

Mast Cells Promote Inflammatory Th17 Cells and Impair Treg Cells Through an IL-1 β and PGE₂ Axis

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Purpose: CD4⁺ effector T cells (Teffs) play a key role in immune responses by infiltrating the sites of inflammation and modulating local leukocyte activity. In turn resident immune cells shape their response. This study aimed to investigate the influence of mast cells (MCs) on Teff biological responses.

Methods: This study examined human MC-Teff interactions, focusing on how MCs shape Teff responses. Flow cytometry, qRT-PCR, and cytokine assays were used to analyze the impact of primary human MCs on the Teff phenotype and function. MC-Teff crosstalk within Crohn's disease patient tissues was assessed using confocal microscopy and advanced image analysis.

Results: MCs promoted the differentiation of Th17 cells, particularly the inflammatory Th17.1 subset, that secretes IFN- γ and GM-CSF. This differentiation was driven by the PGE₂ and IL-1 β axis. Additionally, MCs disrupted the phenotype and impaired the suppressive function of regulatory T cells (Tregs) through PGE₂, skewing the Th17/Treg balance. The analysis of biopsies from patients with Crohn's disease indicated that this MC/Teff crosstalk may play a role in the pathogenesis of auto-inflammatory processes.

Conclusion: MCs influence CD4⁺ T cell responses by fostering pro-inflammatory Th17 differentiation while impairing Treg function. This interaction underpins a Th17/Treg imbalance, which is significant in auto-inflammatory diseases such as Crohn's disease, positioning MCs as critical drivers of disease pathogenesis.

Plain language summary: The immune system functions through complex interactions between different types of cells to protect the body from harm. CD4⁺ T cells are crucial in managing inflammation by directing other immune cells. Mast cells, found in tissues such as the skin and gut, are among the first to respond to potential threats and can influence T cell behavior. This study examined how mast cells and T cells interact in vitro, particularly in Crohn's disease, a condition in which the immune system causes gut inflammation.

We used laboratory techniques to study how mast cells affect T cells, examining their behavior and communication. We also studied tissue samples from individuals with Crohn's to determine how these interactions occur in real life.

We found that mast cells encourage the development of inflammatory T cells called Th17, particularly a type known as Th17.1, which produce strong inflammatory signals. This process relies on specific molecules such as PGE₂ and IL-1 β . Simultaneously, mast cells weaken the function of regulatory T cells (Tregs), which normally help control inflammation. This leads to an imbalance between Th17 cells and Tregs, tipping the immune system toward excessive inflammation. In tissue samples from Crohn's disease patients, we found evidence of this imbalance, suggesting that mast cells play a major role in driving harmful inflammation.

These findings help us understand why inflammation becomes uncontrollable in diseases such as Crohn's disease. They also suggest that targeting mast cells could be a promising strategy for new treatments.

Keywords: mast cells, helper T cells, prostaglandin E2, IL-1 β , Th17, inflammatory Th17.1 cells, Crohn disease, IL-17, IFN- γ

Introduction

Mast cells (MCs) are tissue-resident cells that are particularly abundant in skin and mucosa. MC responses to innate stimuli (complement component C5a, alarmins such as IL-33) or antibody-targeted antigens are well documented.^{1–3} MCs swiftly release numerous prestored mediators by degranulation or *de novo* synthesize cytokines, chemokines and eicosanoids according to their triggered receptors^{1,4} allowing them to participate in inflammatory response initiation, defense against pathogens, venom detoxification, tissue repair or interact with the adaptive immune system, most notably CD4⁺ T cells.^{2,5}

Upon TCR engagement in the lymph nodes, naive CD4⁺ T cells can differentiate into different subsets, including the four major T effector cell (Teff) subsets: Th1 (T helper) type 1, Th2, Th17 and induced regulatory T (Treg) cells, according to the signals received during the differentiation process.^{6,7} CD4⁺ Teffs then migrate to inflamed tissues, where they shape the local immune and non-immune cell responses. Understanding how Teff functions are induced or shaped in tissues is an area of continuous investigation.⁸ For instance, local TCR engagement allows Th1 cells to produce cytokines,^{9,10} EGFR-expressing Th2 cells infiltrating the lungs produce IL-13 in response to IL-33¹¹ and when exposed to serum amyloid A, Th17 cells up-regulate IL-17 in the *lamina propria*.¹² Th cells also show plasticity (*eg*, enlargement of the panel of cytokines produced), allowing them to adapt and fine-tune the immune response.^{13–15}

Upon tissue infiltration, CD4⁺ Teffs encounter MCs that reside near the blood vessels and participate to their recruitment. Both cognate and non-cognate interactions can occur, leading to reciprocal activation.¹⁶ We recently showed that activated memory CD4⁺ T cells induce the production of a vast array of molecules in MCs, endowing them with an APC role and to produce several pro-inflammatory mediators that can stimulate T cells.¹⁷ Because CD4⁺ T cells show plasticity and need to be reactivated locally to produce cytokines,^{6,10} MCs appear to be potential candidates for shaping Teff responses. MCs are often associated with type 2 immune responses,^{2,18,19} but it has been reported that MCs favor Th17 responses indirectly by acting on dendritic cells²⁰ or directly by promoting IL-17 and GM-CSF production in Th cells in the context of experimental autoimmune encephalomyelitis.²¹ In contrast, Lotfi-Emran et al reported that human MCs could induce Th1 recall responses upon cognate interaction with Teffs²² and Matsui et al showed that MC priming with peptidoglycan fosters Th1 development.²³ These reports suggest that MCs, according to their microenvironment, can mold Teff function. We have recently shown that activated CD4⁺ Teffs are potent MC activators. These Teff-activated MCs upregulate several molecules dedicated to T cell activation.¹⁷ This raises the question of whether MCs that have been primed by CD4⁺ Teffs can influence in turn Teff responses. In this study, we focused on MC/CD4⁺ memory T cell interplay, independently of any other polarizing factor, to investigate how Teff-licensed MCs shape Teff responses.

Materials and Methods

Human Primary MC Cultures

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of anonymous healthy volunteer donors (aged 18–60 years) provided by the French Blood Establishment (EFS, Toulouse, France). CD34⁺ precursor cells were isolated from (peripheral blood mononuclear cells (PBMCs; EasySep Human CD34 Positive Selection Kit, STEMCELL Technologies). CD34⁺ cells were grown in 48-Well Flat-Bottom Plate in StemSpanTM medium (STEMCELL Technologies) supplemented with recombinant human IL-6 (50 ng/mL; Peprotech), human IL-3 (10 ng/mL; Peprotech) and 3% supernatant of CHO transfectants secreting murine SCF (a gift from Dr. P. Dubreuil, Marseille, France, 3% correspond to ~50 ng/mL SCF) for one week. Cells were then grown in IMDM Glutamax I, sodium pyruvate, 2-mercaptoethanol, 0.5% BSA, Insulin-transferrin selenium, penicillin/streptomycin (100 U/mL / 100 µg/mL) (all from Invitrogen), IL-6 (50 ng/mL), and 3% supernatant of CHO transfectants secreting murine SCF for 8 weeks, and tested phenotypically (Tryptase⁺, CD117⁺, FcεRI⁺) and functionally (β-hexosaminidase release in response to FcεRI crosslinking) before use in experiments. Only primary cell lines showing more than 95% CD117⁺/FcεRI⁺ cells were used in experiments.

Human Studies

Formalin-fixed paraffin-embedded (FFPE) colon sections from patients with Crohn disease were selected after pathological examination (n=5). Normal colon biopsies from patients were used as controls (n=5). Samples were collected at the

Institut Universitaire du Cancer de Toulouse-Oncopole (IUCT-O) and processed at the CRB Cancer des Hôpitaux de Toulouse, following the standard ethical procedures (Declaration of Helsinki), after obtaining written informed consent from all patients. In accordance with French law, the “CRB Cancer des Hôpitaux de Toulouse” collection was reported to the Ministry of Higher Education and Research (DC-2009-989, AC-2008-820) and approved by the Toulouse hospital ethics committee. Clinical and biological annotations of the samples have been declared to the “Comité National Informatique et Libertés” (CNIL—French Data Protection Authority).

