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Modulation of immune responses by elexacaftor/tezacaftor/ivacaftor therapy in cystic fibrosis: data from a compassionate use program

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Abstract

Background Elexacaftor-tezacaftor-ivacaftor (ETI) improves clinical outcomes in people with Cystic Fibrosis (pwCF), with possible anti-inflammatory properties. However, the molecular mechanisms underlying these effects remain unclear. This study investigates ETI's anti-inflammatory and immunomodulatory activity, focusing on essential signaling pathways.

Methods Forty-nine pwCF were followed for 24 months. PwCF underwent clinical and pulmonary function assessment along with sweat test chloride measurement. Blood samples were analyzed for red and white blood cell counts and C-reactive protein (CRP). Plasma cytokines were quantified and phospho-kinase arrays and western blotting were used to assess protein phosphorylation in peripheral blood mononuclear cells (PBMC) from pwCF and healthy controls, pre- and post-ETI. Gene expression was evaluated in patient-derived PBMC and CF bronchial epithelial cells in vitro.

Results ETI treatment significantly improved percent-predicted forced expiratory volume in 1 s (ppFEV1) and reduced intravenous antibiotic use. Inflammatory markers (including CRP) and circulating leukocytes decreased, especially lymphocytes and monocytes. Six of 27 pro-inflammatory cytokines were significantly downregulated. ETI strongly inhibited Signal Transducer and Activator of Transcription 5 (STAT5) phosphorylation in PBMC and CF epithelial cells, both in vivo and in vitro. This correlated with reduced interleukin IL-6, IL-8, and TNF-α mRNA levels. Pharmacological inhibition of JAK/STAT mimicked ETI effects on cytokine expression, supporting STAT5 as an important player involved in CF chronic inflammation.

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Conclusion Long-term ETI treatment confirms clinical benefits and exerts measurable immunomodulatory effects, partially via inhibition of JAK/STAT signaling. These findings support its broader impact beyond CFTR correction. Further studies are warranted to explore long-term immunological outcomes, especially in younger patients initiating early therapy.

Keywords Cystic fibrosis, Inflammation, CFTR modulators, Elexacaftor, STAT5

Background

Cystic Fibrosis (CF) is an inherited autosomal recessive disorder caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (*CFTR*) gene, which encodes a chloride and bicarbonate channel [1].

Over 2,000 *CFTR* gene variants have been identified so far. The most common pathogenic mutation, F508del *CFTR*, leads to improper folding of the CFTR protein in the endoplasmic reticulum (ER), thereby impairing its trafficking to the plasma membrane. CFTR is widely expressed in epithelial tissues and plays a crucial role in maintaining epithelial surface hydration. Consequently, CF manifests as a multisystem disease, affecting exocrine pancreatic, hepatic, intestinal, and pulmonary functions [2].

Reduced airway surface liquid (ASL) volume contributes to cellular stress in the respiratory tract, leading to mucus accumulation, hypoxia, and enhanced susceptibility to opportunistic infections, primarily *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *and Staphylococcus aureus.* Even in the absence of infection, CF airway epithelial cells exhibit a constitutive pro-inflammatory state, characterized by elevated production of cytokines and chemokines such as interleukin (IL)-6 and IL-8. Indeed, CF is often described as a sterile neutrophilic inflammatory disorder of the airways [3].

Upon bacterial colonization, inflammation is further exacerbated by massive neutrophil recruitment, elevated systemic levels of inflammatory mediators, and delayed neutrophil apoptosis, which sustains their pro-inflammatory activity. Neutrophils also release high amounts of neutrophil extracellular traps (NETs), composed of DNA and proteases, along with oxidants such as myeloperoxidase. This chronic inflammatory environment drives progressive lung tissue damage [4–6].

The introduction of CFTR modulator therapies, including potentiators (e.g., ivacaftor) and correctors (e.g., elexacaftor and tezacaftor), has led to significant improvements in clinical outcomes and life expectancy for people with cystic fibrosis (pwCF) over the past two decades, especially those carrying the F508del mutation in *CFTR*. Several clinical trials and real-world studies have documented significant gains in respiratory function, reduced pulmonary exacerbations, and improved nutritional status as measured by the body mass index (BMI). Nonetheless, elexacaftor/tezacaftor/ivacaftor (ETI) treatment has been associated with adverse events, including psychiatric symptoms, elevated liver enzymes, skin rashes, and gastrointestinal disturbances in both pediatric and adult populations [7–9].

Despite these clinical advances, it remains unclear whether ETI therapy can effectively modulate the chronic inflammatory state characteristic of CF. Several studies have reported reduced systemic inflammation markers, such as C-reactive protein (CRP), absolute neutrophil count (ANC), IL-18, IL-1β, and tumor necrosis factor (TNF)-α, from 3 to 12 months after ETI treatment [10–12]. Others described reduction in immunoglobulins (IgG, IgA) and serum gamma-globulin levels, as well as improvements in macrophage-mediated phagocytosis, bacterial killing, and efferocytosis following one year of therapy [13, 14]. Importantly, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway has been proposed as a key regulator of ETI-associated anti-inflammatory responses in pediatric pwCF [15].

Conversely, other studies have reported modest or no significant changes in pro-inflammatory cytokine levels, including IL-6 and IL-8, after 12 months of ETI, especially in larger patient cohorts [14, 15]. One study on 57 pwCF carrying the F508del mutation showed a reduction in systemic inflammation and altered immune phenotypes following ETI, though residual immune activation remained when compared to healthy controls [16]. Lung-specific data from sputum samples supported a local anti-inflammatory role of ETI, showing reduced neutrophil elastase, IL-1β, IL-8, and bacterial burden. Yet, these findings were not consistently replicated, and follow-up studies reported no significant impact on IL-6 or IL-8 levels after 3 and 12 months of therapy [17, 18].

Taken together, the current evidence suggests that while ETI may exert partial anti-inflammatory and immunomodulatory actions, principally in lung tissues, its overall impact on systemic inflammation remains controversial and not fully understood. These inconsistencies may be due, at least partially, to limited duration of most clinical trials (3–12 months) and heterogeneity of patient populations. Moreover, the molecular mechanisms driving these immunological changes are still largely undefined.

Therefore, the purpose of this study is to investigate the long-term role of ETI in modulating inflammation, as well as its clinical impact in pwCF with severe respiratory disease.

In this context, we present new clinical and ex vivo data supporting a partial anti-inflammatory role for long-term ETI treatment in pwCF and propose the JAK/STAT signaling pathway as a potential molecular target mediating this effect.

Materials and methods

Patient recruitment

Between October 2019 and June 2021, 49 pwCF (M = 23,47%, F = 76,53%; mean age 36.3 ± 10.1 years, range 17.9–52.8) who were routinely followed at the Cystic Fibrosis Center, Verona, started ETI therapy on a compassionate-use program. Dosage regimens were prescribed according to the package leaflet and the requirements of the Italian Medicines Agency (AIFA) at that time for patients > 12 years: elexacaftor 200 mg once daily, tezacaftor 100 mg once daily, ivacaftor 150 mg every 12 h. Clinical evaluations, hematological assessments, and pulmonary function tests using forced spirometry were systematically performed at ETI initiation and during follow-up visits at 3, 6, 12, and 24 months after treatment. Sweat test was recorded before ETI initiation and at 12 and 24 months after. Information on the number of antibiotic treatments for pulmonary exacerbations was prospectively collected and recorded in patient chart.

