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(54) Title: USE OF INTRA-TUMORAL CD8 TEMRA CELLS FOR PREDICTING RESPONSE OF A CANCER PATIENT TO CHECKPOINT INHIBITOR THERAPY

(57) Abstract: The invention relates to the field of predicting (clinical) response of a cancer patient to a therapy comprising an immune checkpoint blocker or to therapy with an immune checkpoint blocker. In particular enriched pre-therapy levels of intra-tumoral CD8 Temra cells and/or intra-tumoral CXCL10-positive macrophages are markers indicative of a positive response. An alternative pre-therapy marker indicative of a positive response relies on detecting enhanced levels of T cell receptor sharing between intra-tumoral and peripheral CD8 Temra cells. The invention therefore refers to methods of e.g. selecting a patient eligible for therapy comprising an immune checkpoint blocker or for therapy with an immune checkpoint blocker including determining the levels of these markers.



USE OF INTRA-TUMORAL CD8 TEMRA CELLS FOR PREDICTING RESPONSE
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FIELD OF THE INVENTION

5 The invention relates to the field of predicting (clinical) response of a cancer patient to a therapy comprising an immune checkpoint blocker or to therapy with an immune checkpoint blocker. In particular enriched pre-therapy levels of intra-tumoral CD8 Temra cells and/or intra-tumoral CXCL10-positive macrophages are markers indicative of a positive response. An alternative pre-therapy marker indicative of a positive response relies on detecting enhanced levels of T cell receptor sharing between
10 intra-tumoral and peripheral CD8 Temra cells. The invention therefore refers to methods of e.g. selecting a patient eligible for therapy comprising an immune checkpoint blocker or for therapy with an immune checkpoint blocker including determining the levels of these markers.

BACKGROUND OF THE INVENTION

15 A tumor is forced to create its own specific "ecosystem" within the surrounding healthy tissue. Many factors and processes are decisive over whether or not a single tumor cell will be able to create, and support, its ecosystem and, therewith, growth. In an attempt to create a simplifying overview, Blank et al. 2016 (Science 352: 658-660) designed a visually appealing "cancer immunogram" in which currently known factors and processes influencing tumor growth/survival are grouped in seven classes of
20 parameters. For each individual patient/tumor, the status of the seven classes of parameters can be plotted, the resulting plot giving insight in treatment options. Somewhat similar to the cancer immunogram, Charoentong et al. 2017 (Cell Reports 18:248-262) designed an immunophenogram/immunophenoscore which provides an as yet to be further validated tool for predicting response of a tumor to immune checkpoint blockade therapy (ICBT; as the majority of cancer
25 patients do not respond to such therapy). Such tools strongly underscore the need to expand knowledge on the status of a tumor or cancer as this, besides potentially leading to identification of new therapeutic targets, aids in deciding on the optimal (available) treatment for each individual tumor.

Identification of biomarkers capable of determining whether a cancer patient is responding to ongoing ICBT is one challenge. An even more daunting challenge is to find tools that can distinguish cancer
30 patients responding to ICBT from cancer patients not responding to ICBT even before start of the ICBT. The latter is of crucial importance in fragile cancer patients for which time for second or third line treatments may be lacking, therewith creating a narrow window-of-opportunity for successful treatment. Choosing the correct treatment upfront is therefore of utmost importance. Furthermore, in earlier stages of the disease, patients are more frequently eligible for curative resection of a tumor. Such

resection is often preceded by ((neo-)adjuvant) therapy, such as ICBT, prior to surgery. Successful ((neo-)adjuvant) therapy minimizes side-effects and/or tumor progression, and therewith minimizes the risk of delay of surgery. Biomarkers that identify patients that will benefit from (neo-)adjuvant therapy are therefore necessary.

- 5 Blood CD8 T_{EMRA} levels have been studied in the context of ICBT for a number of cancer types (head and neck squamous cell carcinoma: Tada et al. 2022, Cancer Immunol Immunother 71:851–863; recurrent/metastatic nasopharyngeal carcinoma: Kwong et al. 2020, J Clin Oncol 38, 15 Suppl, Abstract e18519; mesothelioma: Mankor et al. 2020, EBioMedicine 62:103040; lung cancer: Kunert et al. 2019, J ImmunoTher Cancer 7:149; Iwahori et al. 2019, Sci Rep 9:2636). Increased pre-treatment blood CD8
- 10 T_{EMRA} levels were reported to be associated with positive ICBT response for NSCLC and mesothelioma. CD8 T_{EMRA} cells are known to be highly mobile between blood and (tumor)tissue (e.g. Zheng et al. 2021, Science 374:abe6474). A low level of “double positive” CD8 T_{EMRA} cells in blood (and thus assumingly also in the tumor) was reported to be beneficial for ICB response in lung cancer – double positive being CD27+ and CD28+ (Lee et al. 2021, J Immunother Cancer 9:e002709, and WO2022/216021).
- 15 TCR repertoire profiles have been linked with ICBT response (e.g. Porciello et al. 2022, J Exp Clin Cancer Res 41:356).

The involvement of CXCL9/10-secreting macrophages in establishing a response to ICBT has been described (House et al. 2020, Clin Cancer Res 26 :487-504).

20 SUMMARY OF THE INVENTION

The present disclosure relates to several methods.

Such methods include methods of selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI, or of predicting the response, the likelihood of response, or responsiveness of a subject having cancer to therapy including an immune checkpoint

- 25 inhibitor (ICI) or to therapy with an ICI, such method comprising quantifying the abundance of CD8 Temra cells in a pre-therapy tumor biopsy sample obtained from the subject, and

selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an

- 30 ICI when the abundance of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to reference pre-therapy tumor biopsy sample CD8 Temra cell abundances that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI.

Such methods further include methods of selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI, or of predicting the response, the likelihood

of response, or responsiveness of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI, such method comprising quantifying the abundance of CD8 Temra cells and of CXCL10-positive macrophages in a pre-therapy tumor biopsy sample obtained from the subject, and

5 selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an ICI when the abundances of CD8 Temra cells and of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample correspond to reference pre-therapy tumor biopsy sample CD8 Temra cell and CXCL10-positive macrophage abundances that are indicative of response of a subject having the cancer
10 to the therapy including an ICI or to the therapy with an ICI.

In the above methods, the abundance of CD8 Temra cells is quantified by means of quantifying the expression of CD8 Temra cell marker genes.

In the above methods, the abundance of CXCL10-positive macrophages is quantified by means of quantifying expression of CXCL10-positive macrophage marker genes.

15 Such marker gene expression can e.g. be analyzed in (m)RNA isolated from the pre-therapy biopsy sample; by means of single cell sequencing of cells of the pre-therapy biopsy sample; or by means of spatial transcriptomic analysis of the pre-therapy biopsy sample.

In the above methods, the abundance of CD8 Temra cells can alternatively be quantified by means of immunohistochemical phenotyping, by spatial phenotyping or spatial proteomics, by means of whole-
20 tissue single-cell imaging of CD8 Temra cells in the pre-therapy biopsy sample.

In the above methods, the abundance of CXCL10-positive macrophages can alternatively be quantified by means of immunohistochemical phenotyping, by spatial phenotyping or spatial proteomics, by means of whole-tissue single-cell imaging of CD8 Temra cells in the pre-therapy biopsy sample.

Any of the above methods may include a further step of determining the level of T cell receptor (TCR)
25 sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample obtained from the same subject. In a further embodiment thereto, such methods may include a step wherein a subject having cancer is selected for therapy including an ICI or for therapy with an ICI, or wherein a subject having cancer is predicted to respond, or likely to respond to therapy including an ICI or to therapy with an ICI when additionally the level of TCR sharing
30 corresponds to reference levels of TCR sharing that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI. In a further embodiment thereto, such methods may include a further step of determining the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in an on-therapy blood sample obtained from the same subject. In a further embodiment thereto, such methods may include a further

step of determining the subject to respond to the therapy including an ICI or to the therapy with an ICI when the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in the on-therapy blood sample is not significantly lower than the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample.

This disclosure further includes methods of selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI, or of predicting the response, the likelihood of response, or responsiveness of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI, such method comprising

determining the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample obtained from the same subject, and

selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an ICI when the level of TCR sharing corresponds to reference levels of TCR sharing that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI.

This disclosure further includes methods of determining response of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI, such method comprising determining the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample obtained from the same subject; determining the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in an on-therapy blood sample obtained from the same subject; determining the subject to respond to the therapy including an ICI or to the therapy with an ICI when the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in the on-therapy blood sample is not significantly lower than the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample.

This disclosure also relates to immune checkpoint inhibitors (ICIs) for use in treating or inhibiting cancer, for use in inhibiting cancer progression, or for use in inhibiting cancer relapse, comprising selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an ICI as determined in or with any of the hereinabove described methods.

This disclosure also relates to diagnostic kits for use in any of the hereinabove described methods wherein such kits are comprising the tools to quantify the abundance of CD8 Temra cells in a pre-therapy biopsy sample, the abundance of CXCL10-positive macrophages in a pre-therapy biopsy sample, or the level of TCR sharing in CD8 Temra cells in a pre-therapy biopsy sample and in a blood sample.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Study design, patient and sample overview. PBMC: peripheral blood mononuclear cells; W0/3/6: week 0/3/6.

FIGURE 2. The intra-tumoral T-cell (CD4+ and CD8+) and NK-cell composition is distinct from the peripheral T-cell (CD4+ and CD8+) and NK-cell composition. (A) Heatmaps showing expression of marker genes used for annotation of intra-tumoral T-cell (CD4+ and CD8+) and NK-cell phenotypes. (B) Heatmaps of showing the expression of marker genes used for annotation of peripheral T-cell (CD4+ and CD8+) and NK-cell cell phenotypes. GD: gamma-delta T-cells. (C) Violin plot of CD8A and CD8B expression in intra-tumoral CD8 Temra and cytotoxic NK cells.

FIGURE 3. Clonally-expanded CD45RA effector-memory CD8 T-cells (CD8 Temra) are associated with response to immune checkpoint inhibitors (CPI) (A) Boxplots depicting relative abundance of intra-tumoral CD8 T-cell phenotypes in CPI treated patients (n=21), calculated per patient and stratified for response. P-values calculated using Mann-Whitney U-test, only p-values <0.05 are shown. R: responder; NR: non-responder.

(B) Boxplots depicting average *PDCD1* (PD1) expression level, calculated per patient (n=31) in each intra-tumoral CD8 T-cell phenotype.

(C) Gini-index of intra-tumoral CD8 T-cells calculated for CPI-treated patients (n=21), calculated per patient and stratified for response. P-values calculated using two-sample T-test or Mann-Whitney U-test, as appropriate, only p-values <0.05 are shown. R: responder; NR: non-responder.

(D) Kaplan-Meier plot of progression free survival in CPI-treated patients (n=20) with high or low (split by median) Gini-index in intra-tumoral CD8 Temra.

FIGURE 4. T-cell receptor (TCR) sharing confirms CD8 Temra as crucial effector T-cells in the tumor micro-environment (TME) of hepatocellular carcinoma. (A) Proportion of TCRs shared between tumor and blood at prior to treatment (PBMC week 0), relative to the total number of TCRs detected, calculated per sample and stratified for response. P values calculated using Mann-Whitney U-test. R: responder; NR: non-responder. (B) Kaplan-Meier plot of progression-free survival in CPI-treated patients (n=14) with high or low (split by median) TCR sharing between tumor and blood. PBMC: peripheral blood mononuclear cells; W0: week 0.

FIGURE 5. Pro-inflammatory PDL1-expressing CXCL10+ macrophages recruit effector-memory T cells into the TME. (A) Boxplots depicting average CD274 (PDL1) expression level in the TME of CPI-treated patients (n=21), calculated per patient in myeloid cells (left) and CXCL10+ macrophages (Macro CXCL10, right), stratified for response. P-values calculated using Mann-Whitney U-test. R: responder; NR: non-responder. (B) Kaplan-Meier plot of progression free survival in CPI-treated patients (n=21) with high or low (split by median) PDL1-expression in CXCL10+ macrophages.

FIGURE 6. Relative expression levels of CD27 and CD28 in (A) intra-tumoral immune cells and (B) peripheral immune cells. Intra-tumoral and peripheral CD8 Temra cells are CD27-negative and CD28-negative (double negative). MAIT: mucosal-associated invariant T cell.

FIGURE 7. Intra-tumoral differentiation of CD8 T-cells. Density of shared T-cells along each indicated CD8 trajectory in CPI-treated patients (n=14) stratified for response. The density plots reflect the relative number of shared T-cells separately for responders versus non-responders along each indicated CD8 trajectory. Shared T-cells are characterized by a TCR found in peripheral blood prior to treatment. P-values reflect the difference in distributions, calculated using the Kolmogorov-Smirnov test.

FIGURE 8. Intra-tumoral differentiation of CD8 T-cells. Top pathways identified using fgSEA on DEGs along CD8 T_{EX} versus CD8 T_{EMRA} trajectory (using *diffEnd* test; TradeSeq) for the REACTOME and GO: Biological processes gene sets. Significantly enriched pathways (adjusted p-value <0.01) were identified and ranked based on enrichment score for each gene set separately. Only the top pathways of each gene set, enriched in each trajectory were retained.

FIGURE 9. Intra-tumoral differentiation of CD8 T-cells. UMAP representation of peripheral T-cells (PBMCs) at week 0 (W0), week 3 (W3) and week 6 characterized by a TCR shared with CD8 T_{EX} (A) and CD8 T_{EMRA} (B) in the pre-treatment TME, stratified for response to CPI (top: responders, bottom: non-responders).

FIGURE 10. Monocytes and macrophages in the TME of HCC. (A) Heatmap displaying the expression of marker genes in the identified monocyte and macrophage phenotypes. (B) Heatmap depicting the expression of functional genes in macrophage subtypes.

FIGURE 11. CD8 T_{EMRA} and CXCL10-positive macrophages (Macro CXCL10) each are predictive biomarkers of response to CPI, but not of response to tyrosine kinase inhibitor (TKI) therapy. Kaplan-Meier plots of progression-free survival (PFS) in 300 CPI-treated (*top*) versus 58 TKI-treated advanced HCC patients (*bottom*) with high versus low (split by median) CD8 T_{EMRA} (*left*) or Macro CXCL10 (*right*) enrichment score, calculated per sample in bulk RNAs.

FIGURE 12. CD8 T_{EMRA} and CXCL10-positive macrophages (Macro CXCL10) each are predictive biomarkers of response to CPI, but not of response to tyrosine kinase inhibitor (TKI) therapy. Scatter plot depicting the correlation between CD8 T_{EMRA} and Macro CXCL10 enrichment scores, calculated per sample in bulk

RNA of 358 advanced HCC patients (as in Figure 11). Spearman correlation coefficient and p-value as indicated.

FIGURE 13. CD8 T_{EMRA} and CXCL10-positive macrophages (Macro CXCL10) combined as predictive biomarker of response to CPI, but not of response to tyrosine kinase inhibitor (TKI) therapy. Kaplan-Meier plots of OS (left) and PFS (*right*) in 300 CPI-treated (*top*) versus 58 TKI-treated advanced HCC patients (*bottom*) (as in Figure 11) with high versus low CPI-response biomarker enrichment score (using optimal cut-off determined by maximally selected rank statistics), calculated per sample in bulk RNA.

DETAILED DESCRIPTION

Despite great efforts to characterize the tumor-microenvironment (TME) of hepatocellular carcinoma (HCC) (Zheng et al. 2017, Cell 169:1342-1356; Zhang et al. 2019; Cell 179:829-845; Ma et al. 2019, Cancer Cell 36:418-430; Ma et al. 2021, J Hepatol 75:1397-1408; Xue et al. 2022, Nature 612:141-147), factors associated with response/resistance to immune checkpoint inhibitors (CPI) remain to be elucidated. PD1-expressing CD8 T-cells have been identified as key effector cells in response to CPI in several tumor types, including breast cancer (Bassez et al. 2021, Nat Med 27:820-832), lung cancer (Thommen et al. 2018, Nat Med 24:994-1004) and melanoma (Sade-Feldman et al. 2018, Cell 175:998-1013), where persistent exposure of CD8 T-cells to tumor antigens will stimulate differentiation towards a dysfunctional, exhausted phenotype. CD8 T-cells express typical exhaustion markers, such as PDCD1 (PD1), upon which their activation status and anti-tumoral cytolytic function are dampened. PD(L)1 blockade reinvigorates the anti-tumoral immune response leading to proliferation of these cytotoxic T-cells that are able to overcome tumor-induced immunosuppression and induce durable clinical benefits. However, in HCC, the role of PD1+ CD8 T cells is controversial. The presence of exhausted PD1+ CD8 T-cells in HCC has been associated with a more aggressive disease biology (Kim et al. 2022, Gastroenterol 155:1936-1950) and poor prognosis (Barsch et al. 2022, J Hepatol 77:397-409). Moreover, in pre-clinical models of non-alcoholic steatohepatitis (NASH)-associated HCC, exhausted, unconventionally activated PD1+ CD8 T-cells were related to impaired tumor surveillance, causing tissue damage and facilitating, rather than inhibiting hepatocarcinogenesis upon anti-PD1 treatment (Pfister et al. 2021, Nature 592:450-456). The differences between HCC and other solid tumor types might be in part explained by the unique immune context of the liver as well as the fact that liver cancer most often develops in a background of chronic inflammation caused by a variety of chronic liver diseases (Llovet et al. 2021, Nat Rev Dis Prim 7:62).

Standard treatments of HCC consist of immune checkpoint inhibitor therapy (ICBT; 15-20% response rate), anti-angiogenic therapy, or a combination of both (~35% response rate). ICB therapy and/or anti-angiogenic therapies have been compared to the previous standard of care treatment (i.e. tyrosine

kinase inhibitors) in large phase III trials. True head-to-head comparisons are missing and will likely never be available. In the fragile HCC population, there is a constant threat of liver decompensation that renders further, e.g. second or third line, treatments often impossible. With this very narrow window-of-opportunity for successful treatment, choosing the correct treatment upfront is of utmost importance.

Work leading to current invention focused on the response of HCC patients to ICBT. The presence of high levels of CD45RA+ effector-memory CD8+ T-cells (referred to hereinafter as CD8 Temra or CD8 T_{EMRA}; PD1-negative) in a pre-treatment biopsy of hepatocellular cancer was found to be indicative of a positive response to ICBT (with non-responders having lower levels of CD8 Temra). Such correlation was not clear when looking only at blood CD8 Temra levels. Furthermore, no such correlation was present for levels intra-tumoral PD1+ CD8+ T-cells, the more commonly recognized key effector cell in ICB response. The tumor CD8 Temra shared T cell receptors (TCRs) with blood CD8 Temra to a high degree, and the proportion of CD8 T_{EMRA} with shared TCRs remained high in responders (compared to non-responders). Both blood and tumor CD8 Temra were negative for both CD27 and CD28. Furthermore, enrichment of PDL1-expressing CXCL10+ macrophages (PDL1+ CXCL10+ macrophages, or PDL1-positive CXCL10-positive macrophages) in ICBT-responsive tumors was found as further biomarker.

The initial work involved single cell transcriptomic (scRNAseq) and T-cell receptor sequencing (scTCRseq) analysis of pre-treatment tissue biopsies and serial (pre- and on-treatment) peripheral blood mononuclear cell samples of HCC patients stratified according to clinical response to ICBT – (n = 37, of which 30 treated with aPDL1, 5 with Tyr kinase inhibitor, and 2 untreated – see Figure 1).

A subsequent validation was done on bulk RNA data of 358 pre-treatment biopsies (n = 253 treated with aPDL1 (atezolizumab) + aVEGF (bevacizumab); n = 47 treated with aPDL1; n = 58 treated with Tyr kinase inhibitor (sorafenib)). Intratumoral CD8 Temra and PDL1+ CXCL10+ macrophages were, individually or combined, confirmed to be associated with longer progression-free survival in patients treated with ICB (-including) therapy, but not in patients treated with the kinase inhibitor.

In a first aspect, the current disclosure relates to methods of/for selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI, such methods comprising

measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CD8 Temra cells in a pre-therapy tumor biopsy sample obtained from the subject, and

selecting a subject having cancer for therapy including an ICI or for therapy with an ICI

when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample is within a range of reference pre-therapy tumor biopsy sample CD8 Temra cell abundances, levels, proportions, frequencies or numbers, wherein the reference pre-therapy tumor biopsy sample CD8 Temra cell abundances, levels, proportions, frequencies or numbers are indicative of

5 response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI; or when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CD8 Temra cell abundances, levels, proportions, frequencies or numbers that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI; or

10 when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CD8 Temra cell abundances, levels, proportions, frequencies or numbers of subjects having the cancer and known to respond to the therapy including an ICI or to the therapy with an ICI.

15 In an alternative first aspect, the current disclosure relates to methods of/for predicting the response, predicting the likelihood of response, or predicting the responsiveness of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI, such methods comprising

measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CD8 Temra cells in a pre-therapy tumor biopsy sample obtained from the subject, and

20 predicting a subject having cancer to respond or likely to respond to therapy including an ICI or to therapy with an ICI

when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample is within a range of reference pre-therapy tumor biopsy sample CD8 Temra cell abundances, levels, proportions, frequencies or numbers, wherein the reference pre-therapy tumor biopsy sample CD8 Temra cell abundances, levels, proportions, frequencies or numbers are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI; or

25 when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CD8 Temra cell abundances, levels, proportions, frequencies or numbers that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI; or

30 when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CD8 Temra cell

abundances, levels, proportions, frequencies or numbers of subjects having the cancer and known to respond to the therapy including an ICI or to the therapy with an ICI.

In a second aspect, the current disclosure relates to methods of/for selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI, such methods comprising

measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in a pre-therapy tumor biopsy sample obtained from the subject, and selecting a subject having cancer for therapy including an ICI or for therapy with

an ICI

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample is within a range of reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers, wherein the reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI; or

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CXCL10-positive macrophages abundances, levels, proportions, frequencies or numbers that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI; or

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CXCL10-positive macrophages abundances, levels, proportions, frequencies or numbers of subjects having the cancer and known to respond to the therapy including an ICI or to the therapy with an ICI.

In an alternative second aspect, the current disclosure relates to methods of/for predicting the response, or predicting the likelihood of response, or predicting the responsiveness of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI, such methods comprising

measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in a pre-therapy tumor biopsy sample obtained from the subject, and predicting a subject having cancer to respond or likely to respond to therapy including an ICI or to therapy with an ICI

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample is within a range of reference CXCL10-positive macrophage

abundances, levels, proportions, frequencies or numbers, wherein the reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI; or

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CXCL10-positive macrophages abundances, levels, proportions, frequencies or numbers that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI; or when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CXCL10-positive macrophages abundances, levels, proportions, frequencies or numbers of subjects having the cancer and known to respond to the therapy including an ICI or to the therapy with an ICI.

In a third aspect, this disclosure relates to methods of selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI, such methods comprising measuring, determining, assessing, quantifying, or analyzing the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample obtained from the same subject, and selecting a subject having cancer for therapy including an ICI or for therapy with an ICI when the level of TCR sharing corresponds to reference levels of TCR sharing that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI.

In an alternative third aspect, this disclosure relates to methods of predicting the response, the likelihood of response, or responsiveness of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI, such method comprising measuring, determining, assessing, quantifying, or analyzing the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample obtained from the same subject, and predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an ICI when the level of TCR sharing corresponds to reference levels of TCR sharing that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI.

In one embodiment to the third aspect, the methods can further include the measuring, determining, assessing, quantifying, or analyzing the level of TCR sharing between the CD8 Temra cells in the pre-

therapy tumor biopsy sample and the CD8 Temra cells in an on-therapy blood sample obtained from the same subject.

In a fourth aspect, this disclosure relates to methods of determining response of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI, such methods comprising

measuring, determining, assessing, quantifying, or analyzing the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample obtained from the same subject;

measuring, determining, assessing, quantifying, or analyzing the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in an on-therapy blood sample obtained from the same subject;

determining the subject to respond to the therapy including an ICI or to the therapy with an ICI when the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in the on-therapy blood sample is not significantly lower than the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample.

The 1st to 4th aspect as described hereinabove can be combined in any way or any order. Such combinations thus include, without being exhaustive:

a) methods relying on measuring, determining, assessing, quantifying, or analyzing CD8 Temra cells and relying on measuring, determining, assessing, quantifying, or analyzing CXCL10-positive macrophages; or

b) methods relying on measuring, determining, assessing, quantifying, or analyzing CD8 Temra cells and relying on pre-therapy TCR sharing between tumoral and peripheral CD8 Temra cells; or

c) methods relying on measuring, determining, assessing, quantifying, or analyzing CXCL10-positive macrophages and pre-therapy TCR sharing between tumoral and peripheral CD8 Temra cells; or

d) methods relying on measuring, determining, assessing, quantifying, or analyzing CD8 Temra cells and relying on measuring, determining, assessing, quantifying, or analyzing CXCL10-positive macrophages, and relying on pre-therapy TCR sharing between tumoral and peripheral CD8 Temra cells.

Furthermore any of the methods of a) to d) can further be combined with on-treatment steps relying on comparing levels of TCR sharing between pre-treatment tumoral CD8 Temra and on-treatment peripheral CD8 Temra and levels of pre-therapy TCR sharing between tumoral and peripheral CD8 Temra cells.

As one example, the methods of a) are exemplified hereinafter. Such methods of/for selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI, are methods comprising

measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CD8 Temra cells and of CXCL10-positive macrophages in a pre-therapy tumor biopsy sample obtained from the subject, and selecting a subject having cancer for therapy including an ICI or for therapy with an ICI

when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample is within a range of reference CD8 Temra abundances, levels, proportions, frequencies or numbers, wherein the reference CD8 Temra abundances, levels, proportions, frequencies or numbers are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI, and

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample is within a range of reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers, wherein the reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI;

or

when the abundance, level, proportion, frequency or number of CD8 Temra cells and the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample each are within a range of reference CD8 Temra cell and of reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers, respectively, wherein the reference CD8 Temra cell abundances, levels, proportions, frequencies or numbers and the reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI;

or

when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CD8 Temra abundances, levels, proportions, frequencies or numbers that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI, and

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CXCL10-positive macrophages abundances, levels, proportions, frequencies or numbers that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI;

or

when the abundances of CD8 Temra cells and of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample correspond to pre-therapy tumor biopsy sample CD8 Temra cell and CXCL10-positive macrophage abundances, respectively, that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI;

or

when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CD8 Temra abundances, levels, proportions, frequencies or numbers of subjects having the cancer and known to respond to the therapy including an ICI or to the therapy with an ICI, and

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CXCL10-positive macrophages abundances, levels, proportions, frequencies or numbers of subjects having the cancer and known to respond to the therapy including an ICI or to the therapy with an ICI;

or

when the abundance, level, proportion, frequency or number of CD8 Temra cells and of CXCL10-positive macrophage in the pre-therapy tumor biopsy sample each corresponds to (reference) pre-therapy tumor biopsy sample CD8 Temra cell abundances, levels, proportions, frequencies or numbers and of CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers of subjects having the cancer and known to respond to the therapy including an ICI or to the therapy with an ICI.

Alternatively, such methods of a) are methods of/for predicting the response, or predicting the likelihood of response, or predicting the responsiveness of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI, and such methods are comprising

measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CD8 Temra cells and of CXCL10-positive macrophages in a pre-therapy tumor biopsy sample obtained from the subject, and predicting a subject having cancer to respond or likely to respond to therapy including an ICI or to therapy with an ICI

when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample is within a range of reference CD8 Temra abundances, levels, proportions, frequencies or numbers, wherein the reference CD8 Temra abundances, levels, proportions, frequencies or numbers are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI, and

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample is within a range of reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers, wherein the reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI;

or

when the abundance, level, proportion, frequency or number of CD8 Temra cells and the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample each are within a range of reference CD8 Temra cell and of reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers, wherein the reference CD8 Temra cell abundances, levels, proportions, frequencies or numbers and the reference CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI;

or

when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CD8 Temra abundances, levels, proportions, frequencies or numbers that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI, and

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CXCL10-positive macrophages abundances, levels, proportions, frequencies or numbers that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI;

or

when the abundances of CD8 Temra cells and of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample correspond to pre-therapy tumor biopsy sample CD8 Temra cell and CXCL10-positive macrophage abundances, respectively, that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI;

or

when the abundance, level, proportion, frequency or number of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CD8 Temra abundances, levels, proportions, frequencies or numbers of subjects having the cancer and known to respond to the therapy including an ICI or to the therapy with an ICI, and

when the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the pre-therapy tumor biopsy sample corresponds to (reference) pre-therapy tumor biopsy sample CXCL10-

positive macrophage abundances, levels, proportions, frequencies or numbers of subjects having the cancer and known to respond to the therapy including an ICI or to the therapy with an ICI;

or

when the abundance, level, proportion, frequency or number of CD8 Temra cells and of CXCL10-positive macrophage in the pre-therapy tumor biopsy sample each corresponds to (reference) pre-therapy tumor biopsy sample CD8 Temra cell abundances, levels, proportions, frequencies or numbers and of CXCL10-positive macrophage abundances, levels, proportions, frequencies or numbers of subjects having the cancer and known to respond to the therapy including an ICI or to the therapy with an ICI.

- 10 The methods of the first and/or second aspect (i.e. first aspect, second aspect, or first and second aspects combined) can further include a step of measuring, determining, assessing, quantifying, or analyzing the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample obtained from the same subject. Such methods can optionally further include a step of selection of a subject having cancer for therapy including an ICI or for therapy with an ICI, or of predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an ICI when (additionally; additional to basing the selection or prediction on pre-therapy tumoral CD8 Temra and/or CXCL10-positive macrophage abundances) the level of TCR sharing corresponds to reference levels of TCR sharing that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI.
- 20 Optionally, such methods can further include a step of measuring, determining, assessing, quantifying, or analyzing the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in an on-therapy blood sample obtained from the same subject. Such methods can further optionally include a step of determining the subject to respond to the therapy including an ICI or to the therapy with an ICI when the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in the on-therapy blood sample is not significantly lower than the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample.

- 30 The methods of the first and/or second aspect can further include a step of measuring, determining, assessing, quantifying, or analyzing the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in an on-therapy blood sample obtained from the same subject. Such methods can optionally further include a step of determining the subject to respond to the therapy including an ICI or to the therapy with an ICI when the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in the on-therapy blood

sample is not significantly lower than the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample.

In a further aspect, the present disclosure relates to an ICI for use in treating or inhibiting cancer in a subject, for use in inhibiting cancer progression in a subject, or for use in inhibiting cancer relapse in a subject, (the use) comprising or including any of the methods or combinations thereof as described hereinabove (methods or combination of methods relating to selection of subjects eligible for or predicted to (likely) respond to a therapy including an ICI or to a therapy with an ICI).

Alternatively, the present disclosure relates to an ICI for use in treating or inhibiting cancer in a subject, for use in inhibiting cancer progression in a subject, or for use in inhibiting cancer relapse in a subject, if/wherein the subject is selected for therapy comprising an ICI or for therapy with an ICI according to any of the relevant methods or combinations thereof as described hereinabove; or if/wherein the subject is predicted to respond or likely to respond to therapy comprising an ICI or to therapy with an ICI according to any of the relevant methods or combinations thereof as described hereinabove.

