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Original Article

Phenotypic and functional characterisation of liver-infiltrating T-cells in a case of CFTR modulator induced drug-induced liver injury

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ABSTRACT

Background: The introduction of CFTR modulators has been transformative for many people with cystic fibrosis (CF). The drugs are generally well tolerated although adverse reactions such as delayed-type T-cell mediated hypersensitivity and drug-induced liver injury (DILI) have been reported. Here, we characterise a novel underlying immunological mechanism driving DILI post Elexacaftor/Tezacaftor/Ivacaftor (ETI) administration.
Methods: Liver-infiltrating T-cells were isolated, post liver biopsy, using a collagenase digestion protocol. Phenotypic analysis of isolated T-cell populations was conducted using flow cytometry and mass cytometry (CyTOF). Lymphocyte transformation tests (LTT) were conducted to detect CFTR modulator-specific T-cell proliferation ([3H]-thymidine). Cytokine analysis was performed using intracellular cytokine staining and enzyme-linked immunospot assays.
Results: Liver function tests and histological examination of liver tissue revealed elevated alanine transaminase levels and evidence of perivenular zone three confluent necrosis and hepatocyte loss with mixed inflammatory infiltrate of lymphocytes. T-cells isolated from a percutaneous liver punch biopsy were associated with a dominant CD8+ cellular phenotype. Phenotypic analysis also revealed a CD45RO+ memory phenotype alongside expression of trafficking and migratory markers, such as CXCR3 and LFA-1. *In vitro* antigen specificity testing demonstrated significant proliferative T-cell responses after exposure to Ivacaftor, Ivacaftor M1 and Ivacaftor M6 metabolites within populations of CD3+ liver-infiltrating T-cells, but not peripheral blood mononuclear cells.
Conclusions: CFTR modulator-specific T-cells appear to be localised to sites of liver injury and not present at high frequencies within peripheral blood. Our findings align with pathomechanisms of immune-mediated DILI, during which drug-activated T-cells migrate towards sites of inflammation, infiltrate tissues and induce hepatotoxicity.

1. Introduction

Cystic fibrosis (CF) is a hereditary condition caused by defects in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) channel. The CFTR channel regulates the transport of salt, chloride ions and fluid across the epithelia [1]. Absent or defective CFTR protein leads to multi-system effects, most notably the respiratory system [2]. The introduction of modulator drugs which can partially correct the expression and function of the CFTR protein, has changed the landscape for up to 90 % of people with *CF*. Elexacaftor/Tezacaftor/-Ivacaftor (ETI) is presently the most widely used CFTR modulator combination and has proved highly effective in reducing pulmonary exacerbations, increasing lung function, weight and quality of life. If started early, treatment has the potential of normalising life expectancy

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[3]. There are presently six compounds available including the correctors Lumacaftor, Elexacaftor, Tezacaftor, Vanzacaftor and the potentiators Ivacaftor and deuterated Ivacaftor.

ETI is generally well tolerated although delayed-type T-cell mediated hypersensitivity, elevation in liver function tests and non-specific drug induced liver injury (DILI) have been reported [4,5]. Immunogenic events attributed to the initiation of CFTR modulator therapy include cutaneous-based reactions, such as maculopapular rash and drug reaction with eosinophilia and systemic symptoms (DRESS) [6–8]. Previously, *in vitro* studies have identified and characterised T-cell involvement within clinical cases of DRESS and skin rash stemming from Lumacaftor administration [6,9]. To date, such studies have focused on immunological responses to Lumacaftor derived from the peripheral blood, with only *in vivo* assays suggestive of T-cell mediated hypersensitivity to Ivacaftor following intradermal testing with IVA/LUM [9].

In this article, we describe an adult with CF, who is homozygous for F508del, presenting with elevated serum alanine transferase (ALT) which peaked at 353 U/L, 14.5 months after starting ETI therapy. Baseline liver ultrasound and liver function tests were normal prior to commencing therapy as was an extended liver screen. ETI was discontinued and ALT slowly settled over the following month. A liver biopsy showed predominant changes associated with central veins and zone three perivenular hepatocytes. Perivenular inflammation was predominantly lymphocytic, with hepatocyte drop out and pigment laden histiocytes observed, most commonly attributed to drug reactions. The offending compounds contained within the fixed CFTR modulator could not be identified using conventional *in vitro* diagnostic tests performed on peripheral blood mononuclear cells (PBMCs).

Due to clinical deterioration, a trial of Tezacaftor/Ivacaftor (TEZ/IVA) was carefully considered. Prior to commencing treatment, a further liver biopsy was undertaken (7 months between biopsies) and reassuringly showed an absence of inflammation or acidophil bodies. Unfortunately, ALT levels increased within a week rising to a peaking level of 364 U/L on day 19, necessitating treatment cessation. One further attempt of introducing Ivacaftor alone followed by increasing doses of TEZ/IVA was successful for 5 months, but ALT increased and peaked at 540 U/L. ALT levels returned to normal a month after drug discontinuation, with a third liver biopsy undertaken 5 weeks after drug discontinuation proving unremarkable. Samples of the second two liver biopsies were collected as part of clinical evaluation and sent for immunological assessment to define the likely drug culprit and drivers of DILI.

2. Methods

2.1. PBMC from human subjects

Venous blood (40 mL) was obtained from the patient following diagnosis. PBMC were isolated by density gradient centrifugation, during which whole blood was layered onto an equal volume of lymphoprep™ (STEMCELL™ Technologies) and then centrifuged for 25 min at 2000 rpm with no brake applied. The resulting buffy coat, comprising the lymphocyte layer, was carefully removed by gentle resuspension using a Pasteur pipette. Cells were washed twice with Hanks Balanced Salt Solution (HBSS) before resuspension in RPMI-based T-cell culture media supplemented with penicillin (100 μg/mL), streptomycin (100 U/mL), holo-transferrin (12.5 mg/mL), HEPES buffer (25 mM), L-glutamine (2 mM) and 5 % human AB serum. All volunteers in this study gave informed consent and ethical approval was obtained from the Liverpool research ethics committee.

Patient PBMCs were used for *in vitro* assays including lymphocyte transformation tests (LTTs) and Enzyme Linked Immunospot (ELISpot) assays. An autologous Epstein-Bar Virus (EBV) transformed B-cell line was generated from patient PBMC and used as antigen presenting cells for functional T-cell assays. Briefly, patient PBMC were cultured with filtered B95.8 supernatant and cyclosporin A (CSA, 1 μg/mL). The EBV-transformed B-cell lines were maintained in F1 culture medium [RMPI-1640, 10 % Foetal Bovine Serum (FBS), HEPES buffer (25 mM), Penicillin/Streptomycin (100 μg/mL) and L-glutamine (2 mM)] and cyclosporin was removed after 3 weeks [10].

