Inflammatory Bowel Diseases, 2022, **28**, 971–976
https://doi.org/10.1093/ibd/izab349
Advance access publication 15 January 2022
Brief Report - Basic Science

Tofacitinib Inhibits Leukocyte Trafficking Across the Intestinal Endothelial Barrier in a Specific Cohort of Ulcerative Colitis Patients

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Lay Summary

The JAK/STAT inhibitor tofacitinib, recently approved for the treatment of ulcerative colitis, is found to modulate the intestinal endothelial barrier functions in directing the leukocyte adhesion and transmigration in ulcerative colitis patients displaying high levels of endothelial STAT3/STAT6 phosphorylation.

Key Words. Ulcerative Colitis, endothelial cells, leukocytes, transcriptomics

Introduction

Ulcerative colitis (UC) is a chronic, relapsing inflammatory condition, which primarily affects the colonic mucosa, and whose etiopathogenesis is still unknown.[1] Immune cell trafficking through the intestinal microvasculature has emerged as a key process in UC pathophysiology, and drugs targeting immune cell mobilization have become potentially relevant for clinical therapy.[1]

New UC therapeutic pipelines include the small-molecule drugs targeting the Janus kinase (JAK) family of tyrosine kinases and the signal transducer and activator of transcription (STAT) family of DNA-binding proteins,[2] which demonstrated significant effects on dampening both the adaptive and innate immune responses in UC.[3] Currently, the only JAK inhibitor approved for the treatment of patients with moderately to severely active UC is tofacitinib, selectively inhibiting the JAK1 and JAK3 and displaying efficacy and safety in phase III clinical trials.[3] Nevertheless, a discrete number of patients do not respond to this drug, and understanding why this happens would be of extreme importance.

In the gut, the impact of tofacitinib has been described only in lymphocytes, such as T cells,[4] macrophages, and intestinal epithelial cells.[5] However, no data describing its effect on the regulation of leukocyte trafficking through the UC-associated endothelium are available yet. Because of the importance of leukocyte-endothelial cell interactions during UC pathogenesis,[1] here we sought to elucidate whether tofacitinib may affect the endothelial barrier in patients with UC, thus modulating the immune cell trafficking.

Methods

Patients and Human Intestinal Microvascular Cell Derivation

UC human intestinal microvascular cells (UC-HIMECs) were isolated from surgical specimens[6] of patients (n = 10) with active UC (endoscopic Mayo score > 1) and an age between 25 and 68 years at the time of surgery (Figure 1A) (Protocol number ICH1908 [id#341] CE Humanitas ex D.M.8/2/2013-183/14). HIMECs were used between passages 2 and 6. For tofacitinib (Pfizer, New York, NY, USA) titration, concentrations from 10 to 300 nM were tested.

Enzyme-Linked Immunosorbent Assay

Tofacitinib-treated (T50) or untreated (UN) UC-HIMECs were lysed and centrifuged. Collected supernatants underwent

Received for publication: September 6, 2021. Editorial Decision: December 6, 2021

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ID	Age	Sex	Endoscopic Mayo score	HIMEC	Treatment
UC1	68	M	3	Responder	Mesalazine/ steroids
UC2	40	F	3	Responder	Vedolizumab/ Mesalazine
UC3	58	M	3	Responder	Mesalazine
UC4	25	M	3	Responder	Apremilast
UC5	61	F	2	Non-responder	Mesalazine
UC6	77	M	2	Non-responder	Vedolizumab/ Mesalazine
UC7	43	F	1	Non-responder	Infliximab/ Mesalazine
UC8	55	M	3	Non-responder	Mesalazine/ Omega3 fatty acids
UC9	54	M	3	Non-responder	Apremilast

