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Full Length Article

Tissue distribution of γδ T cell subsets in oesophageal adenocarcinoma

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ABSTRACT

The global obesity epidemic is contributing to increased prevalence of diseases fuelled by chronic inflammation, including cancer. Oesophageal adenocarcinoma (OAC) is an obesity-associated malignancy with increasing prevalence, dismal prognosis, and severely dysregulated immune processes. We previously reported that αβ T cells migrate to omentum and liver in OAC and contribute to inflammation in these tissues. Here, we assessed the tissue distribution and phenotype of gamma/delta (γδ) T cells in the blood, omentum, liver and tumour of OAC patients.

Our data show that the Vδ1 and Vδ3 subsets of γδ T cells are most prevalent in omentum and liver of OAC patients. Furthermore, γδ T cells are predominantly pro-inflammatory in these tissues, and co-express IFN-γ and IL-17. Moreover, γδ T cells exhibit cytotoxic capabilities in OAC omentum and liver.

This study provides the first indication that γδ T cells contribute to obesity-associated inflammation in OAC and might be exploited therapeutically.

1. Introduction

Global obesity rates have reached epidemic proportions and the associated health burden is a major concern in the Western world, affecting 41 million children and 1.9 billion adults [www.who.int]. One of many adverse consequences of obesity is an increased risk of certain cancers, with up to 20% of cancer deaths being attributable to obesity [1,2].

Adenocarcinoma of the oesophagus (OAC), a cancer increasing in incidence several fold in parallel with the increased incidence of obesity in western society, is an aggressive obesity-associated cancer with a dismal 5-year survival rate of only ~19% and a treatment response rate of <30% [3–5]. For OAC, two factors are considered important in fuelling cancer risk in obesity, first a strong association with gastro-oesophageal reflux disease (GORD), and the second from myriad effects of the adipose tissue itself, in particular visceral adipose tissue (VAT) [6–10]. The omentum forms the largest component of the VAT compartment and in an obese setting both omentum and liver are enriched with inflammatory macrophages and T cells which mediate chronic and pathological inflammation [10–12]. In OAC, we have previously established that conventional T cells are key players in pathological omental and hepatic inflammation and we hypothesise that their recruitment to these tissues occurs at the expense of their infiltration of tumour [10,12,13]. At a time when the importance of immune contexture for effective anti-tumour immunity has emerged and the abundance of lymphocytes in tumours has been linked with a favourable prognosis, immunotherapy has become the fourth pillar of cancer treatment [14,15]. We propose that preferential migration of anti-tumour immune

Abbreviations: Gamma/delta (γδ), T cells; V delta-1, −2, −3, (Vδ1, Vδ2, Vδ3); oesophageal adenocarcinoma, (OAC); chemokine, (C—C motif); ligand 20, (CCL20); chemokine receptor 6, (CCR6); chemoradiotherapy, (CRT); T cell receptor, (TCR); treatment, (tx); visceral adipose tissue, (VAT); visceral fat area, (VFA); stromal vascular fraction, (SVF); peripheral blood mononuclear cells, (PBMC).

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Table 1
Demographic information of OAC patient cohort undergoing surgical resection.

$N = 36$	
Age (range years)	65.75 (43–84)
Sex ratio (M:F)	26:6
Tumour stage	
T0	3
T1	4
T2	4
T3	17
T4	4
Nodal status[a]	
N0	13
N1	10
N2	4
N3	4
Mean BMI (kg/m^2)	27.39
Underweight (BMI < 20)	2
Normal weight (BMI 20–24.9)	4
Overweight (BMI 25–29.9)	17
Obese (BMI >30)	9
Mean waist circumference (cm) (range)	97.23 (82–115)
Mean visceral fat area (VFA) (cm^2) (range)	179.75 (52.25–369.26)
Received neoadjuvant treatment	81.25%

[a] Nodal status was not available for one patient.

cells to the omentum and liver is a unique challenge for obesity-associated cancer because it both fuels tumour-promoting inflammation and compromises immune infiltration of tumour [12,13,16]. A detailed mapping of immune cell phenotypes may uncover key pathways in obesity-associated carcinogenesis, as well as potential targets in cancer therapy. In this context, gamma delta (γδ) T cells, a potent anti-tumour T cell subset and the focus of several immunotherapy clinical trials, may be of relevance [17,18].

γδ T cells are a heterogeneous type of innate-like or unconventional T cell, typically constituting 1–5% of circulating T lymphocytes and are characterized by expression of the γδ T cell receptor (TCR) rather than the αβ TCR expressed by conventional T cells [19–22]. γδ T cells can elicit potent cytolytic and cytokine-driven activities and studies report their heterogenous abundance in oesophageal tumours [23]. γδ T cells have been implicated in inflammation and thermogenesis in the adipose tissue of obese mice [24–26]. Importantly, γδ T cell abundance in tumours, including OAC, has a strong favourable association with patient survival [15]. Their potent anti-tumour potential has led to the development of several γδ T cell-based immunotherapies, yet clinical trials to date have shown mixed success [17,18]. Therefore, improving our understanding of the anatomical positioning and inflammatory profiles of γδ T cells in cancer may harness the most potent and cytotoxic subsets of these cells and improve current immunotherapeutic approaches. A greater understanding of anti-tumour effector cells is particularly important in the challenging setting of obesity-associated cancer, in which tissue compartments outside of the tumour may recruit these potent tumour killers, as we have described for other immune cells in OAC [13,16,27].

In the setting of obesity-associated cancer, γδ T cells have been identified as contributors to tumourigenic inflammation in colorectal cancer but the role of these unconventional T cells has not yet been described in the pathological inflammation underlying OAC [28]. Here for the first time, we have utilised OAC patient tissue as a clinically relevant ex vivo model of obesity and inflammation-driven cancer to characterise γδ T cell frequencies and function in the blood, omentum, liver and tumour.

2. Materials & methods

2.1. Ethical approval

This study was carried out in accordance with the Declaration of Helsinki ethical guidelines for biomedical research involving human subjects. Ethical approval was granted from the St James's Hospital Research Ethics Committee (SJH). All specimens and data were collected with prior informed consent, from patients attending St James's Hospital. Patient samples and data were pseudonymised by biobank managers (EKF and AB) to protect the privacy rights of the patients.

2.2. Patient specimens

Thirty-two patients with confirmed OAC undergoing surgical resection at the National Oesophageal and Gastric Centre, St James's Hospital Dublin were consented and enrolled in this study between 2014 and 2019. The patient group included 26 males and 6 females, representative of the male predominance in OAC, with an average age of 65.75 years [Table 1]. The mean BMI at time of surgery was 27.39 kg/m^2, and visceral fat area (VFA) was 179.75 cm^2. The patient cohort was similar in ethnic background. Neo-adjuvant CRT was administered to 81.25% of patients. Blood, omentum, liver and tumour specimens were obtained from consenting patients at time of surgical resection and after neo-adjuvant treatment. A further 39 whole blood and 12 tumour biopsies were collected from OAC patients prior to neoadjuvant CRT for comparison to this post-treatment cohort.

