



Differential Pathogenic Th17 Profile in Mesenteric Lymph Nodes of Crohn's Disease and Ulcerative Colitis Patients

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The drug targets IL23 and IL12 regulate pathogenicity and plasticity of intestinal Th17 cells in Crohn's disease (CD) and ulcerative colitis (UC), the two most common inflammatory bowel diseases (IBD). However, studies examining Th17 dysregulation in mesenteric lymph nodes (mLNs) of these patients are rare. We showed that in mLNs, CD could be distinguished from UC by increased frequencies of CCR6⁺CXCR3⁻RORγ⁺Tbet⁻CD4⁺ (Th17) memory T cells enriched in CD62L^{low} effector memory T cells (T_{EM}), and their differentially expressed molecular profile. Th17 T_{EM} cells (expressing *IL17A*, *IL17F*, *RORC*, and *STAT3*) displayed a higher pathogenic/cytotoxic (*IL23R*, *IL18RAP*, and *GZMB*, *CD160*, *PRF1*) gene signature in CD relative to UC, while non-pathogenic/regulatory genes (*IL9*, *FOXP3*, *CTLA4*) were more elevated in UC. In both CD and UC, IL12 but not IL23, augmented IFNγ expression in Th17 T_{EM} and switched their molecular profile toward an ex-Th17 (Th1*)-biased transcriptomic signature (increased *IFNG*, and decreased *TCF7*, *IL17A*), suggesting that Th17 plasticity occurs in mLNs before their recruitment to inflamed colon. We propose that differences observed between Th17 cell frequencies and their molecular profile in CD and UC might have implications in understanding disease pathogenesis, and thus, therapeutic management of patients with IBD.

Keywords: Th17 cells, mesenteric lymph nodes, inflammatory bowel disease, human, plasticity

INTRODUCTION

Lymph nodes (LNs) are the key sites to initiate an effector response and generate memory T cells. However, human lymphoid tissue samples available for research use is quite limited. Recent studies examined several organs of deceased healthy donors, which expanded our knowledge on T cell compartmentalization throughout the body under homeostatic conditions (1, 2). Naïve vs. memory CD4⁺ T cell balance shifts with age, with memory cells gaining numbers in adulthood in mucosal and lymphoid tissues (3). Mesenteric lymph nodes (mLNs) home gut migratory DCs that dictate the type of helper T (Th) responses by driving naïve T cell polarization toward Th1, Th2, Th9, Th17, T follicular helper (T_{fh}) or regulatory T (T_{reg}) cells, each specialized in immunogenic or tolerogenic

immune responses (4). Migratory DCs further induce the expression of gut homing receptors such as CCR9 or $\alpha 4\beta 7$ on antigen-specific T cells, which enable their return to the intestine (5). MLNs are thus considered a primary site for generation of mucosal Th responses, including Th17 cells that are important contributors to gut homeostasis. Indeed, an essential role was demonstrated for murine mLNs in the generation of pro-inflammatory IL17A⁺CD4⁺ T cells that are found in the small intestine (6). However, Takebayashi et al. found that absence of mLNs did not affect IL17 cytokine production by CD4⁺ T cells isolated from the colonic lamina propria in murine IBD models (7). Furthermore, it is proposed that Th17 cells are generated in the gut and rarely observed in mLNs and Peyer's patches (8). Studies investigating Th cells in mLNs of patients with inflammatory bowel diseases (IBD) remain scarce (9–12).

Crohn's disease (CD) and ulcerative colitis (UC) are the two most frequent chronic, remitting and relapsing IBD forms (13). Both diseases share common features but are distinct entities with CD developing in the entire gastrointestinal tract and UC in colon and rectum. The immune mechanisms that govern UC and CD disease process include the recruitment of pathogenic Th17 cells in the gut. Pathogenicity of mucosal Th17 cells is not defined by IL17 secretion *per se* but rather by their plastic nature, a hallmark of IBD (14). Th17 conversion to ex-Th17 (Th1*) is predominantly controlled by two pro-inflammatory cytokines IL12 and IL23 that share a common p40 chain (15). Yet, the potential contribution of mLNs to the generation of pre-committed pathogenic Th17 cells during intestinal inflammation in CD and UC remains to be investigated. In the present study, we examined the distribution of memory Th17 cells in the mLNs of UC and CD patients, their molecular characteristics, and determined their plasticity in response to IL12 and IL23.