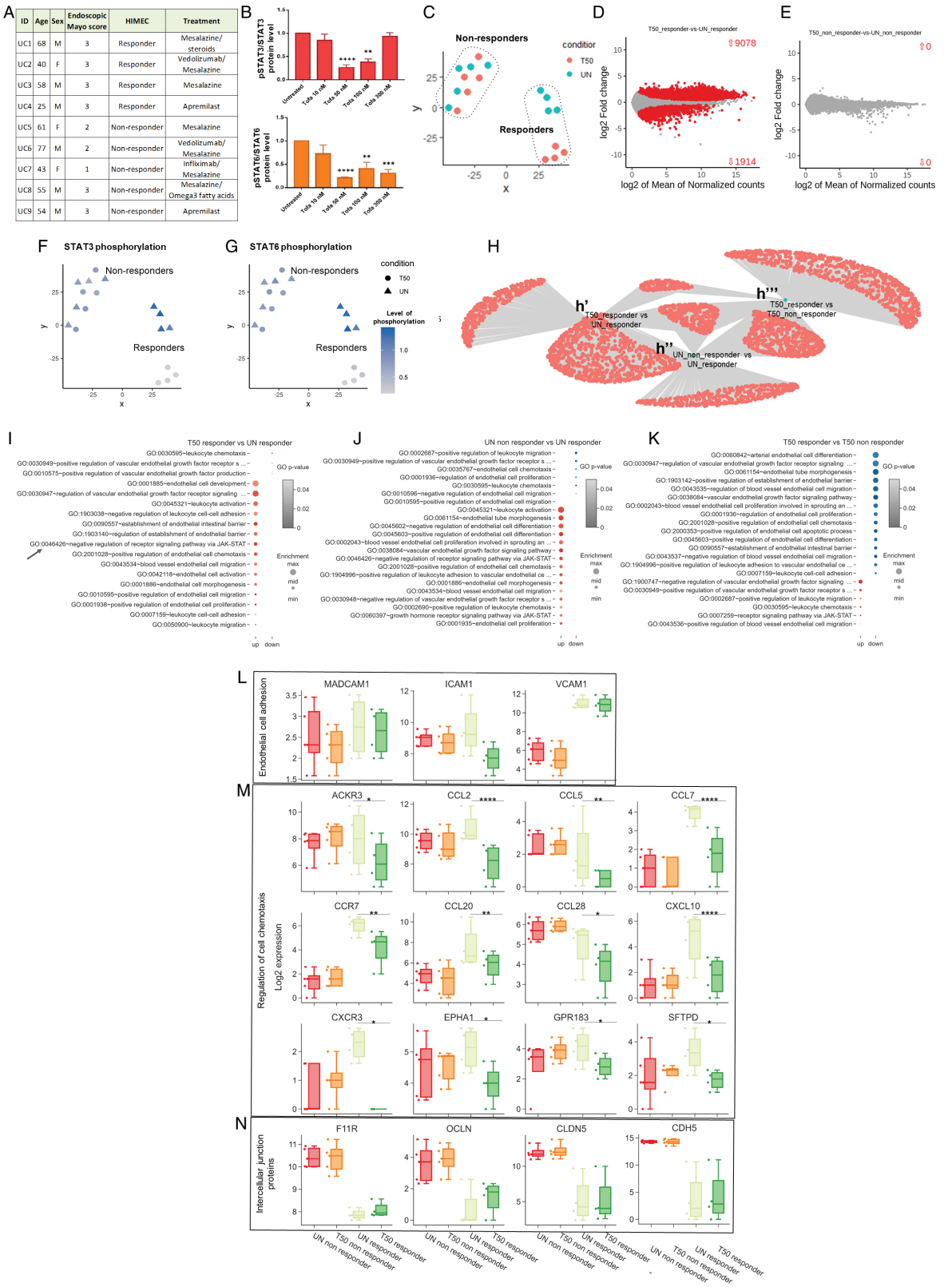

FIGURE 1. Tofacitinib shapes the transcriptomic state of ulcerative colitis human intestinal microvascular cells (UC-HIMECs). A, Table showing patients' characteristics. B, Bar graphs showing the phosphorylated STAT3 (pSTAT3) and phosphorylated STAT6 (pSTAT6) protein levels in UC-HIMECs upon tofacitinib (Tofa) treatment at the indicated conditions. Data are displayed as mean ± SEM. $^{**}P < .01$; $^{***}P < .005$; $^{****}P < .001$. C, Sample dispersion within the t-distributed stochastic neighbor embedding multidimensional scaling space colored by condition, showing nonresponder and responder UC-HIMEC clusters upon Tofa 50 nM (T50) treatment. D and E, MA plots showing differential gene expression results in the indicated comparisons. Red dots represent genes being differentially expressed with high statistical significance (false discovery rate $< 1 \times 10^{-10}$). The number of differentially modulated genes and their trend of expression are indicated in red. F and G, Sample dispersion within the t-distributed stochastic neighbor embedding multidimensional scaling space colored by condition, showing nonresponder and responder UC-HIMEC clusters in accordance with the phosphorylated

enzyme-linked immunosorbent assay (ELISA) to determine STAT3 (STAT3 Total/Phospho-Human InstantOne ELISA Kit; Thermo Fisher Scientific, Waltham, MA, USA; cat. 58-86103-11) and STAT6 (Human Phospho-STAT6 [Y641] ELISA; RayBiotech, Norcross, GA, USA; cat. PEL-STAT6-Y641-5) protein concentrations, according to the manufacturers' instructions.

RNA Sequencing and Transcriptomics

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). Library preparation, RNA sequencing (RNA-Seq), and transcriptomics profiling were performed as previously described.[7] RNA-Seq raw data can be found at *GSE165636* accession ID.

Peripheral Blood Mononuclear Cell Isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy donors by density gradient centrifugation with Ficoll. The PBMC-containing ring was collected and washed with RPMI 1640 medium (Thermo Fisher Scientific). Isolated PBMCs were freshly used for transmigration and adhesion experiments.