2.3. Sample preparation

Peripheral blood was collected in EDTA tubes (BD). Omental adipose tissue samples (5 g) were enzymatically digested with collagenase type II (Sigma) to obtain the stromal vascular fraction (SVF) as previously described [12,29]. Liver samples (<0.1 g from each patient) and tumour biopsies were digested with collagenase type IV (Sigma) as previously described [12,29,30]. Adipose tissue conditioned media (ACM) and liver conditioned media (LCM) were prepared as previously described [10,16]. Tumour tissue conditioned media (TCM) was prepared by culturing a tumour biopsy in 1 ml M199 media supplemented with 1% gentamicin at 37 °C, 5% CO_2 for 24 h.

2.4. Cell labelling and flow cytometry

Peripheral blood, SVF, intrahepatic immune cells and intratumoural immune cells were stained with fluorochrome-conjugated monoclonal antibodies (mAb) specific for human PerCP-labelled CD3, Pe-Cy7-labelled IL-10 (BioLegend), PE-labelled Vδ1, FITC-labelled Vδ2, APC-labelled γδ-TCR (Miltenyi Biotec), APC-Cy7-labelled CD45 (BD Bioscience), V500-labelled IFN-γ (BD), FITC-labelled IL-17, (eBiosciences), PE-labelled CD107a (BD Pharmingen), BV510-labelled CCR6 (BD Horizon) and APC-labelled Vδ3 (Beckman Coulter). Red cells were lysed with BD lysis buffer (BD Bioscience), as per manufacturer's recommendations.

2.5. Measurement of intracellular cytokine production and CD107a

For intracellular cytokine staining, peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Lymphoprep (Stemcell Technologies). PBMC, SVF and intrahepatic immune cells were stimulated with 50 ng/ml of phorbal myristate acetate and 1 μg/ml of ionomycin (PMA/I, Sigma) for 1 h, followed by the addition of 1 μg/ml of monensin (BioLegend) for a further 3 h in the presence of CD107a antibody. Cells were stained with mAbs specific for human surface markers (CD3, γδ-TCR), then fixed with 4% paraformaldehyde (PFA, Sigma), permeabilized with 0.2% saponin and stained with mAbs specific for the cytokines IFN-γ, IL-10 and IL-17A. Cells were acquired using FACS Canto II flow cytometer (BD Bioscience) and analysed using FlowJo Version 10 (Tree Star) software.

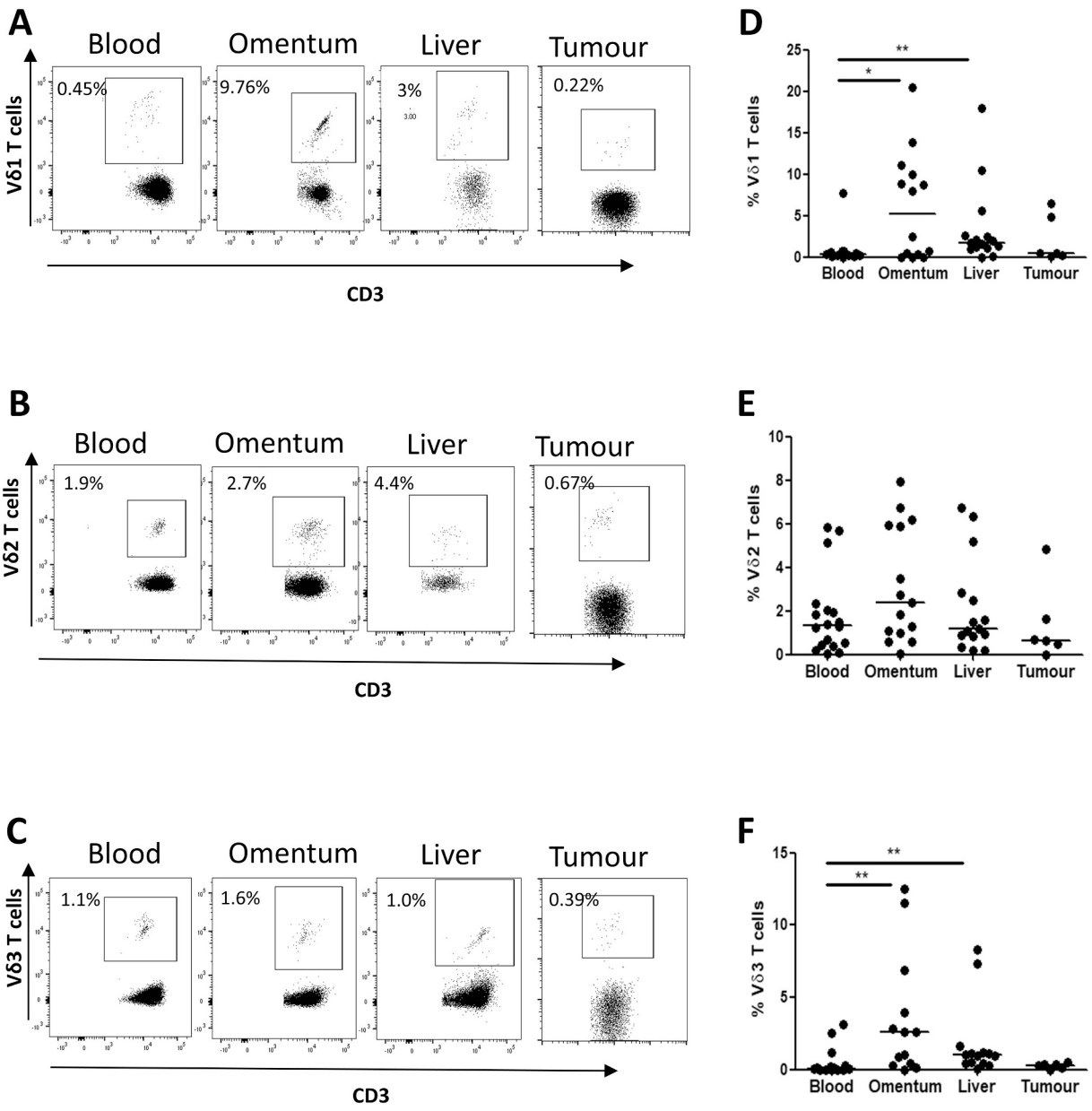

Fig. 1. Vδ1 and Vδ3 T cells are abundant in the omentum and liver of OAC patients. γδ T cell subset frequencies were analysed by flow cytometry in the whole blood, omentum, liver and tumour of a total of 26 patients. A, B, C: Representative flow cytometry dot plots of Vδ1, Vδ2 and Vδ3 T cells in blood, omentum, liver and tumour. D, E, F: Scatter plots show percentages of Vδ1, Vδ2 and Vδ3 T cells in blood, omentum, liver and tumour of a total of 26 OAC patients. *$p < 0.05$, **$p < 0.01$ using one-way ANOVA (Kruskal-Wallis test with Dunns post-test comparison).

