Supplementary information

Prognostic role of intratumoral $CD8^+$/$FoxP3^+$ lymphocyte ratio in patients with resected colorectal cancer liver metastasis

Kostandinos Sideras, Boris Galjart, Angela Vasaturo, Alexander Pedroza-Gonzalez, Katharina Biermann, Shanta Mancham, Alex L. Nigg, Bettina Hansen, Hans J.A. Stoop, Guoying Zhou, , Cornelis Verhoef, Stefan Sleijfer, Dave Sprengers, Jaap Kwekkeboom, Marco J. Bruno

Table of contents

Supplementary methods

TIL isolation

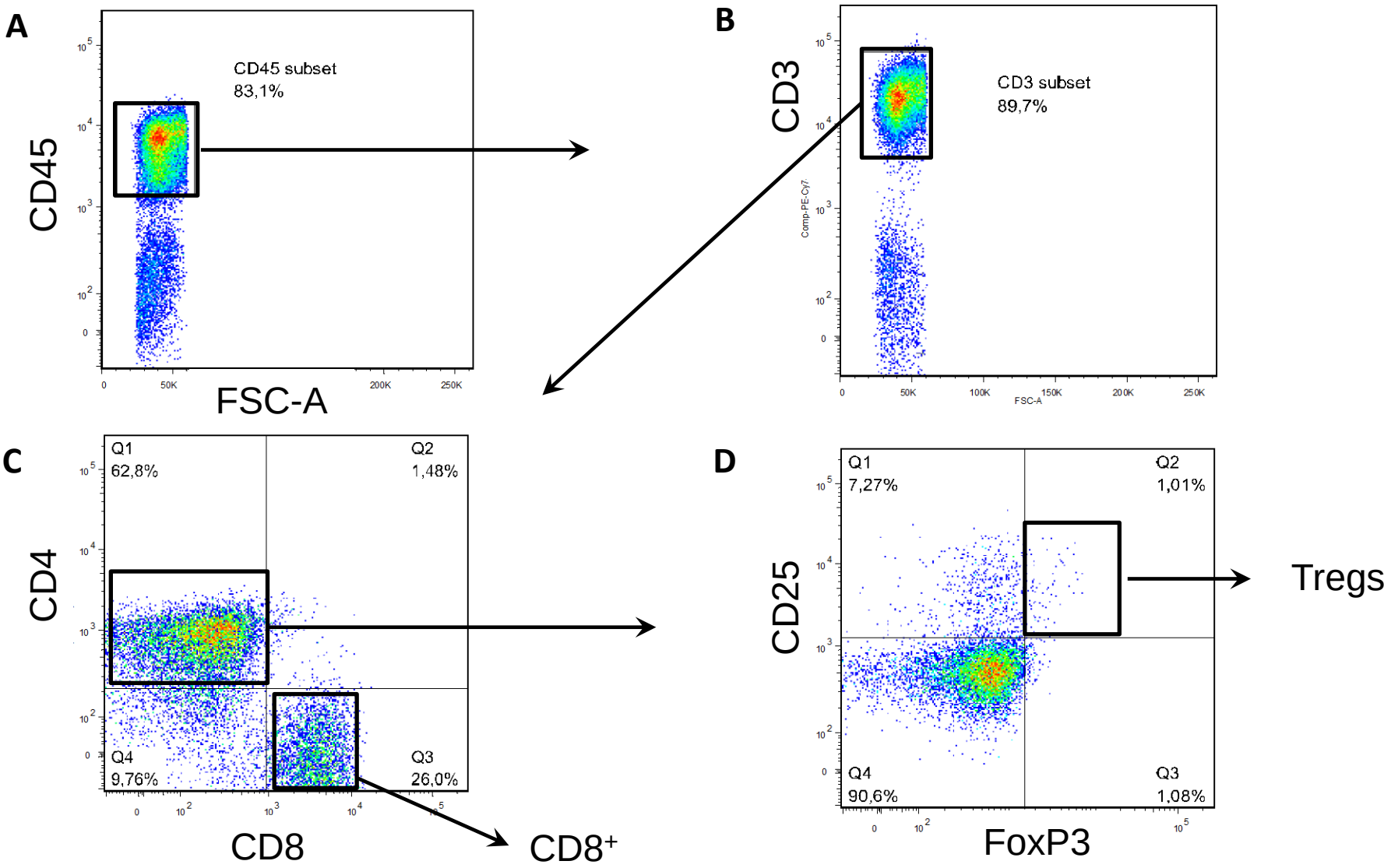
Single cell TILs were isolated from tumor tissue via tissue digestion as previously described [1, 2]. Briefly, fresh tumor tissue was cut into small pieces and digested with 0.5 mg/mL of collagenase (Sigma-Aldrich, St. Louis, MO) and 0.1 mg/mL of DNase I (Roche, Indianapolis, IN) for 30 minutes at 37 oC. Cell suspensions were filtered through cell strainers and mononuclear cells were obtained by Ficoll density gradient centrifugation. Viability was determined by trypan blue exclusion.

