High-resolution single-cell atlas reveals diversity and plasticity of tissue-resident neutrophils in non-small cell lung cancer

Highlights

- High-resolution single-cell atlas of the tumor microenvironment (TME) in NSCLC
- Histological tumor subtypes and driver genes imprint specific cellular TME patterns
- In-depth characterization of tissue-resident neutrophil (TRN) subpopulations
- TRN gene signature identifies patients refractory to treatment with PD-L1 inhibitor

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In brief

Salcher et al. integrate non-small cell lung cancer (NSCLC) single-cell datasets, refine patient stratification, and reveal histological and genotypic associations with tumor microenvironment (TME) composition. Single-cell sequencing of cells with low mRNA content identifies tissue-resident neutrophil (TRN) subpopulations with non-canonical functional properties. TRN gene signature is associated with immune-checkpoint inhibitor treatment failure.
High-resolution single-cell atlas reveals diversity and plasticity of tissue-resident neutrophils in non-small cell lung cancer

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SUMMARY

Non-small cell lung cancer (NSCLC) is characterized by molecular heterogeneity with diverse immune cell infiltration patterns, which has been linked to therapy sensitivity and resistance. However, full understanding of how immune cell phenotypes vary across different patient subgroups is lacking. Here, we dissect the NSCLC tumor microenvironment at high resolution by integrating 1,283,972 single cells from 556 samples and 318 patients across 29 datasets, including our dataset capturing cells with low mRNA content. We stratify patients into immune-deserted, B cell, T cell, and myeloid cell subtypes. Using bulk samples with genomic and clinical information, we identify cellular components associated with tumor histology and genotypes. We then focus on the analysis of tissue-resident neutrophils (TRNs) and uncover distinct subpopulations that acquire new functional properties in the tissue microenvironment, providing evidence for the plasticity of TRNs. Finally, we show that a TRN-derived gene signature is associated with anti-programmed cell death ligand 1 (PD-L1) treatment failure.

INTRODUCTION

Non-small cell lung cancer (NSCLC) is a highly aggressive and heterogenous disease with diverse histological subtypes and distinct mutational signatures,1 which accounts for an annual global death rate of 1.8 million patients.2 The technical advances in single-cell RNA sequencing (scRNA-seq) technologies enabled the dissection of the complex NSCLC tumor microenvironment (TME) in different stages, and numerous scRNA-seq NSCLC studies have identified a hitherto underestimated TME heterogeneity in early and advanced disease.3–15 Furthermore, these studies highlighted the importance of small cell populations in governing essential biological pathways such as immune cell activation or trafficking by tumor endothelial cells.6 However, a major limitation of these studies is the limited number of analyzed patient samples per study. Moreover, the lack of genomic data as well as long-term follow-up data prevents comprehensive dissection of the biological heterogeneity and its potential contribution to therapy resistance and survival outcome.

Technical and methodological variations between the different studies result in significant inconsistencies and knowledge gaps. As such, not all cell types (e.g., neutrophilic granulocytes) have been portrayed in the same depth and extension yet, posing an unmet need to characterize these populations as well. In NSCLC, it is well accepted that next to cancer cells, leukocytes...
Figure 1. Schematic outline of the overall concept used in this study
(A) Summary of the data integration and analysis workflow.
(B) Overview of the core NSCLC atlas and the epithelial, immune, and stromal/endothelial components depicted as uniform manifold approximation and projection (UMAP) plots.
(C) Fractions of depicted cell types per scRNA-seq platform.
(D) UMAP of the UKIM-V dataset (n = 17) colored by cell type.
(E) Core atlas extended by Leader11 and UKIM-V-2 datasets.

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compose the majority of cells within the TME.\(^{12,14}\) Particularly since immunotherapy is routinely used in clinical practice, in-depth characterization of the cancer immune cell compartment has been intensively pushed forward, and diverse cellular subsets have been profiled.\(^{5,6,11}\) Previous compositional analyses by flow cytometry as well as histological work ups suggested that neutrophils compose a significant proportion of all tumor-resident leukocytes, with an estimated abundance ranging from 8% to 20%.\(^{14,16,17}\) Intriguingly, when looking at the scRNA-seq studies in NSCLC published over the last years, neutrophils are clearly underrepresented. This discrepancy is most likely based on technical issues rather than on biological phenomena, but its clarification is of immense importance for our fundamental immunological understanding of NSCLC and for potential translational clinical investigations. This notion is underscored by pre-clinical data suggesting that neutrophils are essential mediators of both anti-tumor inflammatory pathways (reviewed in Shaul and Fridlender\(^{18}\)), including the potential of neutrophils to limit lymphocyte trafficking into malignant tumors, thereby limiting efficacies of programmed cell death 1 (PD-1) inhibition.\(^{19}\) Correlative studies in patients with NSCLC have linked the neutrophil-lymphocyte ratio with clinical outcome and response to immunotherapy.\(^{19-21}\) Additionally, pre-clinical evidence strongly supports the use of neutrophil-depleting agents (e.g., CXCR2 antagonists) as an adjunction to immune-checkpoint inhibitors.\(^{19}\)

To overcome the above-mentioned hurdles, we compiled major publicly available datasets into a comprehensive NSCLC scRNA-seq atlas covering 232 patients with NSCLC and 86 non-cancer controls. Additionally, given the scarcity of neutrophil single-cell data, we complemented the atlas by analyzing samples from 17 patients with NSCLC using a platform that captures cells with very low transcript count and carried out deep characterization of tissue-resident neutrophils (TRNs) including both tumor-associated neutrophils (TANs) and normal adjacent tissue-associated neutrophils (NANs).

## RESULTS

### Generation of a core large-scale NSCLC single-cell atlas

We first developed a core NSCLC atlas by compiling scRNA-seq data from 19 studies and 21 datasets comprising 505 samples from 298 patients (Figure 1A). This comprehensive NSCLC single-cell atlas integrates expert-curated, quality-assured, and pre-analyzed transcriptomic data from publicly available studies as well as our own dataset (UKIM-V) in early and advanced stage NSCLC of any histology (see STAR methods; Figures S1A–S1K).

Important study characteristics are summarized in Table S1. In total, the core atlas includes transcriptomic data from 212 patients with NSCLC and 86 control individuals, comprising 196 tumor samples and 168 non-tumor control samples. Of the 212 patients with NSCLC, 156 were histopathologically annotated as lung adenocarcinoma (LUAD), 41 as lung squamous cell carcinoma (LUSC), and 15 were not otherwise specified (NSCLC NOS). NSCLC samples include tissue of the primary tumor (n = 176) or metastasis (n = 45) that were obtained either by surgical resection or by computed tomography- and bronchoscopy-guided biopsies. We clustered the disease stages of the patients with NSCLC as early (UICC stage I–II) versus advanced (UICC III–IV) diseases, as not all studies provided sufficient information on tumor stages. Among the control samples, 89 were derived from distant non-malignant tissue of patients with lung tumors (annotated as normal_adjacent), of which 65 have a patient-matched tumor sample. Further, 10 samples were derived from non-tumor-affected lymph nodes of patients with NSCLC (annotated as normal) and 79 samples from patients without evident lung cancer history (annotated as normal). Of the control patients, 18 had a history of chronic obstructive pulmonary disease (COPD). Overall, the core atlas integrates 898,422 single cells, which are annotated to 12 coarse cell-type identities and 44 major cell subtypes or cell states (e.g., dividing cells) based on previously established canonical single-cell signatures (Figure S1A) including 169,223 epithelial cells, 670,409 immune cells, and 58,790 stromal and endothelial cells (Figure 1B). We also annotated important CD8+ T cell subclusters (terminally exhausted, activated, effector memory, naïve, natural killer [NK]-like, dividing) using previously reported marker genes\(^{22}\) (Figure S1J). The cell-type composition for each dataset, the tissue of origin, and the patients within the core atlas are shown in Figures S1B and S1C.

Previous scRNA-seq studies discriminated the clinically relevant types of LUSC and LUAD. The UKIM-V dataset was histopathologically classified by routine pathologists followed by independent review by expert lung pathologists (S.P. and C.K.). Cancer cells (in total, 83,439 cells, from primary tumor and metastatic tissue) in the atlas showed high heterogeneity of their transcriptomic profiles (Figure 1B). Due to the large patient cohort, we were able to apply high-resolution lung cancer cell classification based on their specific marker gene expression signatures (Figure S1D).

We divided the following main clusters LUSC (KRT5, KRT6A, KRT17, SOX2, NTRK2, TP63); LUAD (CD24, MUC1, KRT7, NAPSA, NKX2-1, MSLN); LUAD with signs of epithelial-to-mesenchymal transition (LUAD EMT) (VIM, SERPINE1, CDH1, MIF); LUAD with neuroendocrine features (LUAD NE) (CHGA, SYP, NCAM1, TUBA1A); LUAD with high expression of mesothelin (MSLN) associated with EMT and metastasis (LUAD MSLN);\(^{23}\) and NSCLC expressing both LUAD and LUSC markers (NSCLC mixed) (MUC1, KRT7, KRT6A, SOX2) (Figures 1B and S1D). There are highly mitotic/proliferative clusters (TOP2A, MKI67) of both LUAD and LUSC that may resemble highly aggressive and invasive cancer cells. The LUAD EMT cells likely resemble an invasive, pro-metastatic cluster characterized by the plasminogen activator PA11 (SERPINE1) or the mesenchymal protein vimentin (VIM) that are both involved in cell adhesion, invasion, and angiogenesis.\(^{24,25}\) Besides, all subclusters showed a high expression of the conserved non-coding RNAs...
MALAT1 and NEAT1 (Figure S1D) that were previously linked to metastasis formation in NSCLC. For all subsequent analyses, we used the histopathological annotation LUAD or LUSC.

**Neutrophils are underrepresented in most scRNA-seq studies**

In both tumor and normal lung tissue of all core atlas samples, the neutrophil cluster (FCGR3B, CSF3R, CXCR2, and G0S2) comprised 8,468 cells with overt low mRNA counts. Overall, neutrophils account for only 1.5% of all atlas cells (Figure S1E). Remarkably, 78% of all atlas neutrophils derive from the UKIM-V dataset (Figures 1C and S1B), in which neutrophils compose 12% of all cells and 18% of all leucocytes, respectively. The remaining neutrophil data originate mainly from four other datasets, in all of which neutrophils comprise less than 6% of all leucocytes. Particularly in those studies using the droplet-based 10x Chromium platform, neutrophils are completely absent or only rarely depicted (Figure S1F). Our comparative flow cytometry analysis demonstrated that neutrophils account for 10%–20% of all leucocytes in NSCLC tumor and normal adjacent tissues (n = 63) (Figure S1G), which is in accordance with previous flow cytometry and histology data.

Thus, low neutrophil abundance seen in previous scRNA-seq datasets suggests an underrepresentation, most likely due to technical issues. Neutrophils are fragile, short-lived cells (circulatory half-life of 7–10 h in humans), are particularly sensitive to handling procedures, and express an exceptionally low amount of mRNA molecules. Comparative analysis of scRNA-seq platforms indicated that the BD Rhapsody workflow captures a notably high number of mRNA molecules per cell and may thus be especially suitable to depict low-mRNA-content cells (Figure S1H). As a consequence, neutrophils represented a major cell cluster in the UKIM-V dataset generated with BD Rhapsody, whereas the low-mRNA-content neutrophil cluster could not be appropriately detected in the datasets generated with 10x Chromium, as also described recently, and only to a very limited extend when applying other platforms (Figure 1C).