2.6. Generation of γδ T cells from healthy donor-derived blood

γδ T cell lines were generated from peripheral blood mononuclear cells (PBMC) isolated from the fresh blood of individuals with hemochromatosis, obtained with consent from St James's Hospital Dublin. PBMC were isolated by density centrifugation over Lymphoprep (StemCell Technologies). PBMC were washed and resuspended in RPMI with Glutamax (Gibco) supplemented with 10% FBS and seeded at a concentration of 4×10^6 PBMC/ml in T75 flasks. Cells were maintained at 37 °C and 5% CO2 overnight. α/βTCR$^+$ T cells were depleted from the PBMC using a CliniMACS (R) TCR α/β-Biotin kit, as per the supplier's instructions (Miltenyi Biotec). The α/β T cell-depleted fraction was then resuspended at a concentration of 7.5×10^6 cells/ml in complete RPMI and cells were plated in a round-bottom 96 well plate. Cells were then stimulated with 1μg/ml anti-CD3 (Clone OKT3, BioLegend), 70 ng/ml of IL-15 (Miltenyi Biotec) and 50 U/ml IL-2. Cytokines were replenished in media every 3–4 days. Purity of the γδ T cell fraction was assessed using flow cytometry.

2.7. Assessing the effects of OAC patient-derived adipose, liver and tumour tissue conditioned medias on intracellular cytokine production and CD107a by Vδ1, Vδ2 and Vδ3 T cells

Expanded γδ T cells were treated with serum-free M199 or ACM, LCM or TCM diluted 1 in 2 with serum-free M199 for a total of 24 h at 37 °C, 5% CO_2. Following 20 h of treatment, all cells were stimulated with 50 ng/ml of PMA (Sigma) and 1 μg/ml of ionomycin (Sigma), followed one hour later by the addition of 1 μg/ml monensin (BioLegend) and PE-labelled CD107a antibody (BD Pharmingen), followed by a further 3 h incubation. Cells were subsequently stained with fluorochrome-conjugated mAbs specific for human APC-Cy7-labelled CD3, BV421-labelled IL-10, Pe-Cy5-labelled IL-17a, Pe-Cy7-labelled

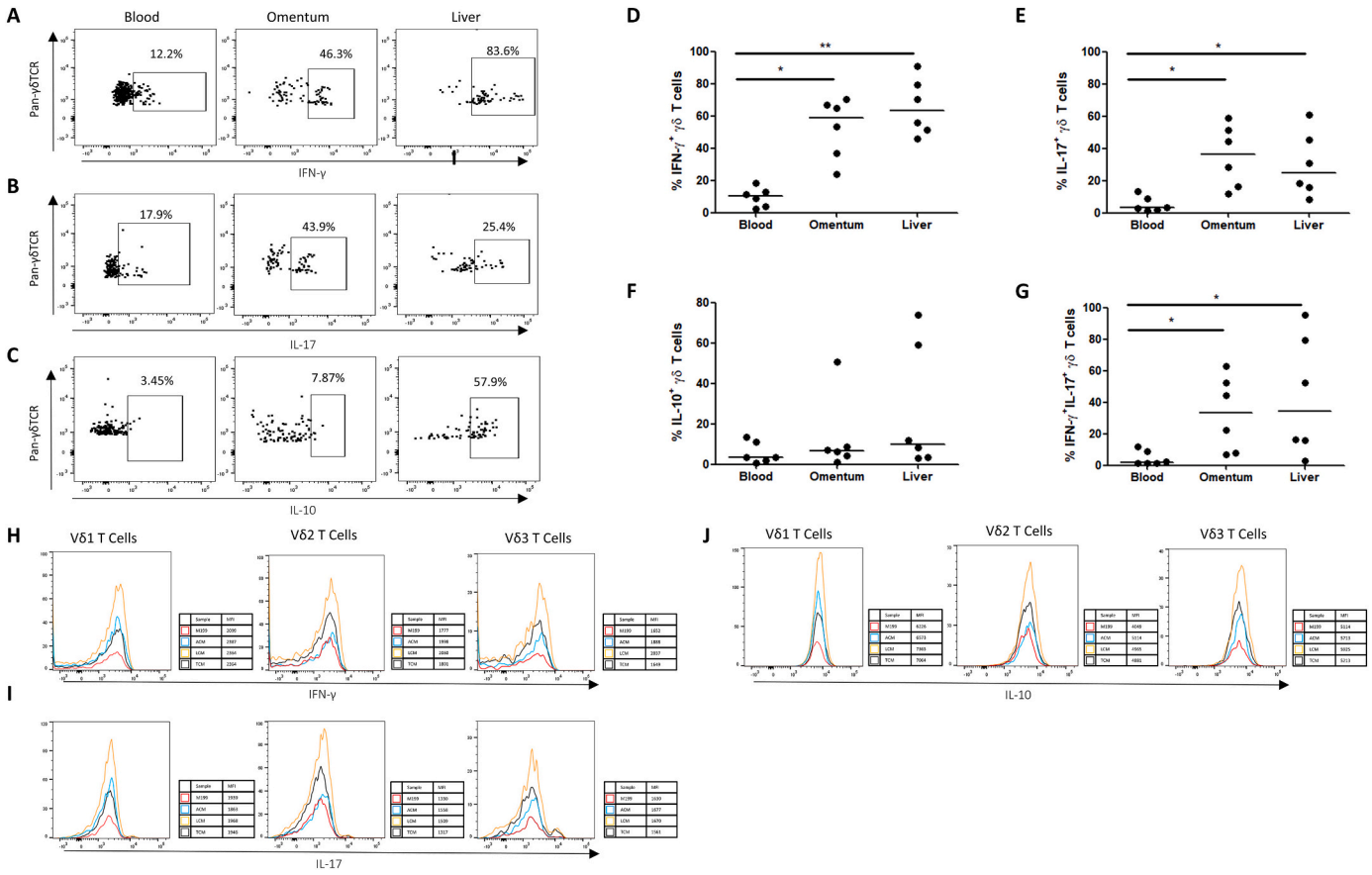

Fig. 2. γδ T cells in omentum and liver of OAC patients express significantly higher levels of IFN-γ and IL-17, compared to their circulating counterparts. PBMC, SVF and intrahepatic immune cells were stimulated with PMA and ionomycin for 4 h in the presence of monensin and IFN-γ, IL-17, and IL-10 expression by γδ T cells was subsequently assessed by flow cytometry. A, B, C: Representative flow cytometry dot plots of IFN-γ^+, IL-17^+, and IL-10^+ γδ T cells in blood, omentum and liver. D, E, F, G: Scatter plots show percentages of IFN-γ^+, IL-17^+, IL-10^+ and IFN-γ^+IL-17^+ γδ T cells in blood, omentum and liver of 6 OAC patients. *$p < 0.05$, **$p < 0.01$ using one-way ANOVA (Kruskal-Wallis test with Dunns post-test comparison). H, I, J: Representative histograms showing IFN-γ (H), IL-17 (I), and IL-10 (J) production by healthy donor blood-derived Vδ1, Vδ2 and Vδ3 T cell lines following exposure to M199 control media (M199, red), OAC patient-derived omental adipose tissue conditioned media (ACM, blue), liver tissue conditioned media (LCM, yellow) or tumour tissue conditioned media (TCM, black).

