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Original Article

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TREM-1$^+$ Macrophages Define a Pathogenic Cell Subset in the Intestine of Crohn's Disease Patients

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Abstract

Background and Aims: Uncontrolled activation of intestinal mononuclear phagocytes [MNPs] drives chronic inflammation in inflammatory bowel disease [IBD]. Triggering receptor expressed on myeloid cells 1 [TREM-1] has been implicated in the pathogenesis of IBD. However, the role of TREM-1$^+$ cell subsets in driving IBD pathology and the link with clinical parameters are not understood. We investigated TREM-1 expression in human intestinal MNP subsets and examined blocking TREM-1 as a potential IBD therapy.

Methods: *TREM-1* gene expression was analysed in intestinal mucosa, enriched epithelial and lamina propria [LP] layers, and purified cells from controls and IBD patients. TREM-1 protein on immune cells was assessed by flow cytometry and immunofluorescence microscopy. Blood monocyte activation was examined by large-scale gene expression using a TREM-1 agonist or LP conditioned media [LP-CM] from patients in the presence or absence of TREM-1 and tumour necrosis factor [TNF] antagonist antibodies.

Results: *TREM-1* gene expression increases in intestinal mucosa from IBD patients and correlates with disease score. TREM-1$^+$ cells, which are mainly immature macrophages and CD11b$^+$ granulocytes, increase among LP cells from Crohn's disease patients and their frequency correlates with inflammatory molecules in LP-CM. LP-CM from Crohn's disease patients induces an inflammatory transcriptome in blood monocytes, including increased *IL-6* expression, which is reduced by simultaneous blocking of TREM-1 and TNF.

Conclusions: High intestinal *TREM-1* expression, reflecting a high frequency of TREM-1$^+$ immature macrophages and TREM-1$^+$CD11b$^+$ granulocytes, is linked to the deleterious inflammatory microenvironment in IBD patients. Therefore, blocking the TREM-1 pathway, especially simultaneously with anti-TNF therapy, has potential as a new IBD therapy.

Key Words: Crohn's disease, TREM-1, macrophages, intestinal inflammation, TNF, IL-6

1. Introduction

Inflammatory bowel disease [IBD], including Crohn's disease and ulcerative colitis [UC], is a chronic disease affecting the gastrointestinal tract and is increasing worldwide.[1,2] Several defects in intestinal homeostasis, including microbiota dysbiosis, epithelial barrier disruption and inflammation, have been linked to IBD. These culminate in the recruitment of immune cells that sustain the inflammatory microenvironment.[3,4] Intestinal mononuclear phagocytes [MNPs], comprising monocytes, macrophages [Mfs] and dendritic cells [DCs], play a critical role in maintaining tissue homeostasis and potentially in the emergence of IBD.[5,6] Indeed, intestinal MNPs internalize and process food and microbiota antigens for presentation to T cells to induce oral tolerance[7–9] but are also critical for triggering immune responses against pathogens.[10,11] Ideally, immune responses to pathogens are self-limiting and lead to resolution of inflammation and rapid return to tissue homeostasis.[12,13] This is exemplified by Mfs that clear apoptotic and dead cells and promote wound healing.[5,14,15] However, repeated and aberrant activation of MNPs can result in a chronic inflammatory microenvironment in IBD patients.[6,16] Thus, MNPs have a key role in cellular pathways that modulate tolerance vs chronic inflammation in IBD.

Cellular receptors expressed by MNPs sense local stimuli and can drive harmful immune responses in IBD patients.[17] One such receptor is the triggering receptor expressed on myeloid cells-1 [TREM-1], which is expressed on innate immune cells such as MNPs and neutrophils.[18–21] TREM-1 activation, after its multimerization at the cell membrane surface,[22] leads to the secretion of proinflammatory cytokines and chemokines, thus amplifying immune responses.[23,24] Moreover, microbial components in the intestinal environment such as lipopolysaccharide [LPS] and peptidoglycan, as well as cytokines such as tumour necrosis factor [TNF], can upregulate TREM-1 expression.[23,24] However, it is still not clear which endogenous ligand[s] are responsible for TREM-1 activation *in vivo*, particularly in human disease.[23–25]

Therefore, on the one hand, TREM-1 plays a critical role in the innate immune response to microbial pathogens while on the other hand it is involved in chronic inflammatory diseases such as IBD.[23,24] In experimental mouse models, TREM-1 activation induces intestinal inflammation and its blockade attenuates colitis severity,[26–28] partially through restoration of impaired autophagy and dysbiosis.[28] In humans, we and others have shown that TREM-1⁺ Mfs are increased in IBD patients, especially in active lesion areas.[26,29,30] Moreover, its engagement induces proinflammatory cytokine secretion by lamina propria [LP] cells,[26,29] which is partly dampened by TREM-1 blockade.[29] Together, these data support a potential role of TREM-1 in perpetuating chronic intestinal inflammation in IBD patients. However, the role of TREM-1⁺ MNP subsets in driving this pathological process, as well as their link with clinical parameters in IBD patients, are not fully understood.

Here we observe that *TREM-1* gene expression increases in intestinal mucosa from IBD patients and correlates with disease activity in UC. We show that high ileal *TREM-1* expression in Crohn's disease is due to an increase frequency of intestinal TREM-1⁺ cells, mainly immature Mfs and CD11b⁺ granulocytes, and correlates with inflammatory molecules in LP conditioned media [LP-CM] prepared from patients. Finally, simultaneous blocking of TREM-1 and TNF decreases LP-CM-induced *IL-6* in blood monocytes compared to blocking TNF alone. Together, the data suggest that blocking TREM-1, especially simultaneously with anti-TNF therapy, could be a potential therapy for some IBD patients.

2. Material and Methods

2.1. Clinical samples

The study was approved by the Regional Ethical Review Board in Gothenburg [permits 040-08 and 085-11] and all participants gave informed written consent [Table 1]. Surgical tissue from Crohn's disease and UC patients, as well as controls, was obtained from patients recruited at the Department of Surgery, Sahlgrenska University Östra Hospital, Gothenburg, Sweden. In the surgical control group, mucosa was acquired from colorectal cancer patients undergoing tumour resection; macroscopically normal tissue furthest from tumor tissue was obtained. Some of the ileal samples in the control group in Figure 1A were biopsies, whereas all other samples were from surgical tissue. The control biopsies [indicated with open blue

Table 1. Patient characteristics.

	Controls	Crohn's disease	UC
Number of patients	26	39	15
Percentage of females	50	56	20
Age in years, median [range]	73 [22–94]	42 [17–79]	39 [24–58]
Disease duration in years, median [range]		13 [0–39]	12 [3–33]
Harvey–Bradshaw index, median [range]		8 [0–24]	
Full Mayo score, median [range]			3 [0–9]
Disease status, active inflammation/remission		33/6	12/3
Type of operation for tissue accessibility			
Screening endoscopy	5		
Colorectal cancer surgery	21		
Crohn's disease or UC surgery		39	15
Samples used, blood/ileum/colon	9/20/8	8/32/7	0/0/15
Treatments, number [%]			
None	26 [100]	1 [3]	2 [13]
5-ASA	0	7 [18]	11 [73]
Anti-TNF	0	22 [56]	6 [40]
Corticosteroids	0	29 [74]	12 [80]
Thiopurines	0	16 [41]	7 [47]

Abbreviations: 5-ASA, 5-aminosalicylic acid; TNF, tumour necrosis factor; UC, ulcerative colitis.

