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## Parallel Profiles of Inflammatory and Effector Memory T Cells in Visceral Fat and Liver of Obesity-Associated Cancer Patients

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3 **Parallel Profiles of Inflammatory and Effector Memory T cells in Visceral**  
4 **Fat and Liver of Obesity-associated Cancer Patients.**

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1  
2 **Abstract**  
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5 In the midst of a worsening obesity epidemic, the incidence of obesity-associated  
6 morbidity, including cancer, diabetes, cardiac and liver disease is increasing. Insights into  
7 mechanisms underlying pathological obesity-associated inflammation are lacking. Both the  
8 omentum, the principal component of visceral fat, and liver of obese individuals are sites of  
9 excessive inflammation, but to date the T cell profiles of both compartments have not been  
10 assessed or compared in a patient cohort with obesity-associated disease.  
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14 We have previously identified that omentum is enriched with inflammatory cytokines,  
15 chemokines and T cells. Here, we compared the inflammatory profile of T cells in the  
16 omentum and liver of patients with the obesity-associated malignancy oesophageal  
17 adenocarcinoma (OAC). Furthermore, we assessed the secreted cytokine profile in OAC  
18 patient serum, omentum and liver to assess systemic and local inflammation.  
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22 We observed parallel T cell cytokine profiles and phenotypes in the omentum and  
23 liver of OAC patients, in particular CD69<sup>+</sup> and inflammatory effector memory T cells. This  
24 study reflects similar processes of inflammation and T cell activation in the omentum and  
25 liver, and may suggest common targets to modulate pathological inflammation at these sites.  
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43 **Keywords**  
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46 Inflammation, obesity, cancer, T cells, liver, omentum  
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## 1. Introduction

Obesity has reached epidemic proportions worldwide and is now estimated to contribute to approximately 3.5 million deaths every year [1, 2, 3, 4]. The association between obesity and cancer is well established and may be associated with the development of 40% of certain malignancies in the US [4, 5, 6]. The expansion of adipose tissue due to obesity results in enhanced angiogenesis, increased infiltration of inflammatory macrophages and T cells and significantly greater release of pro-inflammatory factors including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12 and IFN- $\gamma$ , all of which contribute to a systemic state of chronic inflammation [7]. Such chronic inflammation and excessive pro-inflammatory cytokine production can result in genomic instability and the initiation of cancer. Furthermore, alterations in key lymphocyte subsets such as CD4<sup>+</sup> and CD8<sup>+</sup> T cells, NK, NKT and regulatory T (Treg) cells have been reported in obese cancer patients, which may negatively impact anti-tumour immune responses [8, 9, 10, 11, 12].

Excess visceral adipose tissue (VAT) is considered a more important risk factor for cancer development, inflammation and dysmetabolism than subcutaneous fat. The omentum, which drains into the liver via the portal circulation, forms the largest component of the VAT compartment. We and others have previously shown that the omentum is enriched with activated pro-inflammatory T cells, now understood to be pivotal in both the establishment and maintenance of adipose tissue inflammation in obesity [8, 13]. Importantly, we have also demonstrated that the omentum and liver are enriched in inflammatory cytokines and chemokines that facilitate T cell migration to these sites [7, 14]. Furthermore, our group reported that chemokine receptor antagonism is a potential novel treatment strategy to reduce T cell infiltration to the omentum and liver [14].

The aim of this study was to ascertain the role of T cells in inflammation within the omentum and liver of patients with an inflammation-driven and obesity-associated cancer,

oesophageal adenocarcinoma (OAC), and to compare T cell phenotypes at these two important sites. As the incidence of OAC is increasing dramatically, paralleling that of the obesity epidemic, it is a highly relevant clinical model with which to study the underlying mechanisms and potential therapeutic targets for obesity-associated inflammation, and lessons learned may also apply to other obesity-associated malignancies. By evaluating whether the inflammatory profile of the T cell infiltrates in liver are similar to those in omentum, novel insights on potential targets relevant to cancer and obesity-associated liver inflammation may be uncovered.

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## 2. Materials and Methods

### Subjects

Twenty four consecutive consenting patients with oesophageal adenocarcinoma, attending the Oesophageal Unit at St. James Hospital, Dublin from 2011 to 2014 were enrolled in this study. The patient cohort was similar in age and race, and 23 had received neo-adjuvant chemo-radiotherapy. The patient group included 22 males and 2 females, representative of the male predominance in oesophageal adenocarcinoma, with an average age of 63.5yrs. The mean BMI was 25.8. Patients provided informed consent for sample acquisition and the study received ethical approval from the St James's Hospital Ethics Review Board. All cancer patients were evaluated by a dietician. Body mass index, waist circumference and anthropometric variables were measured as described previously [8]. Visceral fat area was assessed by computer tomography as previously described, with more than 160 cm<sup>2</sup> and 80cm<sup>2</sup> defining visceral obesity in males and females, respectively [15].