IFN-γ (BioLegend), FITC-labelled Vδ2, VioGreen-γδ-TCR (Miltenyi Biotec) and APC-labelled Vδ3 (Beckman Coulter). Intracellular staining was performed using FIX&PERM Cell Fixation and Permeabilization Kit (Nordic MUBio). Cells were acquired using the CANTO II (BD Biosciences) flow cytometer and analysed using FlowJo software (Tree Star).

2.8. Assessing the effects of OAC patient-derived adipose, liver and tumour tissue conditioned medias on γδ T cell cytotoxicity

OE33 cells were grown to confluence in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C, 5% CO_2. Expanded γδ T cells were treated with serum-free M199 media or ACM, LCM or TCM diluted 1 in 2 with serum-free M199 for a total of 24 h at 37 °C, 5% CO_2. After 20 h of incubation, cells were either stimulated with 50 ng/ml of PMA (Sigma) and 1 μg/ml of ionomycin (Sigma) for the final four hours, or left unstimulated. OE33 cells were stained with CFSE (ImmunoChemistry Technologies) and seeded at a density of 200,000 cells/ml of RPMI supplemented with 10% FBS and 1% penicillin/streptomycin. Following treatments, γδ T cells were resuspended in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin and co-cultured with OE33 cells for four hours, at an effector:target ratio of 10:1. Cytotoxicity was quantified with the Cell-mediated Cytotoxicity Assay (ImmunoChemistry Technologies). SR-FLICA and 7-AAD were used to quantify apoptotic and dead cells, respectively. An apoptosis-positive control was included, where OE33 cells were incubated for four hours with 4 μg/mL camptothecin. For necrosis positive controls, an aliquot of target cells were incubated at 56 °C for 6 min. Cells were acquired using the CANTO II (BD Biosciences) flow cytometer and analysed using FlowJo software (Tree Star). The percentage of specific lysis was calculated as (% dead cells-spontaneous dead)/(100-spontaneous dead) [31].

2.9. Statistical analyses

Statistical analysis was carried out using Prism GraphPad Version 5.0. Differences between groups were assessed using one-way ANOVA where appropriate. Differences between obese and non-obese cohorts were assessed by unpaired *t*-test. Pearson correlations were performed to assess correlations between BMI and γδ T cell subset frequency and functionality. *p* values <0.05 were considered as significant.

3. Results

3.1. Abundance of Vδ1 and Vδ3 T cells in the omentum and liver of OAC patients

To ascertain the tissue distribution of the three main subsets of human γδ T cells (Vδ1, Vδ2 and Vδ3) in OAC patients, flow cytometry was used to quantify the frequencies of these cells in OAC whole blood, omentum, liver and tumour (Fig. 1). Doublet events were excluded after gating on lymphocytes and γδ T cell subsets were expressed as a