Ethical approval was obtained from the Ethics Committees of the Azienda Ospedaliera Universitaria Integrata, Verona (approval no. 216CET) and Sapienza University of Rome (territorial Lazio CE approval N.5943). All studies were conducted in accordance with the Declaration of Helsinki. Samples from fifty healthy subjects of comparable age (M = 50%, F = 50%; mean age 34.7 ± 10.7, range 19–50) were included. All participating patients and subjects provided signed informed consent before enrollment, and 5 ml of peripheral blood was collected from each subject.

Hematologic laboratory assessment

White-blood-cell count with differential, hemoglobin, hematocrit, total red blood cell (RBC) count, and platelet count were measured in the CF cohort at baseline and after 12 and 24 months of ETI using the XN-9000 fully automated hematology analyzer (Sysmex Europe GmbH, Norderstedt, Germany) at the Service of Laboratory Medicine, Azienda Ospedaliera Universitaria Integrata of Verona, in accordance with current diagnostic guidelines.

Cell cultures

Peripheral-blood mononuclear cells (PBMC) were isolated by Ficoll-Paque Plus density-gradient centrifugation (400 g, 25 min, room temperature) and maintained in RPMI-1640 (Thermo Fisher, Waltham, MA, USA) supplemented with 20% FBS for 24 h. CF patient-derived bronchial epithelial cells (CFBE41o-), homozygous for F508del *CFTR*, and healthy control bronchial epithelial cells (16HBE14o-) were cultured in collagen IV-coated flasks using Eagle's Minimum Essential Medium (EMEM; Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% FBS, 1% L-glutamine, and essential amino acids. Bronchial epithelial cells were maintained in culture for a maximum of two weeks and subsequently cryopreserved in liquid nitrogen. Cells were incubated with 0.1% dimethyl sulfoxide (DMSO, vehicle) or elexacaftor (VX-445, 3 μM), tezacaftor (VX-661, 3 μM), and ivacaftor (VX-770, 1 μM) for 24 h at 37 °C, 5% CO_2. Alternatively, cells were incubated with the clinically approved JAK/STAT inhibitor ruxolitinib (3 μM) for 1 h. Following treatments, cells were stimulated with IL-6 (20ng/ml) for 30 min, or TNF-α (50 ng/ml) for additional 4 h.

Bioplex analysis in plasma

Plasma was obtained by centrifuging whole blood at 2,300 rpm for 10 min at RT, followed by 3,000 rpm for 15 min at 4 °C, and stored at -80 °C. Pro-inflammatory mediators were quantified with the Human Cytokine 27-plex Assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions, using a Luminex xMAP platform. Data were analyzed with Bio-Plex Manager software (Bio-Rad).

Proteome profiler array

Total proteins were extracted from patient-derived PBMC following the protocol of the Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN) and quantified using the Qubit Protein Assay (Thermo Fisher). A total of 200 μg of total proteins were processed. Membrane spots were developed by chemiluminescence (ChemiDoc, Bio-Rad), and spot densitometry was quantified using ImageJ software v1.54 (NIH, Bethesda, MD, USA).

Western blot

Cells were lysed in RIPA buffer containing protease inhibitors (Complete protease inhibitor cocktail, Roche, Basel, Switzerland) supplemented with sodium orthovanadate (1 mM). Proteins were separated on 6% acrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). After blocking (5% BSA or 5% milk in TBS-T), membranes were probed with anti-phospho-STAT5 (Y694/Y699), anti-STAT5, anti-phospho-STAT2 (Y690), anti-STAT2 (Bio-techne, Minneapolis, MN) or anti-β-actin antibodies (Sigma-Aldrich), followed by HRP-conjugated secondary antibodies. Signals were visualized with Clarity Max ECL (Bio-Rad) and quantified by densitometry using ImageJ software v 1.54 (NIH).

Table 1 Clinical and genetic features of CF patients enrolled in this study

Compassionate program	
Participants, n	49
Age, years, mean ± SD	36.3 (10.1)
Female, n (%)	26 (53.1)
CFTR genotype, n (%)	
Phe508del/Phe508del	14 (28.6)
Phe508del/other	35 (71.4)
Baseline colonization, n (%)	
Pseudomonas aeruginosa	32 (65)
MRSA	7 (14)
Staphylococcus aureus	5 (10)
Achromobacter xylosoxidans	4 (8)
Scedosporium apiospermum	3 (6)
Burkholderia cepacia	2 (4)
Ex vivo experiments	
Participants, n	4
Age, years, mean ± SD	8 (4.9)
Female, n (%)	1 (25%)
CFTR genotype, n (%)	
Phe508del/Phe508del	1 (25%)
Phe508del/other	3 (75%)

MRSA, Methicillin-Resistant Staphylococcus Aureus

qPCR

Total RNA was extracted with the High Pure RNA Isolation Kit (Roche) and reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). qPCR was performed with PowerUp SYBR Master Mix (Thermo Fisher) and QuantiTect primer assays (Qiagen, Hilden, Germany) for GAPDH (Hs_GAPDH_1_SG, NM_001256799) and target genes IL-1β (Hs_IL1B_1_SG, NM_000576), IL-6 (Hs_IL6_1_SG, NM_000565), IL-8 (Hs_CXCL8_1_SG, NM_000584), TNF-α (Hs_TNF_1_SG, NM_000594), STAT5a (Hs_STAT5A_1_SG, NM_003152), STAT2 (Hs_STAT2_1_SG, NM_198332). Reactions (25 ng cDNA) were run on a QIAquant 96-plex (Qiagen). Relative expression was calculated by the ΔΔCt method using GAPDH as a reference.

In-Silico promoter analysis

Transcription-factor binding sites were identified with LASAGNA-Search 2.0 [19] then visualized by SnapGene Viewer v.7.2.1. (GSL Biotech LLC, Boston, MA), in the following proximal (-600 bp) gene promoters: C-C Motif Chemokine Ligand 5 (CCL5), C–X–C Motif Chemokine Ligand 8 (CXCL8), Granulocyte Colony-Stimulating Factor (CSF3), IL1B, IL6, IL9, IL10, IL17, C-C Motif Chemokine Ligand 3 (CCL3), TNF, and Vascular Endothelial Growth Factor (VEGF).

Statistical analysis

Continuous variables were tested for normality with the Shapiro-Wilk test. Normally distributed data were compared with two-tailed paired Student's t-test. Non-parametric data were analyzed with Wilcoxon signed-rank test. Analyses were performed in GraphPad Prism 8 (Boston, MA, USA), or SigmaPlot 14 (Palo Alto, CA, USA). A two-sided p-value < 0.05 was considered significant.