### Sample Preparation

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden). Omental samples (10 g) were digested enzymatically as previously described to obtain stromal vascular fraction (SVF) [8]. Liver samples were processed as described previously with slight modifications [16]. Briefly, liver tissue (1 g) was digested enzymatically in a 50 ml tube containing 25 ml Disruption Enzyme Mix Solution (50 ml HBSS + 0.025 g Collagenase type IV (0.05%) + 100 µl DNase I (10 mg/ml) (0.002 %) + 1 ml FCS + 1 ml 30 % BSA). Tissue was incubated for 35 minutes on a shaking incubator at 37°C at 180 RPM before being passed through a 70 µm polypropylene filter (Filcon; BD Bioscience, San Jose, California, USA) to discard debris. Cells were washed twice with HBSS and centrifuged at 1300 RPM for 3 minutes. The cell pellet was resuspended in 0.87% ammonium chloride solution to lyse contaminating

1 erythrocytes. The remaining intrahepatic immune cells were counted and assessed for  
2 viability by trypan blue. Adipose conditioned media (ACM) was prepared as previously  
3 described, briefly omentum was minced using a scalpel, adding 10 ml of serum-free M199  
4 media per 5 g of omentum and incubating at 37°C, 5% CO<sub>2</sub> for 72 hours. Following 72 hours,  
5 the media was passed through a 70 µm polypropylene filter to discard debris [7]. Liver  
6 conditioned media (LCM) was prepared as previously described by mincing liver using a  
7 scalpel, adding 350 µl of serum-free M199 media per 0.1 g of liver and incubating at 37°C,  
8 5% CO<sub>2</sub> for 72 hours. Following 72 hours, the media was passed through a 70 µm  
9 polypropylene to remove debris [14].  
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### 21 **Antibodies and flow cytometry**

22 Freshly-isolated PBMC, SVF and intrahepatic immune cells were stained with monoclonal  
23 antibodies (mAbs) specific for human surface markers (CD3, CD4, CD8, CD45RA, CD69;  
24 BD Biosciences, Oxford, UK), CD27 (eBioscience, Hatfield, UK) and CD62L (Abcam,  
25 Cambridge, UK). Gating strategy for surface markers is outlined in supplemental Figure 1A.  
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27 For intracellular cytokine staining cells were stimulated with 10 ng/ml of phorbol myristate  
28 acetate and 1 µg/ml of ionomycin (PMA/I) for 1 hour, followed by the addition of 10 µg/ml  
29 of brefeldin A for a further 3 hours. Cells were stained with mAbs specific for human surface  
30 markers CD3 and CD8 for 30 minutes. As human CD4 cannot be reliably detected following  
31 treatment with PMA, CD4<sup>+</sup> T cells were represented by CD3<sup>+</sup>CD8<sup>-</sup> cells, the vast majority of  
32 this population is made up of CD4<sup>+</sup> T cells, however it will also contain minor populations of  
33 innate lymphocytes. Cells were fixed and permeabilised, then stained with mAbs specific for  
34 the cytokines IFN-γ, TNF-α and IL-10 (BD Biosciences, Oxford, UK) and IL-17  
35 (eBioscience, Hatfield, UK). Gating strategy for intracellular cytokines is outlined in  
36 supplemental Figure 1B. Cells were acquired using a CyAn ADP flow cytometer (Beckman  
37 Coulter) and analysed with FlowJo software (TreeStar Inc.).  
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### **Quantification of cytokine levels in serum, omentum and liver**

The V-PLEX™ human cytokine plate (Meso Scale Discovery) was used to detect the levels of IL-1 $\beta$ , IL-6, IL-12, GM-CSF, IL-4, IL-2, IL-7 and IL-15 in the serum, ACM and LCM of OAC patients according to the manufacturer's instructions and read using a MesoScale Diagnostics Sector S600.

### **Statistical analyses**

Statistical analysis was carried out using Prism GraphPad Version 5.0. Differences between groups were assessed using two-tailed paired or unpaired t tests where appropriate. p values of <0.05 were considered to be significant.