MATERIALS AND METHODS

Human Clinical Samples

MLNs were collected from surgical resections. This study included 25 patients with CD and 9 patients with UC (clinical information is shown in **Supplementary Table 1**). No histological data or bacterial infections suggested a differential diagnosis.

Cell Purification and Analysis

MLNs were digested mechanically to obtain cellular suspensions (11). Antibodies used for flow cytometry are listed in **Supplementary Table 2**. Their respective Fluorescence minus one (FMO) or isotype controls are shown in **Supplementary Figure 1**. FCS Express 6 (DeNovo Software) or *t*-SNE (*t*-Distributed Stochastic Neighbor Embedding) plugin available in FlowJo version 10.5.3 (FlowJo, LLC) (16) were used for data analysis.

Cell Sorting and Culture

CD62L^{low}CD45RO⁺CD45RA⁻CD25⁻CD8⁻CD4⁺ T cell subsets: CCR6⁺CXCR3⁻, CCR6⁺CXCR3⁺, and CCR6⁻CXCR3⁺ were FACS sorted for functional

studies according to the gating strategy depicted in **Figure 2A**. Transcriptomic studies examined sorted CCR6⁺CXCR3⁻CD62L^{low}CD45RO⁺CD45RA⁻CD25⁻CD8⁻CD4⁺ T cells treated in the absence or presence of IL12. Cell isolation was performed using FACS Aria II cell sorter and data were analyzed using FACS Diva 6 (BD Biosciences).

The three purified CD4⁺ T cell subsets were stimulated with anti-CD3/CD28 beads (Miltenyi Biotec) and cultured with or without IL12 (20 ng/ml, R&D system) or IL23 (10 ng/ml, R&D system) for 6 days. Cultures were performed in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin; 20,000–50,000 cells per well. For intracytoplasmic staining, PMA-ionomycin was added for 6 h in cell cultures and Brefeldin A for the last 3 h, cells were then fixed and stained with CD3 monoclonal antibody followed by intracytoplasmic staining for IL17 and IFN γ .

NanoString

NanoString was performed at the LDI Molecular Pathology Research Core. RNA was isolated using the NucleoSpin RNA extraction protocol followed by nCounter Low RNA Input Amplification Protocol (nanoString).

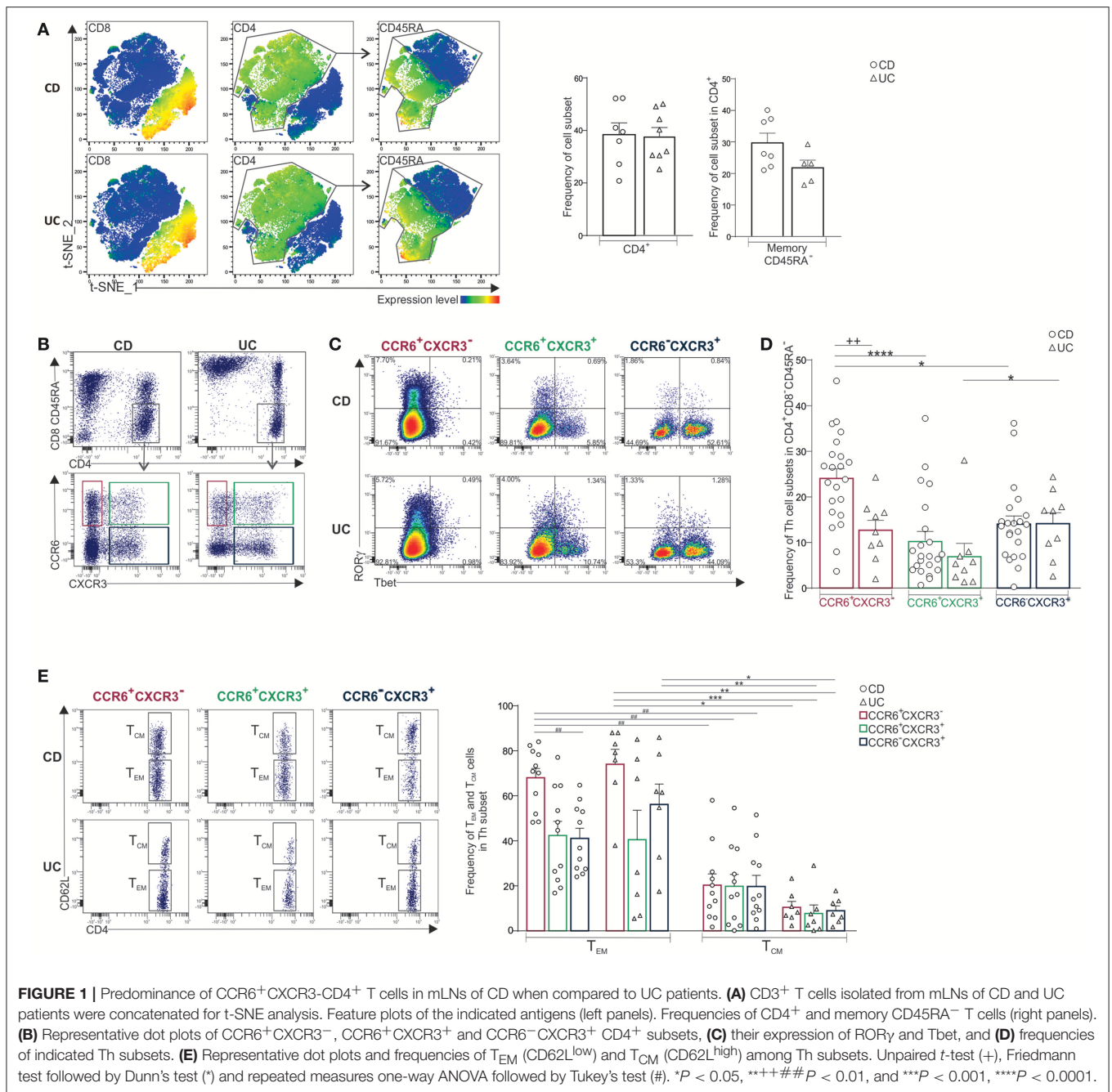
Differential gene expression was assessed using the NanoString Human Immunology v2 panel according to the manufacturer's specifications. In brief, amplified RNA was used for Sample Preparation. The samples were then processed with the nCounter Preparation Station to purify the hybridized targets and affix them to the cartridge for imaging using the nCounter Digital Analyzer (CCD camera). Barcodes were counted for each target molecule at High Resolution.