**scRNA-seq of low-mRNA-content cells**

Due to high clinical relevance of neutrophils and the need for their better in-depth transcriptomic characterization, we used the advantage of the BD Rhapsody platform in depicting cells with low mRNA content for further analysis. As our initial dataset included only three patients, we next performed scRNA-seq of an additional 14 patients with NSCLC to increase the statistical weight of our cohort (UKIM-V cohort). In total, our dataset contains tumor and adjacent normal lung tissue from 17 patients (6 male, 11 female) undergoing lobectomy for treatment-naive NSCLC (12 LUAD, 5 LUSC). Cells were freshly isolated, processed, and sequenced as described in detail in the STAR methods. The UKIM-V dataset (1 and 2) comprises 122,902 cells that cluster into all main lung cell types defined by the expression of specific marker genes (Figure 1D). Neutrophils are characterized as a cell cluster with exceptionally low mRNA content and thus a relatively low number of detected transcripts, but due to the relatively high number of mRNA molecules captured per cell (unique molecular identifier [UMI] counts in epithelial cells: 8,938), we could readily depict these cells. The 15,190 neutrophils identified in the UKIM-V dataset were derived from control lung (n = 6,378) and corresponding tumor tissue (n = 8,812).

**Extension of the core single-cell atlas by transfer learning**

To combine the strength of the core atlas with our own data including neutrophils, we used the recently developed transfer-learning method scArches, which enables the extension of the core atlas using additional current, as well as future, datasets. We mapped our second UKIM-V dataset as well as one recently published dataset comprising 288,157 cells onto the atlas (Figure 1E). The cell-type annotations were transferred from the core atlas to the two new datasets on the basis of transcriptomic similarity in the batch-corrected joint embedding (see STAR methods). This extended atlas now integrates 29 datasets from 19 studies and comprises 1,283,972 cells, 44 cell types, 556 samples, and 318 patients, resulting in 1.75 billion expression values. The overall cell-type composition of the extended atlas is shown in Figure S1I, and the patient numbers per cell type are shown in Table S2. All subsequent analyses were carried out on the extended atlas dataset unless otherwise specified (Table S3).

**Identification of changes in cell-type compositions in distinct histological or genetic tumor types and tumor stages is of utmost importance as it can highlight hetero-cellular interactions and possibly enable association(s) with therapy response. However, detecting shifts in cell-type composition using scRNA-seq data is challenging due to the inherent bias present in cell-type compositions and low sample sizes. We therefore adopted a Bayesian model for identifying changes in cell-type compositions while controlling for the false discovery rate (scCODA tool). scCODA requires setting a reference cell type that is assumed to be constant between conditions. When comparing cellular composition in LUSC and LUAD in primary tumor tissue with cancer cells as reference cell type, we found a significantly higher proportion of neutrophils in LUSC, whereas macrophages, CD4+ T cells, alveolar cells type 2, and transitional club/AT2 were more abundant in LUAD (Figure 1F). The analysis of the cell-type compositions in early-stage compared with advanced-stage NSCLC tumors showed a higher abundance of cDC2 in the early stage (Figure S1K). To validate the findings, we carried out orthogonal validation using an external cohort of 110 patients with NSCLC (55 LUAD and 55 LUSC) and immunohistochemistry staining for neutrophils, CD4+ T cells, and macrophages. The validation results confirmed the findings (Figure 1G) and further support histology-specific TME characteristics.

**Single-cell composition of the TME reveals distinct NSCLC tumor immune phenotypes**

Next, we stratified patients with NSCLC based on infiltration patterns of their TME using the extended atlas. Unsupervised clustering on batch-corrected cell-type fractions revealed four distinct tumor immune phenotypes (Figure 2A): (1) immune-deserted (ID) tumors (i.e., no significant immune cell infiltration but a high cancer cell fraction); (2) the subtype of tumors with B cell dominance (B; B cell, plasma cell, mast cells); (3) the subtype of tumors with myeloid dominance (M; macrophage/monocyte); and (4) the subtype of tumors with T cell dominance (T; CD8+, CD4+, T regulatory cells). The affiliation of UKIM-V
patients to myeloid cell and T cell subtypes was validated using flow cytometry (Figures S2A and S2B). Across the strata, most patients of the B cell, myeloid cell, and T cell subtypes showed LUAD histology, while half of the patients with LUSC were over-represented in the ID subtype (Figure S2C). Neutrophils were excluded from patient stratification since they are under-represented in most datasets. Using logistic regression, we did not find any association of the different patient strata to tumor stages (early versus advanced) or sex.

To identify tumor-cell-based TME imprinting characteristics, we next analyzed differentially enriched pathways\(^3^1\) in the cancer cells of each of the four immune phenotypes. The ID subtype showed significant downregulation (false discovery rate [FDR] <0.1) of the androgen pathway (Figure 2B). Previously, androgen receptor signaling has been shown to suppress programmed cell death ligand 1 (PD-L1) transcription in hepatocellular carcinoma (HCC) cells and may thus exert immune stimulatory effects.\(^3^2\) Analysis of differentially expressed transcription factors\(^3^3,^3^4\) in the cancer cells of each subtype showed a significant downregulation of FOXO4 in the ID subtype (Figure S2C). As previously reported, FOXO transcription factors are essential mediators of immune cell homeostasis,\(^3^5\) and FOXO4 downregulation could thus promote an ID phenotype.

We then applied the tool CytoSig\(^3^6^\) to define enriched cytokine signaling signatures in cancer cells of each immune phenotype (Figure 2C). CytoSig analyzes defined cytokine signatures that are differentially expressed when a cell is exposed to a specific cytokine (that is name giving for the respective cytokine signature). As expected, most signatures are downregulated in the ID group; solely, the signature of the tolerogenic cytokine interleukin-4 (IL-4), a modulator of T regulatory cell-mediated immune suppression,\(^3^7\) was significantly elevated (Figure 2C). We found a significant upregulation of interferon type I–III signatures in the myeloid subgroup, suggesting a particularly important role of macrophages/monocytes in interferon signaling in the TME (Figure 2C).

Analysis of cell-cell communication reveals hetero-cellular crosstalk in the TME

Using the CellPhoneDB ligand-receptor complexes database, we next assessed differences in the hetero-cellular crosstalk of cancer cells toward diverse immune cells among the two major histotypes LUAD and LUSC by analyzing the top 10 differentially expressed cancer cell ligands (Figure 3A; the top 30 ligands are shown in Figure S3A). Overall, in both histologies, cancer cellular interactions were directed to diverse immune cell subtypes with different targets. In LUAD, we found a prominent upregulation of the KDR-VEGFA axis from cancer cells toward neutrophils, macrophages/monocytes, mast cells, and classical dendritic cells (cDCs), potentially implicating immunosuppressive signaling by
Figure 3. Cellular crosstalk analysis

(A) Circos plot of the cellular crosstalk of cancer cells toward the major immune cells in LUAD versus LUSC. Shown are the top 10 differentially expressed cancer cell ligands. Red interactions are upregulated in LUAD, and blue interactions are upregulated in LUSC.

(B) Cancer-immune cell crosstalk in each patient subtype. Top panel: differentially expressed ligands of cancer cells in each subtype (B, M, T, ID) (DESeq2 on pseudo-bulk, FDR < 0.1). Bottom panel: respective receptors and the expression by cell type. Dot sizes and colors refer to the fraction of cells expressing the receptor and gene expression, respectively, averaged over all patients. Dots are only shown for receptors that are expressed in at least 10% of the cells of the respective cell types.

See also Figure S3.
cancer cells in this histotype. Other major LUAD pathways involve the immunosuppressive macrophage scavenger receptor MARCO as well as ADGRE5-CDS5 signaling, associated with migration and invasion. Conversely, in LUSC cancer cells, there is upregulation of pro-migratory SPP1 signaling that has previously been reported as upregulated in lung cancer tissue particularly of squamous histology, as well as an upregulation of Jagged1 (JAG1), which induces NOTCH, thereby promoting tumor progression and regulating the tumor immune microenvironment via, e.g., neutrophil recruitment.

Next, we investigated the crossstalk of cancer cells to immune cells within the patient immune subtypes T cell, B cell, myeloid cell, and ID by analyzing differentially expressed cancer cell ligands (Figure 3B). While downregulated in the ID subgroup, B, M, and lesser T cell subgroups showed upregulated signaling of several chemokines (CXCL9/10/11, CCL3/13/18) to their cognate receptors on T and myeloid cell subsets, suggesting that cancer-cell-secreted chemokine gradients contribute to immune infiltration.

Integration of bulk RNA-seq data reveals genotype-immune phenotype associations
scRNA-seq provides an unprecedented view on the cellular heterogeneity in the TME. However, the majority of the scRNA-seq studies lack both cancer genotype information and survival data. The TCGA reference dataset includes this information together with bulk RNA-seq data. Using the recently published computational method SCISSOR, we evaluated the association of atlas-derived cell-type transcriptomic signatures with genotype and survival data from the TCGA reference dataset including 1,026 patients (UICC I-IV, LUAD, and LUSC). In a previous pan-cancer study using bulk RNA-seq data, we have shown that genomic features including mutational load, tumor heterogeneity, and specific driver genes determine immune phenotypes. Here, the high resolution of the single-cell NSCLC atlas enabled an in-depth analysis of these determinants. EGFR, TP53, KRAS, and STK11 mutations showed distinct immune infiltrates (Figures 4A–4D, S4A, and S4B). For example, cDC1 and cDC2 showed opposite infiltration patterns in patients with LUAD with mutations of either EGFR (high cDC infiltration, as reported previously) or KRAS and STK11 (low cDC infiltration) (Figures 4A–4D). Conversely, TP53- and STK11-mutated genotypes were associated with CD8 T infiltration, which is not seen in EGFR- or KRAS-mutated tumors (Figures 4C, 4D, S4A, and S4B). High CD8 T cell infiltration in TP53-mutated LUAD has also been described previously. Hence, our single-cell view of the TME provides further evidence for the link between the genetic makeup of the tumor, the histology, and the respective immune contexture. Given the importance of the driver genes in terms of treatment decisions, we confirmed our findings by orthogonal validation of the genotype-immunophenotype associations using two external cohorts (n = 19 and n = 37) (Figures S4C and S4D).

We next analyzed the cell-type transcriptomic signatures and their association with survival in 1,026 patients from the TCGA cohort. Overall, patients with NSCLC with B cell-rich tumors showed a prominent association with improved survival, whereas neutrophils were the strongest negative survival predictor (Figure 4E) and were, together with monocytes, the only immune cell types that were negative predictors in both LUAD and LUSC (Figures 4E, S4E, and S4F). To support our finding, we used an independent method based on deconvolution using bulk RNA-seq data and confirmed that B cells are indeed associated with better prognosis, albeit significantly only for LUAD (Figures 4F, S4G, and S4H), which has also been proposed in multiple previous studies (reviewed in Patel et al.). Finally, the analysis of the CD8 T cell subtypes showed that naive CD8 T cells were the strongest predictor for improved survival (Figure 4G).