### 3. Results

*The omentum and liver of OAC patients contain significant proportions of activated effector memory T cells.*

Peripheral blood mononuclear cells (PBMC), stromal vascular fraction (SVF) of omentum and intrahepatic immune cells from 11 OAC patients were fluorescently labelled with antibodies against CD3, CD4, CD8, CD45RA, CD27, CD62L, CD69 for subsequent flow cytometric analysis. Percentages of naïve ( $CD45RA^+CD27^+$ )  $CD8^+$  T cells (Blood 42.4%; Omentum 10.9%; Liver 8.7%) were significantly reduced in both the omentum and liver compared with blood, while there were no significant differences observed in the  $CD4^+$  T cell population (Figure 1a). Significantly higher percentages of effector memory ( $CD45RA^-CD27^-$ )  $CD4^+$  (Blood 23.9%; Omentum 56.6%; Liver 51.1%) and  $CD8^+$  (Blood 8.3%; Omentum 37.6%; Liver 34.7%) T cells were observed in the omentum and/or liver of OAC patients compared to blood (Figure 1a).

There were significantly lower percentages of  $CD62L^+CD4^+$  (Blood 81.7%; Omentum 19.2%; Liver 13.3%) and  $CD62L^+CD8^+$  (Blood 49.3%; Omentum 19.7%; Liver 15.4%) T cells, while significantly higher percentages of  $CD69^+CD4^+$  (Blood 4.8%; Omentum 36.9%; Liver 42.9%) and  $CD8^+$  (Blood 4.9%; Omentum 31.9%; Liver 52.8%) T cells were detected in both the omentum and liver of our OAC study cohort, compared to blood (Figure 1b). Interestingly, no statistically significant differences were observed in the frequencies of effector memory, naïve or  $CD69^+$  T cells between the omentum and liver of the OAC cohort thus highlighting the striking similarities of T cell repertoires in these tissues, compared to the circulation (Figure 1).

*The omentum and liver of OAC patients contain significant proportions of inflammatory  $CD4^+$  and  $CD8^+$  T cells.*

1 PBMC, SVF and intrahepatic immune cells from 15 OAC patients were fluorescently  
2 labelled with antibodies against CD3, CD8, IFN- $\gamma$ , TNF- $\alpha$ , IL-17 and IL-10 for subsequent  
3 flow cytometric analysis.  
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7 The percentages of CD4<sup>+</sup> (CD3<sup>+</sup>CD8<sup>-</sup>) IFN- $\gamma$ <sup>+</sup> (Blood 3.1%; Omentum 30.6%; Liver 21.2%),  
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10 TNF- $\alpha$ <sup>+</sup> (Blood 6.3%; Omentum 25.2%; Liver 28.6%), IL-17<sup>+</sup> (Blood 0.5%; Omentum 3.7%;  
11  
12 Liver 5.1%) and CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> (Blood 12.7%; Omentum 39.1%; Liver 35.5%), CD8<sup>+</sup>TNF- $\alpha$ <sup>+</sup>  
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14 (Blood 8.1%; Omentum 45.8%; Liver 29.4%) and CD8<sup>+</sup>IL-17<sup>+</sup> (Blood 0.9%; Omentum 3.7%;  
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16 Liver 3.8%) T cells were significantly elevated in both the omentum and liver of OAC  
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18 patients, compared to blood, but frequencies of IL-10-producing T cells were not  
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20 significantly different (Figure 2). Statistical analysis of cytokine-producing T cell subsets  
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22 between the omentum and liver revealed no significant differences thus further demonstrating  
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24 the marked concordance between these tissues in inflammatory profile (Figure 2).  
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### 32 ***High levels of secreted inflammatory cytokines from both OAC omentum and liver***

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34 Having profiled cytokine expression by T cells in the blood, omentum and liver, we then  
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36 quantified secreted inflammatory factors from the serum, adipose and liver conditioned media  
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38 (ACM and LCM respectively) of 14 OAC patients by multiplex ELISA to determine the  
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40 inflammatory profile of the multi-cellular tissue. The levels of secreted IL-1 $\beta$ , IL-12, GM-  
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42 CSF, IL-6, IL-2, IL-4 and IL-15 were significantly higher in both ACM and LCM compared  
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44 to serum confirming that the liver is very comparable to the omentum in terms of secreted  
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46 inflammatory cytokines with the exception of IL-15 levels which were significantly higher in  
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48 the ACM of OAC patients, compared to LCM (Figure 3).  
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#### 4. Discussion