NanoString Statistical Analysis

The mRNA expression matrix for 583 genes was normalized using a list of house-keeping genes including *ABCF1*, *ALAS1*, *EEF1G*, *G6PD*, *GUSB*, *HTPRT1*, *OAZ1*, *POLR2A*, *PPIA*, *RPL19*, *TBP*, *TUBB*. However, it excluded *GAPDH* for having a high expression SD in our dataset. Subsequent PCA analysis revealed that the house-keeping normalized data was primarily clustered by diseases (UC and CD) which is of biological significance. In order to validate the inclusion of a patient covariable in the association model, we performed normalization using the R program (17): R limma (18) and EdgeR (19, 20) library that removed the effect of the patient identity on the PCA expression pattern. The resulting PCA analysis graph showed the samples being clustered by conditions (control and IL12) for which we want to analyze the expression.

A differential expression analysis was done with the R limma package with three contrast matrices:

1. ContUC vs. ContCD (Differential expression analysis between Control samples from UC and CD)
2. IL12CD vs. ContCD (Different expression analysis between IL12 stimulated cell vs. control for CD)
3. IL12UC vs. ContUC (Different expression analysis between IL12 stimulated cell vs. control for UC)



The association model included the contrast sample condition plus a covariate for the patient identity to reflect what was detected on the PCA analysis.

Graphics and visualization of the differential expression analysis metrics were done using the gplots, ggplot2, and beanplot libraries.

Statistical Analysis

Statistical analysis was performed with Prism version 6 (GraphPad Software). Data were checked for normality using

Shapiro-Wilk test and then the appropriate test was applied as indicated. For all tests, 1 symbol means *P* < 0.05, 2 symbols mean *P* < 0.01, and 3 symbols mean *P* < 0.001. Bar graphs are shown as mean ± SEM.