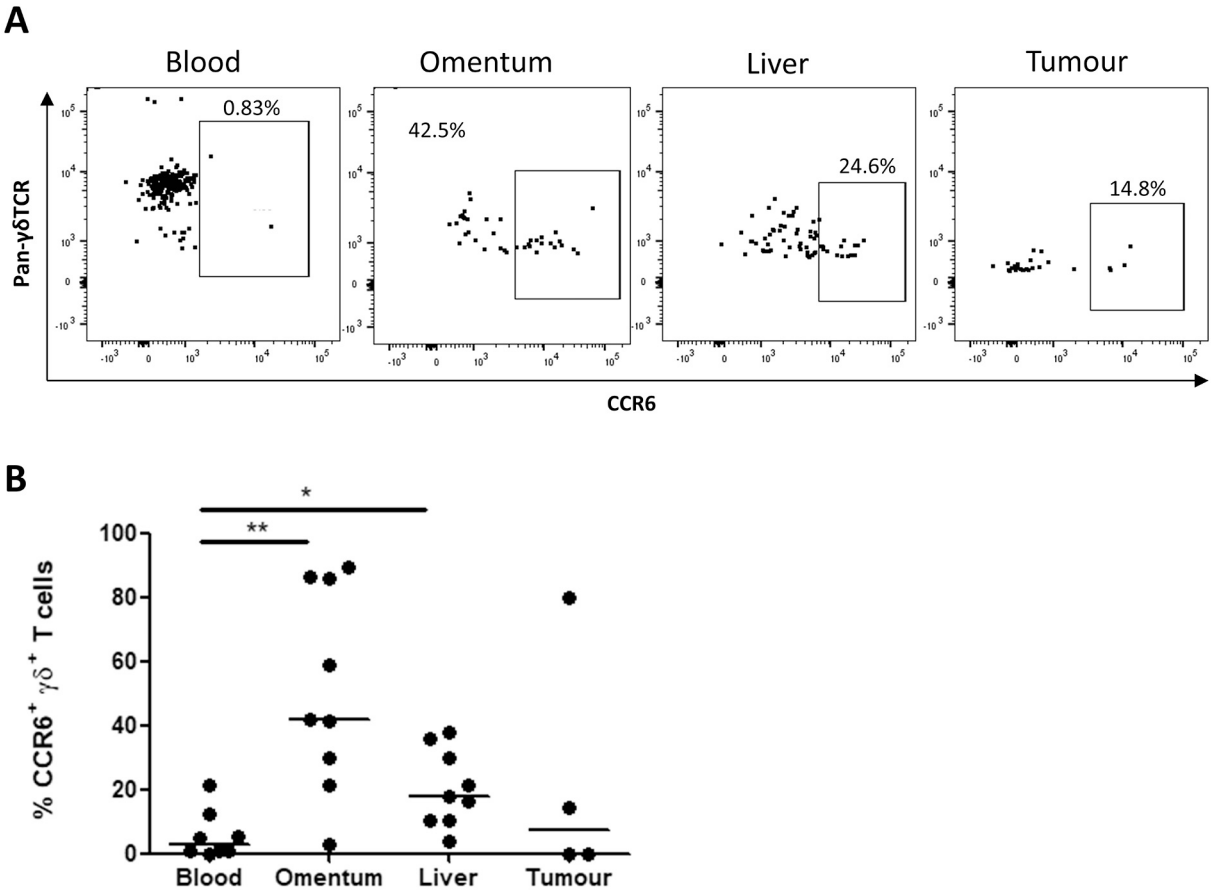

Fig. 3. CCR6 expression is significantly higher in γδ T cells in the omentum and liver of OAC patients, compared to blood.
CCR6 expression was analysed by flow cytometry on γδ T cells from whole blood, omentum, liver and tumour of a total of 9 OAC patients. A: Representative flow cytometry dot plots of CCR6$^+$ γδ T cells from whole blood, omentum, liver and tumour. B: Scatter plot shows percentages of CCR6$^+$ γδ T cells in OAC patient blood, omentum, liver and tumour. *$p < 0.05$, **$p < 0.01$ using one-way ANOVA (Kruskal-Wallis test with Dunns post-test comparison).

percentage of total CD3$^+$ cells. Our data revealed a relatively low prevalence of all three γδ T cell subsets in both the peripheral blood (Mean ± S.E. Vδ1$^+$: 0.76 ± 0.41%; Vδ2$^+$: 1.81 ± 0.43%; and Vδ3$^+$ T cells: 0.46 ± 0.22%, $n = 18$) and tumours of OAC patients (Vδ1$^+$: 2.1 ± 1.14%; Vδ2$^+$: 1.38 ± 0.72%; and Vδ3$^+$ T cells: 0.26 ± 0.07%, $n = 6$) (Fig. 1). In contrast, we observed significantly higher frequencies of Vδ1 and Vδ3 T cells in OAC omentum (Vδ1$^+$: 6.06 ± 1.72%, $p < 0.05$; and Vδ3$^+$ T cells: 3.5 ± 1.17%, $p < 0.01$, $n = 14$) and liver tissues (Vδ1$^+$: 3.4 ± 1.23%, $p < 0.01$; and Vδ3$^+$ T cells: 1.81 ± 0.69%, $p < 0.01$, $n = 15$), compared to blood (Fig. 1A, C). Interestingly, Vδ2$^+$ T cell frequencies were not significantly higher in omentum and liver, compared to blood or tumour (Blood: 1.81 ± 0.43% vs Omentum: 3.17 ± 0.68% vs Liver: 2.14 ± 0.22% vs Tumour: 1.38%, Fig. 1B).

3.2. Omental and hepatic γδ T cells in OAC patients are predominantly pro-inflammatory and co-express IFN-γ and IL-17

Previous work by our group has shown that effector T cells are key players in omental and hepatic inflammation in OAC [12]. We therefore sought to evaluate the inflammatory profile of γδ T cells derived from OAC omentum and liver. Upon stimulation with PMA/I, significantly higher frequencies of IFN-γ-expressing γδ T cells were observed in OAC patient omentum and liver, compared to blood (Blood: 9.7 ± 2.7% vs Omentum: 52.6 ± 8.22% vs Liver: 65.7% ± 13.1%, $p < 0.05$, $n = 6$, Fig. 2A, D). In addition, we detected significantly higher frequencies of IL-17-producing γδ T cells in OAC patient omentum and liver, compared to blood (Blood: 5.3 ± 3.4% vs Omentum: 35 ± 5.8% vs Liver: 29.9 ± 15.4% *$p < 0.05$, $n = 6$, Fig. 2B, E). Interestingly, analysis of γδ T cells co-expressing IFN-γ and IL-17 revealed that these pro-inflammatory subsets were also significantly enriched in OAC omentum and liver, compared to blood (Blood: 4.6 ± 1.3% vs Omentum: 32.9 ± 8.6% vs Liver: 43.7% ± 15.6, $p < 0.05$, $n = 6$, Fig. 2G). In contrast, frequencies of IL-10-producing γδ T cells were not significantly different between the compartments of blood, omentum and liver (Fig. 2F, n = 6). Interestingly, exposure of healthy donor blood-derived Vδ1, Vδ2 and Vδ3 T cell lines to the tissue conditioned media generated from OAC omentum, liver or tumour did not significantly alter the pro-inflammatory cytokine profile of these individual γδ T cell subsets (Fig. 2H, I, J).

3.3. High CCR6 expression by γδ T cells in the omentum and liver of OAC patients

CCR6$^+$ γδ T cells have been established as potent IL-17 producers and key players in inflammation-driven CRC [28]. To determine if the pro-inflammatory γδ T cells in OAC omentum and liver expressed this chemokine receptor, the frequencies of CCR6$^+$ γδ T cells in the blood, omentum, liver and tumour of a total of 9 OAC patients were quantified by flow cytometry (Fig. 3A). Our data revealed significantly higher frequencies of CCR6$^+$ γδ T cells in OAC omentum (50.8 ± 10.43% *$p < 0.01$) and liver (20.4 ± 3.93%, *$p < 0.05$), compared to blood (5.8 ± 2.63%, Fig. 3B).

3.4. γδ T cells in the omentum and liver of OAC patients have greater degranulation capacity compared to their circulating counterparts