Thus, for example, the present disclosure relates to an ICI for use in treating or inhibiting cancer, for use in inhibiting cancer progression, or for use in inhibiting cancer relapse, (the use) comprising or including for instance

quantifying the abundance of CD8 Temra cells in a pre-therapy tumor biopsy sample obtained from the subject, and

selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an ICI when the abundance of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to pre-therapy tumor biopsy sample CD8 Temra cell abundances that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI.

In a further aspect, the present disclosure relates to use of an ICI for manufacturing/in the manufacture of a medicament for treating or inhibiting cancer in a subject, for inhibiting cancer progression in a subject, or for inhibiting cancer relapse in a subject, comprising or including any of the methods or combinations thereof as described hereinabove (methods or combination of methods relating to selection of subjects eligible for or predicted to (likely) respond to a therapy including an ICI or to a therapy with an ICI).

Alternatively, the present disclosure relates to use of an ICI for manufacturing/in the manufacture of a medicament for treating or inhibiting cancer in a subject, for inhibiting cancer progression in a subject, or for inhibiting cancer relapse in a subject, wherein the subject is selected for therapy comprising an ICI

or for therapy with an ICI according to any of the relevant methods or combinations thereof as described hereinabove; or wherein the subject is predicted to respond or likely to respond to therapy comprising an ICI or to therapy with an ICI according to any of the relevant methods or combinations thereof as described hereinabove.

Thus, for example, the present disclosure relates to use of an ICI for manufacturing/in the manufacture of a medicament for treating or inhibiting cancer in a subject, for inhibiting cancer progression in a subject, or for inhibiting cancer relapse in a subject, comprising or including for instance quantifying the abundance of CD8 Temra cells in a pre-therapy tumor biopsy sample obtained from the subject, and

selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an ICI when the abundance of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to pre-therapy tumor biopsy sample CD8 Temra cell abundances that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI.

In a further aspect, the present disclosure relates to methods of treating or inhibiting cancer in a subject, of inhibiting cancer progression in a subject, or of inhibiting cancer relapse in a subject, such methods comprising or including any of the methods or combinations thereof as described hereinabove (methods or combination of methods relating to selection of subjects eligible for or predicted to (likely) respond to a therapy including an ICI or to a therapy with an ICI), and further comprising or including administering to a selected subject a therapy including an ICI or a therapy with an ICI, or comprising or including administering a therapy including an ICI or a therapy with an ICI to a subject predicted or likely predicted to respond to such therapy. Alternatively, the present disclosure relates to methods of treating or inhibiting cancer in a subject, of inhibiting cancer progression in a subject, or of inhibiting cancer relapse in a subject, wherein the subject is selected for therapy comprising an ICI or for therapy with an ICI according to any of the relevant methods or combinations thereof as described hereinabove; or wherein the subject is predicted to respond or likely to respond to therapy comprising an ICI or to therapy with an ICI according to any of the relevant methods or combinations thereof as described hereinabove. With administering a (or an effective dose of a) therapy including an ICI or a (or an effective dose of a) therapy with an ICI, the cancer, cancer progression or cancer relapse in a subject is treated or inhibited.

Thus, for example, the present disclosure relates to methods of treating or inhibiting cancer in a subject, of inhibiting cancer progression in a subject, or of inhibiting cancer relapse in a subject, such methods comprising or including for instance

quantifying the abundance of CD8 Temra cells in a pre-therapy tumor biopsy sample obtained from the subject,

selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an

5 ICI when the abundance of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to pre-therapy tumor biopsy sample CD8 Temra cell abundances that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI, and administering to a selected subject a therapy including an ICI or a therapy with an ICI.

10 Alternatively, this disclosure relates to methods of administering to a subject a therapy including an ICI or a therapy with an ICI such as for treating or inhibiting cancer in the subject, for inhibiting cancer progression in the subject, or for inhibiting cancer relapse in the subject, such methods comprising or including any of the methods or combinations thereof as described hereinabove (methods or combination of methods relating to selection of subjects eligible for or predicted to (likely) respond to a

15 therapy including an ICI or to a therapy with an ICI), and further comprising or including administering to a selected subject a therapy including an ICI or a therapy with an ICI, or comprising or including administering a therapy including an ICI or a therapy with an ICI to a subject predicted or likely predicted to respond to such therapy. Alternatively, in such methods of treating or inhibiting cancer in a subject, of inhibiting cancer progression in a subject, or of inhibiting cancer relapse in a subject, the subject is

20 selected for therapy comprising an ICI or for therapy with an ICI according to any of the relevant methods or combinations thereof as described hereinabove; or the subject is predicted to respond or likely to respond to therapy comprising an ICI or to therapy with an ICI according to any of the relevant methods or combinations thereof as described hereinabove.

With administering a (or an effective dose of a) therapy including an ICI or a (or an effective dose of a)

25 therapy with an ICI, the cancer, cancer progression or cancer relapse in a subject is treated or inhibited. Thus, for example, the present disclosure relates to methods of administering to a subject a therapy including an ICI or a therapy with an ICI such as for treating or inhibiting cancer in the subject, for inhibiting cancer progression in the subject, or for inhibiting cancer relapse in the subject, such methods comprising or including for instance

30 quantifying the abundance of CD8 Temra cells in a pre-therapy tumor biopsy sample obtained from the subject,

selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an ICI when the abundance of CD8 Temra cells in the pre-therapy tumor biopsy sample corresponds to pre-

therapy tumor biopsy sample CD8 Temra cell abundances that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI, and administering to a selected subject a therapy including an ICI or a therapy with an ICI.

- 5 In one embodiment to the aspects implying measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CD8 Temra cells, measurement, determination, assessment, quantification or analysis of the abundance of CD8 Temra cells is by means of differential gene expression analysis, such as in/of RNA (such as in/of bulk RNA) isolated from the pre-therapy biopsy sample; by means of single cell sequencing (RNA (cDNA) sequencing) such as of cells of
10 the pre-therapy biopsy sample; or by means of spatial transcriptomics (e.g. FISH or sequencing) analysis such as in/of a pre-therapy biopsy sample. Alternatively, the abundance, level, proportion, frequency or number of CD8 Temra cells is measured, determined, assessed, quantified, or analyzed by means of differential gene expression analysis, such as in/of RNA (such as in/of bulk RNA) isolated from the pre-therapy biopsy sample; by means of single cell sequencing (RNA (cDNA) sequencing) such as of cells of
15 the pre-therapy biopsy sample; or by means of spatial transcriptomics (e.g. FISH or sequencing) analysis such as in/of a pre-therapy biopsy sample. In particular, the measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CD8 Temra cells in the sample obtained from a subject having cancer is performed *in vitro* in or on the said sample.
- 20 Some of the above-described aspects or embodiments imply measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CD8 Temra cells in a blood sample, or in any derivative or processed form of a blood sample still comprising CD8 Temra cells. The abundance, level, proportion, frequency or number of CD8 Temra cells therein is measured, determined, assessed, quantified, or analyzed by means of differential gene expression analysis, such as
25 in/of RNA (such as in/of bulk RNA) isolated from the pre- or on-therapy blood sample (or derivative or processed form thereof) or by means of single cell sequencing (RNA (cDNA) sequencing) such as of cells of the pre- or on-therapy blood sample (or derivative or processed form thereof). Spatial transcriptomics (e.g. FISH or sequencing) analysis is less applicable.
- 30 In a further embodiment thereto, such differential gene expression analysis or spatial transcriptomics analysis is performed on/with a set of (human) marker genes chosen from GZMH (protein granzyme H; UniProt protein alternative names: CCP-X, cathepsin G-like 2, cytotoxic T-lymphocyte proteinase, cytotoxic serine protease C (CSP-C); UniProt gene name synonyms CGL2, CTSG2), GNLY (protein granulysin; UniProt protein alternative names: lymphokine LAG-2, protein NKG5, T-cell activation protein

519; UniProt gene name synonyms LAG2, NKG5, TLA519), NKG7 (protein natural killer cell granule protein 7; UniProt protein alternative names: G-CSF-induced gene 1 protein (GIG-1 protein), granule membrane protein of 17 kDa (GMP-17), natural killer cell protein 7, p15-TIA-1; UniProt gene synonym GIG1), FGF2 (protein fibroblast growth factor binding protein 2; UniProt protein alternative names: FGF-BP2, FGF-binding protein 2; 37 kDa killer-specific secretory protein (Jsp37), HBp17-related protein (HBp17-RP); UniProt gene name synonym KSP37), GZMB (protein granzyme B; UniProt protein alternative names: C11, CTLA-1, cathepsin G-like 1 (CTSG1), cytotoxic T-lymphocyte proteinase 2, fragmentin-2; UniProt gene name synonyms CGL1, CSPB, CTLA1, GRB), CST7 (protein cystatin 7; UniProt protein alternative names: cystatin-like metastasis-associated protein (CMAP), leukocystatin; no UniProt gene name synonyms), CCL5 (protein C-C motif chemokine 5; UniProt protein alternative names: EoCP, eosinophil chemotactic cytokine, SIS-delta, small-inducible cytokine A5, T cell-specific protein P228 (TCP228), T cell-specific protein RANTES; UniProt gene name synonyms D17S136E, SCYA5), PRF1 (protein perforin 1; UniProt protein alternative names: cytolysin, lymphocyte pore-forming protein (PFP); UniProt gene name synonym PFP), CX3CR1 (protein CX3C chemokine receptor 1; UniProt protein alternative names: beta-chemokine receptor-like 1, fractalkine receptor 1, G-protein coupled receptor 13, V28; UniProt gene name synonyms CMKBRL1, GPR13), CTSW (protein cathepsin W; UniProt protein alternative names: lymphopain; no UniProt gene name synonyms), GZMA (protein granzyme A; UniProt protein alternative names: CTL tryptase 1, cytotoxic T-lymphocyte proteinase 1, fragmentin-1, granzyme-1, Hanukkah factor; UniProt gene name synonyms CTLA3, HFSP), KLRD1 (protein natural killer cells antigen CD94; UniProt protein alternative names: KP43, killer cell lectin-like receptor subfamily D member 1, NK cell receptor; UniProt gene name synonym CD94), GZMM (protein granzyme M; UniProt protein alternative names: Met-1 serine protease, Met-ase, natural killer cell granular protease; UniProt gene name synonym MET1), CD3D (protein T-cell surface glycoprotein CD3 delta chain; UniProt protein alternative names: CD3d; UniProt gene name synonym T3D), CD8A (protein T-cell surface glycoprotein CD8 alpha chain; UniProt protein alternative names: T-lymphocyte differentiation antigen T8/Leu-2; UniProt gene name synonym MAL), CD8B (protein T-cell surface glycoprotein CD8 beta chain; UniProt protein alternative names: CD8b; UniProt gene name synonym CD8B1), CD52 (protein CAMPATH-1 antigen; UniProt protein alternative names: CDw52, Cambridge pathology 1 antigen, epididymal secretory protein E5, human epididymis-specific protein 5 (He5); UniProt gene name synonyms CDW52, HE5), PTPRC (protein receptor-type tyrosine phosphatase C; UniProt protein alternative names: leukocyte common antigen (L-CA), T200; UniProt gene name synonym CD45), CD3G (protein T-cell surface glycoprotein CD3 gamma chain; UniProt protein alternative names: ; UniProt gene name synonym T3G), HCST (protein hematopoietic cell signal transducer; UniProt protein alternative names: DNAX-activation protein 10, membrane protein DAP10, transmembrane adapter protein KAP10; UniProt

gene name synonyms DAP10, KAP10, PIK3AP), CD3E (protein T-cell surface glycoprotein CD3 epsilon chain; UniProt protein alternative names: T-cell surface antigen T3/Leu-4 epsilon chain; UniProt gene name synonym T3E), PLEK (protein pleckstrin; UniProt protein alternative names: platelet 47 kDa protein (p47); UniProt gene name synonym P47), KLRG1 (protein killer cell lectin-like receptor subfamily G member 1; UniProt protein alternative names: C-type lectin domain family 15 member A, ITIM-containing receptor MAFA-L, MAFA-like receptor, mast cell function-associated antigen; UniProt gene name synonyms CLEC15A, MAFA, MAFAL), RAC2 (protein Ras-related C3 botulinum toxin substrate 2; UniProt protein alternative names: GX, small G protein, p21-Rac2 ; no UniProt gene name synonyms), LCK (protein tyrosine-protein kinase Lck; UniProt protein alternative names: leukocyte C-terminal Src kinase (LSK), lymphocyte cell-specific protein-tyrosine kinase, protein YT16, proto-oncogene Lck, T cell-specific protein-tyrosine kinase, p56-LCK; no UniProt gene name synonyms), CD247 (protein T-cell surface glycoprotein CD3 zeta chain; UniProt protein alternative names: CD247; UniProt gene name synonyms CD3Z, T3Z, TCRZ), HOPX (protein homeodomain-only protein; UniProt protein alternative names: lung cancer-associated Y protein, not expressed in choriocarcinoma protein 1, odd homeobox protein 1; UniProt gene name synonyms HOD, HOP, LAGY, NECC1, OB1), KLRK1 (protein killer cell lectin-like receptor subfamily K member 1; UniProt protein alternative names: NKG2-D type II integral membrane protein, NK cell receptor D, NKG2-D-activating NK receptor, CD314; UniProt gene name synonyms D12S2489E, NKG2D), BIN2 (protein bridging integrator 2; UniProt protein alternative names: breast cancer-associated protein 1; UniProt gene name synonym BRAP1), S100A4 (protein S100-A4; UniProt protein alternative names: calvasculin, metastasin, placental calcium-binding protein, protein Mts1, S100 calcium-binding protein A4; UniProt gene name synonyms CAPL, MTS1), CORO1A (protein coronin; no UniProt protein alternative names; no UniProt gene name synonyms), IL2RG (protein cytokine receptor common subunit gamma; UniProt protein alternative names: interleukin-2 receptor subunit gamma (IL-2 receptor subunit gamma, IL-2R subunit gamma), gammaC, p64; no UniProt gene name synonyms), ITGB2 (protein integrin beta-2; UniProt protein alternative names: cell surface adhesion glycoproteins LFA-1/CR3/p150,95 subunit beta, CD18; UniProt gene name synonyms CD18, MFI7), IFITM1 (protein interferon-induced transmembrane protein 1; UniProt protein alternative names: dispanin subfamily A member 2a (DSPA2a), interferon-induced protein 17, interferon-inducible protein 9-27, Leu-13 antigen, CD225; UniProt gene name synonyms CD225, IFI17), EMP3 (protein epithelial membrane protein 3; UniProt protein alternative names: hematopoietic neural membrane protein 1 (HNMP-1), protein YMP; UniProt gene name synonym YMP), TRBC1 (protein T cell receptor beta constant 1; no UniProt protein alternative names; no UniProt gene name synonyms), SPON2 (protein spondin 2; UniProt protein alternative names: differentially expressed in cancerous and non-cancerous lung cells 1 (DIL-1), mindin; UniProt gene name synonym DIL1), and FLNA (protein filamin A; UniProt protein

alternative names: actin-binding protein 280 (ABP-280), alpha-filamin, endothelial acting-binding protein, filamin-1, non-muscle filamin; UniProt gene name synonyms FLN, FLN1).

In particular the set of CD8 Temra cell marker genes comprises at least CX3CR1, FGFBP2, and CD8 (CD8A and/or CD8B), and optionally a further gene chosen from GZMB, GNLY, NKG7 and PRF1. Alternatively, the set of CD8 Temra cell marker genes comprises at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 or all 38 of the above-listed genes. Alternatively, the set of CD8 Temra cell marker genes comprises at least CX3CR1, FGFBP2, and CD8 (CD8A and/or CD8B), and optionally a further gene chosen from GZMH, GNLY, NKG7, GZMB, CST7, CCL5, PRF1, CTSW, GZMA, KLRD1, GZMM, CD3D, CD52, PTPRC, CD3G, HCST, CD3E, PLEK, KLRG1, RAC2, LCK, CD247, HOPX, KRLK1, BIN2, S100A4, CORO1A, IL2RG, ITGB2, IFITM1, EMP3, TRBC1, SPON2, and FLNA.

Alternatively, the set of CD8 Temra cell marker genes comprises at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 or all 36 genes chosen from CX3CR1, FGFBP2, CD8 (CD8A and/or CD8B), GZMH, GNLY, NKG7, GZMB, CST7, CCL5, PRF1, CTSW, GZMA, KLRD1, GZMM, CD3D, CD52, PTPRC, CD3G, HCST, CD3E, PLEK, KLRG1, RAC2, LCK, CD247, HOPX, KRLK1, BIN2, S100A4, CORO1A, IL2RG, ITGB2, IFITM1, EMP3, TRBC1, and FLNA. In one embodiment, these 4 comprise CX3CR1, FGFBP2, and CD8 (CD8A and/or CD8B), and optionally a further gene chosen from GZMH, GNLY, NKG7, GZMB, CST7, CCL5, PRF1, CTSW, GZMA, KLRD1, GZMM, CD3D, CD52, PTPRC, CD3G, HCST, CD3E, PLEK, KLRG1, RAC2, LCK, CD247, HOPX, KRLK1, BIN2, S100A4, CORO1A, IL2RG, ITGB2, IFITM1, EMP3, TRBC1, and FLNA.

In one embodiment to the aspects implying measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CXCL10-positive macrophages, measurement, determination, assessment, quantification or analysis of the abundance of CXCL10-positive macrophages is by means of differential gene expression analysis, such as in/of RNA (such as in/of bulk RNA) isolated from the pre-therapy biopsy sample; by means of single cell sequencing (RNA (cDNA) sequencing) such as of cells of the pre-therapy biopsy sample; or by means of spatial transcriptomics (e.g. FISH or sequencing) analysis such as in/of a pre-therapy biopsy sample. Alternatively, the abundance, level, proportion, frequency or number of CXCL10-positive macrophages is measured, determined, assessed, quantified, or analyzed by means of differential gene expression analysis, such as in/of RNA (such as in/of bulk RNA) isolated from the pre-therapy biopsy sample; by means of single cell sequencing (RNA (cDNA) sequencing) such as of cells of the pre-therapy biopsy sample; or by means of spatial transcriptomics (e.g. FISH or sequencing) analysis such as in/of a pre-therapy biopsy sample. In particular, the measuring, determining, assessing, quantifying, or analyzing the abundance, level,

proportion, frequency or number of CXCL10-positive macrophages in the sample obtained from a subject having cancer is performed *in vitro* in or on the said sample.

In a further embodiment thereto, such differential gene expression analysis or spatial transcriptomics analysis is performed on/with a set of (human) marker genes chosen from CXCL10 (protein C-X-C motif chemokine 10; UniProt protein alternative names: 10 kDa interferon gamma-induced protein (gamma-IP10, IP-10), small inducible cytokine B10; UniProt gene synonyms INP10, SCYB10), CXCL9 (protein C-X-C motif chemokine 9; UniProt protein alternative names: gamma interferon inducible monokine, monokine induced by interferon gamma (HuMIG, MIG), small inducible cytokine B9; UniProt gene synonyms CMK, MIG, SCYB9), GBP1 (protein guanylate-binding protein 1; UniProt protein alternative names: GTP-binding protein 1 (GBP-1), interferon-induced guanylate binding protein 1; no UniProt gene synonyms), TYMP (protein thymidine phosphorylase; UniProt protein alternative names: TP, gliostatin, platelet-derived endothelial cell growth factor (PD-ECGF), TdRPase; UniProt gene synonym ECGF1), CALHM6 (protein calcium homeostasis modulator protein 6; UniProt protein alternative names: protein FAM26F ; UniProt gene synonyms C6orf187, FAM26F), CCL2 (protein C-C motif chemokine 2; UniProt protein alternative names: HC11, monocyte chemoattractant protein 1, monocyte chemotactic and activating factor (MCAF), monocyte chemotactic protein 1 (MCP-1), monocyte secretory protein JE, small-inducible cytokine A2; UniProt gene synonyms MCP1, SCYA2), TNFSF13B (protein tumor necrosis factor receptor superfamily member 17; UniProt protein alternative names: CD257, B lymphocyte stimulator (BLyS), B-cell activating factor, BAFF, dendritic cell-derived TNF-like molecule, TNF- and APOL-related leukocyte expressed ligand (TALL-1); UniProt gene synonyms BAFF, BLYS, TALL1, TNFSF20, ZTNF4), WARS (protein tryptophan-tRNA ligase, cytoplasmic; UniProt protein alternative names: interferon-induced protein 53 (IFP53), tryptophanyl-tRNA synthetase (TrpRS, hWRS); UniProt gene synonyms IFI53, WRS), CCL8 (protein C-C motif chemokine 8; UniProt protein alternative names: HC14, monocyte chemoattractant protein 2, monocyte chemotactic protein 2 (MCP-2), small-inducible cytokine A8; UniProt gene synonyms MCP2, SCYA10, SCYA8), IL4I1 (protein L-amino acid oxidase; UniProt protein alternative names: interleukin-4 induced protein 1, protein Fig-1; UniProt gene synonym FIG1), ICAM1 (protein intercellular adhesion molecule 1; UniProt protein alternative names: major group rhinovirus receptor, CD54; no UniProt gene synonyms), LILRB4 (protein leukocyte immunoglobulin-like receptor subfamily B member 4; UniProt protein alternative names: CD85 antigen-like family member K, immunoglobulin-like transcript 3 (ILT-3), leukocyte immunoglobulin-like receptor 5 (LIR-5), monocyte inhibitory receptor HM18, CD85k; UniProt gene synonyms ILT3, LIR5), CXCL11 (protein C-X-C motif chemokine 11; UniProt protein alternative names: beta-R1, H174, interferon gamma-inducible protein 9 (IP-9), interferon-inducible T-cell alpha chemoattractant (I-TAC), small-inducible cytokine B11; UniProt

gene synonyms ITAC, SCYB11, SCYB9B), SOD2 (protein superoxide dismutase [Mn], mitochondrial; no UniProt protein alternative names; ; no UniProt gene synonyms), LAP3 (protein cytosol aminopeptidase; UniProt protein alternative names: cysteinylglycine-S-conjugate dipeptidase by similarity, leucine aminopeptidase 3 imported (LAP-3), leucyl aminopeptidase by similarity, peptidase S imported, proline aminopeptidase by similarity, prolyl aminopeptidase; UniProt synonyms LAPEP, PEPS), STAT1 (protein signal transducer and activator of transcription 1-alpha/beta; UniProt protein alternative names: transcription factor ISGF-3 components p91/p84; no UniProt gene synonyms), GBP5 (protein guanylate-binding protein 5; UniProt protein alternative names: GBP-TA antigen 1, GTP-binding protein 5, guanidine nucleotide-binding protein 5; no UniProt gene synonyms) and CD80 (protein T-lymphocyte activation antigen CD80 ; UniProt protein alternative names: activation B7-1 antigen, BB1, CTLA-4 counter receptor B7.1 (B7); UniProt gene synonyms CD28LG, CD28LG1, LAB7). In particular the set of marker genes for CXCL10-positive macrophages comprises at least 4 of the above-listed genes. Alternatively, the set of CD8 Temra cell marker genes comprises at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or all of 18 of the above-listed genes. Alternatively, the set of marker genes for CXCL10-positive macrophages comprises at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or all 16 genes chosen from CXCL10, CXCL9, GBP1, TYMP, CALHM6, CCL2, TNFSF13B, WARS, CCL8, IL4I1, ICAM1, LILRB4, CXCL11, SOD2, LAP3, and STAT1.

In one embodiment to the aspects implying measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CD8 Temra cells, measurement, determination, assessment, quantification or analysis of the abundance of CD8 Temra cells is by means of histopathological or immunohistochemical phenotyping, by spatial phenotyping or spatial proteomics, or by means of whole-tissue single-cell imaging from the pre-therapy biopsy sample. Alternatively, the abundance, level, proportion, frequency or number of CD8 Temra cells is measured, determined, assessed, quantified, or analyzed by means of differential gene expression analysis, such as in RNA isolated from the pre-therapy biopsy sample. Protein markers that can be used in these settings include CD8, CD45RA, CX3CR1, PRF1 and/or GZMB.

In one embodiment to the aspects implying measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CXCL10-positive macrophages, measurement, determination, assessment, quantification or analysis of the abundance of CXCL10-positive macrophages is by means of histopathological or immunohistochemical phenotyping, by spatial phenotyping or spatial proteomics, or by means of whole-tissue single-cell imaging from the pre-therapy biopsy sample. Alternatively, the abundance, level, proportion, frequency or number of CXCL10-positive macrophages

is measured, determined, assessed, quantified, or analyzed by means of differential gene expression analysis, such as in RNA isolated from the pre-therapy biopsy sample.

Protein markers that can be used in these settings include CD68, CXCL10, and/or PDL1.

Some of the above-described aspects or embodiments imply measuring, determining, assessing, quantifying, or analyzing the level of T cell receptor (TCR) TCR sharing between pre-therapy biopsy CD8 Temra cells and peripheral (blood) CD8 Temra cells. The level of TCR sharing or the proportion of shared TCR sequences can be calculated by dividing the number of shared TCRs between pre-therapy biopsy CD8 Temra cells and peripheral (blood) CD8 Temra cells by the total number of TCRs. An alternative way of indicating the level of TCR sharing relies on the Gini coefficient or Gini index (measuring inequality among values of a frequency distribution; a Gini index of zero indicates perfect equality whereas a Gini index of one indicates maximal equality) which can e.g. be calculated using the ineq (v0.2-13) package in R and captures the distribution of T-cells across the TCR repertoire. This value ranges between 0 and 1, and the closer it is to 1 the less equal the distribution of clonotypes is (Thomas et al. 2013, Proc Natl Acad Sci USA 110: 1839–1844). In particular, the measuring, determining, assessing, quantifying, or analyzing the level of T cell receptor (TCR) TCR on CD8 Temra cells in a sample obtained from a subject having cancer is performed *in vitro* in or on the said sample.

In a further aspect, the current disclosure relates to methods, such methods comprising:

- obtaining a tumor biopsy from a subject having cancer prior to start of anti-tumor therapy;
- measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CD8 Temra cells in the biopsy sample;
- optionally, measuring, determining, assessing, quantifying, or analyzing the abundance, level, proportion, frequency or number of CXCL10-positive macrophages in the biopsy sample;
- optionally, obtaining a blood sample from the subject having cancer prior to start of anti-tumor therapy and measuring, determining, assessing, quantifying, or analyzing the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the biopsy sample and the CD8 Temra cells in the blood sample;
- optionally, obtaining an on anti-tumor therapy blood sample from the subject having cancer and measuring, determining, assessing, quantifying, or analyzing the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the biopsy sample and the CD8 Temra cells in the on anti-tumor therapy blood sample.

In particular, such methods are methods of/for analyzing a biological sample of a subject having cancer; in particular the biological sample is at least a biopsy sample (more in particular a biopsy sample obtained from the subject prior to start of anti-tumor therapy) and optionally a blood sample (from the same

subject). In particular to these methods, the tumor or cancer is a liver tumor, or is hepatocellular carcinoma.

In particular to these methods, the abundance, level, proportion, frequency or number of CD8 Temra cells or of CXCL10-positive macrophages is measured, determined, assessed, quantified, or analyzed by differential gene expression analysis, such as in/of RNA (such as in/of bulk RNA) isolated from the biopsy sample; by means of single cell sequencing (RNA (cDNA) sequencing) such as of cells of the biopsy sample; by means of spatial transcriptomics (e.g. FISH or sequencing) analysis such as in/of the biopsy sample; by means of histopathological or immunohistochemical phenotyping, by spatial phenotyping or spatial proteomics, or by means of whole-tissue single-cell imaging of the biopsy sample.

In particular the set of CD8 Temra cell marker genes comprises at least CX3CR1, FGFBP2, and CD8 (CD8A and/or CD8B), and optionally a further gene chosen from GZMB, GNLY, NKG7 and PRF1; or other combinations of marker genes as described hereinabove; whereas protein markers that can be used include CD8, CD45RA, CX3CR1, PRF1 and/or GZMB.

In particular the set of marker genes for CXCL10-positive macrophages comprises at least 4 of the genes chosen from CXCL10, CXCL9, GBP1, TYMP, CALHM6, CCL2, TNFSF13B, WARS, CCL8, IL4I1, ICAM1, LILRB4, CXCL11, SOD2, LAP3, and STAT1; or other combinations of marker genes as described hereinabove; whereas protein markers that can be used in these settings include CD68, CXCL10, and/or PDL1.

In particular, the level of TCR sharing or the proportion of shared TCR sequences can be calculated by dividing the number of shared TCRs between biopsy CD8 Temra cells and peripheral (blood) CD8 Temra cells by the total number of TCRs. An alternative way of indicating the level of TCR sharing relies on the Gini coefficient or Gini index (measuring inequality among values of a frequency distribution; a Gini index of zero indicates perfect equality whereas a Gini index of one indicates maximal equality) which can e.g. be calculated using the ineq (v0.2-13) package in R and captures the distribution of T-cells across the TCR repertoire. This value ranges between 0 and 1, and the closer it is to 1 the less equal the distribution of clonotypes is (Thomas et al. 2013, Proc Natl Acad Sci USA 110: 1839–1844).

CD8 T cells

Many different types of CD8 T cells have been defined and include CD8 Tn (naïve CD8 T-cells), CD8 Trm (tissue resident memory CD8 T-cells), CD8 Tcm (central memory CD8 T-cells), CD8 Tem (effector memory CD8 T-cells), CD8 Teff (effector CD8 T-cells), CD8 Tscm (stem cell memory CD8 T-cells), CD8 Temra (effector memory CD8 T-cells re-expressing CD45RA), CD8 Tex (exhausted CD8 T-cells), CD8 Tpm (peripheral memory CD8 T-cells) and proliferating CD8 T-cells (Martin et al. 2018, Front Immunol 9:2692; Reiser et al. 2016, J Immunol Res 2016:8941260; Zheng et al. 2021, Science 374:abe6474). As an example, extracellular markers (phenotypic markers) of CD8 Tn cells include CD45RA⁺ (CD45RA-positive),

CD45RO- (CD45RO-negative), CD25+ (CD25-positive; for Treg cells), CD62L+ (L-Selectin+; CD62L-positive), CCR-7+ (CCR-7-positive). Upon differentiation of CD8 Tn to CD8 Tem, the status of these markers changes to CD45RA- (CD45RA-negative), CD45RO+ (CD45RO-positive), CD62L- (CD62L-negative), CCR-7- (CCR-7-negative) (e.g. Golubovskaya et al. 2016, Cancers 8:8030036). Flow cytometry was used "to determine the proportion of T cell subsets (naïve [TN]; CD45RA+ CCR7+, central memory [TCM]; CD45RA-CCR7+, effector memory [TEM]; CD45RA-CCR7-, and terminally differentiated effector memory (TEMRA); CD45RA+ CCR7-), activation status (CD38+ and Ki-67+), and expression of immune checkpoint molecules (CTLA-4, PD-1, Lag-3, and Tim-3) for CD4+ and CD8+ T cells" (Tada et al. 2022, Cancer Immunol Immunother 71:851-863). Such phenotypic markers in general allow different types of immune cell subsets to be detected, sorted, analyzed, etc. Alternatively, expression levels of such phenotypic (and other) markers can be used for detection of different types of immune cells.