Results

Clinical aspects of ETI response

The clinical and genetic characteristics of the study population are summarized in Table 1. Pulmonary function improved significantly 12 months after initiation of ETI and this clinical benefit was maintained at 24 months (Table 2). Absolute changes in ppFEV1 and percent predicted Forced Vital Capacity (ppFVC) from baseline were + 14.7 and + 14.2% points at 12 months, and + 18.2 and + 15.7% points at 24 months, respectively (both $p < 0.001$), mirroring findings from phase III clinical trials and real-world studies [20–22].

Table 2 Changes in clinical parameters from baseline to 12 and 24 months of ETI therapy

Characteristics	Baseline	12 months			24 months		
	Median (IQR)	Median (IQR)	Change from baseline	*p* value	Median	Change from baseline	*p* value
$ppFEV_1$ (%)	37 (33.0–43.0)	52.0 (44.0-63.2)	13.0 (9.0–21.0)	< 0.001	53.0 (46.0–66.0)	13.0 (10.0–23.0)	< 0.001
ppFVC (%)	63.0 (52.0–70.0)	83.0 (72.7–95.2)	16.0 (10.0–27.0)	< 0.001	81.0 (69.5–93.5)	18.0 (10.0–24.0)	< 0.001
Sweat chloride (mmol/L)	97.0 (85-108.5)	33.0 (25.0-42.7)	-61.0 (-73.0- -49.0)	< 0.001	36.0 (29.0–44.0)	-62.0 (-74.0-51.5)	< 0.001
BMI	19.7 (17.9–22.0)	21.5 (19.8–23.3)	1.8 (0.9–2.4)	< 0.001	21.4 (20.0-23.1)	1.7 (0.2–2.8)	< 0.001
Antibiotic treatments for PEx in previous 12 months, mean (range)	4.0 (3.0–5.0)	0.0 (0.0–1.0)	-4.0 (-5.0- -3.0)	< 0.001	1.0 (0.0–1.0)	-3.0 (-5.0- -2.0)	< 0.001
CFQ-R RD	72.2 (62.5–83.2)	94.4 (83.3–94.4)	16.7 (11.1–27.3)	< 0.001	88.9 (83.3–94.4)	11.1 (0-27.7)	< 0.001

Data are expressed as median (IQR). The p value is referred to change from baseline (Wilcoxon signed-rank tests). IQR, interquartile range

The BMI also rose steadily throughout treatment (Table 2). While previous real-life data reported variable reductions in sweat-chloride concentration [23, 24], our cohort showed a significant, consistent decline at 12 months that persisted to 24 months, with minimal inter-individual variability. Although the need for intravenous antibiotic therapy markedly decreased over the study period (Table 2), we did not observe a clear reduction in the type of chronic lung colonization after ETI treatment.

ETI treatment reduced CRP plasma levels and circulating leucocytes

It has been recently reported that a six-month ETI treatment can reduce ANC while increasing eosinophil numbers in peripheral blood [25]. In contrast, another study described an ETI-associated reduction in ANC without any effect on eosinophil levels [11]. A third study reported a significant reduction in IgE levels following ETI initiation, without a change in eosinophil count [26].

To investigate the potential immunomodulatory activity of ETI, we obtained peripheral blood samples from 49 adults with CF at baseline and again after 12 and 24 months of therapy. Comprehensive blood counts were performed, including lymphocytes, neutrophils, eosinophils, and monocytes. ETI treatment was associated with a broad reduction in erythrocytes, platelets, and total leukocytes, driven chiefly by declines in monocyte and lymphocyte counts. Eosinophil numbers remained unchanged. ANC fell slightly but not significantly. CRP levels dropped significantly after 12 months and remained almost normalized after 24 months (Fig. 1).

ETI exerted a partial anti-inflammatory effect by reducing plasma cytokine and chemokine levels

Emerging data from clinical studies on the systemic anti-inflammatory effects of ETI in pwCF remain conflicting. Some studies reported reductions in pro-inflammatory cytokines (e.g., IL-1, IL-6) and chemokines (e.g., IL-8), while others describe modest or undetectable changes in plasma samples from treated cohorts. To obtain a more comprehensive understanding of the systemic inflammatory profile, we quantified the levels of 27 soluble inflammatory mediators in plasma samples collected from pwCF before and after 12–24 months of ETI treatment, and compared them with plasma from 50 age-matched healthy controls. PwCF exhibited a baseline pro-inflammatory state, with elevated plasma levels of IL-1Rα, IL-4, IL-6, IL-7, IL-8, IL-9, IL-15, IL-17, TNF-α, IFN-γ, IP-10, Monocyte Chemoattractant and Activating Factor (MCAF), Eotaxin, VEGF, Macrophage Inflammatory Protein-1 alpha (MIP-1α), Macrophage Inflammatory Protein-1 beta (MIP-1β), and G-CSF (Fig. 2 and Supplementary Fig. 1). Following ETI treatment, six pro-inflammatory mediators, including IL-6, IL-8, IL-17, MIP-1α, G-CSF, and TNF-α, were significantly decreased after just 12 months, and this trend was maintained at 24 months. These results support a partial but sustained anti-inflammatory effect of ETI in pwCF.

ETI inhibits STAT2 and STAT5 activation

The observed alterations in cytokine and chemokine profiles suggest that ETI may exert broader effects through modulation of intracellular signaling pathways. This