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2 Obesity is associated with an imbalance of inflammatory factors and immune cell infiltrates  
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4 in the omentum and liver, which not only may be relevant to cancer risk, but also liver  
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6 inflammation in the form of non-alcoholic steatohepatitis (NASH) and fatty liver disease  
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8 (NAFLD) [17]. Activated and inflammatory Th1 cells, natural killer (NK) cells and NKT  
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10 cells are present in large numbers in normal healthy liver, and the healthy gut has been  
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12 identified as a site of “controlled inflammation”, in which lymphocytes are functionally and  
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14 phenotypically distinct to those in the circulation [18]. However, alterations in CD8<sup>+</sup> T cells  
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16 and NKT cells have been reported in non-alcoholic steatohepatitis and cancer in obese  
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18 murine models [19, 20, 21, 22, 23]. In human cancer patients, the prevalence of such immune  
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20 subsets in the VAT and liver has the potential to disrupt tumour surveillance as well as  
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22 promoting tumourigenic inflammation. Previously, we have shown in OAC patients that  
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24 activated and pro-inflammatory T cells and pro-inflammatory cytokines are enriched in the  
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26 omentum, the primary depot of VAT [8]. Furthermore, we have demonstrated that T cell  
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28 enrichments in omentum is most likely due to enhanced T cell recruitment to this tissue and  
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30 that the MIP-1 $\alpha$  chemokine pathway, among others, guides T cell migration to both omentum  
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32 and liver [14]. Here, we investigated whether the enhanced T cell chemotaxis to OAC liver  
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34 that was previously described, results in an abundance of pro-inflammatory T cells in this  
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36 tissue, in parallel to what is observed in the omentum [8, 14]. We expanded our studies to  
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38 elucidate the frequencies of TNF- $\alpha$ , IL-17 and IL-10 producing T cells in the omentum and  
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40 liver. The key finding is the concordance between omentum and liver in this cancer cohort,  
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42 with both Th1 and Th17 cells present in abundance and a reduced proportion of IL-10<sup>+</sup> T  
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44 cells. There was also a striking concordance in the presence of activated and effector memory  
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46 T cell populations at the two tissue sites. Together with our previous findings, these data  
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48 suggest that similar chemokine gradients are recruiting activated and inflammatory T cells to  
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1 both the omentum and liver, which in turn may contribute to local and systemic inflammation  
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5 Of the cytokines assessed in this study many are already known contributors to  
6 obesity-associated inflammation, including GM-CSF, IL-1 $\beta$ , IL-6 and IL-12 [7, 24, 25, 26,  
7 27]. Furthermore, IL-6 has been implicated in the development of NAFLD in human obesity,  
8 while IL-1 $\beta$  is involved in progression from steatosis to steatohepatitis [28, 29]. Interestingly,  
9 levels of IL-2 and IL-4 were detected in our patient cohort but could not be detected in a  
10 previous study examining adipose tissue resident CD4<sup>+</sup> and CD8<sup>+</sup> T cells, this may be due to  
11 enhanced trafficking of non-resident T cells producing these cytokines in the omentum [30].  
12 Previous studies have implicated IL-15 in decreased lipid deposition and have reported that  
13 obese individuals have reduced serum levels compared to normal weight adults [31].  
14 However, to our knowledge, the present study is the first to report abundant levels of this T  
15 and NK cell stimulating cytokine in human omentum. While the abundance of IL-1 $\beta$ , IL-6,  
16 IL-12 and IL-2 was mirrored in matched omentum and liver samples, both of these tissues  
17 contained equally low levels of IL-7.  
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36 There is important therapeutic relevance for these novel findings as we have shown  
37 for the first time that Th1 and Th17 cells are likely contributors to pathological inflammation  
38 in the omentum and liver of patients with obesity-associated cancer, and that naive T cells  
39 and IL-10 producing T cells subsets are not prevalent at these sites. Importantly, these data  
40 suggest that strategies such as chemokine receptor antagonism targeting the recruitment of  
41 such inflammatory cells to the omentum of obese patients will also reduce their prevalence in  
42 liver and have direct consequences for NAFLD.  
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53 In conclusion, this study has for the first time compared the inflammatory profile of  
54 the omentum and liver, utilising a clinically relevant model of obesity and inflammation-  
55 associated cancer, oesophageal adenocarcinoma. Our novel findings demonstrate that these  
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1 proximal tissues are enriched in both inflammatory cells and secreted factors that are  
2 conducive to obesity-associated inflammation and numerous associated pathologies,  
3 including cancer. This suggests that therapeutic strategies should target inflammation at both  
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5 omental and hepatic sites in obese patients.  
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11 **Informed consent:** Informed consent was obtained from all individual participants included  
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13 in the study.  
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16 **Ethical approval:** “All procedures performed in studies involving human participants were  
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18 in accordance with the ethical standards of the institutional and/or national research  
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20 committee and with the 1964 Helsinki declaration and its later amendments or comparable  
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22 ethical standards.”  
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31 **Conflict of Interest:** The authors declare that they have no conflict of interest.  
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## Figure Legends