The context of the present disclosure focuses in part on CD8 Temra cells and makes a difference between intra-tumoral CD8 Temra cells (expressing e.g. CD69, CCL3 and/or CCL4) and peripheral CD8 Temra cells (expressing e.g. CD52). A detailed pan-cancer single-cell based immune landscape was reported by Zheng et al. 2021 (Science 374:abe6474) and provides amongst other information on CD8 Temra cells.

CD45RA and CD45RO are expressed isoforms of CD45, a membrane-bound tyrosine phosphatase. Alternative splicing leads to the long or high molecular weight CD45RA isoform or to the short or low molecular weight CD45RO isoform. Expression of CD45RA and CD45RO appears mutually exclusive, although co-expression is possible and thought to occur during transitioning from one state to another (e.g. Kandel et al. 2022, Cells 11:1844). As indicated above, naïve T cells are usually defined as CD45RA positive (CD45RA+) and CD45RO negative (CD45RO-) whereas memory T cells are CD45RO positive (CD45RO+) and CD45RA negative (CD45RA-). CD8 Temra cells are memory T cells that have re-started expression of CD45RA. CD8 Temra cells are also referred to as terminally differentiated effector memory CD8 T-cells (e.g. Yang et al. 2019, Blood 134 Supplement 1:2329).

Gene expression and quantification of gene expression

The term "level of expression" or "expression level" generally refers to the amount of an expressed (bio)marker (marker and biomarker are used interchangeably herein) in a biological sample. "Expression" generally refers to the process by which information (e.g., gene- encoded and/or epigenetic information) is converted into the structures present and operating in the cell. Therefore, as used herein, "expression" may refer to transcription into a polynucleotide, translation into a polypeptide, or even polynucleotide modifications (e.g. alternative splicing) and/or polypeptide modifications (e.g., posttranslational modification of a polypeptide). Fragments of the transcribed polynucleotide, the translated polypeptide, or polynucleotide and/or polypeptide modifications (e.g., posttranslational modification of a

polypeptide) are also regarded as expressed whether they originate from a transcript generated by alternative splicing or a degraded transcript, or from a post-translational processing of the polypeptide, e.g., by proteolysis. "Expressed genes" include those that are transcribed into a polynucleotide as mRNA and then translated into a polypeptide, and also those that are transcribed into RNA but not translated into a polypeptide (for example, transfer and ribosomal RNAs, long non-coding RNA, microRNA or miRNA).

"Increased/higher expression," "increased/higher expression level," "increased/higher levels," "elevated expression," "elevated expression levels," or "elevated levels" refers to an increased/higher expression or to increased/higher levels of a (bio)marker in an individual relative to a suitable control or standard.

The term "detection" includes any means of detecting, including direct and indirect detection. The term "marker" or "biomarker" as used herein refers to an indicator molecule or set of indicator molecules (e.g., predictive, diagnostic, and/or prognostic indicator), which can be detected in a sample. The biomarker may be a predictive biomarker and serve as an indicator of the likelihood of sensitivity or benefit to therapeutic treatment of a patient having a particular disease or disorder (e.g., a proliferative cell disorder (e.g., cancer)) to treatment (e.g. with an immune checkpoint blocker). Biomarkers in general include, but are not limited to, polynucleotides (e.g., DNA and/or RNA (e.g., mRNA)), polynucleotide copy number alterations (e.g., DNA copy numbers), polypeptides, polypeptide and polynucleotide modifications (e.g., post-translational modifications, nucleotide substitutions, nucleotide insertions or deletions (indels)), carbohydrates, and/or glycolipid-based molecular markers. In some embodiments, a biomarker is a gene. The "amount" or "level" of a biomarker, as used herein, is a detectable level in a biological sample. These can be measured by methods known to one skilled in the art and also disclosed herein.

In first instance, methodologies for determining gene expression by means of determining transcript levels, also referred to as transcriptome analysis or analysis of the transcriptome, is described in more detail. Any such gene detection or gene expression detection method is starting from an analyte nucleic acid (i.e. the nucleic acid of interest (which does not necessarily need to be the whole nucleic acid of interest, parts of such nucleic acids can suffice for determining expression) and of which the amount is to be determined) and may be defined as comprising one or more steps of, for instance,

- a step of isolating RNA from a (biological) sample (wherein a fraction of the isolated RNA is the analyte strand);
- a step of reverse transcribing the RNA obtained from the biological sample into DNA;
- a step of amplifying the isolated DNA; and/or

- a step of quantifying the isolated RNA, the DNA obtained after reverse transcription, or the amplified DNA.

In case an amplified DNA is quantified, this quantification step can be performed concurrent with the amplification of the DNA, or is performed after the amplification of the DNA.

- 5 The quantification of gene expression or the determination of gene expression levels may be based on at least one of an amplification reaction, a sequencing reaction, a melting reaction, a hybridization reaction or a reverse hybridization reaction. Quantification of gene expression can further involve a normalization step, wherein levels of expression of a gene of interest are normalized to e.g. levels of expression of a housekeeping gene or of a gene of which the expression is relatively constant under
- 10 different conditions.

This disclosure covers methods which include measurement, determination, assessment, analysis, detection or quantification of nucleic acids corresponding to one or more (bio)markers as defined herein (more specifically CD8 Temra cell marker genes, CXCL10-positive macrophage marker genes, TCRs). In

15 any of these methods the detection can comprise a step such as a nucleic acid amplification reaction, a nucleic acid sequencing reaction, a melting reaction, a hybridization reaction to a nucleic acid, or a reverse hybridization reaction to a nucleic acid, or a combination of such steps.

Often one or more artificial, man-made, or non-naturally occurring oligonucleotide is used in such method. In particular, such oligonucleotides can comprise besides ribonucleic acid monomers or

20 deoxyribonucleic acid monomers: one or more modified nucleotide bases, one or more modified nucleotide sugars, one or more labelled nucleotides, one or more peptide nucleic acid monomers, one or more locked nucleic acid monomers, the backbone of such oligonucleotide can be modified, and/or non-glycosidic bonds may link two adjacent nucleotides. Such oligonucleotides may further comprise a modification for attachment to a solid support, e.g., an amine-, thiol-, 3'-propanolamine or acrydite-

25 modification of the oligonucleotide, or may comprise the addition of a homopolymeric tail (for instance an oligo(dT)-tail added enzymatically via a terminal transferase enzyme or added synthetically) to the oligonucleotide. If said homopolymeric tail is positioned at the 3'-terminus of the oligonucleotide or if any other 3'-terminal modification preventing enzymatic extension is incorporated in the oligonucleotide, the priming capacity of the oligonucleotide can be decreased or abolished. Such

30 oligonucleotides may also comprise a hairpin structure at either end. Terminal extension of such oligonucleotide may be useful for, e.g., specifically hybridizing with another nucleic acid molecule (e.g. when functioning as capture probe), and/or for facilitating attachment of said oligonucleotide to a solid support, and/or for modification of said tailed oligonucleotide by an enzyme, ribozyme or DNAzyme. Such oligonucleotides may be modified in order to detect (the levels of) a target nucleotide sequence

and/or to facilitate in any way such detection. Such modifications include labelling with a single label, with two different labels (for instance two fluorophores or one fluorophore and one quencher), the attachment of a different 'universal' tail to two probes or primers hybridizing adjacent or in close proximity to each other with the target nucleotide sequence, the incorporation of a target-specific sequence in a hairpin oligonucleotide (for instance Molecular Beacon-type primer), the tailing of such a hairpin oligonucleotide with a 'universal' tail (for instance Sunrise-type probe and Amplifluor™ -type primer). A special type of hairpin oligonucleotide incorporates in the hairpin a sequence capable of hybridizing to part of the newly amplified target DNA. Amplification of the hairpin is prevented by the incorporation of a blocking nonamplifiable monomer (such as hexethylene glycol). A fluorescent signal is generated after opening of the hairpin due to hybridization of the hairpin loop with the amplified target DNA. This type of hairpin oligonucleotide is known as scorpion primers (Whitcombe et al. 1999, Nat Biotechnol 17:804-807). Another special type of oligonucleotide is a padlock oligonucleotide (or circularizable, open circle, or C-oligonucleotide) that are used in RCA (rolling circle amplification). Such oligonucleotides may also comprise a 3'-terminal mismatching nucleotide and/or, optionally, a 3'-proximal mismatching nucleotide, which can be particularly useful for performing polymorphism-specific PCR and LCR (ligase chain reaction) or any modification of PCR or LCR. Such oligonucleotide may comprise or consist of at least and/or comprise or consist of up to 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200 or more contiguous nucleotides.

The analyte nucleic acid, in particular the analyte nucleic acid of a biomarker of interest can be any type of nucleic acid, which will be dependent on the manipulation steps (such as isolation and/or purification and/or duplication, multiplication or amplification) applied to the nucleic acid of the gene of interest in the biological sample; as such it can be DNA, RNA, cDNA, may comprise modified nucleotides, or may be hybrids of DNA and/or RNA and/or modified nucleotides, and can be single- or double-stranded or may be a triplex-forming nucleic acid.

The artificial, man-made, non-naturally occurring oligonucleotide(s) as applied in the above detection methods can be probe(s) or a primer(s), or a combination of both.

A probe capable of specifically hybridizing with a target nucleic acid is an oligonucleotide mainly hybridizing to one specific nucleic acid sequence in a mixture of many different nucleic acid sequences. Specific hybridization is meant to result, upon detection of the specifically formed hybrids, in a signal-to-noise ratio (wherein the signal represents specific hybridization and the noise represents unspecific hybridization) sufficiently high to enable unambiguous detection of said specific hybrids. In a specific case specific hybridization allows discrimination of up to a single nucleotide mismatch between the

probe and the target nucleic acids. Conditions allowing specific hybridization generally are stringent but can obviously be varied depending on the complexity (size, GC-content, overall identity, etc.) of the probe(s) and/or target nucleic acid molecules. Specificity of a probe in hybridizing with a nucleic acid can be improved by introducing modified nucleotides in said probe.

5 A primer capable of directing specific amplification of a target nucleic acid is the at least one oligonucleotide in a nucleic acid amplification reaction mixture that is required to obtain specific amplification of a target nucleic acid. Nucleic acid amplification can be linear or exponential and can result in an amplified single nucleic acid of a single- or double-stranded nucleic acid or can result in both strands of a double-stranded nucleic acid. Specificity of a primer in directing amplification of a nucleic
10 acid can be improved by introducing modified nucleotides in said primer. The fact that a primer does not have to match exactly with the corresponding template or target sequence to warrant specific amplification of said template or target sequence is amply documented in literature (for instance: Kwok et al. 1990, Nucl Acids Res 18:999-1005. Primers as short as 8 nucleotides in length have been applied successfully in directing specific amplification of a target nucleic acid molecule (e.g. Majzoub et al. 1983,
15 J Biol Chem 258:14061-14064).

A nucleotide is meant to include any naturally occurring nucleotide as well as any modified nucleotide wherein said modification can occur in the structure of the nucleotide base (modification relative to A, T, G, C, or U) and/or in the structure of the nucleotide sugar (modification relative to ribose or deoxyribose). Any of the modifications can be introduced in a nucleic acid or oligonucleotide to
20 increase/decrease stability and/or reactivity of the nucleic acid or oligonucleotide and/or for other purposes such as labelling of the nucleic acid or oligonucleotide. Modified nucleotides include phosphorothioates, alkylphosphorothioates, methylphosphonate, phosphoramidate, peptide nucleic acid monomers and locked nucleic acid monomers, cyclic nucleotides, and labelled nucleotides (i.e. nucleotides conjugated to a label which can be isotopic ($<^{32}>P$, $<^{35}>S$, etc.) or non-isotopic (biotin,
25 digoxigenin, phosphorescent labels, fluorescent labels, fluorescence quenching moiety, etc.)). Other modifications are described higher (see description on oligonucleotides).

Nucleotide acid amplification is meant to include all methods resulting in multiplication of the number of a target nucleic acid. Nucleotide sequence amplification methods include the polymerase chain reaction (PCR; DNA amplification), strand displacement amplification (SDA; DNA amplification),
30 transcription-based amplification system (TAS; RNA amplification), self-sustained sequence replication (3SR; RNA amplification), nucleic acid sequence-based amplification (NASBA; RNA amplification), transcription-mediated amplification (TMA; RNA amplification), Qbeta-replicase-mediated amplification and run-off transcription. During amplification, the amplified products can be conveniently labeled either using labeled primers or by incorporating labeled nucleotides.

The most widely spread nucleotide sequence amplification technique is PCR. The target DNA is exponentially amplified. Many methods rely on PCR including AFLP (amplified fragment length polymorphism), IRS-PCR (interspersed repetitive sequence PCR), iPCR (inverse PCR), RAPD (rapid amplification of polymorphic DNA), RT-PCR (reverse transcription PCR) and real-time PCR. RT-PCR can be performed with a single thermostable enzyme having both reverse transcriptase and DNA polymerase activity (Myers et al. 1991, Biochem 30:7661-7666). Alternatively, a single tube-reaction with two enzymes (reverse transcriptase and thermostable DNA polymerase) is possible (Cusi et al. 1994, Biotechniques 17:1034-1036).

Solid phases, solid matrices or solid supports on which molecules, e.g., nucleic acids, analyte nucleic acids and/or oligonucleotides as described hereinabove, may be bound (or captured, absorbed, adsorbed, linked, coated, immobilized; covalently or non-covalently) comprise beads or the wells or cups of microtiter plates, or may be in other forms, such as solid or hollow rods or pipettes, particles, e.g., from 0.1 μm to 5 mm in diameter (e.g. "latex" particles, protein particles, or any other synthetic or natural particulate material), microspheres or beads (e.g. protein A beads, magnetic beads). A solid phase may be of a plastic or polymeric material such as nitrocellulose, polyvinyl chloride, polystyrene, polyamide, polyvinylidene fluoride or other synthetic polymers. Other solid phases include membranes, sheets, strips, films and coatings of any porous, fibrous or bibulous material such as nylon, polyvinyl chloride or another synthetic polymer, a natural polymer (or a derivative thereof) such as cellulose (or a derivative thereof such as cellulose acetate or nitrocellulose). Fibers or slides of glass, fused silica or quartz are other examples of solid supports. Paper, e.g., diazotized paper may also be applied as solid phase. Clearly, molecules such as nucleic acids, analyte nucleic acids and/or oligonucleotides as described hereinabove, may be bound, captured, absorbed, adsorbed, linked or coated to any solid phase suitable for use in hybridization assay (irrespective of the format, for instance capture assay, reverse hybridization assay, or dynamic allele-specific hybridization (DASH)). Said molecules, such as nucleic acids, analyte nucleic acids and/or oligonucleotides as described hereinabove, can be present on a solid phase in defined zones such as spots or lines. Such solid phases may be incorporated in a component such as a cartridge of e.g. an assay device. Any of the solid phases described above can be developed, e.g. automatically developed in an assay device.

Quantification of amplified DNA can be performed concurrent with or during the amplification. Techniques include real-time PCR or (semi-)quantitative polymerase chain reaction (qPCR). One common method includes measurement of a non-sequence specific fluorescent dye (e.g. SYBR Green) intercalating in any double-stranded DNA. Quantification of multiple amplicons with different melting points can be followed simultaneously by means of following or analyzing the melting reaction (melting curve analysis or melt curve analysis; which can be performed at high resolution, see, e.g. Wittwer et al.

2003, Clin Chem 843-860; an alternative method is denaturing gel gradient electrophoresis, DGGE; both methods were compared in e.g. Tindall et al. 2009, Hum Mutat 30:857-859).

Another common method includes measurement of sequence-specific labelled probe bound to its complementary sequence; such probe also carries a quencher and the label is only measurable upon
5 exonucleolytic release from the probe (hydrolysis probes such as TaqMan probes) or upon hybridization with the target sequence (hairpin probes such as molecular beacons which carry an internally quenched fluorophore whose fluorescence is restored upon unfolding the hairpin). This latter method allows for multiplexing by e.g. using mixtures of probes each tagged with a different label e.g. fluorescing at a different wavelength.

10 Exciton-controlled hybridization-sensitive fluorescent oligonucleotide (ECHO) probes also allow for multiplexing. The hybridization-sensitive fluorescence emission of ECHO probes and the further modification of probes have made possible multicolor RNA imaging in living cells and facile detection of gene polymorphisms (Okamoto 2011, Chem Soc Rev, 40:5815–5828).

Other methods of quantifying expression include SAGE (Serial Analysis of Gene Expression) and MPSS
15 (Massively Parallel Signature Sequencing), each involving reverse-transcription of RNA.

With "assaying" or "determining" or "detecting" and the like (e.g. assessing, measuring) is meant that a biological sample, suspected of comprising a target nucleic acid (such as a nucleic acid of a biomarker of interest as described herein), is processed as to generate a readable signal in case the target nucleic acid is actually present in the biological sample. Such processing may include, as described above, a step of
20 producing an analyte nucleic acid. Simple detection of a produced readable signal indicates the presence of a target or analyte nucleic acid in the biological sample. When in addition the amplitude of the produced readable signal is determined, this allows for quantification of levels of a target or analyte nucleic acid as present in a biological sample.

In particular, the readable signal may be a signal-to-noise ratio (wherein the signal represents specific
25 detection and the noise represents unspecific detection) of an assay optimized to yield signal-to-noise ratios sufficiently high to enable unambiguous detection and/or quantification of the target nucleic acid. The noise signal, or background signal, can be determined e.g. on biological samples not comprising the target or analyte nucleic acid of interest, e.g. control samples, or comprising the required reference level of the target or analyte nucleic acid of interest, e.g. reference samples. Such noise or background signal
30 may also serve as comparator value for determining an increase or decrease of the level of a target or analyte nucleic acid in the biological sample, e.g. in a biological sample taken from a subject suffering from a disease or disorder, further e.g. before start of a treatment and during treatment.

The readable signal may be produced with all required components in solution or may be produced with some of the required components in solution and some bound to a solid support. Said signals include,

e.g., fluorescent signals, (chemi)luminescent signals, phosphorescence signals, radiation signals, light or color signals, optical density signals, hybridization signals, mass spectrometric signals, spectrometric signals, chromatographic signals, electric signals, electronic signals, electrophoretic signals, real-time PCR signals, PCR signals, LCR signals, Invader-assay signals, sequencing signals (by any method such as Sanger dideoxy sequencing, pyrosequencing, 454 sequencing, single-base extension sequencing, sequencing by ligation, sequencing by synthesis, “next-generation” sequencing (NGS) (van Dijk et al. 2014, Trends Genet 30:418-426)), nanopore sequencing, melting curve signals etc. An assay may be run automatically or semi-automatically in an assay device. In view of its relatively low costs compared to e.g. very costly cancer therapies, NGS is finding its way to routine clinical care (Ratner 2018, Nature Biotechnol 36:484).

Specific hybridization of an oligonucleotide (whether or not comprising one or more modified nucleotides) to its target sequence is to be understood to occur under stringent conditions as generally known in the art (e.g. Sambrook et al. 1989. Molecular Cloning. A laboratory manual. CSHL Press). However, depending to the hybridization solution (SSC, SSPE, etc.), oligonucleotides should be hybridized at their appropriate temperature in order to attain sufficient specificity. In order to allow hybridization to occur, the target nucleic acid molecules are generally thermally, chemically (e.g. by NaOH) or electrochemically denatured to melt a double strand into two single strands and/or to remove hairpins or other secondary structures from single stranded nucleic acids. The stringency of hybridization is influenced by conditions such as temperature, salt concentration and hybridization buffer composition. High stringency conditions for hybridization include high temperature and/or low salt concentration (salts include NaCl and Na₃-citrate) and/or the inclusion of formamide in the hybridization buffer and/or lowering the concentration of compounds such as SDS (detergent) in the hybridization buffer and/or exclusion of compounds such as dextran sulfate or polyethylene glycol (promoting molecular crowding) from the hybridization buffer. Conventional hybridization conditions are described in e.g. Sambrook et al. 1989 (Molecular Cloning. A laboratory manual. CSHL Press) but the skilled craftsman will appreciate that numerous different hybridization conditions can be designed in function of the known or the expected homology and/or length of the nucleic acid sequence. Generally, for hybridizations with DNA oligonucleotides without formamide, a temperature of 68 DEG C, and for hybridization with formamide, 50% (v/v), a temperature of 42 DEG C is recommended. For hybridizations with oligonucleotides, the optimal conditions (formamide concentration and/or temperature) depend on the length and base composition of the probe and must be determined individually. In general, optimal hybridization for oligonucleotides of about 10 to 50 bases in length occurs approximately 5 DEG C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched sequences to hybridize and can therefor result in reduced specificity. When using

RNA oligonucleotides with formamide (50% v/v) it is recommend to use a hybridization temperature of 68 DEG C for detection of target RNA and of 50 DEG C for detection of target DNA. Alternatively, a high SDS hybridization solution can be utilized (Church et al. 1984, Proc Natl Acad Sci USA 81:1991-1995). The specificity of hybridization can furthermore be ensured through the presence of a crosslinking moiety on the oligonucleotide (e.g. Huan et al. 2000, Biotechniques 28: 254-255; WO00/14281). Said crosslinking moiety enables covalent linking of the oligonucleotide with the target nucleotide sequence and hence allows stringent washing conditions. Such a crosslinking oligonucleotide can furthermore comprise another label suitable for detection/quantification of the oligonucleotide hybridized to the target.

RPKM (Reads Per Kilobase Million) is often used as measure for expression. FPKM (Fragments Per Kilobase Million) is very similar to RPKM; whereas RPKM was designed for single-end RNA-seq (every read corresponded to a single sequenced fragment), FPKM was designed for paired-end RNA-seq. With paired-end RNA-seq, two reads can correspond to a single fragment, or, if one read in the pair did not map, one read can correspond to a single fragment. The only difference between RPKM and FPKM is that FPKM takes into account that two reads can map to one fragment (and so it doesn't count this fragment twice). When using RNA-seq, reporting or results often is in RPKM (Reads Per Kilobase Million) or FPKM (Fragments Per Kilobase Million). Whatever metric used (another alternative for example is TPM (Transcripts Per Kilobase Million)), such metric is attempting to normalize for sequencing depth and gene length and provide a measure for quantifying transcript levels/gene expression/expression units.

Next to methodologies for determining gene expression by means of determining transcript levels (transcriptome analysis), it is also possible to quantify gene expression by means of proteomic analysis (proteome analysis or analysis of the proteome). Classical proteomic analysis methods include ELISA, western blotting, mass spectrometry, chromatographic separation, immunohistochemistry, cell sorting (based on cell surface marker(s)) etc. Although not necessarily required, it can be advantageous to rely on multiplexed cytometry methods that can be performed directly on, e.g., a section of a cancer tissue biopsy (Formalin-Fixed Paraffin-Embedded (FFPE), fresh frozen (FF), ...). Multiplexed cytometry methods, as well as some predictive cancer biomarkers identified using such methodology, have been reviewed by e.g. Fan et al. 2020 (Cancer Communications 40:135-153) and have emerged with the advent of more sophisticated imaging techniques (e.g. cyclic immunofluorescence, tyramide-based immunofluorescence, epitope-targeted mass spectrometry, RNA detection) and standardized quantification methodologies. Such multiplexed cytometry methods include multiplex immunocytochemistry (mICH), imaging mass spectrometry, multiplexed ion beam imaging, chipcytometry, nucleotide (DNA/RNA)-barcoding-based mICH, and digital spacing profiling. Another

technique involving proteomic analysis is Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq).

Sequencing

Polynucleotide sequence determination is a common step in some of the methods as applied in the current disclosure.

In general, sequencing methods may include, but are not limited to: high-throughput sequencing, pyrosequencing, sequencing-by-synthesis, single-molecule sequencing, nanopore sequencing, semiconductor sequencing, sequencing-by-ligation, sequencing-by-hybridization, RNA-Seq (Illumina), Digital Gene Expression (Helicos), Next generation sequencing, Single Molecule Sequencing by Synthesis (SMSS) (Helicos), massively- parallel sequencing, Clonal Single Molecule Array (Solexa), shotgun sequencing, Maxam- Gilbert or Sanger sequencing, primer walking, sequencing using PacBio, SOLiD, Ion Torrent, or Nanopore platforms, short read sequencing, long read sequencing, and any other sequencing methods known in the art. The sequencing method can be massively parallel sequencing, that is, simultaneously (or in rapid succession) sequencing any of at least 100, 1000, 10,000, 100,000, 1 million, 10 million, 100 million, 1 billion, or 10 billion polynucleotide molecules.

Certain DNA sequencing methods may rely on the capture of polynucleotides of interest such as to enrich for these sequences of interest. Polynucleotide or sequence capture typically involves the use of oligonucleotide probes that hybridize to the polynucleotide or sequence of interest. A probe set strategy can involve tiling the probes across a region of interest (complete or partial tiling of the target sequence with probes). Such probes can be, e.g., 10 to 400 or about 400 bases long, 10 to 300 or about 300 bases long, 10 to 200 or about 200 bases long, 10 to 100 or about 100 bases long, 10 to 80 or about 80 bases long, 10 to 60 or about 60 bases long, Such probes may comprise at least one or a set of oligonucleotides of 10 to 60 bases or nucleotides long and/or comprise at least one or a set of oligonucleotides of 15 to 120 bases or nucleotides long. Any set of such oligonucleotide probes can have a depth of about 0.1x, 0.2x, 0.3x, 0.4 x, 0.5x, 0.1x to 0.5x, 1x 2x, 3x, 4x, 5x, 6x, 8x, 9x, 10x, 15x, 20x, 50x or more. Enriched nucleic acid molecules can be representative of a nucleic acid features of interest such as, but not necessarily limited to the CD8 Temra cell markers and CXCL10-positive macrophage markers as described herein.

Sequencing depth refers to the number of times a locus is covered by a sequence read aligned to the locus. A locus can be as small as a nucleotide, as large as a chromosome arm, or as large as the entire genome. Sequencing depth can be expressed as e.g. 10x, 50x, 100x, where “x” refers to the number of times a locus is covered by a sequence read. Sequencing depth can also be applied to multiple loci, or to the whole genome, in which case “x” can refer to the mean number of times the loci, or whole genome, is sequenced. Ultra-deep sequencing refers to a sequencing depth of at least 100x.

Shallow whole genome sequencing, low coverage whole genome sequencing, or ultra-low pass whole genome sequencing in general refers to short-read sequencing of genomes at low coverage, typically less than 3x coverage, less than 2x coverage, less than 1x coverage, such as 0.1x to 1x coverage, such as 0.1x to 0.8x coverage, such as 0.1x to 0.6x coverage, such as 0.1x to 0.5x coverage, such as 0.1x to 0.4x coverage, such as 0.1x to 0.3x coverage, such as 0.9x coverage, 0.8x coverage, 0.7x coverage, 0.6x coverage, 0.5x coverage, 0.4x coverage, 0.3x coverage, 0.2x coverage or 0.1x coverage, such as 0.1x coverage or less. Sequencing coverage can also be expressed as average sequencing coverage. Low coverage in the context of sequencing thus can also refer to typically on average less than 3x coverage, on average less than 2x coverage, on average less than 1x coverage, such as on average 0.1x to 1x coverage, such as on average 0.1x to 0.8x coverage, such as on average 0.1x to 0.6x coverage, such as on average 0.1x to 0.5x coverage, such as on average 0.1x to 0.4x coverage, such as on average 0.1x to 0.3x coverage, such as on average 0.9x coverage, on average 0.8x coverage, on average 0.7x coverage, on average 0.6x coverage, on average 0.5x coverage, on average 0.4x coverage, on average 0.3x coverage, on average 0.2x coverage or on average 0.1x coverage, such as on average 0.1x coverage or less.

By performing shallow whole genome sequencing, low coverage whole genome sequencing, or ultra-low pass whole genome sequencing, each sample is subjected to a small amount of sequencing, allowing application of whole genome sequencing to many samples at low cost per sample.

A sequence read is a string of nucleotides sequenced from a part or all of a nucleic acid molecule. A sequence read may be a short string of nucleotides (e.g. 20 to 150 nucleotides, around 50 nucleotides) sequenced from a nucleic acid (fragment). Sequence reads may be obtained at one end of a nucleic acid (fragment) or from both ends of a nucleic acid (fragment). Sequence reads may be obtained by e.g. applying a sequencing technique to the nucleic acid (fragment), by hybridization arrays or capture probes, by amplification techniques (e.g. PCR, linear amplification, isothermal amplification) such as amplification techniques using a single primer.

Thus, obtaining information from the nucleic acid molecules present in a biological sample may include a step of preparing a sequencing library using the nucleic acid molecules isolated from the biological sample. The preparation of such sequencing library may include a step of DNA amplification, or may, alternatively, not include a step of DNA amplification. Obtaining information from the nucleic acid molecules present in a biological sample may include obtaining DNA sequence reads. Obtaining information from the nucleic acid molecules (e.g. cfDNA molecules) present in a biological sample may include the step of aligning the plurality of (DNA) sequence reads to a reference genome to determine the genomic positions of each (individual) sequence read of the plurality of sequence reads. In view of

the size of the reference genome and the number of sequence reads in a plurality of sequence reads, the sequence reads are optionally received at a computer system.

Spatial detection methods

Spatial proteomics

A good overview of spatial detection methods is provided by Lewis et al. 2021 (Nature Methods 18:997-1012) with Figures 3 to 5 therein summarizing the described methods and their performance.

The most classical spatial detection methods are histopathological staining (e.g. hematoxylin and eosin staining) and immunohistochemical staining. Histopathological staining provides information on different tissue structures and possible abnormalities therein. Immunohistochemical (IHC) staining involves binding of antibodies to target proteins of interest, usually these (primary) antibodies are unlabeled and (primary) antibodies bound to its target in e.g. a tissue section are subsequently detected by binding of a labeled, e.g. fluorescently labeled, (secondary) antibody that binds to the (primary) antibody bound to the target protein of interest. In multiplexed IHC (mIHC) usually up to 4 or 5 target proteins of interest can be detected simultaneously. In modern mIHC, iterative cycles of target protein of interest detection are applied. This involves successive cycles of antibody binding and stripping of the antibody or stripping or bleaching of the antibody-labels. Alternatively, a pool of DNA-barcoded antibodies is applied and iterative hybridization with differently labeled oligonucleotides is performed. As a result, some of these techniques can detect up to 100 different proteins can be detected in a single tissue sample.

Non-iterative methods of target protein of interest detection involve binding of metal isotope-labeled antibodies that are subsequently detected by mass spectrometry upon release from a sample by means of tissue ablation with a laser beam (IMC: imaging mass cytometry) or tissue ionization with an ion beam (MIBI: multiplexed ion beam imaging). These techniques can detect up to 40 different proteins can be detected in a single tissue sample. IMC also allows for detection of an RNA target of interest.