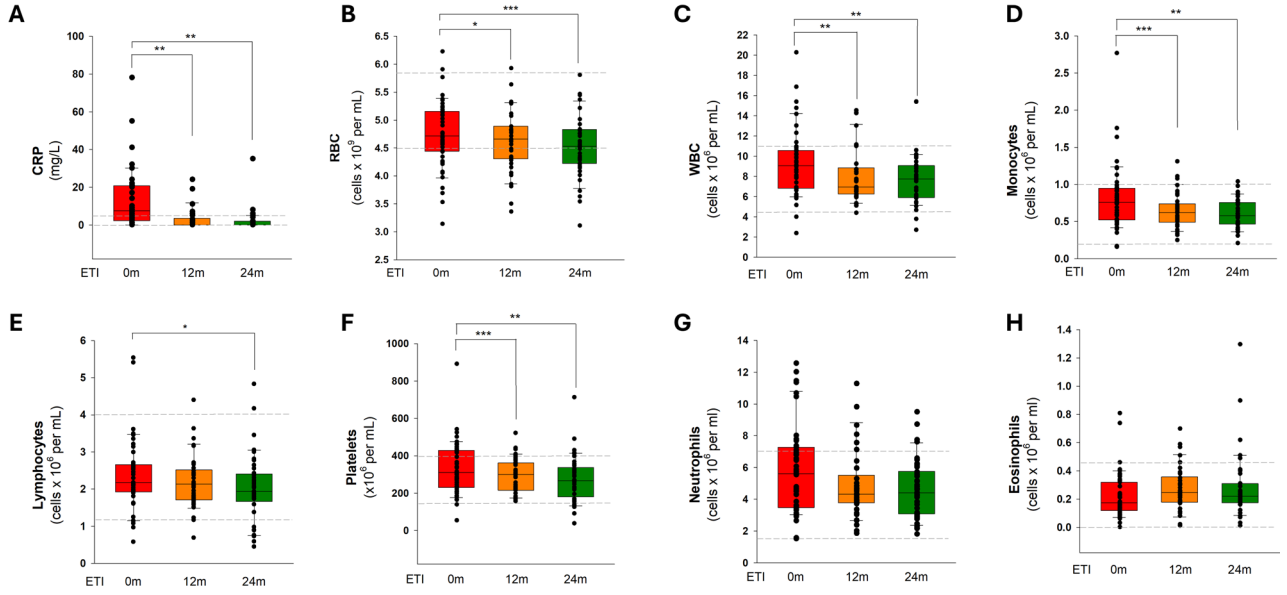

Fig. 1 Changes in clinical inflammatory markers following ETI therapy.Box-and-whisker plots showing variations in (**A**) C-reactive protein (CRP), (**B**) red blood cell (RBC), (**C**) white blood cell (WBC), (**D**) monocyte, (**E**) lymphocyte, (**F**) platelet count, (**G**) neutrophil, and (**H**) eosinophil counts measured at baseline (0 m) and after 12 (12 m) and 24 months (24 m) of ETI therapy in pwCF (n = 49). Normal ranges of each measurement are indicated by dotted grey lines. Statistical analysis was performed using two-tailed paired t-test (*p < 0.05, **p < 0.01, ***p < 0.001)

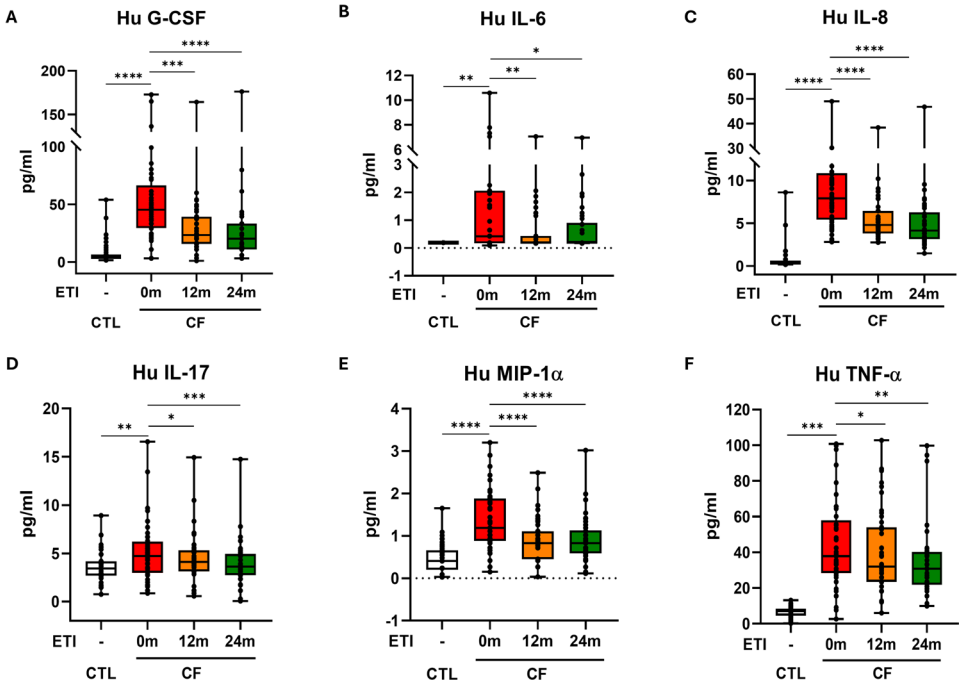

Fig. 2 Inflammatory mediators significantly modulated in plasma of pwCF following ETI therapy. Box-and-whisker plots of G-CSF (**A**), IL-6 (**B**), IL-8 (**C**), IL-17 (**D**), MIP-1α (**E**), and TNF-α (**F**) levels measured by Bioplex analysis in plasma samples from healthy controls (CTL, $n=50$) and pwCF ($n=49$) with similar ages, before (0 m) and after 12–24 months of ETI treatment. Comparison between healthy donors and CF patients was analyzed by Mann–Whitney unpaired t-test. Measurements before and after treatment were analyzed by mixed-model one-way ANOVA (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

hypothesis is supported by previous studies demonstrating ETI-induced changes in gene and protein expression profiles [15, 27, 28]. To further explore this aspect, we analyzed kinase phosphorylation in PBMC collected from three pwCF before and after 3 months of ETI treatment. We specifically chose this early time point because kinase inhibition is typically a rapid and early event, often detectable before downstream modulation of cytokine and chemokine release. Using a phospho-kinase array, we observed significant downregulation of phospho-STAT2 (Y690) and phospho-STAT5 (Y694/Y699) following ETI exposure (Fig. 3A), findings that were validated by western blot analysis (Fig. 3B-F). However, a partial reduction in total STAT2 and STAT5 protein amount was also observed. Notably, STAT5A and STAT2 mRNA levels were both significantly reduced after 3 months of ETI in patient-derived PBMCs (Fig. 3G). These findings suggest that ETI may exert broader transcriptional effects rather than acting exclusively through CFTR modulation.

To confirm that ETI treatment was responsible for the inhibition of the JAK/STAT pathway, we incubated PBMC isolated from younger, treatment-naïve pwCF (homozygous for F508del *CFTR*) ex vivo with ETI for 24 h. Remarkably, this short exposure alone nearly abolished the disease-associated increase in STAT5 phosphorylation, resetting p-STAT5 to the low basal levels seen in healthy donors (Fig. 4A-B). The speed and magnitude of this effect suggest that kinase and transcription factor inhibition is an immediate, cell-intrinsic response to ETI, preceding any downstream shifts in cytokine or chemokine release. Unfortunately, pSTAT2 signals were undetectable in PBMCs cultured ex vivo, precluding further analyses and comparisons. Resting STAT activity was scarcely detectable in control cells, underscoring the CF-specific hyperactivation of the pathway and the ability of ETI to rapidly normalize a key inflammatory signal.

IL-6 is widely recognized as a key upstream cytokine that activates the JAK/STAT signaling pathway. Accordingly, the ETI effect on activation of STAT2 and STAT5 proteins was investigated in PBMC freshly isolated from healthy donors upon IL-6 stimulation. However, ETI treatment did not significantly reduce p-STAT5 and p-STAT2 levels in healthy donor cells (Fig. 4C-E).

To determine whether this response was confined to leukocytes, we treated bronchial epithelial cells expressing either wild-type *CFTR* (16HBE14o-) or F508del *CFTR* (CFBE41o-) with ETI in vitro. Again, ETI sharply curtailed STAT5 and STAT2 phosphorylation in both lines, reducing p-STAT5 in CFBE41o- cells down to wild-type levels (Fig. 5A-D). These findings revealed a broad, cross-cell type STAT2 and STAT5 dampening effect of ETI in CF cells, highlighting its capacity to swiftly re-establish normal signaling across multiple models. Again, ETI showed no significant effect on healthy 16HBE14o- cell lines following JAK/STAT activation (Fig. 5E-G).