Study Approval

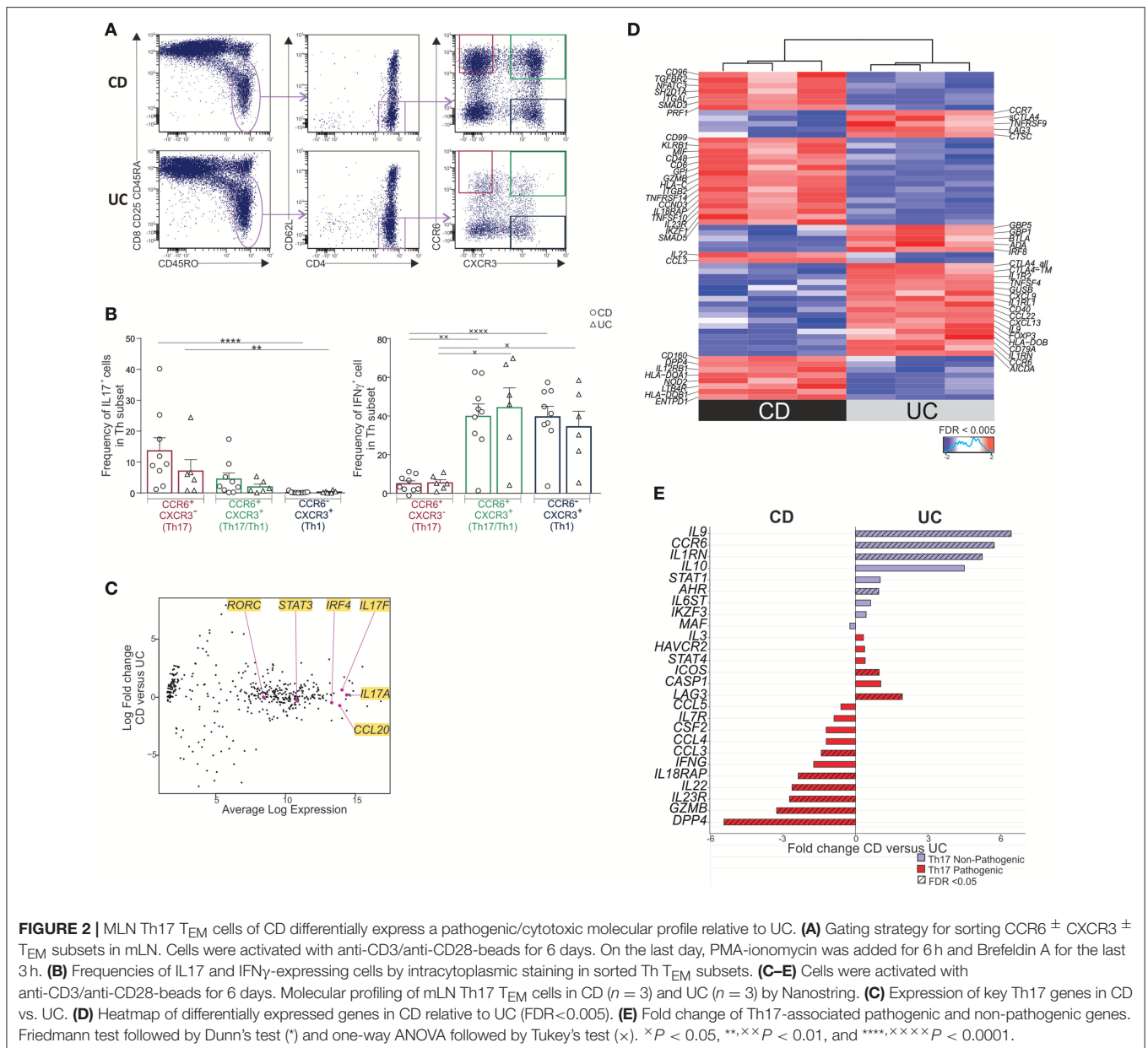
This study was approved by the Institutional Ethics Research Committee of the Center Hospitalier de l'Université de Montréal (CER-CHUM). The patients provided written consent to the study protocol.

RESULTS

Predominance of CCR6⁺CXCR3⁻CD4⁺ T Cells in mLNs of CD When Compared to UC Patients

The human T cell compartment is heterogeneous with variable distribution in different mucosal and lymphoid tissues (3) that is further altered upon inflammation. We investigated whether the distribution of CD4⁺ T cell subsets in inflamed mLNs distinguished CD from UC. The percentage of CD4⁺ T cells and memory CD45RA⁻CD4⁺ T cells was similar in both diseases (Figure 1A). Memory CD4⁺ T cells were next stratified using CCR6 and CXCR3 which are Th17 and Th1-associated markers, respectively (Figure 1B) (21). Accordingly,

CCR6⁺CXCR3⁻CD4⁺ T cells expressed RORγ but not Tbet, and conversely, CCR6⁻CXCR3⁺CD4⁺ T cells expressed Tbet but not RORγ (Figure 1C). CCR6⁺CXCR3⁺CD4⁺ T cells co-expressed RORγ and Tbet. Interestingly, the percentage of memory CCR6⁺CXCR3⁻CD4⁺ T cells was significantly higher in CD relative to UC, and additionally, it predominated over both CCR6⁻CXCR3⁺ and CCR6⁺CXCR3⁺ CD4⁺ T cell subsets in CD only (Figure 1D). However, there were no differences between CD and UC in the frequencies of CCR6⁻CXCR3⁺ or CCR6⁺CXCR3⁺ CD4⁺ T cells (Figure 1D). Memory Th cell subsets were further subdivided into CD62L^{low} effector memory (T_{EM}) and CD62L^{high} central memory (T_{CM}) T cells. As expected, inflamed mLNs comprised more T_{EM} than T_{CM} cells among all Th subsets examined (Figure 1E).