2.2. Isolation of liver infiltrating T-cells

Percutaneous liver biopsies were obtained from the patient diagnosed with DILI. Liver tissue was incubated with HBSS supplemented with collagenase IV (500 mg/L), DNase I (50 μg/L) and foetal bovine serum (FBS) for 15 min. The sample was filtered through a 30-μm-nylon mesh filter prior to centrifugation (500 g for 10 min). The cell pellet was resuspended in RPMI-based T-cell culture medium and supplemented tri-weekly with interleukin-2 (IL-2; 200 IU/mL) to facilitate T-cell expansion.

2.3. Lymphocyte transformation test

Lymphocyte transformation tests (LTT) were conducted to detect CFTR modulator and piperacillin (positive control drug)-specific T-cell proliferation according to the protocol described by Pichler and Tilch [11]. Briefly, patient PBMC were seeded in a U-bottomed 96 well-plate in triplicate cultures at a density of 1.5×10^5 cells/well and incubated with in RPMI-based T-cell culture medium with CFTR modulator panel (Ivacaftor, 2.5 μM; Ivacaftor M1, 5 μM; Ivacaftor M6, 10 μM; Tezacaftor, 10 μM; Tezacaftor M1, 10 μM; Tezacaftor M2, 50 μM; Elexacaftor, 10 μM; ETI, 1 μg/mL; Tezacaftor/ivacaftor, 1 μg/mL), piperacillin (0–2 mM) or Phytohemagglutinin-L (PHA; 10 μg/mL) for 5 days. Following incubation, [3H]-thymidine (0.5 μCi/well) was added and cultures were incubated for a further 16 h. [3H]-thymidine incorporated cultures were harvested onto printed fibreglass filter mats using a cell harvester (Automated Harvester 96 Tomtec MK III) and dried at 80 °C. Fibreglass mats were heat sealed inside Wallac sample bags with MeltiLex scintillator sheets using a Wallac 1495–021 Microsealer (Perkin Elmer, Waltham, MA, USA). Radioactivity was quantified using a MicroBeta 2450 microplate counter and readouts were given by counts per minute (cpm).

2.4. In vitro antigen specificity testing

Liver infiltrating T-cells isolated from the DILI patient (5×10^4 cells/well) were co-incubated with autologous EBV-transformed B-cells (1×10^4 cells/well) and either cell culture medium (negative control), CFTR modulator and metabolites panel (Ivacaftor, 2.5 μM; Ivacaftor M1, 5 μM; Ivacaftor M6, 10 μM; Tezacaftor, 10 μM; Tezacaftor M1, 10 μm; Tezacaftor M2, 50 μM; Elexacaftor, 10 μM; Symkevi, 1–2 μg/mL; ETI 1–2 μg/mL) or PHA (10 μg/mL) for 48 h. For the final 16 h tritiated thymidine was added as previously described in order to determine CFTR mediated proliferation of liver infiltrating T-cells.

2.5. Cytokine release assays (ELISpot)

To assess secretion of Interferon-γ (IFNγ) from activated patient PBMC, ELISpot assays were performed per the manufacturer's instructions (Mabtech, Sweden). PBMC (1.5×10^5 cells/well) were incubated with RPMI-based T-cell culture medium, the CFTR modulators of interest or PHA-L for 48 h. ELISpot plates were pre-coated with the cytokines of interest (IFNγ; 15 μg/mL). Following incubation, corresponding biotin-conjugated detection antibodies and streptavidin conjugated alkaline phosphatase (strep-ALP) were used for assay development. The plate was imaged using an AID ELISpot reader (Cadima Madical, Stourbridge, UK) and spot forming units (SFU) were calculated to determine IFNγ secretion. The same process was followed using liver infiltrating T-cells (5×10^4 cells/well) with the inclusion of autologous EBV-transformed B-cells (1×10^4 cells/well).

2.6. Intracellular cytokine staining

Intracellular cytokine staining (ICS) was performed using liver infiltrating T-cells to investigate cytokine production in response to the CFTR modulators. Liver infiltrating T-cells (1×10^6 cells/well) were incubated with autologous EBV-derived B-cells and CFTR modulators of interest or positive control; phorbol 12-myristate 13-acetate and ionomycin (PMAI) in the presence of costimulatory anti-CD28d and anti-CD49d antibodies (BD Biosciences; 1 μg/mL) for 1 hour (37 °C, 5 % CO_2). Following the incubation, brefeldin A (10 μg/mL) was added and the cells were further incubated for 16 h (37 °C, 5 % CO_2). Cells were stained with CD3 (Cat. No. 300,448, 1:50 dilution), CD4 (Cat. No. 555,346, 1:30 dilution), CD8 (Cat. No. 301,036, 1:100 dilution) and CD45RO (Cat. No. 304,222, 1:50 dilution) to allow analysis of specific T-cell subsets. To measure the production of intracellular cytokines, cells were incubated with anti-IFNγ (Cat. No. 554,701, 1:200 dilution), anti-Granzyme B (GB) (Cat. No. 372,214, 1:100 dilution) and anti-TNFα (Cat. No. 12-7349-81, 1:100 dilution) fluorophore conjugated antibodies. The BD FACSCanto II was used to measure fluorescence and FlowJo® version 10 software was used for data analysis.

2.7. Flow cytometry and cellular phenotyping

Surface marker expression for DILI liver infiltrating T-cells was investigated via flow cytometry. To identify individual immune-cell populations, liver infiltrating T-cells were stained with fluorophore conjugated antibodies to investigate expression of CD3 (Cat. No. 555,342), CD45RO (Cat. No. 304,222), CD4 (Cat. No. 317,443), CD8 (Cat. No. 301,036), CD45RA (Cat. No. E11286–1631), CCR5 (Cat. No. E17013–101), CCR7 (Cat. No. 11–1979–41), CXCR3 (Cat. No 353,719), CXCR6 (Cat. No. 356,006), CD69 (Cat. No. 310,912) and LFA-1 (Cat. No. E1–2200–1632). Fluorescence was measured using the BD FACSCanto II and FlowJo® version 10 software was used for data analysis.