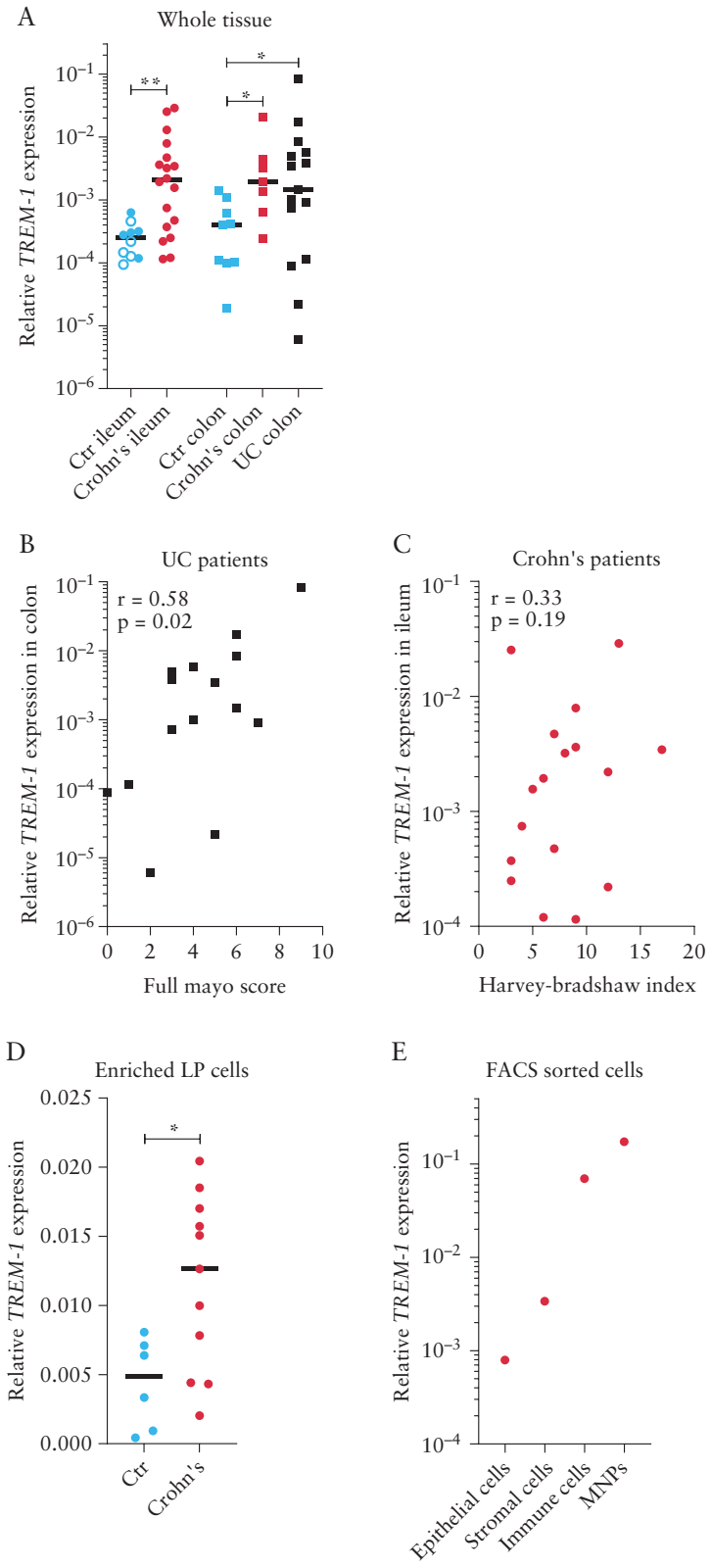

Figure 1. Intestinal mucosal *TREM-1* expression is increased in IBD patients, is attributed largely to MNPs and is linked to tissue disorders. [A] *TREM-1* gene expression determined by RT-PCR in intestinal mucosa of the indicated patient groups. Open blue circles represent ileal biopsies from controls as described in the Section 2.1. [B] Correlation between the full Mayo score from UC patients and colonic mucosal *TREM-1* expression. [C] Correlation between the Harvey–Bradshaw Index from Crohn's disease patients and ileal mucosal *TREM-1* expression. [D] *TREM-1* expression in enriched LP cells. [E] *TREM-1* expression in cells purified by fluorescence-activated cell sorting [FACS] as shown in Supplementary Figure 2A. Given the paucity of cells available in primary tissue, cells from four Crohn's disease patients were pooled before RNA extraction to get enough cDNA for each of the sorted cell populations. [A, D] Small horizontal lines indicate the median. Differences between the control group and patient groups were assessed by two-sided Mann-Whitney test; *p vs Ctr. [B, C] Correlations were assessed by Spearman's test. Abbreviations: Ctr, controls; LP, lamina propria; MNPs, mononuclear phagocytes; UC, ulcerative colitis; FACS sorted cells, cells purified using fluorescence-activated cell sorting.

circles in Figure 1A] were obtained from individuals undergoing endoscopy for health screening and were recruited at the Endoscopy unit, Sahlgrenska University Hospital. Buffy coats were obtained from anonymous healthy blood donors at Clinical Immunology and Transfusion Medicine, Sahlgrenska University Hospital.

2.2. Intestinal mucosa, tissue dissociation and cell enrichment

Fresh intestinal mucosa was collected in PBS and transported on ice [for maximum of 1 h]. The mucosa was stripped of muscle and fat and cut into small pieces. A piece of mucosa was put in RNAlater Stabilization Solution [Thermo Fisher Scientific] and stored at −80°C for gene expression analysis by RT-PCR [see below]. To obtain enriched epithelial [Epi] cells, mucosal tissues were washed with HBSS-EDTA (Ca^{2+} and $Mg2^{+}$ -free HBSS [Thermo Fisher Scientific] containing 2% FBS [Biological Industries], 15 mM Hepes [Thermo Fisher Scientific] and 2 mM EDTA [VWR]) 3–5 times for 15 min each at 37°C. Then, to obtain enriched LP cells, mucosa was incubated with stirring at 37°C for 90–120 min [depending on the size] with RPMI-digestion media (RPMI 1640 with GlutaMAX [Thermo Fisher Scientific] containing 10% FBS, 15 mM Hepes, 2.5 mM $CaCl_2$, 60 Kunitz units/mL DNase I [Sigma-Aldrich] and 40 CDU/mL collagenase D [Sigma-Aldrich] or 0.1 mg/mL Liberase DL [Roche]). Following digestion, the crude LP cell suspension was further dissociated using a gentleMACS C Tube in combination with the GentleMACS Dissociator [Miltenyi Biotec] for 3 × 1 min. Both Epi and LP cell suspensions were filtered through a 150-µm polyamide mesh [Saatifil] followed by filtration with a 100-µm and/or a 40-µm cell strainer [Corning, Sigma-Aldrich]. Filtered suspensions were washed in PBS and erythrocytes were removed by incubation with RBC lysis buffer [154 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA in water] for 10 min at room temperature followed by a final wash in PBS. Viable cells were counted on KOVA slides [KOVA International] after Trypan blue staining [Thermo Fisher Scientific]. For gene expression analysis, cells from Epi and LP suspensions were resuspended in Buffer RLT [Qiagen] + 1% β-mercaptoethanol [Sigma-Aldrich] and stored at −80°C. Remaining suspensions were used for cell culture, flow cytometry and cell sorting as described below.

2.3. RNA extraction from FFPE tissue

Formalin-fixed paraffin-embedded [FFPE] tissue from ileum of controls and Crohn's disease patients was sectioned directly into microcentrifuge tubes as curls [10-µm sections]. The curls were deparaffinized with xylene and RNA was extracted using a RNeasy FFPE Kit [Qiagen] according to the manufacturer's instructions. RNA concentration and quality were assessed using both NanoDrop 1000 Spectrophotometer [Thermo Fisher Scientific] and Agilent 2200 TapeStation System [Agilent Technologies]. Gene expression was analysed by NanoString nCounter technology [see below].

2.4. Peripheral blood CD14⁺ monocyte isolation

Peripheral blood mononuclear cells [PBMCs] were isolated from buffy coats by density gradient centrifugation on Ficoll-Paque PREMIUM [GE Healthcare]. After washing in PBS and removal of erythrocytes as described above, $CD14^{+}$ monocytes were isolated using $CD14^{+}$ magnetic beads (EasySep Human CD14 Positive Selection Kit II [Stemcell Technologies, Canada]). Purity was evaluated on BD LSRFortessa X-20 and was > 90%.

2.5. Cell culture

Cells from LP suspensions were seeded at 37°C in Nunc 96-well polystyrene round-bottom plates [Thermo Fisher Scientific] with RPMI-culture media (RPMI 1640 with GlutaMAX containing 10% FBS, 15 mM HEPES and 50 µg/mL gentamycin [Thermo Fisher Scientific]) at 1×10^6 cells/mL. After 22 h of incubation, supernatants were collected after centrifugation at 2000 rpm for 5 min and filtering through a 0.2-µm filter [Corning]. To prevent protein degradation, protease inhibitor cocktail 50× [Promega] was added and supernatants were stored at −80°C for protein quantification or as conditioned media [LP-CM].

Magnetically isolated $CD14^{+}$ monocytes were seeded at 37°C in Nunc 96-well round-bottom plates with RPMI-culture media at 1×10^6 cells/mL. Monocytes were then cultured with or without peptidoglycan from *Staphylococcus aureus* [Invivogen] [as a positive control], human TREM-1 agonist, human TREM-1 antagonist, anti-TNF adalimumab biosimilar or appropriate isotype control antibody [Supplementary Table 1]. In some conditions, monocytes were also cultured with LP-CM [40% in RPMI-culture media]. After 5 h of culture, plates were centrifuged for 5 min at 1500 rpm and monocytes were lysed in diluted Buffer RLT [1/3 Buffer RLT + 1% β-mercaptoethanol and 2/3 water] and stored at −80°C for gene expression analysis by NanoString nCounter technology or RT-PCR as below.

2.6. Protein quantification

Protein detection in LP supernatants was performed using a multiplex Proximity Extension Assay for 92 inflammation-related proteins using the Inflammation Panel from Olink Proteomics. NPX, Normalized Protein eXpression, is Olink's arbitrary unit in $\log_2$ scale for relative quantification. NPX values were converted to linear data, using the formula 2^{NPX}, before further statistical analysis. TNF concentration in LP supernatants was also measured by enzyme-linked immunosorbent assay [ELISA] (DY210 [R&D Systems]) according to the manufacturer's recommendations.

2.7. Flow cytometry and cell sorting

Epi and LP cell suspensions were resuspended in FACS buffer [PBS with 3% FBS, 15 mM Hepes and 5 mM EDTA] and incubated with FcR blocking reagent and LIVE/DEAD according to the manufacturer's protocol [Thermo Fisher Scientific] for 10 min at room temperature in the dark. After washing in FACS buffer, cells were stained with fluorescence-conjugated antibodies [Supplementary Table 1] for 30 min at 4°C in the dark. Cells were acquired using a BD LSRFortessa X-20 and data were analysed with FlowJo 10.6.1 software [BD Biosciences]. For gene expression analyses by RT-PCR [see below], cell populations were sorted using a BD FACSAria Fusion, washed once with PBS, resuspended in Buffer RLT + 1% β-mercaptoethanol and stored at −80°C.

2.8. Multicolour immunofluorescence microscopy

FFPE blocks of ileum from controls and Crohn's disease patients were cut into 4-µm sections and mounted on Superfrost Plus microscope slides [Thermo Fisher Scientific] at the Clinical Pathology unit, Sahlgrenska University Hospital. Slides were deparaffinized with xylene and rehydrated with successive baths of ethanol and water. Antigens were unmasked using a 2100 Antigen Retriever [Aptum Biologics]. Slides were then sequentially stained with antibodies against TREM-1, CD163, CD11c and Pan-CK [Supplementary Table 1] using an Opal 7-Color Manual IHC Kit [Akoya Biosciences]

according to the manufacturer's recommendations. Subsequently, cell nuclei were stained with spectral DAPI [Akoya Biosciences] and slides were mounted with ProLong Glass Antifade Mountant [Thermo Fisher Scientific]. Stained sections were scanned with a Metasystem automated slide scanner [MetaSystems, Germany] equipped with SpectraSplit filter system for extended multicolour imaging [Kromnigon] and a Carl Zeiss AxioImager.Z2 microscope [Carl Zeiss]. Image analysis was performed with Zeiss ZEN 3.1 software [Carl Zeiss].

2.9. RNA extraction and RT-PCR analysis

Total RNA from whole intestinal mucosa or enriched Epi and LP cells was extracted using NucleoSpin RNA Mini kit [Macherey-Nagel] or RNeasy Mini Kit [Qiagen]. Total RNA from sorted cells and blood CD14$^+$ monocytes was extracted using RNeasy Micro Kit [Qiagen]. cDNA was produced using Quantiscript Reverse Transcriptase [Qiagen]. Forward and reverse primers [Supplementary Table 1] were developed and produced by OligoArchitect Oline [Sigma-Aldrich]. QuantiFast SYBR Green PCR Master Mix [Qiagen] was utilized for quantitative RT-PCR using an Applied Biosystems 7300 Real-Time PCR System [Thermo Fisher Scientific]. Relative gene expression was normalized to the housekeeping gene *RPLP0* and analysed using the $2^{-\Delta Ct}$ method.