TRNs acquire new properties in the TME
One unique feature of the large-scale NSCLC atlas we assembled is the enrichment with single-cell expression profiles from neutrophils generated using samples from 17 patients with NSCLC, so we focused on the deep characterization of these cells. TANs are known as a very heterogeneous cell population with both anti- and pro-tumorigenic properties. Likely due to technical reasons, the characterization of NANs lags even further behind that of TANs. To overcome this insufficient TRN characterization, we here characterized the transcriptomic signatures of TANs and NANs in NSCLC using the extended atlas (Figures S5A and S5B). Neutrophils were more abundant in patients with LUSC compared with those with LUAD (Figure S5A), as described previously, which we confirmed in two external cohorts by flow cytometry analysis (47 LUAD and 16 LUSC) (Figure S5C) and immunohistochemistry (55 LUAD and 55 LUSC) (Figure 1G). The overall TAN phenotype was characterized by high expression of OLR1 (LOX-1), VEGFA, CD83, ICAM1, and CXCR4 and low expression of CXCR1, CXCR2, PTGS2, SELT (CD62L), CSF3R, and FGR3B (CD16B) (Figure 5D), confirming previously reported signature genes. The TAN characteristic gene set included expression patterns of both established
Figure 5. Characterization of tissue-resident neutrophils using scRNA-seq
(A and B) UMAP of tissue-resident neutrophils (TRNs) from the extended atlas, (A) classified into tumor-associated neutrophils (TANs) and normal-adjacent associated neutrophils (NANs) and (B) colored by histology (as defined by histopathological assessment).
(C) Neutrophil fractions (as percentage of leucocytes) by flow cytometry of LUAD and LUSC tumor tissue (LUAD n = 47, LUSC n = 16; Wilcoxon test, **p < 0.01). The horizontal line represents the median, and whiskers extend to the interquartile range.
(D) Candidate TAN genes. Each dot refers to a patient with at least 10 neutrophils in both NAN and TAN groups. Lines indicate the mean per study. p values are derived from a paired t test and adjusted for FDR.
(E) Expression levels of VEGFA in various cell types in primary tumor samples. Each dot represents a patient with at least 10 cells (median values, boxes represent the interquartile range [IQR], whisker data points within 1.5 times the IQR).
(F) Transcription factor analysis of TAN versus NAN using DoRothEA. Each dot represents a single patient, and bars are the mean of all patients. p values are derived using a paired t test and are FDR adjusted. Shown are transcription factors with a mean score difference >0.2 and an FDR <0.1.
(G) Comparison between tumor and normal-adjacent samples for selected candidate genes using flow cytometry. Each dot represents a patient that was not part of the scRNA-seq dataset. Paired Wilcoxon test, *p < 0.05, ****p < 0.0001. CD16: the horizontal line represents the median, and whiskers extend to the IQR.
(H) Selected multiplex immunofluorescence (M-IF) staining of LOX-1 (red) and pancytokeratin (green) in tumor tissue and matched normal-adjacent lung tissue of a patient with LUSC. Scale bar: 100 μm.
See also Figure S5.
Figure 6. Tissue-resident neutrophil subtypes in NSCLC
(A) UMAP of all TRNs colored by TAN and NAN subclusters. The neutrophil clusters derive from 85 patients, 42 of whom have >10 neutrophils.
(B) Top 5 markers for each TAN and NAN cluster. The marker gene quality is reflected by the area under the receiver operator characteristics curve (AUROC; 1 = marker gene perfectly distinguished the respective cluster from other clusters in all patients; AUROC 0.5 = no better than random).

(legend continued on next page)
In a recent study, remarkable neutrophil adaptability to different tissue environments was shown,\(^{51}\) suggesting that while transient, TRNs acquire new properties and function within tissue. The neutrophil phenotype differs in dependence on spatial-, temporal-, and disease-specific clues\(^ {52}\) as well as during the evolution from bone-marrow-resident immature (CXCR4\(^ {\text{high}}\), CXCR2\(^ {\text{low}}\), CD16\(^ {\text{low}}\), CD62L\(^ {\text{low}}\), MME\(^ {\text{low}}\)), to circulating/mature (CXCR2\(^ {\text{high}}\), CD16\(^ {\text{high}}\), CD62L\(^ {\text{high}}\)), to aged/senescent neutrophils (CXCR4\(^ {\text{high}}\)).\(^ {53-55}\) However, none of these markers are specific for a certain maturation state. Relative to TANs, matched NANs in our dataset showed high expression of established neutrophil maturity markers (SELL, PTSG2, CXCR2, CXCR1, FGGR3B, MME) as well as canonical neutrophil markers (S100A8, S100A9, S100A12) (Figures 5D and S5B). While downregulation of these markers in TANs suggests immaturity, we could not identify a clear expression pattern of previously suggested immaturity signatures.\(^ {55,56}\) Notably, CXCR1 and CXCR2 have previously been reported as TAN markers in NSCLC;\(^ {1,12}\) however, our analysis revealed elevated CXCR1/ CXCR2 expression in NANs (Figure 5D). Conversely, low expression of SELL (CD62L) and CXCR2 (both downregulated in aged neutrophils)\(^ {56}\) (Figure 5D) and high expression of the known neutrophil activation markers CD83 (an inhibitory immune checkpoint),\(^ {58,59}\) the atypical chemokine receptor CCR2\(^ {60}\) Icam1 (CD54),\(^ {57}\) and C15orf48 (a mitochondrial transcript upregulated during inflammation)\(^ {51}\) as well as several cytokines (CCL3, CCL4L2, CCL4, CCL2) (Figures 5D and S5B) support an aged/chronically activated/exhausted TAN phenotype.\(^ {62}\)

Neutrophils support the pro-angiogenic switch in cancer via release of VEGF and other pro-angiogenic factors (reviewed in Ozel et al.\(^ {53}\)). Our atlas provided evidence that neutrophils represent a major source for VEGF expression within the NSCLC TME (Figure 5E). A highly differentially expressed TAN marker of major interest is lectin-type oxidized LDL receptor 1 (LOX-1) encoded by the OLR1 gene that is known as main receptor for oxidized low-density lipoprotein (LDL)\(^ {64}\) (Figures 5D and S5B). OLR1 has been described as a putative marker to distinguish normal peripheral blood neutrophils (LOX-1\(^ +\)) from polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs),\(^ {65}\) respectively. However, concordant to our results, OLR1 expression by TANs has been previously described,\(^ {12}\) and our comparative analysis to matched NANs underlined the tumor specificity of this marker. Moreover, peroxisome proliferator-activated receptor gamma (PPARG), a nuclear receptor and direct transcriptional regulator of OLR1,\(^ {66}\) was elevated in TANs (Figure 5F). Concordantly, flow cytometry analysis of tissue from patients with NSCLC (n = 7) confirmed elevated LOX-1 (OLR1) expression in TANs (Figure 5G). We could further validate the transcriptomic TAN signature at the protein level by flow cytometry, including elevated expression of CD83 (n = 7) and CD54 (ICAM1) (n = 6) and lower expression of CD181 (CXCR1), CD62L (SELL) (n = 8), and CD16 (n = 35) (Figure 5G).

We additionally performed multiplex immunofluorescence staining of paraffin-embedded NSCLC tumor tissue and patient-matched normal adjacent lung tissue. Co-staining of LOX-1 and CXCR2 suggested LOX-1 as neutrophil marker (Figure S5C) (of note, CXCR2 also marks MDSCs\(^ {67}\)). We found infiltration of LOX-1\(^ +\) cells in tumor tissue but not in adjacent normal-lung tissue (Figure 5H), underscoring the cancer-tissue specificity of this marker.

**Plasticity and non-canonical functional properties of TRNs**

Previous studies have proposed transcriptomic subclusters of neutrophils in NSCLC.\(^ {4}\) However, a distinct subclassification and in-depth characterization of TRN including TANs and NANs in NSCLC has not been described so far. We applied unsupervised Leiden clustering on all atlas neutrophils (n = 19,166), separating four TAN subsets (TAN-1 to TAN-4) and three NAN subsets (NAN-1 to NAN-3) (Figure 6A) that are backed by multiple datasets and multiple patients (Figure S6A). Marker gene selection revealed an extensive phenotypic heterogeneity among the clusters and allowed identification of marker genes for each subcluster, of which the top 5 are given in Figure 6F. The TAN signature genes described above (OLR1, VEGFA, CXCR4, CD83) showed relative homogenous expression among all TAN subclusters (Figure S6B). Overall, NAN clusters showed a predominance in LUAD and TAN clusters in LUSC tumors (Figures S6C and S6D).

Specific NAN-1 genes included the alarmin S100A12, a known marker of activated proinflammatory neutrophils,\(^ {68}\) and the
NETosis co-factor PADI4, as well as pro-angiogenic markers (PROK2, MMP9). The NAN-2 cluster is pretty similar to the NAN-1 cluster but showed reduced expression of some NAN-1-specific genes (e.g., ST100A12, PADI4, MMP9). NAN-3 shows strong expression of interferon-stimulated genes (GBP1, GBP5, IFIT2) (Figure 6B). TAN-1 shows high expression of interleukin 1 receptor antagonist (IL1RN), a known marker of activated neutrophils that negatively regulates IL-8 secretion to control excessive neutrophil inflammatory activity, as well as of the potent NF-κB activator RIPK2 and CD44, regulating cell recruitment and adhesion (Figure 6B). These finding support the concept that the plasticity of neutrophils is profoundly shaped by the NSCLC TME that attracts and activates neutrophils.

The TAN-2 subcluster was characterized by the expression of the major histocompatibility complex (MHC) class II genes HLA-DRA, CD74, HLA-DMB, and HLA-DRB1, indicating a phenotype with an immunogenic antigen-presenting feature. Both CD74 and HLA-DRA are also expressed in the other TAN clusters, albeit at lower levels (Figure 6B). To validate the scRNA-seq findings, we analyzed samples from an additional 11 patients using flow cytometry, confirming the antigen-presenting phenotype as seen by an upregulation of HLA-DR on TANs in NSCLC compared with neutrophils from normal adjacent lung tissue (Figure 6C). Of note, the transition to HLA-DR+ neutrophils was accompanied by a shift toward the identified TAN signature (elevated expression of CD83 and LOX-1 and lower expression of CD181 [CXCR1], CD62L [SELL], and CD16) in neutrophils derived from NSCLC tumor tissue (Figure S6E).

The TAN-3 subcluster was characterized by a high expression of proinflammatory cytokines (C15orf48, CCL3, CCL4, CSTB) as well as galectin 3 (LGALS3), which is associated with neutrophil activation and emigration (Figure 6B). Finally, TAN-4 showed high expression of ribosomal genes (such as RPS12, RPL3, RPN2, RPL23) (Figure 6B) similar to a neutrophil cluster identified in patients with severe COVID-19. This may suggest the highly plastic phenotype of TAN-4 eventually transitioning to other cell phenotypes, as described previously for tumor endothelial cells.