Concurrent quantitation of more than 40 proteins of interest is furthermore possible by quantitative analysis (involving sequencing) of oligonucleotides cleaved off from oligo-nucleotide labeled primary antibodies, this in a technique called digital spatial profiling (DSP). DSP also allows for detection of an RNA target of interest. These spatial proteomic techniques have been summarized in e.g. Figure 3 of Lewis et al. 2021 (Nature Methods 18:997-1012).

Spatial transcriptomics

Spatial transcriptomics rely on direct detection of transcripts of interest with fluorescently labeled probes, the technology called fluorescent in situ hybridization (FISH). Many different FISH-based spatial transcriptomic methods have been developed and include iterative hybridization or bleaching or

destruction of the labeled probes. Some of the FISH-based spatial transcriptomic methods allow for detection of up to 10000 different transcripts (summarized in e.g. Figure 4 of Lewis et al. 2021 (Nature Methods 18:997-1012). Another series of spatial transcriptomic methods involve sequencing. *In vitro* methods (e.g. on a tissue sample) include laser capture microdissection (LCM) methods, methods including an mRNA capture step, and microfluidic-based methods – all have been reported to allow for detection of 10000 or more targets. In situ methods include in situ sequencing and fluorescence in situ sequencing methods. The sequencing technique can rely on sequence-by-ligation or sequence-by-hybridization methodologies. Again, some of these methods have been reported to allow for detection of 10000 or more targets (incompletely summarized in e.g. Figure 5 of Lewis et al. 2021 (Nature Methods 18:997-1012)).

Standard or control / standard or control expression level

Standards or controls for the expression level (at transcriptomic level or at proteomic level) of a biomarker gene as listed above (including any of the CD8 Temra cell markers, any of the CXCL10-positive macrophage markers, level of TCR sharing) can be defined in some alternative ways.

In one embodiment, such standard or control expression level refers to a pre-determined range of expression levels/standard values. Typically such ranges are defined after collecting a set of expression levels of a gene X (including any of the CD8 Temra cell markers, any of the CXCL10-positive macrophage markers, level of TCR sharing) as determined in a suitable number of cancer patients. As outlined in the Examples herein, the expression of the biomarker genes as listed above (i.e. any of the CD8 Temra cell markers or CXCL10-positive macrophage markers) is higher in future responders to therapy comprising an immune checkpoint inhibitor (ICI) or to therapy with an ICI versus/compared to in future non-responders to such therapy. From these, suitable standard/control expression levels or expression level ranges can be determined.

The expression level of a gene X as determined in any of the above methods for a cancer patient potentially eligible for treatment with a therapy comprising an immune checkpoint inhibitor (ICI) or to a therapy with an ICI (the test subject) can alternatively be compared with the expression level at a similar time-point of the same gene X in a cancer patient or set of cancer patients known as (subsequent) responder(s) (or non-responder(s)) to the therapy comprising an immune checkpoint inhibitor (ICI) or to the therapy with an ICI (the control subject(s)). If the expression level of the gene X in the test subject is (roughly/about) equal to the expression level in a responding control subject, or is higher than the expression level in a non-responding control subject, then the test subject is predicted to be a responder to the immunotherapy or immunogenic therapy. If the expression level of the gene X in the test subject is (roughly/about) equal to the expression level in a non-responding control subject, or is lower than the

expression level in a non-responding control subject, then the test subject is predicted to be a non-responder to the immunotherapy or immunogenic therapy.

In particular, the expression level of a gene X is determined by normalization relative to expression of e.g. a housekeeping gene or set of housekeeping genes. Any diagnostic kit or device designed to operate according to any of the above-listed methods of the invention (see further) therefore includes the option/possibility to determine, assess, measure, quantify expression of one or more household genes in addition to the means to determine, assess, measure, quantify expression of one or more of the above-listed biomarker genes predictive for outcome of therapy comprising an immune checkpoint inhibitor (ICI) or of therapy with an ICI in a subject having cancer, or predictive for (early) response or predictive for (early) response of a subject having cancer to therapy comprising an immune checkpoint inhibitor (ICI) or to therapy with an ICI.

The higher expression of individual or combined biomarker genes as listed above in future responders to therapy comprising an immune checkpoint inhibitor (ICI) or to therapy with an ICI versus/compared to in future non-responders to therapy comprising an immune checkpoint inhibitor (ICI) or to therapy with an ICI can be higher with 5% or more, 10% or more, 15% or more, 20% or more, 25% or more, 30% or more, 35% or more, 40% or more, 45% or more, 50% or more, 55% or more, 60% or more, 65% or more, 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 100% or more; or with up to 10%, up to 20%, of up to 30%, of up to 40%, of up to 50%, of up to 60%, of up to 70%, of up to 80%, of up to 90%, or with up to 100% or more. In case of a 100% higher analyte strand number of an individual biomarker gene, the expression of that individual biomarker has doubled, or increased 2-fold. The higher analyte strand number of an individual biomarker can further be 1.1-fold higher, 1.2-fold higher, 1.3-fold higher, 1.4-fold higher, 1.5-fold higher, 1.6-fold higher, 1.7-fold higher, 1.8-fold higher, 1.9-fold higher, 2-fold higher, 2.1-fold higher, 2.2-fold higher, 2.3-fold higher, 2.4-fold higher, 2.5-fold higher, 2.6-fold higher, 2.7-fold higher, 2.8-fold higher, 2.9-fold higher, 3-fold higher, more than 3-fold higher, 3.5-fold higher, 4-fold higher, more than 4-fold higher, between 3-fold and 4-fold higher, 4.5-fold higher, 5-fold higher, more than 5-fold higher, between 2-fold and 5-fold higher, between 3-fold and 5-fold higher, between 3-fold and 5-fold higher, 6-fold higher, more than 6-fold higher, between 2-fold and 6-fold higher, between 3-fold and 6-fold higher, between 4-fold and 6-fold higher, 7-fold higher, more than 7-fold higher, 8-fold higher, more than 8-fold higher, 9-fold higher, more than 9-fold higher, 10-fold higher, up to 10-fold higher, more than 10-fold higher, between 2-fold and 10-fold higher, between 3-fold and 10-fold higher, between 4-fold and 10-fold higher, between 5-fold and 10-fold higher, between 6-fold and 10-fold higher, between 7-fold and 10-fold higher, between 8-fold and 10-fold higher, substantially more than 10-fold higher, between 10-fold and 15-fold higher, up to 15-fold higher, between 10-fold and 20-fold higher, up to 20-fold higher, substantially more than 20-fold in

higher crease such as up to 25-fold higher, up to 30-fold higher, up to 40-fold higher, or up to 50-fold higher.

Tumor, cancer, neoplasm

5 The terms tumor and cancer are sometimes used interchangeably but can be distinguished from each other. A tumor refers to “a mass” which can be benign (more or less harmless) or malignant (cancerous). A cancer is a threatening type of tumor. A tumor is sometimes referred to as a neoplasm: an abnormal cell growth, usually faster compared to growth of normal cells. Benign tumors or neoplasms are nonmalignant/non-cancerous, are usually localized and usually do not spread/metastasize to other
10 locations. Because of their size, they can affect neighboring organs and may therefore need removal and/or treatment. A cancer, malignant tumor or malignant neoplasm is cancerous in nature, can metastasize, and sometimes re-occurs at the site from which it was removed (relapse). The initial site where a cancer starts to develop gives rise to the primary cancer. When cancer cells break away from the primary cancer (“seed”), they can move (via blood or lymph fluid) to another site even remote from
15 the initial site. If the other site allows settlement and growth of these moving cancer cells, a new cancer, called secondary cancer, can emerge (“soil”). The process leading to secondary cancer is also termed metastasis, and secondary cancers are also termed metastases. For instance, liver cancer can arise as primary cancer, but can also be a secondary cancer originating from a primary breast cancer, bowel cancer or lung cancer; some types of cancer show an organ-specific pattern of metastasis. Most cancer
20 deaths are in fact caused by metastases, rather than by primary tumors (Chambers et al. 2002, Nature Rev Cancer2:563-572).

Cancer is referred to in general terms herein. More specifically, the cancer is hepatocellular carcinoma (HCC), such as advanced HCC (aHCC). Hepatocellular carcinoma (HCC) is the most common form of liver cancer and one of the few neoplasms with increasing incidence and mortality worldwide (Sung et al.
25 2021, CA Cancer J Clin 71:209-249) . The majority of HCC patients (50- 60%) eventually evolve to an advanced stage (aHCC) requiring systemic treatment (Llovet et al. 2021, Nat Rev Dis Prim 7:6). Recently, immune checkpoint inhibitors (ICI; alternatively termed immune checkpoint blockers or ICBs; or checkpoint inhibitors or CPIs) have dramatically changed the treatment landscape of aHCC. In front line clinical trials, patients receiving combinations of PD(L)1 inhibition with anti-angiogenic drugs have a
30 median overall survival (OS) of 20 months (Finn et al. 2020, N Engl J Med 382:1894-1905; Cheng et al. 2022, J Hepatol 76:862-873; Ren et al. 2021, Lancet Oncol 22:977-990; Qin et al. 2022, Annals of Oncology 33 (suppl_7): S808-S869, Camrelizumab (C) plus rivoceranib (R) vs. sorafenib (S) as first-line therapy for unresectable hepatocellular carcinoma (uHCC): A randomized, phase III trial), which is almost double compared to results achieved using tyrosine kinase inhibitors (TKI) (Llovet et al. 2008, N Engl J

Med 359:378-390; Kudo et al. 2018, Lancet 391:1163-1173). Response to CPI in aHCC can be durable, with some patients experiencing long-term disease remission and even cure. However, in trials assessing CPI monotherapy, objective response was observed in only 14-17% of patients (Yau et al. 2022, Lancet Oncol 23:77-90; Qin et al. 2022, Ann Oncol 33 Suppl 7:S808-S869, Final analysis of RATIONALE-301: Randomized, phase III study of tislelizumab versus sorafenib as first-line treatment for unresectable hepatocellular carcinoma; Abou-Alfa et al. 2022, NEJM Evid 78:1-12; Finn et al. 2020, J Clin Oncol 38:193-202; Lee et al. 2020, Lancet Oncol 21:808-820).

Treatment / therapeutically effective amount

"Treatment"/"treating" refers to any rate of reduction, delaying or retardation of the progress of the disease or disorder, or a single symptom thereof, compared to the progress or expected progress of the disease or disorder, or single symptom thereof, when left untreated. This implies that a therapeutic modality on its own may not result in a complete or partial response (or may even not result in any response), but may, in particular when combined with other therapeutic modalities, contribute to a complete or partial response (e.g. by rendering the disease or disorder more sensitive to therapy). More desirable, the treatment results in no/zero progress of the disease or disorder, or single symptom thereof (i.e. "inhibition" or "inhibition of progression"), or even in any rate of regression of the already developed disease or disorder, or single symptom thereof. "Suppression/suppressing" can in this context be used as alternative for "treatment/treating". Treatment/treating also refers to achieving a significant amelioration of one or more clinical symptoms associated with a disease or disorder, or of any single symptom thereof. Depending on the situation, the significant amelioration may be scored quantitatively or qualitatively. Qualitative criteria may e.g. by patient well-being. In the case of quantitative evaluation, the significant amelioration is typically a 10% or more, a 20% or more, a 25% or more, a 30% or more, a 40% or more, a 50% or more, a 60% or more, a 70% or more, a 75% or more, a 80% or more, a 95% or more, or a 100% improvement over the situation prior to treatment. The time-frame over which the improvement is evaluated will depend on the type of criteria/disease observed and can be determined by the person skilled in the art.

A "therapeutically effective amount" refers to an amount of a therapeutic agent to treat or prevent a disease or disorder in a mammal. In the case of cancers, the therapeutically effective amount of the therapeutic agent may reduce the number of cancer cells; reduce the primary tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, (progression of) tumor growth; and/or relieve to some extent one or more of the symptoms associated with the disorder. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or

cytotoxic. For cancer therapy, efficacy in vivo can, e.g., be measured by assessing the duration of survival (e.g. overall survival), time to disease progression (TTP), response rates (e.g., complete response and partial response, stable disease), length of progression-free survival, duration of response, and/or quality of life. The term “effective amount” refers to the dosing regimen of the agent (e.g. antagonist as described herein) or composition comprising the agent (e.g. medicament or pharmaceutical composition). The effective amount will generally depend on and/or will need adjustment to the mode of contacting or administration. The effective amount of the agent or composition comprising the agent is the amount required to obtain the desired clinical outcome or therapeutic effect without causing significant or unnecessary toxic effects (often expressed as maximum tolerable dose, MTD). To obtain or maintain the effective amount, the agent or composition comprising the agent may be administered as a single dose or in multiple doses. The effective amount may further vary depending on the severity of the condition that needs to be treated; this may depend on the overall health and physical condition of the mammal or patient and usually the treating doctor’s or physician’s assessment will be required to establish what is the effective amount. The effective amount may further be obtained by a combination of different types of contacting or administration.

The aspects and embodiments described above in general may comprise the administration of one or more therapeutic compounds to a mammal in need thereof, i.e., harboring a tumor, cancer or neoplasm in need of treatment. In general a (therapeutically) effective amount of (a) therapeutic compound(s) is administered to the mammal in need thereof in order to obtain the described clinical response(s).

“Administering” means any mode of contacting that results in interaction between an agent (e.g. a therapeutic compound) or composition comprising the agent (such as a medicament or pharmaceutical composition) and an object (e.g. cell, tissue, organ, body lumen) with which said agent or composition is contacted. The interaction between the agent or composition and the object can occur starting immediately or nearly immediately with the administration of the agent or composition, can occur over an extended time period (starting immediately or nearly immediately with the administration of the agent or composition), or can be delayed relative to the time of administration of the agent or composition. More specifically the “contacting” results in delivering an effective amount of the agent or composition comprising the agent to the object.

Outcome of or response to the immunotherapy or the immunogenic therapy

The response to a therapy including an ICI or to a therapy with an ICI in one embodiment is a clinical response such as duration of survival (e.g. overall survival), time to disease progression (TTP), response rates (e.g., complete response and partial response, stable disease, no response), length of progression-free survival, duration of response, quality of life, but can likewise be expressed in terms of having a

therapeutic effect (such as treating/treatment cancer, inhibiting tumor progression or relapse, inhibiting tumor metastasis, and the like). The (bio)markers as used in the current methods in particular are useful for dividing the subjects having cancer into likely responders to therapy including an ICI or to therapy with an ICI on the one hand, and into likely non-responders to therapy including an ICI or to therapy with an ICI on the other hand.

Kits

In view of all above, kits, such as diagnostic or prognostic kits or kits of parts, can be designed that are tailored to enable any of the methods/uses disclosed herein, such as:

- methods of/for selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI as described herein,
- methods of/for predicting the response, predicting the likelihood of response, or predicting the responsiveness of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI as described herein,
- methods of determining response of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI as described herein,
- use of an ICI for manufacturing/in the manufacture of a medicament for treating or inhibiting cancer in a subject, for inhibiting cancer progression in a subject, or for inhibiting cancer relapse in a subject as described herein,
- such methods/uses relying on measuring, determining, assessing, quantifying, or analyzing CD8 Temra cells and/or relying on measuring, determining, assessing, quantifying, or analyzing CXCL10-positive macrophages; and/or relying on pre-therapy TCR sharing between tumoral and peripheral CD8 Temra cells; and/or relying on measuring, determining, assessing, quantifying, or analyzing CXCL10-positive macrophages and pre-therapy TCR sharing between tumoral and peripheral CD8 Temra cells; and/or relying on measuring, determining, assessing, quantifying, or analyzing CD8 Temra cells and relying on measuring, determining, assessing, quantifying, or analyzing CXCL10-positive macrophages, and relying on pre-therapy TCR sharing between tumoral and peripheral CD8 Temra cells.

Such kits can be comprising the tools to quantify the abundance of CD8 Temra cells in a pre-therapy biopsy sample, the abundance of CXCL10-positive macrophages in a pre-therapy biopsy sample, or the level of TCR sharing in CD8 Temra cells in a pre-therapy biopsy sample and in a blood sample.

Such kits in particular comprise the tools to detect the marker genes and/or proteins as described herein (in particular CD8 Temra cell marker genes and/or proteins, and/or CXCL10-positive macrophage marker genes and/or proteins), or to detect TCR sharing as described herein.

For detection of marker gene expression or TCR sharing, oligonucleotides can be designed. The oligonucleotides can be primers and/or probes. In particular, the primers and/or probes are labeled primers and/or probes; primers and/or probes comprising non-naturally occurring nucleotides; hairpin or structurally locked primers and/or probes; or a combination thereof. In particular, the oligonucleotides comprise a sequence specifically hybridizing to e.g. a CD8 Temra cell marker gene or to a CXCL10-positive macrophage marker gene, or to e.g. TCR genes. In particular, such oligonucleotide is comprising least one modified or non-naturally occurring nucleotide (as described hereinabove). Further in particular, the oligonucleotide may be part of a primer and probe set, of which set at least one primer or probe is comprising a sequence specifically hybridizing to the envisaged marker gene. Such kits/diagnostic kits can alternatively comprise a multi-membered set of oligonucleotides, wherein each member of the set comprises at least one modified or non-naturally occurring nucleotide and a sequence specifically hybridizing to one of the envisaged marker gene. Such kits/diagnostic kits can alternatively comprise a plurality of separate primer and probe sets, wherein each set is comprising a primer or probe comprising of which at least one of the primer or probe is comprising a modified or non-naturally occurring nucleotide, and wherein each set comprises a primer or probe of which at least one of the primer or probe is comprising a sequence specifically hybridizing to one of the envisaged marker genes. A non-naturally occurring nucleotide may be a nucleotide that is chemically different from a nucleotide present in a living cell (such as a labelled nucleotide), or may be a chemically naturally occurring nucleotide but which is mutated relative to the natural target nucleic acid on which the oligonucleotide is specifically hybridizing.

For detection of marker protein expression, a panel of suitable antibodies can be compiled. In particular, such antibodies can be coupled, decorated or linked with a detectable label or moiety. More in particular such label is removable or is bleachable (enabling iterative marker protein detection with antibodies to different marker proteins), or is a metallic label (enabling mass spectrometry-based detection). More in particular such label is a "barcode" oligonucleotide.

Depending on the method of detection of the marker gene as described herein, such kits may further comprise reagents such as reagents required for extraction of DNA from cells, reagents for amplification of DNA, hybridization reagents, DNA intercalating dyes, reaction vials, and a kit instruction manual or a kit manual. In one specific embodiment, such kits/diagnostic kits are including the tools for detecting the status of, in total, at most 250 genes including CD8 Temra cell marker genes and/or CXCL10-positive macrophage marker genes as described herein, or at most 225, 200, 175, 150, 125, 111, 110, 105, 100, 95, 90, 85, 80, 79, 78, 77, 76, 75, 74, 73, 72, 71, 70, 69, 68, 67, 66, 65, 64, 63, 62, 61, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4 genes, including at least

4 CD8 Temra cell marker genes or at least 4 CXCL10-positive macrophage marker genes as described herein.

Depending on the method of detection of the marker proteins as described herein, such kits may further comprise reagents such as labelled secondary antibodies (in case the primary antibody/ies to the marker protein(s) of interest is/are not labelled), reagents enabling optimal detection of the label, reagents for removal or bleaching of the label, reaction vials and a kit instruction manual or a kit manual.

In a further specific embodiment, such kits/diagnostics kit contain the components and/or reagents enabling multiplex analysis of marker gene expression or marker protein expression.

In a further specific embodiment, such kits/diagnostic kits contain

Immune checkpoint inhibitors/blockers (ICIs/ICBs/CPIs)

Immune checkpoints antagonists or inhibitors as referred to herein include the cell surface protein cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed cell death protein-1 (PD-1) and their respective ligands. CTLA-4 binds to its co-receptor B7-1 (CD80) or B7-2 (CD86); PD-1 binds to its ligands PD-L1 (B7-H10) and PD-L2 (B7-DC). Other immune checkpoint inhibitors include the adenosine A2A receptor (A2AR), B7-H3 (or CD276), B7-H4 (or VTCN1), BTLA (or CD272), IDO (indoleamine 2,3-dioxygenase), KIR (killer-cell immunoglobulin-like receptor), LAG3 (lymphocyte activation gene-3), NOX2 (nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoform 2), TIM3 (T-cell immunoglobulin domain and mucin domain 3), VISTA (V-domain Ig suppressor of T cell activation), SIGLEC7 (sialic acid-binding immunoglobulin-type lectin 7, or CD328) and SIGLEC9 (sialic acid-binding immunoglobulin-type lectin 9, or CD329).

In any of the above aspects, embodiments, and kits, the therapy comprising an ICI or therapy with an ICI can in particular be a therapy comprising a combination in any way of two immune checkpoint inhibitors.

In one embodiment these are each inhibiting a different immune checkpoint or a different immune checkpoint-ligand interaction. For instance, when an inhibitor of PD1 is selected as a first immune checkpoint inhibitor, the second immune checkpoint inhibitor could be an inhibitor of PDL1 or an inhibitor of PDL2. Such first and second immune checkpoint inhibitor are each inhibiting a different immune checkpoint protein. In a further non-limiting example, an inhibitor of PD1 is selected as a first immune checkpoint inhibitor, and as second immune checkpoint inhibitor an inhibitor different from an inhibitor of PDL1 and different from an inhibitor of PDL2 is selected, e.g. an inhibitor of CTLA-4 is selected. In this latter example, the first and second immune checkpoint inhibitor are not only each inhibiting a different immune checkpoint, but also each inhibiting a different immune checkpoint-ligand interaction.

An overview of clinical developments in the field of immune checkpoint therapy is given by Fan et al. 2019 (Oncology Reports 41:3-14). Immune checkpoint inhibitors include, but are not limited to anti-PD-1, anti-PD-L1 or anti-CTLA-4 antibodies.

PD1

5 Aliases of PD1 provided in GeneCards® include PDCD1; Programmed Cell Death 1; Systemic Lupus Erythematosus Susceptibility 2; PD-1; CD279; HPD-1; SLEB2; and HPD-L. The genomic locations for the PDCD1 gene are *chr2:241,849,881-241,858,908* (in GRCh38/hg38) and *chr2:242,792,033-242,801,060* (in GRCh37/hg19). The GenBank reference PD1 mRNA sequence is known under accession no. NM_005018.3. Approved PD1-inhibiting antibodies include nivolumab, pembrolizumab, and
 10 cemiplimab; PD1-inhibiting antibodies under development include CT-011 (pidilizumab) and therapy with PD1-inhibiting antibodies is referred to herein as α -PD-1 therapy or α -PD1 therapy. PD1 siRNA and shRNA products are available through e.g. Origene.

PD-L1

Aliases of PD-L1 provided in GeneCards® include CD274, Programmed Cell Death 1 Ligand 1, B7 Homolog
 15 1, B7H1, PDL1, PDCD1 Ligand 1, PDCD1LG1, PDCD1L1, HPD-L1, B7-H1, B7-H, and Programmed Death Ligand 1. The genomic locations for the PDCD1 gene are *chr9:5,450,503-5,470,567* (in GRCh38/hg38) and *chr9:5,450,503-5,470,567* (in GRCh37/hg19). The GenBank reference PD1 mRNA sequence is known under accession no. NM_001267706.1, NM_001314029.2 and NM_014143.4. Approved PD-L1-inhibiting antibodies include atezolizumab, avelumab, and durvalumab. PD-L1 siRNA and shRNA products are
 20 available through e.g. Origene.

CTLA4

Aliases of CTLA4 provided in GeneCards® include Cytotoxic T-Lymphocyte Associated Protein 4; CTLA-4; CD152; Insulin-Dependent Diabetes Mellitus 12; Cytotoxic T-Lymphocyte Protein 4; Celiac Disease 3; GSE; Ligand And Transmembrane Spliced Cytotoxic T Lymphocyte Associated Antigen 4; Cytotoxic T
 25 Lymphocyte Associated Antigen 4 Short Spliced Form; Cytotoxic T-Lymphocyte-Associated Serine Esterase-4; Cytotoxic T-Lymphocyte-Associated Antigen 4; CELIAC3; IDDM12; ALPS5; and GRD4.

The genomic locations for the CTLA4 gene are *chr2:203,867,771-203,873,965* (in GRCh38/hg38) and *chr2:204,732,509-204,738,683* (in GRCh37/hg19). The GenBank reference CTLA4 mRNA sequences are known under accession nos. NM_001037631.3 and NM_005214.5. Approved CTLA4-inhibiting
 30 antibodies include ipilumab; CTLA4-inhibiting antibodies under development include tremelimumab; therapy with CTLA4-inhibiting antibodies is referred to herein as α -CTLA4 therapy. CTLA4 siRNA and shRNA products are available through e.g. Origene.

Immunotherapy / immunotherapeutic compound or agent

Immunotherapy in general is defined as a treatment comprising administration of an immunotherapeutic compound or agent that supports (including activation or reactivation) the body's own immune system to help fight a disease, more specifically cancer in the context of the current invention. Immunotherapeutic treatment as used herein refers to the reactivation and/or stimulation and/or reconstitution of the immune response of a mammal towards a condition such as a tumor, cancer or neoplasm evading and/or escaping and/or suppressing normal immune surveillance. The reactivation and/or stimulation and/or reconstitution of the immune response of a mammal in turn in part results in an increase in elimination of tumorous, cancerous or neoplastic cells by the mammal's immune system (anticancer, antitumor or anti-neoplasm immune response; adaptive immune response to the tumor, cancer or neoplasm).

Immunotherapeutic agents include antibodies, in particular monoclonal antibodies, employed as (targeted) anti-cancer agents include alemtuzumab (chronic lymphocytic leukemia), bevacizumab (colorectal cancer), cetuximab (colorectal cancer, head and neck cancer), denosumab (solid tumor's bony metastases), gemtuzumab (acute myelogenous leukemia), ipilumab (melanoma), ofatumumab (chronic lymphocytic leukemia), panitumumab (colorectal cancer), rituximab (Non-Hodgkin lymphoma), tositumomab (Non-Hodgkin lymphoma) and trastuzumab (breast cancer). Other antibodies include for instance abagovomab (ovarian cancer), adecatumumab (prostate and breast cancer), afutuzumab (lymphoma), amatuximab, apolizumab (hematological cancers), blinatumomab, cixutumumab (solid tumors), dacetuzumab (hematologic cancers), elotuzumab (multiple myeloma), farletuzumab (ovarian cancer), intetumumab (solid tumors), muatuzumab (colorectal, lung and stomach cancer), onartuzumab, parsatuzumab, pritumumab (brain cancer), tremelimumab, ublituximab, veltuzumab (non-Hodgkin's lymphoma), votumumab (colorectal tumors), zatuximab and anti-placental growth factor antibodies such as described in WO 2006/099698.

Immunotherapeutic agents of particular interest further include immune checkpoint inhibitors (such as anti-PD-1, anti-PD-L1 or anti-CTLA-4 antibodies; detailed hereinafter), bispecific antibodies bridging a cancer cell and an immune cell, dendritic cell vaccines, CAR-T cells, oncolytic viruses, RNA vaccines, and so on. Immunotherapy is a promising new area of cancer therapeutics and several immunotherapies are being evaluated preclinically as well as in clinical trials and have demonstrated promising activity (Callahan et al. 2013, J Leukoc Biol 94:41-53; Page et al. 2014, Annu Rev Med 65:185-202). However, not all the patients are sensitive to immune checkpoint blockade and sometimes PD-1 or PD-L1 blocking antibodies accelerate tumor progression. An overview of clinical developments in the field of immune checkpoint therapy is given by Fan et al. 2019 (Oncology Reports 41:3-14). Combinatorial cancer treatments that include chemotherapies can achieve higher rates of disease control by impinging on

distinct elements of tumor biology to obtain synergistic antitumor effects. It is now accepted that certain chemotherapies can increase tumor immunity by inducing immunogenic cell death and by promoting escape in cancer immunoediting, such therapies are therefore called immunogenic therapies as they provoke an immunogenic response. Drug moieties known to induce immunogenic cell death include

5 bleomycin, bortezomib, cyclophosphamide, doxorubicin, epirubicin, idarubicin, mafosfamide, mitoxantrone, oxaliplatin, and patupilone (Bezu et al. 2015, Front Immunol 6:187). Other forms of immunotherapy include chimeric antigen receptor (CAR) T-cell therapy in which allogenic T-cells are adapted to recognize a tumoral neo-antigen and oncolytic viruses preferentially infecting and killing cancer cells. Treatment with RNA, e.g. encoding MLKL, is a further means of provoking an immunogenic

10 response (Van Hoecke et al. 2018, Nat Commun 9:3417), as well as vaccination with neo-epitopes (Brennick et al. 2017, Immunotherapy 9:361-371).

Further anti-tumor agents include those described in general terms in the sections "Inhibition of a target of interest", and "Pharmacological knock-down of a protein of interest" included herein, and wherein the target or protein of interest can be any known anti-cancer target or protein.

Computer / computer system

A computer or computer system as mentioned herein may utilize one or more subsystems. A computer or computer system may be a single computer apparatus comprising the one or more subsystems (e.g. internal components), or may be multiple computers or multiple computer apparatuses each being a

20 subsystem, and optionally, each comprising one or more own subsystems. Desktops, laptops, mainframe servers, tablets, mobile phones etc. all are computers or computer systems. The subsystems are usually interconnected and include a (central) processor (single-core processor, multi-core processor on a same integrated chip, or multiple processing units on a single circuit board or networked) capable of executing instructions, an input/output (I/O) controller, and a storage device (external, internal, peripheral, cloud,

25 any medium readable by a computer or computer system). Input devices include keyboards, scanners, a computer mouse, camera, microphone, etc. In particular, the input device is a data collection or data generating device (which by itself may comprise a computer or computer system), such as a polynucleotide sequencing device (whether automated or not). Collected or generated data are fed to a computer or computer system designed to analyze the collected or generated data; this may be an

30 ordinary computer system on which data analyzing software is installed (on a storage device) or which is capable of accessing data analyzing software (e.g. installed in or transmitted from a network) and whereby the processor of the computer system is instructed by the data analysis software on how to process the collected or generated data fed to the computer system, and how to display these via a display adapter to an output device. Output devices are further subsystems and comprise printers,

monitors, computer readable medium. Input and output devices are usually connected to a computer or computer system via input/output ports to one another or via a network.

The specific combination of hardware and software allows implementation of e.g. analysis of data generated by a polynucleotide sequencing device, expression analysis device, spatial transcriptomics device or spatial proteomics device. Different software packages (proprietary or open source) can be run on a computer or computer system to achieve the desired degree of data analysis. Output of one computerized data analysis can be the input of a subsequent computerized data analysis step, hence creating an analysis pipeline. Software components can be written in different codes (e.g. Java, C, C++, Perl, Python) as long as the computer processor is able to execute the functions of the software component.