2.10. NanoString nCounter analysis

NanoString nCounter analysis [NanoString Technologies] was performed at the Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden. For analysis of FFPE curls from ileum, differential gene expression was assessed using the Human Fibrosis V2 Panel [NanoString nCounter; 770 genes including ten housekeeping genes], in which a Panel Plus containing 30 genes of interest was added [Supplementary Table 1]. For analysis of peripheral blood CD14$^+$ monocytes, differential gene expression was assessed using the Human Myeloid Innate Immunity V2 Panel [NanoString nCounter; 770 genes including 40 housekeeping genes]. Graphics and visualization of gene expression were done using nSolver 4.0 Analysis Software [NanoString Technologies].

2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0 [GraphPad Software]. Paired and unpaired comparisons were determined by non-parametric Wilcoxon and Mann-Whitney tests, respectively. Non-parametric Spearman's tests were used to evaluate correlations. Differences were considered significant at $p < 0.05$ [$*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$].

3. Results

3.1. Intestinal mucosal *TREM-1* expression is increased in IBD patients, is attributed largely to Mfs and neutrophils and is linked to tissue disorders

Analysis of whole tissue *TREM-1* gene expression in fresh mucosa revealed increased expression in the colon of both Crohn's disease and UC patients and in the ileum of Crohn's disease patients compared to their respective controls [Figure 1A]. Of note, there was no statistical difference in *TREM-1* expression between ileum and colon in controls or in Crohn's disease patients [Figure 1A]. To gain insight into interpatient variability, we evaluated several parameters of the patients [Table 1] in relation to their whole tissue *TREM-1* expression. First, we did not observe any effect of sex, age, disease duration or smoking status [data not shown]. We also performed analyses based on treatments. As the number of different treatment regimens/combinations is great, and treatments are also heterogeneous regarding timing and duration, we compared only two groups for each treatment [with or without]. We did not observe a statistically significant effect on *TREM-1* expression due to use of corticosteroids, thiopurines, 5-aminosalicylic acid [5-ASA] or anti-TNF [data not shown]. However, whole tissue *TREM-1* expression from UC patients significantly correlated with their full Mayo score [Figure 1B] while in the ileum of Crohn's disease patients, whole tissue *TREM-1* expression tended to correlate with the Harvey–Bradshaw Index [HBI] [Figure 1C].

To further investigate the impact of high *TREM-1* expression on the intestinal mucosal disorder in Crohn's disease patients, we evaluated the correlation between *TREM-1* and more than 800 genes related to inflammation and fibrosis in the same sample. To this end, using NanoString nCounter technology, we used quantitative RNA expression of whole ileal mucosa from an additional cohort of controls and Crohn's disease patients. First, the NanoString nCounter technology confirmed that whole tissue *TREM-1* expression increases in the ileum of Crohn's disease patients compared to controls [Supplementary Figure 1A] but did not significantly correlate with HBI [Supplementary Figure 1B]. Then, *TREM-1* expression in Crohn's disease patients positively correlates with 117 genes related to the immune system [based on Biological Processes from Gene Ontology] [Supplementary Figure 1C].[31] By contrast, *TREM-1* expression negatively correlated with 75 genes related to metabolic processes [Supplementary Figure 1D].

To delineate the cell type[s] linked to the pathological pathways associated with high *TREM-1* expression in patients, we analysed which cell types express the surface molecules encoded by the genes that positively correlate with *TREM-1*. This was based on both Cell Type Annotation from NanoString nCounter technology and the Blood Atlas from The Human Protein Atlas [http://www.proteinatlas.org].[32] In both cases, the genes encoding surface molecules were highly expressed by neutrophils and monocytes/macrophages, including *CSF3R*, *FCAR*, *FCGR3A/B*, *FPR1*, *IL1R1*, *TLR2* and *CD14* [data not shown]. We next used fresh ileal mucosa from controls and Crohn's disease patients and analysed *TREM-1* expression in Epi and LP layers. Overall, *TREM-1* was expressed more in the LP fraction compared to the paired Epi fraction in both controls and Crohn's disease patients [data not shown]. Among Crohn's disease patients, *TREM-1* expression increased in the LP fraction compared to controls [Figure 1D]. Then, by analysing cells purified by fluorescence-activated cell sorting [FACS] from ileal mucosa of Crohn's disease patients [Supplementary Figure 2A], we showed that *TREM-1* is mainly expressed by CD45$^+$ immune cells, particularly MNPs, rather than by epithelial cells or stromal cells [Figure 1E]. Overall, the data suggest that high intestinal *TREM-1* expression, mainly by MNPs and neutrophils, is linked to immunopathology and tissue disorders.

3.2. Intestinal MNP frequency increases in Crohn's disease patients due to accumulation of immature Mfs

To define which intestinal MNP subset[s] are involved in the *TREM-1* increase in Crohn's disease ileum, we analysed TREM-1 expression on defined MNP populations by flow cytometry [Supplementary Figure 2B].[6,33] First, no difference in the frequency of CD45$^+$ immune cells among live LP cells from ileum was observed between controls and Crohn's disease patients

[~ 60% in both groups, data not shown]. In addition, among CD45⁺ immune cells, there was no difference in the frequency of non-lymphocytes [CD3⁻CD19⁻CD56⁻] [Supplementary Figure 2E]. By contrast, the frequency of MNPs [Supplementary Figure 2A] was increased in Crohn's disease patients compared to controls [Figure 2B]. Therefore, we analysed the frequency of the main subsets of cells among MNPs, which are conventional DCs [cDCs] and Mfs, based on CD11c and CD14 expression, as well as the less well-defined CD11c⁻CD14⁻ population that comprises plasmacytoid dendritic cells, among others [Figure 2A and Supplementary Figure 2B]. No difference in frequency was apparent between controls and Crohn's disease patients regarding cDCs among MNPs [Figure 2C], while the frequency of cDCs increased slightly among CD45⁺ cells due to an increase of the cDC2 subset in Crohn's disease patients [Supplementary Figure 2F,G]. By contrast, the CD11c⁻CD14⁻ cells, among MNPs [Figure 2C] and among CD45⁺ cells [Supplementary Figure 2H], were lower in Crohn's disease patients.

In contrast, there was a significant increase in Mf frequency among MNPs and among CD45⁺ cells [Figure 2C, D] in Crohn's disease patients compared to controls. This prompted us to further investigate the frequency of the recently described intestinal Mf subsets [Supplementary Figure 2B].[6,33] First, based on the expression of CD11c, the bulk Mf population can be separated into newly recruited monocytes that express higher CD11c, hereafter called immature Mfs, and fully differentiated resident Mfs that express lower CD11c, hereafter called mature Mfs [Figure 2A].[33] We found that the frequency of immature Mfs increases in Crohn's disease patients compared to controls [Figure 2E] while there was no difference in the frequency of mature Mfs [Figure 2F]. Thus, the ratio of immature/mature Mfs increases by 20 times in Crohn's disease patients compared to controls [Figure 2G]. More precisely, it has been recently described that intestinal Mfs comprise four subsets, two immature subsets called Mf1 and Mf2, and two mature subsets named Mf3 and Mf4 [Figure 2H and Supplementary Figure 2B].[33] There is a maturation waterfall from Mf1 to Mf3 while the Mf4 subset is present in the submucosa and muscularis propria.[33] Here, for the first time, we show that the frequency of both Mf1 and Mf2 subsets increase in Crohn's disease patients compared to controls [Figure 2I] while there is no difference in the frequency of Mf3 and Mf4 [Figure 2J]. Together these data show increased intestinal Mf frequency in Crohn's disease patients due to a higher recruitment of blood monocytes that, based on surface phenotype, have not matured in the tissue.

3.3. Intestinal TREM-1⁺ cells increase in Crohn's disease patients and are mainly immature Mfs and CD11b⁺ granulocytes

To confirm the increase of TREM-1 at the protein level, we analysed its expression by flow cytometry [Supplementary Figure 2B–D]. Overall, the frequency of TREM-1⁺ cells increased among CD45⁺ cells in Crohn's disease patients compared to controls [Figure 3A]. Moreover, the frequency of TREM-1⁺CD11b⁺ granulocytes was higher in Crohn's disease ileum [Figure 3B] and represent the majority of TREM-1⁺ cells in both controls and Crohn's disease patients [~60%]. Among CD45⁺ cells, the frequency of TREM-1⁺ Mfs was also increased in Crohn's disease patients [Figure 3B] while the frequency of the other TREM-1⁺ cells, mainly cDCs, did not change. Finally, the frequency of TREM-1⁺ cells correlated with *TREM-1* gene expression in LP cells [Figure 3C].

To define the intestinal MNP subset[s] comprising the increase in TREM-1⁺ cells, we assessed TREM-1 expression on each MNP subset by flow cytometry [Supplementary Figure 2B]. In both Crohn's disease patients and controls, a high frequency of TREM-1⁺ cells was found among the immature Mf subsets, Mf1 and Mf2 [Figure 3D, E]. In contrast, the mature Mf subsets Mf3 and Mf4 had low expression of TREM-1 [Figure 3D, E]. In particular, there was a progressive decrease of TREM-1 expression from Mf1 to Mf3 [Figure 3D, E], suggesting that TREM-1 is highly expressed on blood monocytes but is reduced during differentiation of intestinal Mfs into mature cells. Of note, a low fraction of the three cDC subsets expressed TREM-1 [Figure 3D] and there was no difference in the frequency of TREM-1⁺ cDC subsets between controls and Crohn's disease patients [data not shown]. Moreover, comparing controls and Crohn's disease patients for a given Mf subset, the fraction of TREM-1⁺ cells was similar [Figure 3E]. Nevertheless, we observed an increased frequency of both TREM-1⁺ Mf1 and TREM-1⁺ Mf2 among CD45⁺ cells in Crohn's disease patients [Figure 3F]. Thus, even if TREM-1 expression did not increase on a specific MNP subset per se during Crohn's disease, increased monocyte recruitment to the intestinal mucosa, and thus accumulation of immature Mfs that highly express TREM-1, resulted in an overall increase of TREM-1⁺ Mfs in the intestine of Crohn's disease patients.