The transcriptional profiling of the neutrophil subsets indicates their remarkable phenotypic plasticity. We therefore performed RNA velocity analysis (see STAR methods) using only the UKIM-V dataset (which includes treatment-naive patients with NSCLC only) since the method requires raw sequencing data. The analysis indicates a transition from NAN-3 to both NAN-2 and NAN-1 (whose transcriptomic signatures are similar). NAN-3 and NAN-2 transition to TAN-2 and TAN-1, respectively, with TAN-1 and TAN-2 transitioning into all TAN subtypes (Figures 6D and partition-based graph abstraction7 in 6E). Interestingly, this TRN evolution seems to follow a one-directory path with TAN-3 as final transition, although at this point we do not know whether TAN-3 could further transit to other cell types. These observations support the hypothesis that TAN phenotypes are substantially modulated by local cues encountered in the TME.

We next investigated the cellular interactions of TRN subsets with CD8+ T cells and cancer cells by analyzing differentially expressed TRN ligands (FDR <0.01, abs(log2FC) >1) of each subset, revealing distinct signaling of NANs versus TANS (Figure 6F). In all TAN subsets, we found VEGFA signaling toward cancer cells, again underlining their important proangiogenic role, as well as SPP1 signaling, which has been associated with an immunosuppressive TME and pro-migratory effects. CD274 (PD-L1) to PDCD1 (PD-1) signaling is significantly upregulated in TAN-2 while being downregulated in NAN-1, proposing CD8+ T cell-inhibitory effects of TAN that accord well with previous observations of impeded immunotherapy responses in neutrophil-rich tumors. Conversely, NANs showed prominent interactions involving genes of the tumor-necrosis family (TNFSF13B, TNFSF10, LTB) that have been previously associated with neutrophil activation.

### DISCUSSION

We built a large-scale atlas of single-cell transcriptomes of NSCLC through integration of 29 datasets spanning over 1,280,000 cells from 556 samples and 318 individuals representing 1.75 billion expression values. Reduction of dataset-specific batch effects due to variation in experimental design and used platforms while retaining biological information resulted in a high-quality reference atlas for NSCLC, offering superior coverage of histological and clinical variables and thereby providing a unique resource for dissecting the cellular diversity in the TME and generating hypotheses. We leveraged the information content of the NSCLC atlas by sequencing additional patient samples using a platform suitable for diagnostic and therapeutic applications.
to depict low-mRNA-content cells, which enabled us to comprehensively characterize the diversity and plasticity of TRNs.

First, we provide a high-resolution view of the TME in NSCLC with 44 major cell types/states and show different cell-type composition patterns in LUAD and LUSC, including more precise functional transcriptomic classification of malignant epithelial cells in both histotypes. The single-cell composition of the TME in NSCLC enabled refined tumor classification and patient stratification into four immune phenotypes: ID, myeloid, B cell, and T cell subtypes. These findings may have important implications for improving cancer immunotherapy in NSCLC. For example, combination therapies that target both myeloid cells and lymphoid cells could represent an immunotherapeutic strategy to treat myeloid subtypes, as shown recently in a melanoma mouse model. Similarly, given the heterogeneity of the intratumoral B cells and the importance of tertiary lymphoid structures, further analysis of the B cell subtype might open promising therapeutic avenues by additional refined B cell targeting.

Second, integration of bulk RNA-seq data from the TCGA NSCLC cohort uncovered cell subsets associated with alterations in major driver genes, such as EGFR, KRAS, STK11, and TP53, in both LUAD and LUSC subtypes, providing further evidence that genetic aberrations in cancer cells dictate the immune contexture of tumors. We validated the findings from the computational analysis using two independent cohorts and immunofluorescence/immunohistochemistry assays, confirming previously published reports on genotype/immunophenotype dependencies. This knowledge could be exploited to derive rationale for personalized therapeutic combination strategies based on the underlying genetic tumor profile.

Third, we provide in-depth characterization of TRNs including both TANs and NANs in human NSCLC. Our dissection of the diversity of TANs suggest that the conflicting reports can be attributed to the different TRN subsets. Of particular interest for cancer immunotherapy is the TAN phenotype with an immunogenic antigen-presenting feature. This observation implies acquisition of antigen-presenting-like properties by neutrophils at the tumor site, as previously reported. Such conversion of neutrophils to antigen-presenting cells may elicit anti-tumor immunity and has recently been shown in a murine model. Identification of targets that can block the transition of the antigen-presenting TAN-2 subset into TAN-1 and TAN-3 or reverse the final phenotypes into TAN-2 phenotype is an important goal for future studies.

Finally, we report that the TRN-derived gene signature has a predictive and prognostic effect of the TRN signals for immunotherapy-treated patients with NSCLC. Using transcriptomic data for patients with NSCLC (n = 439) from two randomized clinical trial cohorts treated with a single anti-PD-L1 antibody (atezolizumab), we provide evidence for the association of TRNs with therapy failure. Although not statistically significant for the chemotherapy arm, the similarity of the graphs for both drugs fits the general paradigm that neutrophil infiltration is associated with worse anti-tumor outcomes.

Beyond these biological insights, the results from this study have also important implications. Specifically, the diversity and plasticity of TRNs shown here further underscore the necessity to reevaluate the rationale for targeting neutrophils to overcome ICI therapy resistance in combination therapies using CXCR1 and CXCR2 antagonists and other inhibitors. As shown here, TANs can acquire antigen-presenting properties, and such conversion of abundant neutrophils to antigen-presenting cells could overcome the limitations of the low abundance of cross-presenting DCs. We advocate that rigorous approaches are required to analyze the impact of the TRN diversity and plasticity on tumor immunity in NSCLC and possibly in other cancers. Our study, however, has several limitations. First, NSCLCs show a great intratumor heterogeneity, and the sampling location (e.g., tumor core versus tumor margin) may affect the cellular composition, particularly the in case of biopsies being compared with tissue pieces from lobe resections. With the exception of one study, all studies incorporated in our atlas applied single regional sampling without annotation of the exact sampling area. Thus, our analyses do not take into account this variable, and we therefore advocate that future studies should include information about the sampling location. Second, in many cases, mRNA is not a definite proof of the extent to which a protein is expressed, and information on both RNA and protein expression is necessary for getting a complete picture of gene regulation and single-cell heterogeneity. In neutrophils, this particularly regards granule protein expression that varies throughout granulopoiesis, not always strictly correlating with mRNA expression. This could partly explain the above-mentioned conflicting literature results of TAN phenotypes. And third, albeit the association of the TRN signature with anti-PD-L1 treatment failure was analyzed using transcriptomic data for patients with NSCLC, prospective studies are required to show that the TRN signature indeed represents a bona fide anti-PD-L1 therapy outcome-predicting marker rather than being a negative prognostic marker.

In conclusion, we provide a NSCLC atlas with single-cell resolution as well as a web portal that enables interactive exploration of the dataset through cell-x-gene (https://luca.icbi.at) that allows visualization of metadata and gene expression. The biological insights we present here and future discoveries arising from the exploitation of the high-resolution NSCLC atlas could provide the basis for developing combination therapies for patients with NSCLC who are not sufficiently responding to immune-checkpoint blockers.

**STAR METHODS**

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          | Antibodies list | See Table S9 |
| Biological samples  | Fresh resections of tumor tissue and adjacent normal lung tissue from NSCLC patients | This paper | N/A |
| Critical commercial assays | BD TuDoR<sup>TM</sup> dissociation reagent | BD Biosciences | Cat#: 661563 |
| BD Pharm Lyse<sup>TM</sup> | BD Biosciences | Cat#: 555899 |
| BD Rhapsody<sup>TM</sup> Cartridge Kit | BD Biosciences | Cat#: 633733 |
| BD Rhapsody<sup>TM</sup> Cartridge Reagent Kit | BD Biosciences | Cat#: 633731 |
| BD<sup>TM</sup> Human Single-Cell Multiplexing Kit | BD Biosciences | Cat#: 633781 |
| BD Rhapsody<sup>TM</sup> WTA Amplification Kit | BD Biosciences | Cat#: 633801 |
| BD Rhapsody<sup>TM</sup> cDNA Kit | BD Biosciences | Cat#: 633773 |
| AMPure XP | Beckman Coulter | Cat#: A63880 |
| Qubit<sup>TM</sup> dsDNA HS Assay Kit | Invitrogen | Cat#: Q32854 |
| High Sensitivity D1000 Reagents | Agilent | Cat#: 5067–5585 |
| High Sensitivity D5000 Reagents | Agilent | Cat#: 5067–5593 |
| High Sensitivity D1000 ScreenTape | Agilent | Cat#: 5067–5584 |
| High Sensitivity D5000 ScreenTape | Agilent | Cat#: 5067–5588 |
| BD Pharmingen<sup>TM</sup> 7-AAD | BD Biosciences | Cat#: 559925 |
| Calcein AM | Invitrogen | Cat#: C1430 |
| Drq7 | BD Biosciences | Cat#: 564904 |
| Opal 7-Color Automated Immunohistochemistry Kit | Akoya Biosciences | Cat#: NEL821001KT |
| BOND Epitope Retrieval 1 | Leica Biosystems | Cat#: AR9961 |
| BOND Epitope Retrieval 2 | Leica Biosystems | Cat#: AR9640 |
| BOND Dewax Solution | Leica Biosystems | Cat#: AR9222 |
| BOND Wash Solution 10x | Leica Biosystems | Cat#: AR9590 |
| Spectral DAPI | Akoya Biosciences | Cat#: FP1490 |
| Prolong Diamond Antifade | Thermo Fisher | Cat#: P36961 |
| BOND Research Detection System | Leica Biosystems | Cat#: DS9455 |
| BOND Titration Kit | Leica Biosystems | Cat#: OPT9049 |
| BD TuDoR<sup>TM</sup> dissociation reagent | BD Biosciences | Cat#: 661563 |
| Spectral DAPI | Akoya Biosciences | Cat#: FP1490 |
| Prolong Diamond Antifade | Thermo Fisher | Cat#: P36961 |
| BOND Research Detection System | Leica Biosystems | Cat#: DS9455 |
| BOND Titration Kit | Leica Biosystems | Cat#: OPT9049 |
| Deposited data | BD Rhapsody dataset (demultiplexed UMI counts) | This study | https://doi.org/10.5281/zenodo.6411867 |
| Processed input data | This study | https://doi.org/10.5281/zenodo.6411867 |
| Results, including intermediate results, core and extended atlas in h5ad format, and scArches model | This study | https://doi.org/10.5281/zenodo.6411867 |
| Core and extended atlas as cell-x-gene instance and h5ad/Seurat v3 files with standardized metadata according to the cell-x-gene schema | This study | https://cellxgene.cziscience.com/collections/edb893ee-4066-4128-9aee-5eb2b038287 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Adams_Kaminski_2020 scRNA-seq dataset (processed) | GEO | GSE136831 |
| Chen_Zhang2020 scRNA-seq dataset (fastq) | SRA | PRJNA634159 |
| Goveia_Carmeliet_2020 (processed) | (Goveia et al., 2020) | https://endotheliomics.shinyapps.io/lung_ectax/ |
| Guo_Zhang_2018 (fastq) | EGA | EGAS00001002430 |
| Habermann_Kropski_2020 (processed) | GEO | GSE135893 |
| He_Fan_2021 (fastq) | NGDC GSA | CRA001963 |
| Kim_Lee_2020 (processed) | GEO | GSE131907 |
| Lambrechts_Thienpont_2018 (fastq) | ArrayExpress | E-MTAB-6149, E-MTAB-6653 |
| Laughney_Massague_2020 (processed) | GEO | GSE123904 |
| Madissoon_Meyer_2020 (processed) | (Madissoon et al., 2019) | https://www.tissuestabilitycellatlas.org/ |
| Maier_Merad_2020 (processed) | (Maier et al., 2020) | https://github.com/effiker/Maier_et_al_nature_2020 |
| Maynard_Bivona_2020 (fastq) | SRA | PRJNA591860 |
| Mayr_Schiller_2020 (processed) | (Mayr et al., 2021) | https://github.com/thelab/2020_Mayr |
| Reyman_Misharin_2018 (processed) | GEO | GSE122960 |
| Travaglini_Krasnow_2020 (processed) | (Travaglini et al., 2020) | https://www.synapse.org/#!Synapse:syn21560406 |
| Vieira_Teichmann_2019 (processed) | GEO | GSE130148 |
| Wu_Zhou_2021 (processed) | GEO | GSE148071 |
| Zillionis_Klein_2019 (processed) | GEO | GSE127465 |
| Leader_Merad_2021 (processed) | GEO | GSE154826 |
| TCGA data RNA-seq and mutation data | GDC | https://portal.gdc.cancer.gov/ |
| TCGA survival data | (Liu et al., 2018) | https://github.com/data2intelligence/CytoSig/ |
| Cytosig signatures | (Efremova et al., 2020; Turei et al., 2016) | Downloaded from https://omnipathdb.org/interactions/?fields=sources,references&genesymbols=1&databases=CellPhoneDB on 2022-04-06 |