As the potent cytolytic activities of γδ T cells are desirable for an

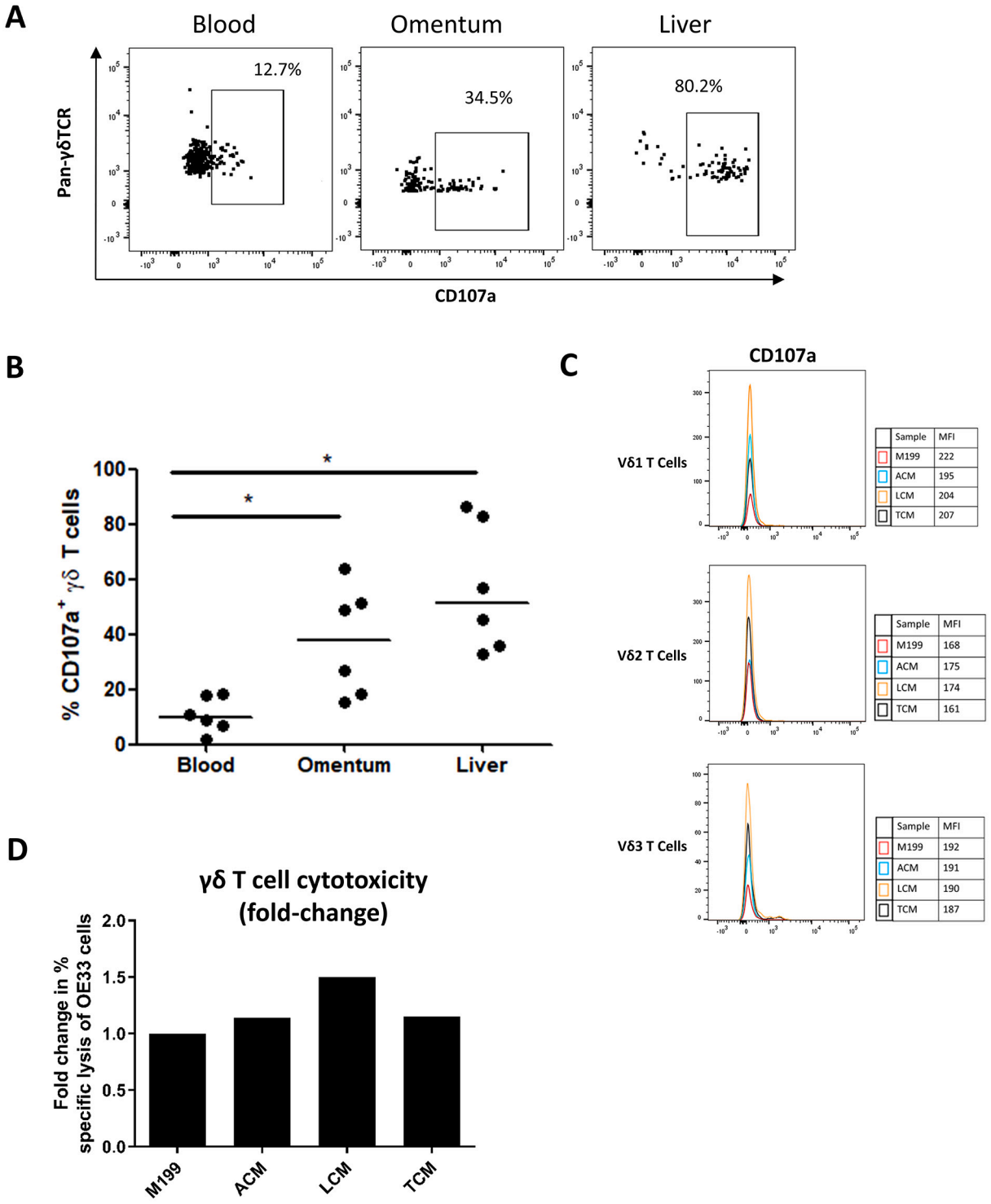

Fig. 4. γδ T cells in omentum and liver of OAC patients express significantly higher levels of degranulation marker CD107a.
PBMC, SVF and intrahepatic immune cells were stimulated with PMA and ionomycin for 4 h in the presence of monensin and CD107a expression by γδ T cells was subsequently assessed by flow cytometry. A: Representative flow cytometry dot plots of $CD107a^+$ γδ T cells in blood, omentum and liver. B: Scatter plot shows percentages of $CD107a^+$ γδ T cells in OAC patient blood, omentum and liver. *$p < 0.05$, **$p < 0.01$ using one-way ANOVA (Kruskal-Wallis test with Dunns post-test comparison). C: Representative histograms showing CD107a expression by healthy donor blood-derived Vδ1, Vδ2 and Vδ3 T cell lines following exposure to M199 control media (M199, red), OAC patient-derived omental adipose tissue conditioned media (ACM, blue), liver tissue conditioned media (LCM, yellow) or tumour tissue conditioned media (TCM, black). D: Bar chart shows fold change of the percentage of specific lysis of OE33 cells following incubation with γδ T cell lines previously exposed to OAC patient-derived ACM, LCM, or TCM, relative to M199 control media.

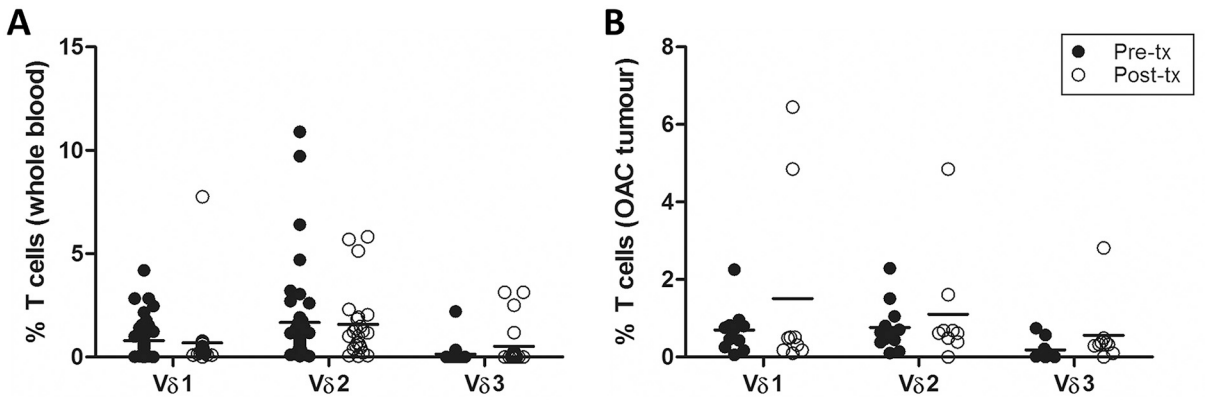

Fig. 5. γδ T cell subset frequencies are unaltered after chemoradiotherapy.
γδ T cell subset frequencies were analysed by flow cytometry in a cohort of OAC patients in whole blood and tumour at a time point prior to neoadjuvant chemoradiotherapy (CRT) and a time point following CRT. A: Scatter plot shows percentages of Vδ1, Vδ2 and Vδ3 T cells in blood of pre-CRT (pre-tx, black circle, $n = 39$) and post-CRT OAC patients (post-tx, white circle, $n = 30$). B: Scatter plot shows percentages of Vδ1, Vδ2 and Vδ3 T cells in tumour of pre-CRT (pre-tx, black circle, $n = 12$) and post-CRT OAC patients (post-tx, white circle, $n = 9$). Data was analysed by one-way ANOVA. Tx = treatment.

effective anti-tumour immune response, we next assessed the cytotoxic potential of the omental and hepatic subsets of these cells in OAC patients, by analysing their expression of the degranulation marker CD107a. The proportions of CD107a-expressing γδ T cells in PBMC, SVF and intrahepatic lymphocytes were quantified by flow cytometry. Following stimulation, our data showed that frequencies of CD107a-expressing γδ T cells were significantly higher in OAC omentum (37.4 ± 5.2%, $p < 0.05$) and liver (56.7 ± 13.5%, $p < 0.05$), compared to blood (10.7 ± 2.3%), ($n = 6$, Fig. 4). Interestingly, exposure of healthy donor blood-derived, expanded Vδ1, Vδ2 and Vδ3 T cells to the tissue conditioned media generated from OAC patient omentum, liver and tumour did not significantly alter degranulation of these individual cell subsets (Fig. 4C). Importantly, when the cytotoxicity of OE33 cells by expanded γδ T cell lines was examined, there were no significant differences in the fold change of the percentage of specific lysis of OE33 cells following incubation with γδ T cell lines previously exposed to OAC patient-derived ACM, LCM, or TCM, relative to the baseline percentage of specific lysis of OE33 cells following incubation with γδ T cell lines previously exposed to M199 control media which was set to 1 (Fold-change of specific lysis, relative to M199 control: M199 -v- ACM -v- LCM -v- TCM; 1 -v- 1.14 -v- 1.5 -v- 1.15. Percentage of specific lysis: M199 -v- ACM -v- LCM -v- TCM; 11.82% -v- 13.5% -v- 17.93% -v- 13.54%. Fig. 4D).