Finally, we analysed TREM-1 protein expression on intestinal cell populations *in situ* using multicolour immunofluorescence microscopy. FFPE ileum slides were stained simultaneously using a five-colour panel to identify nuclei [DAPI], epithelial cells [Pan-CK], Mfs [CD163], Mfs/cDCs [CD11c] and TREM-1 [Supplementary Figure 3A]. When the fluorescence intensity of each antibody was quantified in ten villi per subject, the intensity of both DAPI and Pan-CK did not differ between controls and Crohn's disease patients [data not shown]. In contrast, CD163, CD11c and TREM-1 intensity were increased in Crohn's disease patients compared to controls [Supplementary Figure 3B]. Moreover, TREM-1 intensity correlated with CD11c intensity in Crohn's disease patients [Supplementary Figure 3C] but not in controls [Supplementary Figure 3D], supporting that immature Mfs, which express CD11c, are a main source of TREM-1 staining in Crohn's disease patients. Overall, these data show that TREM-1 protein was mostly expressed by immature Mfs and correlated with Mf markers *in situ* in Crohn's disease patients. Finally, we noted that the intensity of TREM-1 protein measured by immunofluorescence tended to correlate with *TREM-1* gene expression measured by NanoString nCounter technology [Supplementary Figure 3E], suggesting the possibility of using protein or gene expression to quantify TREM-1 in FFPE tissue from IBD patients.

3.4. TNF release by LP cells increases in Crohn's disease patients and strongly correlates with the frequency of TREM-1⁺ cells

To understand if the accumulation of intestinal TREM-1⁺ cells is associated with the pathology in Crohn's disease, we analysed their link to TNF production by isolated LP cells. We observed that TNF content in LP-CM of Crohn's disease patients was higher relative to controls [Figure 4A]. Moreover, patients with the highest TNF content in LP-CM are those with both the highest *TREM-1* gene expression [Figure 4B] and the highest frequency of TREM-1⁺ cells among LP cells [Figure 4C]. Furthermore, a correlation matrix between flow cytometry, RT-PCR and ELISA showed that TNF content in LP-CM positively correlated with the frequency of Mfs [Figure 4D; far right column]. In particular, it correlated with the frequency of immature

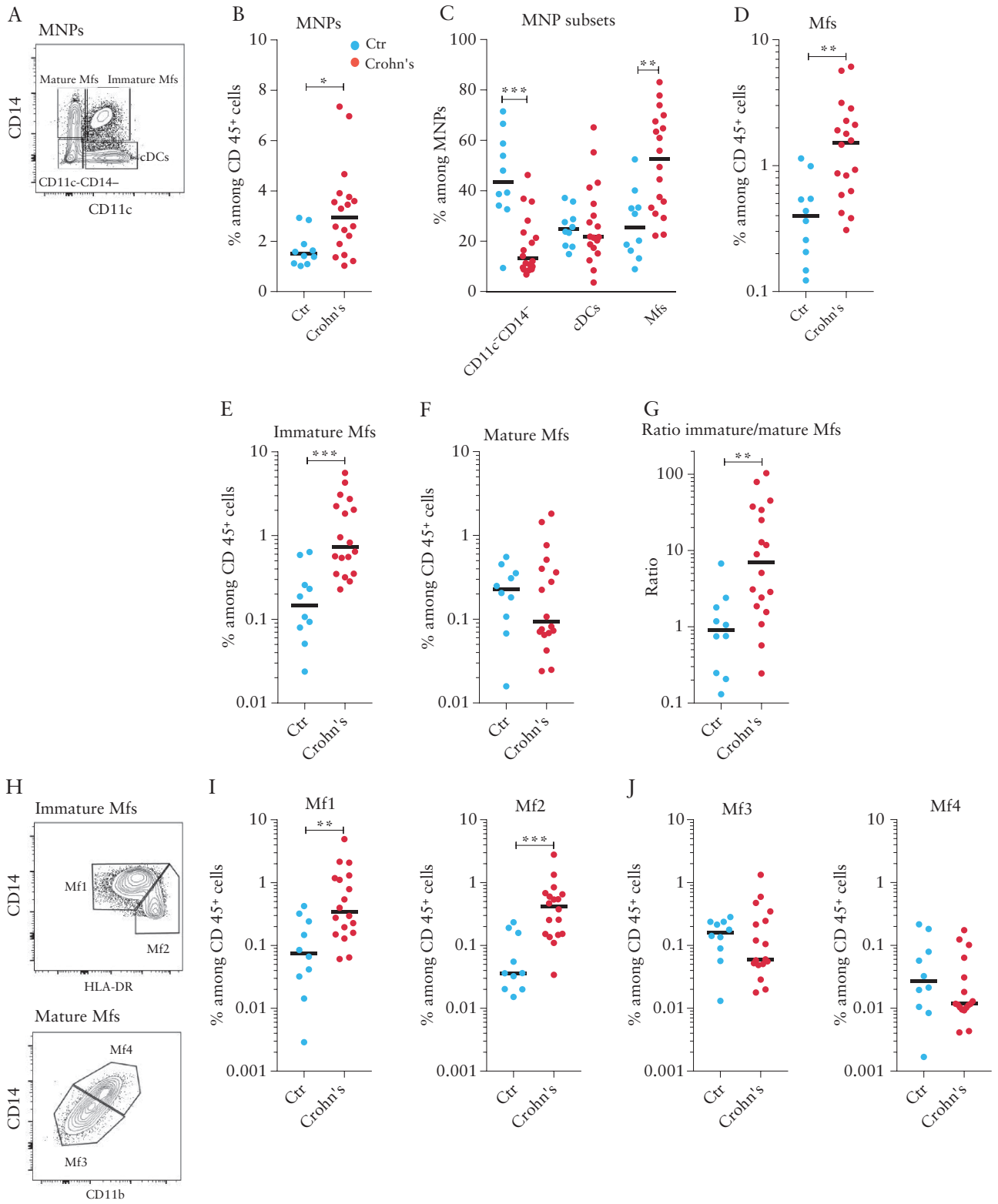

Figure 2. Intestinal MNP frequency increases in Crohn's disease patients due to accumulation of immature Mfs. [A] Gating strategy for flow cytometry analysis to determine the frequency of MNP subsets shown in B–G. The dot plot is from one Crohn's disease patient representative of the cohort. [B–F, I, J] The frequency of cells determined by flow cytometry as a percentage of CD45+ cells or MNPs as indicated. [G] The ratio between the frequency of immature Mfs to mature Mfs from the same subject. [H] Gating strategy for flow cytometry analysis of immature [upper plot] and mature [lower plot] Mf subsets used for the data in I and J. Dot plots are from one Crohn's disease patient representative of the cohort. [B–G, I, J] Small horizontal lines indicate the median. Differences between the control group and Crohn's disease patient group were assessed by two-sided Mann-Whitney tests; *p vs Ctr. Abbreviations: cDC, conventional dendritic cells; Ctr, controls; Mfs, macrophages; MNPs, mononuclear phagocytes.

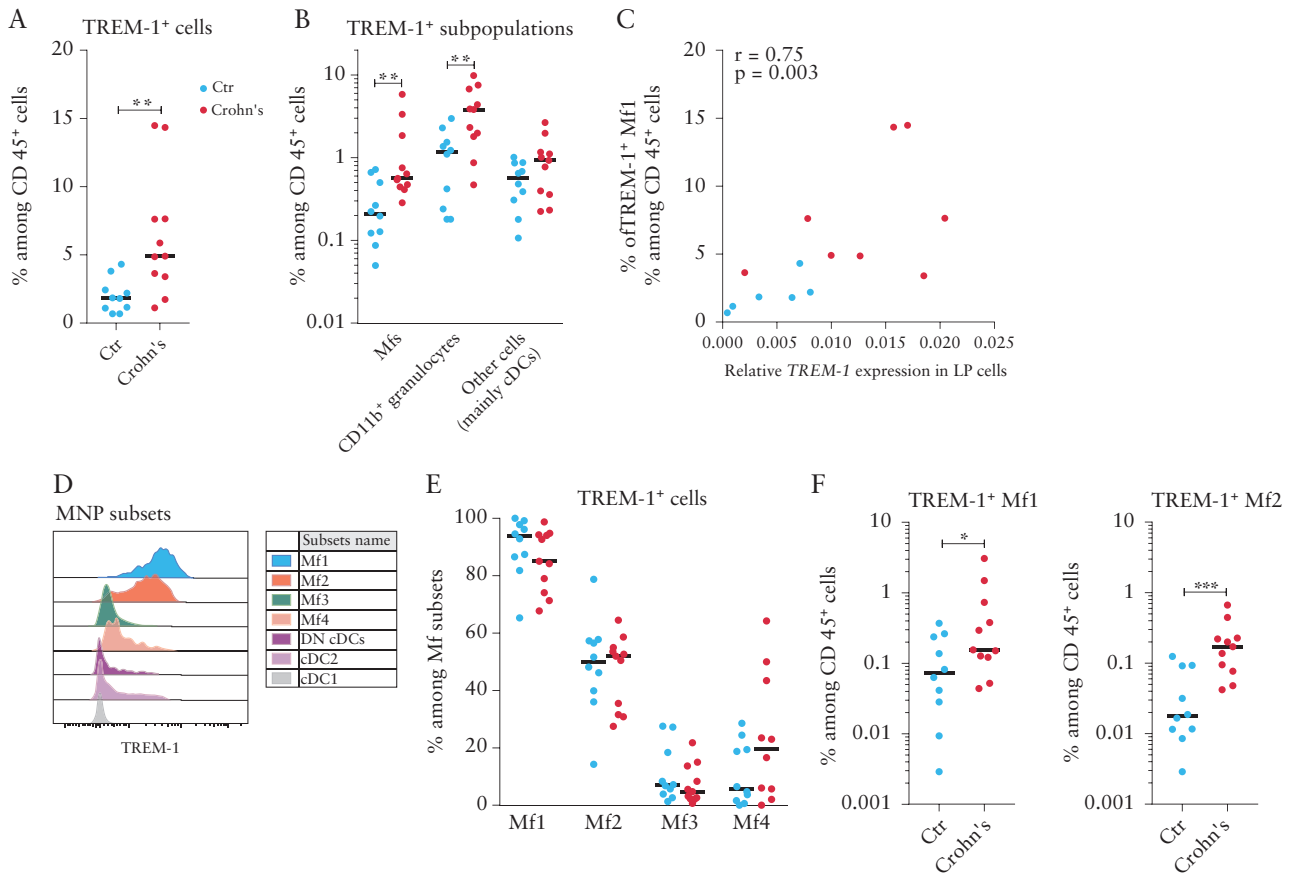

Figure 3. Intestinal TREM-1⁺ cells increase in Crohn's disease patients and are mainly immature Mfs and CD11b⁺ granulocytes. The percentage of TREM-1⁺ cells or TREM-1⁺ subpopulations among CD45⁺ cells [A, B, F], or among Mf subsets [E] are shown. Frequencies were determined by flow cytometry as in Supplementary Figure 2. [C] Correlation between *TREM-1* gene expression in LP cells and the frequency of TREM-1⁺ cells. [D] Histogram showing fluorescence intensity of TREM-1 on the indicated MNP subsets from a representative Crohn's disease patient. [A, B, E, F] Small horizontal lines indicate the median. Differences between the control group and Crohn's disease patient group were assessed by two-sided Mann-Whitney tests; **p* vs Ctr. [C] The correlation was assessed by Spearman's test. Abbreviations: cDC, conventional dendritic cells; Ctr, controls; LP, lamina propria; Mfs, macrophages; MNPs, mononuclear phagocytes.