**Software and algorithms**

- Seven Bridges - BD Rhapsody™ WTA Analysis Pipeline
- FlowJo™
- Mantra Snap
- inForm Tissue Analysis
- GraphPad Prism
- Cellranger v5.0.0
- nf-core RNA-seq pipeline v3.0
- Nextflow v22.04.5
- Singularity/Aphtainer v3.7.0–1.el7
- Nextflow workflow to reproduce this study

Software packages used for scRNA-seq analysis are packaged as singularity containers and available on zenodo

- Seven Bridges - BD Rhapsody™ WTA Analysis Pipeline
- FlowJo™
- Mantra Snap
- inForm Tissue Analysis

(Continued on next page)
RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zlatko Trajanoski (zlatko.trajanoski@i-med.ac.at)

Materials availability
The study did not generate new unique reagents.

Data and code availability
- Processed scRNA-seq data from this study has been deposited on Zenodo as listed in the key resources table. Raw data is not made available due to privacy concerns.
- Processed scRNA-seq data from other studies has been deposited on Zenodo. The original study identifiers are listed in the key resources table.
- Final and intermediate results of the computational analysis are made available on Zenodo.
- All code to reproduce this study is wrapped into a nextflow workflow and publicly available on Github. All software dependencies are made available as singularity containers. Some of the algorithms employed (scVI, scANVI, UMAP) involve stochastic processes that require specific hardware for exact reproducibility (see key resources table).
- Microscopy data reported in this paper will be shared by the lead contact upon request. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects
Samples of NSCLC tumor tissues and matched adjacent normal lung tissues (more than 5 cm distance to the tumor) were obtained from surgical specimens of patients undergoing resection at the Department of Visceral, Transplant and Thoracic Surgery (VTT), Medical University Innsbruck, Austria, and in collaboration with the INNPATH GmbH, Innsbruck, Austria, after obtaining informed consent in accordance with a protocol reviewed and approved by the Institutional Review Board at the Medical University Innsbruck, Austria (study code: AN214-0293 342/4.5). Demographic details are provided in Table S6.

METHOD DETAILS

Preparation of NSCLC tissue and normal lung tissue
Surgically resected NSCLC tumor tissues and adjacent normal tissues were minced into small pieces (<1 mm) on ice and enzymatically digested with agitation for 30 min at 37 °C using the BD TuDoR dissociation reagent (BD Biosciences). The obtained single-cell solution was sieved through a 70 μm cell strainer (Corning) and red blood cells were removed using the BD Pharm Lyse lysing solution (BD Biosciences). Cells were counted and viability assessed with the BD Rhapsody scRNA-seq platform (BD Biosciences) using Calcein-AM (Invitrogen) and Draq7 (BD Biosciences).

BD Rhapsody library preparation and sequencing
Freshly isolated single-cells were immediately processed with the BD Rhapsody scRNA-seq platform (BD Biosciences). The BD Single-Cell Multiplexing Kit (BD Biosciences) was used to combine and load two samples (tumor tissue and normal adjacent tissue) onto a single BD Rhapsody cartridge (BD Biosciences). Sample-tag staining was performed according to the manufacturer's
protocol (sample-tag staining at room temperature for 20 min and washing by centrifugation at 400 g for 5 min). Single-cell isolation in microfluidics (cell load: 20 min incubation at room temperature) with subsequent cell-lysis and capturing of poly-adenylated mRNA molecules with barcoded, magnetic capture-beads was performed according to the manufacturer’s instructions. Beads were magnetically retrieved from the microfluidics, pooled into a single tube before reverse transcription. Unique molecular identifiers (UMIs) were added to the cDNA molecules during cDNA synthesis. Whole transcriptome amplification (WTA) and sample-tag sequencing libraries were generated according to the BD Rhapsody single-cell whole-transcriptome amplification workflow. The quantity and quality of the sequencing libraries was analyzed with the Qubit dsDNA HS (High Sensitivity) assay kit (Invitrogen) and the 4200 TapeStation (Agilent) system. Libraries were sequenced on the NovaSeq 6000 system (Illumina) targeting a sequencing depth of 50,000 reads/cell.

**Flow cytometry**
Cells isolated from surgically resected NSCLC tumor tissues and adjacent normal tissues were stained with a backbone cocktail of 12 antibodies (CD56, CD3, CD8, CD4, CD45, HLA-DR, CD31, CD14, CD15, CD326, CD19, CD16) which, was complemented either with an additional 8 antibodies (CD28, CD38, CD123, CD34, CD161, CD193, TCRgd, CD90) to define all cell populations, or several mixtures of up to three antibodies (CD54, CD83, CD49b, CD62L, LOX-1, CD181) for a detailed characterization of neutrophils, at pre-titrated concentrations. All source data of the aforementioned antibodies is provided in the key resources table, applied flourochromes are listed in Table S7. After washing and addition of 5 μL 7-AAD, the cells were measured on a FACSymphony A5 flow cytometer (BD Biosciences). Data were analyzed using FlowJo v10.7 software. For details of the gating strategy see Figure S7.

**Multiplex immunofluorescence**
NSCLC tumor and tumor-adjacent tissue samples were fixed in 4% formalin for 6–72 h and embedded in paraffin. Four-micrometer sections were used for immunofluorescence staining. Immunofluorescence staining on formalin-fixed paraffin-embedded (FFPE) tissue was performed using the Opal 7-Color Automated Immunohistochemistry Kit (cat: NEL821001KT, Akoya Biosciences, Menlo Park, USA). A multiplex panel of immune markers was developed with antibodies against: CD16 (clone EPR22409-124, Abcam), CD8 (clone C8/144B, Dako), CD68 (clone PG-M1, Dako), CD3 (polyclonal, Dako), CD20 (clone L26, Dako), cytokeratin (clone AE1/AE3, Dako; clone C-11, Abcam). In additional settings antibodies were used against: CD16 (clone EPR22409-124, Abcam), CXCR2 (clone EPR22301-103, Abcam), OLR1 (polyclonal, Sigma Life Science) (further information given in Table S7). The staining procedure was performed using an automated staining system (BOND-RX, Leica Biosystems). All markers were sequentially applied and paired with respective Opal fluorophores (Table S7). To visualize cell nuclei, the tissue was stained with 4',6-diamidino-2-phenylindole (spectral DAPI, Akoya Biosciences). Stained slides were scanned using Mantra 2 Quantitative Pathology Workstation (Akoya Biosciences) and representative images from each tissue were acquired with the Mantra Snap software v1.0.4. Spectral unmixing, multispectral image analysis and cell phenotyping was carried out using the inForm Tissue Analysis Software v2.4.10 (Akoya Biosciences). In short, DAPI staining was used to segment cells. The perinuclear area (the defined 4-pixel area around nuclei) was therefore defined to be cell cytoplasm. Thereafter, the total cell area was evaluated for nucleic/cytoplasmic/membrane marker expression. The inForm build-in algorithm for cell phenotyping was used to define the intensity threshold for the positivity of each marker individually and each cell was characterized/phenotyped by presence/absence of the marker.

**Immunohistochemistry**
Staining of the validation cohort was approved by the Internal Review Board of the University of Luebeck (file number 16-277). Tissue microarrays (TMA) were constructed from formalin-fixed paraffin-embedded (FFPE) tumor blocks originating from surgical samples. In short, for TMA construction each sample was represented in triplicates of 0.6 mm diameter cores. A tumor sample was incorporated in further analysis if at least one core was evaluable. The validation cohort included 55 chemo-naive LUAD and LUSC, respectively, with no history of previous malignancies or history of receiving chemotherapy or radiotherapy. Immunohistochemistry (IHC) staining was performed according to the manufacturer’s instructions, using the Ventana Discovery (Ventana Medical System) automated staining system. Slides were incubated with a primary antibody against CD4 (CD4 SP35, Ventana, RTU) to detect CD4+ lymphocytes and with a primary antibody against CD68 (KP1, Ventana, RTU) to detect macrophages (Table S7). They were further stained with chloroacetate-esterase (Naphthol-AS-D-chloracetate, Serva) to highlight neutrophils. To evaluate the immune cell infiltration, CD4+ lymphocytes, macrophages and neutrophils were counted in three high power fields (HPFs) per core, meaning that up to nine HPFs per case were assessed. Two experienced pathologists (SP and CK) performed an independent evaluation of the slides.

Staining of CXCR2 to detect neutrophils was performed using the rabbit antibody against CXCR2 (EPR2301-103, Abcam) diluted in a Primary Antibody Diluent Buffer (Primary antibody diluent, Leica Biosystems). The staining protocol included a standard antigen retrieval step with CC1/pH9 buffer (Discovery CC1, Ventana), incubation with the primary antibody for 0.5 h at room temperature (RT). Antibody staining was detected by DAB. Neutrophils were counted in 5 HPFs.

