulated condition [negative control]. D) Bortezomib and butyrate reduce inflammatory cytokine production by CD4⁺ T_{RM} cells in CD. Supernatants from cell cultures from [C] were analysed for production of the indicated cytokines by enzyme-linked immunosorbent assay. E) Bortezomib and butyrate do not affect cell viability. Cells from [C] were counted with trypan blue to verify viability after stimulation and co-culture, data are presented as the percentage of live cells in the unstimulated condition. Experiments were performed with three biological replicates; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 by the unpaired Student's *t* test or [D] analysis of variance. *P* < 0.0001. the unpaired students *t*-test or the [D] ANOVA.

Figure 4. Continued

in the circulation. These cells have a unique genetic profile consistent with their identification as a distinct T cell subset.^{14–16} The biology of T_{RM} cells suggests that they are reactive to enteric flora, potentially providing a functional link between the intestinal dysbiosis in CD and the dysregulated immune response, which drive disease. We found that CD4⁺ T_{RM} cells are enriched in quiescent and active CD, avidly produce TNF α and IL-17A, and are the major mucosal T cell source of TNF α . Furthermore, these cells express PRDM1, and treatment with PRDM1 inhibitors is associated with impaired induction of IL17A and TNF.

Human studies and murine models have strongly implicated the IL-17-producing subset of T cells [T_h17 cells] in the pathogenesis of CD.^{1,28} Consistent with this, agents that target IL-23, which is necessary for the development of T_h17 cells, are effective in CD.⁴³ However, the anti-IL-17 trials were prematurely terminated as they were not only ineffective but may worsen disease.^{30,32} Although IL-17 is the defining cytokine of T_h17 cells, T_h17 cells can induce

pathology via IL-23-dependent but IL-17-independent mechanisms such as GM-CSF production.⁴⁴ In addition, there is evidence that IL-17 promotes epithelial cell restitution after injury.^{45,46} Whereas controls largely lacked T_h17 CD4⁺ T_{RM} cells, substantial fractions of CD4⁺ T_{RM} cells in CD were not only IL-17A⁺, but many of these were also TNF α ⁺. Thus, there were substantial increases in double-producing TNF α ⁺ T_h17 CD4⁺ T_{RM} cells in CD.

Given the clearly pathogenic role of TNF α in CD disease, our data suggest the possibility that TNF α production may be one mechanism for the pathogenicity of T_h17 cells. In contrast to TNF α ⁺ IL-17A⁺ CD4⁺ T_{RM} cells [double-positive cells], large fractions of CD4⁺ T_{RM} cells in controls were TNF α ⁺ single-positive. This finding is consistent with data showing that many mucosal TNF α ⁺ T cells are reactive to the enteric flora.^{37,42} Collectively, therefore, the increased frequency of TNF α ⁺ CD4⁺ single-positive T_{RM} cells in CD raises the intriguing possibility that intestinal dysbiosis promotes the accumulation or maintenance of activated enteric reactive CD4⁺ T_{RM}

cells. Indeed CD69, a key marker of T_{RM} cells, is one of the most differentially regulated genes between anti-TNF responders and non-responders.⁴⁷

Although large fractions of $CD4^+ T_{RM}$ cells in controls and patients produced TNF α , our initial gating [Figure 3A] does not preclude the possibility that other T cell subsets are the major source of TNF α in the intestinal mucosa. Thus, we stimulated T cells and gated on live TNF α^+ cells [Figure 4A] in order to identify the major TNF α -producing T cell subsets. This demonstrated the somewhat unexpected finding that T_{RM} cells were a major source of mucosal T cell-produced TNF α . Before the discovery of T_{RM} cells, T_{EM} cells were thought to be the major effector population in the intestine. Consistent with an activated immune state in CD, we found higher fractions of TNF α^+ effector T cells [T_{RM} and T_{EM}] in CD compared with controls.

An important consideration in our findings, as with all ex vivo experiments, is that they may not reflect the behaviour of cells in vivo. Thus, although we have demonstrated that $CD4^+ T_{RM}$ cells are a major source of TNF α , it is possible that $CD4^+ T_{RM}$ cells in vivo are held in a quiescent state by the tissue microenvironment. It is also possible that the exuberant cytokine production by T_{RM} cells in CD represents reactive epiphenomena of active disease.