Mfs, particularly the Mf1 subset and TREM-1⁺ Mfs, but not with mature Mfs [Figure 4D]. TNF content in LP-CM is also strongly correlated with the frequency of TREM-1⁺CD11b⁺ granulocytes, but much less so with CD11b⁺ granulocytes lacking TREM-1 [Figure 4D]. Together, the data suggest that TREM-1⁺ immature Mfs and TREM-1⁺CD11b⁺ granulocytes could contribute to the TNF content in the ileum of Crohn's disease patients.

3.5. The intestinal microenvironment of Crohn's disease patients promotes an inflammatory transcriptome in blood monocytes

Next, we assessed the signalling pathways downstream of TREM-1 that may influence proinflammatory cytokine and chemokine production, with the goal to identify factors in inflamed intestinal LP that could influence monocyte differentiation upon their recruitment to the intestine. Indeed, the LP-CM from inflamed intestinal tissue of Crohn's disease patients contains TREM-1 ligands that correlate with proinflammatory cytokines with the potential to activate immature Mfs.[29] We thus investigated the signalling downstream of TREM-1 exposed to LP-CM from Crohn's disease patients and a TREM-1 agonistic antibody. To this end, we used healthy human blood CD14⁺ monocytes, which highly express TREM-1,[29,34] and migrate continually to the intestine.[33,35] First, TREM-1 agonist antibody induced several pathways promoting monocyte activation [Figure 5A] including *CCL2* and *CCL7* gene expression [Table 2a]. Then, using LP-CM from controls to mimic the homeostatic intestinal microenvironment, we observed that control LP-CM induced mainly genes involved in angiogenesis and extracellular matrix remodelling [Figure 5B], such as VEGFA and MMP19 [Table 2b], in blood monocytes. By contrast, using LP-CM from Crohn's disease patients to mimic the inflamed intestinal microenvironment, genes mainly involved in inflammation [Figure 5C], including cytokines and chemokines [Table 2c], were induced. Control LP-CM and Crohn's disease LP-CM induced some pathways in common, but importantly Crohn's disease LP-CM induced a more inflammatory transcriptome in blood monocytes [Table 2d]. Of note, by comparing CD14⁺ monocytes treated with the TREM-1 agonist [Table 2a] or Crohn's disease LP-CM [Table 2c], *RGS1* is the only gene among the top ten increased genes common to both. However, all of the top ten genes that increased with the TREM-1 agonist are also increased at least by two-fold with Crohn's disease LP-CM [Table 3a]. Likewise, most of the top 10 genes that increased with Crohn's disease LP-CM are also increased with the TREM-1 agonist [Table 3b]. Thus, despite differences in the actual fold change, both the TREM-1 agonist and Crohn's disease LP-CM induce numerous identical genes, supporting that Crohn's disease LP-CM contains TREM-1 ligands with the potential to activate pathways downstream of TREM-1 in CD14⁺ monocytes.

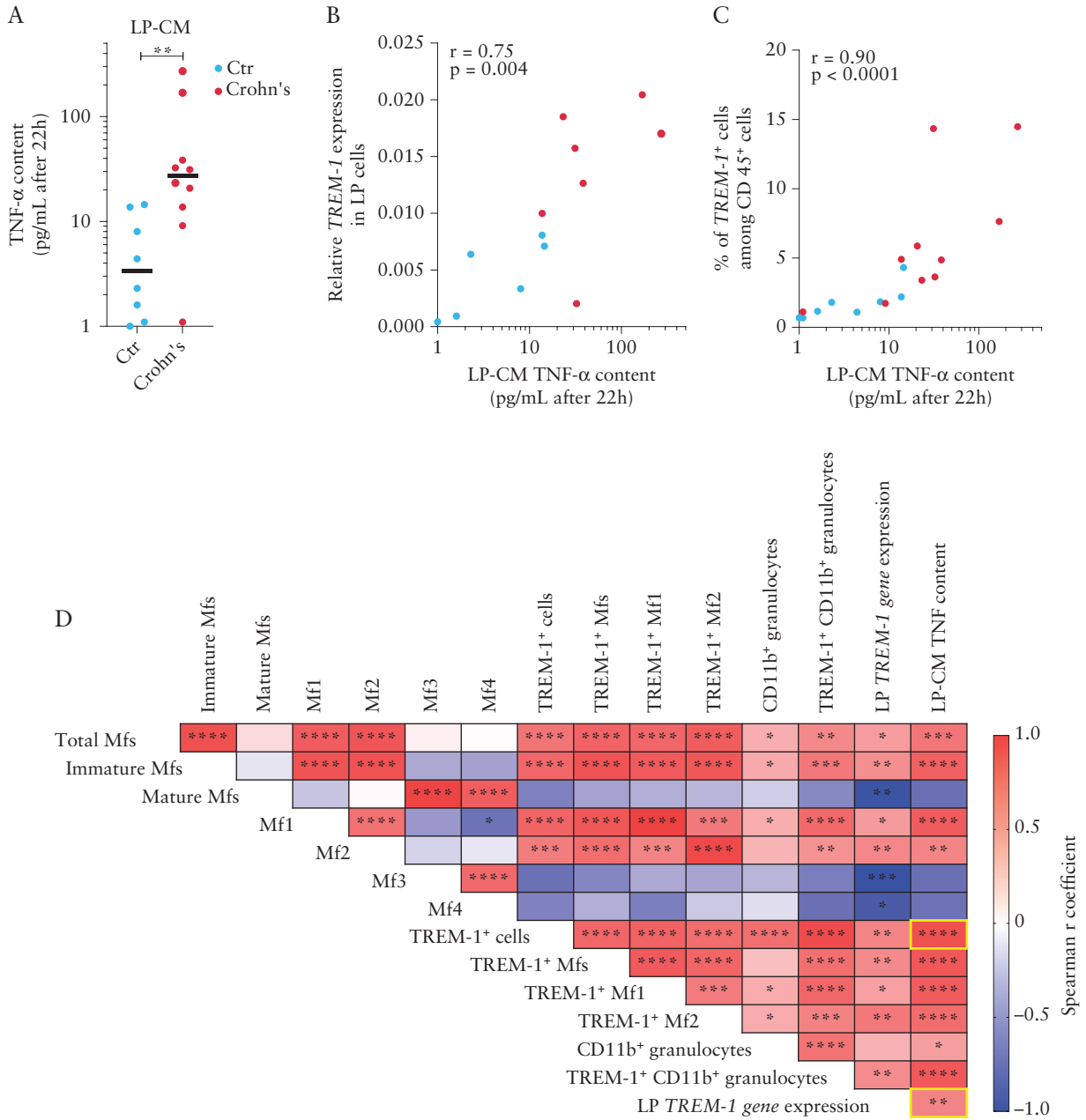

Figure 4. TNF release by LP cells increases in Crohn's disease patients and strongly correlates with the frequency of TREM-1⁺ cells. [A] TNF content in LP conditioned media [LP-CM] after 22 h of *ex vivo* culture. [B] Correlation between LP-CM TNF content and *TREM-1* gene expression in LP cells. [C] Correlation between LP-CM TNF content and the frequency of TREM-1⁺ cells. [D] Correlation matrix between the frequency of cells determined by flow cytometry, *TREM-1* gene expression in LP cells and LP-CM TNF content from controls and Crohn's disease patients. The colour in each square indicates the strength of the correlation according to the scale at the right, with red being a strong positive correlation. Squares outlined in yellow indicate the correlations shown in B and C. [A] Small horizontal lines indicate the median. The difference between the control group and Crohn's disease patient group was assessed by two-sided Mann-Whitney tests; **p* vs Ctr. [B–D] Correlations were assessed by Spearman's test. Abbreviations: Ctr, controls; LP-CM, lamina propria conditioned media; Mfs, macrophages.

To investigate if the inflammatory transcriptome induced in monocytes by Crohn's disease LP-CM was due to a higher content of proinflammatory molecules compared to control LP-CM, we analysed proteins in the LP-CM using a multiplex immunoassay related to inflammation [see section 2.6]. Among the 92 proteins analysed, 38 were increased in Crohn's disease LP-CM by a minimum of two-fold compared to control LP-CM [Figure 5D]. Notably, each of these 38 proteins that increased in Crohn's disease LP-CM correlated with all of the others in LP-CM, with the only exceptions being IL-24 and CCL13 [data not shown]. This suggests that the protein content in LP-CM from each subject reflected their inflammatory status, i.e. high for Crohn's disease patients and low/absent for controls, regardless of the protein analysed. To further explore the data, we generated an 'LP-CM inflammation score' by averaging the content of the 38 increased proteins from the LP-CM of each subject [Figure 5E]. This LP-CM inflammation score, which is based on protein levels, correlated with expression of some of the top ten genes whose expression increased in monocytes treated with Crohn's

A

TREM-1 agonist versus IgG1

Pathways	Signature score
Growth factor signaling	9,5
Cytokine signaling	8
Lymphocyte activation	7,8
Cell migration and adhesion	5,5
Metabolism	5,5
Chemokine signaling	4,6
Angiogenesis	4,5
Fc receptor signalling	4,3
ECM remodeling	4,1
Differentiation and maintenance of myeloid cells	4
Complement activation	3,6
TLR signaling	3,5
TH1 activation	3
Antigen presentation	2,2
Pathogen response	2
T-cell activation and checkpoint signaling	0,5
TH2 activation	0
Interferon signaling	−0,3
Cell cycle and apoptosis	−1,3