Adhesion and Transmigration Assays

UC-HIMECs were seeded on fibronectin-coated 6-well transwell permeable membranes with 3 µm pore size (Corning Costar, Cambridge, MA, USA) and cultured in endothelial cell growth medium (EGM-2; Lonza, Basel, Switzerland). HIMEC monolayers were then treated with 50 nM tofacitinib (Pfizer) or left untreated. Upon 17 hours' incubation, tofacitinib was washed out, and 5×10^5 PBMCs were seeded on HIMEC monolayers for 6 hours (adhesion experiment) or 24 hours (transmigration experiment). PBMCs that had adhered or transmigrated in the lower chamber of the transwell were then collected, counted, and analyzed by flow cytometry.

Flow Cytometry Analysis

PBMCs in adhesion onto, or transmigrated across, T50 or UN UC-HIMECs were collected and washed in RPMI medium supplemented with 5% fetal calf serum, 10 mM HEPES. Cells were stained for T cell and non–T cell populations analysis with a panel of specific markers, as described in Figure 2.

Cells were then fixed in 1% paraformaldehyde and acquired using the BD LSRFortessa cell analyzer and BD FACSDiva software (BD, Franklin Lakes, NJ, USA). Analysis was performed with FlowJo 10 (FlowJo, Ashland, OR, USA). Compensated, biexponentially transformed signals were subjected to machine learning–driven automatic gating with opencyto.[8] Cell type quantification and statistical analysis were performed with FlowCore.[9] Multidimensional scaling and k-means clustering were performed with umap (https://cran.r-project.org/web/packages/umap) and stats (https://www.rdocumentation.org/packages/stats/).

Statistics

Statistical analyses were performed within the R 4.0.1 environment (R Foundation for Statistical Computing, Vienna, Austria). Mean comparisons between experimental groups were analyzed with either 2-tailed unpaired Student's *t* test or 1- or 2-way analysis of variance with Bonferroni's correction. *P* values ≤.05 were considered statistically significant.