2.8. Mass cytometry (HELIOS®)

The cellular phenotype of patient derived T-cells was further assessed using a mass cytometry protocol, according methodologies previously described by Lin et al. [12]. Briefly, patient liver-infiltrating T-cells were incubated with RPMI-based T-cell culture medium or PMAI positive control in the presence of costimulatory anti-CD28d and anti-CD49d (BD Biosciences; 1 μg/mL) for 1 hour (37 °C, 5 % CO_2). Brefeldin-A (10 μg/mL) was added and incubated for a further 6 h (37 °C, 5 % CO_2). Cells were incubated with a combination of two anti-CD45 barcodes; 86 Cd (1:150) 110 Cd (1:150), 111 Cd (1:150) on ice for 30 min. To analyse cell populations, liver-infiltrating T-cells were incubated with anti-CD3 (141 Pr), anti-CD45RA (143 Nd), anti-CD4 (145 Nd), anti-CD8a (146 Nd) and anti-CD45RO (150 Nd) metal labelled antibodies. Additionally, for the study of regulatory T-cell populations from whole PBMC following Ivacaftor reintroduction, PBMC were incubated with anti-FOXP3 (159 Tb). Data was acquired using the Helios® CyTOF Mass Cytometer and analysed using Cytobank software.

3. Results

3.1. Liver function tests and histological examination of liver tissue revealed elevated alanine transaminase levels and lymphocyte infiltration

LFTs were conducted to assess serum total bilirubin levels, serum ALT levels and serum alkaline phosphatase (ALP) levels (Fig. 1A). Elevated serum ALT levels (353 IU/L) were detected 14.5 months after starting ETI and at each additional timepoint at which percutaneous liver punch biopsies were collected for clinical and immunological evaluation (Fig. 1A). Clinical examination of the percutaneous liver punch biopsy collected after the adverse reaction to ETI revealed localised zone three inflammation (perivenular) with hepatocyte dropout. Significantly, inflammation was predominantly lymphocytic in nature. Portal tracts were unremarkable, demonstrating preserved architecture and an absence of inflammatory infiltrates, further supporting hepatocellular injury in favour of cholestatic injury. Staining also confirmed the presence of activated macrophages in zone three. No iron accumulation, copper-associated protein or alpha-1 antitrypsin deficiency was detected (Fig. 1B).

3.2. T-cells isolated from percutaneous liver punch biopsy were associated with a dominant CD8+ cellular phenotype

Cell suspensions were prepared from a percutaneous liver biopsy obtained from a CF patient with ETI-DILI following collagenase digestion (Fig. 2). Following CD3+ purification, cellular fractions were imaged using light microscopy (20X) to establish phenotypic and morphological traits (Fig. 2A). The sample demonstrated red blood cell contamination and heterogenous populations of liver-resident and liver-infiltrating cells in the unseparated and CD3- cellular fractions. This may include, but is not limited to, Kupffer cells, liver-resident macrophages and liver sinusoidal endothelial cells. Notably, no morphological evidence was observed for the presence of hepatocytes or dendritic cells. The purified CD3+ cellular fraction demonstrated cellular morphology consistent with T-lymphocytes.

Phenotypic co-receptor analysis conducted using flow cytometry revealed that following CD3+ purification, cells isolated from the ETI-DILI biopsy demonstrated CD3+ purity (99.7 %). Furthermore, the T-cell phenotype was found to be skewed in favour of CD8+ *T*-cells (67.5 %) compared to CD4+ (32.5 %) (Fig. 2B). Additionally, 3D clustering analysis acquired by mass cytometry confirmed the dominant CD8+ phenotype at single-cell resolution (Fig. 2C).

3.3. Phenotypic analysis of infiltrating T cells reveals a CD45RO+ memory phenotype alongside the expression of trafficking and migratory markers

Further flow cytometric and 3D clustering analysis (tSNE) was performed on the liver-infiltrating T-cell population to assess memory cellular phenotype and chemokine receptor expression (Fig. 3). The isolated CD3+ *T*-cell population consisted almost exclusively of $CD45RO^+$ memory T cells, with <5 % expressing the naïve marker CD45RA+ (Fig. 3A and B). Interestingly, memory T-cells isolated from the ETI-DILI punch biopsy were found to express high levels of CXCR3 (96.5 %), associated with CD8+ *T*-cell recruitment to sites of inflammation and tissue damage. CD3+CD45RO+ populations were also observed to highly express the activation maker CD69 (89.9 %), and LFA-1 (96.4 %), associated with cellular trafficking and migration.

3.4. In vitro T-cell assays demonstrate the functionality and proliferative capacity of liver infiltrating T-cells

The functionality of liver-infiltrating T-cells was investigated following stimulation with the mitogen PHA-L (Supplementary Fig. 1). Liver infiltrating T-cells secreted Th1 (IFNγ) and Th2 (IL-10 and IL-13) cytokines in addition to cytotoxic and cytolytic effector molecules (granzyme B and perforin) after graded mitogen stimulation (2.5 & 10 μg/mL) (Supplementary Fig. 1A). The proliferative capacity of T-cells was observed to diminish following long-term culture and mitogen exposure, with stimulation index (SI) values decreasing >60 fold after a culture period of 40 days (Day 20, SI = >300; Day 40, SI > 4.5) (Supplementary Fig. 1B). ICS analysis assessed intracellular cytokine release from CD4+CD45RO+ and CD8+CD45RO+ memory T-cells in response to PMAI. Both T-cell populations were associated with the release of high levels of IFNγ (>50 % of T-cell population) and TNF-α (>80 % of T-cell population), with lower levels of granzyme B release detected (>20 % of T-cell population).