Coculture Experiments

1×10^5 memory CD4⁺ T cells freshly purified from PBMC by negative selection and magnetic separation (EasySep™ Human Memory CD4⁺ T cell enrichment Kit, STEMCELL Technologies) were co-cultured at 1:1 ratio for 6 days with anti-CD3/CD28-coated beads (Dynabeads, Life Technologies) at 1 bead: 10 T cells in RPMI 1640 supplemented with 10% serum replacement medium (knockout medium, Life Technologies), GlutaMAX-I, sodium pyruvate, 2-mercaptoethanol, and 1% supernatant of CHO transfectants secreting murine SCF. Cocultures were treated as indicated with either indometacin (100 µmol/L, Sigma-Aldrich), PGE₂ (11–300 ng/mL, Cayman Chemical), PGD₂ (11–300 ng/mL, Cayman Chemical), 15d-PGJ₂ (11–300 ng/mL, Cayman Chemical), butaprost (10 µmol/L, Cayman Chemical), sulprostone (10 nmol/L, Cayman Chemical), L902,688 (12 nmol/L, Cayman Chemical), and IL-1Ra (100 ng/mL, BioLegend). For the proliferation assay, memory CD4⁺ T cells were stained using the CellTrace™ Violet Cell Proliferation Kit (Thermo Fisher Scientific) according to the manufacturer’s recommendations. Tregs were purified from CD4⁺ memory T cells using magnetic separation (EasySep™ Human CD4⁺CD127^{low}CD25⁺ Regulatory T Cell Isolation Kit, STEMCELL Technologies) and labelled with CellTrace™ Violet. CTV-Labelled Tregs were reincorporated into their non-Treg counterparts. Reagent list and Polyunsaturated fatty acids (PUFAs) quantitation method are provided in the [Supplementary Materials 1](#).

Flow Cytometry and Cell Sorting

Cell surface staining with fluorochrome-labelled primary antibodies was performed in PBS 1% FCS 1% human serum (HS) at the concentration recommended by the manufacturer at 4°C for 30 min. Cell viability was ascertained by labeling the cells with fixable viability dyes (eBioscience).

For T cell cytokine intracellular staining, cells were stimulated with PMA (50 ng/mL) and ionomycin (1 µg/mL) in the presence of GolgiPlug™ and GolgiStop™ (BD Biosciences) for 5 h. After washing in PBS, cells were incubated with anti-CD117 (104D2) to exclude MCs and viability dye (eBioscience™ Fixable Viability Dye eFluor™ 780) in PBS 1% FCS 1% HS at 4°C for 30 min. Cells were washed with PBS and next fixed in PFA 4% for 10 min. At RT and permeabilized with PBS 1% FCS 1% HS 0.1% saponin (permeabilization buffer) for 10 min. The cells were then incubated with the following antibodies in permeabilization buffer for 45 min at room temperature: anti-IL-17A (BL168), anti-IFN-γ (B27), anti-IL-4 (8D4-8), anti-IL-22 (22URTI), anti-granzyme B (GB11), and anti-GM-CSF (BVD2-21C11). The cells were then washed with PBS and analyzed using flow cytometry.

Treg cells in co-cultures were identified by cell surface staining with anti-CD117 (104D2), anti-CD127 (HIL-7R-M21), anti-CD25 (M-A251), anti-LAP (TW4-2F8), and viability dye (eBioscience™ Fixable Viability Dye eFluor™ 780), followed by Foxp3 staining with anti-FoxP3 (259D/C7) using the Human FoxP3 buffer set (BD Biosciences) according to the manufacturer’s recommendations.

For T helper cell subset sorting, effector/memory CD4⁺ T cells were labeled with anti-CXCR3 (1C6/CXCR3), anti-CCR4 (L291H4), anti-CCR6 (G034E3), anti-CCR10 (#314305) and viability dye (eBioscience™ Fixable Viability Dye eFluor™ 780). Cell sorting was performed with indicated gating strategy by using a custom configuration FACSARIA cell sorter.

All flow cytometry experiments were acquired using a custom configuration Fortessa flow cytometer and FACS Diva software (BD Biosciences) and analyzed with FlowJo V10.4.2 software (TreeStar). Cell sorting was performed by using a custom configuration FACSARIA cell sorter (BD Biosciences) or FACSMelody cell sorter (BD Biosciences).

Cytokine Quantitation

After 6 days of co-culture, CD4⁺ T cells were sorted using FACS and re-stimulated with PMA (50 ng/mL) and ionomycin (1 µg/mL) for 5 h. The supernatants were collected and stored at -80°C. IFN-γ, IL-4, and IL-17 concentrations were measured using a bead-based multiplex assay (LEGENDplex™, BioLegend), according to the manufacturer's recommendations. Flow cytometric analysis was performed using the MACSQuant® Analyzer 10 instrument (Miltenyi Biotec).

Quantitative Real-Time PCR in MC/T Cell Cocultures

RNA extracted for RNAseq Libraries preparation was also used for RT-qPCR analysis (RNAseq validation). For transcription factor analysis, total RNA was extracted from 200 000 T cells (sorted at day 6 of coculture using the phenol chloroform protocol), and RNA concentration was determined using a Clariostar multi-mode plate reader (BMG Labtech). Reverse transcription of total RNA was performed using the SuperScript™ VILO™ cDNA Synthesis Kit according to the manufacturer's recommendations (thermo Fisher scientific). Real-time PCR was performed with Master Mix Fast Advanced TaqMan™ (ThermoFisher Scientific) using a StepOnePlus™ Real-Time PCR detection system (Applied Biosystems™). The following primers were used: TBX21 (Hs00894392_m1), GATA3 (Hs00231122_m1), FOXP3 (Hs01085834_m1) and RORC (Hs01076112_m1). Gene expression was normalized as n-fold difference to the housekeeping gene GAPDH (Hs03929097_g1) based on the $2^{-\Delta\Delta C_t}$ method. Relative quantification of gene expression was performed using StepOne Software v2.3 (Applied Biosystems™).

Immunofluorescence and Confocal Microscopy

MC and CD4⁺ memory T cells were co-cultured with or without anti-CD3/CD28 coated beads for 48 h. Cells were plated on poly-D-lysine (Sigma)-coated slides and were fixed in PFA 4% for 10 min at RT, and permeabilized with PBS 1% BSA 0.1% saponin for 5 minutes. Cells were stained with mouse anti-chymase (IgG1k, clone B7), goat anti-CD4 (polyclonal Ab) and rabbit anti-IL-1β (polyclonal Ab) at RT for 1h in PBS 1% BSA 0.1% saponin. Cells were next incubated with matched secondary antibodies: donkey anti-rabbit IgG (H+L) Alexa Fluor 555 (polyclonal), donkey anti-mouse IgG (H+L) Alexa Fluor 647 (polyclonal), donkey anti-goat IgG (H+L), and Alexa Fluor 488 (polyclonal). Cells were next counterstained with DAPI (1 µg/mL).

For IF studies of human colon biopsies, 3µm thick sections of FFPE tissues were provided by the Biological Resource Center of the CHU Toulouse (CRB). The sections were first rehydrated in successive bathes of: Xylène (20 min). / 100% ethanol (20 min). / 95% ethanol (5 min). / 70% ethanol (5 min). / 50% ethanol (5 min). / PBS (5 min). Antigen retrieval was performed using a citrate buffer (pH 6; Sigma-Aldrich). Sections were saturated for 30 min in PBS 10% Human Serum 0.3% Saponin and incubated at 4°C with primary antibody overnight in PBS 10% Human Serum 0.3% Saponin. The sections were then washed four times and incubated with secondary antibody for 2 h at RT. Before covering, the samples were washed thrice and incubated for 5 min with DAPI (1 µg/mL). The following primary antibodies were used: Mouse (IgG1) anti-Human-Tryptase (Clone AA1), goat anti-Human CD4 (polyclonal), Rabbit anti-Human Cleaved-IL-1β(D3A3Z), Rabbit anti-Cox2 (D5H5). The following secondary antibodies from Thermo Fischer Scientific were used: Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (polyclonal), Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (polyclonal), Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (polyclonal). Images were acquired using Zeiss LSM 780 confocal microscope. The acquired images were analyzed using Zen (Carl Zeiss Microscopy) and ImageJ software.

Statistical Analysis

Statistical tests were performed using the GraphPad Prism V9 software (GraphPad Software, Inc). Test performed are indicated in the figure legends. Nonparametric tests were performed when the group distribution failed to achieve normality or homogeneity of variance. All p values are two-sided, (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001 and ns, not significant).