Results

To explore the effect of tofacitinib on intestinal endothelial barrier functions in patients with UC, UC-derived primary intestinal microvascular endothelial cells (UC-HIMECs) were used as an in vitro system. Upon treatment of UC-HIMECs with different tofacitinib concentrations, we identified the 50 nM as the most effective dose in reducing both STAT3 and STAT6 (Figure 1B) phosphorylation. Next, T50 and UN UC-HIMECs were analyzed by RNA-Seq, and t-distributed stochastic neighbor embedding clustering showed that, while tofacitinib modulated the transcriptomic profile of some UC patient–derived HIMECs (hereafter referred to as responder HIMECs), it exerted no effect in another subgroup of UC-derived endothelial cells (hereafter referred to as nonresponder HIMECs), at least at the gene expression level (Figure 1C). Indeed, the differential gene expression analysis pointed out a high number of differentially modulated genes between T50 responder UC-HIMECs and the UN responder cells (Figure 1D); on the contrary, no significantly modulated genes were observed between T50 and UN nonresponder UC-HIMECs (Figure 1E). In accordance with the main known effect of tofacitinib, responder UC-HIMECs strongly decreased the levels of STAT3/STAT6 phosphorylation upon tofacitinib treatment, as compared with the nonresponder cells (Figure 1F, 1G), thus not only confirming its efficacy in some UC patients, but also providing an explanation as for why other subjects may be refractory to this drug. These results indicate that the tofacitinib-mediated inhibition of JAK/STAT signaling shapes the transcriptomic profile of UC-derived HIMECs. Interestingly, UC-HIMECs displaying intermediate levels of STAT3 and STAT6 phosphorylation did not respond to tofacitinib in terms of modulation of transcriptomic profile. Notably, by analyzing patients' clinical characteristics, we observed that, while different drug treatments did not correlate with HIMEC sensitivity to tofacitinib, the phosphorylation levels were directly correlated with HIMEC responsiveness to the inhibitor (phospho-STAT6: R = 0.96402944, $P = 7.01 \times 10^{-6}$; phospho-STAT3: R = 0.942900511 $P = 4.34 \times 10^{-5}$).

To explore the differences between responder and nonresponder UC-HIMECs to tofacitinib treatment in terms of modulated endothelial cell–specific biological processes (BPs), we next performed the Gene Ontology (GO) analysis. Tofacitinib treatment modulated a high number of GO categories in UC-HIMECs, displayed as GO clouds and GO plots (Figure 1H-1K). Because the aim of our study was to

STAT3 and STAT6 protein levels upon T50 treatment. H, Gene Ontology (GO) category cloud plot showing the numerosity (red dots) and sharing (gray lines) of differentially modulated biological processes between the indicated comparisons (H'-H'''). I-K, GO plots showing the enrichment of the GO categories in the indicated comparisons. L-N, Box plots showing the expression profiles of the DEGs belonging to endothelial cell adhesion, regulation of cell chemotaxis, and intercellular junction categories in responder and nonresponder UC-HIMECs upon Tofa treatment. Data are displayed as mean ± SEM. *$P < .05$; **$P < .01$; ***$P < .005$; ****$P < .001$. UN, untreated.