**Figure 1: The omentum and liver of OAC patients are enriched with activated, effector memory T cells.** PBMC, stromal vascular fraction (SVF) and intrahepatic immune cells isolated from the blood and omentum of 11 OAC patients and the liver of 8 OAC patients were fluorescently labelled with antibodies against CD3, CD4, CD8, CD45RA, CD27, CD62L and CD69. **a:** Bar charts showing that the mean percentages of CD45RA<sup>+</sup>CD27<sup>+</sup> CD8<sup>+</sup> T cells (naïve, top right) are lower while CD45RA<sup>-</sup>CD27<sup>-</sup> (effector memory, bottom) CD4<sup>+</sup> (left) and CD8<sup>+</sup> (right) T cells are higher in the omentum and liver, compared to blood. **b:** Bar charts showing mean percentages of CD62L<sup>+</sup> (naïve, top) CD4<sup>+</sup> (left) and CD8<sup>+</sup> (right) T cells are lower while CD69<sup>+</sup> (activated, bottom) CD4<sup>+</sup> (left) and CD8<sup>+</sup> (right) T cells are expanded in omentum and liver, compared to the blood of OAC patients. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

**Figure 2: The omentum and liver of OAC patients are enriched with pro-inflammatory T cells.** PBMC, SVF and intrahepatic immune cells isolated from the blood of 15 and omentum and liver of 14 OAC patients were fluorescently labelled with antibodies against CD3, CD8, IFN- $\gamma$ , TNF- $\alpha$ , IL-17 and IL-10 and analyzed by flow cytometry. Bar charts showing mean percentages of IFN- $\gamma$ <sup>+</sup>, TNF- $\alpha$ <sup>+</sup> and IL-17<sup>+</sup> CD8<sup>-</sup> CD3<sup>+</sup> (left) and CD8<sup>+</sup> CD3<sup>+</sup> (right) T cells are significantly higher in omentum and liver, compared to the blood of OAC patients while significant differences are not observed in the IL-10<sup>+</sup> fractions. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

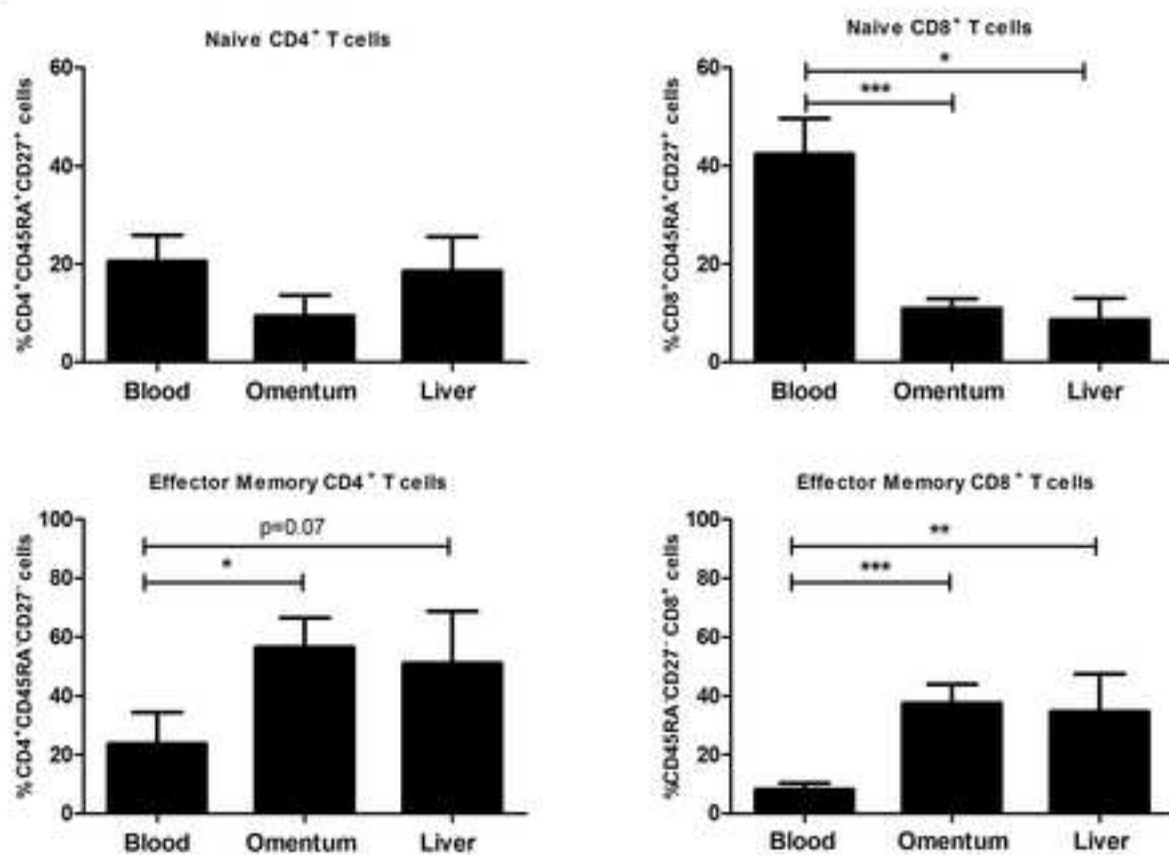
**Figure 3: The omentum and liver of OAC patients are enriched with secreted inflammatory factors.** The secreted levels of IL-6, IL-1 $\beta$ , GMCSF, IL-12, IL-2, IL-4, IL-7 and IL-15 were quantified in the serum, adipose conditioned media (ACM) and liver conditioned media (LCM) of 14 EAC patients using MSD ELISA. Bar charts showing the mean concentrations of IL-6, IL-1 $\beta$ , GMCSF, IL-12, IL-2, IL-4 and IL-15 are significantly