However, only in CD the frequencies of CCR6⁺CXCR3⁻ T_{EM} cells were significantly higher than CCR6⁻CXCR3⁺ T_{EM} cells.

Noteworthy, mLN CD4⁺ T cells also comprised minor T subpopulations that were equally distributed in CD and UC, they included T_{reg} (CD25⁺Foxp3⁺), and invariant T cells (**Supplementary Figure 2A**). The latter comprised MAIT (93–97%) (TCRV α 7.2⁺TCRV α 4.24⁻), $\gamma\delta$ T (1.5–3%) (TCRV δ ⁺TCRV α 7.2⁻) and iNKT (1.5–3%) (TCRV α 7.2⁻TCRV α 4.24⁺) cells. T_{reg} and invariant T cell subpopulations were more represented in the CD4⁺ compared to CD8⁺ compartment (**Supplementary Figures 2A–C**). Within these invariant subpopulations, only 20% of cells expressed CCR6 (**Supplementary Figure 2D**). Furthermore, T follicular helper cells (T_{fh}) were detected as rare ICOS⁺CXCR5⁺Ki-67⁺ cells in both diseases (**Supplementary Figure 2E**).

Taken together, CD mLNs comprised more CCR6⁺CXCR3⁻CD4⁺ T cells relative to UC and T_{EM} cells predominated over T_{CM} population.

MLN Th17 T_{EM} Cells Differentially Expressed a Pathogenic/Cytotoxic Molecular Profile in CD Relative to UC

Next, we thought to compare the cytokine and molecular profile of CCR6⁺CXCR3⁻ CD62L^{low}CD4⁺ T cells (Th17 T_{EM}) in mLNs of UC and CD patients. To this end, mLN Th17 T_{EM}, purified as depicted in **Figure 2A**, expressed high IL17 and low IFN γ while CCR6⁺CXCR3⁺ CD62L^{low}CD4⁺ T cells (Th17/Th1 T_{EM}) produced both, and CCR6⁻CXCR3⁺CD62L^{low}CD4⁺ T (Th1 T_{EM}) cells secreted IFN γ only (**Figure 2B**). However, unlike with unfractionated CD4⁺ T cells (10), no significant differences were noted in the frequencies of IL17 or IFN γ -producing cells in purified Th T_{EM} subsets between CD and UC patients. Th17 T_{EM} identity was further confirmed at the molecular level by equally elevated expression of *IL17A*, *IL17E*, *RORC*, *STAT3*, and *CCL20* in CD and UC (**Figure 2C**) (4, 14, 22).

Unexpectedly, mLN Th17 T_{EM} subset in CD was distinguished by a set of differentially expressed genes when compared to UC (**Figure 2D** (FDR < 0.005) and **Supplementary Table 3**). In fact, *IL23R*, *CCL3*, *IL22*, *DPP4*, *GZMB*, and *IL18RAP*, reported to be associated with a pathogenic Th17 signature in humans and mice (22–24), were over-expressed in Th17 T_{EM} from CD relative to UC (**Figure 2E**). *GZMB* and *IL18RAP* along with *PRF1*, *CSF1*, *CD160*, *CXCR6*, *CD3E*, *KLRB1* further delineated a pro-inflammatory/cytotoxic Th profile in CD relative to UC (**Figures 2D,E** and **Supplementary Table 3**) (25, 26). In contrast, Th17 T_{EM} in UC, when compared to CD, had a greater expression of *IL9*, *IL10*, *IL1RN*, *CTLA4*, and *FOXP3*, genes that are considered non-pathogenic or regulatory (23, 24, 27). Interestingly, augmented *IL9* along with low *CD96* and *DPP4* expression (**Figure 2D**) observed in UC relative to CD mimics a Th9 pro-inflammatory profile associated with chronic intestinal inflammation in mice (28, 29). Moreover, a Th9-biased profile has also been reported in UC mucosa (30).

