**Supplementary Information**

**Table Titles**

Supplementary Table 1. NSCLC patients baseline characteristics.

Supplementary Table 2. Response to treatment of advanced NSCLC patients.

Supplementary Table 3. Results of multivariate Cox regression model.

Supplementary Table 4. Metal-conjugated mAbs used for mass cytometry studies.

Supplementary Table 5. Metal-conjugated mAbs used for IMC studies.

**Supplementary Figure legends**

**Fig S1:** **A)** Scheme of immune TME analysis strategy. Tumoral (Tu) and non-tumoral (non-Tu) lung tissue was collected from surgical specimens after macroscopical examination of the tissue by a pathologist. For each specimen, a fragment was formalin-fixed and paraffin embedded (FFPE) for tissue imaging (QIF or IMC) analysis. The remainder of the tissue was directly processed for single-cell CyTOF analysis. **B-C)** Association between CD8+ T cell **(B)** and CD4+ T cell **(C)** levels in non-Tu lung tissues by multiplex QIF and CyTOF (n=25).

**Fig S2:** Metal-conjugated mAbs against inducible markers were titrated using resting vs stimulated PBMC. The optimal antibody concentration was assayed by serial dilutions and determined by the median mass intensity (MMI) signal to noise ratio from internal positive and negative controls. Heatmaps (in the left of each panel) depict the MMI for each marker at different serial dilutions in stimulated (S) or unstimulated (U) PBMC in the different immune populations. X indicates the highest concentration level, which was 0.01ug/ul for all markers, with the exception of LAG-3, TIM-3, CD137, and KLRG-1, that 0.02ug/ul was used; Charts depict the marker MMI for positive (blue) and negative (orange) controls. Ratio is indicated with a grey line. 2D-plots depict the expression levels in the negative and positive controls, at the chosen concentration indicated with a red dashed line box.

**Fig S3:** **A)** Density t-SNE plot of CD45+ compartment from tumor (Tu) and non-tumor (non-Tu) lung tissues, with populations indicated. **B)** t-SNE plot of CD45+ compartment from Tu and non-Tu tissues overlaid with the expression of selected markers.

**Fig S4:** Lymphoid and non-lymphoid immune subsets ratio across non-tumor and paired tumor tissues analyzed by CyTOF. P-value was determined by paired student’s t test. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001.

**Fig S5:** From 20 non-Tu and 20 paired Tu cases, 20,824 CD3+ cells were analyzed using the CITRUS method. Visual representation of unsupervised hierarchical clustering from all CD3+ immune cells obtained from non-Tu and Tu paired lung tissues. **A)** CD8+ compartments are contoured on the basis of canonical lineage markers CD8 and CD4. The colour scale indicates the median intensity of CD4 and CD8 expression. **B)** CD8+ subsets compartments are contoured on the basis of all phenotypical markers included in the analysis and depicted in C). The colour scale indicates the median intensity of CD45RO expression. **C)** Graphs illustrate each marker expression across the CD8+ clusters. The color scale indicates the median intensity of each depicted marker. D) Frequency of the three CD8+ subsets across peripheral blood mononuclear cells (PBMC), non-tumoral lung tissue and tumoral lung tissue from eight NSCLC patients. Bar plots show mean ± SEM.

**Fig S6:** **A)** Expression of depicted markers in Ebo CD8 TIL Ki-67 high and Ki-67 low analysed by CyTOF. P-value was determined by paired student’s t test. \*\*, p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001. **B)** Density t-SNE plot of Ebo CD8 TIL. Colours depict the intensity of each indicated marker.