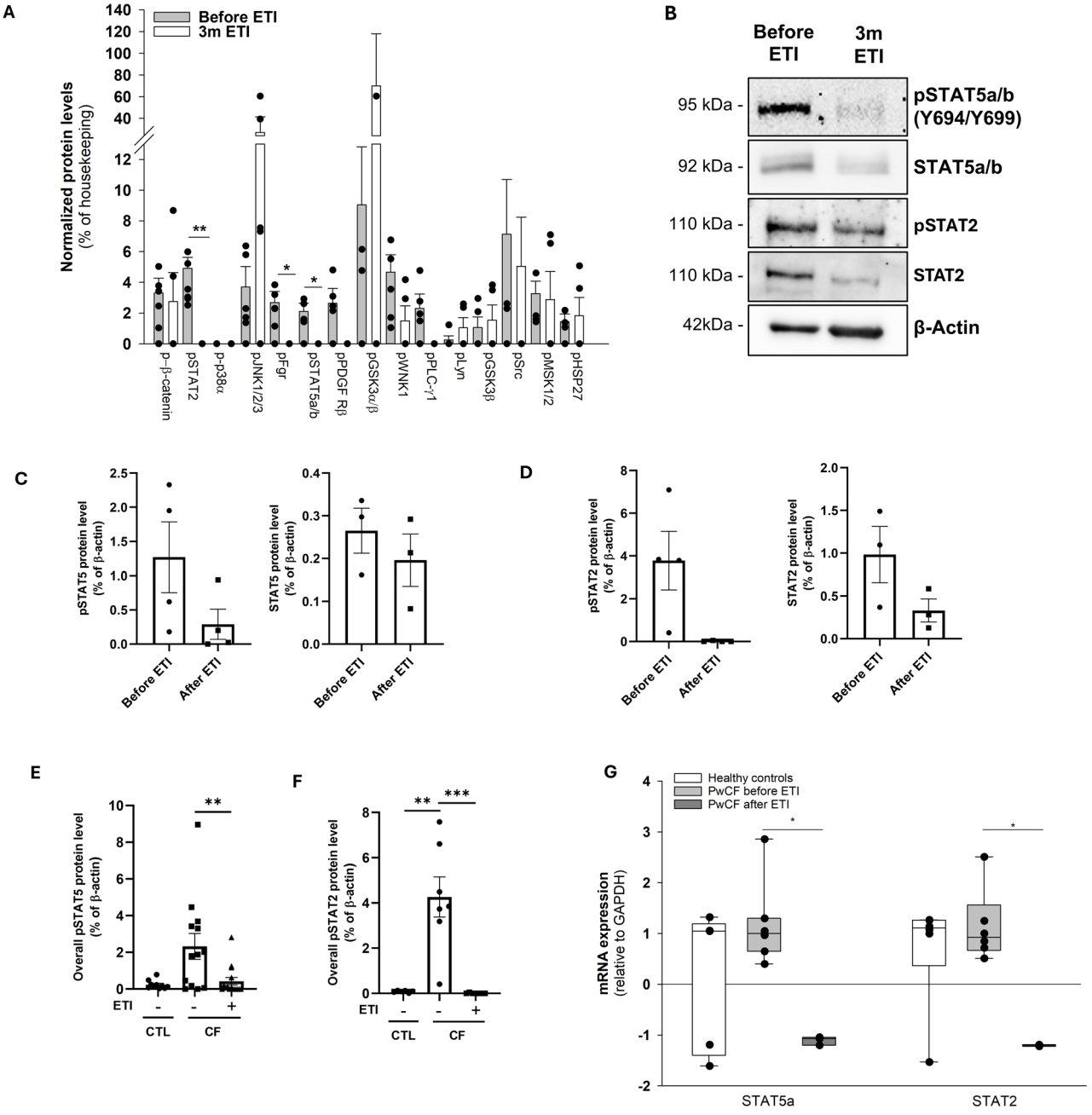

Fig. 3 Phospho-kinase array and western blot validation of p-STAT inhibition in PBMC following ETI treatment. **A**) Phospho-kinase array, (**B**-**D**) western blot analysis of PBMC collected from pwCF before and after 3 months of in vivo ETI therapy. **E**-**F**) Densitometric analysis of total western blot experiments in PBMC isolated from pwCF. **G**) RT-qPCR of stat5a and stat2 in PBMC collected from healthy donors and pwCF before after 3 months of ETI therapy

We also sought to determine whether inhibition of the JAK/STAT pathway is correlated with the clinical improvement observed in patients with CF after ETI therapy. We obtained a limited number of ex vivo clinical specimens that could be freshly collected under optimal preservation conditions, in order to minimize analytical bias. Of note, three of the four patients who exhibited a clear reduction in p-STAT2 and p-STAT5 levels in PBMCs following ETI therapy also showed a concomitant, marked improvement in FEV1 and FVC (Supplementary Fig. 2).

JAK/STAT inhibition downregulates pro-inflammatory mediators similarly to ETI

The immunomodulatory and oncogenic functions of STAT5 are well established [29]. In addition, STAT5 has been documented as an amplifier of macrophage pro-inflammatory gene expression in mice, driving the