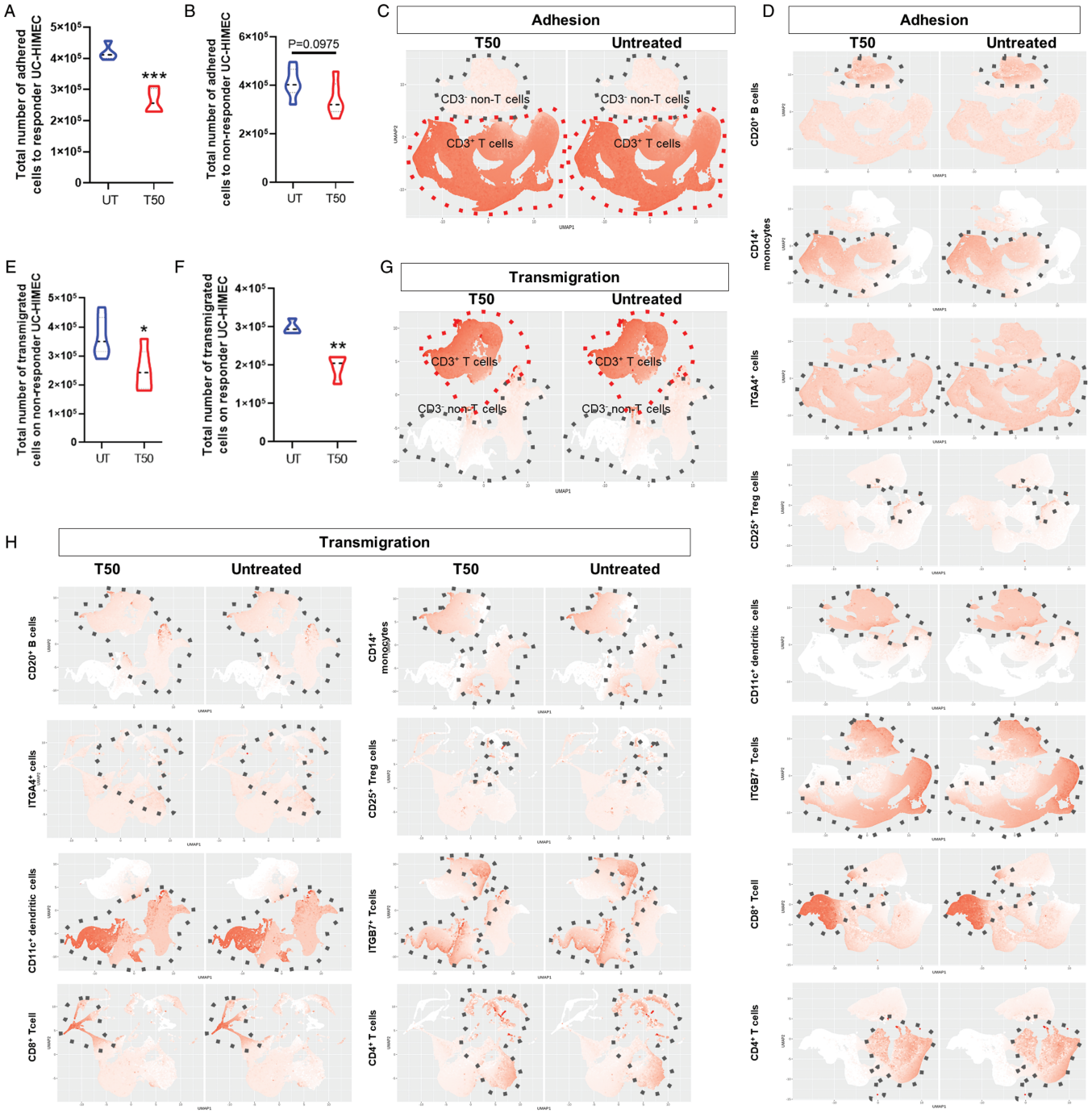

FIGURE 2. Tofacitinib impacts the leukocyte adhesion and transmigration across the ulcerative colitis (UC) endothelial barrier. A and B, Violin plots showing the total number of adhered peripheral blood mononuclear cells onto responder and nonresponder UC human intestinal microvascular cells (UC-HIMECs). *P****<.005. C and D, Single-cell dispersion plots within the UMAP multidimensional scaling space, divided by treatment, of cytofluorimetric data detecting both adhered T cells and non T cells, along with different leukocyte populations. For the T cell immunophenotyping panel, cells were stained with anti-human CD3 (BV650; BioLegend; cat.317324; 1:40), anti-human CD4 (BV605; BioLegend; cat.317438; 1:333), anti-human CD8 (BV780; BD; cat.563823; 1:158), anti-human CD25 (PE; BD; cat.555432; 1:40), anti-human CD127 (BV421; BioLegend; cat. 351309; 1:158), anti-human CCR7 (PE-CF594; BD; 562381; 1:80), and anti-human CD45RO (BV711; BD; cat.563723; 1:20) antibodies. For immunophenotyping non–T cells, cells were stained with anti-human CD3 (BV650; BioLegend; cat.317324; 1:40), anti-human HLA-DR (PE; BD; cat.555812; 1:158), anti-human CD56 (PE-CF594; BD; cat.562289; 1:80), anti-human CD14 (PerCp-Cy5.5; BioLegend; cat.301824; 1:666), anti-humanCD11c (BV711; BD; cat.563130; 1:20), anti-human CD123 (PE-Cy7; BD; cat.560826; 1:158), and anti-human CD20 (Pacific Blue; BioLegend; cat.302328; 1:1428). Dashed-line areas include cells positive to the indicated markers. E and F, Violin plots showing the total number of transmigrated peripheral blood mononuclear cells across responder and nonresponder UC-HIMEC layers. *$P < .05$; **$P < .01$. G and H, Single-cell dispersion plots within the UMAP multidimensional scaling space, divided by treatment, of cytofluorimetric data detecting both transmigrated T cells and non–T cells, along with different leukocyte populations. Dashed-line areas include cells positive to the indicated markers.

investigate the effect on tofacitinib-sensitive UC-HIMECs, we focused our attention on the most enriched categories in T50 compared with the UN responder UC-HIMECs (Figure 1H' and 1I). Besides confirming the negative regulation of JAK/STAT signaling in T50 compared with the UN responder UC-HIMECs (Figure 1I, black arrow), we also observed that BPs related to the negative regulation of leukocyte cell-cell adhesion, the establishment of endothelial intestinal barrier, and the regulation of establishment