In conclusion, Th17 T_{EM} cells are associated with a pathogenic/cytotoxic molecular profile in CD and a non-pathogenic/regulatory profile in UC.

IL12 Shifts mLN Th17 T_{EM} Cells Toward ex-Th17 (Th1*) in CD and UC

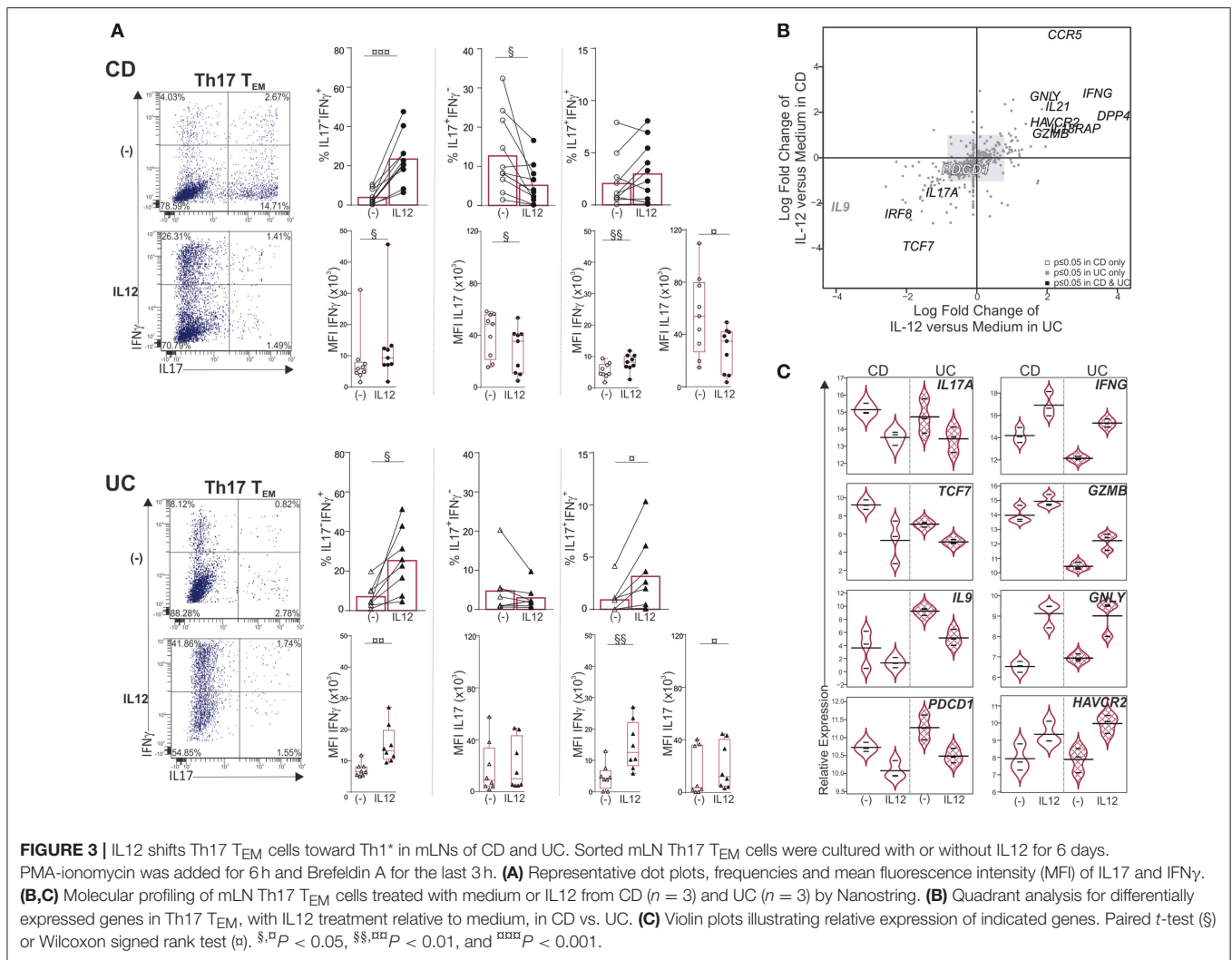
IL23 favors Th17 effector function while IL12 down-regulates IL17 and promotes IFN γ expression in circulating and intestinal Th17 cells (21, 23, 31, 32). Furthermore, mucosal pathogenic Th17 cells that contribute to IBD pathogenesis are best defined by their ability to acquire IFN γ , and thus, ultimately switch to Th1* (23). We therefore asked whether Th17 T_{EM} in mLNs could be shifted toward Th1*. Th17 T_{EM} exposure to IL12 increased the percentage of IL17⁻IFN γ ⁺ cells as well as IFN γ production per cell, as measured by the mean fluorescence intensity (MFI), in both CD and UC (**Figure 3A**). Frequencies of IL17⁺IFN γ ⁻ cells were significantly reduced by IL12 in CD only, further demonstrating a shift of Th17 T_{EM} cells to Th1*. In addition, we noticed that IL12 augmented frequencies of IL17⁺IFN γ ⁺ cells in 7 out of 9 CD, and 6 out of 8 UC samples. In contrast, IFN γ and IL17 expression was not significantly modified by IL12 in Th17/Th1 T_{EM}, and, IFN γ expression was marginally increased in Th1 T_{EM} in UC only (**Supplementary Figure 3A**).