Results

MCs Promote Th17/Treg Imbalance and the Emergence of Pro-Inflammatory Th17.1 Cells

We recently showed that upon co-culture with activated memory $CD4^+$ T cells, human MCs express several soluble (pro-inflammatory cytokines and COX-2-dependent eicosanoids) or membrane cues (costimulatory molecules) known to influence T cell responses.¹⁷ This capacity acquired by MCs during co-culture raises questions regarding the action they have in return on the biological functions of $CD4^+$ effector T cells (Teffs). To answer this question, we set up a co-culture system in which MCs, differentiated from $CD34^+$ precursors isolated from healthy blood donors, were cultured with allogeneic $CD45RO^+CD45RA^-$ memory $CD4^+$ T cells stimulated with CD3/CD28-coated beads. We first measured the proliferation and activation (monitored by the activation marker CD25 expression) kinetics of memory $CD4^+$ T cells. MCs greatly enhanced $CD4^+$ T cell proliferation (Figure 1A). CD25 expression on the $CD4^+$ T cell surface was slightly increased in the presence of MCs at the beginning of the co-culture but reached similar levels after the fifth day (Figure 1B). On day 6 of co-culture, $CD4^+$ T cell subsets were analyzed based on their intracellular cytokine content. Th cell subsets were defined according to the panel of cytokines produced: Th1 as $IFN-\gamma^+/IL-17^-/IL-22^-$, Th2 as $IFN-\gamma^-/IL-4^+$, Th17 as $IFN-\gamma^-/IL-17^+$, Th17.1 as $IFN-\gamma^+/IL-17^+$. Tregs were gated as $FOXP3^+$, latency-associated peptide (LAP)⁺, $CD25^+$ and $CD127^{low}$ (Figure 1C). Since TCR activation induces in Tregs the expression of transforming growth factor (TGF)- β , which remains associated with its propeptide LAP, as membrane-bound LAP,²⁴ we used LAP expression as an indicator of TGF- β production. Because Ag-experienced $CD4^+$ T lymphocytes isolated from human blood may have very different priming histories, we analyzed more than 70 combinations of MC/ $CD4^+$ Teffs from different donors. As shown in Figure 1C and D, the frequencies of Th1, Th2, Th17 and Th17.1 were increased in the presence of MCs, while Treg frequency was reduced. Analysis of these subset cell numbers indicated that MCs increased the number of cytokine-producing Th1, Th2, and Th17 cell subsets, whereas they slightly decreased the number of LAP⁺ Tregs (Figure S1). Flow cytometry analysis was confirmed by the increase in IL-4 and IL-17 amounts released by $CD4^+$ T cells when cultured in the presence of MCs (Figure 1E). Expression of Th master transcription factors quantified by RTqPCR on FACS-isolated $CD4^+$ T cells showed that whilst RORC expression was increased in the presence of MCs, FOXP3 was decreased (Figure 1F).

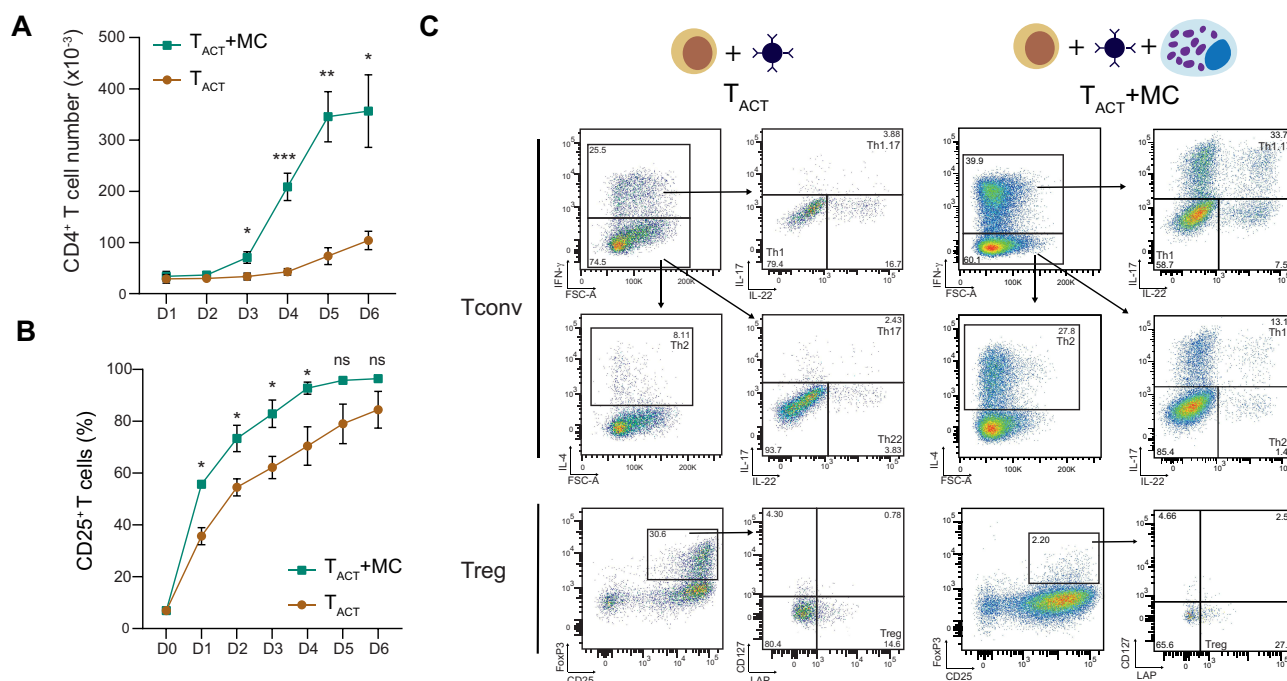


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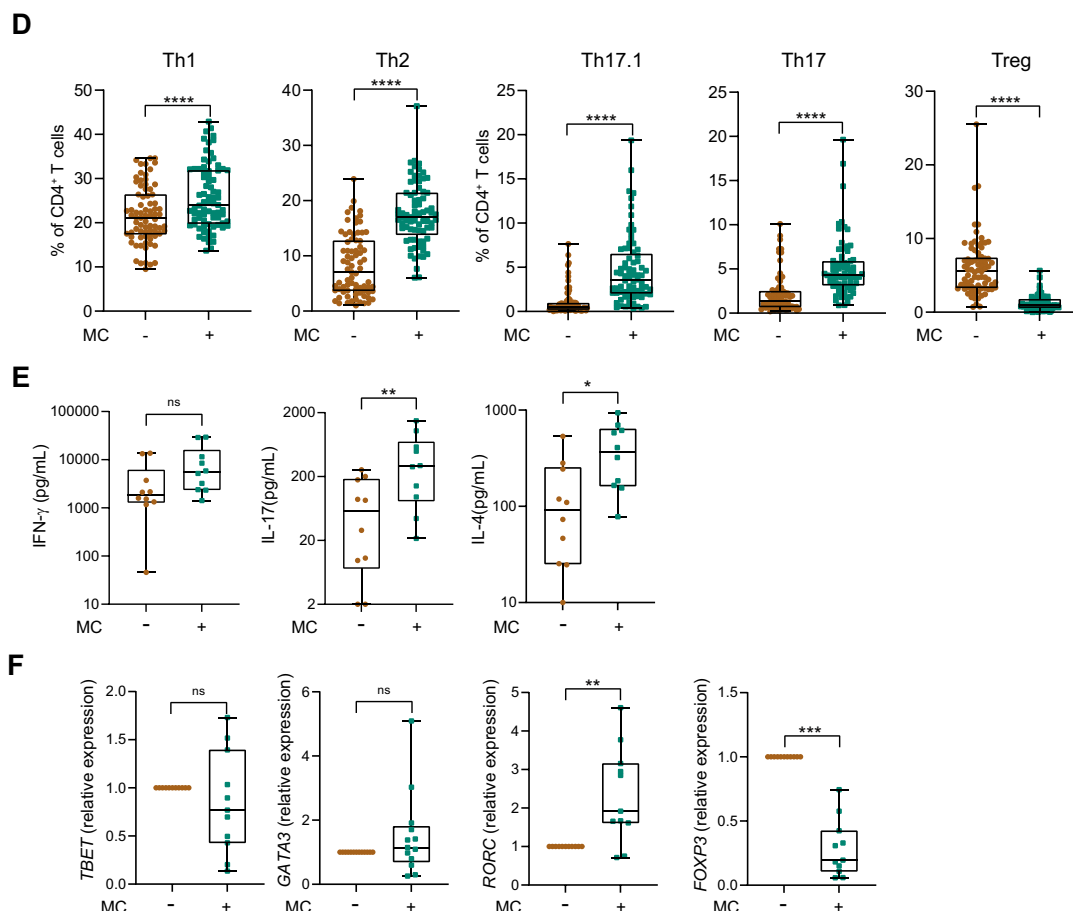