The methods of the invention may be computer-implemented methods, or methods that are assisted or supported by a computer or by a computer system. For instance, information reflecting determining, detecting, assaying, assessing or analyzing biomarker expression or biomarker expression levels obtained from a sample is received by at least one first processor, and/or is provided in user readable format by at least one/another processor. The same or a further processor may be calculating e.g. relative biomarker expression or biomarker expression level (such as relative to a control or standard) from the information received. The one or more processors may be coupled to random access memory operating under control of or in conjunction with a computer operating system. The processors may be included in one or more servers, clusters, or other computers or hardware resources, or may be implemented using cloud-based resources. The operating system may be, for example, a distribution of the LinuxTM operating system, the UnixTM operating system, or other open- source or proprietary operating system or platform. Processors may communicate with data storage devices, such as a database stored on a hard drive or drive array, to access or store program instructions other data. Processors may further communicate via a network interface, which in turn may communicate via the one or more networks, such as the Internet or other public or private networks, such that a query or other request may be received from a client, or other device or service. Such computer-implemented methods (or such methods that are assisted or supported by a computer) may be provided as a kit or as part of a kit. The bioinformatics software required to perform (part of) the computer-implemented methods, i.e. a computer program product, may also be part of a kit, or may be provided as an individual product. A computer product may also consist of a computer readable medium which is storing any of the instructions, computer program, or bioinformatics software enabling a computer system to perform at least one of the analysis of the herein described methods and/or to perform at least one calculation (e.g. (spatial) biomarker expression or biomarker expression level) as described herein.

Other Definitions

The present invention is described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes. Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated. Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Press, Plainsview, New York (2012); and Ausubel et al., current Protocols in Molecular Biology (Supplement 100), John Wiley & Sons, New York (2012), for definitions and terms of the art. The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

It is to be understood that although particular embodiments, specific configurations as well as materials and/or molecules, have been discussed herein for cells and methods according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention. The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting the application. The application is limited only by the claims.

In referring to genes or proteins herein, distinction between their respective annotation is not always made hereinabove or hereinafter.

The content of the documents cited herein are incorporated by reference.

EXAMPLES**EXAMPLE 1. Establishing a cell atlas representing the tumor-microenvironment and peripheral immune system of advanced HCC (aHCC)**

Subjecting 31 pre-treatment aHCC tissue biopsies to scRNAseq (Figure 1; Table 1), we obtained high quality transcriptomic data from 84 825 single-cells. Subsequent analysis involving dimensionality reduction and clustering identified several clusters, assigned to T-cells and NK-cells (26%), B-cells (2%), myeloid cells (17%) and stromal cell types (13.5%) based on marker gene expression. We also identified a proliferative cluster, which mainly consisted of proliferating T-cells and a large cluster of HCC cancer cells (40%) expressing both genes associated with normal liver function (*ALB*, *HP*, *FGA*, *FGB*) and liver cancer (*AFP*, *SPINK1*, *GPC3*, *AKR1C1*). Inferring copy number variations (CNV) from the scRNAseq data (Tickle et al. 2019, *inferCNV of the Trinity CTAT Project*; Klarman Cell Observatory, Broad Institute of MIT and Harvard, Cambridge, MA, USA; github.com/broadinstitute/inferCNV) confirmed the malignant origin of the HCC cluster by displaying CNV alterations previously described in HCC (Schulze et al. 2015, *Nat Genet* 47:505-511). There was no evidence of cluster bias based on biopsy type, treatment or underlying liver disease in immune cells and stromal cell types. Similarly, single-cell profiling of serial on-treatment (week 0-3-6) PBMC samples (n=58), yielded high-quality transcriptomic data for 286 806 PBMCs, annotated to their respective cell types using marker genes. Together, these data provide a unique cell atlas of both the TME and peripheral immune system of aHCC patients treated with CPI, an invaluable resource to further current knowledge.

Table 1. Patient demographics, tumour characteristics and treatment data

	n=37
Age in years; median (range)	70 (24-84)
Male sex – no. (%)	29 (78)
Cirrhosis – no. (%)	22 (59)
Underlying liver disease	
Alcohol – no. (%)	16 (43)
Obesity-related fatty-liver disease – no. (%)	6 (16)
Viral hepatitis - no. (%)	2 (5)
Adenoma – no. (%)	1 (3)
Other – no. (%)	3 (8)
Unknown/None – no. (%)	9 (24)
Previous treatment for HCC – no. (%)	14 (38)
Liver transplantation – no. (%)	2 (5)
Local-regional – no. (%)	8 (22)
Systemic – no. (%)	2 (11)

TKI – no.	1
Chemotherapy – no.	1
Systemic + Local-regional – no. (%)	2 (11)
TKI + resection – no.	1
TKI + chemotherapy + resection – no.	1
AFP	
AFP in ng/mL – median (range)	8,40 (1,2-400800)
AFP >10 ng/mL – no. (%)	16 (43)
AFP >400 ng/mL – no. (%)	9 (24)
Child-Pugh Score	
Class A – no. (%)	34 (92)
Class B – no. (%)	3 (8)
Tumour type	
HCC – no. (%)	36 (97)
Mixed – no. (%)	1 (3)
Tumour characteristics at baseline	
Multinodular disease – no. (%)	29 (78)
Extrahepatic spread – no. (%)	12 (32)
Macrovascular invasion – no. (%)	13 (35)
Treatment	
Tyrosine-kinase inhibitor (TKI) – no. (%)	5 (14)
Sorafenib – no.	3
Lenvatinib – no.	2
Checkpoint inhibitor (CPI) – no. (%)	30 (81)
Nivolumab – no.	5
Pembrolizumab – no.	1
Atezolizumab – no.	2
Atezolizumab/Bevacizumab - no.	21
Atezolizumab/Cabozantinib - no.	1
Untreated - no. (%)	2 (4)

HCC = Hepatocellular carcinoma; TKI = Tyrosine-kinase inhibitor; CPI = checkpoint inhibitor based treatment; AFP = alpha-foetoprotein.

EXAMPLE 2. The intra-tumoral T-/NK-cell composition is distinct from the peripheral T-/NK-cell composition

First, we explored the T-/NK-cell compartment of the TME and peripheral blood in more detail. Subclustering a total of 17 656 intra-tumoral T-/NK-cells and 155 213 peripheral T-/NK-cells (54% of PBMCs) separately, we identified several phenotypes of CD4 T-cells, CD8 T-cells and natural killer cells (NK cells) (Figure 2A, B). Notably, the peripheral T-/NK-cell distribution differed from the composition of intra-tumoral T-/NK-cells. Comparatively, naive (CD4 T_N and CD8 T_N) T-cells and CD4 central memory

(CD4 T_{CM}) T-cells occupied a larger share in peripheral blood, while CD4 regulatory T-cells (CD4 T_{REG}) and CD8 effector memory T-cells (CD8 T_{EM}; *GZMK*) were over-represented in the TME. Furthermore, CD4 (CD4 CXCL13) and CD8 (CD8 T_{EX}) 'exhausted' T-cells were unique to the TME and characterized by the highest expression of *PDCD1* (PD1) and other known exhaustion markers. Importantly, CD8 T_{EX} expressed the highest levels of *IFNG* along with a number of cytotoxic markers (*PRF1*, *NKG7*; Figure 2A), despite their 'exhaustion' phenotype, supporting their denomination as 'antigen-experienced' T-cells (Bassez et al. 2021, Nat Med 27:820-832). On the other hand, CD4 CXCL13 have been previously described as 'exhausted' CD4 T-cells in HCC (Zheng et al. 2017, Cell 169:1342-1356; Zhang et al. 2019, Cell 179:829-845). Though this cluster was very small in aHCC, we know from previous studies (Bassez et al. 2021, Nat Med 27:820-832) that it consists of both Th1 CD4 T-cells (*IFNG*, *CXCR3*) and follicular-helper CD4 T-cells (*BCL6*, *CD200*) (Helmink et al. 2020, Nature 577:549-555). Finally, based on the expression of typical marker genes (*CX3CR1*, *SPON2*, *FGFBP2*; Figure 2A), we identified CD45RA effector-memory T-cells (CD8 T_{EMRA}) both in the TME and in peripheral blood. We confirmed their expression of *CD45RA* at the protein level using TotalSeq-C data. Importantly, CD8 T_{EMRA} were phenotypically similar and clustering close to the cytotoxic NK-cells, but distinguishable based on their expression of CD8 (*CD8A*, *CD8B*; Figure 2C) and the detection of a productive T-cell receptor (TCR) sequences.

By combining scRNAseq and scTCRseq, we identified 10 517 T-cells carrying 7569 unique TCRs in the TME, while 104 165 peripheral T-cells carried 79 103 unique TCR sequences. We identified TCR clonotypes based on identical TCR sequences, and defined dominant clonotypes as TCRs shared by >5 T-cells. In intra-tumoral T-cells, dominant clonotypes were concentrated within effector (CD8 T_{EM}, CD8 T_{EMRA}) and 'antigen-experienced' T-cell clusters (CD8 T_{EX}, CD4 CXCL13), while non-dominant clonotypes were mostly found in naive, memory or regulatory T-cell subtypes. Similarly, dominant peripheral T-cell clonotypes, in line with their phenotypical counterparts in the tumour, were concentrated within peripheral effector T-cells (CD8 T_{EM}, CD8 T_{EMRA}). These findings were replicated when defining dominant clonotypes as TCRs representing 1% or more of the TCR repertoire in order to account for the number of T-cells detected.

In short, though phenotypically distinct, both CD8 effector T-cells (CD8 T_{EM}, CD8 T_{EMRA}) and CD8 exhausted T-cells (CD8 T_{EX}) were characterized by dominant T-cell clonotypes both in the tumour and in peripheral blood.

EXAMPLE 3. Clonally-expanded CD45RA effector-memory CD8 T-cells are associated with response to immune checkpoint inhibitors (CPI)

Having established the cell atlas in Example 1, and based on the findings in Example 2, we focused on exploring the intra-tumoral CD8 T-cell compartment in more detail according to clinical response to CPI.

Comparing relative abundancies of various T-cell phenotypes in the TME, we found that CD8 T_{EMRA} were more abundant in responding tumours compared to non-responders (p=0.03; Figure 3A). Of note, though CD8 T_{EX} expressed the highest levels of the therapeutic target *PDCD1* (PD1; Figure 3B), their presence (*i.e.* relative abundancies) in the TME did not differ according to response (Figure 3A).

Differential gene expression and pathway analysis of CD8 T-cells in the TME revealed upregulation of cytotoxic genes (*GZMB*, *GNLY*, *PRF1*, *GZMH*) and typical CD8 T_{EMRA} markers (*FGFBP2*, *CX3CR1*, *FCGR3A*), suggesting that CD8 T_{EMRA} play an important role in achieving durable response to CPI. In contrast, non-responding tumours were more memory-like (*FOS*) and, surprisingly, upregulated *GZMK*, a typical CD8 T_{EM} marker. Responding tumours were also characterized by a more clonal pre-treatment TCR repertoire while non-responders displayed a richer and more diverse, non-clonal baseline TCR repertoire. Calculating the Gini-index, which takes both TCR evenness (1-clonality) and TCR richness into account, for each CD8 T-cell phenotype, intra-tumoral CD8 T_{EM}, CD8 T_{EMRA} and CD8 T_{EX} were most clonally-expanded. Importantly, when stratifying for response to CPI, CD8 T_{EMRA} in responding tumours had a significantly higher Gini-index compared to non-responders (p=0.004; Figure 3C) and high levels of clonally expanded CD8 T_{EMRA} in the TME were associated with significantly longer PFS upon CPI therapy (median PFS 12 versus 4 months; p=0.027; Figure 3D). In contrast, we did not detect significant differences in clonal expansion of CD8 T_{EM} and CD8 T_{EX} when comparing responders to non-responders. These findings suggest that response to CPI in aHCC is facilitated by clonally expanded CD8 T_{EMRA} residing within the TME prior to treatment.

EXAMPLE 4. TCR sharing confirms CD8 T_{EMRA} as crucial effector T-cells in the TME of aHCC

Intra-tumoral T-cells can share identical TCR sequences with T-cells residing in peripheral blood (Valpione et al. 2020, Nat Cancer 1:210-221; Wu et al. 2020, Nature 579:274-278). As such T-cells are more likely to be tumor-reactive, we explored TCRs shared between tumors and PBMCs in 14 CPI-treated patients, 7 of which were CPI-responders. We focussed specifically on those TCR sequences present in tumor and peripheral blood prior to treatment initiation (PBMC week 0), hypothesizing that these shared TCRs represent a baseline immune response, directed at and driven by the tumour. A total of 242 unique shared, potentially 'tumor-specific', TCRs were detected, representing approximately 6.5% of all TCRs detected in the tumor compared to 0.4% of all TCRs detected in peripheral blood. In order to correct for the number of T-cells detected in each sample, we calculated the proportion of shared TCRs relative to the total number of TCRs detected in PBMCs and found that responders displayed a higher degree of TCR sharing (on average 2.5%; p=0.008; Figure 4A). Similar trends were detected in proportions of peripheral T-cells carrying TCRs shared between tumor and PBMCs at week 0, relative to the total number of T-cells detected in peripheral blood, calculated per sample and stratified for response.

Importantly, increased TCR sharing was associated with significantly longer PFS (median PFS 11 versus 4 months; $p=0.036$; Figure 4B), supporting our hypothesis that TCR sharing may indeed identify the fraction of intra-tumoral T-cells that truly target the tumour.

Linking these shared TCRs to their T-cell phenotype in the TME revealed that the majority represented CD8 T-cells, concentrated within CD8 effector subtypes (CD8 T_{EM} and CD8 T_{EMRA}). In fact, 52% of CD8 T_{EMRA} and 20% of CD8 T_{EM} in the TME were characterized by a TCR also detected in peripheral blood prior to treatment, while CD8 T_{EX} displayed far less TCR sharing with peripheral blood (8%). In line with these findings, differential gene expression, demonstrated an overexpression of CD8 (*CD8A* and *CD8B*) and cytotoxic markers (*GZMA*, *GZMB*, *PRF1*), as well as typical CD8 T_{EMRA} markers (*CX3CR1*, *FCGR3A*) in intra-tumoral T-cells with shared TCRs ($n=471$ shared T-cells). In contrast, T-cells carrying a TCR found exclusively in the tumour were enriched for exhaustion markers (*TIGIT*, *CTLA4*) and regulatory genes (*FOXP3*, *TNFRSF4*, *TNFRSF18*). Importantly, while shared CD8 T_{EM} were present in the TME of both responders and non-responders (approximately 10% of all CD8 T_{EM} in both groups), shared CD8 T_{EMRA} were almost exclusively seen in responding tumours (40% of all intra-tumoral CD8 T_{EMRA} in responders, compared to 4.6% in non-responders).

CD8 T_{EMRA} have been described as 'recently-activated' CD8 effector-memory T-cells (Zhang et al. 2018, Nature 564:268-272). They do not express *PDCD1* or other markers traditionally associated with antigen-experience. Instead they re-express *CD45RA* after antigenic stimulation (Sallusto et al. 1999, Nature 401:708-712; Tian et al. 2017, Nat Commun 8:1473). They are considered a sentinel-like T-cell phenotype that patrols inflammatory sites of frequent antigenic encounter (Henson et al. 2012, Curr Opin Immunol 24:476-481). They are endowed with potent cytolytic properties based on their high expression of cytotoxic markers (*PRF1*, *NKG7*, *GZMA*, *GZMB*, *GZMH*, *GNLY*) that relies on direct interaction between the T-cell and its target cell and constitutively express receptors that direct their migration to inflamed tissue (*CX3CR1*) (Henson et al. 2012, Curr Opin Immunol 24:476-481).

EXAMPLE 5. Interaction with tumor-antigens drives intra-tumoral differentiation towards CD8 T_{EMRA}

In order to gain insights into the origins of CD8 T_{EMRA} in the TME, we computed pseudotime trajectories of intra-tumoral CD8 T-cells using Slingshot (Street et al. 2018, BMC Genomics 19:1-16). We considered CD8 naive T-cells (CD8 T_N) as the root of the trajectory because they had the highest TCR richness. In line with previous reports (Bassez et al. 2021, Nat Med 27:820-832; Zheng et al. 2017, Cell 169:1342-1356), naive T-cells were connected to T_{EM} cells and then diverged into three distinct trajectories, connecting naive and T_{EM} T-cells to T_{RM}, naive and T_{EM} T-cells to T_{EMRA}, and naive and T_{EM} T-cells to T_{EX}. TCR richness decreased along each of these trajectories. CD8 T_{EM} displayed most TCR clonotype overlap with T_{EX}, but also with T_{EMRA} and T_{RM}, while there was almost no TCR overlap between T-cells belonging to different

lineages, supporting the validity of the three trajectories. Profiling marker genes along each trajectory confirmed their functional annotation.

When plotting the densities of T-cells along each trajectory, we found striking differences between responders and non-responders. Intra-tumoral CD8 T-cells in responders were capable of evolving towards more differentiated phenotypes, an effect that was most pronounced in the T_{EMRA} trajectory, while non-responders seemed frozen at an earlier stage of the pseudotime ($p < 0.001$). There was a steady increase in Gini-index along both the T_{EMRA} and T_{EX} trajectories of responding tumours. Importantly, when assessing the density of shared T-cells (i.e. T-cells characterized by a TCR found in PBMC at week 0) along the CD8 trajectories, we found these were clearly enriched towards the end of the T_{EMRA} trajectory in responders ($p < 0.0001$; Figure 7). In contrast, along the T_{EX} trajectory, the greatest T-cell density was seen at the T_{EM} stage in both responders and non-responders.

We then used TradeSeq (Van den Berge et al. 2020, Nat Commun 11:1-13) to identify sets of genes differentially expressed along the T_{EMRA} versus T_{EX} trajectories (using *diffEnd* test). A total of 14 pathways from the REACTOME gene sets and 76 pathways from the 'GO: biological processes' gene sets were significantly enriched in the CD8 T_{EMRA} and T_{EX} trajectories, respectively. Importantly, the T_{EMRA} trajectory was dominated by pathways related to innate-like immunity (Figure 8), reflecting their role as potent effector T-cells that eliminate cancer cells through direct cytotoxicity. In contrast, the T_{EX} trajectory was enriched in pathways involved in *IFNG* signalling and immune cell activation and differentiation. In order to understand which factors drive this dual differentiation, we again used TradeSeq to assess differences in expression patterns before and after the point where the trajectories diverge (using *earlyDEG* test) and found a total of 467 pathways were enriched. Importantly, pathways involved in antigen-binding were top ranked, suggesting that further differentiation requires direct interaction with antigens.

Finally, to study the on-treatment immune response, we used shared TCR clonotypes present in PBMCs and the TME prior to treatment, linked them to their phenotype in peripheral blood and tracked their evolution during treatment in PBMCs sampled during treatment (week 0-3-6). Firstly, the 264 unique TCRs characterizing CD8 T_{EMRA} in the TME were found predominantly in peripheral CD8 T_{EMRA} . Prior to treatment they represented 26.3% of all CD8 peripheral T-cells in responders (834 out of 3165 peripheral CD8 T-cells), compared to just 3.9% in non-responders (Figure 9B). Tracking their evolution during treatment, in non-responders, we observed a threefold decrease from week 0 to week 6 (3.9% to 1.3%, Figure 9B), while in responders their presence remained stable during treatment (26.3% to 23.8%). This was in stark contrast to the 566 unique TCRs found in CD8 T_{EX} in the TME that were found in less than 0.5% of CD8 peripheral T-cells in responders and non-responders alike (Figure 9A). Moreover, these TCRs found in intra-tumoral CD8 T_{EX} did not emerge in peripheral blood during treatment.

Taken together, these data suggest that while CD8 T_{EM} are present in the TME of both responders and non-responders alike, upon stimulation by tumoral antigens, CD8 T_{EM} differentiate into CD8 T_{EMRA} in responders specifically, potentially resulting in direct anti-tumour cytotoxicity. Furthermore, CD8 T_{EMRA} display significant TCR sharing with peripheral blood in responders, and continue to do so upon treatment with CPI, in line with their patrolling phenotype. In contrast, intra-tumoral differentiation from CD8 T_{EM} to CD8 T_{EX} occurs equally in responders and non-responders to CPI, suggesting that they might be bystander T-cells directed at non-tumor antigens. This is reinforced by the fact that CD8 T_{EX} do not share TCRs with blood prior to treatment, nor do they appear during treatment with CPI.

EXAMPLE 6. Pro-inflammatory PDL1-expressing CXCL10+ macrophages are associated with response to CPI

While intra-tumoral CD8 T_{EMRA} seem essential for subsequent response to CPI, they do not express PD1. Therefore, we wondered whether the true target of CPI in HCC might be found in PDL1-expressing cells. Expression of *CD274* (PDL1) in the TME was generally low, but clearly detected in myeloid cells. Therefore, we subclustered the 14 173 myeloid cells into monocytes/macrophages (n=12 513) and dendritic cells (DC; n=765). Within the monocyte/macrophage compartment, we identified several tumour-associated macrophage (TAM) subtypes (Figure 10A), of which the majority expressed high levels of anti-inflammatory markers, suggesting a predominantly immunosuppressive baseline TME in aHCC (Figure 10B). However, we also identified pro-inflammatory CXCL10+ TAMs (Macro CXCL10) characterized by high expression of genes involved in T-cell recruitment (*CXCL9*, *CXCL10*) and interferon-gamma signalling (*STAT1*, *IDO1*, *GBP1*) (Figure 10B).

Comparing relative abundancies of the various TAM subtypes between responding and non-responding tumours, we did not detect significant differences. However, differential gene expression showed an enrichment of genes involved in T-cell recruitment (*CXCL9*, *CXCL10*; adjusted p-value <0.01) in the macrophage compartment of responding tumours, while non-responders were enriched in immunosuppressive markers (*GPNMB*, *CCL18*, *CD5L*). Responding tumours also displayed higher levels of *CCL2*, which is a potent monocyte-attracting chemokine (Jin et al. 2021, Front Oncol 11:722916; Gschwandtner et al. 2019, Front Immunol 10:2759), but is also involved in recruitment of other immune cells into the TME. Finally, responders displayed higher expression of *SPP1* and *IL32*, markers previously associated with response to CPI in lung cancer (Leader et al. 2021, Cancer Cell 39: 1594-1609) and melanoma (Gruber et al. 2020, JCI Insight 5: e138772), respectively. Additional pathway analysis confirmed that macrophages of responding tumours were enriched in pro-inflammatory pathways. Importantly, on average, myeloid cells from responders expressed significantly higher levels of *CD274* (Figure 5A). More specifically, *CD274* expression was highest in Macro CXCL10, and Macro CXCL10

derived from responding tumours displayed higher *CD274* expression (Figure 5A). High PDL1 expression in Macro CXCL10 was also associated with longer PFS (median PFS not estimable versus 6 months; $p=0.055$; Figure 5B). Taken together, response to CPI is associated with an activated, pro-inflammatory, PDL1-expressing myeloid component in the pre-treatment TME.

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EXAMPLE 7. PDL1-expressing CXCL10+ macrophages recruit effector-memory T-cells into the TME

Tumor-associated macrophages have been associated with recruitment of peripheral T-cells into the TME (Ardighieri et al. 2021, Front Immunol 12:690201). Therefore, we used CellChat (Jin et al. 2021, Nat Commun 12:1-20) to predict receptor-ligand interactions between myeloid cells and T-cells. Firstly, calculating the significant interactions between immune cell types in the TME separately for responders and non-responders, we found that overall, responders displayed more interaction possibilities. Focussing on the CXCL signalling pathway network, we found predicted interactions between liver-resident macrophages (Kupffer cells) and T-cells in all patients, regardless of response. In contrast, when compared to non-responders, responding tumours displayed more predicted interactions between CXCL10+ macrophages and the T-cell compartment. While the CXCL12/CXCR4 interaction, which originated almost exclusively from Kupffer cells, was found equally in responders and non-responders, the *CXCL9/10/11* and *CXCR3* ligand-receptor pairs were significantly enriched in responders compared to non-responders.

In the TME, *CXCR3* was prominently expressed in several activated T-cell subtypes (i.e. CD4 T_{H1} and CD8 T_{EX} , in addition to CD4 T_{EM} and CD8 T_{EM} , but not in CD8 T_{EMRA} . Along the T_{EMRA} trajectory, *CXCR3* expression reached its peak at the effector-memory state, while in peripheral T-cells, *CXCR3* was expressed mostly in effector-memory T-cells. Based on these findings, we hypothesized that the interaction between *CXCR3* and its ligands *CXCL9/10/11* plays an important role in recruiting peripheral CD8 effector-memory T-cells (CD8 T_{EM}) into the TME. To confirm this, we again used CellChat to explore receptor-ligand interactions between the intra-tumoral myeloid cells and peripheral CD8 T-cells. Indeed, taking all cell-cell communication networks into account, we found that CD8 peripheral T-cells were the most dominant signalling 'receivers' in responders, with higher levels of incoming signals compared to their phenotypical counterparts in non-responding tumours. Furthermore, within the CXCL-signalling pathway, there were more predicted interactions between intra-tumoral CXCL10+ macrophages and peripheral CD8 T_{EM} in responders compared to non-responders.

Overall, this supports the notion that the intra-tumoral myeloid compartment, which is characterized by upregulated expression of *CXCL9/10/11* in responders, is crucial for the recruitment and activation of *CXCR3*+ effector-memory T-cells into the TME and has a potentially decisive role in determining response to immunotherapy.

EXAMPLE 8. CD8 T_{EMRA} and CXCL10+ macrophages as predictive biomarkers of response to CPI in HCC

Using scRNAseq, we identified clonally expanded, cytotoxic CD8 T_{EMRA} as effector cells driving response to CPI, while CXCL10+ macrophages (Macro CXCL10) function as gatekeepers responsible for the recruitment of primed peripheral T-cells. Next, we aimed to validate these single-cell derived findings and explore the potential of CD8 T_{EMRA} and Macro CXCL10 as predictive biomarkers of response to CPI in aHCC. Calculating a per sample CD8 T_{EMRA} and Macro CXCL10 enrichment score in transcriptomic data of 358 pre-treatment tumour biopsies of aHCC patients treated with atezolizumab +/- bevacizumab (n=300) versus sorafenib (n=58), we found that high CD8 T_{EMRA} and Macro CXCL10 enrichment scores were associated with significantly longer PFS in CPI-treated patients (Figure 11), but not in sorafenib-treated patients (Figure 11). Furthermore, the presence of CD8 T_{EMRA} and Macro CXCL10 in the TME was strongly correlated (R=0.85; p<0.00001), supporting the notion that they populate the TME of aHCC simultaneously (Figure 12). Indeed, combining CD8 T_{EMRA} and Macro CXCL10 marker genes into a single gene set, we found that CPI-treated patients with a high enrichment score for the CPI-response biomarker had significantly longer OS and PFS (p=0.022 and p<0.0001, respectively), an association that was not seen in sorafenib treated patients (Figure 13). Taken together, the combined presence of CD8 T_{EMRA} and Macro CXCL10 in the pre-treatment TME of aHCC patients is associated with improved outcomes upon CPI treatment, specifically, validating the single-cell derived findings and underlining the potential value of the CPI-response biomarker as a predictive biomarker of response to CPI in aHCC.

EXAMPLE 9. Markers for CD8 T_{EMRA} and CXCL10+ macrophages

CD8 T_{EMRA} (n=36) and Macro CXCL10 (n=16) marker genes were selected based on differential gene expression analysis of single cell RNA sequencing data and used to deconvolute bulk RNAseq data. The CD8 T_{EMRA} marker genes include "GZMH", "GNLY", "NKG7", "FGFBP2", "GZMB", "CST7", "CCL5", "PRF1", "CX3CR1", "CTSW", "GZMA", "KLRD1", "GZMM", "CD3D", "CD8A", "CD52", "PTPRC", "CD3G", "HCST", "CD3E", "PLEK", "KLRG1", "RAC2", "LCK", "CD247", "HOPX", "KRLK1", "BIN2", "S100A4", "CORO1A", "IL2RG", "ITGB2", "IFITM1", "EMP3", "TRBC1", "FLNA", and optionally CD8B and SPON2. The CXCL10-positive macrophage marker genes include "CXCL10", "CXCL9", "GBP1", "TYMP", "CALHM6", "CCL2", "TNFSF13B", "WARS", "CCL8", "IL4I1", "ICAM1", "LILRB4", "CXCL11", "SOD2", "LAP3", "STAT1", and optionally GBP5 and CD80.

Each gene set was used to calculate per sample enrichment scores for CD8 T_{EMRA} and CXCL10-positive macrophage, respectively using single-sample Gene Set Enrichment Analysis (ssGSEA) function from the R-package 'GSVA' (version 1.38.2). For each cell type, samples were divided into two groups: high versus

low enrichment score (split by median) and the Kaplan-Meier method was used to estimate and compare PFS between groups using the log-rank test (Figure 11).

Finally, we combined the CD8 T_{EMRA} and CXCL10-positive macrophage markers genes into a CPI response biomarker, comprising a total of 52 genes. Calculating the per sample bulk RNAseq enrichment score (ssGSEA), two groups with high (biomarker^{high}) versus low (biomarker^{low}) enrichment score for the CPI response biomarker were delineated using maximally selected rank statistics as described in the R package 'maxstat' (version 0.7.25). Kaplan-Meier curves for overall survival (OS) and progression-free survival (PFS) were generated for CPI- and sorafenib-treated patients, respectively. Significant differences between groups (biomarker^{high} vs biomarker^{low}) were evaluated using the log-rank test. All analyses were done using the R packages 'survival' (version 3.3.1) and 'survminer' (version 0.4.9).

EXAMPLE 10. Discussion

Our study represents the first, homogenous single-cell atlas of both the TME and peripheral immune system of aHCC patients treated with CPI, allowing the correlation of single-cell readouts with durable and clinically-meaningful response for the first time.

Within the pre-treatment TME of aHCC, we observed how PD1-negative, CD45RA effector-memory CD8 T-cells (CD8 T_{EMRA}) play a pivotal role in facilitating response to CPI. This clearly differs from other cancer types, where instead of CD8 T_{EMRA}, PD-1 expressing CD8 T_{EX} have repeatedly been identified as key effector cells in response to CPI. Although both CD8 T_{EX} and CD8 T_{EMRA} contained clonally-expanded T-cells, it was the CD8 T_{EMRA}, that were more abundant in the pre-treatment TME of responding tumours. We also found typical CD8 T_{EMRA} genes (*CX3CR1*, *SPON2* and *FCGR3A*) to be overexpressed in intra-tumoral T-cells from responders compared to non-responders and high levels of clonally-expanded CD8 T_{EMRA}, not CD8 T_{EX}, were associated with significantly longer PFS upon CPI-treatment. Additionally, CD8 T_{EMRA} displayed the highest degree of TCR sharing with peripheral blood, a phenomenon almost exclusively observed in responders which persisted on treatment, potentially suggesting that CD8 T_{EMRA} are targeting tumour-specific antigens. Finally, using trajectory analyses, we observed how intra-tumoral CD8 T-cells in responders were capable of evolving towards more differentiated phenotypes, an effect most pronounced in the T_{EMRA} trajectory, while non-responders seemed frozen at an earlier stage of the pseudo-time. T-cells carrying a TCR shared between tumor and blood were also enriched towards the end of the T_{EMRA} trajectory in responders, specifically. In contrast, PD1-expressing CD8 T_{EX} were not more abundant or more clonal in responders compared to non-responders. Along the T_{EX} trajectory, the greatest T-cell density was seen at the T_{EM} stage both in responders and non-responders and their TCRs were not found in blood prior to treatment, nor did they appear during therapy. This suggests that against the backdrop of the immunosuppressive milieu of the liver, PD1-expressing CD8 T-cells do not

become activated during response to CPI, as they do in other cancer types (Bassez et al. 2021, Nat Med 27:820-832; Sade-Feldman 2018, Cell 175:998-1013).