Furthermore, Th17 conversion to Th1* under the influence of IL12 was associated with *IL17A*, *TCF7* and *IL9* downregulation while pro-inflammatory and cytotoxic gene expression (*IFNG*, *IL21*, *GZMB*, *DPP4*, *GZMB*) increased in both CD and UC (**Figures 3B,C**). *TCF7* downregulation was consistent with the emergence of IL17⁻IFN γ ⁺ (Th1*) cells and increase of Th1 genes (33, 34). *IFNG* and *HAVCR2* gene expression, which were augmented, best defined Th1-like T cells in colorectal cancer (35), while *PDCD1*, an immune checkpoint inhibitor, was decreased. The gene encoding *IL17E*, that promotes colitis in mice (36), was not inhibited upon IL12 stimulation; reinforcing the concept that IL12 induces the generation of pathogenic Th1* cells in inflamed mLNs. IL12 is likely contributed by mature DCs that accumulate in mLNs of CD (37); these cells also produce IL23 along with IL12 (10). Unlike exposure to IL12, modulation of IL17 and IFN γ expression was unchanged in Th17, Th17/Th1 and Th1 T_{EM} cells in response to IL23 (**Supplementary Figure 3B**). Failure of IL23 to augment IL17 or IFN γ in Th17 T_{EM} cells was not attributed to absence of IL23 receptor since *IL23R* was expressed in CD, and at higher levels relative to UC (**Figure 2D**).

Taken together, IL12 but not IL23 promotes plasticity of mLN Th17 T_{EM} cells.

DISCUSSION

It is established that mucosal Th cells regulate gut homeostasis and inflammation, but few reports examined mLNs of IBD patients. Overall, the present study revealed that CD and UC could be distinguished by examining the frequencies and molecular profile of Th17 cells in mLNs. mLNs in CD were characterized by a predominant Th17 T_{EM} population displaying a pathogenic/cytotoxic gene signature relative to Th17 T_{EM} cells in UC that expressed a profile biased toward regulatory genes.



Under IL12 exposure, mLN Th17 T_{EM} cells from both CD and UC shifted toward a Th1* cytokine and molecular profile, suggesting that Th17 plasticity is taking place in this inductive site before T cell homing to gut tissues.

A previous study indicated that frequencies of IL-17⁺ cells are augmented in CD when compared to UC using plastic-coated CD3/CD28 activated unfractionated mLN CD4⁺ T cells (10). We showed here that the proportion of IL17⁺ cells was similar in both diseases using anti-CD3/CD28 beads activated CCR6⁺CXCR3⁻ effector memory CD4⁺ T cells while the expression of IL17 per cell (MFI IL17) tended to augment in CD.

Pathogenicity of Th17 cells is best defined by their capacity to elicit disease after *in vivo* adoptive transfer, their selected gene expression profile and their plastic nature (23, 24, 38). In mice, Th17 cells gradually progress to a pre-Th1 effector phenotype in the LN and, to a Th17/Th1-like effector phenotype in non-lymphoid tissues (39), suggesting that Th17 conversion is already initiated in LN. Indeed in CD mLN, Th17 T_{EM} cells displaying a pathogenic, “pre-Th1” inflammatory gene (*IFNG*, *HAVCR2*, *CD26*) profile (22) corroborate the progression

observed in mice LN. Furthermore, Th17 T_{EM} cells isolated from the mLN of IBD patients converted to Th1* under the influence of IL12. Th17 program inhibition by IL12, as shown here by decreased *TCF7* expression (34), also depends on *Eomes* up-regulation that inhibits *RORC2* and *IL17A* expression while maintaining *IFNG* (40). Interestingly, *Eomes*-expressing Th1* and Th1 cells have a more stable phenotype and do not up-regulate IL17 under the influence of IL1 β , IL6, IL23, and TGF β Th17-polarizing cytokines (40, 41), consistent with a lack of modulation of IL17 and IFN γ expression observed in CD and UC Th1 T_{EM} cells. The limitation of our study is that *EOMES* was not part of the nanostring expression matrix.