Figure 1 MCs promote Th17/Treg imbalance. Freshly isolated memory CD4⁺ T cells from healthy blood donors were stimulated with anti-CD3/CD28 coated beads (T_{ACT}) for 6 days in the presence (+MCs) or the absence (Ctrl) of MCs. **(A and B)** Kinetics of CD4⁺ T cell numbers **(A)** and CD25 expression in CD4⁺ T cells **(B)**. Pooled data from 9 **(A)** and 6 **(B)** independent experiments (mean $\pm$ SEM, two-way ANOVA with Dunnett post hoc test). **(C)** Gating strategy for the identification of Th cell subsets (Tconv) and Treg at day 6 of coculture. Shown are dot plot gated on singlet, live, CD4⁺ CD117⁻ cells. **(D)** Frequency of Th1, Th2, Th17, Th17.1 and Treg at day 6 of culture. Each point represents a combination of MCs and memory CD4⁺ T cells originating from different healthy donors ($n=77$ from 31 independent experiments). **(E)** After 6 days of coculture, CD4⁺ T cells were FACS-sorted and then restimulated with PMA and ionomycin ($n=9-12$). Amounts of cytokines measured in supernatants as assessed by multiplex bead-based assay. **(F)** Transcription factor expression in CD4⁺ T cells as assessed by RT-qPCR. Data are presented as box and whiskers plot, each point represents a MC/T cell pair. Paired Wilcoxon signed-rank test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns not significant.

Analysis of the IL-17-producing CD4⁺ T cell population showed that the fraction of Th17.1 (*ie* IFN- γ ⁺) among IL-17⁺ cells had doubled in the presence of MCs (**Figure 2A**). The pro-inflammatory phenotype of Th17.1 cells induced by MCs was confirmed by the increased expression of IL-22, GM-CSF and Granzyme B known to be strongly expressed in pathogenic Th17.1 (**Figure 2B**).²⁵ The analysis of intracellular cytokines showed that Th17 and Th17.1 subsets generated in the presence of MCs remained stable after 12 days of coculture (**Figure 2C**). Thus, MCs may imbalance Treg/Th17 steadiness by inducing pro-inflammatory Th17 lymphocytes while reducing Treg frequency.

MCs Alter Both Phenotype and Function of Treg

To assess the extent to which MCs alter Treg compartment, Tregs were sorted from freshly isolated CD4⁺ T cells, stained with the cell tracker CTV and then reincorporated with their Tconv counterparts to be cocultured with MCs (**Figure 3A**). After 6 days of coculture, both the frequency and number of CTV-labelled cells were not altered, but the percentage and number of Foxp3⁺CD25⁺CD127^{low}LAP⁺ cells in the CTV⁺ cell population were significantly reduced (**Figure 3B–D**). Moreover, LAP expression level in CTV⁺ cells was strongly decreased after coculture with MCs suggesting a reduced ability to produce TGF β (**Figure 3E**). Treg suppression assay, performed with CTV⁺ cells sorted after 6 days of coculture with MCs, showed a significant reduction of their immunosuppressive activity (**Figure 3F**). These results indicate that MCs alter Treg phenotype and impair their immunosuppressive potency. Because Treg cells can differentiate into Th17 cells,^{26,27} we analyzed the

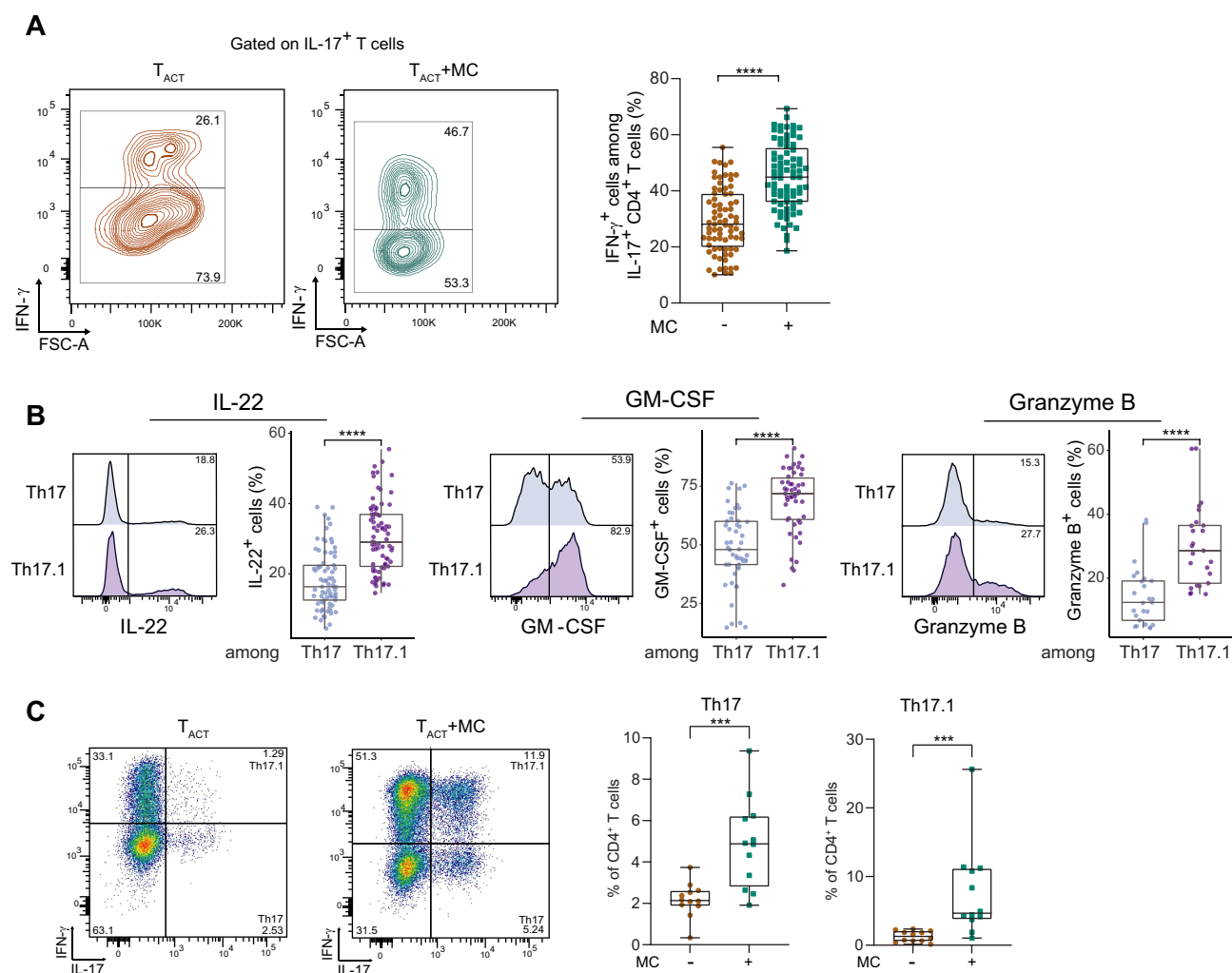


Figure 2 MCs promote Th17 cell commitment toward pro-inflammatory Th17.1 lymphocytes. Freshly isolated memory CD4⁺ T cells from healthy blood donors were stimulated with anti-CD3/CD28 coated beads for 6 days in the presence (+MCs) or the absence (Ctrl) of MCs. **(A)** Frequency of IFN- γ ⁺ cells among IL-17⁺ CD4⁺ T cells at day 6 of coculture, representative dot plots and pooled data. **(B)** Representative histograms and pooled data (n=25 to 77) from flow cytometry analysis of the frequency of Th17 and Th17.1 cells expressing IL-22, GM-CSF or Granzyme B at day 6 of coculture with MCs. **(C)** Frequency of Th17 and Th17.1 cells at day 12 of coculture as assessed by flow cytometry (n=12). Data are presented as box and whiskers plot, each point represents a combining MC/T cell pair. Paired Wilcoxon signed-rank test, ****p < 0.001, ***p < 0.0001, ns not significant.

frequency of CTV⁺ T lymphocytes producing IL-17 and/or IFN- γ . Although MCs promoted the emergence of Th17 cells (Figure 3G and H), the proportion of Th17 cells originating from Tregs remained very low (Figure 3I).