We also analysed the data by parameters of disease activity. In this regard, we did not find significant correlations between the disease duration, HarveyBradshaw index [HBAI], and C-reactive protein [CRP] levels and the percentage of $CD4^+ T_{RM}$ cells, nor the fraction of IL-17A $^+$ and TNF α^+ $CD4^+ T_{RM}$ cells [Supplementary Figure 5A–C, available as Supplementary data at ECCO-JCC online]. Interestingly, however, there was a trend towards a weak positive correlation between cytokine production and the fraction of $CD4^+ T_{RM}$ cells [Supplementary Figure 5D, available as Supplementary data at ECCO-JCC online]. However, these data should be interpreted with caution. Foremost, our study was not powered to detect differences between degrees of CD activity. Second, it is well known that HBAI does not correlate with histology. Third, CRP values of our subjects may be falsely low, as many received steroids around the time of surgery. For these reasons, we relied on the histological assessment of active disease, and in that regard, our data truly reflect distinctions between active CD and healthy tissue.

Furthermore, controls in our cohort were on average older than CD subjects, and it is possible that age and gender affect our findings [Box 1]. We thus examined the distribution and cytokine production of $CD4^+ T_{RM}$ cells by gender and age. Gender did not alter the frequencies of $CD4^+ T_{RM}$ cells in controls or CD subjects [Supplementary Figure 6A, available as Supplementary data at ECCO-JCC online]. Moreover, the frequencies of [total] $CD4^+ T_{RM}$ cells and TNF α^+ and IL-17A $^+$ $CD4^+ T_{RM}$ cells did not vary significantly with age, in either control or Crohn's subjects [Supplementary Figure 6B, C, available as Supplementary data at ECCO-JCC online]. These data are consistent with previously published results that found that the proportions of $CD4^+ T_{RM}$ cells stabilised with age.⁹

Advances in drug delivery have permitted the development of agents that target intracellular signalling pathways. For example, the Janus kinase inhibitors have proven to be highly successful in ulcerative colitis, and ROR γ t inhibitors are in the early stages of development.⁴⁸ These advances have expanded therapeutic targets from cytokines and surface receptors to transcription factors and cell signaling, making it all the more important to identify selectively expressed pathways. In this regard, we found that T_{RM} cells express PRDM1, and that suppression of PRDM1 is associated with impaired induction of IL17A and TNF α by $CD4^+ T_{RM}$ cells. PRDM1

confers pathogenicity to peripheral T_H17 cells in animal models by transcriptional control of *IL17a* and *IL23r*.⁴¹ Furthermore, genetic pathway analysis in humans predicts involvement in inflammatory bowel disease [IBD] and also that PRDM1 binds to the *IL7R*, *IL23R*, and *CD69* promoter regions.⁴⁹

These results, coupled with the literature, raise the intriguing possibility that PRDM1 may have a functional role in $CD4^+ T_{RM}$ cells. Importantly however, these data are correlative and do not establish causality. Butyrate and bortezomib likely inhibit PRDM1 via histone-deacetylase [HDAC] pathways.^{38,39} Butyrate has been extensively implicated as a microbiota-produced anti-inflammatory metabolite which likely functions via multiple distinct pathways, including inducing T cell-derived IL-10 and T_H1 and T-regulatory cell pathways.^{50,51} Therefore, our results do not rule out these other pathways for butyrate [and bortezomib] induced $CD4^+ T_{RM}$ cell suppression. Indeed, these results argue for future studies that use unbiased methods to assess how these agents suppress $CD4^+ T_{RM}$ cell function.

Collectively, therefore, these findings lay the groundwork to elucidate the role of T_{RM} cells more broadly in CD, and raise the intriguing possibility that T_{RM} cells may be manipulated or targeted for therapeutic benefit.

Funding

This work was supported by the American Gastroenterological Association-Pfizer pilot award in Inflammatory Bowel Diseases [SB].

Conflict of Interest

None declared.

Author Contributions

SB designed the study, drafted the manuscript, and analysed all data; AH, GH, and MZ performed all experiments; NB and CB recruited all patients; JK, BM, and MMM provided support of patient inclusion and patient selection; MZ, HG, WZ, PH, RS, NK, and JK helped plan the study and experiments and provided support for data analysis. All authors have reviewed the manuscript.

Supplementary Data

Supplementary data are available at ECCO-JCC online.

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