B

Ctr LP-CM versus IgG1/IgG4

Pathways	Signature score
Angiogenesis	5,2
ECM remodeling	4
T-cell activation and checkpoint signaling	3,5
Cytokine signaling	3
Complement activation	2,5
Cell migration and adhesion	2,2
Lymphocyte activation	2,2
Differentiation and maintenance of myeloid cells	2
TH2 activation	2
TH1 activation	1,5
Interferon signaling	0,8
Cell cycle and apoptosis	0,3
Antigen presentation	0,2
TLR signaling	0
Chemokine signaling	−0,1
Metabolism	−1,5
Pathogen response	−2
Growth factor signaling	−3
Fc receptor signaling	−3,5

C

Crohn's LP-CM versusIgG1/IgG4

Pathways	Signature score
Cytokine signaling	8
ECM remodeling	6
Angiogenesis	5
TLR signaling	4,8
Pathogen response	4,2
Chemokine signaling	4
Growth factor signaling	4
Complement activation	0,1
Fc receptor signaling	0,1
T-cell activation and checkpoint signaling	0,1
TH2 activation	−0,2
TH1 activation	−0,5
Differentiation and maintenance of myeloid cells	−2
Lymphocyte activation	−2,3
Cell migration and adhesion	−2,4
Cell cycle and apoptosis	−2,5
Antigen presentation	−3,2
Interferon signaling	−3,2
Metabolism	−5,5

D

Crohn's LP-CM

Protein	Fold over Ctr LP-CM
CXCL5	404.8
CCL4	124.1
CCL3	95.5
MMP-1	66.9
TNF	65.7
OSM	57.7
IL-6	54.4
MCP-1	51.1
CXCL1	48.7
IL-12B	39.0
LIF	34.9
MMP-10	34.6
MCP-3	33.7
CSF-1	22.5
CXCL9	21.7
uPA	12.7
CXCL10	11.7
IL-8	10.7
CXCL6	10.7
VEGFA	9.4
IL-17A	8.7
CCL20	8.0
MCP-2	6.8
IL-1 alpha	6.8
HGF	6.6
CD40	6.6
CCL19	5.8
TNFRSF9	5.8
EN-RAGE	5.5
IL-18	4.3
ADA	4.1
IL-10	3.6
IL-24	3.4
CCL23	2.8
LAP TGF-beta-1	2.7
MCP-4	2.5
IL-18R1	2.2
IL-33	2.0

E

Sample annotation	LP-CM inflammation score
Ctr A	3.0
Ctr B	96.5
Ctr C	76.6
Crohn's A	1987.0
Crohn's B	836.4
Crohn's C	1600.0

F

LP-CM inflammation score

Gene	Correlation
IL6	
CXCL5	*
CXCL1	
CXCL3	
IL1B	
IL1A	
CCL4	
CXCL2	*
SERPINB2	*
CCL3L1	

1.0 0.5 0 −0.5 −1.0 Spearman r coefficient

G

LP-CM inflammation score

Frequency among CD45⁺ cells	Correlation
Mfs	
Mature Mfs	
Immature Mfs	*
Mf1	*
Mf2	
Mf3	
Mf4	
TREM-1⁺ cells	**
TREM-1⁺ Mfs	*

1.0 0.5 0 −0.5 −1.0 Spearman r coefficient

Figure 5. The intestinal microenvironment of Crohn's disease patients promotes an inflammatory transcriptome in blood monocytes. [A–C] Differentially expressed transcriptomic pathways in blood CD14⁺ monocytes from healthy donors treated for 5 h with [A] TREM-1 agonist antibody [5 μg/mL], [B] LP-CM from three controls or [C] LP-CM from three Crohn's disease patients. Quantification of gene expression was done with the NanoString nCounter Human Myeloid Innate Immunity V2 Panel. Pathway signature scores condense each sample's gene expression profile into a small set of pathway scores. An experiment can then be explored through the lens of pathway signature scores instead of in the much higher-dimension lens of gene expression values. Pathway signature scores are fit using the first principal component of each gene set's data. They are orientated such that an increasing score corresponds to mostly increasing expression [specifically, each pathway score has positive weights for at least half its genes]. Covariates plots compare pathway signature scores to covariates. Red triangles and green triangles help to show the relative increase and decrease in pathways, respectively, compared to the control condition. [D] The 38 molecules that increased by a minimum two-fold in LP-CM from the three Crohn's disease patients compared to LP-CM from the three controls. The fold change

disease LP-CM compared to control LP-CM [Figure 5F; Table 2d]. In addition, similar to that shown above for TNF content in LP-CM measured by ELISA [Figure 4C, D], the LP-CM inflammation score also correlated with the accumulation of immature Mfs and TREM-1$^+$ cells in intestinal mucosa [Figure 5G]. Collectively, our data show that factors released by LP cells of Crohn's disease patients induce an inflammatory transcriptome in blood CD14$^+$ monocytes that correlates with the frequency of TREM-1$^+$ Mfs in intestinal mucosa.

3.6. *IL-6* induced in blood monocytes by Crohn's disease LP-CM is partially reduced by TREM-1/TNF double blockade

To further investigate the pathways induced by TREM-1 that, in the presence of factors in Crohn's disease LP-CM, amplify the inflammatory signals involved in monocyte activation, TREM-1 signalling in blood CD14$^+$ monocytes was blocked using an antagonist antibody. Broad transcriptional analysis using NanoString nCounter technology in a pilot experiment revealed a decrease in many transcription pathways involved in monocyte activation, such as pathogen response, cytokine signaling, chemokine signaling and TLR signalling [Figure 6A]. Among the top genes decreased by blocking TREM-1 were *IL-6*, *CCL3* and *CCL4* [Figure 6B; Table 2e below horizontal line]. This is in stark contrast to the increased transcription of these same genes in monocytes exposed to the TREM-1 agonist or Crohn's disease LP-CM [Table 3b, c], suggesting that factors in Crohn's disease LP-CM can trigger the TREM-1 receptor and result in increased *IL-6*, *CCL4* and *CCL3* transcription. We extended the analysis of the effect of blocking TREM-1 on these three genes using LP-CM from six additional Crohn's disease patients. LP-CM from this new group of patients did not, however, significantly reduce expression of *IL-6*, *CCL3* or *CCL4* after TREM-1 blockade [Figure 6C–E]. As TREM-1 is a receptor that amplifies other inflammatory signals,[23,24] the effect of blocking TREM-1 may be underestimated as additional pathways, initiated by other cytokines in the LP-CM, may be simultaneously influencing monocyte activation. To this end, we focused on TNF, which correlates with TREM-1-expressing monocytes [Figure 4] and is readily used as a clinical treatment for IBD. We thus simultaneously blocked TREM-1 and TNF using a research-grade adalimumab antibody. While blocking TNF alone dampened *CCL4* and *IL-6* expression induced in blood monocytes by LP-CM from several of the patients [Figure 6D, E], simultaneous blocking of TREM-1 and TNF had an additional effect on decreasing *IL-6* expression [Figure 6E]. Of note, monocytes treated with Crohn's disease LP-CM in the presence of adalimumab alone, TREM-1 antagonist alone or combined adalimumab + TREM-1 antagonist did not result in statistically significant differences in expression of the apoptosis-related genes *CASP3*, *CASP8* or *BCL2* between the different treatments [Supplementary Figure 4]. These data suggest that the additional reduction in *IL-6* expression observed between the single adalimumab treatment vs combined adalimumab and TREM-1 antagonist [Figure 6E] is not linked to increased apoptosis in the single vs dual treatments. In summary, the TREM-1 pathway may act in concert with other factors in the intestine of Crohn's disease patients, such as TNF, to influence the inflammatory environment by, for example, inducing *IL-6* expression in newly recruited monocytes.

4. Discussion

Blocking the TREM-1 pathway in experimental mouse models of colitis reduces inflammation and colitis severity,[26–28,36] but little is known about TREM-1 in human intestinal inflammation and the effect of TREM-1 blockade in IBD patients. We show that *TREM-1* gene expression increases in intestinal mucosa from IBD patients including the colon of UC patients, where it correlates with the full Mayo score, and in the ileum and colon of Crohn's disease patients. In the latter cohort of patients, ileal *TREM-1* expression also tends to correlate with HBI. The correlation of UC but not Crohn's disease patients to their disease activity index could reflect disease-specific differences. For example, UC patients with active disease are always characterized by inflammation while Crohn's disease is more heterogeneous and Crohn's disease patients can also present strictures, fibrosis or fistulas.[1,37] Furthermore, the full Mayo score used for UC patients includes an endoscopic score while for Crohn's disease patients we had access to only HBI, which poorly reflects endoscopic activity. However, the increase of ileal *TREM-1* expression in Crohn's disease patients positively correlates with genes related to immune response and inflammation, and more specifically to surface markers of neutrophils and monocytes/macrophages. Consistent with this, analysis of enriched cell fractions and purified cells showed that *TREM-1* was largely expressed by immune cells in the LP, particularly MNPs.

At the protein level, using both flow cytometry and multicolour immunofluorescence microscopy, we show that among ileal mucosal MNPs, immature Mfs, which are newly recruited blood monocytes,[6,33,35] increase in Crohn's disease patients while there was no difference in the frequency of mature Mfs, consistent with previous findings.[6] Moreover, using a recent gating strategy to define intestinal Mf subsets as two immature and two mature subsets,[6,33] we compared the four intestinal Mf subsets between controls and Crohn's disease patients for the first time. We show that the increase of immature Mfs is due to an accumulation of both Mf1 and Mf2 subsets. We also observed that TREM-1$^+$ cells are increased in Crohn's disease patients compared to controls, and TREM-1 intensity in Crohn's disease ileum *in situ* significantly correlates with CD11c, which is highly expressed on immature Mfs that infiltrate inflamed intestine.[33] Moreover, among MNPs, immature Mfs, but not mature Mfs, highly express TREM-1 resulting in increased TREM-1$^+$ Mf1 and TREM-1$^+$ Mf2 in Crohn's disease ileum. Of note, comparing controls and Crohn's disease patients for a given Mf subset, the fraction of TREM-1$^+$ cells was similar. Taken together, these data suggest that each intestinal MNP subset has a specific TREM-1 protein level that is not affected by the disease per se. Corroborating this, there was no difference in *TREM-1* gene expression in PBMCs between controls and Crohn's disease patients [data not shown]. Finally, TREM-1$^+$CD11b$^+$ granulocytes,

is based on the mean of the three values per group. [E] LP-CM inflammation score for each sample. This score is the mean of the linear NPX values [see Section 2.6] of the 38 proteins shown in D as a way to get a relative quantification of the inflammation status of the individuals analysed. [F] Correlations between the LP-CM inflammation scores and the top ten increased genes in human blood CD14$^+$ monocytes treated with the Crohn's disease LP-CM compared to cells treated with the control LP-CM [see Table 2d]. [G] Correlations between the LP-CM inflammation scores and the frequency of cells in the same individual determined by flow cytometry [gated as in Supplementary Figure 2]. [F, G] Correlations were assessed by Spearman's test. Abbreviations: Ctr, controls; LP-CM, lamina propria conditioned media; Mfs, macrophages.