Certainly, it seems that CD8 T_{EMRA} are able to overcome immunosuppression within the liver TME as our findings point towards CD8 T_{EMRA} as the main candidate effector cell type of anti-tumoral immunity upon CPI treatment in aHCC. While CD8 T_{EMRA} have been previously identified in the TME of early stage HCC patients (Zheng et al. 2017, Cell 169:1342-1356; Zhang et al. 2019, Cell 179:829-845; Xue et al. 2022, Nature 612:141-147), their role in response to CPI has never been described. Interestingly, CD8 T_{EMRA} are more abundant and more clonally-expanded in aHCC compared to our observations in other cancer types (Bassez et al. 2021, Nat Med 27:820-832; Qian et al. 2020, Cell Research 30:745-762). Notably, CD8 T_{EMRA} do not express the typical exhaustion markers associated with activation and antigen-experience, nor do they express markers associated with a TME enriched for high cytokine expression or marked interferon gamma signalling. Instead, they re-express CD45RA upon antigen stimulation and are characterized by an NK-like functional phenotype, endowed with potent cytolytic properties that are mediated by the release of lytic granules and rely on direct interaction with target cells.

Nonetheless, the PD1 negative status of CD8 T_{EMRA} suggests that they may not be the direct therapeutic targets of CPI. Indeed, within the myeloid compartment, we identify activated, pro-inflammatory, PDL1-expressing CXCL10+ macrophages as essential regulators. Analogously to the suppressive role of PD1 in T-cells, PDL1 is an inhibitory activation marker for macrophages, designed to prevent uncontrolled inflammation (Hartley et al. 2018, Cancer Immunol Res 6:1260-1273). Pre-clinical research has shown that upon CPI treatment, PDL1-expressing myeloid cells proliferate and are activated (Hartley et al. 2018, Cancer Immunol Res 6:1260-1273; Bar et al. 2020, JCI Insight 5: e129353). In line with this, we found that CXCL10+ macrophages in CPI-responders express higher levels of PDL1 and this was associated with better outcomes upon treatment, suggesting that CPI treatment may lead to increased activation of PDL1-expressing CXCL10+ macrophages, releasing their chemokines (CXCL9/10/11) into the TME. The importance of CXCL9/10/11 in the therapeutic efficacy of CPI, by their role in T-cell recruitment, has been previously described in other cancer types (House et al. 2020, Clin Cancer Res 26:487-504; Mikuchi et al. 2015, Nat Commun 6:7458; Seitz et al. 2022, Br J Cancer 126:1470-1480). Intriguingly, CXCR3, the main target of CXCL9/10/11, was expressed predominantly in peripheral T_{EM} and along the T_{EMRA} trajectory CXCR3 expression reached its peak during the effector-memory phase. This suggests that increasing CXCL10+ macrophage activity may lead to more efficient and continued peripheral T-cell recruitment, replenishing the intra-tumoural CXCR3+ T_{EM} population. Subsequently, within the tumour and upon antigen stimulation, these CXCR3+ T_{EM} preferentially differentiate towards PD-1 negative CD8 T_{EMRA}, the key effectors of direct anti-tumour cytotoxicity within the TME. Indeed, the presence of CD8 T_{EMRA} and Macro CXCL10 in the pre-treatment TME was strongly linked and we demonstrate that their combined

presence is associated with improved outcomes upon CPI-treatment, specifically, confirming their value as predictive biomarkers response to CPI in aHCC.

In conclusion, we propose a novel paradigm, where response to CPI in aHCC is driven by clonally expanded, cytotoxic CD8 T_{EMRA} characterized by a high degree of TCR sharing with peripheral blood and present in the tumour prior to therapy. PDL1-expressing CXCL10+ macrophages are positioned as essential gatekeepers in the TME, interacting with the peripheral T-cell compartment to ensure effective T-cell recruitment into the TME. While the single-cell resolution was essential for explorative purposes, we demonstrate the predictive value of CD45RA effector-memory CD8 T-cells and CXCL10+ macrophages as biomarkers of response to CPI in aHCC using bulk RNAseq.

EXAMPLE 11. Methods

11.1. Patients and methods

Between December 2018 and January 2022, all patients diagnosed with aHCC and eligible for systemic treatment at the University Hospitals Leuven, were invited to participate in our study. Clinical eligibility was based on good performance status (ECOG 0-1) and adequate hematologic and end-organ function. Selection of systemic treatment was at the discretion of the treating physician, guided by clinical practice guidelines, individual patient eligibility and treatment availability at time of inclusion. Radiological response was evaluated by computed tomography (CT) or magnetic resonance imaging (MRI) approximately every 3 months, according to standard clinical practice and assessed by an independent radiologist using the modified RECIST criteria (Llovet & Lencioni 2020, J Hepatol 72: 288-306). Response was defined as objective response (partial or complete response) at 3 months or disease control (stable disease) during at least 6 months after treatment initiation.

Prospective sample collection included a fresh tissue biopsy before start of treatment and serial PBMC samples collected prior to and during treatment (week 0-3-6). For two patients, two biopsies from the same tumour nodule were taken. All samples were subjected to simultaneous scRNAseq and scTCRseq, as previously described (Bassez et al. 2021, Nat Med 27:820-832; Qian et al. 2020, Cell Research 30:745-762; Lambrechts et al. 2018, Nat Med 24:1277-1289). scRNAseq data from all available samples was used for clustering and annotation of single cells into their respective tumoural and peripheral cell (phenotypes). As we specifically aimed to explore the effect of CPI, all subsequent comparative analyses focussed on CPI-treated patients, stratified according to clinical response. The study was approved by the Ethics Committee of University Hospitals Leuven (UZ/KUL, S62548). All patients gave written informed consent for the use of their samples for research purposes.

11.2. Sample collection and processing

Fresh tissue biopsies (n=31) were obtained via diagnostic needle biopsy with 18-G needles prior to start of systemic therapy and immediately subjected to single-cell dissociation as previously described (Bassez et al. 2021, Nat Med 27:820-832; Qian et al. 2020, Cell Research 30:745-762; Lambrechts et al. 2018, Nat Med 24:1277-1289). The tissue samples were first mechanically dissociated using a scalpel, followed by enzymatic dissociation in digestion medium (2 mg ml⁻¹ Collagenase P (Sigma Aldrich) and 0.2 mg ml⁻¹ DNase I (Roche) in DMEM (Thermo Fisher Scientific)). Red blood cells were removed from the cell suspension using red blood cell lysis buffer (Roche), and the cells were filtered using a 40-µm Flowmi tip strainer (VWR). The number of living cells was determined using a LUNA automated cell counter (Logos Biosystems). Peripheral blood mononuclear cells (PBMCs) were extracted from serial peripheral blood samples (n=58) by immunomagnetic negative selection using 'EasySep™ Direct Human PBMC Isolation Kit' (Stemcell Technologies). Red blood cells were removed using red blood cell lysis buffer (Roche). Cells were filtered using a 40-µm Flowmi tip strainer (VWR), the number of living cells was determined using a LUNA automated cell counter (Logos Biosystems) and stored at -80°C (in FBS+10% DMSO) for simultaneous thawing at a later timepoint.

Sixteen PBMC samples were thawed simultaneously by adding DMEM stepwise, cells were filtered using 40-µm Flowmi tip strainer (VWR) and the number of living cells was counted using a LUNA automated cell counter (Logos Biosystems). Up to 1 million cells from two different samples were pooled together in 50/50 proportion. The pooling matrix was designed in such a way to allow for bio-informatic identification of samples after sequencing (see below for details). Simultaneous epitope measurement was performed on 27 PBMC samples. First, the cells were incubated on ice with 5 µl of Fc receptor blocking solution (TruStain Fcγ from Biolegend) for 10 minutes. Next, 15.9 µl of TotalSeq-C (Biolegend) 162 antibody-oligo pool (1:1,000 diluted in labeling buffer (PBS+1%BSA) until 100 µm; full list in Suppl. Dataset 1), followed by another 30-min incubation on ice. Cells were washed three times with labeling buffer and filtered through a 40-µm Flowmi strainer.

11.3. Single-cell RNA-sequencing, T-cell repertoire profiling and cell surface epitope profiling

We performed single-cell TCR-seq and 5' gene expression profiling on the single-cell suspensions derived from fresh tissue biopsies and PBMC samples using Chromium Single Cell V(D)J Solution from 10X Genomics according to the manufacturer's instructions. Up to 5000 cells per sample for biopsies and up to 20 000 live cells for pooled PBMC samples were loaded onto a 10X Genomics chip to generate cell-barcoded 5' gene expression libraries. For two patients, we obtained two biopsies from the same tumour nodule for which separate libraries were generated. The libraries were sequenced on an Illumina NextSeq and/or NovaSeq6000 system, and mapped to the GRCh38 human reference genome using Cell Ranger (10x Genomics). V(D)J enriched libraries were sequenced on an Illumina HiSeq4000 system

and TCR alignment and annotation were achieved with CellRanger VDJ (10x Genomics; Version 3.1.0). Additional epitope profiling was performed by TotalSeq-C (Biolegend) on a subset of PBMC samples. These samples were processed as described above, with the addition of a separate library of barcode-tagged antibodies for each cell. The RNA-derived 'Gene Expression library' was mapped to the GRCh38 human reference genome using CellRanger (10x Genomics) as described above, while the protein-derived 'Antibody Capture library' was mapped to the whole TotalSeq-C antibody list.

11.4. PBMC patient-ID assignment using Souporecell

As described above, PBMC samples were pooled, loading 2 samples per lane in the 10X Genomics chip, in a 50/50 proportion. The Souporecell tool (Heaton et al. 2019, bioRxiv 2019:699637) was used to assign each cell back to its sample of origin. In short, the tool first remaps the scRNAseq data of the input samples using the Minimap2 mapper. The remapped data is then analyzed for variants on a per cell basis, followed by clustering based on co-occurring variants to assign each cell a probability of belonging to each of the clusters. Cells carrying co-occurring variants are assigned to a sample specific cluster. Each run was designed in such a way that each cell cluster in Souporecell could easily be linked back to the corresponding sampleID.

11.5. Single-cell gene expression analysis (scRNAseq)

Raw gene expression matrices generated by CellRanger (10x Genomics) were analyzed further using Seurat 4 (Hao et al. 2021, Cell 184:3573-3587) using default parameters unless otherwise specified. All samples were merged into a single Seurat object. Barcodes expressing <200 and >6000 genes and <400 unique molecular identifiers (UMIs) were removed. All cells with >50% mitochondrial RNA content were removed as they likely represent dying cells. Previous single-cell studies in liver and liver cancer have used varying cut-off from 30% (Ramachandran et al. 2019, Nature 575:512-518) to up to 50% (Xue et al. 2022, Nature 612:141-147; MacParland et al. 2018, Nat Commun 9:1-21). Gradually decreasing the threshold from 50% to 30% primarily removed annotated cancer cells or low quality cells in downstream analysis, leaving the immune cells unchanged.

A total of 91 169 cells ([63-10094] cells per sample) with on average 1251 genes per cell and 4483 unique transcripts per cell were retained and normalized (using *NormalizeData* function). The 2000 most variable genes were identified (using *FindVariableFeatures* function) and principal component analysis (PCA) was applied to reduce dimensionality after regressing for the number of UMIs, percentage of mitochondrial RNA and cell cycle genes (S and G2M scores, calculated using *CellCycleScoring* function). The 25 most informative principal components (PCs) were retained for clustering and Uniform Manifold Approximation and Projection for dimension reduction (UMAP). However, the resulting UMAP revealed clustering based on sample-specific variation. To correct for this, the Harmony algorithm (Korsunsky et al. 2019, Nat Methods 16:1289-1296) was applied to regress out sample-specific effects (using the first

25 PCs), resulting in a well-integrated dataset. As expected, malignant cell clusters were patient-specific, while non-malignant clusters contained cells derived from different patients. There was no evidence of cluster bias based on biopsy type, treatment group or underlying liver disease in immune cells and stromal cell types. The data were clustered using *FindNeighbours* and *FindClusters* functions. The resulting two-dimensional UMAP representation of the dataset consisted of distinct major cell types, identified and annotated based on the expression of known marker genes.

11.6. scRNAseq clustering leading to cell subtypes in tissue biopsies

To subcluster T- and NK-cells into their respective phenotypes, we subset T-/NK-cells annotated at the major cell type level. We applied the same process as described above, with an additional removal of TCR genes prior to the identification of variable features, in order to avoid clustering based on TCR genes. We first used marker genes to identify CD4+ T-cells, CD8+ T-cells, NK-cells and proliferating cells. Subsequently, applying an identical process, we subclustered the CD4+ T-cells, CD8+ T-cells and proliferative cluster separately into their cellular (sub-)phenotypes. Finally, all subsets were merged back into one annotated T-/NK-cell Seurat object for further downstream analyses. Similarly, myeloid cells were annotated into dendritic cells versus monocytes/macrophages. Dendritic cells were merged with plasmacytoid dendritic cells for subclustering and annotation. Monocytes and macrophages were subclustered separately into their respective phenotypes.

11.7. Single-cell copy number analysis in tumour biopsies

Copy number variations (CNV) were assessed with the R package *inferCNV* (Tickle et al. 2019, *inferCNV of the Trinity CTAT Project*; Klarman Cell Observatory, Broad Institute of MIT and Harvard, Cambridge, MA, USA; github.com/broadinstitute/inferCNV), designed to infer CNVs from tumoural scRNAseq data. *InferCNV* compares the expression of genes in malignant cells to the expression in cells annotated as non-malignant. T-/NK-cells, B-cells and myeloid cells were used as a reference for non-malignant cells.

11.8. Integration, clustering and annotation of scRNAseq and TotalSeqC data in PBMC samples

Raw gene expression matrices from the PBMC samples were generated using CellRanger 3.1 (10x Genomics) and analyzed using Seurat 4 (Hao et al. 2021, Cell 184:3573-3587). One Seurat object was generated with the scRNA-seq data and the antibodies present in the antibody pool (Suppl. Dataset 1). All barcodes expressing <200 and >6000 genes, <400 UMIs and >15% mitochondrial DNA content were removed. Next, we used the Soupcore clusters to link each barcode to its sample of origin. All cells classified with insufficient confidence were removed. A total of 286 806 cells ([564-11 609] cells per samples) with on average 1053 genes per cell and 2596 unique transcripts per cell were retained.

The PBMC subset with only scRNAseq data available was processed similarly to the scRNAseq data from pre-treatment biopsies. For PBMC samples with RNA and antibody data available all features were reported as variable features. The data were normalized by Centered-Log-Normalisation (CLR) using the

second margin (using *NormalizeData*) and subsequently scaled for ADT-count. Finally, dimensionality reduction was performed using PCA and the UMAP representation was generated.

Next, the RNA- and ADT-assays were combined using a 'weighted nearest neighbor' analysis (Hao et al. 2021, Cell 184:3573-3587). In short, a new 'integrated' assay was generated using
5 *FindMultiModalNeighbours* function by assigning a weight to each cell based on the relative contribution of the RNA- versus ADT-assay to the clustering process. A new UMAP was generated using the integrated dataset and clustering analysis was performed (using *FindCluster* function with the Smart Local Moving algorithm).

Subsequently, the PBMC subset without ADT-data was projected onto the 'integrated' assay. Combining
10 both the RNA- and ADT-assay, allowed for a better biological separation of cells based on ADT-data (e.g. CD4+ versus CD8+ T-cells). Manual annotation was performed iteratively based on marker gene expression. First, the major peripheral cell types were annotated, followed by annotation of peripheral T-/NK-cells into their respective phenotypes.

11.9. Differential gene expression and pathway analysis

Differentially expressed genes (DEG) were identified using Wilcoxon's test with the *FindMarkers* function from Seurat without a threshold for log fold-change (logFC) and for expression in a minimum fraction of cells. The obtained p-values were corrected for multiple testing (Bonferroni). The R package clusterProfiler 4.0 (Wu et al. 2021, Innov 2:100141) was then used to perform Over-Representation-Analysis (ORA) on the significant DEG (logFC > abs(0.25) and adjusted p-value < 0.01) for the 'Hallmarks
20 of cancer' gene sets. The resulting list of enriched gene sets was filtered for adjusted p-values (using Benjamini-Hochberg method) < 0.01.

11.10. Assessing the TCR repertoire using V(D)J analysis

In the biopsies, we detected 11 967 out of 17 656 T-/NK-cells with a productive TCR sequence, meaning that they could be joined in the proper reading frame by V(D)J recombination without a premature stop
25 codon, enabling the expression of a complete TCR alpha or beta-chain for downstream analysis. Excluding NK-cells, gamma-delta T-cells and MAIT cells (restrictive TCR), 90% of annotated T-cells annotated carried a productive TCRs, resulting in a total of 10 517 annotated T-cells with a productive TCR, carrying a total of 7569 unique TCRs. Of note, one sample was removed from further TCR analysis, as we did not detect annotated T-cells carrying productive TCRs.

30 In PBMCs, we detected 117 339 out of 164 691 T-/NK-cells carrying a TCR sequence. Again, we considered only productive TCR sequences. Excluding NK-cells, gamma-delta T-cells and MAIT cells (restrictive TCR), 90% of peripheral T-cells carried a productive TCRs. A total of 16 TCR sequences were shared between at least two patients. The majority were single TRA or single TRB sequences. One full-chain TRA/TRB TCR was shared between samples pooled in the same lane and therefore most probably

due to incorrect assignment by Souporecell. All TCR sequences shared between patients were removed for further analysis. This resulted in a total of 104 165 annotated peripheral T-cells, carrying 79 103 unique TCRs.

Next, TCR clonotypes were defined as TCRs with the same complementarity-determining region 3 (CDR3) nucleotide sequences. Dominant clonotypes were defined as 1) TCRs shared by 5 or more T-cells and 2) clonotypes representing at least 1% of the TCR repertoire in each sample. Clonality was defined as the complement of evenness (1-evenness), as previously described (Bassez et al. 2021, Nat Med 27:820-832; Riaz et al. 2017, Cell 171:934-949), where evenness represents the normalized Shannon entropy. The evenness value lies between 0 and 1, with a high value indicating a more equal distribution of TCRs and a low value indicating TCR skewing due to clonal expansion. TCR richness was defined as the number of unique TCRs divided by the total number of cells with a unique TCR and was calculated as a metric for clonotype diversity. Finally, the Gini-index was calculated using the *ineq* (v0.2-13) package in R and captures the distribution of T-cells across the TCR repertoire. The value ranges between 0 and 1. The higher the Gini-index, the less equal the distribution of the clonotypes. Each TCR metric (clonality, evenness, richness, Gini-index) were calculated per T-cell phenotype in each sample.

11.11. TCR sharing between intra-tumoural T-cells and peripheral T-cells

For fourteen patients both pre-treatment tumour biopsies and serial PBMC samples were available. In this overlap-dataset, we detected a total of 77 931 annotated T-cells, carrying 60 280 unique TCRs. A total of 242, 227 and 192 TCRs were shared between biopsy and PBMC at week 0, week 3 and week 6, respectively. The proportion of shared TCR in peripheral blood was calculated by dividing the number of shared TCRs between pre-treatment biopsy and PBMCs by the total number of TCRs detected in each sample. Similarly, the proportion of shared peripheral T-cells was calculated by dividing the number of Tcells carrying TCRs shared between pre-treatment biopsy and PBMCs by the total number of Tcells carrying a productive TCR in each sample.

11.12. Trajectory Inference Analysis

The R package Slingshot v2.2.0 (Street et al. 2018, BMC Genomics 19:1-16) was used to define computationally imputed pseudotime trajectories of CD8+ T cells in the TME. Naive T cells (CD8 T_N) were considered as the root state when calculating the trajectories and the pseudotime. To visualize the degree of overlap in TCR clonotypes between T-cell phenotypes, the connection weight for each pair of T-cell phenotypes was calculated as the shared number of unique TCRs divided by the total number of unique TCRs in the T-cell phenotype located first on the trajectory (=shared TCR weight). We then used TradeSeq v1.7.07 (Van den Berge et al. 2020, Nat Commun 11:1-13) to identify DEGs between trajectories. We first used the *diffEnd* function, a between-lineage test to identify DEGs between the terminally differentiated ends of each trajectory. Then, we used the *earlyDEG* function using knots 1-6,

to assess the differences in expression patterns early on in the CD8 trajectories. The DEG lists were then used as input for GSEA using the R package f-GSEA for the REACTOME and GO: Biological Processes gene sets. Only significantly enriched pathways were retained (adjusted p-value <0.01).

11.13. Predicting cell-to-cell interactions in scRNAseq data

The CellChat (v1.1.3) algorithm (Jin et al. 2021, Nat Commun 12:1-20) was used to predict cell-cell interactions between cell types in scRNAseq data, using default parameters with following exceptions: the number of permutations used was 10 000 and cell-cell interactions between cell types were not considered when less than 15 cells represented a group. We focused on significant cell-cell interactions between immune cell types in responders and non-responders, separately (p-value <0.01).

11.14. Estimating progression free survival

We calculated the progression free survival probability for three features associated with response: 1) Gini-index in CD8 T_{EMRA} in pre-treatment biopsies, 2) TCR sharing between tumour and peripheral blood (PBMC Week 0) and 3) PDL1-expression in CXCL10+ tumour-associated macrophages (Macro CXCL10). For each metric, patients were grouped into two groups compared to the median (high versus low). The Kaplan-Meier method was used to estimate progression free survival curves and the log-rank test was used to assess significant differences between groups. Analyses were done using the R packages 'survival' (version 3.3.1) and 'survminer' (version 0.4.9).

11.15. Validating single-cell findings in bulk RNA seq dataset of 358 advanced HCC patients

In order to validate single-cell derived insights and explore the potential of CD8 T_{EMRA} and Macro CXCL10 as predictive biomarkers of response to CPI, we used CD8 T_{EMRA} (n=36) and Macro CXCL10 (n=16) marker genes in order to deconvolute a publicly available bulk RNAseq dataset (Zhu et al. 2022, Nat Med 28:1599-1611) (EGAS0001005503; DA00468). Overall, 358 prospectively collected tumour samples of advanced HCC patients treated with atezolizumab/bevacizumab (n=253), atezolizumab (n=47) or sorafenib (n=58) in the context of the phase Ib (GO30140; arms A and F) (Lee et al. 2020, Lancet Oncol 21:808-820) and phase III clinical trials (IMBrave150) (Finn et al. 2020, N Engl J Med 382:1894-1905; Cheng et al. 2022, J Hepatol 76:862-873) were available. For details on study design and patient cohorts refer to the original publication (Zhu et al. 2022, Nat Med 28:1599-1611). The per sample raw RNA read files and associated clinical data were downloaded from the European Genome Archive (EGAS0001005503; DA00468). The raw read files were mapped to the human reference genome (refdata-gex-GRCh38-2020-A) using the STAR aligner (STAR.2.7.2a) in paired-end mode. Gene counts per sample were then computed using featureCounts (Subread toolkit) and the RNA counts were normalized based on the trimmed mean of M-values (TMM) method using the R-package edgeR (version 3.3.2). The resulting effective library size was used for downstream analysis.

CLAIMS

1. A method of selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI, or of predicting the response, the likelihood of response, or
5 responsiveness of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI)
or to therapy with an ICI, such method comprising
quantifying the abundance of CD8 Temra cells in a pre-therapy tumor biopsy sample obtained from
the subject, and
selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting
10 a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy
with an ICI when the abundance of CD8 Temra cells in the pre-therapy tumor biopsy sample
corresponds to reference pre-therapy tumor biopsy sample CD8 Temra cell abundances that are
indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy
with an ICI.
15
2. A method of selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI, or of predicting the response, the likelihood of response, or
responsiveness of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI)
or to therapy with an ICI, such method comprising
20 quantifying the abundance of CD8 Temra cells and of CXCL10-positive macrophages in a pre-therapy
tumor biopsy sample obtained from the subject, and
selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting
a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy
with an ICI when the abundances of CD8 Temra cells and of CXCL10-positive macrophages in the
25 pre-therapy tumor biopsy sample correspond to reference pre-therapy tumor biopsy sample CD8
Temra cell and CXCL10-positive macrophage abundances that are indicative of response of a subject
having the cancer to the therapy including an ICI or to the therapy with an ICI.
3. The method according to claim 1 or 2 wherein the abundance of CD8 Temra cells is quantified by
30 means of quantifying the expression of CD8 Temra cell marker genes.
4. The method according to claim 2 wherein the abundance of CXCL10-positive macrophages is
quantified by means of quantifying expression of CXCL10-positive macrophage marker genes.

5. The method according to claim 3 or 4 wherein marker gene expression is analyzed in (m)RNA isolated from the pre-therapy biopsy sample; by means of single cell sequencing of cells of the pre-therapy biopsy sample; or by means of spatial transcriptomic analysis of the pre-therapy biopsy sample.
- 5 6. The method according to claim 1 or 2 wherein the abundance of CD8 Temra cells is quantified by means of immunohistochemical phenotyping, by spatial phenotyping or spatial proteomics, by means of whole-tissue single-cell imaging of CD8 Temra cells in the pre-therapy biopsy sample.
- 10 7. The method according to claim 2 wherein the abundance of CXCL10-positive macrophages is quantified by means of immunohistochemical phenotyping, by spatial phenotyping or spatial proteomics, by means of whole-tissue single-cell imaging of CD8 Temra cells in the pre-therapy biopsy sample.
- 15 8. The method according to any one of claims 1 to 7 further including determining the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample obtained from the same subject.
- 20 9. The method according to claim 8 wherein a subject having cancer is selected for therapy including an ICI or for therapy with an ICI, or wherein a subject having cancer is predicted to respond, or likely to respond to therapy including an ICI or to therapy with an ICI when additionally the level of TCR sharing corresponds to reference levels of TCR sharing that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI.
- 25 10. The method according to claim 8 or 9 further including determining the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in an on-therapy blood sample obtained from the same subject.
- 30 11. The method according to claim 10 further including determining the subject to respond to the therapy including an ICI or to the therapy with an ICI when the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in the on-therapy blood sample is not significantly lower than the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample.
- 35 12. A method of selecting a subject having cancer for therapy including an immune checkpoint inhibitor (ICI) or for therapy with an ICI, or of predicting the response, the likelihood of response, or

responsiveness of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI, such method comprising

determining the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample obtained from the same subject, and

selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an ICI when the level of TCR sharing corresponds to reference levels of TCR sharing that are indicative of response of a subject having the cancer to the therapy including an ICI or to the therapy with an ICI.

13. A method of determining response of a subject having cancer to therapy including an immune checkpoint inhibitor (ICI) or to therapy with an ICI, such method comprising determining the level of T cell receptor (TCR) sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample obtained from the same subject; determining the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in an on-therapy blood sample obtained from the same subject; determining the subject to respond to the therapy including an ICI or to the therapy with an ICI when the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in the on-therapy blood sample is not significantly lower than the level of TCR sharing between the CD8 Temra cells in the pre-therapy tumor biopsy sample and the CD8 Temra cells in a pre-therapy blood sample.

14. An immune checkpoint inhibitor (ICI) for use in treating or inhibiting cancer, for use in inhibiting cancer progression, or for use in inhibiting cancer relapse, comprising selecting a subject having cancer for therapy including an ICI or for therapy with an ICI, or predicting a subject having cancer to respond, or likely to respond to therapy including an ICI or to therapy with an ICI as determined in a method according to any one of claims 1 to 13.

15. A diagnostic kit for use in a method according to any one of claims 1 to 13 wherein the kit is comprising the tools to quantify the abundance of CD8 Temra cells in a pre-therapy biopsy sample, the abundance of CXCL10-positive macrophages in a pre-therapy biopsy sample, or the level of TCR sharing in CD8 Temra cells in a pre-therapy biopsy sample and in a blood sample.