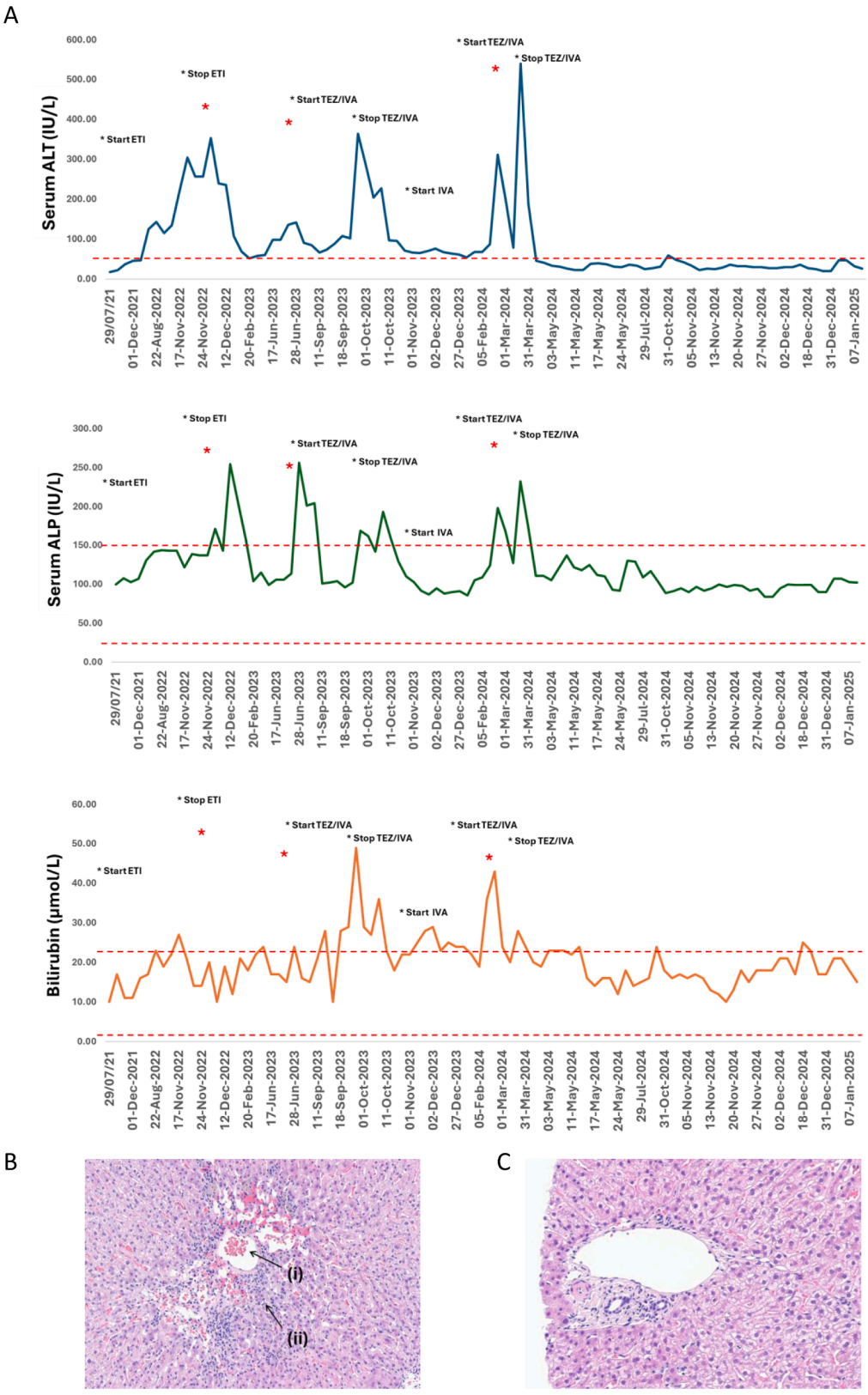


Fig. 1. Liver function tests (LFTs) and H&E Staining of liver tissue collected from a patient with ETI-drug-induced liver injury (DILI). A) LFTs were performed and monitored throughout treatment with CFTR modulators. Treatment start dates are highlighted (*) with treatment cessation required due to elevated LFTs. Serum total bilirubin levels (reference range: 2–21 µmol/L), serum alanine transaminase (ALT) levels (reference range: < 40 IU/L) and serum alkaline phosphatase (ALP) levels reference range: 30–150 IU/L) were assessed. Liver punch biopsies were collected at the timepoints highlighted by * in December 2022, July 2023 and March 2024. B) Central vein with evidence of perivenular zone three confluent necrosis with hepatocyte loss (i) and a mixed inflammatory infiltrate which is lymphocyte predominant (ii) with some plasma cells and scattered pigmented/debris laden macrophages present. C) A normal appearing portal tract containing portal vein, hepatic artery and bile duct with no significant increase in portal inflammation.