IL-1 β Plays a Pivotal Role in the Induction of Th17.1 but Not in the Treg Impairment

We previously showed that upon co-culture with activated Teff, MC produced polarizing cytokines such as IL-1 β , IL-6 and TNF but not IL-12 or IL-23.¹⁷ We focused on IL-1 β which is known to participate in the induction of IL-17⁺ Th subsets²⁸ and analyzed its production during co-culture. We observed that IL-1 β was produced only upon MC-activated Teff interplay, and peaked at 48 h of co-culture (Figure 4A). Confocal microscopy analysis showed that IL-1 β was expressed in MCs as well as in CD4⁺ T lymphocytes (Figure 4B and C). The addition of the IL-1 receptor antagonist (IL-1Ra) in the co-culture reduced the frequencies of Th17 and Th17.1 cell subsets without affecting the Treg subset (Figure 4D and E). In addition, the frequency of Th17.1 among IL-17-producing Th cells was reduced by IL-1Ra treatment (Figure 4F), indicating that IL-1 β was a key factor in the induction of Th17.1 cell subsets by MCs. The production of both IFN- γ and IL-17 by Teff was reduced by IL-1Ra treatment (Figure 4G). Collectively, these results indicated that IL-1 β is a key factor in the induction of pro-inflammatory Th17.1 lymphocytes by MCs.

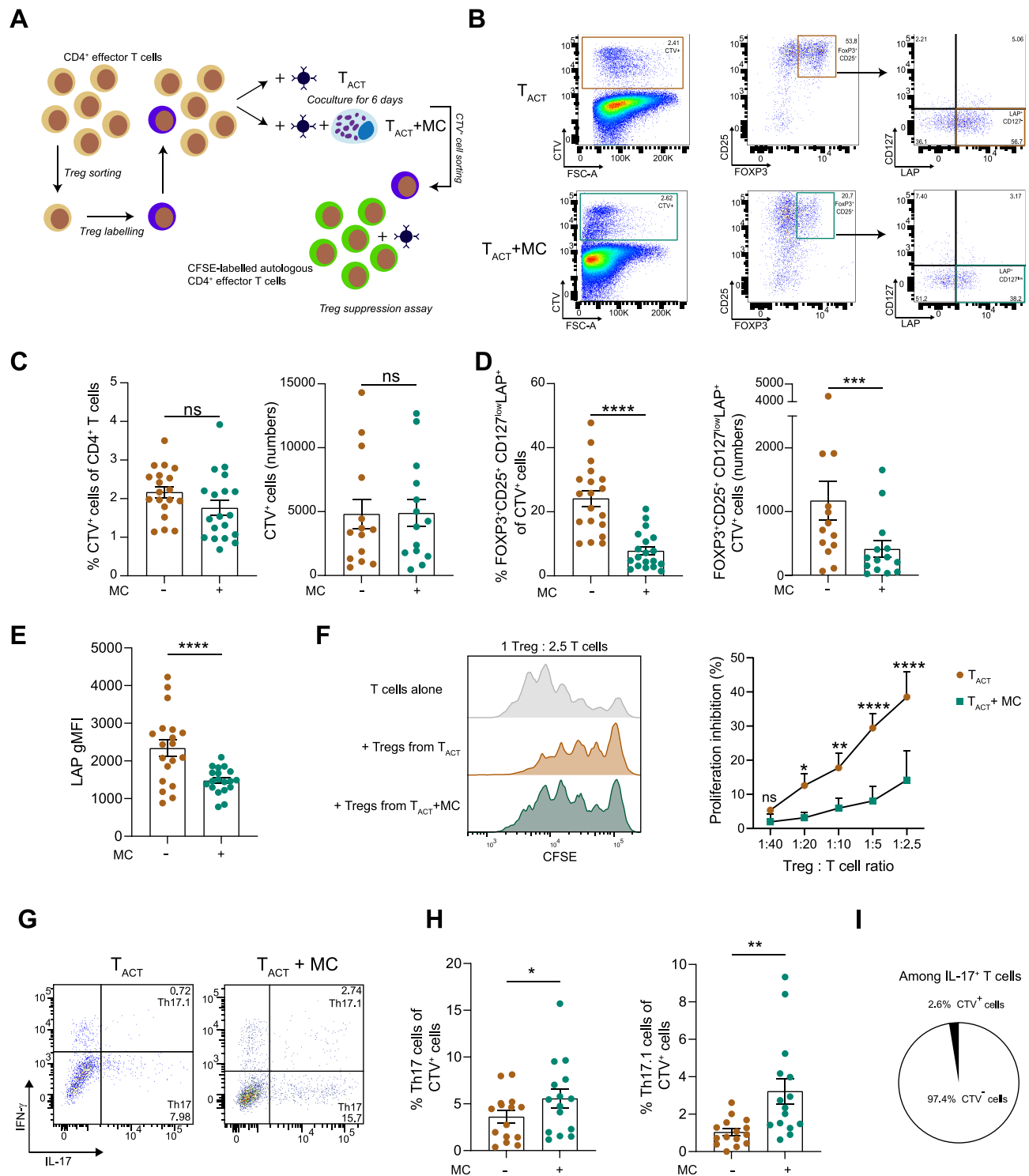


Figure 3 MCs alter both phenotype and function of Treg. **(A)** Experimental design, Tregs (CD4⁺CD127^{low}CD25⁺) were isolated with magnetic cell sorting and labelled with CTV cell tracer, reincorporated with their Tconv counterparts and next cocultured with MCs for 6 days. Representative dot plots **(B)** and pooled data (n=14-19) of the frequency and cell number of CTV⁺ cells **(C)** and CTV⁺ cells exhibiting Treg phenotype **(D)**. **(E)** LAP expression (geomean of fluorescence intensity) in CTV⁺ cell. **(A-F)** CTV⁺ CD4⁺ T cells were FACS-sorted at day 6 of culture and immunosuppression assay was performed using autologous CFSE-labeled memory CD4⁺ T cells. The figure depicts representative histograms (left panel) and inhibition of the proliferation of memory CD4⁺ T cells (right panel) from 5 independent experiments. Proliferation of CFSE⁺ CD4⁺ T cells was assessed by FACS at day 4 (mean ± SEM, two-way ANOVA using Sidak's multiple comparisons test). **(G and H)** Frequency of IFN-γ- and IL-17-producing CTV⁺ cells at day 6 of coculture following PMA/ionomycin restimulation. Representative dotplots **(G)** and pooled data **(H)** from 10 independent experiments. Data are presented as box and whiskers plot, each point represents a combining MC/T cell pair (n=15). **(I)** Proportion of CTV⁺ and CTV⁻ cells among IL-17⁺ CD4⁺ T cells. Paired Wilcoxon signed-rank test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns not significant.