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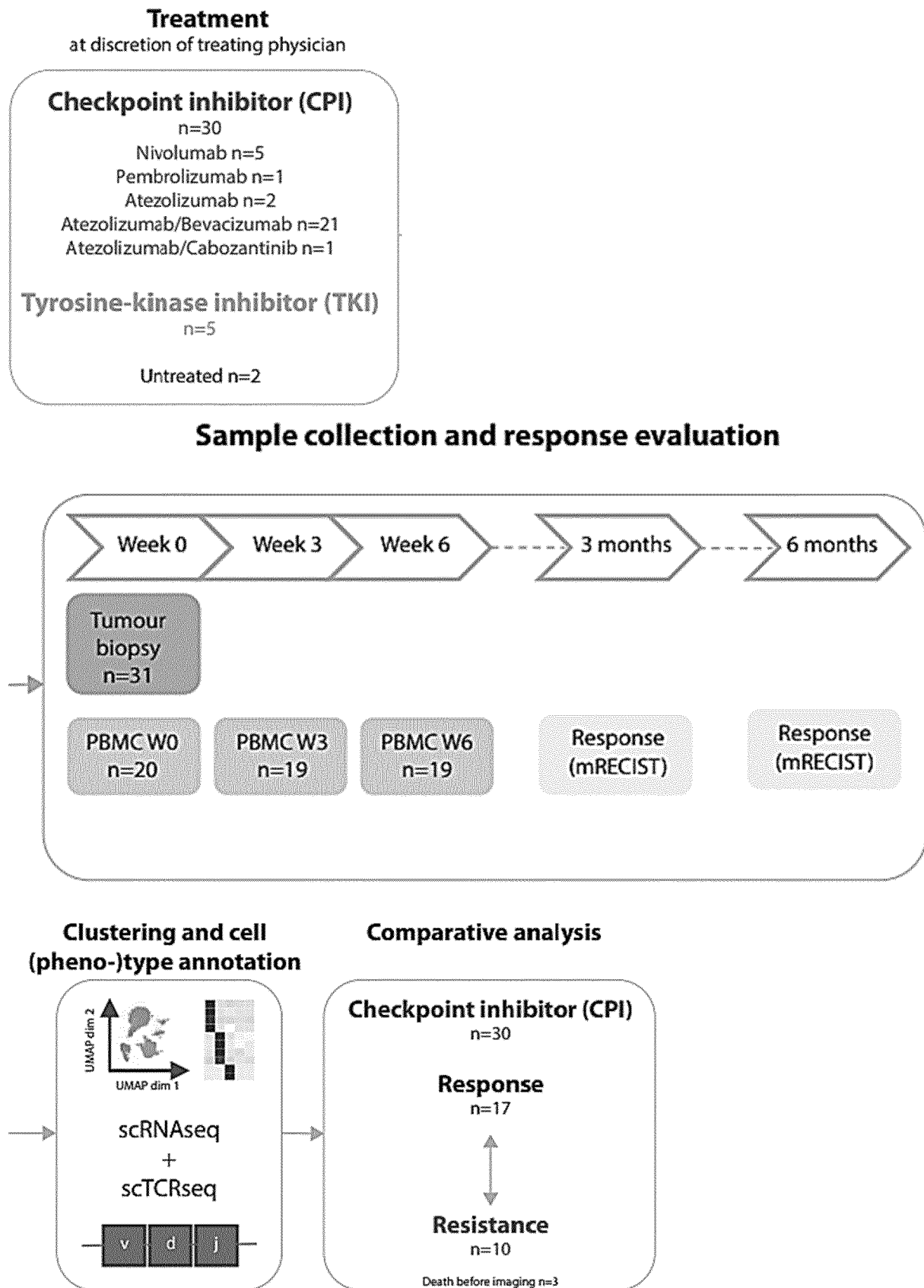


FIGURE 1

A

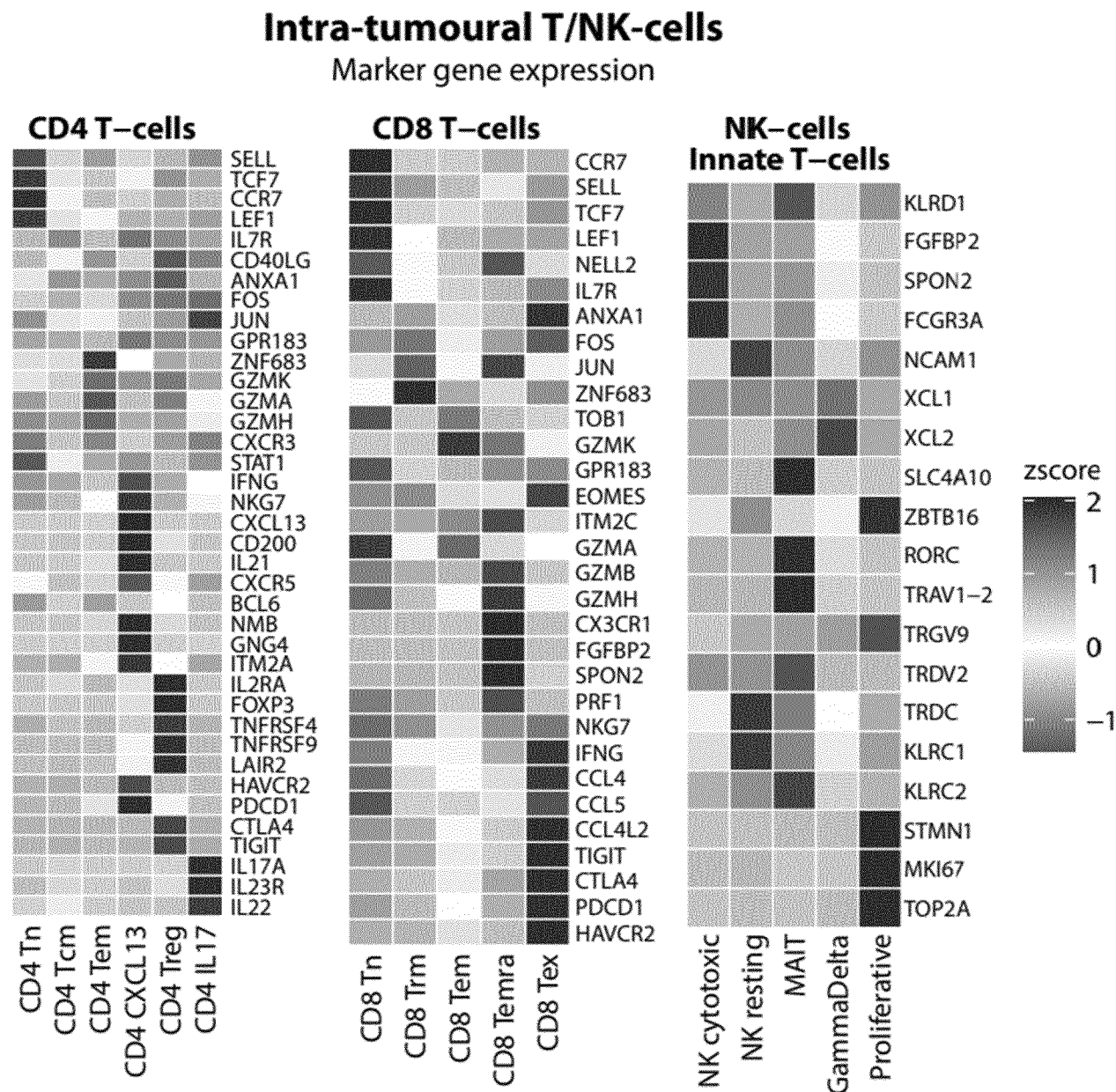
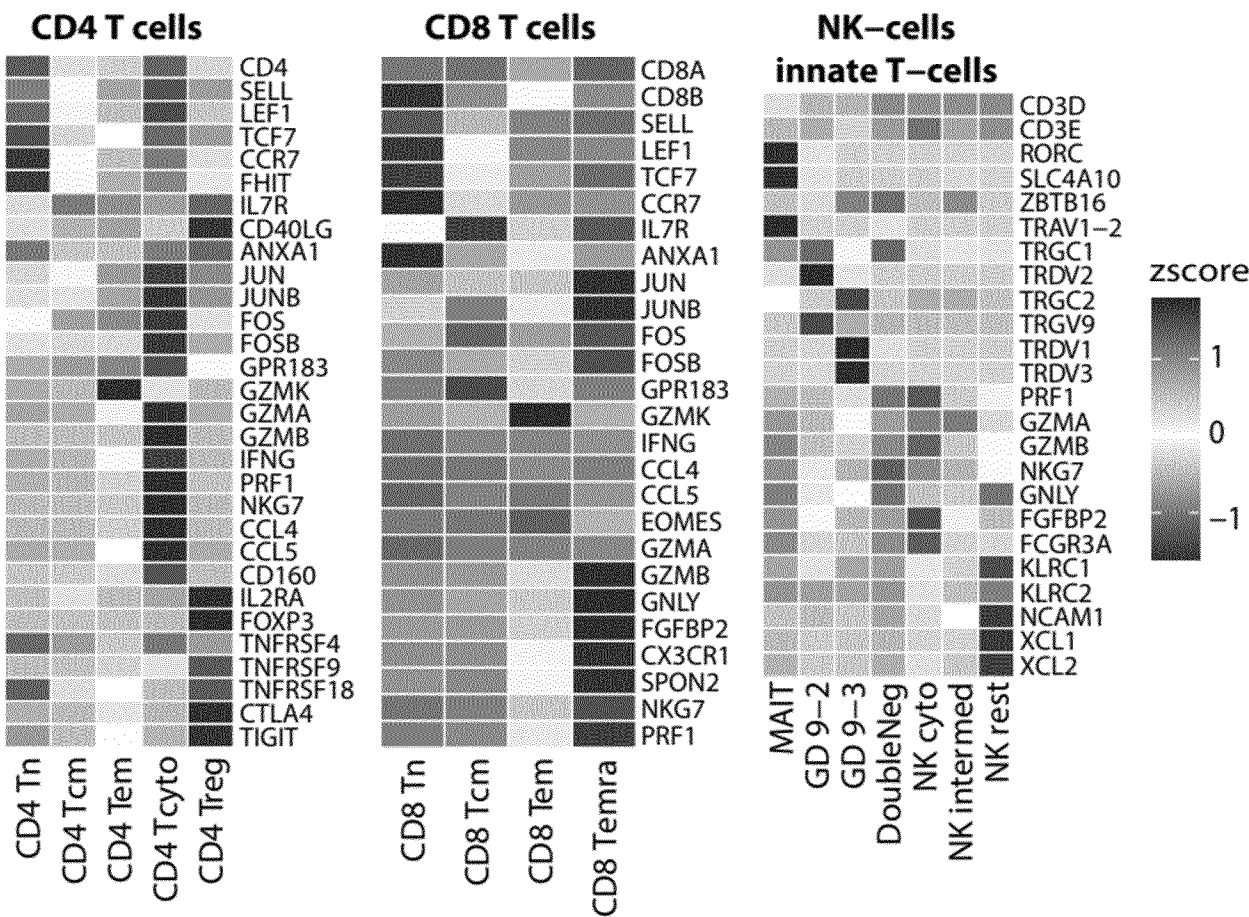


FIGURE 2

B

Peripheral T/NK-cells
Marker gene expression



C

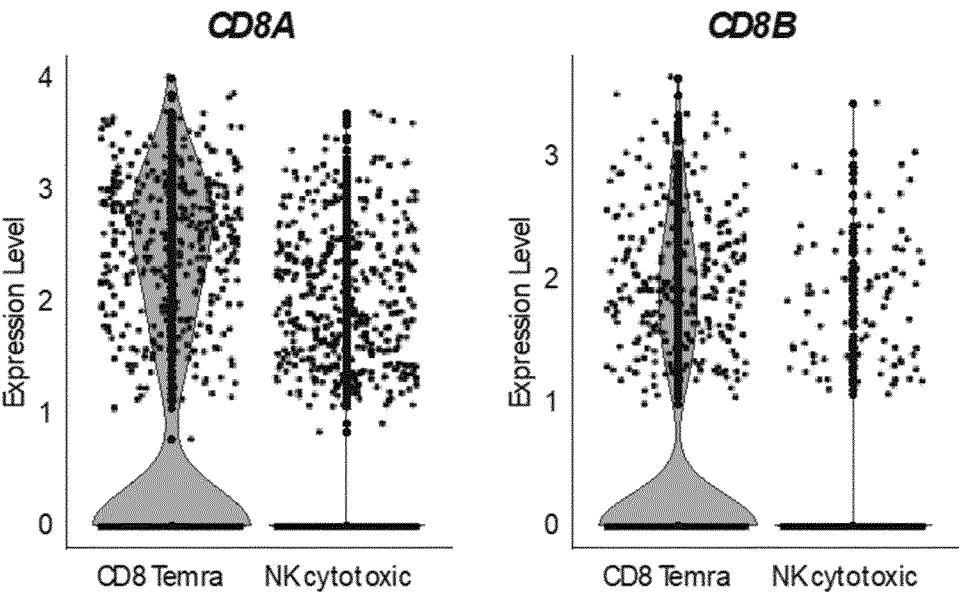
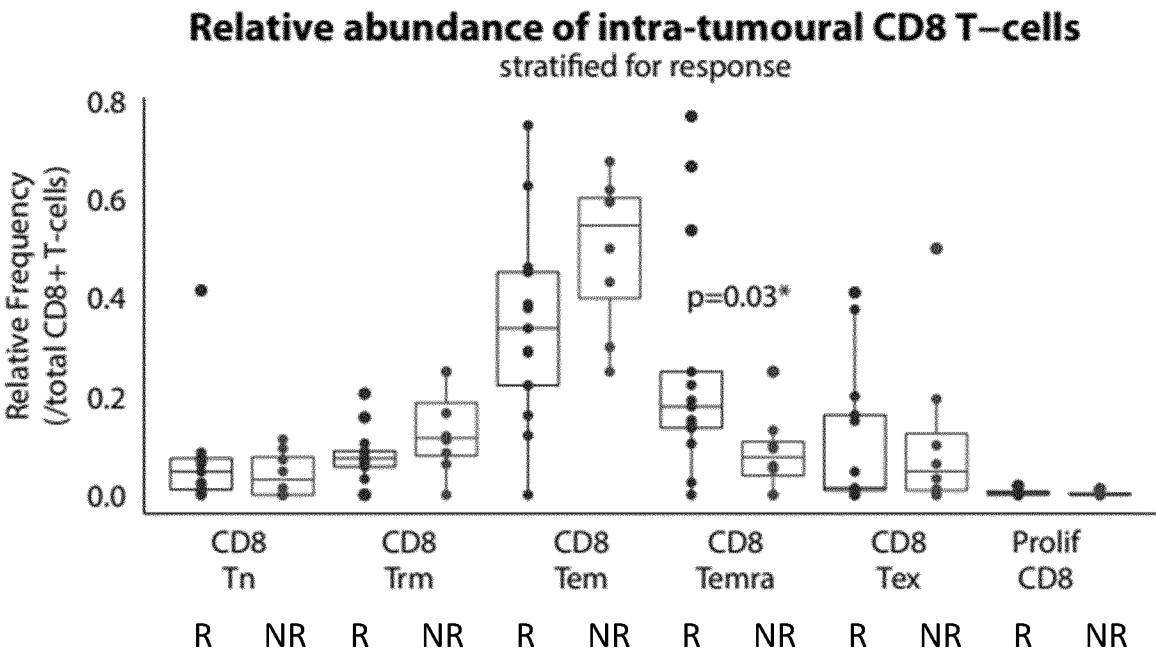


FIGURE 2 - continued

A



B

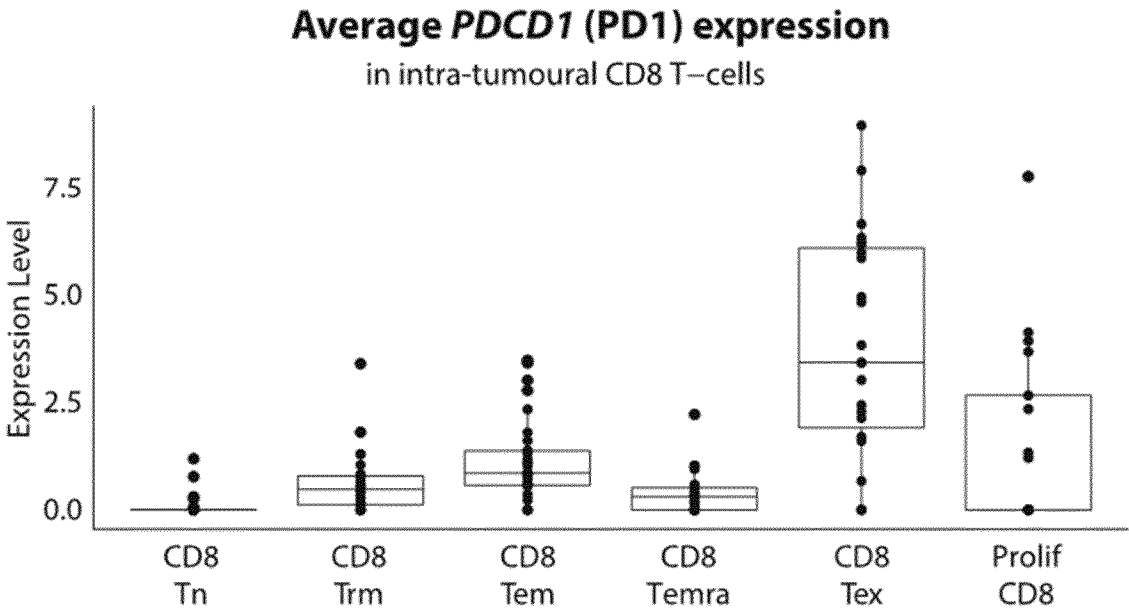
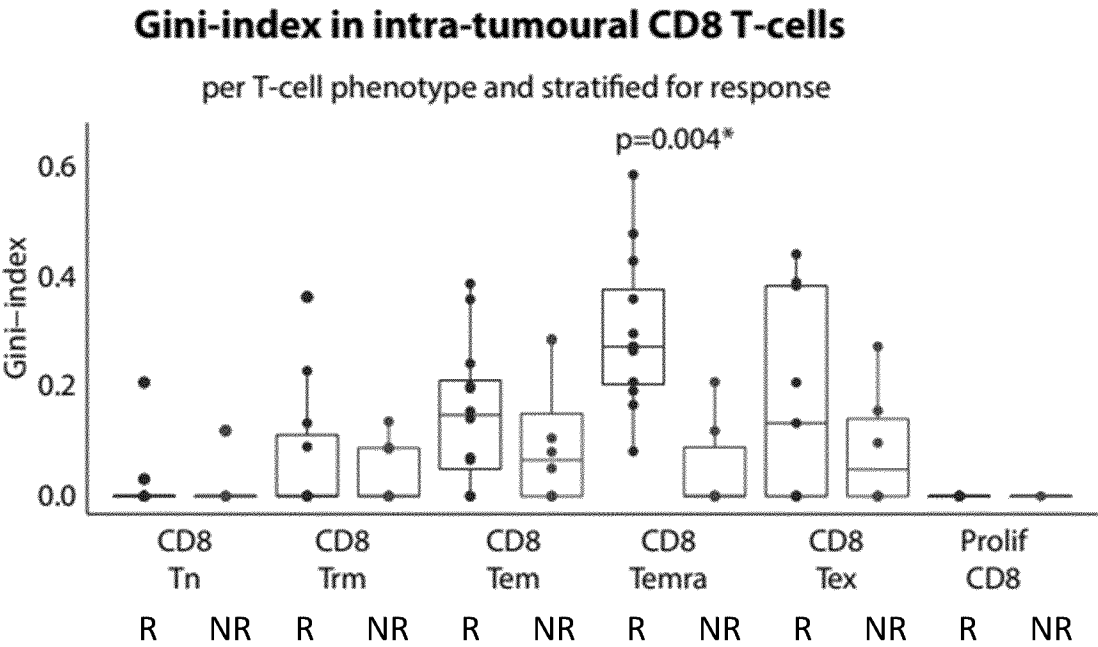


FIGURE 3

C



D

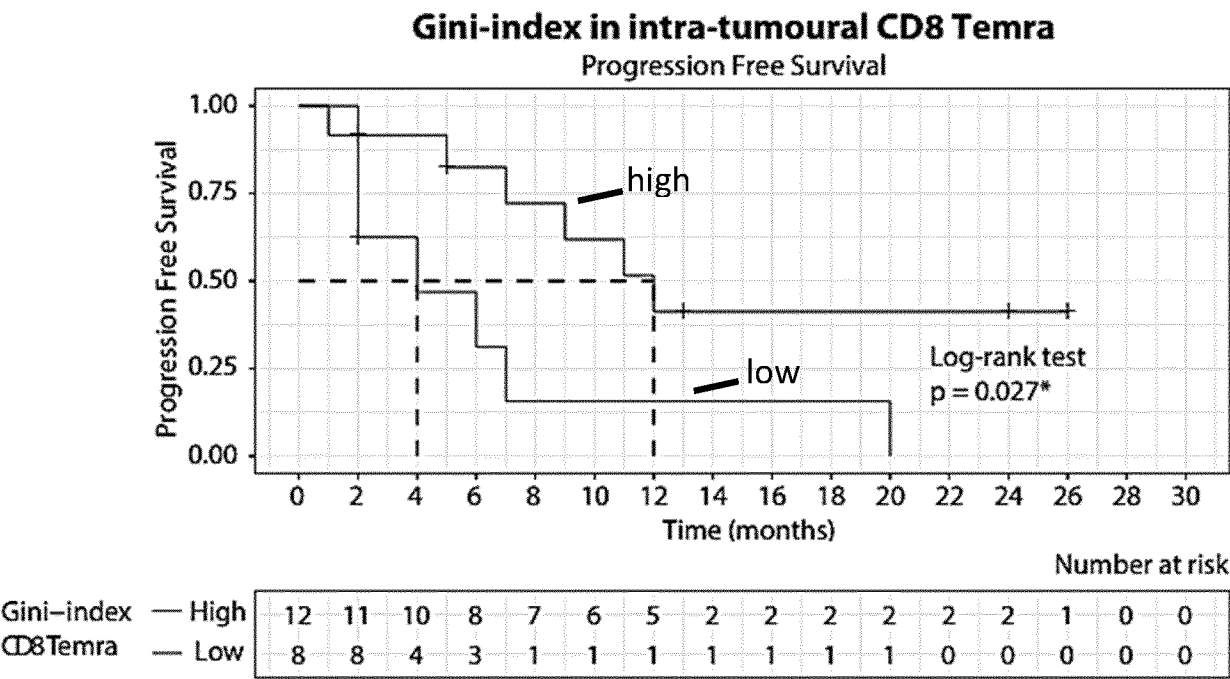
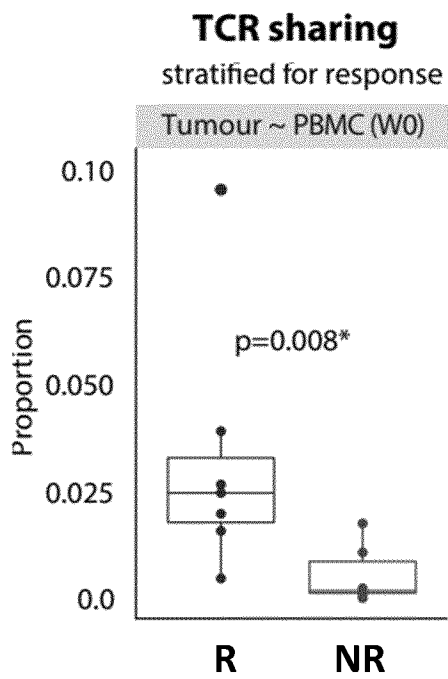