3.5. Intratumoural γδ T cell frequencies do not significantly change following chemoradiotherapy in OAC patients

As our study data were generated from OAC patient samples obtained at time of surgical resection which is a time point after chemoradiotherapy (CRT), we investigated an additional cohort of OAC patients at a time point prior to CRT to ascertain whether γδ T cell subset frequencies were affected by CRT, as has been reported for other cell types [32]. We quantified the frequencies of all three γδ T cell subsets in the blood and tumours of OAC patients before and after CRT and found no significant differences [Fig. 5] as has also been noted for other unconventional T cells such as mucosal associated invariant T (MAIT) cells [29].

3.6. Significantly higher γδ T cell frequencies in omentum and liver of obese OAC patients while CD107a$^+$ and IFNγ$^+$ γδ T cell prevalence in OAC liver positively correlates with BMI

To assess the impact of obesity on the tissue distribution of γδ T cell subsets in OAC, the frequencies and functionality of γδ T cells were compared between non-obese and obese patients. Our data reveal significantly higher Vδ2 T cells within the omentum (Non-obese: 2.404 ± 0.648% vs Obese: 5.79 ± 1.58%, $p = 0.0386$, Fig. 6B) and liver (Non-obese: 1.354 ± 0.81% vs Obese: 4.04 ± 1.98%, $p = 0.028$, Fig. 6C) of obese OAC patients, compared to non-obese. Furthermore, frequencies of intrahepatic Vδ2 T cells significantly increase in prevalence with increasing BMI in our OAC patient cohort ($r = 0.6694$, $p = 0.0088$, $n = 14$, Fig. 6C). Most interestingly, the frequencies of both pro-inflammatory IFN-γ-producing and CD107a$^+$ γδ T cells in OAC liver significantly correlated with BMI suggesting that obesity is accompanied by enrichments of pro-inflammatory and cytotoxic γδ T cells in the liver of OAC patients (IFN-γ: $r = 0.8497$, $p = 0.0322$, $n = 6$, Fig. 6D and CD107a: $r = 0.7930$, $p = 0.05$, n = 6, Fig. 6D).

4. Discussion

At a time when immunotherapy has become the fourth pillar of cancer treatment, the burgeoning health burden of obesity-associated cancers such as OAC presents a unique challenge to tumour immunologists. Accordingly, an understanding of the immune environment in the tumour site, as well as in tissues that may fuel carcinogenesis such as the omentum and liver, has become a topic of great interest and importance. For OAC, the accumulation of pro-inflammatory and anti-tumour T cell subsets in the extratumoural tissues of the omentum and liver has the potential to significantly compromise tumour surveillance while also fuelling pathological inflammation [10,12]. Herein, we elucidated the frequencies, inflammatory profiles and cytotoxic potential of γδ T cells in the blood, omentum, liver and tumour of OAC patients, and revealed several novel findings.

First, our data show that the Vδ1 and Vδ3 subsets of γδ T cells are more abundant in the omentum and liver of OAC patients compared to blood and tumour in the same patients. Furthermore, while we observed that Vδ2 T cells are not found in the same abundance as the Vδ1 and the Vδ3 subsets in OAC omentum, we have identified that obese OAC patients have significantly higher omental and intrahepatic Vδ2 T cell frequencies compared to their non-obese counterparts. Second, and similar to their αβ counterparts, we have identified that γδ T cells predominantly express Th1 and Th17 cytokines in OAC omentum and liver [12]. Hepatic γδ T cells have previously been established as Th1 biased with the majority of cells producing IFN-γ, TNF-α and IL-2 in ex vivo studies [34]. Here, we have observed that the frequencies of such Th1-biased IFN-γ$^+$ γδ T cells in the liver of OAC patients significantly correlate with increasing BMI, suggesting that obesity accentuates this phenotype. Furthermore, IL-17-producing γδ T cells have been shown to regulate adipose tissue regulatory T cell homeostasis and thermogenesis in the adipose tissue of obese mice [26,28]. Notwithstanding, our data provide the first reports of parallel profiles of γδ T cells co-expressing IL-