**Fig S7: A)** Heatmap showing differentially expressed genes between Ebo, Em and Eff CD8+ TIL subsets. **B)** TCRα clonalities are shown by whisker-plots(the lines indicate median values and the boxes interquartile range (IQR) values). Data from sorting cell subsets sequence results which are over 10% frequency clonalities of Ebo, Em and Eff are shown. **C)** The relative abundance of TCRα clonotypes of Ebo, Em and Eff, the Large clonalities ranges are from 10% to 100%, Medium clonalities ranges are from 1% to 0.1% and Small clonalities range is lower than 0.1%. The clonotypes are shown from all sorting cell subsets combine together. **D)** Heatmap showing associated “exhaustion marker” genes in Ebo, Em and Eff CD8+ TIL subsets.

**Fig S8:** **A)** Validation of anti-cleaved-caspase-3. Jurkat cells were treated with DMSO (untreated) or 1uM of camptothecin (treated) for 18h to induce apoptosis. The cells were fixed with formalin and processed to make FFPE cell pellets. The expression of cleaved caspase-3 was measured using QIF. The number in the bars represents the number of replicates used in the study. QIF staining of cleaved caspase-3 in Jurkat cells treated with or without Camptothecin. **B)** Quantitative measurement of cleaved caspase-3 in Jurkat cells treated with or without camptothecin. **C)** Representative image showing IMC staining for indicated markers of a NSCLC case. Each marker is represented by a different colour as indicated in the panel. White arrows point the cells with CD45RO and cleaved caspase 3 expression. Green arrows point CD45RA cells without cleaved caspase 3.

**Fig S9**: QIF of tumor tissue at the moment of (baseline) and two weeks after engraftment in immunodeficient NSG mice. Representative fluorescence microphotographs showing CK (green) and PD-L1 (red).

**Fig S10: A)** Age (left), CD8 TIL abundance (middle) and histology subtype (right) distribution across patients with different clinical stages. **B)** Correlation of Ebo CD8 TIL frequency with age (left) and CD8 abundance (right). **C)** Ebo frequency in squamous and non-squamous NSCLC cases. **D)** Ebo frequency in NSCLC cases with tertiary lymphoid structures (TLS+) or without (TLS-). **E)** Representative H-E image of an identified TLS. TLS were identified as highly organized lymphoid aggregates containing vessels that exhibited definite HEV features (plump and cuboidal endothelial cells). Arrow indicates the highly organized lymphoid aggregates. T, tumor; S, stroma. ns, not significant.

**Fig S11:** Density t-SNE plots of singlets from NSCLC cases overlaid with the expression of indicated markers.

**Fig S12: A)** Representative image showing IMC staining of a NSCLC case and Ebo CD8+ TIL identification alternative strategy, using Ki-67 instead of EOMES. Each marker is represented by a different colour as indicated in the panel. Orange squares indicate the Ebo CD8+ TIL selected, based in the CD8+ CD45RO+ CD45RA- KI-67+ expression of the markers. White squares indicate non-Ebo CD8+ TILs. **B)** Bar dot-plots depict percentages of Ebo CD8+ TIL population over total CD8+ TILs in patients with durable or non-durable clinical benefit to anti-PD therapy. **C)** Kaplan-Meier survival curves for 35 NSCLC patients treated with anti-PD therapy. Patients were divided in two groups based in tumors harbouring Ebo ≥14.2% (High) or Ebo<14.2% (Low) of total CD8 TILs. P-value was determined by the log-rank test. Ebo, Effector burn-out. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001.

**Fig S13: A)** Manual gating strategy mimicking CITRUS clustering following the indicated steps. **B)** Panels depict the expression levels of indicated markers in gated Ebo, Em and Eff CD8+ TIL subsets. **C)** Sorting gating strategy used by FACS. **D)** Panels depict the expression levels of indicated markers in FACS sorted Eff, Em and Ebo CD8+ TIL subsets.

**Fig S14:** Panels depict the expression levels of indicated markers Eff, Em and Ebo CD8+ TIL subsets gated by IMC using the following strategy: Ebo (CD8+, CD45RO+, CD45RA-, EOMES+), Em (CD8+, CD45RO+, CD45RA-, EOMES-) and Eff (CD8+, CD45RO-, CD45RA+).