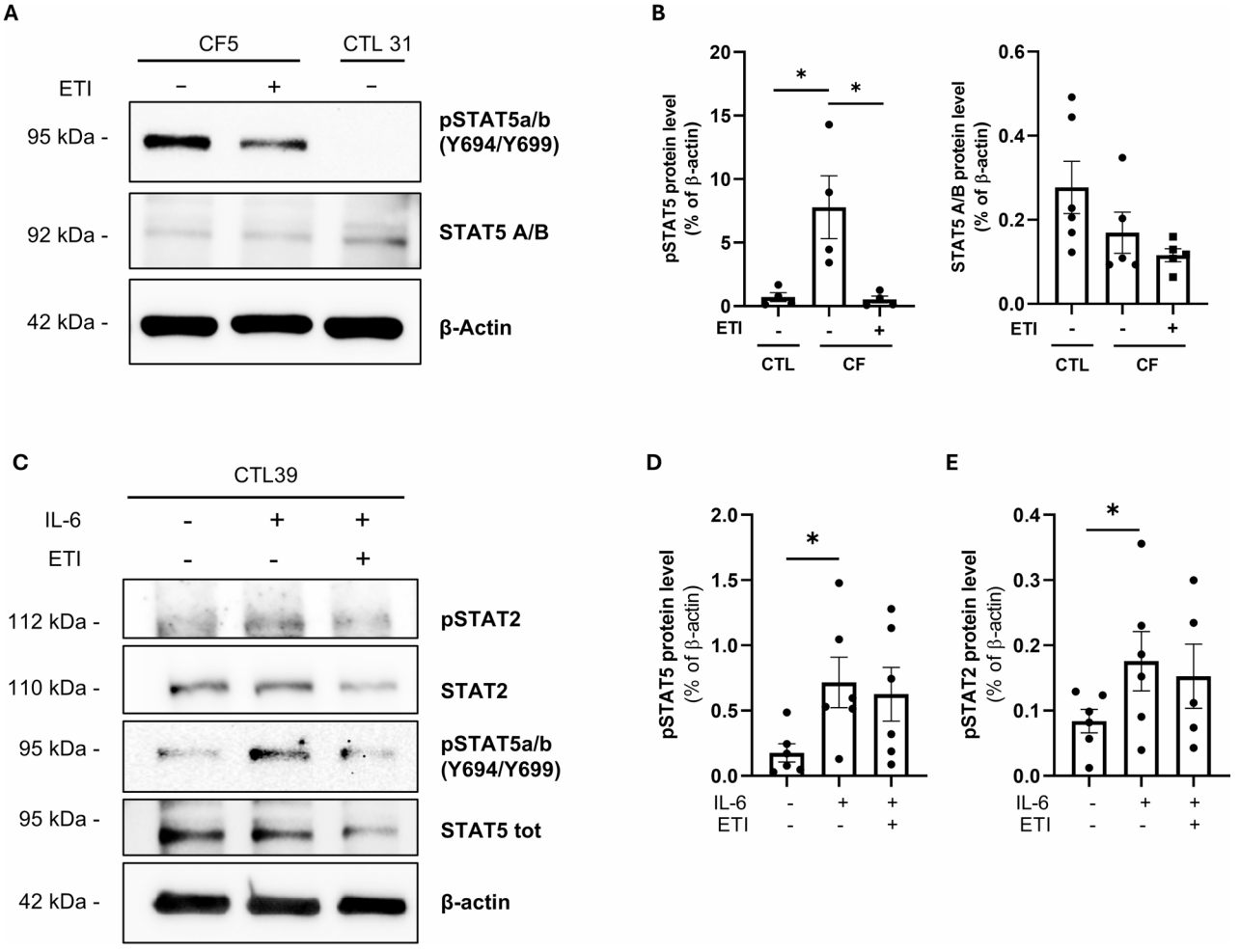

Fig. 4 Ex vivo assessment of ETI-mediated modulation of STAT2 and STAT5 phosphorylation in PBMC. (**A**) Representative western blot analysis of p-STAT5 A/B and total STAT5 A/B in PBMC derived from a healthy control (CTL31) and pwCF (CF5), treated ex vivo with or without ETI. (**B**) Densitometry analysis of western blots as depicted in panel A (n = 6). (**C**) Representative western blot analysis of p-STAT5 A/B, total STAT5 A/B, p-STAT2 and total STAT2 in PBMC from a healthy donor (CTL39), treated ex vivo with ETI or DMSO and stimulated with IL-6. **D**-**E**) Densitometry analysis of western blot as in panel C (n = 6)

activation of NF-κB p50-dependent IL-6 expression [30]. Again, STAT5 can sustain a peculiar pro-inflammatory cell program inducing IL-6, TNFα, and IL-8 gene expression in human macrophages. Reinforcing these findings, STAT5 gene silencing or pharmacological inhibition attenuated the expression of these cytokines in vitro [31]. However, the role of STAT5 in regulating inflammatory gene expression in CF has not yet been comprehensively explored. Because STAT proteins are pivotal transcription factors in multiple signaling pathways, we quantified mRNA levels of several pro-inflammatory mediators that are strongly affected in CF PBMC after ETI treatment, in particular IL-6, IL-8 and TNF-α. As expected, IL-6, IL-8, and TNF-α transcripts were significantly reduced following ETI exposure in PBMC from CF patients after three months of ETI therapy (Fig. 6A).

To investigate whether STAT2 and STAT5 may drive these transcriptional changes, we screened the promoters of these cytokines and chemokines for STAT-binding sites with LASAGNA-Search 2.0 [19]. Interestingly, STAT2 and STAT5 motifs were identified within the proximal promoter regions of IL-8 and TNF-α, underscoring the potential of STATs to regulate these soluble mediators. By contrast, only a single consensus sequence for STAT5 was detected in the promoter region of IL-6 (Fig. 6B).

To determine whether dysregulated JAK/STAT signaling can partially drive inflammatory gene expression in CF, possibly in tissues beyond circulating leukocytes, we compared the effects of the clinically approved JAK inhibitor ruxolitinib with those of ETI in CFBE41o- (homozygous for F508del *CFTR*) and healthy control 16HBE14o- bronchial epithelial cells subjected to a pro-inflammatory challenge (TNF-α). Ruxolitinib markedly decreased TNF-α-induced IL-6 and IL-8 expression (-31% and −42%, respectively) in CFBE41o-, mirroring