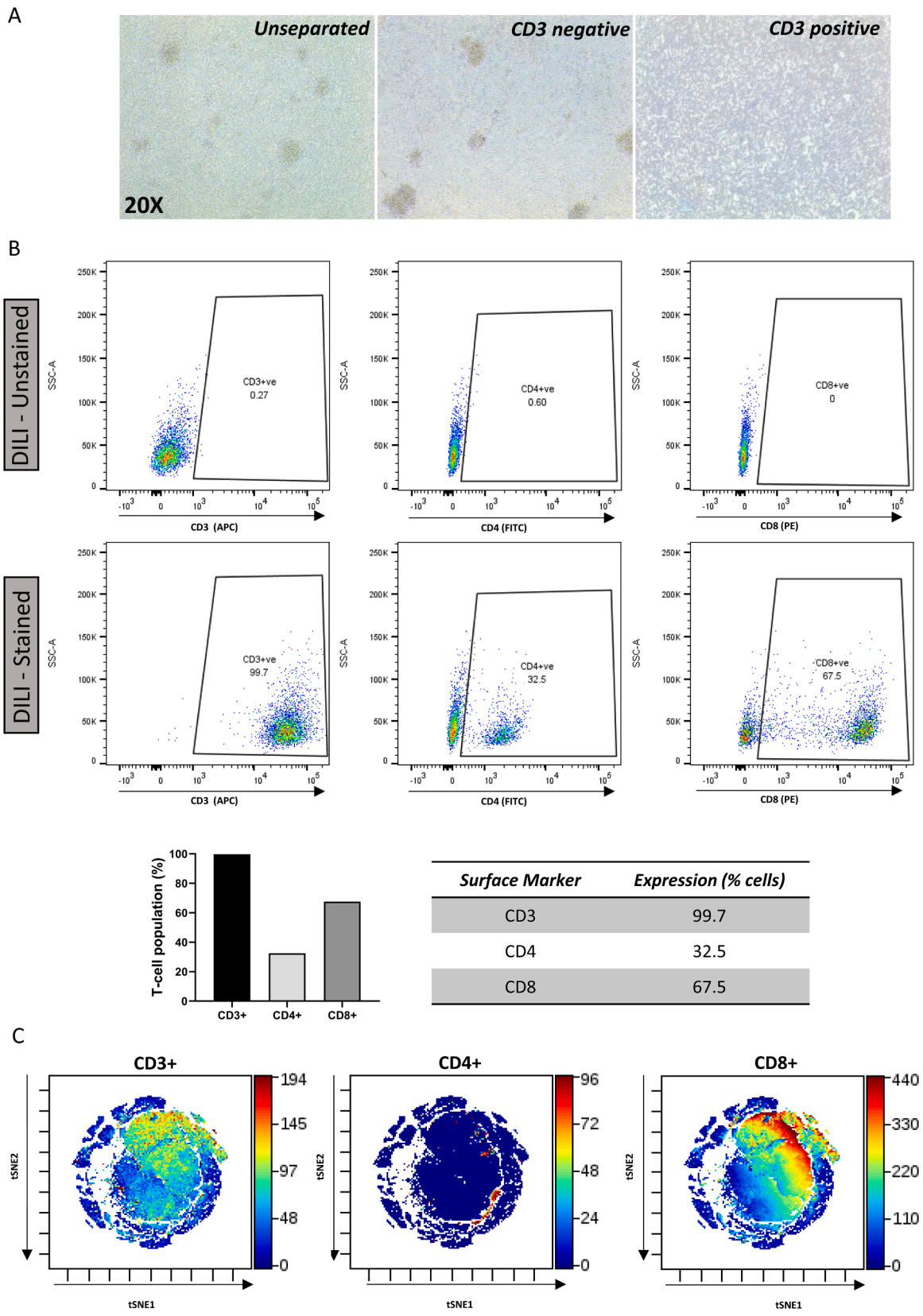
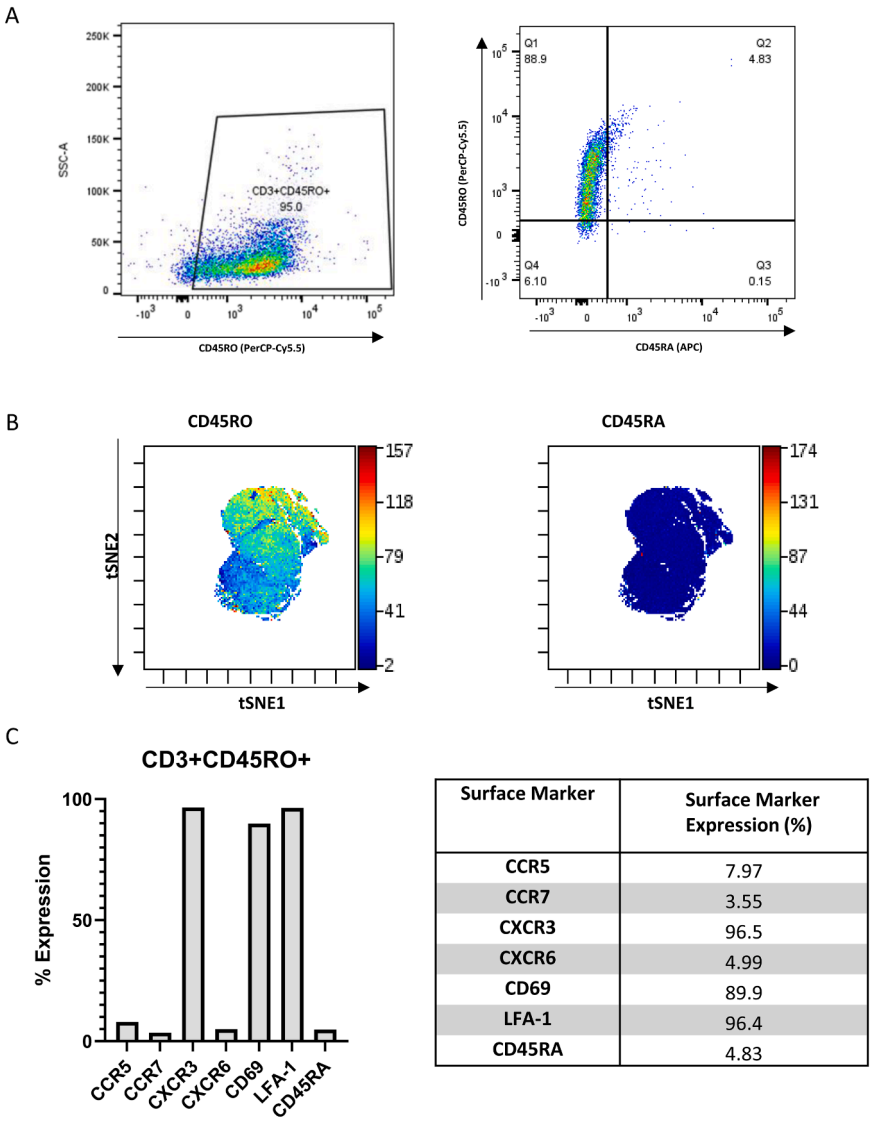


Fig. 2. Confirmation of T-cell isolation from liver biopsy obtained from ETI-DILI patient following CFTR modulator therapy. A) Light microscopy imaging of immune cells isolated from ETI-DILI patient punch biopsy seeded in a 48-well plate for the comparison of unseparated, CD3- and CD3+ cell populations at 20X magnification. B) Gating strategy for surface marker analysis of Liver infiltrating T-cells using an unstained sample. Liver infiltrating T-cells were incubated with anti-CD3+/APC, anti-CD4+/FITC and anti-CD8+/PE fluorophore conjugated antibodies before flow cytometry analysis to compare the expression in liver infiltrating T-cells isolated from the DILI patient biopsy. C) Mass cytometry was used to visualise the tSNE maps showing the T-cell subsets, CD3+, CD4+ and CD8+ isolated from the DILI liver biopsy.



Surface Marker	Surface Marker Expression (%)
CCR5	7.97
CCR7	3.55
CXCR3	96.5
CXCR6	4.99
CD69	89.9
LFA-1	96.4
CD45RA	4.83

Fig. 3. Characterisation of memory T-cells isolated from a liver punch biopsy collected from a CF patient with CFTR modulator-induced liver injury. A) T-cells isolated from the Kaftrio-DILI liver biopsy were incubated with anti-CD3+/PE, anti-CD45RO+/PerCP-Cy5.5 and anti-CD45RA+/APC fluorophore conjugated antibodies and flow cytometry was used to identify the memory T-cells (CD3+CD45RO+) and compare the expression of memory markers (CD45RO+) and naïve markers (CD45RA). B) Mass cytometry analysis was performed to visualise the tSNE maps exploring memory subsets, (CD45RO+) and naïve subsets (CD45RA+) of T-cells isolated from the liver biopsy. C) Flow cytometric analysis was used to determine the percentage expression of surface markers on memory liver infiltrating T-cells (CD3+CD45RO+).

3.5. CFTR modulator-specific T-cells are localised to sites of liver injury and not present at high frequencies within peripheral blood

Liver-infiltrating T-cells isolated from the ETI-DILI biopsy were co-cultured with drug and autologous irradiated EBV-transformed B-cells to determine drug-specific proliferation (Fig. 4). Significant proliferative responses were detected to Ivacaftor ($P = 0.009$), Ivacaftor M1 ($P = 0.0002$) and M6 ($P = 0.045$) metabolites (Fig. 4A). No T-cell responses were observed to the other CFTR modulator compounds (TEZ/ELEX) or the fixed CFTR modulator therapies, TEZ/IVA and ETI. Cytokine analysis (IFNγ ELISpot) of CFTR modulator stimulated CD3+ *T*-cells was associated with IFNγ release after co-incubation with Ivacaftor (Maximal SFU = 320), Ivacaftor M1 (Maximal SFU = 417) and Ivacaftor M6 (Maximal SFU = 212) at multiple graded drug concentrations (Fig. 4B). The strongest T-cell response was observed to the Ivacaftor M1 metabolite in both proliferation and IFNγ secretion assays, followed by Ivacaftor and Ivacaftor M6 (Fig. 5A and B). This was also observed in ICS analysis, with intracellular release of IFNγ, TNF-α and granzyme B detected after drug stimulation (Supplementary Fig. 4).