**Generation of the core atlas**
The data for the core atlas was from previously published NSCLC studies,3–8,10,12,15,90,91 our own data (n = 3), and 7 studies for control purpose.91–97 The selected studies were published between July 2018 and May 2021 and the incorporated datasets were generated with six different sequencing platforms, including the most commonly applied 10x Chromium (10x Genomics) as well as
Smart-seq2, GEXSCOPE (Singleron), inDrop and Drop-Seq. We further integrated our own data generated with the microwell-based BD Rhapsody scRNA-seq platform (BD Biosciences). We specifically selected studies using comparable protocols for sample processing and data generation, such as sequencing of whole cells. We did not exclude studies that applied flow cytometry-based cell-sorting prior to sequencing, as these incorporate relevant information on rare cell types. From non-NSCLC studies we exclusively included those parts of the published data that were relevant for our atlas: from the Madissoon dataset we only included lung samples, from Adams, Reyfman, Habermann, Veira Braga, and Mayr datasets we only used the control samples (including normal lung tissue of tumor patients, which we termed “normal_adjacent” or lung tissue of organ donors without history of pulmonary disease). From the Adams et al. dataset we also included data from patients (n = 18) with chronic obstructive pulmonary disease (COPD) as chronic inflammatory pulmonary disease cohort with an increased lung cancer risk.

Preprocessing and quality control of scRNA-seq data
We distinguish between studies (i.e. a scientific publication) and datasets (i.e. scRNA-seq samples that were generated using the same sample preparation and the same experimental platform). Each study may contain one or multiple datasets. Demultiplexed FASTQ files of the UKIM-V datasets were merged and processed using the Seven Bridges Genomics cloud server with the BD Rhapsody WTA Analysis Pipeline. Samples from the studies Chen, Zhang 2020, Guo, Zhang 2018, He, Fan 2021, Lambrechts, Thienpont 2018 and Maynard_Bivona 2020 were obtained as raw fastq files from the identifiers specified in the key resources table. Smart-seq2 data were processed using the nf-core RNA-seq pipeline with the GRCh38 reference genome and GENCODE v33 annotations. 10x datasets were processed with cellranger v5.0.0 (10x Genomics) and the GRCh38-2020-A reference database as provided by 10x Genomics. All other datasets were obtained as count tables from their respective identifiers. All datasets were loaded into AnnData containers with consistent structure. Quality control was performed with scanpy by thresholding the number of detected genes, counts and the fraction of mitochondrial reads. Thresholds were determined per dataset by visual inspection of the distributions and are listed in Table S8.

Integration of scRNA-seq datasets
Individual datasets were merged into a single AnnData object. Since genome annotations partly differed between the datasets, we re-mapped gene identifiers on the latest version of HGNC gene symbols using the https://mygene.info API. In case of duplicate gene symbols, the one with the maximum read count was retained. If gene symbols were missing from a dataset, the values were filled with zeros. Gene symbols that were missing in more than 5 datasets (25%) were excluded altogether.

We integrated the datasets using the scANVI algorithm as it has been demonstrated to be one of the top-performing methods for atlas-level integration and to scale to >1M cells. Since scANVI requires cell-type annotations for at least one of the input datasets, we manually annotated two “seed” datasets based on unsupervised clustering as described below. We chose Lambrechts_Thienpont_2018_6653 and Maynard_Bivona_2020 as seed datasets as they were not experimentally enriched for specific cell-types and were sequenced on two platforms with very different characteristics (10x and Smart-seq2). Raw counts were used as input for scANVI. The Smart-seq2 counts were scaled by the gene length as recommended on the scvi-tools website. The scANVI model was initialized with a pre-trained scVI model as recommended in the scvi-tools tutorial. The scVI model was trained on the 6000 most highly variable genes as determined with scanpy’s `highly_variable_genes` with parameters `flavor="seurat_v3" and batch_key="dataset"`. Each sample was considered as an individual batch for both scVI and scANVI. Other than that the algorithms were run with default parameters.

Doublet-detection
For droplet-based scRNA-seq datasets we ran the SOLO algorithm to computationally detect multiplets. We chose SOLO over other doublet detection methods as it is readily integrated into scvi-tools, and was found to be one of the top-performing methods in an independent benchmark. We used the SOLO implementation from scvi-tools and initialized SOLO with a pre-trained scVI model.

Unsupervised clustering and cell-type annotation
We computed UMAP embeddings and unsupervised Leiden-clustering with scanpy based on a cell-cell neighborhood graph derived from scANVI latent space. Coarse, lineage-specific clusters were iteratively sub-clustered to identify cell-types at a more fine-grained resolution. Cell type clusters were annotated based on previously reported marker genes (Figure S1A). CD8+ T cell subclusters were annotated based on gene sets from Oliveira et al.

Integrating additional datasets
Two datasets, Leader_Merad_2021 and UKIM-V-2, were added after the completion and annotation of the core atlas. The datasets (“query”) were projected onto the atlas (“reference”) using scArches as implemented in scvi-tools. scVI and scANVI models were re-trained on the fully annotated, doublet-filtered core atlas, with the parameters recommended for scArches: `use_layer_norm="both", use_batch_norm="none", encode_covariates=True, dropout_rate=0.2, and n_layers=2`. Gene-symbols of the query datasets were re-mapped as described above and missing gene symbols filled with zeros. For each query dataset, scArches yielded an embedding in the same latent space as the core atlas. Based on the joint latent space, a neighborhood graph and UMAP embedding were computed for the “extended” atlas. Cell-types were annotated automatically, based on a majority vote of nearest
neighbors. To this end, let $C$ be the pairwise weighted connectivity matrix of the scanpy neighborhood graph computed on the scArches embedding. Then, the transitive connectivity matrix $C'$ (i.e., including connections to neighbors of neighbors) is defined as $C' = C \cdot C$ where the dot operator refers to the matrix product. Let further $Q$ be the set of all query cells, $R$ the set of all reference cells, and $T$ the set of all cell-types. Then, for every cell $q \in Q$ the cell-type is determined as

$$
\arg\max_{t \in T} \sum_{r \in R} \sigma(t, r) C_{qr}
$$

where the indicator function $\sigma(t, r)$ is 1 if cell $r$ is of type $t$ and 0 otherwise. The transitive connectivity matrix $C'$ was chosen over $C$ to increase robustness by increasing the number of neighbors, and to ensure that every cell from the query has connection to a cell in the reference.

**Comparing cell-type abundances**

Since comparing cell-type fractions between groups is challenging due to different characteristics of the datasets and the inherent compositional nature of cell-type fractions, we applied the scCODA model, which addresses this issue. We were interested in the differences between conditions (LUAD vs. LUSC). To this end, we ran the scCODA model with the formula $\sim \text{condition} + \text{tumor\_stage} + \text{dataset}$ with 500,000 iterations using “cancer cells” as the reference cell-type, where $\text{tumor\_stage}$ is a binary vector classifying datasets into early (stages I-II) and advanced (stages III-IV), and dataset is a categorical vector encoding the different datasets. For the comparison, we excluded the Guo_Zhang_2018 dataset, which only contains T cells. The final result shows credible effects with a false-discovery-rate (FDR) of 0.1.

**Patient stratification**

We stratified patients into immune phenotypes based on immune cell-type fractions. We selected all patients with primary tumor samples and excluded the Guo_Zhang_2018 dataset, because it contains only T cells. Neutrophil fractions were excluded, since they are not appropriately captured in the majority of datasets. Cell-type fractions of primary tumor samples were loaded into a patient × cell-type AnnData container. Dataset-specific batch-effects were removed using a linear model as implemented in scanpy.pp.regress_out. Patients were clustered using graph-based Leiden clustering with the “correlation” distance metric for computing the neighborhood graph. Patient clusters were labeled according to their predominant cell-types. In addition to the histological subtypes based on the annotation of the original datasets, we annotated tumor types based on the transcriptomics data according to the most abundant cancer cell cluster.

**RNA velocity analysis**

We performed RNA-velocity analysis on the UKIM-V dataset using velocyto.py and scvelo. BAM files as generated by the BD Rhapsody WTA analysis pipeline were preprocessed with samtools to make them compatible with velocyto.py (see preprocessing/bd_rhapsody/velocyto.nf in our git repository for more details). Loom files generated by velocyto.py were loaded into scvelo to estimate and visualize RNA velocities according to the scvelo tutorial. Partition-based graph abstraction (PAGA) was computed based on the RNA velocity graph, using neutrophil subclusters as grouping variable and the option $\text{minimum\_spanning\_tree}=$False. The result was visualized as a graph showing the transition confidences as directed edges.

**Differential gene expression testing**

We used DESeq2 on pseudo-bulk samples for differential expression testing which has been demonstrated to perform well and properly correct for false discoveries. For each cell-type and patient, we summed up transcript counts for each gene. Pseudo-bulk samples consisting of fewer than 10 cells were discarded. We compared primary tumor samples from LUAD vs. LUSC (condition), primary tumor samples from the patient subtypes M/B/T/desert (group), NANs vs. TANs (cell_type_tan_nan), and Neutrophil clusters (NAN1-3, TAN1-4), including the dataset as a covariate. For comparisons between multiple groups, we used contrasts with sum-to-zero coding. p-values were adjusted for multiple hypothesis testing with independent hypothesis weighting (IHW). Scores were aggregated into pseudobulk samples by computing the mean of each variable for each patient and cell-type. Samples consisting of less than 10 cells were discarded. For each variable, we fitted a model with the formulas $\sim \text{condition} + \text{dataset} + \text{tumor\_stage}$ or $\sim \text{group} + \text{dataset} + \text{condition} + \text{tumor\_stage}$, respectively. Coefficients were obtained from the linear model and p-values calculated with the f-test. p-values were adjusted for multiple testing with the Benjamini-Hochberg procedure.
**Cellphoneodb analysis**

We used the cellphoneodb (CPDB) database\(^{123}\) as obtained from omnipathdb\(^{124}\) to investigate differences in cell-to-cell communication in primary tumor samples. The original CPDB algorithm performs statistical comparisons based on a permutation test which is designed to find differences between cell-types. For our study, on the other hand, we were interested in differences between conditions, using patients as biological replicates. Therefore, we followed an approach similar to the `degs_analysis` mode recently added to CPDB v3\(^{126}\). For each cell-type of interest, we considered the list of significantly differentially expressed signaling molecules in CPDB (ligands or receptors, for outgoing and incoming interactions, respectively). For each of these differentially expressed signaling molecules and for each cell-type, we determined interaction partners that are potentially affected by that change, as those that are expressed in at least 10% of the cells in a certain cell-type. Differentially expressed signaling molecules were determined with DESeq2 as described above. The fraction of cells expressing a signaling molecule was computed as the mean of fractions per patient, to avoid biases due to different cell-counts per patient.

**SCISSOR analysis**

We used SCISSOR\(^{126}\) to associate phenotypic data from bulk RNA-seq experiments with our single-cell data. TCGA mutation and gene expression data was obtained from the GDC portal, survival data from.\(^{127}\) SCISSOR was run on primary tumor cells of each patient individually according to the SCISSOR tutorial using mutation data (logistic regression) and overall survival (cox-regression) as dependent variables. A grid search for the alpha-parameter was performed in \(2^{-i/2}\) with \(i \in \{24, 23, \ldots, 2\}\) and a cutoff parameter of 0.3. 21 of 176 samples with low overall cell count failed during SCISSOR’s Seurat-preprocessing step and were excluded from the subsequent analysis. For each patient and cell-type, we computed the fraction of `scissor + cells` (i.e., positively associated with a mutation or worse survival), `scissor- cells` (i.e., negatively associated), and `neutral cells` and added a pseudo-count of 0.01. A sample was excluded from a cell-type if it contributed \(< 10\) cells. For each cell-type, we computed the log2-ratio of `scissor +` and `scissor-` cells as the mean fraction of `scissor + cells` vs. the mean fraction of `scissor-` cells. Significant differences were determined by comparing the fractions of `scissor +` and `scissor-` cells with a paired wilcoxon test with `zero_method="psplit"` as implemented in the scipy package. p-values were Benjamini-Hochberg-adjusted and considered significant at an FDR <0.01.