1 higher in the ACM and LCM of 14 EAC patients while concentrations of IL-7 are lower,  
2 compared to serum. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.  
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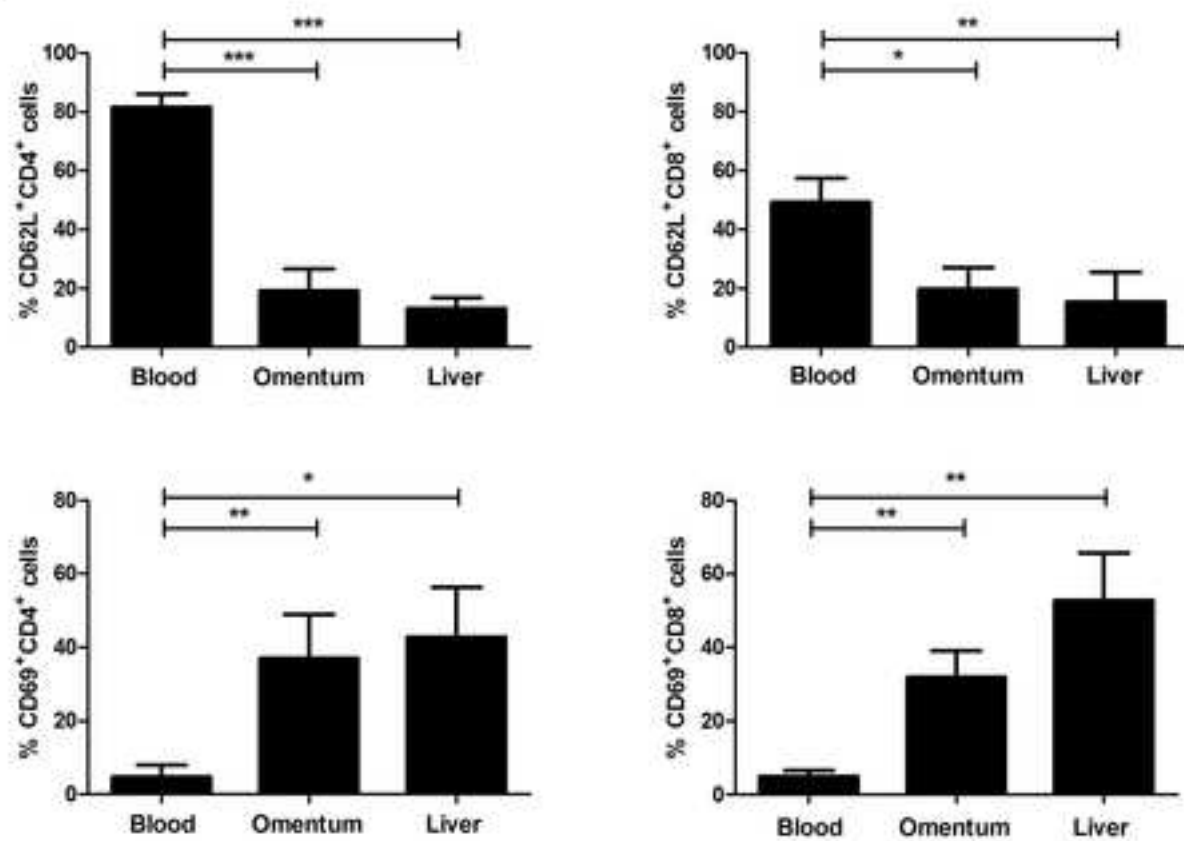
4 **Supplemental Data Figure 1: Representative dotplots showing the gating strategy for**  
5 **surface marker expression and intracellular cytokine expression by T lymphocytes.**  
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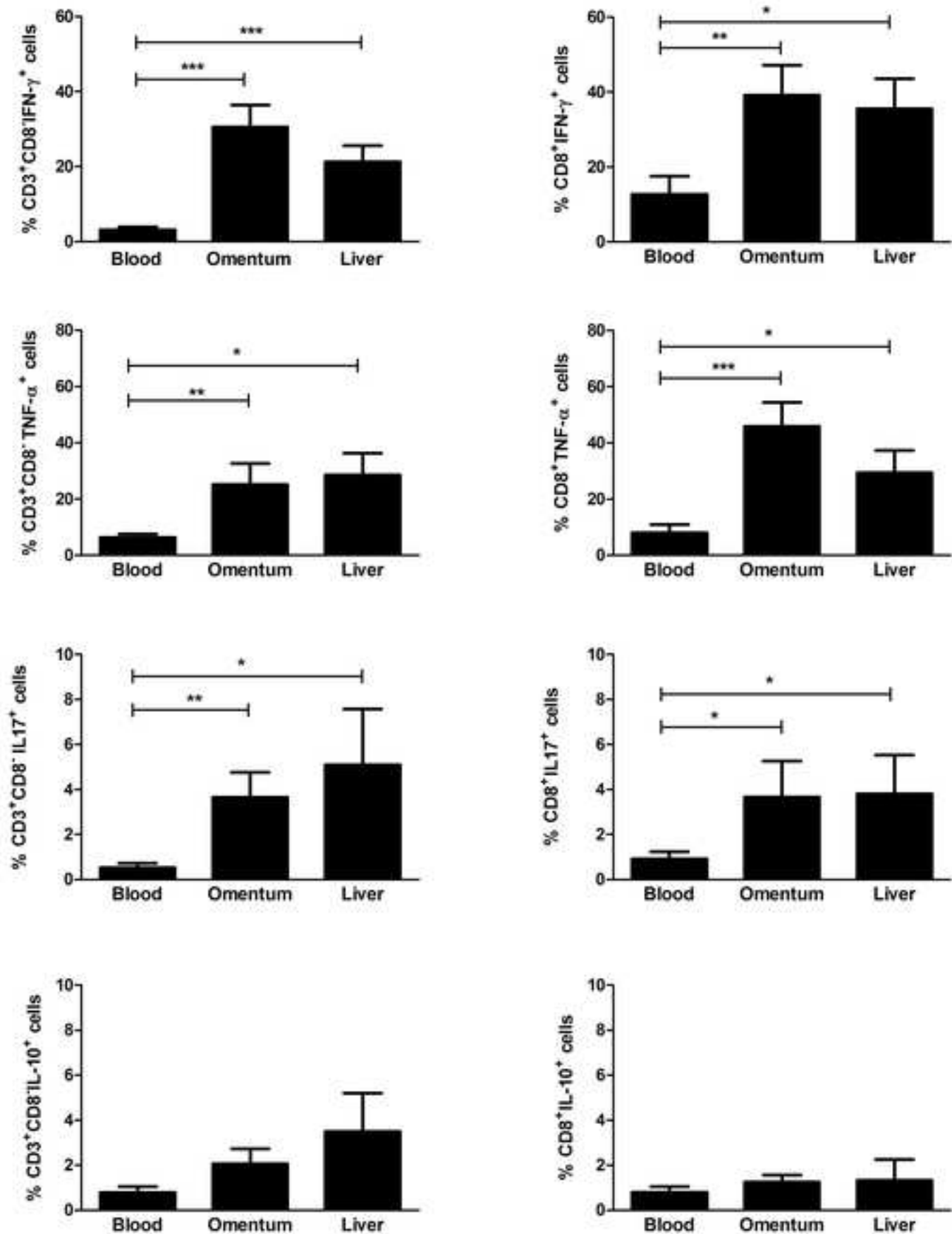