In vitro diagnostic T-cell assays (LTT) were performed using immune cells isolated from PBMC collected from the ETI-DILI CF patient (Fig. 4C and D). No T-cell proliferation was detected with LTT assays after 5-day exposure to pure CFTR modulator compounds and metabolites (Ivacaftor, Ivacaftor M1, Ivacaftor M6, Tezacaftor, Tezacaftor M1, Tezacaftor M2 and Elexacaftor) and fixed CFTR modulator therapies, such as ETI and TEZ/IVA (Fig. 4C). The positive control, PHA-L, successfully induced T-cell proliferation (SI > 40), confirming assay viability. Proliferative T-cell responses were detected to Ivacaftor M1 (average SI > 6), although this was limited to single experimental replicates only (Supplementary Fig. 3). Cytokine release assays (IFNγ ELISpot) also yielded negative results following stimulation with pure CFTR modulator compounds and fixed CFTR modulator therapy (Fig. 4D). *In vitro* diagnostic T-cell assays were conducted on patients identified as ETI tolerant and piperacillin sensitised to confirm that the assays were sensitive to detect drug-specific T-cell responses. (Supplementary Fig. 2). In each CF patient ($n = 3$), high levels of piperacillin-specific T-

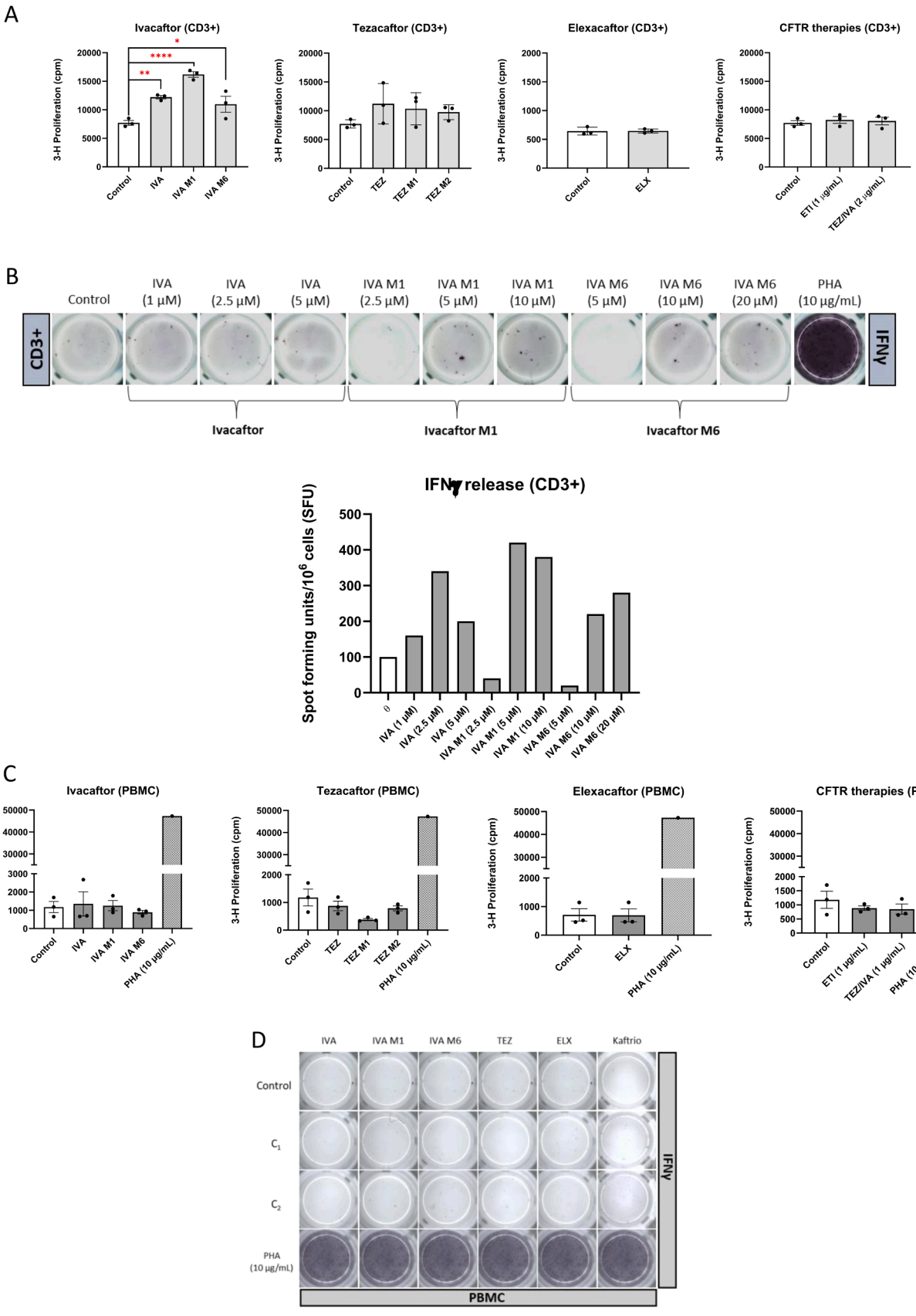


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Fig. 4. *In vitro* T-cell assays for the detection of CFTR modulator-specific T-cell responses. A) *In vitro* antigen specificity testing using CD3+ *T*-cells isolated from punch biopsy. Isolated liver infiltrating T-cells were co-cultured with irradiated autologous EBV-transformed B-cells and pure CFTR modulator compounds and metabolites (Ivacaftor, 2.5 μM; Ivacaftor M1, 5 μM; Ivacaftor M6, 10 μM; Tezacaftor, 10 μM; Tezacaftor M1, 10 μM; Tezacaftor M2, 50 μM; Elexacaftor, 10 μM; ETI, 1 μg/mL; Symkevi, 2 μg/mL). Statistical significance was determined using a student's *t*-test (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$). B) Cytokine release (IFNγ) was assessed by ELISpot assay after 48 h incubation with Ivacaftor and metabolites. Spot forming units of activated CD3+ liver-infiltrating cells were analysed per 10^6 cells. C) Proliferation based LTT (PBMC) assessing T-cell responses to pure CFTR modulator compounds (Ivacaftor, 2.5 μM; Ivacaftor M1, 5 μM; Ivacaftor M6, 10 μM; Tezacaftor, 10 μM; Tezacaftor M1, 10 μM; Tezacaftor M2, 50 μM; Elexacaftor, 10 μM) and CFTR modulator therapies (ETI, 1 μg/mL; Symkevi, 1 μg/mL). D) Cytokine release (IFNγ) assessed by ELISpot assay after 48 h incubation with pure CFTR modulator compounds (Ivacaftor, 2.5–5 μM; Ivacaftor M1, 2.5–5 μM; Ivacaftor M6, 10–20 μM; Tezacaftor, 10–20 μM; Elexacaftor, 5–10 μM) and CFTR modulator therapies (Kaftrio, 1–2 μg/mL).