**TRN clusters**

For an unbiased discovery of TRN subtypes, we performed unsupervised clustering of all cells annotated as neutrophils. The neighborhood graph was computed with `scanpy.pp.neighbors` with \(n_{neighbors}=30\) based on the scANVI latent space. Clusters were determined with `scanpy.tl.leiden` with \(resolution=0.75\). Two subclusters dominated by cells from normal adjacent tissue were labeled normal-associated neutrophils (NAN) 1, 2, and 3, whereas four subclusters of cells from primary tumor samples were labeled tumor-associated neutrophils (TAN) 1, 2, 3 and 4.

**TRN signatures**

Gene signatures for TRN and TRN clusters were determined based on fold-change (FC), specific fold-change (sFC), and area under the receiver operator characteristics curve (AUROC), applying an approach previously used to find cell-type specific marker genes.\(^{127}\) We have previously shown the resulting gene signatures to be highly specific for their respective cell-types.\(^{128}\) To avoid marker genes being biased towards samples contributing more cells than on average we aggregated single cells to pseudo-bulk samples\(^{129}\) by patient before deriving marker genes. For each set of marker genes derived, pseudo-bulk samples were generated by summing up raw counts for each patient and cell-type of interest. The resulting samples were normalized to counts per million (CPM) and log2-transformed with \(\log_{2}(adata\_tl\_score\_genes\_tl)\). Pseudo-bulk samples consisting of fewer than 10 cells were discarded. For each gene and cell-type, FC and sFC were computed as described in\(^{127}\). AUROC was computed using `roc_auc_score` as implemented in scikit-learn. For identifying marker genes for the 7 neutrophil subclusters, we applied a permissive cutoff of sFC >1 and FC > 1.5 and ranked genes by AUROC. For the TAN and NAN signature used to compute signature scores in bulk RNA-seq data, we empirically determined optimal cut-offs by grid search and cross-validation: First, the single-cell input data were randomly split by patients into 80% training data and 20% independent test set. On the training data, five-fold cross validation was performed. On the training set of each fold, metrics were computed as described above and all possible combinations of sFC \(\in \{0.5, 0.6, \ldots, 2.9\}\), FC \(\in \{0.5, 0.6, \ldots, 2.9\}\) and AUROC \(\in \{0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 0.96, 0.97\}\) were tested, resulting in a total of 5,000 possible signatures. On the test set of each fold, a pseudo-bulk sample per patient (mixing all cell-types) was generated and the true fraction of the cell-type of interest calculated. The quality of each signature was evaluated as the Pearson correlation between the signature score (see section “Signature scoring in bulk RNA-seq samples” below) and the true cell-type fraction. The cut-off with the highest average correlation across the five folds was chosen as optimal. Finally, the signature was re-calculated on the entire training set using the optimal cut-off, and a final Pearson correlation determined on the independent test set. We defined a TRN signature to capture Neutrophils independent of their subtype as the union of the TAN and the NAN signature genes.

**Signature scoring in bulk RNA-seq samples**

Signature scores in scRNA-seq data were computed using `scanpy.tl.score_genes`. Bulk RNA-seq primary tumor samples of TCGA LUAD and LUSC were retrieved as TPM from the GDC portal. Bulk RNA-seq samples from NSCLC patients treated with atezolizumab (anti-PD-L1) or docetaxel (chemotherapy) from the POPLAR\(^{23}\) and OAK\(^{20}\) trials were retrieved using the accession numbers reported in.\(^{81}\) Similar to an approach previously described,\(^{11}\) enrichment scores for our TRN signatures were calculated.
as follows: For all signature genes, z-scores were computed across all samples from a dataset. The final signature score was defined as the mean of the z-scores of the signature genes for each sample. Associations of the TRN signature with response to immunotherapy or chemotherapy in the POPLAR and OAK datasets was tested using logistic regression in R with the formula \textit{response} \sim \textit{signature\_score} + \textit{tumor\_type} + \textit{dataset}, where \textit{tumor\_type} represents LUAD and LUSC encoded as a binary vector.

**Survival analysis**

Survival analysis was performed using CoxPH-regression as implemented in the R package \textit{survival}. Kaplan-Meyer plots were created using the R package \textit{survminer}, showing the top 25% vs. bottom 25% of samples stratified by signature score. B cell fractions in TCGA samples were estimated using EPIC\textsuperscript{129} as implemented in \textit{immunedecov}, as we have previously shown EPIC to be one of the best performing methods on B cells.\textsuperscript{129} Cox-regression was performed on B cell fractions (TCGA data) with the formula \textit{survival} \sim \textit{signature\_score} + \textit{ajcc\_stage} + \textit{age}, where \textit{ajcc\_stage} is a categorical vector with tumor stages I-IV. For neutrophil fractions (POPLAR + OAK data) the formula \textit{survival} \sim \textit{signature\_score} + \textit{dataset} + \textit{treatment} was used. For comparisons comprising the entire NSCLC cohort (i.e. both LUAD and LUSC), \textit{tumor\_type} was included as an additional covariate.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was performed using the statsmodels library in Python (scRNA-seq data) or GraphPad Prism (flow cytometry and imaging data) using a linear model, t-test or wilcoxon test as appropriate. Single cell-data were aggregated into pseudobulk samples by biological replicates. Compositional analysis of cell-type fractions was performed using scCODA; survival analysis using CoxPH regression in R. P-values for untargeted analyses (DE genes, TFs, or pathways) were FDR-adjusted. Significance levels and more details on the statistical tests are indicated in the figure captions.

**ADDITIONAL RESOURCES**

The single-cell atlas can be assessed via cell-x-gene (https://luca.icbi.at), a web-based viewer for single-cell datasets that allows visualization of metadata and gene expression.
Supplemental information

High-resolution single-cell atlas reveals diversity and plasticity of tissue-resident neutrophils in non-small cell lung cancer

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Figure S1: related to Figure 1. Composition of the NSCLC single-cell atlas.

(A) Dotplot of cell type marker genes used for cell-type annotation.

(B) Fractions of cell types, sample origins and conditions per study (extended atlas).

(C) Relative cell type proportions by tissue origin in the core atlas. The depicted fractions are the average of all patients, independent of their cell-count.

(D) Marker genes for cancer cell classification.

(E) Cell type fractions in the extended atlas.

(F) Mean neutrophil fraction per sequencing platform across all datasets.

(G) Flow-cytometry of neutrophils (shown as percentage of leucocytes) in tumor tissue and patient-matched normal-adjacent tissue (n=63; Paired Wilcoxon test, **p<0.01). The horizontal line represents the median, whiskers extend to the inter-quartile range.

(H) Number of reads (Smart-seq2) or UMIs (other platforms) in epithelial cells by sequencing platform. The central line denotes the median, boxes represent the interquartile range (IQR) and whiskers extend to the most extreme values within 1.5 * IQR. Points outside 1.5 * IQR are shown as outliers.

(I) UMAP of the extended atlas, colored by the medium resolution cell-type annotation used for most data analyses.

(J) UMAP of CD8$^+$ T cell subclusters according to gene signatures published by Oliveira et al.\textsuperscript{22}.

(K) Cell type composition by tumor stage (early, late) calculated with scCODA (Bayesian model for differential composition analysis) using cancer cells as reference cell-type (assumed to be constant between conditions), including tumor type (LUAD, LUSC) as a covariate and running 500,000 Markov-chain monte carlo iterations. FDR=0.1.
Table S2: related to Figure 1. Patient number per cell type.

| Cell type                          | Number of patients with $\geq 5$ | $\geq 10$ | $\geq 30$ |
|------------------------------------|-----------------------------------|-----------|-----------|
| Alveolar cell type 1               | 133                               | 113       | 69        |
| Alveolar cell type 2               | 172                               | 161       | 122       |
| B cell                             | 226                               | 196       | 164       |
| B cell dividing                    | 26                                | 16        | 6         |
| cDC1                               | 131                               | 99        | 32        |
| cDC2                               | 256                               | 231       | 172       |
| Ciliated                           | 175                               | 140       | 87        |
| Club                               | 101                               | 54        | 21        |
| DC mature                          | 133                               | 79        | 21        |
| Endothelial cell arterial          | 108                               | 65        | 33        |
| Endothelial cell capillary         | 118                               | 93        | 54        |
| Endothelial cell lymphatic         | 129                               | 90        | 37        |
| Endothelial cell venous            | 207                               | 171       | 96        |
| Fibroblast adventitial             | 123                               | 80        | 33        |
| Fibroblast alveolar                | 107                               | 75        | 38        |
| Fibroblast peribronchial           | 115                               | 78        | 38        |
| Macrophage                         | 291                               | 273       | 242       |
| Macrophage alveolar                | 228                               | 201       | 177       |
| Mast cell                          | 205                               | 178       | 120       |
| Mesothelial                        | 31                                | 18        | 2         |
| Monocyte classical                 | 276                               | 257       | 222       |
| Monocyte non-classical             | 149                               | 120       | 74        |
| Myeloid dividing                   | 195                               | 153       | 76        |
| Neutrophils                        | 60                                | 48        | 35        |
| NK cell                            | 238                               | 219       | 186       |
| NK cell dividing                   | 71                                | 28        | 3         |
| pDC                                | 151                               | 113       | 55        |
| Pericyte                           | 83                                | 52        | 22        |
| Plasma cell                        | 218                               | 186       | 131       |
| Plasma cell dividing               | 44                                | 26        | 6         |
| ROS1+ healthy epithelial           | 38                                | 29        | 16        |
| Smooth muscle cell                 | 85                                | 48        | 25        |
| Stromal dividing                   | 14                                | 9         | 3         |
| T cell CD4                         | 269                               | 253       | 209       |
| T cell CD4 dividing                | 141                               | 99        | 32        |
| T cell CD8 activated               | 144                               | 122       | 76        |
| T cell CD8 dividing                | 109                               | 71        | 27        |
| T cell CD8 effector memory         | 252                               | 223       | 193       |
| T cell CD8 naive                   | 183                               | 156       | 115       |
| T cell CD8 terminally exhausted    | 166                               | 131       | 89        |
| T cell NK-like                     | 192                               | 163       | 125       |
| T cell regulatory                  | 216                               | 192       | 150       |
| Transitional club/AT2              | 193                               | 159       | 106       |
| Cell type                      | 5   | 10  | 30  |
|-------------------------------|-----|-----|-----|
| Tumor cells LUAD              | 225 | 192 | 136 |
| Tumor cells LUAD EMT          | 43  | 26  | 13  |
| Tumor cells LUAD mitotic      | 98  | 64  | 23  |
| Tumor cells LUAD MSLN         | 6   | 3   | 1   |
| Tumor cells LUAD NE           | 6   | 3   | 3   |
| Tumor cells LUSC              | 86  | 62  | 36  |
| Tumor cells LUSC mitotic      | 79  | 60  | 32  |
| Tumor cells NSCLC mixed       | 24  | 19  | 12  |
Table S3: related to Figure 1. Sample disposition.