Table 2. The intestinal microenvironment of Crohn's disease patients promotes an inflammatory transcriptome in blood monocytes, which is partly reduced by TREM-1 blockade.

[a] TREM-1 agonist		[b] Ctr LP-CM		[c] Crohn's disease LP-CM		[d] Crohn's disease LP-CM		[e] Crohn's disease LP-CM + TREM-1 antagonist	
Gene	Fold over IgG1	Gene	Fold over IgG1/IgG4	Gene	Fold over IgG1/IgG4	Gene	Fold over Ctr LP-CM + IgG1/IgG4	Gene	Fold over Crohn's disease LP-CM + IgG1/IgG4
IL1RN	71.5	*RGS1*	56.31	*IL6*	1207.86	*IL6*	134.80	*CCL7*	1.92
MMP1	64.52	*MIF*	38.23	*CCL20*	201.19	*CXCL5*	109.03	*BMP6*	1.68
GEM	24.28	*VEGFA*	22.44	*CXCL5*	199.72	*CXCL1*	51.75	*SPRY2*	1.63
THBD	21.49	*NRIP3*	21.77	*IL1B*	168.37	*CXCL3*	34.00	*EDN1*	1.63
CCL7	19.00	*OSM*	18.98	*IL1A*	149.74	*IL1B*	23.83	*MMP1*	1.55
RGS1	14.54	*FLT1*	18.56	*CXCL1*	65.89	*IL1A*	22.70	*CD36*	1.51
CREM	14.54	*IL1RN*	16.18	*PTGS2*	65.87	*CCL4*	20.81	*FOSB*	1.40
CCL2	13.37	*MMP19*	14.93	*CXCL3*	47.58	*CXCL2*	20.37	*IL1R1*	1.39
MMP19	12.95	*TUBA4A*	14.27	*RGS1*	47.16	*SERPINB2*	16.15	*MMP19*	1.37
NR4A1	11.66	*GEM*	14.17	*FLT1*	38.41	*CCL3*	14.60	*LIPA*	1.34
PDGFB	0.32	*ITGAL*	0.10	*EGR2*	0.10	*KLF4*	0.23	*TNFAIP8*	0.63
S100A8	0.31	*CCR2*	0.10	*CCL24*	0.09	*CYSLTR1*	0.23	*BIRC3*	0.63
TLR1	0.29	*IKBKE*	0.10	*CCR2*	0.09	*TUBA1A*	0.23	*CYBB*	0.62
SEMA4A	0.29	*CD36*	0.08	*ITGAL*	0.08	*SELPLG*	0.22	*RIN2*	0.61
BIRC3	0.28	*IL27RA*	0.07	*IL27RA*	0.06	*FOSB*	0.20	*CD14*	0.61
RGL1	0.26	*CCL24*	0.04	*CEBPA*	0.05	*SMARCD3*	0.20	*IL6*	0.61
CD14	0.24	*RASAL1*	0.02	*RGL1*	0.04	*HES4*	0.17	*TNFRSF9*	0.57
TLR8	0.23	*RGL1*	0.02	*RASAL1*	0.03	*CD244*	0.15	*CCL3*	0.56
CYBB	0.21	*EGR2*	0.02	*CD36*	0.03	*PROK2*	0.15	*CCL3L1*	0.56
CCR2	0.18	*CYP1B1*	0.01	*CYP1B1*	0.01	*TUBA4A*	0.11	*CCL4*	0.49

[a–e] The top ten increased [above the line] and decreased [below the line] genes in human blood CD14+ monocytes treated for 5 h with [a] TREM-1 agonist antibody [5 μg/mL], [b–d] LP-CM from three controls or three Crohn's disease patients alone or [e] the LP-CM from the same three Crohn's disease patients plus TREM-1 antagonist antibody [1 μg/mL] relative to monocytes from the same donor treated with isotype control antibodies. Quantification of gene expression was done with the NanoString nCounter Human Myeloid Innate Immunity V2 Panel. [a–e] $n = 3$ for each condition. The fold over is based on the mean of the three values per condition. Abbreviations: Ctr, controls; LP-CM, lamina propria conditioned media.

Table 3. TREM-1 agonist and Crohn's disease LP-CM induce numerous identical genes in blood monocytes.

[a] TREM-1 agonist[a]		Crohn's disease LP-CM	[b] Crohn's disease LP-CM[b]		TREM-1 agonist	[c] TREM-1 agonist		Crohn's disease LP-CM
Gene	Fold over IgG1	Fold over IgG1/IgG4	Gene	Fold over IgG1/IgG4	Fold over IgG1	Gene	Fold over IgG1	Fold over IgG1/IgG4
IL1RN	71.5	34.92	*IL6*	1207.86	1.56	*CCL3*	5.21	6.15
MMP1	64.52	14.99	*CCL20*	201.19	3.05	*CCL4*	1.84	2.84
GEM	24.28	8.82	*CXCL5*	199.72	9.88			
THBD	21.49	7.10	*IL1B*	168.37	5.08			
CCL7	19.00	6.41	*IL1A*	149.74	1.47			
RGS1	14.54	47.16	*CXCL1*	65.89	1.11			
CREM	14.54	5.14	*PTGS2*	65.87	5.64			
CCL2	13.37	9.51	*CXCL3*	47.58	3.45			
MMP19	12.95	20.70	*RGS1*	47.16	14.54			
NR4A1	11.66	2.34	*FLT1*	38.41	6.20			

[a] The top ten increased genes in human blood CD14$^+$ monocytes treated for 5 h with TREM-1 agonist antibody [5 µg/mL] from Table 2a and the fold over of the same genes after treatment with Crohn's disease LP-CM. [b] The top ten increased genes in human blood CD14$^+$ monocytes treated for 5 h with Crohn's disease LP-CM from Table 2c and the fold over of the same genes after treatment with TREM-1 agonist antibody. [c] *CCL3* and *CCL4* fold over in human blood CD14$^+$ monocytes treated for 5 h with TREM-1 agonist antibody or Crohn's disease LP-CM. Quantification of gene expression was done with the NanoString nCounter Human Myeloid Innate Immunity V2 Panel. [a–c] $n = 3$ for each condition. The fold over is based on the mean of three values per condition. Abbreviation: LP-CM, lamina propria conditioned media.

[a]The values for TREM-1 agonist in [a] are from Table 2a to allow direct comparison of the fold over for the same gene induced with Crohn's disease LP-CM.

[b]The values for Crohn's disease LP-CM in b] are from Table 2c to allow direct comparison of the fold over for the same gene induced with TREM-1 agonist.

certainly neutrophils,[29] are also increased in the ileum of Crohn's disease patients.

The high TREM-1 expression on both Mf1 and Mf2 subsets, which increase in the ileum of Crohn's disease patients, differed from the significantly lower TREM-1 expression on the mature Mf subsets. Indeed, we show that TREM-1 expression decreases during Mf maturation, as shown in mouse models.[38] The downregulation of surface TREM-1 on mature Mfs could be due to anti-inflammatory molecules present in the intestinal microenvironment, such as IL-10 and TGF-β.[19,38] Although both IL-10 and LAP/TGF-β1 increased in Crohn's disease LP-CM compared to controls [Figure 5D], their inflammation-dampening functions seem overwhelmed by the inflammatory molecules present. In experimental mouse models, intestinal inflammation did not block monocyte differentiation but rather instructed newly recruited blood monocytes to adopt an inflammatory transcriptome immediately upon their entry into tissue.[39] Interestingly, TREM-1 played a central role in initial signalling towards this inflammatory state in immature Mfs.[39]

The increase of intestinal TREM-1$^+$ cells correlates with the content of inflammatory cytokines in LP-CM, including TNF, which is highly produced by immature Mfs.[33,40] These correlations raise several possibilities including that TREM-1$^+$ cells can produce TNF, or induce TNF-producing cells, or are recruited at the same time as TNF-producing cells. In addition to TNF, LP-CM from Crohn's disease patients contained other proteins that increased relative to control LP-CM. These include IL-6, which has pleotropic proinflammatory functions and is implicated in IBD,[4,41] and chemokines, particularly those that influence the recruitment and trafficking of monocytes and neutrophils including CXCL5, CCL4, CCL3, CCL2 and CXCL1. Two MMPs associated with extracellular matrix remodelling and tissue damage, MMP-1 and MMP-10, were also highly increased in Crohn's disease LP-CM as was OSM. This corroborates previous findings showing that *OSM* gene expression increases in IBD patients, amplifies intestinal inflammation through OSMR-bearing stromal cells and is associated with anti-TNF resistance.[42,43]

Using human blood CD14$^+$ monocytes treated with a TREM-1 agonist antibody, we showed that triggering TREM-1 signalling induces pathways promoting monocyte activation and chemokine expression such as *CCL2* and *CCL7*, which are involved in immune cell recruitment. That the TREM-1 pathway can be triggered in human intestine is supported by data showing increased levels of a potential endogenous TREM-1 ligand, PGLYRP-1, in inflamed intestine of IBD patients.[29] Intestinal Mfs could thus be activated by endogenous TREM-1 ligands and exacerbate the pathogenesis of Crohn's disease. Moreover, by using LP-CM to mimic the intestinal microenvironment, we showed that *IL-6*, *CXCL5*, *CXCL1*, *CCL4* and *CCL3* are among the top ten increased genes in blood CD14$^+$ monocytes treated with Crohn's disease LP-CM compared to control LP-CM [Table 2d], supporting the protein data [Figure 5D]. Collectively our data suggest that TREM-1 can contribute to IBD pathogenesis through the accumulation of TREM-1$^+$ immature Mfs and TREM-1$^+$CD11b$^+$ granulocytes that are activated in intestinal mucosa of Crohn's disease patients and amplify inflammation, promote tissue damage and increase immune cell recruitment.