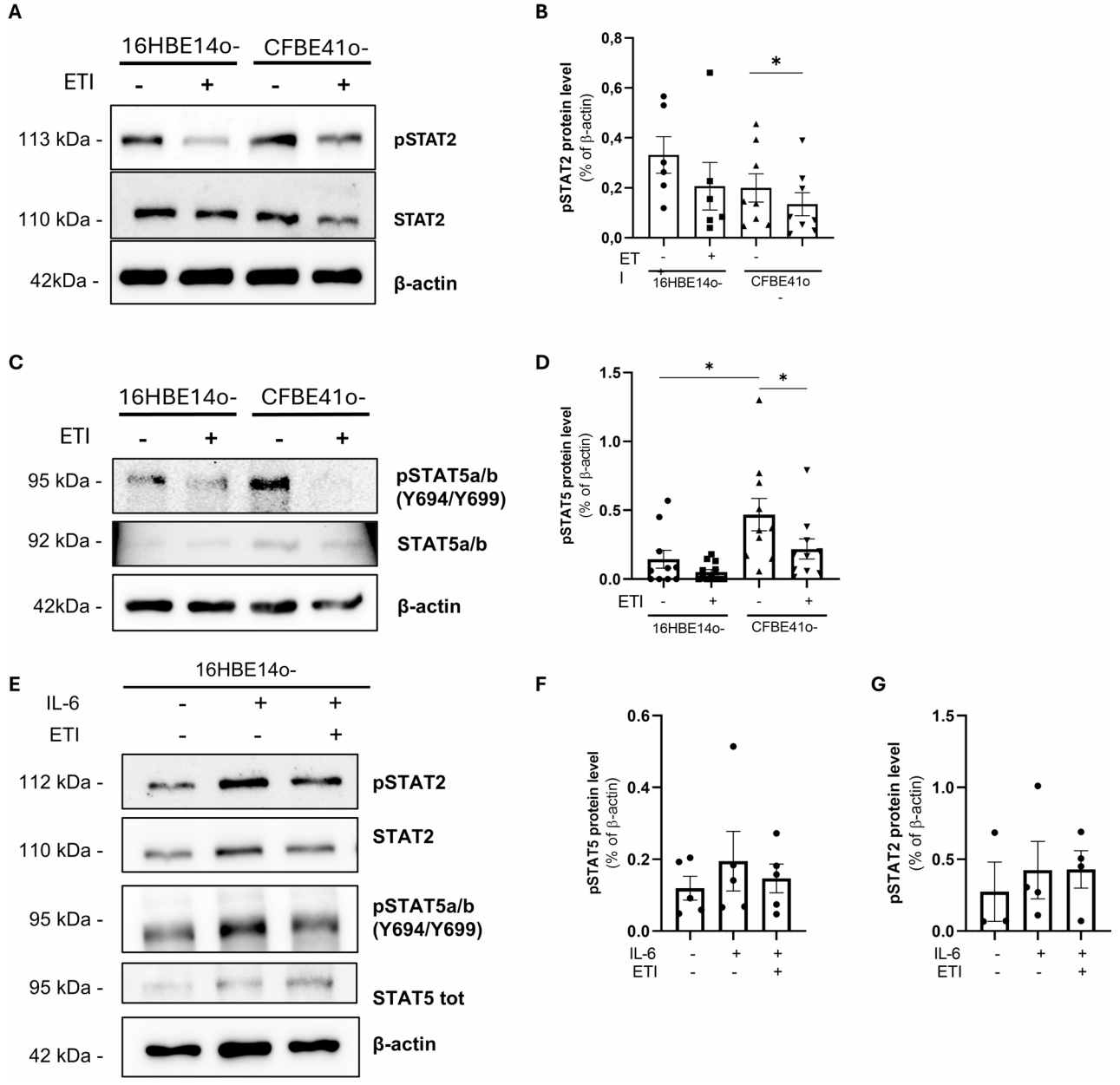

Fig. 5 Western blot validation of pSTAT2 and pSTAT5 inhibition in bronchial epithelial cell lines following ETI treatment.(**A**) Representative western blot of pSTAT2 in CF (CFBE41o-) and healthy (16HBE14o-) bronchial epithelial cell lines in the absence (DMSO, vehicle) or presence of ETI treatment for 24 h. (**B**) Densitometry analysis of pSTAT2 in western blots as depicted in panel A (n = 8). (**C**) Representative western blot of pSTAT5 in CF (CFBE41o-) and healthy (16HBE14o-) bronchial epithelial cell lines in the absence (DMSO, vehicle) or presence of ETI treatment for 24 h. (**D**) Densitometric quantification of pSTAT5 in western blots as depicted in panel C (n = 10). (**E**) Representative western blot of pSTAT2, STAT2, pSTAT5, and STAT5 in 16HBE14o- cells in the absence (DMSO, vehicle) or presence of ETI treatment for 24 h, then stimulated with IL-6 (20 ng/ml) for 30 min. **F**-**G**) Densitometry analysis of pSTAT2 and pSTAT5, respectively, in western blots as depicted in panel E (n = 5). Data are expressed as mean ± SEM. Statistical significance was assessed using the Mann-Whitney rank-sum test or Wilcoxon matched-pairs signed-rank test. *p < 0.05, **p < 0.01

the effect of ETI (-35% and – 25%, respectively, Fig. 6C). Conversely, IL-6 mRNA expression was not diminished in TNFa-stimulated 16HBE14o – control cells after ruxolitinib or ETI, whereas IL-8 was upregulated upon ETI treatment (Fig. 6D). These data suggest that the JAK/STAT pathway is involved in the CF inflammatory response, whereas it appears to play a much less prominent role in non-CF cells.

Discussion

The results of this clinical study confirm the long-term beneficial effects of ETI in pwCF carrying at least one *CFTR* allele with the F508del mutation. As observed in

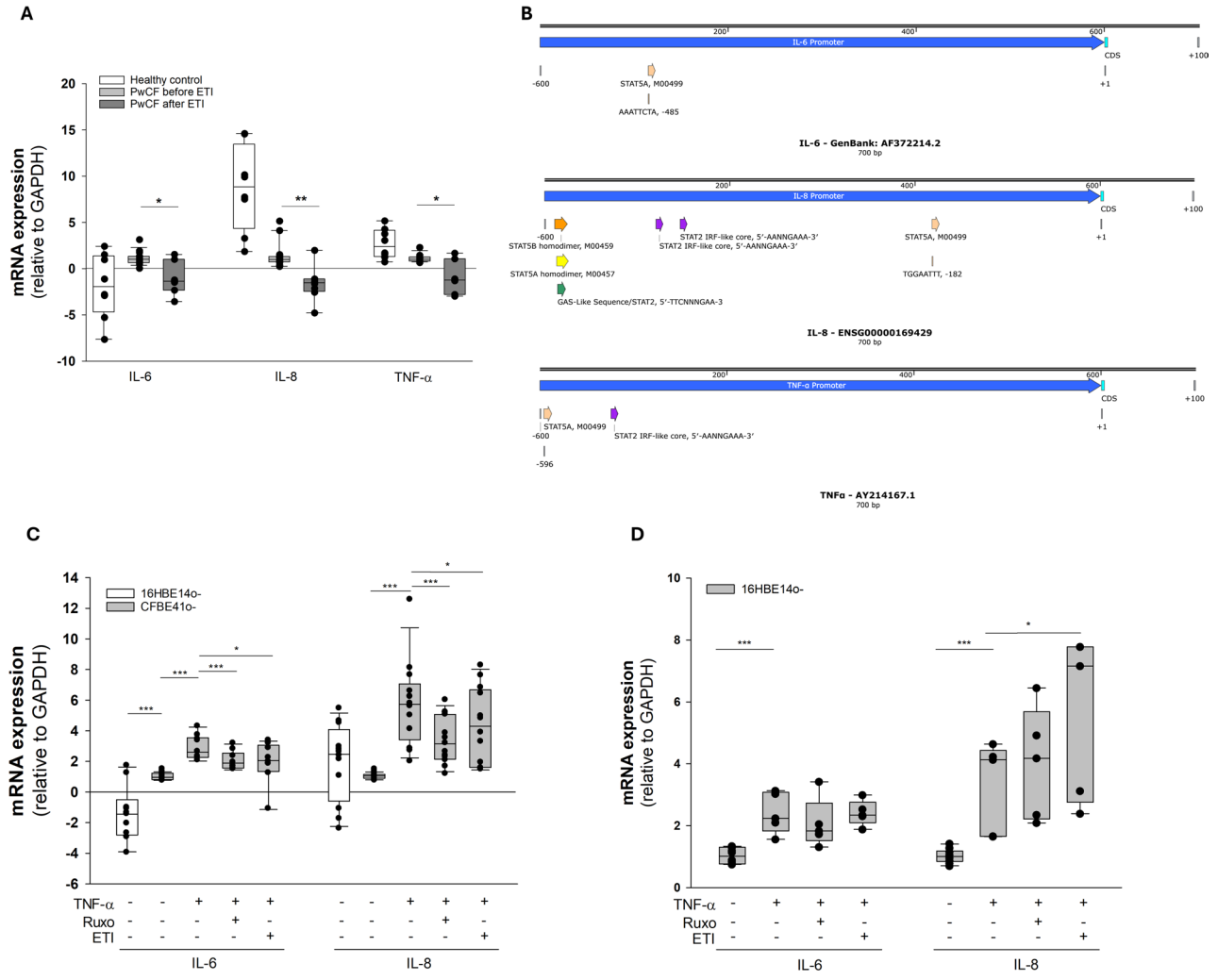

Fig. 6 ETI modulates mRNA expression of pro-inflammatory markers in vivo and in vitro. (**A**) qPCR analysis of mRNA expression levels of inflammatory markers (IL-6, IL-8, and TNF-α) in PBMC from pwCF before and after three-month ETI therapy. (**B**) In silico prediction of STAT2 and STAT5 consensus sequences in proximal promoter regions (600 bp) of pro-inflammatory mediator genes (IL-6, IL-8, and TNF-α). (**C**) qPCR quantification of IL-6 and IL-8 mRNA expression in 16HBE14o- (white boxes) and CFBE41o- (grey boxes) cells treated with TNF-α (50 ng/ml), Ruxolitinib (Ruxo, 3 μM), or ETI (n = 11). (**D**) qPCR quantification of IL-6 and IL-8 mRNA expression in healthy 16HBE14o- cells treated with DMSO (vehicle control) or ETI for 24 h, or with ruxolitinib for 1 h, and then stimulated with TNF-α (50 ng/ml) (n = 5). Statistical analysis was performed using the Mann-Whitney rank-sum test or paired Student t test (*p < 0.05, **p < 0.01, ***p < 0.001)