of endothelial barrier were among the most enriched in T50 UC-HIMECs compared with UN UC-HIMECs (Figure 1I). By investigating the single gene expression, among the endothelial adhesion molecule–encoding transcripts, ICAM1 was found to be downregulated in T50 responding UC-HIMECs by comparison with the UN UC-HIMECs, whereas in the nonresponder cells, no significant differences were observed (Figure 1L). As an additional piece of evidence, among the most downregulated BPs, the leukocyte chemotaxis process was found (Figure 1I). Accordingly, the expression levels of chemokines- and signals-encoding genes annotated in the cell chemotaxis BPs, namely *CXCL10*, *CCL2*, *CCL7*, *CCR7*, *CCL5*, *CCL20*, *SFTPD*, *CXCR3*, *ACKR3*, *GPR183*, *EPHA1*, and *CCL28*, were reduced in T50 responding cells by comparison with the UN cells, with no statistically relevant differences in nonresponder cells upon treatment (Figure 1M). Additionally, we observed that the *CDH5* (Ve-Cadherin) and *F11R* (JAM-A) *adherens* protein-encoding transcripts and the *OCLN* (occludin) and *CLDN5* (claudin 5) tight junction protein-encoding transcripts, while at different levels between responder and nonresponder UC-HIMECs, were not differentially regulated upon JAK/STAT inhibition (Figure 1N), thus excluding these junction proteins as involved in the tofacitinib-mediated mechanism.

These pieces of evidence altogether led us to hypothesize that the main effect exerted by tofacitinib on UC-derived endothelial cell functions was the inhibition of leukocyte adhesion and their chemokine-mediated recruitment and, as a consequence, their transmigration across the intestinal endothelial barrier. To functionally validate the transcriptomic results, we set up an in vitro model mimicking the leukocyte adhesion and extravasation across the intestinal endothelial barriert.[10]

Either responder or nonresponder UC-HIMECs were pretreated with 50 nM tofacitinib or left untreated as a control. Then, the tofacitinib was washed out and the pretreated or untreated HIMECs were co-cultured with human PBMCs in a transwell-based system. Interestingly, only PBMCs (including both T cell and non–T cell populations) co-cultured with tofacitinib-pretreated responder UC-HIMECs, and not those cocultured with the pretreated nonresponder cells, displayed decreased ability to adhere to the endothelial layer (Figure 2A, 2B), with no specific effect on the different immune cell subpopulations analyzed (Figure 2C, 2D).