7 **a (Left-Right):** For surface marker quantification, forward scatter and side scatter were first  
8 used to gate on lymphocytes, followed by gating of CD8<sup>+</sup> CD4<sup>-</sup> lymphocytes and CD8<sup>-</sup> CD4<sup>+</sup>  
9 lymphocytes. CD62L was then quantified as a percentage of either CD8<sup>+</sup> CD4<sup>-</sup> lymphocytes  
10 or CD8<sup>-</sup> CD4<sup>+</sup> lymphocytes. **b (Left-Right):** For intracellular cytokine quantification, forward  
11 scatter and side scatter were first used to gate on lymphocyte gate, followed by gating of  
12 CD8<sup>+</sup> CD3<sup>+</sup> lymphocytes and CD8<sup>-</sup> CD3<sup>+</sup> lymphocytes. TNF- $\alpha$ <sup>+</sup> cells were then quantified as  
13 a percentage of CD8<sup>+</sup> CD3<sup>+</sup> lymphocytes or CD8<sup>-</sup> CD3<sup>+</sup> lymphocytes.  
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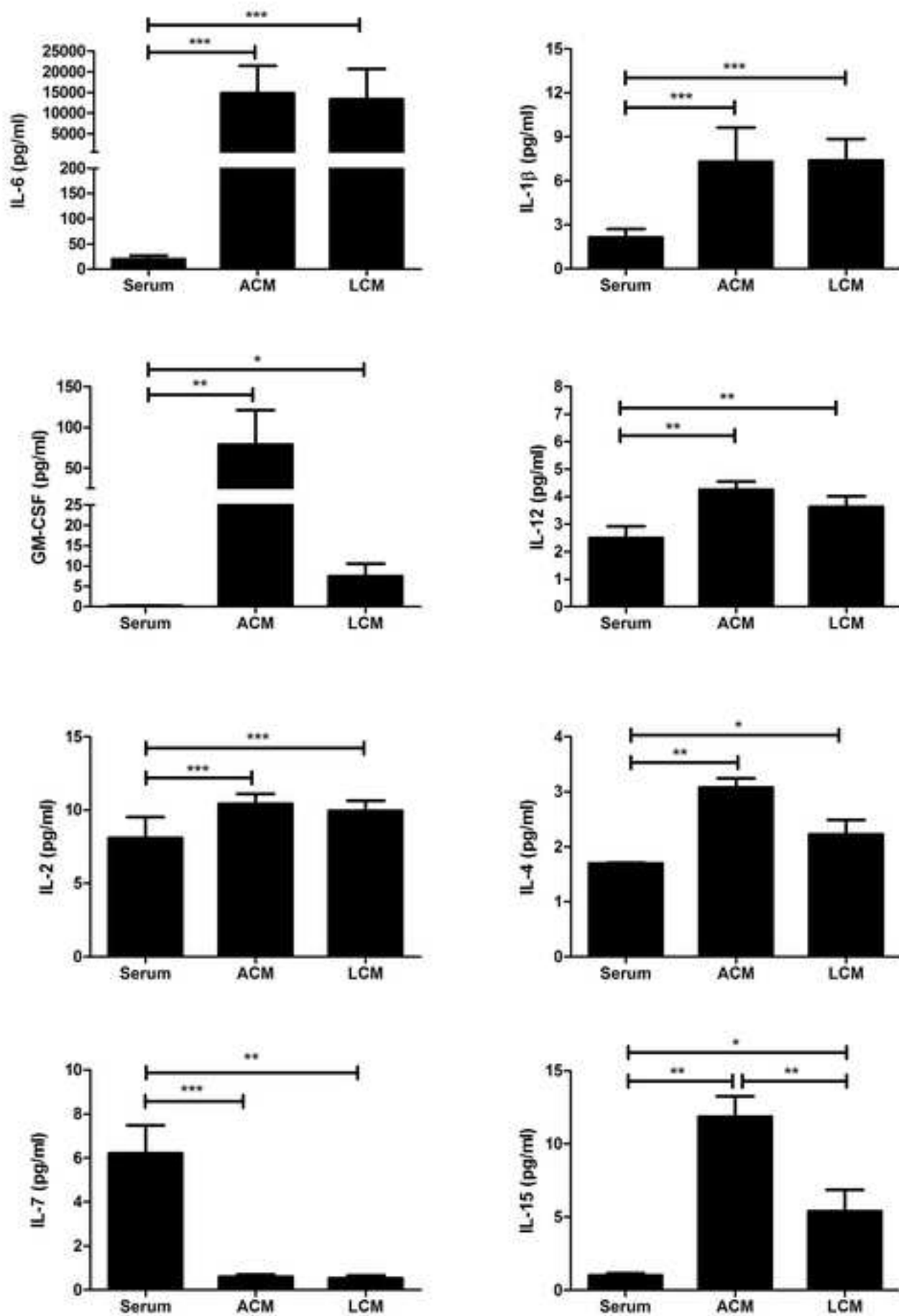
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## SUPPLEMENTAL Figure 1