FIGURE 3 - continued

A



B

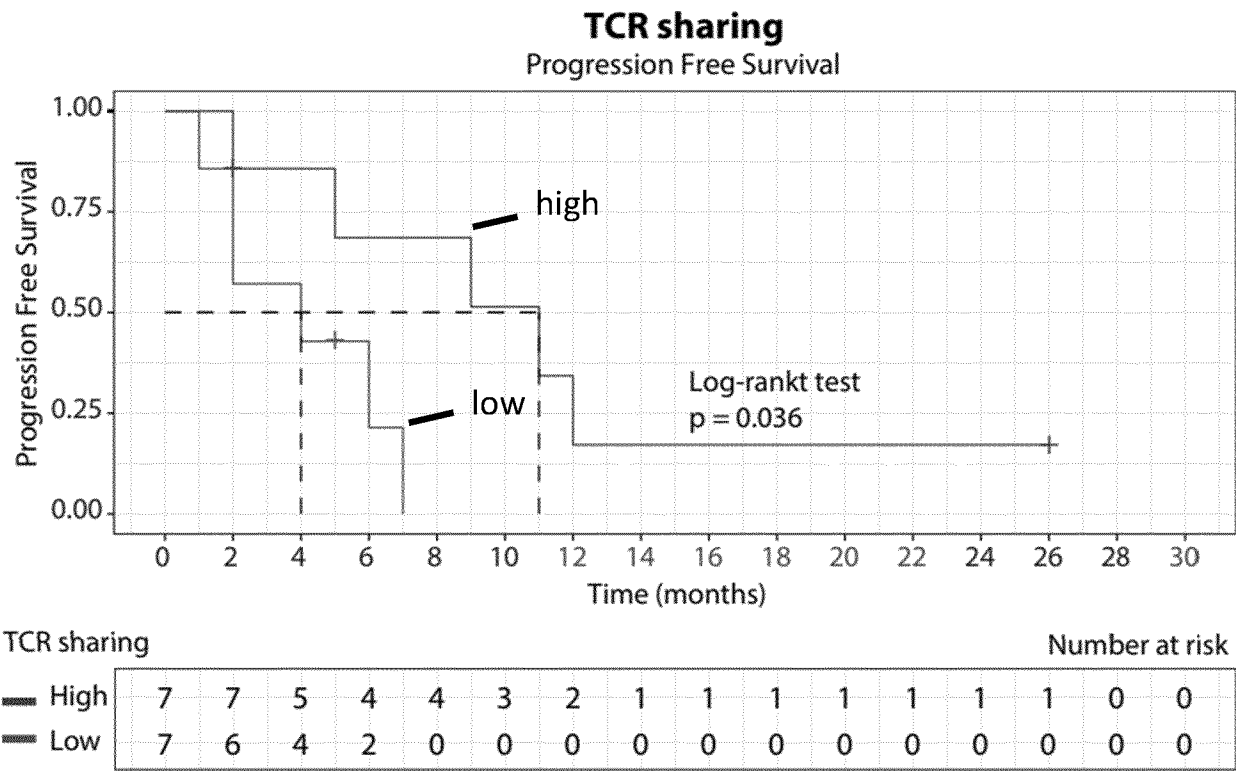


FIGURE 4

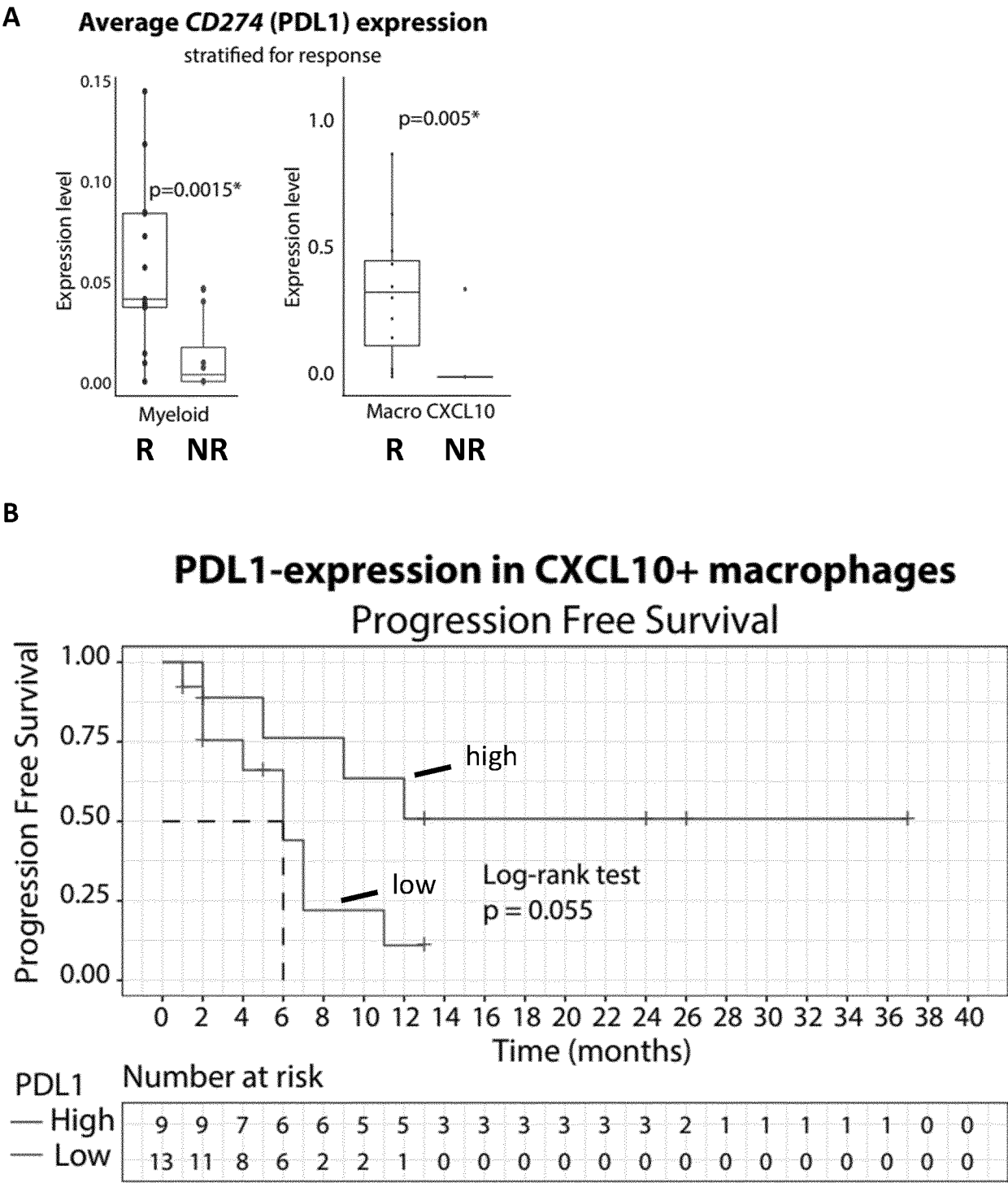


FIGURE 5

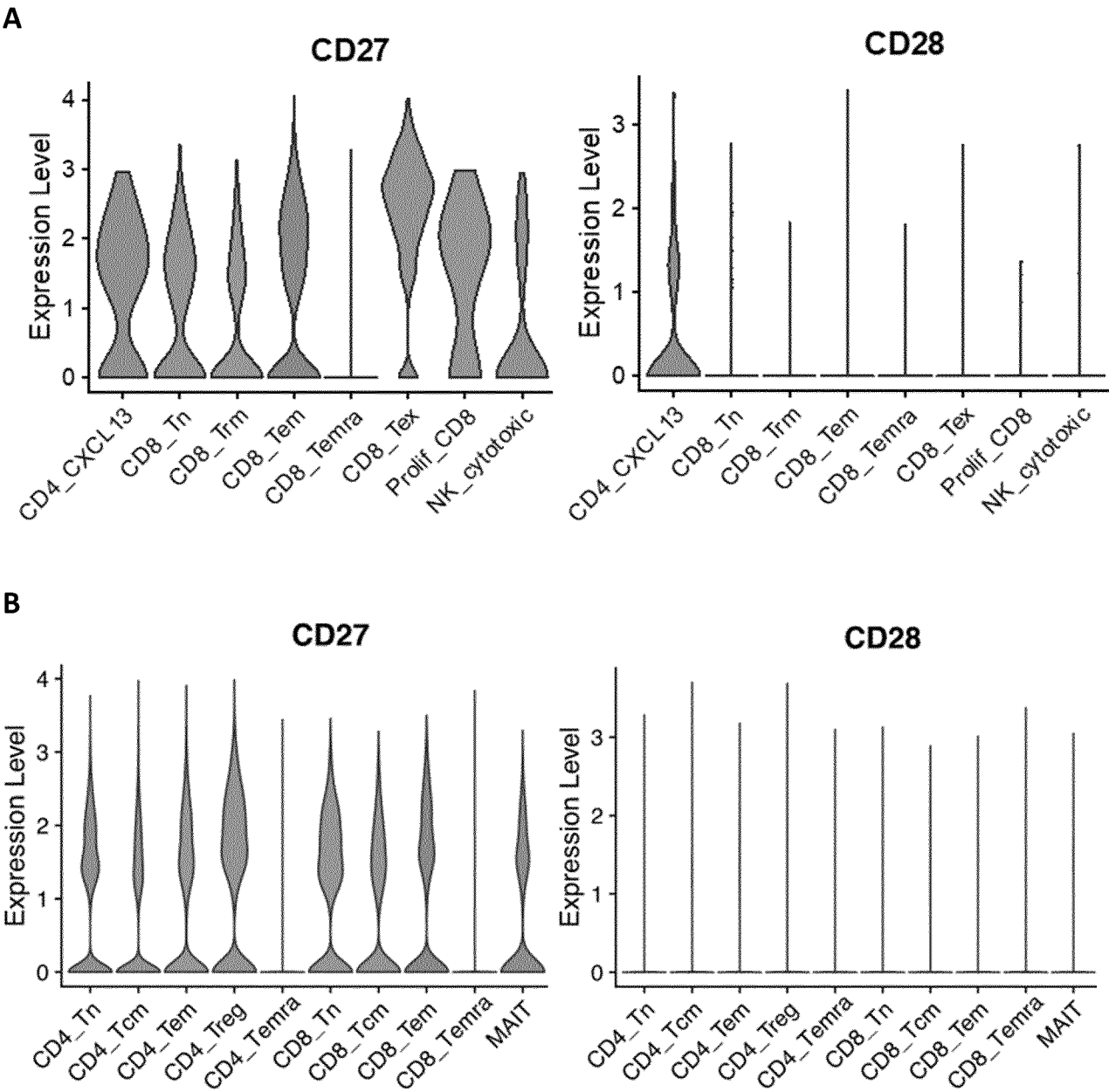


FIGURE 6

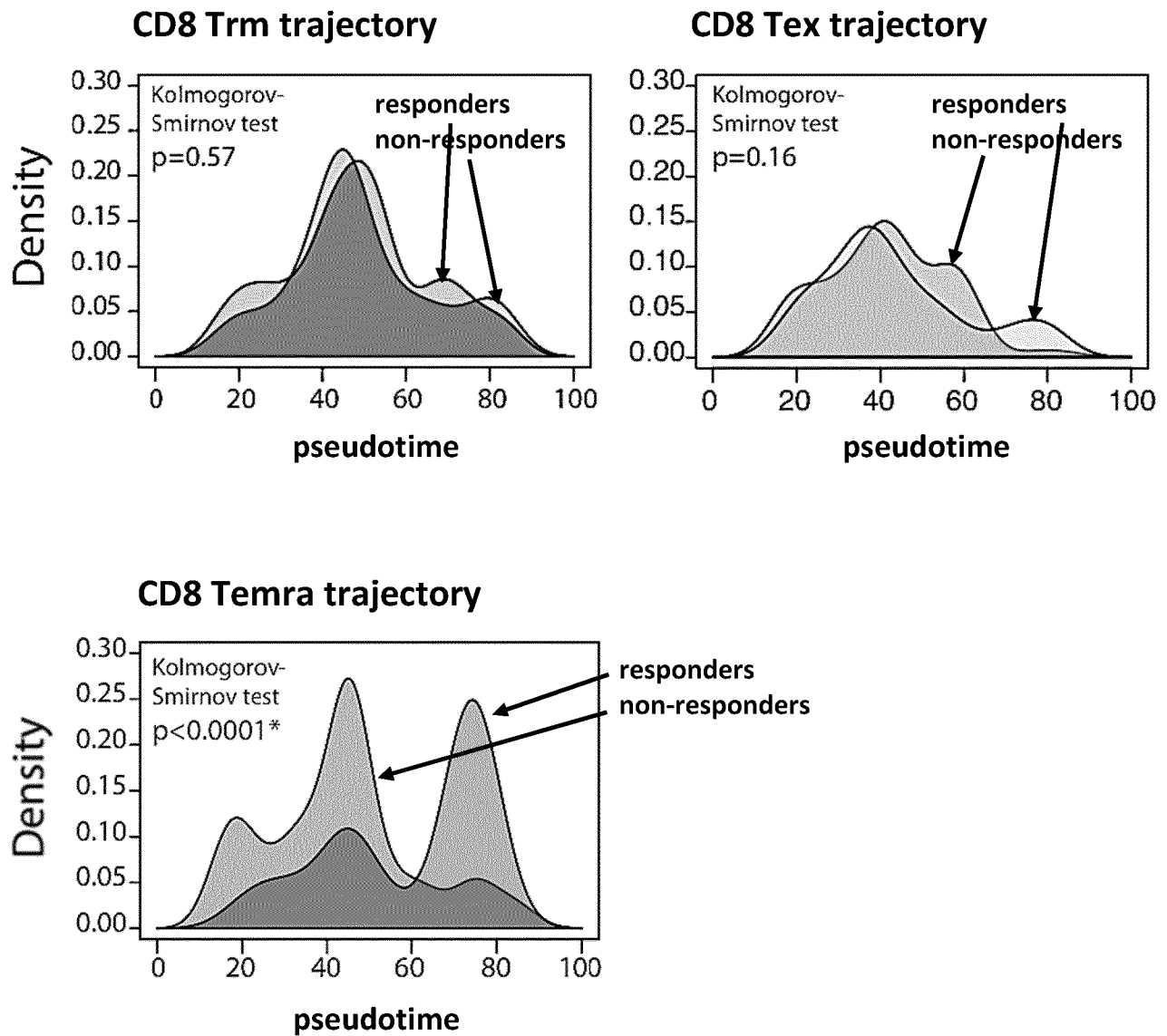


FIGURE 7

CD8 Tex vs CD8 Temra trajectory

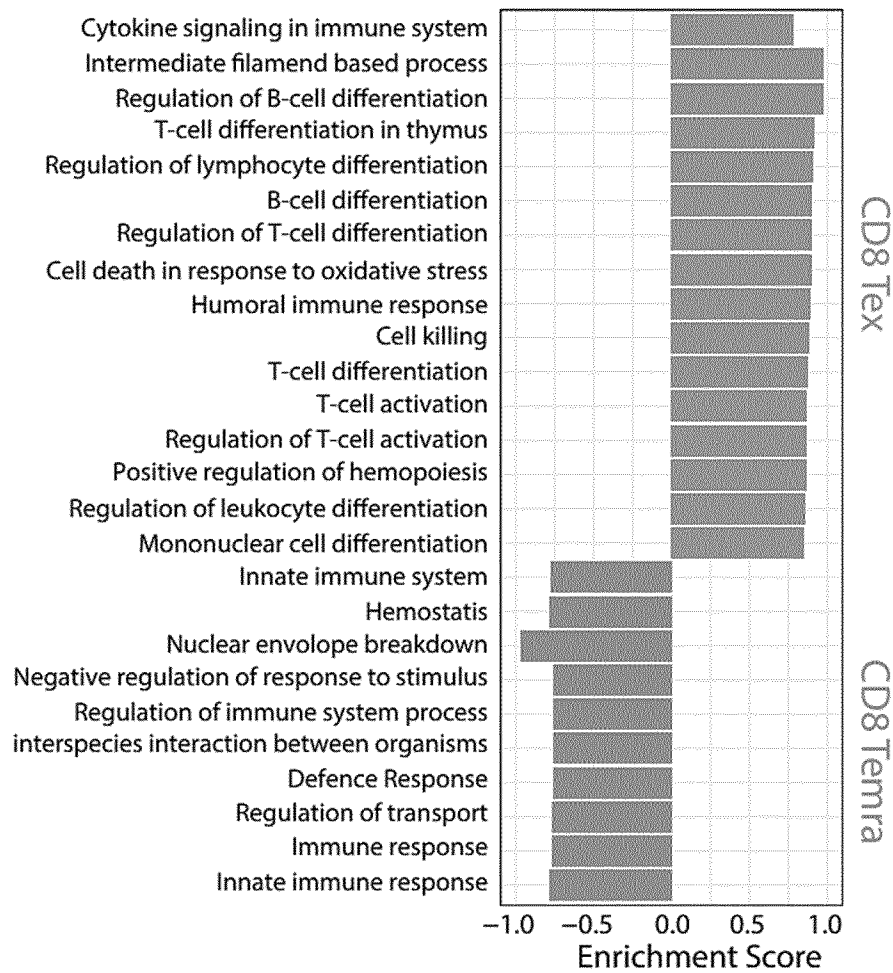


FIGURE 8

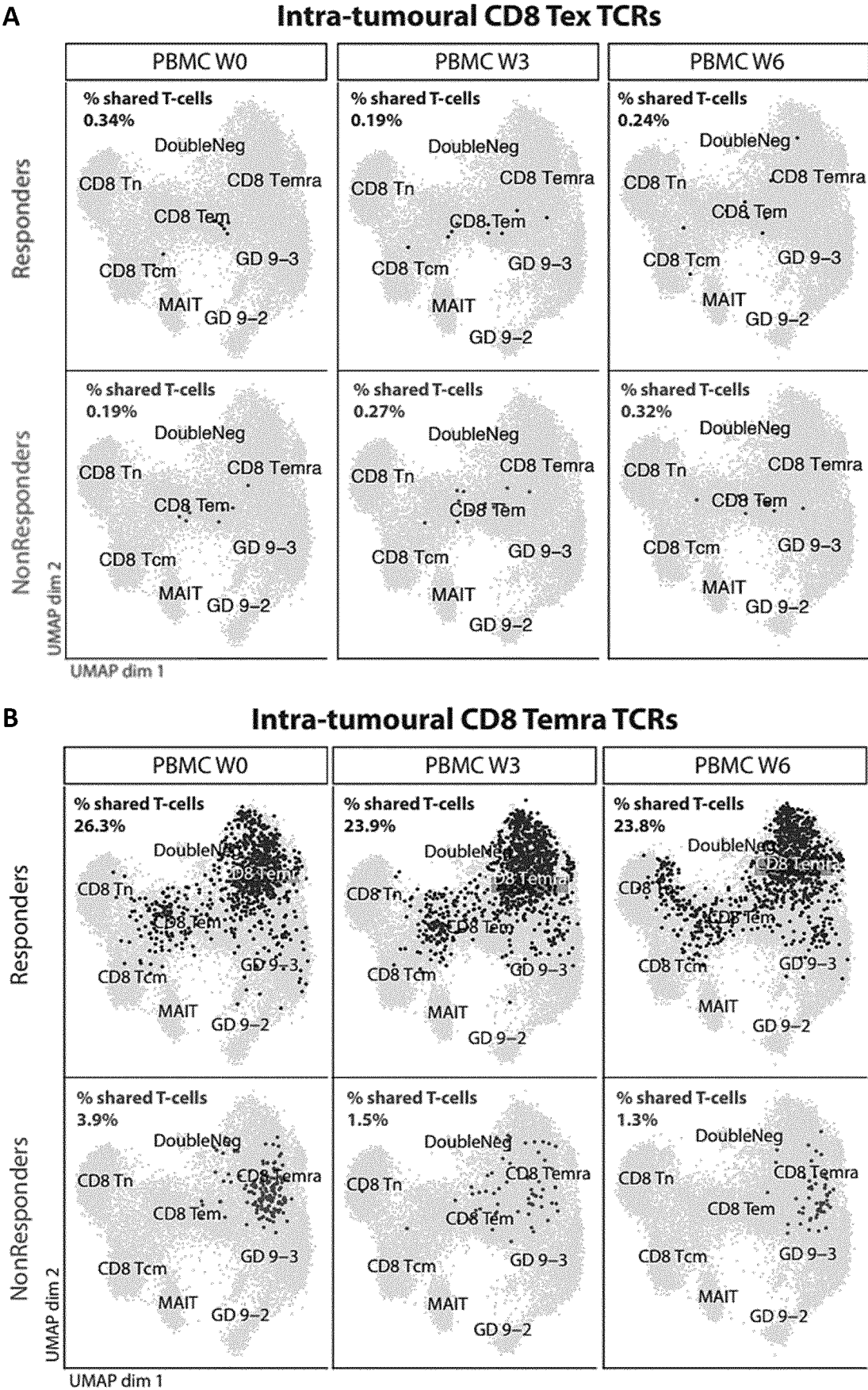


FIGURE 9

A **Monocytes/macrophages in the TME**

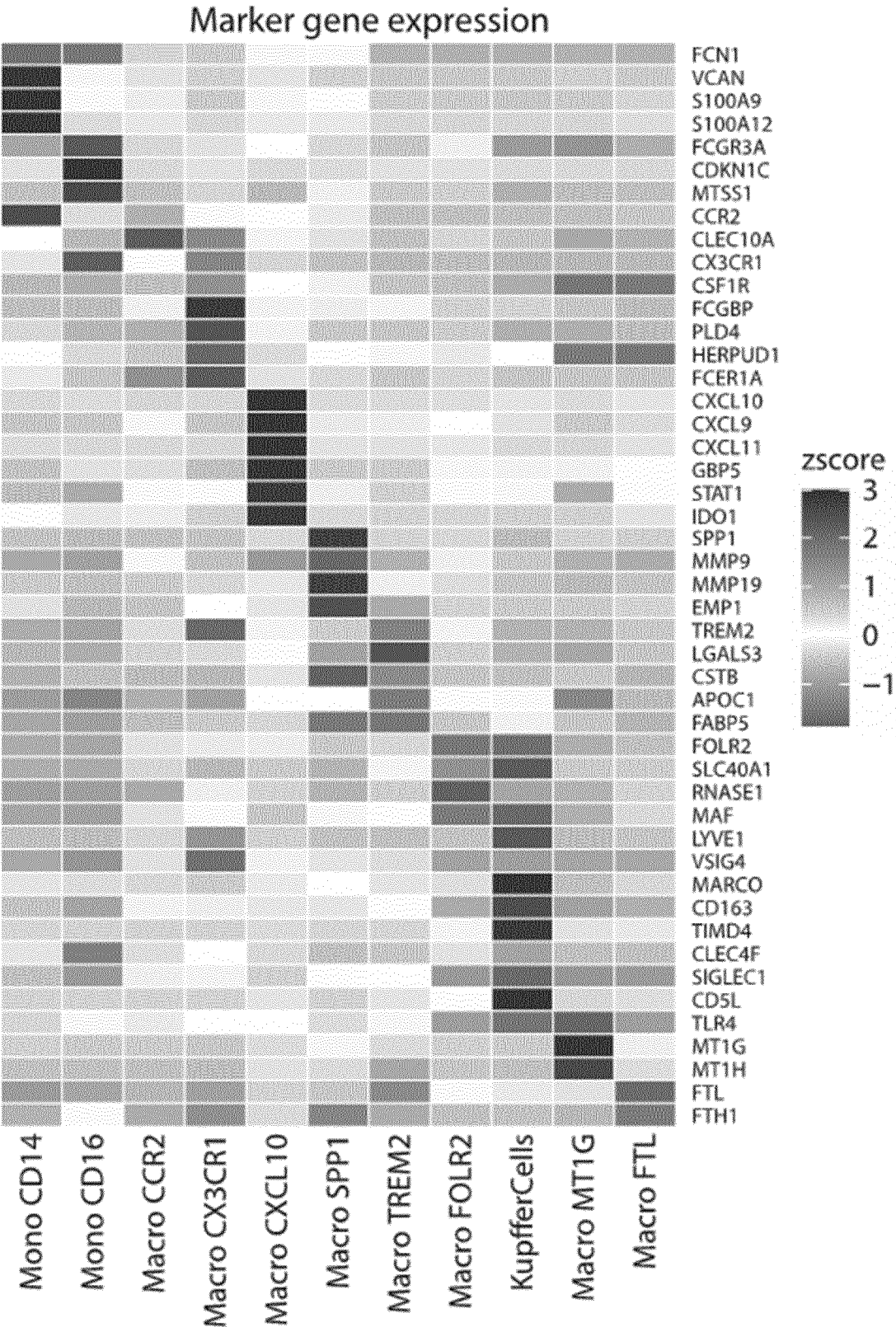


FIGURE 10

B

Macrophages in the TME

Functional markers

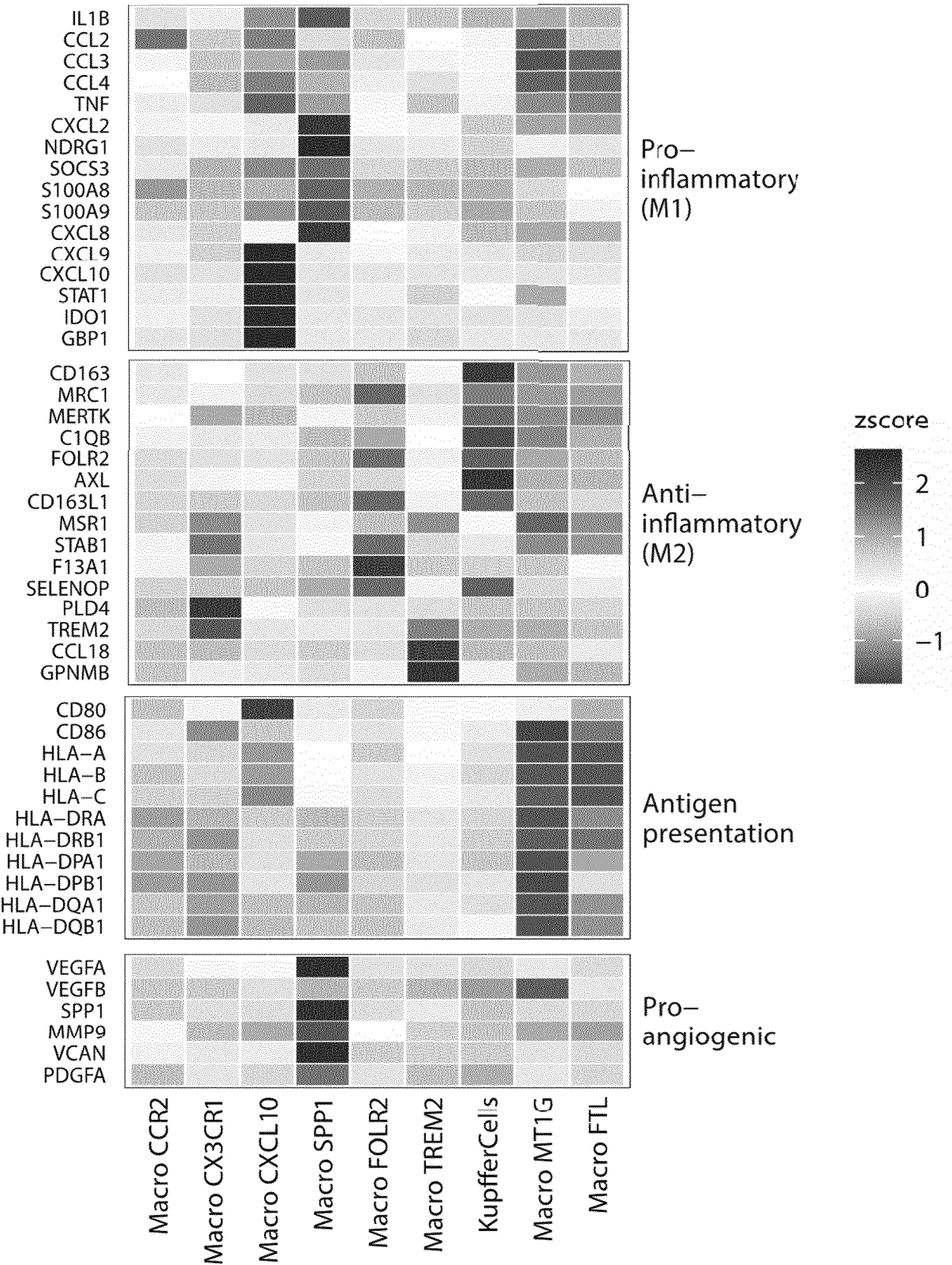
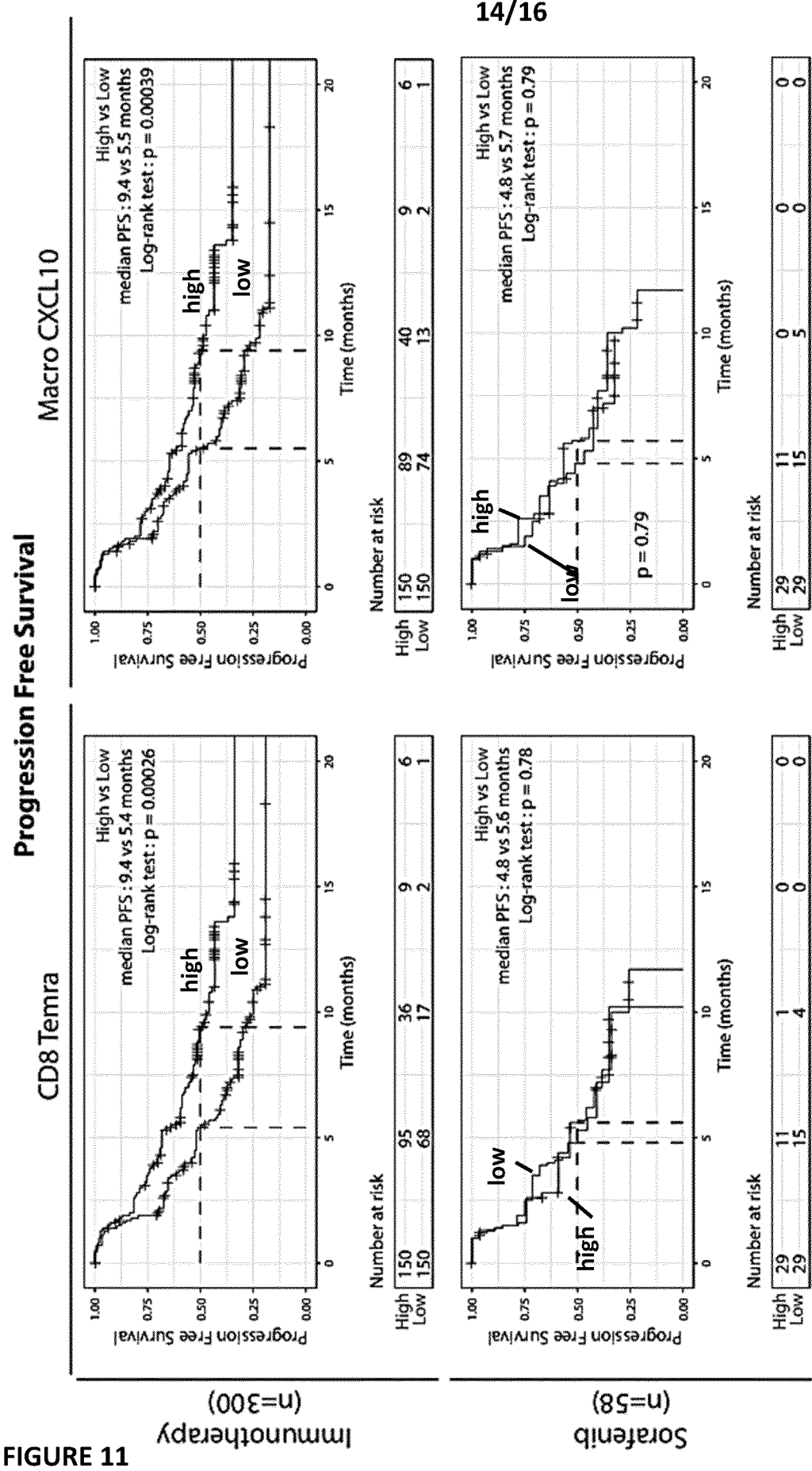


FIGURE 10 – continued



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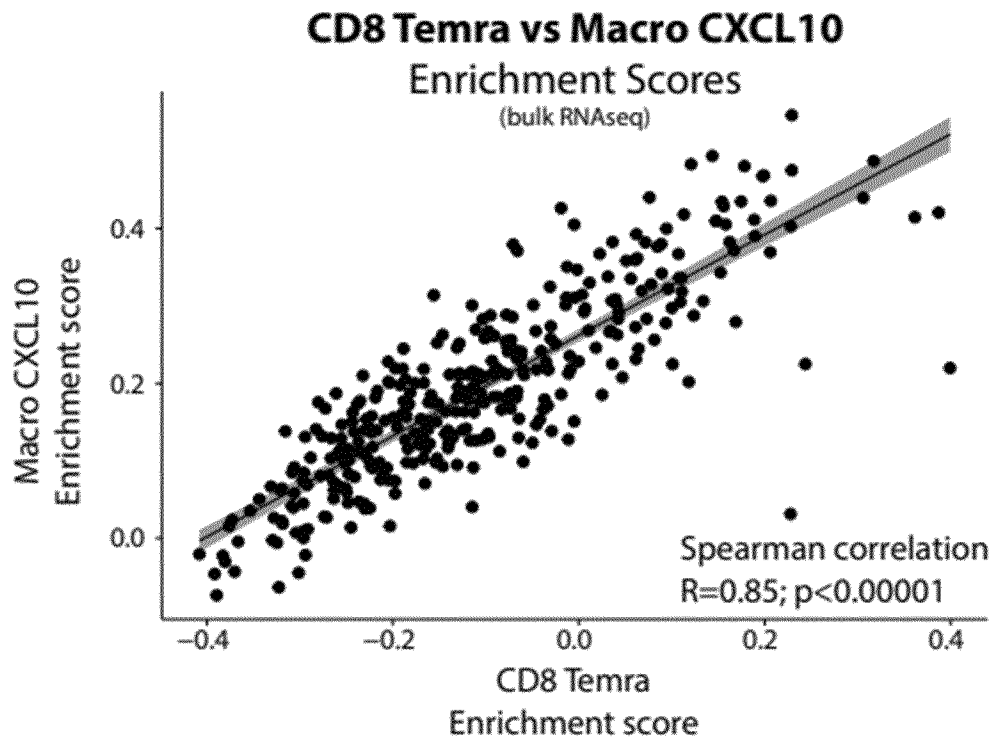
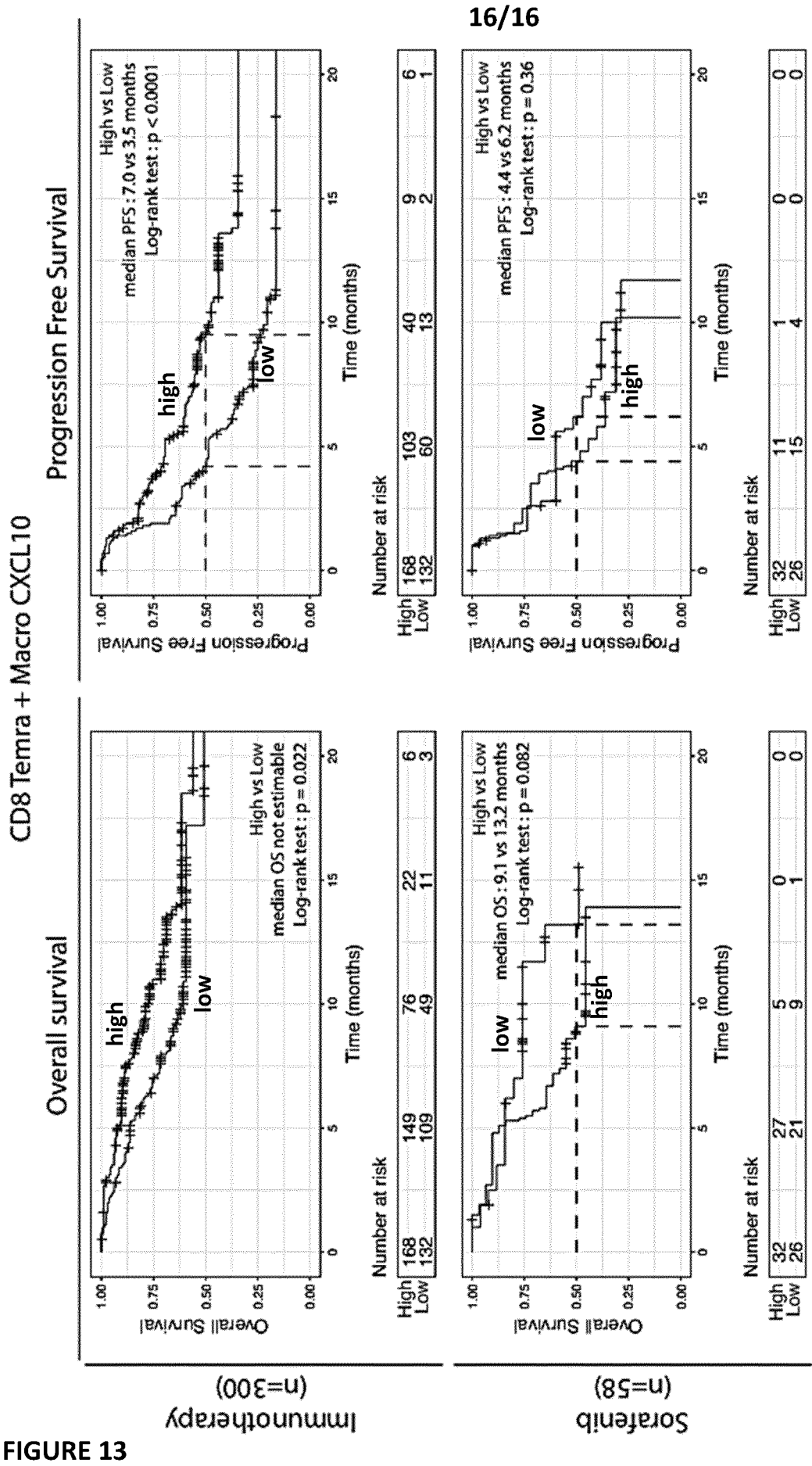


FIGURE 12



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/065306

A. CLASSIFICATION OF SUBJECT MATTER INV. G01N33/574 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CAPPUYNS SARAH ET AL: "PD1-negative CD45RA effector-memory CD8 T-cells are essential for response to checkpoint inhibition in advanced hepatocellular carcinoma", JOURNAL OF HEPATOLOGY , vol. 78, no. Supplement 1 1 June 2023 (2023-06-01), pages s524-s525, XP093096978, AMSTERDAM, NL ISSN: 0168-8278 Retrieved from the Internet: URL:https://pdf.sciencedirectassets.com/272181/1-s2.0-S0168827823X0004X/1-s2.0-S0168827823012825/main.pdf?X-Amz-Security-Token=IQoJb3JpZ2luX2VjENf////////wEaCXVzLWVhc3QtMSJHMEUCIckbwcelHj skg2uNcBva3mrw1QEgWPd4lrKxHXIZx33aAiEAYwiLEPHY9/cQes6ztXemTXDS2 -/-	1 - 15
☒ Further documents are listed in the continuation of Box C.	☒ See patent family annex.	
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 18 June 2024		Date of mailing of the international search report 27/06/2024
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer C.F. Angioni

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/065306

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	uXX47hB2L+4BgyVSYUqvAUI//////////ARAFGgw wNTkwMDM1N the whole document -----	
X	PRINCIPE NICOLA ET AL: "Tumor Infiltrating Effector Memory Antigen-Specific CD8+ T Cells Predict Response to Immune Checkpoint Therapy", FRONTIERS IN IMMUNOLOGY , vol. 11 1 November 2020 (2020-11-01), XP093058726, DOI: 10.3389/fimmu.2020.584423 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/artic les/PMC7688517/pdf/fimmu-11-584423.pdf	1,3,5,6, 8-15
A	the whole document In particular: Title; Abstract; Materials and methods; Figures 1-5; Discussion. -----	2,4,7
X	BASSEZ AYSE ET AL: "A single-cell map of intratumoral changes during anti-PD1 treatment of patients with breast cancer", NATURE MEDICINE, NATURE PUBLISHING GROUP US, NEW YORK, vol. 27, no. 5, 1 May 2021 (2021-05-01), pages 820-832, XP037452982, ISSN: 1078-8956, DOI: 10.1038/s41591-021-01323-8 [retrieved on 2021-05-06]	1,3,5,6, 8-15
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A	the whole document In particular: Title; Abstract; Methods; Figures 1-4; Pages 12 and 13, bridging paragraph. ----- -/-	2,4,7

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/065306

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	WO 2022/216021 A1 (UNIV NAT CHONNAM IND FOUND [KR]) 13 October 2022 (2022-10-13) the whole document See experimental data in the examples (in machine translation). -----	1, 3, 5, 6, 8-15 2, 4, 7

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2024/065306

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2022216021 A1	13-10-2022	EP 4321625 A1 WO 2022216021 A1	14-02-2024 13-10-2022