Flow Cytometry Analysis

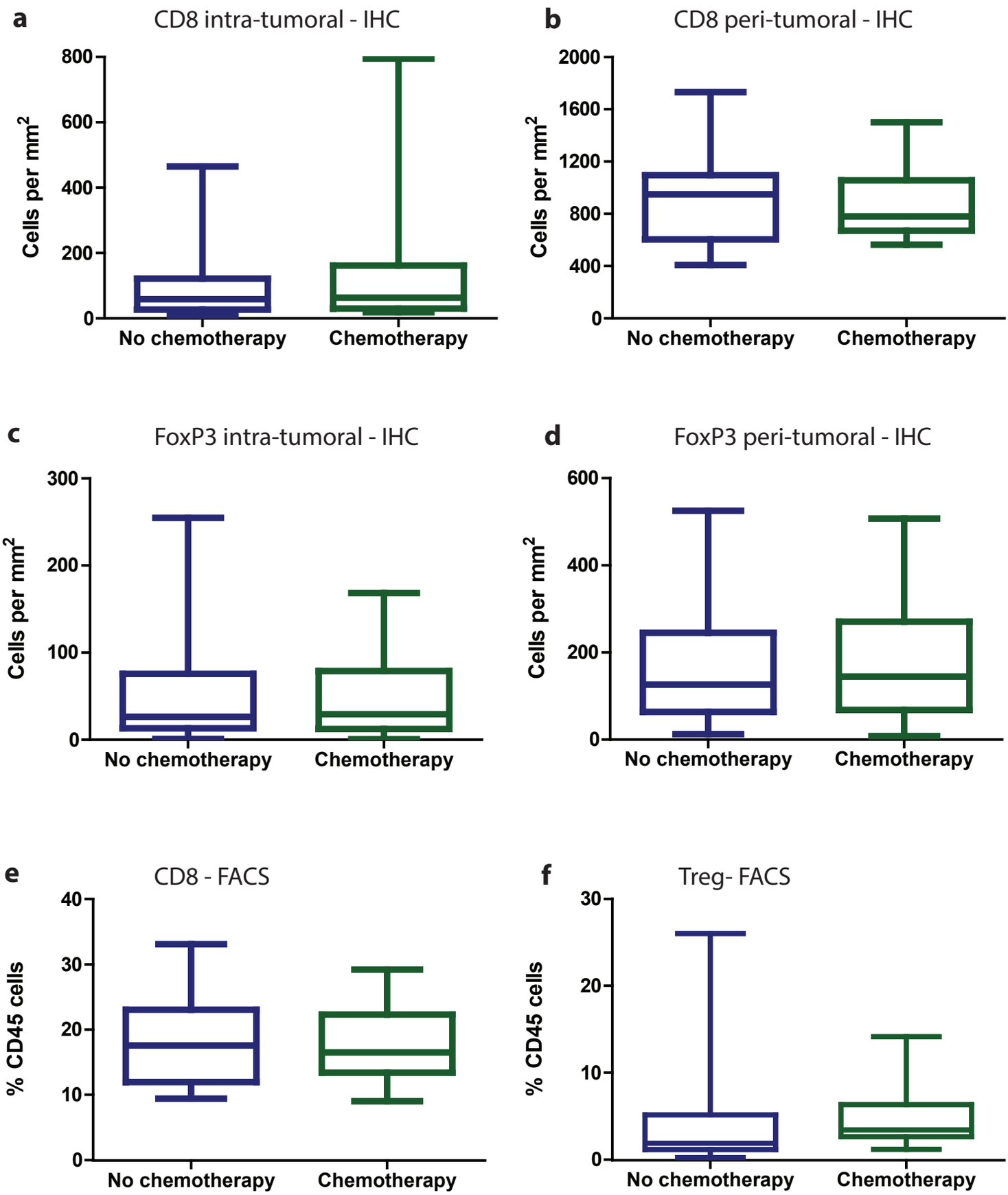
Mononuclear cells isolated from tumor tissue were analyzed for expression of surface and intracellular markers using the following anti-human antibodies: PerCP-labeled anti-CD8 (SK-1) and APC-H7-labeled anti-CD4 (SK3) from BD Biosciences (Erembodegem, Belgium); APC-labeled anti-FoxP3 (PCH101), PeCy7-labeled anti-CD3 (UCTH1), eFluor®450-labeled anti-CD45 (HI30) and eFluor®450-labeled anti-CD25 (BC96) from e-bioscience (Vienna, Austria). Cells were incubated with the antibodies for 30 min at 4 oC in the dark, then washed and fixed with 1% paraformaldehyde. For the intracellular FoxP3 staining, cells were incubated with the FoxP3 antibody using the nuclear staining buffer set from e-biosciences. Dead cells were excluded by using the LIVE/DEAD fixable dead cell stain kit with aqua fluorescent reactive dye (Invitrogen, Paisley, UK). Cells were analyzed in a FACSCanto II system (BD Biosciences, San Diego, CA). The gating strategy has been previously described [1]. Numbers of CD8+ TILs were defined as the proportion of CD8+ cells within live CD45+ cells, while numbers of T-regulatory cells were defined as the proportion of CD4+ CD25+ FoxP3+ within live CD45+ cells.