previous studies, patients with CF exhibited clinical benefits upon ETI treatment initiation, including improvements in pulmonary function and clinical status. Despite recruited patients had advanced lung disease, the effects of ETI on pulmonary function were also evident in the presence of infections, reducing the need for intravenous antibiotic therapy. Although several previous studies reported variable effects of ETI on sweat chloride concentrations, we observed a clear and constant reduction of chloride concentration in our study cohort.

Crucially, this work represents an example of reverse translational research, a bedside-to-bench strategy that applies focused laboratory experimentation to unravel the biological mechanisms behind clinical observations [32]. Our study follows this paradigm by moving from the unexpected clinical anti-inflammatory effects observed in pwCF back into cellular and molecular studies.

In vivo, both RBCs and leukocytes, including lymphocytes and monocytes, were significantly reduced by ETI. This effect also included a significant reduction in the platelet count. Thus, our study reported an immunomodulatory effect of ETI, confirming prior evidence that CFTR modulation reshapes immune-cell dynamics, modifying neutrophil and eosinophil counts [16, 25, 33, 34].

To the best of our knowledge, this study is the first to show that JAK/STAT signaling contributes to the

systemic anti-inflammatory and immunomodulatory effects of ETI.

Among JAK/STAT isoforms, p-STAT2 and p-STAT5 showed the greatest suppression in PBMC upon ETI treatment. STAT5 integrates cytokine and growth-factor signals that promote proliferation, differentiation, and survival, and its constitutive activation is a hallmark of several malignancies, where it supports tumor fitness and enhances regulatory T-cell activity [29]. ETI inhibited both *STAT5A* mRNA expression and STAT5 phosphorylation in PBMC and bronchial epithelial cells, suggesting a targeted anti-inflammatory mechanism that could overlap with oncogenic pathways and warrants long-term surveillance [35]. Although STAT2 is canonically activated by type I interferons [36], emerging data link this factor to epithelial-to-mesenchymal transition (EMT), metastasis, and chemoresistance [37, 38]. Similarly to STAT5, STAT2 may induce IL-6 expression either directly or indirectly via NF-κB [30, 39]. Thus, its inhibition may disrupt pro-inflammatory feed-forward loops. Previous work has implicated NF-κB blockade in ETI activity [15, 40]. However, cytokine expression is regulated by complexes of transcription factors that collaborate with NF-kB in enhancing gene expression [41]. Here, only 6 out of 27 cytokines and chemokines, mostly IL-6, IL-8, and G-CSF, were significantly modulated, indicating a focused rather than global immunosuppressive effect. Persistent IL-6/JAK/STAT signaling can trigger EMT via transcription factors such as TWIST1 [42]. Dysfunctional CFTR promotes TWIST1-dependent up-regulation of vimentin, collagen I, and fibronectin, whereas lumacaftor/ivacaftor reverses this phenotype [43]. The present data add STAT5 to the CF inflammatory network, implying that ETI may curb EMT-driven airway remodeling and chronic inflammation. In contrast, data obtained from healthy donor PBMCs and from IL-6-stimulated bronchial epithelial cells 16HBE14o – indicate that ETI does not reduce STAT2 or STAT5 phosphorylation in non-CF cells. Notably, ETI instead increased IL-8 mRNA expression in 16HBE14o – cells. Collectively, these findings suggest that ETI-mediated modulation of JAK/STAT signaling is context-dependent, likely occurring downstream of CFTR correction, or predominantly within the pro-inflammatory, dysregulated microenvironment associated with CFTR deficiency. A major limitation of our study is that all enrolled participants had advanced lung disease, creating an inflammatory milieu that may not fully represent the wider CF population. Crucially, however, we still detected elevated p-STAT5 in primary PBMC from younger, treatment-naive individuals, demonstrating that STAT5 hyper-activation is an intrinsic hallmark of CF rather than a secondary result of end-stage lung damage. To clarify the contribution of the STAT5 pathway to systemic pathology, larger cohorts with milder disease and sampling of extra-pulmonary tissues should be tested. Whether STATs proteins are hyper-activated in CF through an IL-6-driven autocrine/paracrine loop or via an intrinsic defect remains unresolved, and the molecular basis by which ETI dampens STAT signaling deserves further investigation. One plausible mechanism is that ETI indirectly reduces JAK activity by tempering cross-linked pathways such as mTOR, which can be chronically over-activated in CF through PTEN down-regulation [44].

Despite these uncertainties, our data reveal two novel insights. First, ETI exerts a selective anti-inflammatory and immunomodulatory action by suppressing STAT2 and STAT5 activity while simultaneously restoring CFTR function. Second, we provide the longest follow-up to date (24 months) and the broadest cytokine panel yet examined, extending beyond the usual IL-1β, IL-6, IL-8 and TNF-α, to map the CF inflammatory landscape. Collectively, these findings identify STAT5 as a previously unrecognized driver of CF inflammation and support the development of adjunct therapies that combine CFTR modulation with anti-inflammatory targeted strategies to curb extra-pulmonary complications and long-term decline in pwCF.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

F.L. designed the clinical study, recruited pwCF, and analyzed the data; A.P. performed most of the experiments, analyzed the data, and prepared figures; I.M. and G.C. accurately collected and cryo-preserved clinical samples and specimens; A.M.H. and G.M. performed experiments and analyzed the data; E.D. and Marica Bordicchia contributed to the collection and cryo-preservation of clinical samples and specimens; G.S., M.T.A., and G.B. performed multiplex cytokine assays in plasma samples; M.R. recruited pwCF and collected human plasma samples; E.F., Monica Borgatti, B.F., and G.L. provided fundamental resources and analyzed data; M.C. conceived the study, supervised clinical assessments, designed the clinical study; V.B. conceived the study, supervised the experimental workflow, wrote the original manuscript. All authors read and reviewed the manuscript.

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Data availability

All data analyzed in this study are provided in the Supplementary Information.

Declarations

Ethics approval and consent to participate
This study has been reviewed and approved by the Ethics Committees of the Azienda Ospedaliera Universitaria Integrata, Verona (approval no. 216CET) and Sapienza University of Rome (territorial Lazio CE approval N.5943). All participating patients and subjects provided signed informed consent before enrollment according to the regulations of the Ethics Committees.

Competing interests
The authors declare no competing interests.

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