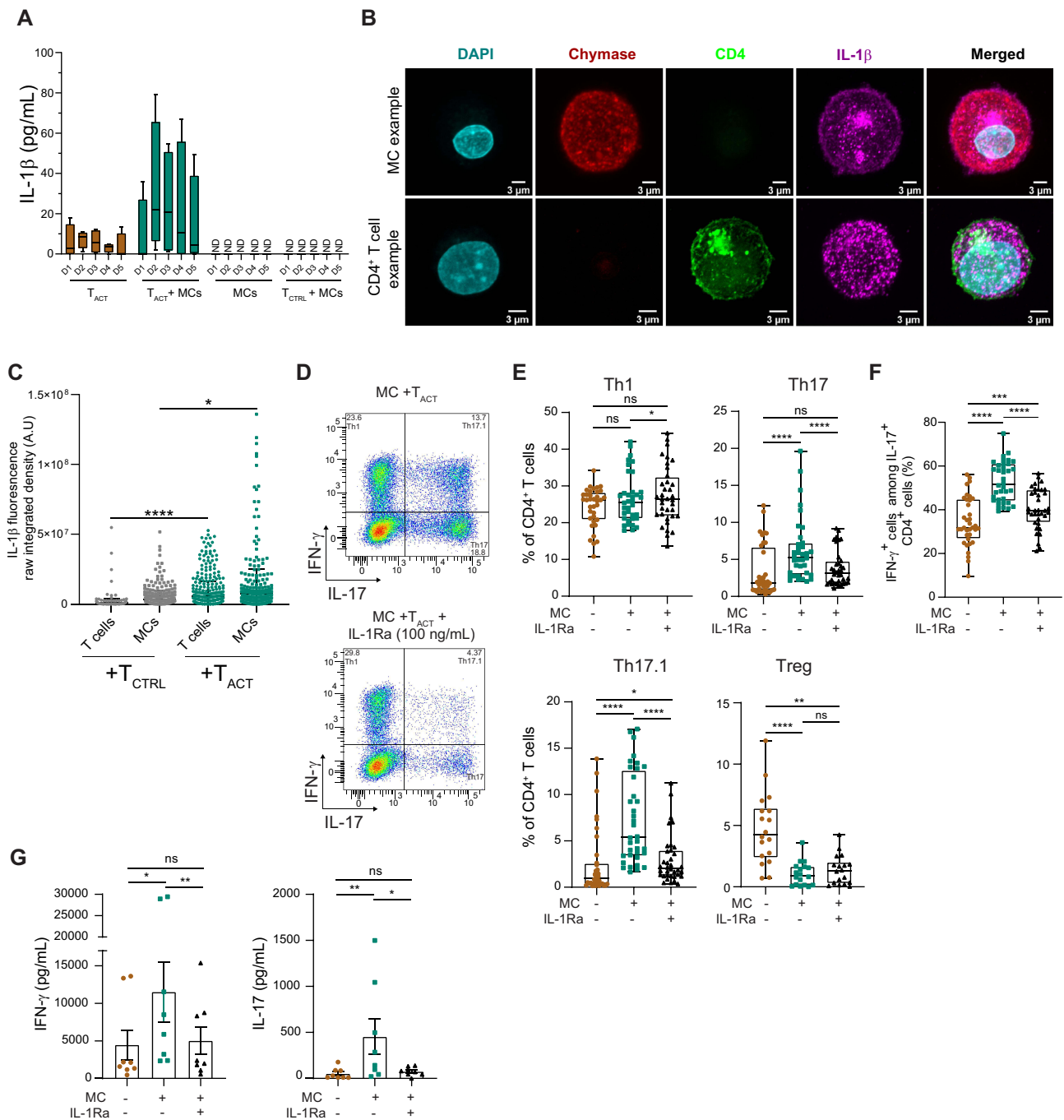


Figure 4 IL-1 β is required for the induction of IL-17 producing Th cell subsets. Memory CD4 $^{+}$ T cells were cultured with or without MCs in the presence of anti-CD3/CD28 coated beads for six days. **(A)** Kinetics of secreted IL-1 β measured in the supernatants under the indicated conditions. Mean $\pm$ SEM from 4 independent experiments. **(B)** Representative confocal microscopy images of IL-1 β $^{+}$ MCs and IL-1 β $^{+}$ CD4 $^{+}$ T cells after 2 days of coculture showing staining (average intensity projections) for chymase (red), IL-1 β (magenta), CD4 (green), and DAPI (cyan). **(C)** Quantification of IL-1 β fluorescence integrated density in MCs and CD4 $^{+}$ T cells (n=3 independent experiments). The Kruskal–Wallis test and pairwise comparisons were performed using Dunn's test. **(D and E)** Representative Dot plots **(D)** and Teff subset frequencies **(E)** on day 6 in IL-1Ra-treated cocultures. **(F)** Percentage of IFN- γ $^{+}$ cells among IL-17 $^{+}$ CD4 $^{+}$ T cells. Each point represents a MC/T cell pair (n=36 from eight independent experiments), and the data are presented as box-and-whisker plots. **(G)** After six days of co-culture, CD4 $^{+}$ T cells were FACS-sorted. Cytokine levels were measured after restimulation with PMA and ionomycin (mean $\pm$ SEM, each point represents a MC/T cell pair, n=8 from three independent experiments) using the Friedman test and pairwise comparisons using Dunn's test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns not significant.

PGE₂ Plays a Pivotal Role in Th17/Treg Imbalance

Since prostaglandins (PGE₂, PGD₂ and 15d-PGJ₂) are produced by MCs upon interaction with activated Teffs¹⁷ and have been shown to shape Treg and Th17 cell differentiation,^{29–32} we investigated whether they play a role in the Treg/Th17 imbalance. The impact of MC-derived eicosanoids on CD4 $^{+}$ T cells was assessed by adding at the beginning of the

coculture, indometacin, an irreversible cyclooxygenase inhibitor that abolishes the production of PGE₂, PGD₂ and 15d-PGJ₂ (Figure S2). Inhibition of cyclooxygenase by indometacin strongly reduced the frequency of Th17 and Th17.1 cell subsets and increased that of Treg (Figure 5A and B). In agreement, the release of IFN- γ and IL-17 as well as the expression of the master transcription factor RORC (Th17) was reduced (Figure 5C and D). The increased frequency of Tregs in the presence of indometacin was substantiated by that of *FOXP3* expression (Figure 5D) and by a recovery of their immunosuppressive ability (Figure 5E).

To identify which COX-2 dependent eicosanoids promote pro-inflammatory IL-17⁺ CD4⁺ T cells, indometacin-treated cocultures were supplemented with increasing amounts of either PGE₂, PGD₂ or 15d-PGJ₂. Although neither PGD₂ nor 15d-PGJ₂ had any effect (Figure S3), the addition of PGE₂ to the co-culture of activated T cells and MCs treated with indometacin restored the frequencies of Th17, Th17.1, and Treg in a dose-dependent manner (Figure 5F and G). Based on the RNAseq dataset showing that activated CD4⁺ T cells express the PGE₂ receptor subclasses EP2, EP3, and EP4,³³ we investigated the role of each PGE₂ receptor subtype by adding specific agonists in co-culture experiments.³⁴ In the presence of indometacin, the effects of PGE₂ were reproduced with butaprost (EP2 agonist) and, to a lesser extent, with L-902,688 (EP4 agonist), but not with sulprostone (EP3 agonist) (Figure 5H and I). Taken together, these results indicate that MC-derived PGE₂ promotes the differentiation of Th17 and Th17.1 lymphocytes and impairs Tregs, mainly through EP2 signaling.

MCs Exhibit a COX-2⁺/IL-1 β ⁺ Profile in Colonic Mucosa of Patients with Crohn Disease

Since the MC/Teff interplay allows the emergence of Th17.1 cells that are involved in immune-mediated inflammatory disease (IMID), we sought evidence of an interaction between MCs and CD4⁺ T cells in biopsies from patients with Crohn's disease. We focused on the mature form of IL-1 β and on the key inducible enzyme involved in prostaglandin biosynthesis, COX-2. High-resolution confocal microscopy tile-scan imaging revealed that MCs (tryptase⁺) were often at the vicinity of CD4⁺ T cells (Figure 6A–C). Quantitative analysis of more than 600 MCs indicated that the mean-distance between MCs and T cells was ~22 μ m, which is compatible with paracrine interactions,³⁵ Figure 6B. Approximately 25% of MCs were in direct contact with CD4⁺ T cells (Figure 6C). The histological analysis of the expression of COX-2 and the mature form of IL-1 β (cleaved IL-1 β) showed that the frequency of MCs expressing COX-2 and mature IL-1 β was dramatically increased in biopsies from Crohn's patients as compared to control samples (50.3% vs 19.3% and 35.8% vs 4.6%, respectively) (Figure 6D–G).

Taken together, these observations suggest that pro-inflammatory MCs, characterized by the expression of COX-2 and IL-1 β , that foster Th17.1 cells are greatly increased in Crohn's disease.

Discussion

We previously showed that activated memory CD4⁺ T cells induce a specific activation phenotype in MCs leading to a propensity to interact with T cells. In this study, we investigated here the effect of these alternatively activated MCs on memory CD4⁺ T cell responses. We used peripheral-blood-derived primary human MCs, which are widely used and found to be transcriptionally close to human tissue-resident MCs³⁶ in co-culture with ex vivo allogeneic CD4⁺ memory T cells isolated from PBMC. We found that MCs activated by Teff changed Th17/Treg equilibrium and induced the emergence of inflammatory Th17.1 cells via a mechanism involving PGE₂ and IL-1 β .