IL23 expression is required on T cells to trigger colitis, which is associated with IFN γ and IL17 co-expression (38). Furthermore, administration of anti-IL23p19 monoclonal antibody attenuates development of colitis in *Abcb1a*^{-/-} mice (38, 42). However, Th17 T_{EM} cells from IBD mLNs did not modulate IL17 and IFN γ expression in response to IL23, differing from increased Th17 responses to IL23 observed in human colonic CD4⁺

T cells from CD patients (43). Failure of IL23 to augment IL17 or IFN γ in mLN Th17 T_{EM} cells was not attributed to absence of IL23 receptor since *IL23R* was expressed in CD, and at higher levels relative to UC. These data suggest that tissue-dependent IL23 responsiveness requires additional signals provided by the cytokine milieu and/or environment, which might be absent or low in mLNs. In fact, serum amyloid A proteins 1 and 2 (SAA1/2), secreted by epithelial cells, have been shown to promote robust IL17A production in ROR γ^+ T cells in the mucosa (44). Moreover, a recent study demonstrates that induction of severe chronic relapsing/remitting UC-like colitis in immunocompetent mice requires not only IL23 and pathogenic CD4⁺ T cells in mLNs and colon, but also intestinal dysbiosis (45).

Owing to the importance of IL23/Th17 axis in IBD pathogenesis, it is not surprising that these cells are deemed to be therapeutic targets. However, their inherent plasticity adds difficulty in targeting them directly in inflammatory settings. Antibodies that block IL12 might be suitable targets, as IL12 promotes Th17 plasticity toward pathogenic Th1* in mucosa (21), and as shown here in mLNs of CD and UC. In fact, anti-IL12p40 drugs are part of the therapeutic arsenal of CD, and clinical trials are ongoing in UC patients (46). However, therapeutic efficacy of both anti-IL12p40 and anti-IL23p19 in ameliorating CD argues for a predominant role for IL23 in disease pathogenesis. Hence, anti-IL23p19 is also in clinical trials for UC (46). Collectively, studying the role of immune cells in IBD mLNs warrants further investigation to better understand differences between CD and UC pathogenesis, and thus, open avenues for personalized medicine.

DATA AVAILABILITY

The nanostring data have been deposited in the Gene expression (GEO) database under accession number GSE131359.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of CRCHUM with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Ethics Research Committee of the Centre Hospitalier de l'Université de Montréal (CER-CHUM).

CONTRIBUTION TO THE FIELD

While being extensively studied in the mucosa, few studies examined helper T cell subsets in the mesenteric lymph

nodes (mLNs) of Crohn's disease (CD) and ulcerative colitis (UC) patients. Lymph nodes are the key sites to initiate an effector response and generate memory T cells, emphasizing the need to investigate immune cells in these lymphoid tissues.

Briefly, we showed that mLNs of CD and UC can be distinguished by frequencies of CXCR3⁻CCR6⁺ Th17 memory T cells, enriched in CD62L^{low} effector memory T cells (T_{EM}), and differentially expressed Th17 T_{EM} molecular profile. Drug targets IL23 and IL12 regulate the pathogenicity and plasticity of intestinal Th17 cells in IBD. Our data further revealed that IL12, but not IL23, shifted mLN Th17 T_{EM} toward a pathogenic Th1* cytokine and molecular profile in both CD and UC, suggesting that Th17 plasticity is taking place in this inductive site before T cell homing to gut tissues.

Therefore, investigation of CD4⁺ helper T cell subsets in the IBD mLNs, which are not easily accessible for research use, has clear implications in further understanding disease pathogenesis and thus, open avenues for personalized medicine.

AUTHOR CONTRIBUTIONS

MB, LC, and MS: Designing research studies. MB, LC, and MR: Conducting experiments. MB, LC, MR, HM, and MS: Acquiring and analyzing data. RW, CR, FS, RL, and GS: Resources. MB, MS, LC, and HM: Writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.01177/full#supplementary-material>

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