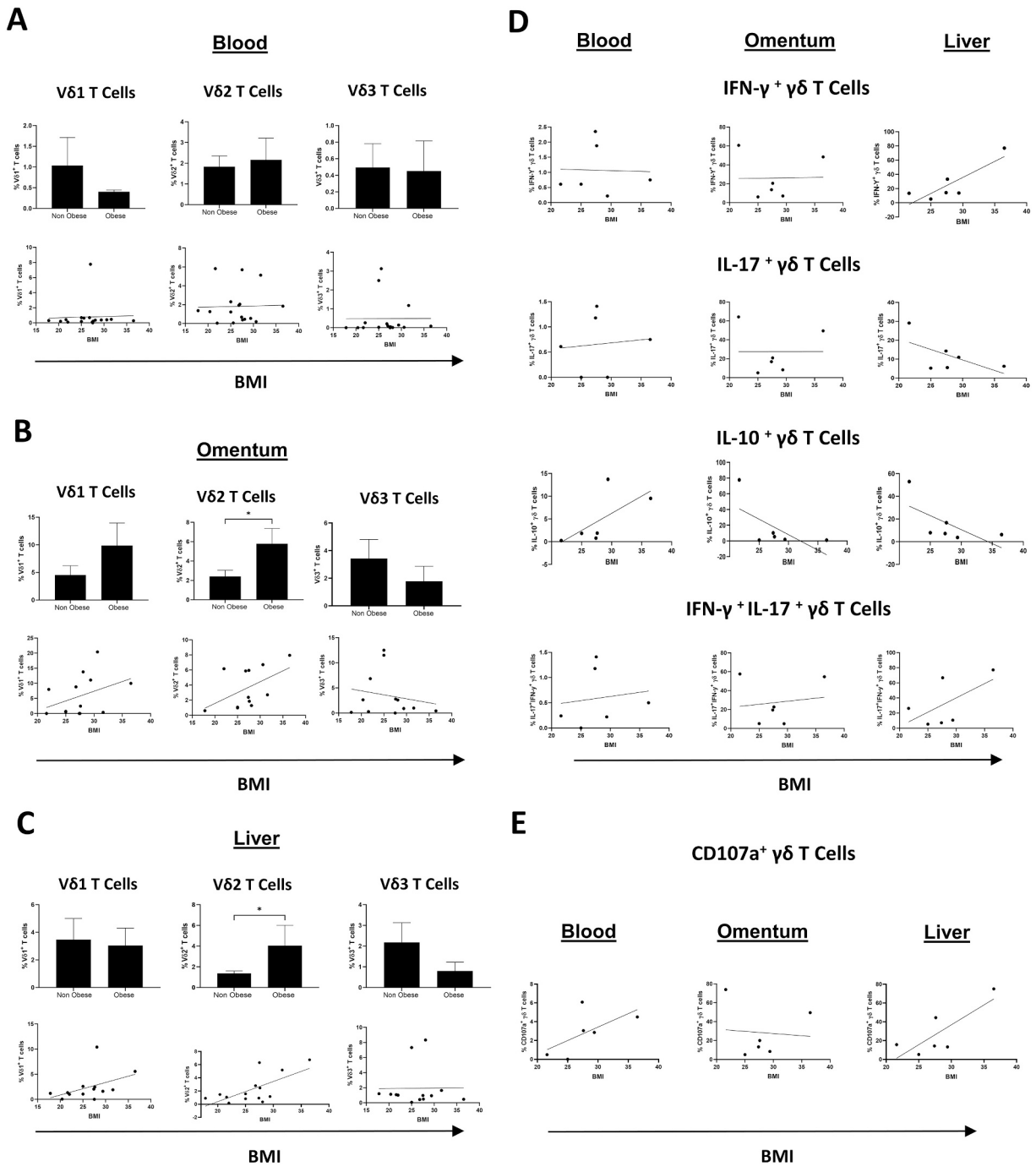

Fig. 6. Significantly higher γδ T cell frequencies in omentum and liver of obese OAC patients while CD107a^{+} and IFNy^{+} γδ T cell prevalence in OAC liver positively correlates with BMI. (A, B, C, top): Bar charts showing the total frequencies of Vδ1, Vδ2 and Vδ3 T cells in the (A) blood, (B) omentum and (C) liver of non-obese (n = 8–10) and obese (n = 3–4) OAC patients. (A, B, C, bottom): Line graphs showing correlations between the frequencies of Vδ1, Vδ2 and Vδ3 T cells in the (A) blood (n = 17–18), (B) omentum (n = 13–14) and (C) liver (n = 14) of OAC patients and their body mass index (BMI). D: Line graphs showing correlations between the frequencies of IFN-γ^{+}, IL-17^{+}, IL-10^{+} and IFN-γ^{+}IL-17^{+} γδ T cells in blood, omentum and liver of OAC patients and their body mass index (BMI). E: Line graphs showing correlations between the frequencies of CD107a^{+} γδ T cells in blood, omentum and liver of OAC patients and their BMI. *$p < 0.05$, **$p < 0.01$ using t-test or Pearson correlation as appropriate.

17 and IFN-γ in the liver and omentum of obesity-associated cancer patients. IL-17-producing γδ T cells have previously been identified as key players in tumourigenic inflammation in colorectal carcinoma and our data provide the first indication of their involvement in adipose tissue and hepatic inflammation in OAC [28]. The reported potential of IL-17-producing γδ T cells to fuel tumourigenesis highlights the importance of further delineating the role of IL-17^{+} IFN-γ^{+} γδ T cells in OAC tumour progression [35]. It must be noted that our cytokine profiling data was generated using PMA/I-stimulated γδ T cells, which is a potent and reliable immune cell stimulant commonly used in immune profiling studies [12,16]. PMA/I may not fully reflect the physiological in situ activation of γδ T cells but importantly, our data is in line with the previous Th1 and Th17 profiles observed in the omentum and liver of OAC patients [12]. Future in vivo experiments can confirm the parallel cytokine profiles of γδ T cells in OAC omentum and liver.

This study also reveals that omental and hepatic γδ T cells exhibit

degranulation potential in OAC patients, which is indicative of their cytotoxic capabilities and suggests that such populations might be desirable at the tumour site. The abundance of γδ T cells in OAC omentum and liver is specific to these sites, as prevalence was relatively low in OAC tumours. Moreover, this effect is not an artefact of treatment-induced depletion, with equally low intratumoural frequencies observed pre- and post-chemoradiotherapy in OAC patients. A plausible thesis is that OAC patients may benefit from therapies harnessing the trafficking of these potent anti-tumour T cells, whose infiltration of tumour has previously been identified as the strongest predictor of good prognosis [15]. Our data also reveal that the frequencies of intrahepatic $CD107a^+$ γδ T cells significantly correlate with increasing BMI suggesting that such therapies might be most appropriate for those cancer patients with the highest obesity status.

A further original finding is the abundance of $CCR6^+$ γδ T cells in omental and hepatic tissues, indicating that the CCL20:CCR6 axis governs the migration of γδ T cells to these sites in OAC patients. We have previously reported abundant levels of omental and hepatic CCL20 in OAC patients, and we suggest that antagonising this chemokine pathway might present an opportunity to therapeutically block γδ T cell accumulation in omentum and liver and allow these cells to migrate preferentially towards other chemotactic signals in OAC tumour [13]. Our group's previous work has also uncovered an abundance of CCL20 in OAC tumour tissue but this was not paralleled by a tumour infiltration of $CCR6^+$ conventional T cells [30]. Here, our data indicate that the CCL20 signal in OAC tumour is not accompanied by a strong $CCR6^+$ γδ T cell infiltrate, at least not one that surpasses that of omentum and liver. As $CCR6^+$ γδ T cell infiltrates in mouse colon are potent IL-17 producers and have been implicated as drivers of inflammation-driven CRC, it is also possible that such pro-inflammatory $CCR6^+$ γδ T cells would be more harmful than beneficial in the OAC tumour microenvironment [28].

In conclusion, we report for the first time the distribution of γδ T cell subsets among the omental, hepatic and tumour tissues of OAC patients, and that the omentum and liver are tightly associated in immune phenotype and signalling, with evidence of significant pro-inflammatory and cytotoxic γδ T cell activity. Our data reveal that the abundance of γδ T cells in omentum and liver parallels the obesity status of the patient thus providing further evidence that dysregulated immune responses in OAC are a consequence of obesity. Overall, these results strongly suggest that γδ T cells contribute to obesity-associated inflammation in OAC. Furthermore, their lower abundance in OAC tumour suggests that their accumulation in omentum and liver may hinder their role in tumour immunosurveillance.

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Declaration of Competing Interest

None.

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