MCs are often associated with type 2 immune responses; however, here, we show that helped MCs may also be prone to drive Th17 responses and dampen Treg cells by producing PGE₂ and IL-1 β upon interaction with activated Teff. Mechanistically, MCs induced IL-17 production by producing PGE₂ and IL-1 β . Our results are in line with a previous report showing a pro-Th17 effect of PGE₂ in Ag-exposed T cells.^{37–40} IL-1 β is a well-known Th17 polarizing factor that has been shown to play a critical role in the reactivation of previously primed effector and memory CD4⁺ T cells by triggering cytokine production.⁴¹ Our results are in line with previous reports showing that co-culture of mouse MCs with CD4⁺ T cells induced GM-CSF in T cells and that MCs exert their influence on T cells through *Il1b* expression.^{21,42} More strikingly, we show that MCs lead to the emergence, via IL1 β and PGE₂, of a Th17.1 cell subset, producing IFN- γ and GM-CSF in combination with IL-17, described as pathogenic in some IMIDs.^{43,44} Although several studies have reported

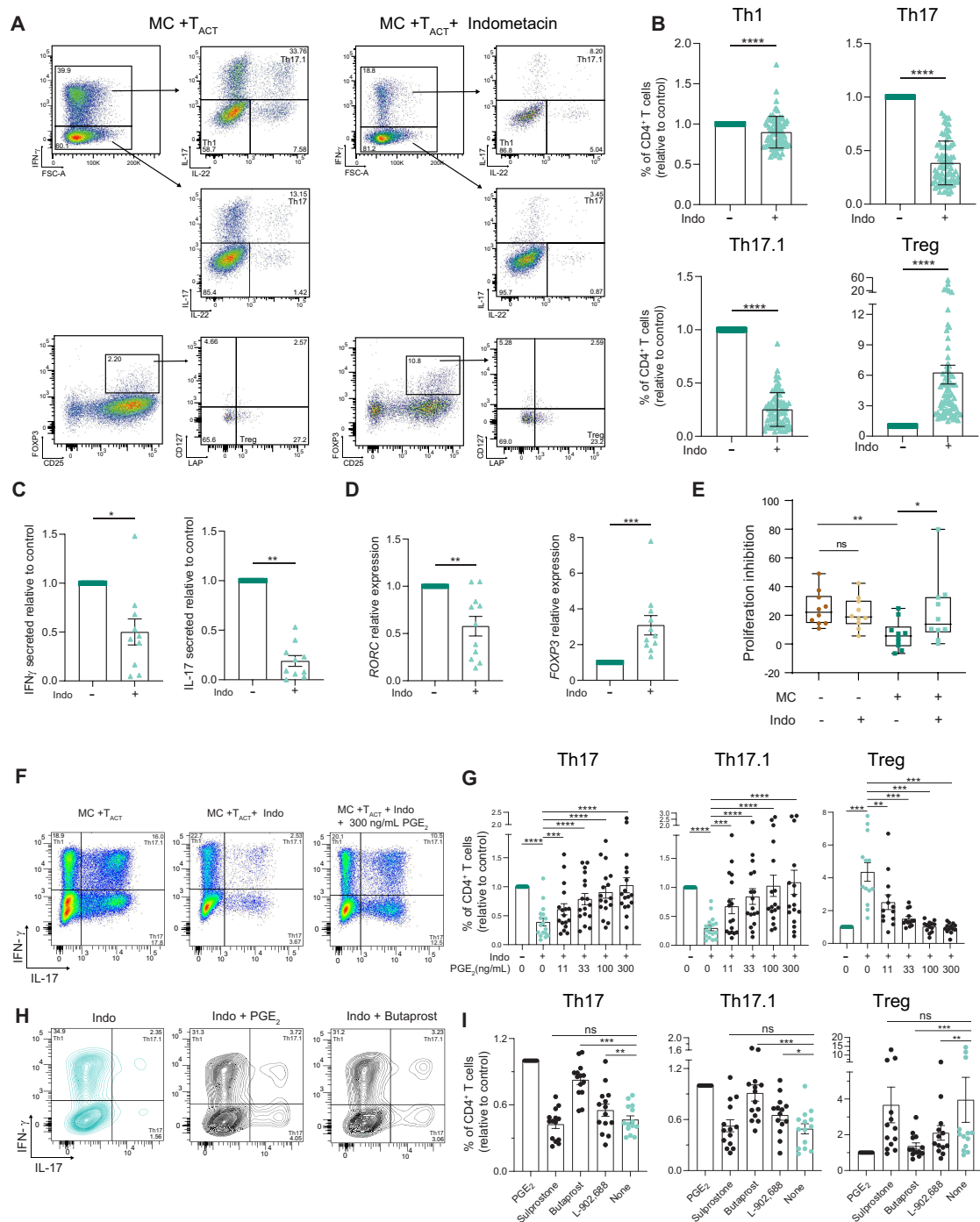


Figure 5 PGE₂ promotes Th17.1/Treg imbalance. Memory CD4⁺ T cells were cultured with MCs in the presence of anti-CD3/CD28 coated beads and treated with indometacin as indicated for 6 days. **(A and B)** Representative dot plots **(A)** and pooled data of the frequency of Th1, Th17, Th17.1 and Treg **(B)** at day 6 of coculture of activated CD4⁺ T cells with MCs in the presence or in the absence of indometacin as assessed by flow cytometry. Th cell subset frequencies are expressed as fold change over the control condition (MC + T_{ACT}), mean ± SEM, each point represents a combining MC/T cell pair (n=77). **(C)** IFN-γ and IL-17 amounts quantified by multiplex bead-based assay after restimulation with PMA and ionomycin (n=9) of CD4⁺ T cells FACS-sorted on day 6 of the coculture. **RORC** and **IL-17A** expression were assessed by RT-qPCR (n=12) **(D)**. **(E)** As detailed in Figure 3, Treg cells were sorted and labelled with CTV cell tracer, reincorporated with their Tconv counterparts and next cocultured with MCs in presence of indometacin for 6 days. CTV⁺ CD4⁺ T cells were FACS-sorted at day 6 of culture and immunosuppression assay was performed using autologous CFSE-labeled memory CD4⁺ T cells for 4 days. The figure depicts the inhibition of proliferation obtained at a ratio of 1 Treg per 5 Tconv (pooled data from 10 experiments, mean ± SEM). **(F and G)** Indometacin-treated cocultures were supplemented with increasing concentrations of PGE₂ and Th cell subsets frequencies were analyzed by flow cytometry at day 6. Dot plots of one representative experiment **(F)**, Th cell subset frequencies are presented as fold change over the control condition (MC + T_{ACT}), mean ± SEM, each point represents an experiment (n=17 from 13 independent experiments) **(G)**. **(H and I)** Indometacin-treated cocultures were supplemented with PGE₂ receptor agonists or 300 ng/mL of PGE₂. Th cell subset frequencies were analyzed by flow cytometry at day 6. Dot plots of one representative experiment **(H)**, Th cell subset frequencies are presented as fold change over the control condition (T_{ACT} + MC with indometacin and 300 ng/mL PGE₂), mean ± SEM, each point represents an experiment (n=14 from 4 independent experiments) **(I)**. Paired Wilcoxon signed-rank test or Friedman test and pairwise comparisons using paired Wilcoxon signed-rank test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns not significant.