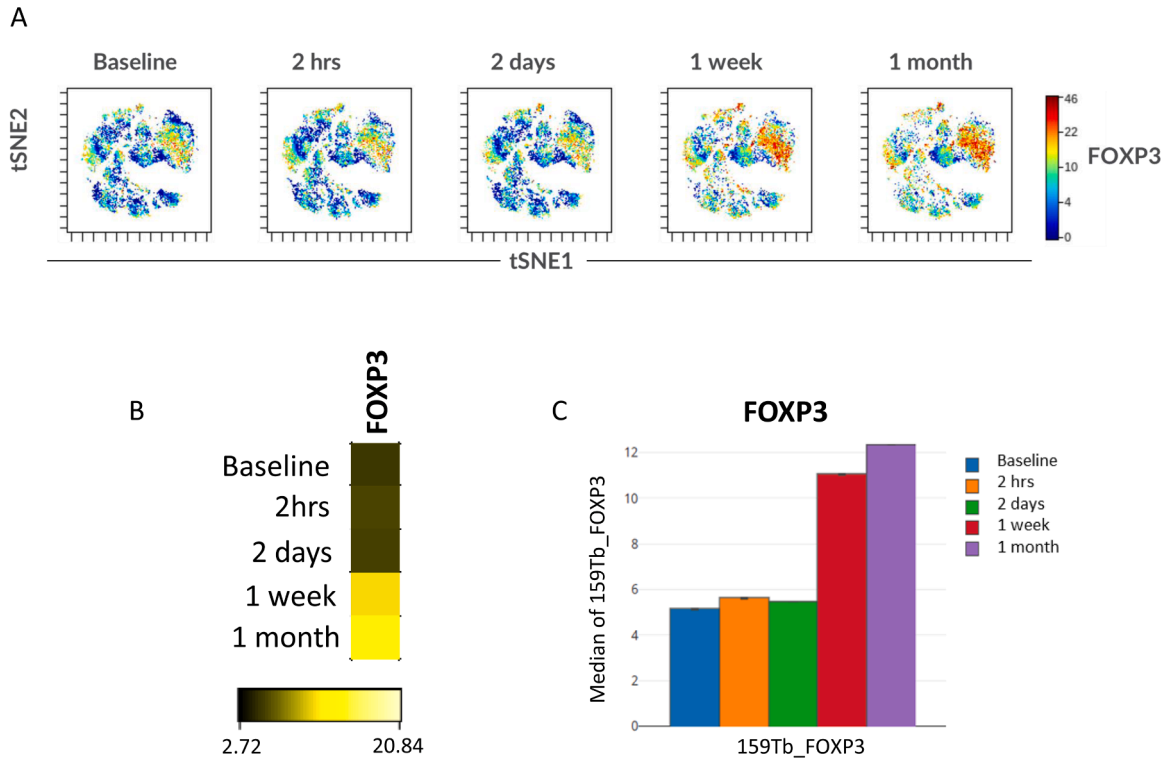

Fig. 5. Mass cytometry analysis of regulatory T-cell populations from live PBMCs following Ivacaftor reintroduction. Mass cytometry (Helios; CyTOF™) was used to investigate FOXP3 expression over the course of Ivacaftor reintroduction (baseline, 2 hrs, 2 days, 1 week and 1 month) to map changes in Treg (FOXP3+) frequency. Data was visualised as tSNE, heatmap and graphs (A, B and C respectively) using the median intensity of the 159Tb_FoxP3 channel.

cell proliferation were detected.

3.6. Expansion of regulatory T-cell populations over a time course of Ivacaftor reintroduction

Mass cytometry was used to investigate the frequency of Tregs (FOXP3+) in PBMC from the ETI-DILI CF patient at multiple time points pre- and post- an Ivacaftor reintroduction regime (Fig. 5). PBMCs from whole venous blood were collected over the course of drug reintroduction (baseline, 2 hrs, 2 days, 1 week and 1 month) before single-cell analysis of immune populations. The frequency of regulatory T-cells (FOXP3+) from live PBMC populations increased >2-fold at timepoints of 1 week and 1 month post drug reintroduction (Fig. 5C).

4. Discussion

CFTR modulators have transformed the quality of life and life expectancy for many patients with CF [13,14] however, adverse reactions to CFTR modulator therapy can result in drug withdrawal. The functional role of T cells in the pathogenesis of delayed-type skin and liver injury has been previously elucidated for a range of drug classes, including β-lactam antibiotics, sulfonamides, glycopeptides, and anticonvulsants [15–19].

Pharmacovigilance data assessing drug safety profiles from both clinical trials and the post-marketing phase have indicated that ETI combination therapy was disproportionately associated with hepatotoxicity [20], with skin and liver injury after ETI treatment accounting for >80 % of all reported immune related adverse events [5]. Here, we describe a case of ETI-DILI for a patient homozygous F508del CFTR mutation. Liver-infiltrating T-cells were isolated and *in vitro* T-cell assays, combined with phenotypic analysis were performed to define and characterise immunological involvement and identify culprit CFTR modulator compounds contributing to DILI diagnosis.

The initial clinical diagnosis of DILI was made following abnormal LFTs and histological examination of a percutaneous liver punch biopsy (Fig. 1). Serum ALTs were found to be significantly elevated and greater than 5-fold above the upper limit of normal (Fig. 1A). The treatment regimen was altered in favour of TEZ/IVA administration, on exposure to dual therapy, the drug was tolerated by the patient for a period of 2 months prior to the resurgence of elevated LFTs. Upon further histological examination after abnormal LFTs in response to TEZ/IVA treatment, the biopsy displayed no evidence of significant ongoing liver injury. Idiosyncratic DILI can be classified as hepatocellular in nature provided ALT levels are 3-fold the upper limit of normal, although some variability can be observed dependent on the therapeutic class in question [21]. Histological examination of the percutaneous liver punch

biopsy collected after the adverse reaction to ETI (Fig. 1B) revealed features consistent with hepatocellular-DILI, specifically the lymphocytic nature of perivenular inflammation and the lack of cholestatic injury.