References

1. Pedroza-Gonzalez A, Verhoef C, Ijzermans JN et al. Activated tumor-infiltrating CD4+ regulatory T cells restrain antitumor immunity in patients with primary or metastatic liver cancer. Hepatology 2013; 57: 183-194.
2. Pedroza-Gonzalez A, Zhou G, Singh SP et al. GITR engagement in combination with CTLA-4 blockade completely abrogates immunosuppression mediated by human liver tumor-derived regulatory T cells ex vivo. Oncoimmunology 2015; 4: e1051297.



Supplementary Figure. 1. Flow cytometric analysis of intra-tumoral lymphocytes from patients with colorectal cancer liver metastasis. (A) Viable (aqua dye-negative) leukocytes were gated based on CD45 expression. (B) T cells were defined based on the expression of CD3. (C) $CD3^+$ T cells were further analyzed for CD4 and CD8. (D) Percentages of Treg cells were then defined as $CD4^+$ $CD25^+$ $FoxP3^+$.



Supplementary Figure 2: Box-plots of TIL populations in relation to wheather patients received (n=17) or did not receive (n=30) prior systemic chemotherapy. Note that no patient received chemotherapy in the 3 month period prior to tumor resection. Also, note that the x-axis range differs based on the population. **a**) CD8 intra-tumoral TIL population by IHC, **b**) CD8 peri-tumoral TIL population by IHC, **c**) FoxP3 intra-tumoral TIL population by IHC, **d**) FoxP3 peri-tumoral TIL population by IHC, **e**) CD8 TIL population by flow cytometry, **f**) CD4/CD25/FoxP3 TIL population by flow cytometry.