Using a similar approach, we next assessed whether this tofacitinib-induced effect on the UC intestinal endothelial layer also impacted leukocyte transmigration. Interestingly, although tofacitinib slightly reduced the transmigration in pretreated nonresponder cells (Figure 2E), the major effect was observed when PBMCs were cocultured with pretreated responder UC-HIMECs, with a strong decrease in leukocyte transmigration induced by tofacitinib treatment (Figure 2F). Also in this case, the effect of tofacitinib on transmigration did not impact specific PBMC populations (Figure 2G, 2H).

These results indicate that tofacitinib reduced JAK/STAT pathway activation in UC-HIMECs, significantly inhibiting the endothelial-leukocyte recruitment and adhesion processes and impacting leukocyte transmigration across responding endothelial cell–formed barriers.

Inhibition of leukocyte adhesion and migration across the endothelial barrier is considered an effective therapeutic approach to treat intestinal inflammation, and many antiadhesion molecules have been developed and approved for the treatment of inflammatory bowel disease.[1]

Discussion

Here, we describe for the first time the action exerted on the intestinal microvasculature by tofacitinib found to affect the UC-HIMEC transcriptomics profile and the leukocyte trafficking through the intestinal endothelial barrier, results consistent with previous studies during lung injury.[11] We also showed that the efficacy of tofacitinib correlates with phosphorylated STAT3/STAT6 levels of UC-HIMECs, which may explain why some patients are refractory to this treatment. However, HIMECs include both hematic and lymphatic intestinal endothelial cells,[10] which determine the influx and efflux of immune cells to and from the inflamed tissue, respectively. Although specific leukocyte subpopulations were not affected in their adhesion to and mobilization through UC-HIMECs by tofacitinib, future studies can elucidate whether differences could be observed in UC-derived hematic and lymphatic cells.

Because our work was primarily focused on the tofacitinib-dependent endothelial cell modulation of leukocyte trafficking, it lacks mechanistic insights about the direct effects of tofacitinib on UC-derived leukocytes during their trafficking across the endothelial barrier. Thus, we strongly believe that this is an important topic to be expanded in the future.

Conclusions

The findings described in our work will contribute to a better understanding of how tofacitinib induces remission in selected UC patients, possibly guiding clinicians on patient stratification and therapeutic decision-making.

Author Contributions

S.D'Alessio, F.U.: conceptualization and writing original draft; S.S., D.F.: data search and acquisition; L.M., L.A.L.: bioinformatics and statistical analysis; L.M., L.P.-B., P.S., S.D'Alessio., F.U., and S.Danese: review and editing; S.D'Alessio. and F.U.: supervision, resources, and funding acquisition.

Funding

This study was funded by Pfizer, under grant # WI229671 to S.D'Alessio. The authors acknowledge the Fondazione Cariplo per la ricerca Biomedica under grant agreement #2018-0112 to F.U. and Fondazione AMICI ONLUS for the research prize 2020 to F.U..

Conflicts of Interest

S.Danese has served as a speaker, consultant, and advisory board member for Schering Plough, Abbott (AbbVie) Laboratories, Merck and Co, UCB Pharma, Ferring, Cellerix, Millennium Takeda, Nycomed, Pharmacosmos, Actelion, Alfa Wasserman, Genentech, Grunenthal, Pfizer, AstraZeneca, Novo Nordisk, Vifor, and Johnson and Johnson. The other authors declare no conflicts of interest.

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