We compared the relative proportion of CD4+ and CD8+ *T*-cells from cellular populations isolated from the ETI-DILI patient [22]. T-cells were associated with a dominant CD8+ cellular phenotype (Fig. 2), which corresponds with current understanding regarding the pathomechanisms of DILI [23]. Further studies were conducted to confirm a CD8+ memory cellular phenotype and measure chemokine receptor expression of isolated CD3+ *T*-cell populations (Fig. 3). Our finding of an almost exclusive CD45RO+ memory T-cell population (95 %) is consistent with the infiltration of drug-exposed lymphocyte infiltration associated with immunological memory (Fig. 3A and B). As expected, the isolated lymphocyte population possessed a degree of heterogenicity, with a small population of naïve T-cells (CD45RA+) identified, in addition to clusters of CD4+ and CD8+ expressing T-cells (Fig. 2D). CD45RO+ memory T-cells are typically enriched in hypersensitive patients and comprise effector, central and tissue-resident memory subsets. These cell populations are associated with chronic or repeated drug exposure, applicable in the case of CFTR modulator therapy, with drug-primed memory T-cells trafficked to the affected tissues after sequential drug exposure [24].

CD3+CD45RO+ memory T-cells were found to express high levels of CXCR3, CD69 and LFA-1 (Fig. 3). The chemokine receptor, CXCR3, is known be upregulated on activated T-cells and controls the localisation of cells to liver tissues, after constitutive expression of the CXCL16 ligand expressed on liver sinusoidal endothelial cells [25]. Furthermore, elevated expression of the adhesion molecule, LFA-1, on the surface of CD3+CD45RO+ memory T-cells demonstrates involvement within cellular trafficking and migratory processes. LFA-1 is an integrin that mediates cell-to-cell and cell-to-extracellular matrix adhesion and is known to play a crucial role in T-cell migration and the formation of immunological synapses [26]. This finding, combined with increased surface expression of CD69, appears to align with pathomechanisms of immune-mediated DILI, during which drug-activated T-cells migrate towards sites of inflammation, infiltrate damaged tissue and induce hepatotoxicity.

T-cells isolated from the ETI-DILI biopsy proliferated and secreted Th1, Th2 cytokines and cytolytic effector molecules in response to the T-cell mitogen, PHA-L (Supplementary Fig. 4A and B). These data were supported using flow cytometry and functionality was further evidenced by the production of IFNγ, granzyme B and TNF-α from memory CD45RO+ populations in response to PMAI. This demonstrates the cytotoxic potential of the isolated T-cell population, associated with the production of pro-inflammatory (IFNγ) and cytolytic (granzyme B, Perforin) cytokines linked to tissue damage and inflammation [27,28].

Liver infiltrating T-cells isolated from the ETI-DILI patient showed significant proliferative responses to Ivacaftor and its two major metabolites (Fig. 4A), supporting the theory that drug responsive T-cells reside at the site of the reaction. The strongest T-cell response was observed to the Ivacaftor M1 metabolite in both proliferation and IFNγ secretion assays, followed by Ivacaftor and Ivacaftor M6 (Fig. 4A and B), further supported by ICS analysis (Supplementary Fig. 4). T-cell responses to metabolites of Ivacaftor are perhaps unsurprising as Ivacaftor is extensively metabolised by CYP3A4 [29]. Responses were not detected to any of the other CFTR modulators, including TEZ/IVA and ETI. At ETI concentrations of 1 μg/mL, the Ivacaftor concentration is 0.38 μM and likely too low to elicit a significant T-cell response. However, due to drug toxicity it was not possible to increase ETI concentrations to achieve comparable Ivacaftor dosage *in vitro*. LTT and ELISpot assays were conducted using ETI-DILI patient PBMC however, no significant responses to any of the CFTR modulators were observed (Fig. 4C and D), making it challenging to draw conclusions from whole PBMC cultures. With the exception of Lumacaftor [6,7], LTT assays conducted to investigate CFTR modulator hypersensitivity are frequently inconclusive and yield negative results in many patients with clinically defined non-immediate allergies to modulator therapy. We hypothesise that this may be a result of a low precursor frequency of circulating CFTR modulator-specific T-cells. This is further supported by data showing strong T-cell responses to CFTR modulators or metabolites limited to a single experimental replicate (Supplementary Fig. 3).

There are likely a range of factors contributing to the development of CFTR modulator hypersensitivity reactions including circulating plasma levels, metabolising status and potential HLA associations. The ETI-DILI patient has been desensitised to ETI and is tolerating 1/2 dose therapy. Due to the importance of CFTR modulators in the treatment of CF, patients are commonly reintroduced to the therapies after a hypersensitivity reaction. It has been reported that following cases of both skin and liver reactions patients are able to tolerate the drugs following a course of desensitisation [30–32]. Interestingly, spontaneous resolution of hypersensitivity has been seen in some patients, where no cessation of treatment is required [5,33]. In the absence of CFTR modulator therapy, patients with CF experience heightened systemic inflammation and impaired regulatory T-cell function, increasing the risk of drug hypersensitivity. However, upon CFTR modulator initiation, Treg function is restored [34], potentially explaining the spontaneous tolerance observed. This phenomenon was demonstrated in this study, following assessment of regulatory T-cell function over a drug reintroduction time-course which showed increased frequency and expansion of Treg populations 1 week post drug reintroduction (Fig. 5).

5. Conclusion

Our findings demonstrate that CFTR modulator responsive T-cells are localised to the liver after resolution of DILI. Although assays performed on the liver infiltrating T-cells identified Ivacaftor and associated metabolites as the likely causative compounds in this hypersensitivity reaction, conventional *in vitro* diagnostic assays were inconclusive. As such, we hypothesise that there is a low frequency of circulating drug responsive T-cells in the blood of patients with ETI-induced DILI. This aligns with our understanding of DILI, confirming that T-cells involved in the reaction migrate to the site of inflammation, inducing hepatotoxicity and tissue damage. This work could be used to inform treatment regimens for patients unable to tolerate CFTR modulators. In future, it will be important to investigate the involvement of systemic inflammation and immune regulation in the tolerance of these compounds following occurrences of hypersensitivity with the aim of developing effective and reproducible desensitisation protocols.

Data availability

Datasets related to this article are available upon request from the corresponding author.

Author contributions

JG, EC and AH conducted the biological experiments. JG, EC, DP, JFR and DJN contributed to conceptualization and experimental design. LG, PW, PT and AC collected patient samples and contributed to the project design. JG and EC contributed equally to the experimental work, data analysis and manuscript drafting. All authors reviewed the manuscript.

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Declaration of competing interest

DP holds a speaker honoraria from Vertex Pharmaceuticals. JFR has

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2025.11.011.

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