In blocking studies, we set out to characterize the pathways downstream of TREM-1 that, in the presence of factors in Crohn's disease LP-CM, amplify TREM-induced monocyte activation. Blocking TREM-1 revealed inflammatory transcriptome downregulation without significant reduction in specific genes. This may be due to the nature of the TREM-1 receptor, which amplifies inflammation in a context where other receptors are the primary

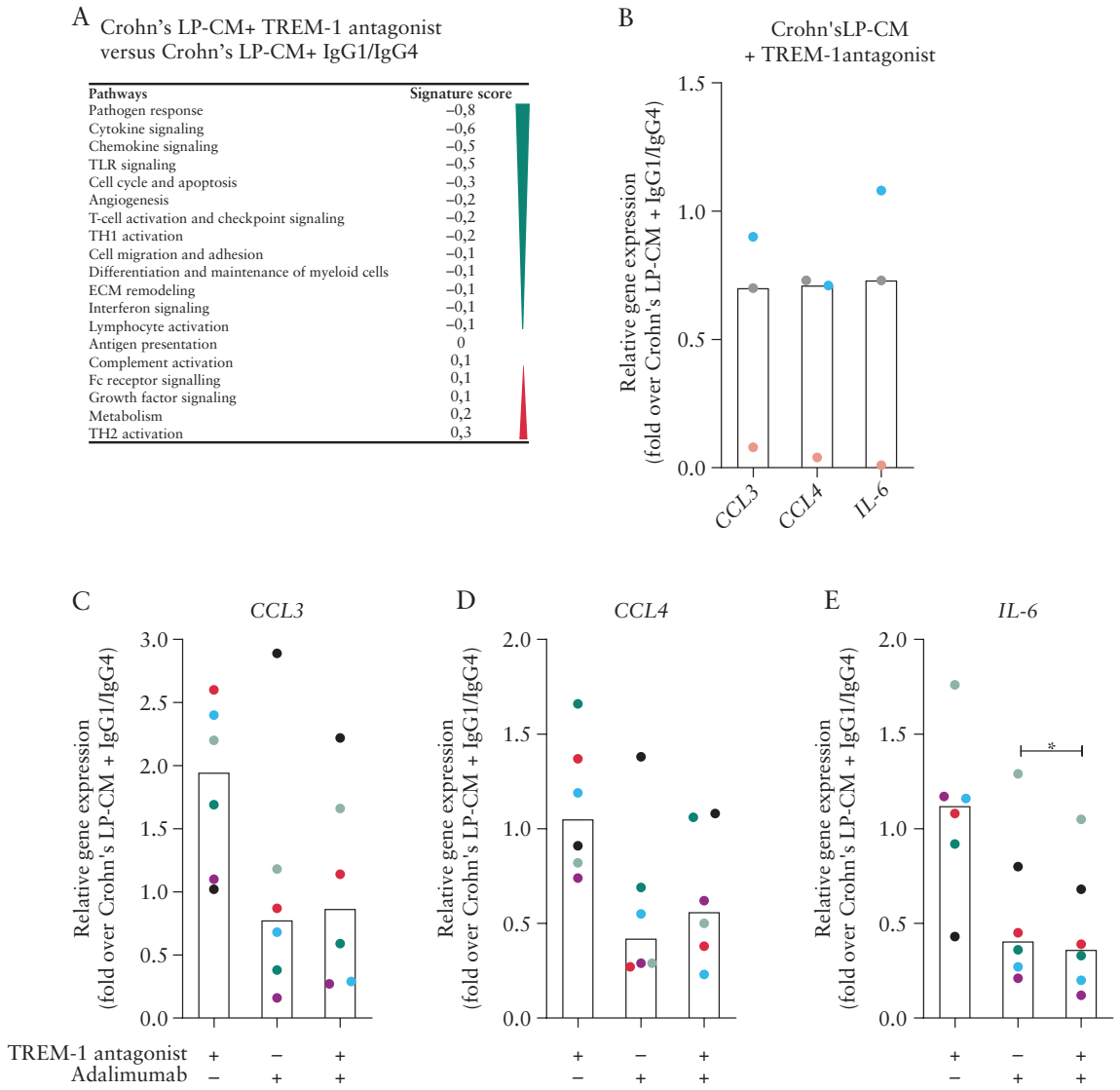

Figure 6. *IL-6* induced in blood monocytes by Crohn's disease LP-CM is partially reduced by TREM-1/TNF double blockade. Differentially expressed [A] transcriptomic pathways or [B] *CCL3*, *CCL4* and *IL-6* expression in blood CD14⁺ monocytes from a healthy donor treated for 5 h with LP-CM from three Crohn's disease patients plus TREM-1 antagonist antibody [1 μg/mL]. Quantification of gene expression was done with the NanoString nCounter Human Myeloid Innate Immunity V2 Panel. Explanation of pathway signature scores is described in the legend to Figure 5. [C–E] *CCL3*, *CCL4* and *IL-6* expression, determined by RT-PCR, in human blood CD14⁺ monocytes treated for 5 h with LP-CM from six additional Crohn's disease patients plus TREM-1 antagonist antibody alone [1 μg/mL], TNF adalimumab antibody alone [1 μg/mL], or both antibodies together. These blocking experiments were done with the appropriate isotype controls, IgG1 and IgG4, as described in Supplementary Table 1 sheet 1. [B–E] Each colour indicates a different individual. [B–E] Data are shown as median values. The difference between paired samples was assessed by a Wilcoxon test; **p* vs adalimumab alone. Abbreviations: LP-CM, lamina propria conditioned media.

drivers of the inflammatory environment.[23,24,29] Indeed, the limited effect of blocking only TREM-1 on monocytes stimulated with Crohn's disease LP-CM is consistent with the large array of inflammatory molecules in Crohn's disease LP-CM that can trigger monocyte activation [Figure 5D], and with the positive correlation of a TREM-1 ligand in IBD intestine with several proinflammatory cytokines including TNF and IL-6.[29] Alternatively, monocytes responding to other types of inflammatory stimuli, such as exposure to microbes or their components, may reveal other effects of blocking TREM-1. Further experiments are needed to understand the signals triggering TREM-1 in IBD patients.

As we show that TNF correlates with TREM-1-expressing monocytes, and anti-TNF treatment is a clinical mainstay[44] with some anti-inflammatory effects on monocytes/Mfs[45,46] and neutrophils[47] from Crohn's disease patients, we simultaneously blocked the TREM-1 and TNF pathways. This combined blocking had an additional effect on decreasing *IL-6* gene expression in monocytes compared to anti-TNF alone. This further supports an inflammation-amplifying role of TREM-1 in the intestinal microenvironment of Crohn's disease patients containing elevated TNF. Moreover, the data suggest TREM-1 antagonists may be a useful supplement to anti-TNF therapy. Given the pleiotropic proinflammatory effects of IL-6, particularly through *trans*-signalling, and its role in driving chronic intestinal inflammation,[4,41] further reducing IL-6 in anti-TNF therapy may have clinical benefits. Moreover, our TREM-1/TNF combined blocking experiment, which was a focused follow-up based on our NanoString data, does not exclude that simultaneously blocking both pathways affects, in addition to *IL-6*, other proinflammatory factors not examined here. Finally, additional benefits of blocking TREM-1 may also be apparent when it is blocked on other TREM-1-expressing

cells; that is, we tested TREM-1 blocking only on blood monocytes, but TREM-1$^+$CD11b$^+$ granulocytes are also increased in the intestinal LP of IBD patients. This raises the possibility that blocking TREM-1 could be even more effective *in vivo* where effects on both immature Mfs and neutrophils would be achieved. In addition, patient heterogeneity in response to blocking TREM-1 needs to be considered. Indeed, it is well established that around 30% of Crohn's disease patients are non-responders to anti-TNF therapy[48,49] and our blocking with adalimumab showed both patient- and gene-specific differences. Moreover, the Crohn's disease patients analysed here had undergone different treatments and had diverse indications for surgical intervention. Investigating a larger patient pool, particularly treatment-naïve patients and anti-TNF responders vs non-responders, could shed additional light on the potential use of blocking TREM-1 as a supplement to other therapies, particularly anti-TNF.

Nevertheless, supporting that TREM-1 could be a potential target in IBD patients, it has been shown that *TREM-1* is among the top 40 down-regulated genes between responders and non-responders to anti-TNF therapy.[50] In addition, Verstockt *et al*. have shown that low *TREM-1* expression in both whole blood and intestinal mucosa can predict anti-TNF therapy responders.[51,52] Moreover, an anti-TREM-1 molecule, called nangibotide, which contains the LR12 peptide and acts as a decoy receptor, has already reached the Phase II clinical stage in septic shock patients.[53,54] Thus, TREM-1 could be a potential biomarker for predicting the effect of anti-TNF therapy and, moreover, blocking TREM-1 signalling in addition to the traditional anti-TNF therapy could be interesting to mitigate intestinal inflammation and disease symptoms in some IBD patients.

Collectively, our data show that TREM-1$^+$ Mfs increase in the ileum of Crohn's disease patients and largely account for the observed increase in TREM-1 at the transcription and protein levels in inflamed intestine, with a contribution by TREM-1$^+$CD11b$^+$ granulocytes, particularly neutrophils.[29] Indeed, at the early stage of mucosal inflammation, neutrophil infiltration can promote tissue repair and resolution of inflammation while chronic neutrophil recruitment in IBD can induce epithelial barrier disruption and inflammation.[55] Overall, our data suggest that high mucosal *TREM-1* expression, attributed to infiltrating neutrophils and immature Mfs in the inflamed intestine, is linked to intestinal destruction in patients, and blocking TREM-1 in combination to anti-TNF therapy may provide clinical benefit to some patients.

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Conflict of Interest

The authors declare no potential conflicts of interest relevant to this article.

Data Availability Statement

All data used are present within this paper and its supplementary information; underlying material will be shared on reasonable request to the corresponding author.

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Author Contributions

M.J.W. and C.C. conceptualized the study, interpreted the data, designed the figures and wrote the manuscript. C.C. performed experiments, analysed the data and compiled the figures. F.G. performed experiments and contributed to project ideas, methodology and data interpretation. F.K.S. performed experiments. S.F.B. contributed to project conceptualization. M.K.M. contributed to human sample collection, specifically the cohort used for whole tissue analysis. L.S. contributed to immunofluorescence microscopy. M.B., L.G.B. and E.B.L. managed the human samples and clinical databases and provided all human surgical samples. The co-authors contributed to manuscript review and editing. M.J.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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