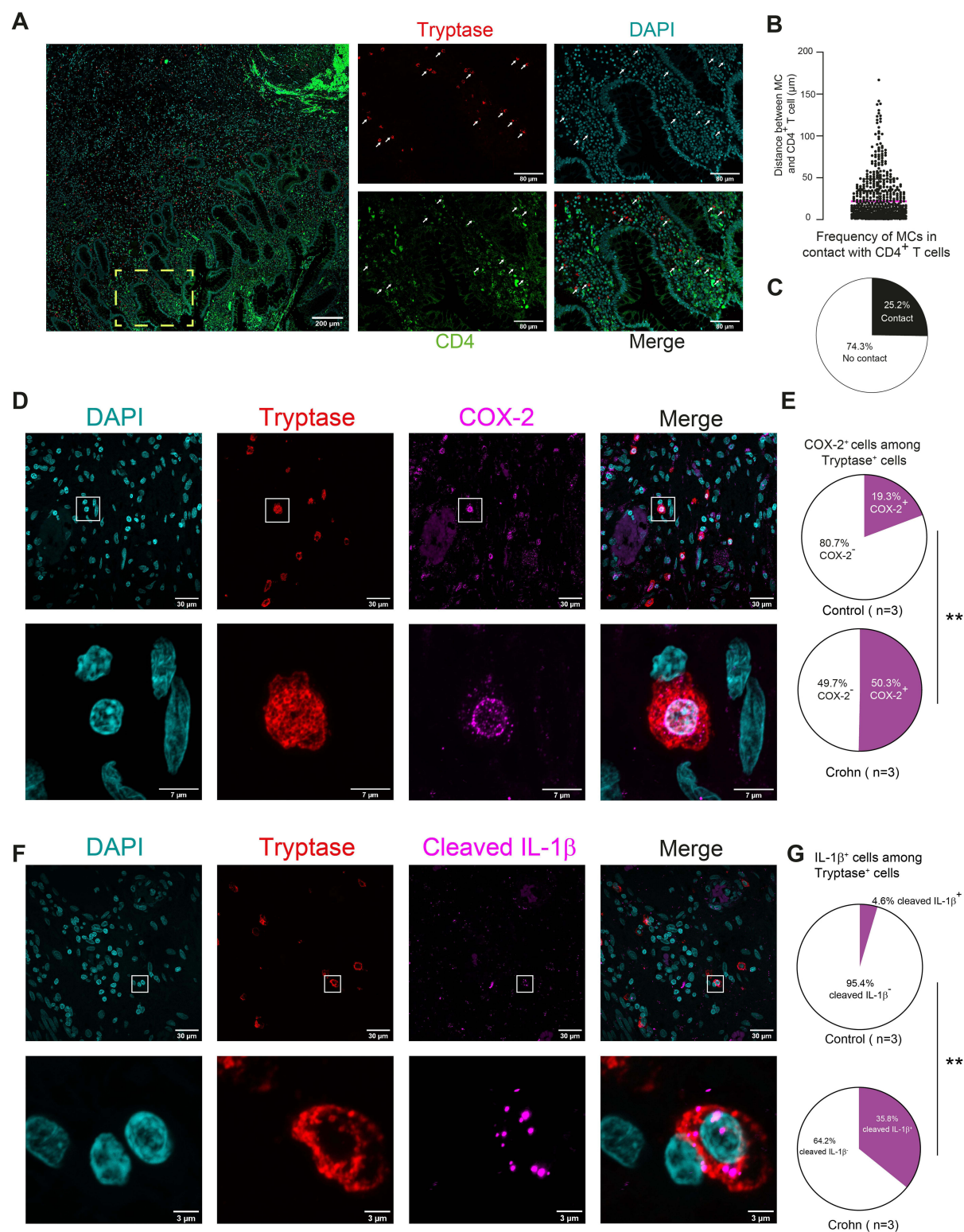


Figure 6 Crohn's disease-associated MCs exhibit a COX-2⁺/IL-1β⁺ profile. (A–C) MC and CD4⁺ T cell distribution in colonic mucosa from patients with Crohn's disease. Paraffin embedded tissues were stained for CD4 and tryptase markers. Representative staining image presented in maximal fluorescence intensity projection, box indicates the region depicted in the right panels, scale bars, scale bars 200 μm (left panel) and 80 μm (right panel). Measure of the distances between randomly selected MCs and their proximal CD4⁺ T cell (n=636 from 6 independent experiments) (B). Percentage of MCs in direct contact with CD4⁺ T cells. MCs of the imaged area were analyzed in 6 independent experiments (C). (D and E) Colonic mucosa from Crohn's disease or control patients were stained for CD4 (green), Tryptase (red), COX-2 (magenta) and DAPI (cyan). Representative images from patient with Crohn's disease, boxes indicate the region depicted in the lower panels (D). Quantification of COX-2⁺ MCs (n=3, E). (F and G) Colonic mucosa from Crohn's disease or control patients were stained for CD4 (green), Tryptase (red), cleaved IL-1β (magenta) and DAPI (cyan). Representative images from patient with Crohn's disease, boxes indicate the region depicted in the lower panels (F). Quantification of COX-2⁺ MCs (n=3, G). Unpaired t-test, ** p<0.01.

that IL-23 promotes the differentiation of Th17.1 cells, we showed here that these cells can also be induced by MCs independently of IL-23, highlighting the complexity of Th17 regulation.

The role of PGE₂ on Treg biology is more challenging to ascertain. Some studies have indicated that PGE₂ may facilitate iTreg differentiation,⁴⁵ whereas others have suggested that it may inhibit Foxp3 induction.^{46,47} Our results demonstrate that, upon stimulation by Teff, MCs alter Treg function via PGE₂ production and EP2 signaling, which is consistent with a previous report indicating that EP2 and EP4 receptors mediate PGE₂ suppression of mouse iTreg differentiation *in vitro*.³⁰ Furthermore, PGE₂ enhances CD4⁺CD25⁺ T regulatory function and confers regulatory activity in human CD4⁺CD25⁺ T cells,⁴⁵ indicating that in addition to PGE₂, MCs provide another signal that dampens Tregs. Nevertheless, it is tempting to speculate that PGE₂ signalling through EP2 induces the production of cyclic AMP in CD4⁺ cells, which has been shown to lead to CREB (cyclic AMP-responsive element binding protein) activation. This, in turn, has been demonstrated to both promote a Th17 gene expression program and reduce Treg proliferation and survival.⁴⁸

The capacity of MCs to promote Th17.1 cells while inhibiting Treg differentiation suggests a detrimental MC-CD4⁺ T cell axis in IMID. Indeed, MCs have been proposed to be involved in IMIDs such as multiple sclerosis, rheumatoid arthritis, type I diabetes or psoriasis.^{49,50} Ulcerative colitis-associated MCs are transcriptionally affected by T cell-derived IFN- γ and TNF in inflamed tissues⁵¹ and we previously showed using *in silico* analysis that a number of genes associated with IBD and other IMIDs overlapped with those upregulated genes in MC activated by Teffs.¹⁷ We found evidences in Crohn's disease, suggesting IL-1 β - and prostaglandin-based MCs/CD4⁺ T cells communication, highlighting the potential of MCs to promote pathogenic Th17 cells. The potential for this mechanism to occur in other IMIDs, such as psoriasis, remains to be demonstrated.

The present study demonstrates that the differentiation of an antigen-experienced CD4⁺ T cell polyclonal population in response to TCR restimulation can be influenced by the presence of MCs. These findings highlight the potential of MCs primed by activated Teff to influence subsequent responses, including the promotion of a proinflammatory Th17/Treg imbalance. This phenomenon may be particularly prevalent in tissues with a high proportion of MCs, such as skin and mucosa. Furthermore, the results indicate that, in the absence of MC priming by alarmins or pathogen-associated molecular patterns, MC/Teff interactions may contribute to the development of autoimmune or autoinflammatory processes.

Consent for Publication

Prior to submission, all listed authors agreed to all the manuscript contents, author lists, orders, and author contribution statements.

Acknowledgments

We thank the CRCT and the Infinity Core Facilities for their assistance.

Funding

This work was supported by grants from the Laboratoire d'Excellence Toulouse Cancer (TOUCAN) (contract ANR11-LABEX) and Ligue Nationale Contre le Cancer (Equipe Labellisée 2018). E.L. was supported by fellowships from the French Ministry of Education and Research, and Ligue Nationale Contre le Cancer. N. C. is the recipient of a grant from ANR (ANR-18-CE14-0039-01). We gratefully acknowledge the Toulouse INSERM Metatoul-Lipidomique Core Facility-MetaboHub ANR-11-INBS-010, where lipidomic analysis was performed, and the platform Aninfimip, an EquipEx ("Equipement d'Excellence"), supported by the French government through the Investments for the Future Program (ANR-11-EQPX-0003).

Disclosure

The authors declare no competing interests. Part of this study has been uploaded to Biorxiv as a preprint: <https://www.biorxiv.org/content/10.1101/2021.07.28.454103v1>

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