| Figure | Dataset | Sample origin |
|--------|---------|---------------|
| 1A     | overview|               |
| 1B     | core atlas | all          |
| 1C     | extended atlas | all        |
| 1D     | UKIM-V | all          |
| 1E     | extended atlas | all        |
| 1F     | extended atlas | primary tumor |
| 1G     | independent Lübeck cohort |          |
| 2A     | extended atlas | primary tumor |
| 2B     | extended atlas (cancer cells) | primary tumor |
| 2C     | extended atlas (cancer cells) | primary tumor |
| 3A     | extended atlas | primary tumor |
| 3B     | extended atlas | primary tumor |
| 4A-E   | extended atlas | primary tumor |
| 4F     | TCGA LUAD/LUSC |               |
| 4G     | extended atlas (CD8+ T cells) | primary tumor |
| 5A-B   | extended atlas (neutrophils) | all        |
| 5C     | independent FACS cohort |           |
| 5D     | extended atlas (neutrophils) | all        |
| 5E     | extended atlas | primary tumor |
| 5F     | extended atlas (neutrophils) | all        |
| 5G     | independent FACS cohort |           |
| 5H     | representative imaging of UKIM-V patient |         |
| 6A-B   | extended atlas (neutrophils) | all        |
| 6C     | independent FACS cohort |           |
| 6D-E   | UKIM-V (neutrophils) | all        |
| 6F     | ligands: extended atlas (neutrophils); receptors: extended atlas | ligands: all; receptors: primary tumor |
| 6G     | extended atlas | all          |
| 6H     | extended atlas (neutrophils) | all        |
| 6I-K   | POPLAR/OAK cohort |            |
| S1A    | core atlas | all          |
| S1B    | extended atlas | all        |
| S1C    | extended atlas | primary tumor + adjacent normal |
| S1D    | core atlas (cancer cells) | primary tumor |
| S1E    | extended atlas | all          |
| S1F    | extended atlas | all          |
| S1G    | independent FACS cohort |            |
| S1H    | extended atlas | all          |
| S1I    | extended atlas | all          |
| S1J    | extended atlas (CD8+ T cells) | all        |
| S1K    | extended atlas | primary tumor |
| S2A    | independent Lübeck cohort |            |
| Figure | Dataset | Sample origin |
|--------|---------|---------------|
| S2B-C  | independent FACS cohort | |
| S2D    | extended atlas | primary tumor |
| S2E    | extended atlas | primary tumor |
| S3A    | extended atlas | primary tumor |
| S4A-B  | extended atlas | primary tumor |
| S4C    | independent IF cohort | |
| S4E-F  | extended atlas | primary tumor |
| S4G-H  | TCGA LUAD/LUSC | |
| S5A    | extended atlas (neutrophils) | primary tumor |
| S5B    | extended atlas (neutrophils) | all |
| S5C    | representative imaging of UKIM-V patient | |
| S6A-E  | extended atlas (neutrophils) | all |
| S6F    | independent FACS cohort | |
| S6G-K  | POPLAR/OAK cohort | |
| S7A    | gating strategy | |
Differential TFs (tumor cells)

| Group | Coef | FDR |
|-------|------|-----|
| B     | 0.2  | <0.001 |
| M     | 0.0  | <0.01  |
| T     | -0.2 | <0.1   |
| desert |     |       |

immune_infiltration

LUAD | LUSC | NSCLC NOS

| Immune Infiltration | LUAD | LUSC | NSCLC NOS |
|--------------------|------|------|-----------|
| B                  |      |      |           |
| M                  |      |      |           |
| T                  |      |      |           |
| desert             |      |      |           |

T/M log2(ratio)
Figure S2: related to Figure 2. Immune phenotypes in histological NSCLC subtypes.

(A) Flow cytometry analysis of CD4$^+$ lymphocytes and macrophages as percentage of all cells in LUAD (n=9) versus LUSC (n=6) tumor samples. The horizontal line represents the median, whiskers extend to the inter-quartile range (Wilcoxon test, **$p<0.01$).

(B) Flow cytometry analysis of the T cell-to myeloid cell ratio (T/M ratio) in tumor tissue (T subtype n=6, M subtype n=4). The horizontal line represents the median, whiskers extend to the inter-quartile range (Wilcoxon test, *$p<0.05$).

(C) Fractions of immune phenotypes in histological subtypes.

(D) Differential of DoRothEA transcription factor signatures in cancer cells between the four immune phenotypes. Heatmap colors indicate the deviation from the overall mean, independent of tumor histology and stage. White dots indicate significant interactions at different false-discovery-rate (FDR) thresholds. P-values have been calculated using a linear model f-test. Only transcription factors with an FDR < 0.1 in at least one patient group are shown.
Figure S3: related to Figure 3. Tumor-immune crosstalk in LUAD vs. LUSC.

(A) Upper panel: top 30 differentially expressed ligands in LUAD vs. LUSC (DESeq2 on pseudo-bulk, FDR < 0.01). Heatmap colors indicate log2 fold changes clipped at ±5, where blue indicates upregulation in LUSC and red indicates upregulation in LUAD. Bottom panel: Respective receptors and the expression by cell type. Dot sizes and colors refers to the fraction of cells expressing the receptor and gene expression, respectively, averaged over all patients. Dots are only shown for receptors that are expressed in at least 10% of the respective cell-types.
Figure S4: related to Figure 4. Association of cellular composition and distinct genotypes and survival in the TCGA data,
(A-B) SCISSOR analysis showing the association of cellular composition and TP53 mutation in LUAD and LUSC derived from the TCGA reference dataset.
(C) Staining of tumor tissue from LUAD patients with KRAS (n=7), EGFR (n=6) or TP53 (n=5) mutation compared to LUAD tumor tissue negative for the respective mutation (n=12). Multiplex immunofluorescence was performed to detect CD4$^+$ T cells, CD8$^+$ T cells, CD20$^+$ B cells, or CD68$^+$ macrophages, respectively. Positive stained cells per 1000 cells are given. CXCR2 expression was analyzed by immunohistochemistry and quantified per high power field in patients with EGFR mutation compared to EGFR wt patients. Analyses related to the corresponding SCISSOR analyses are shown. The horizontal line represents the median, whiskers extend to the inter-quartile range (Wilcoxon test, ***p<0.001).
(D) Immunohistochemistry staining of neutrophils (ASD$^+$ cells) quantified per high power field in patients with EGFR mutation (n=11) compared to EGFR wt patients (n=26). The horizontal line represents the median, whiskers extend to the inter-quartile range (Wilcoxon test, *p<0.05).
(E-F) Association of cellular composition with overall survival for LUSC and LUAD patients.
(G-H) Kaplan-Meyer plot of LUAD and LUSC patients with high (top 25%) and low (bottom 25%) B cell fractions of TCGA lung cancer patients as determined by deconvolution with EPIC. P-value has been determined using CoxPH-regression using tumor stage and age as covariates.
Figure S5: related to Figure 5. Characterization of tissue-resident neutrophils.

(A) Neutrophil fraction in LUSC vs. LUAD (extended atlas). P-value derived using linear model f-test including dataset as a covariate.

(B) Expression of top 30 marker genes (AUROC > 0.75) for NANs and TANs. Every dot is the log2 fold change on a single patient. Bars show the average log2-fold change.

(C) Multiplex immunofluorescence co-staining of CXCR2 (red), LOX-1 (yellow) and pan-cytokeratin (blue) in LUSC tumor tissue. Scale bar = 100 µm.
Figure S6: related to Figure 6. Tissue-resident neutrophil subtypes in NSCLC.

(A) TRN subclusters by the contributing datasets. Left column shows the number of patients with >10 neutrophils in the respective study, the heatmap depicts the number of cells per neutrophil cluster. Data of 43 patients with each > 10 neutrophils.

(B) TAN/NAN candidate marker gene expression by neutrophil subclusters. Expression values are the mean across pseudobulk samples by patient.

(C) NAN and TAN fractions in LUAD versus LUSC. Each dot refers to a patient with at least 10 neutrophils. P-values are derived from a t-test and not adjusted for multiple testing. In all boxplots, the central line denotes the median. Boxes represent the interquartile range (IQR) of the data, whiskers extend to the most extreme data points within 1.5 times the IQR.

(D) Neutrophils subcluster fractions in LUAD versus LUSC. Each dot refers to a patient with at least 10 neutrophils. P-values are derived from a t-test and not adjusted for multiple testing. In all boxplots, the central line denotes the median. Boxes represent the interquartile range (IQR) of the data, whiskers extend to the most extreme data points within 1.5 times the IQR.

(E) Flow cytometry analysis demonstrating the correlation between HLA-DR expression and CD83, LOX-1, CD181, CD62L or CD16 expression, respectively. Representative analysis of neutrophils derived from NSCLC normal-adjacent tissue (blue) and tumor tissue (red) are shown.

(F) Kaplan-Meyer plot of LUSC patients form the POPLAR (Fehrenbacher et al., 2016) and OAK (Rittmeyer et al., 2017) cohorts treated with atezolizumab with high (top 25%) and low (bottom 25%) TRN signature score. P-value has been determined using CoxPH-regression.

(G) Kaplan-Meyer plot of LUAD patients form the POPLAR (Fehrenbacher et al., 2016) and OAK (Rittmeyer et al., 2017) cohorts treated with atezolizumab with high (top 25%) and low (bottom 25%) TRN signature score. P-value has been determined using CoxPH-regression.

(H) Kaplan-Meyer plot comparing patients treated with atezolizumab with high (top 25%) and low (bottom 25%) NAN signature scores. P-value has been determined using CoxPH-regression including cohort and histology as covariates.

(I) Kaplan-Meyer plot comparing patients treated with atezolizumab with high (top 25%) and low (bottom 25%) TAN signature scores. P-value has been determined using CoxPH-regression including cohort and histology as covariates.

(J) Predictive value of cell-type signatures in bulk RNA-seq data from the OAK and POPLAR cohorts of NSCLC patients treated with atezolizumab (anti-PD-L1). The bar charts show cell-type signatures that are associated with worse (log hazard ratio > 0) or better (log hazard ratio < 0) survival at an FDR < 0.1. The hazard ratio and p-values have been determined using CoxPH regression including cohort and histology as covariates.
Table S4: related to Figure 6. Genes signatures.

| TRN signature (n=38) | NAN signature (n=20) | TAN signature (n=18) |
|---------------------|----------------------|----------------------|
| AGO4                | AGO4                 | CCR3                 |
| ARG1                | ARG1                 | CCRL2                |
| CCR3                | CYP4F3               | DDIT3                |
| CCRL2               | ERGIC1               | FLOT1                |
| CYP4F3              | FLOT2                | HIF1A                |
| DDIT3               | FRAT2                | IRAK2                |
| ERGIC1              | LRP10                | MAFF                 |
| FLOT1               | MGAM                 | MAP1LC3B2            |
| FLOT2               | MMP25                | MCOLN1               |
| FRAT2               | MSRB1                | NBN                  |
| HIF1A               | NDEL1                | NOD2                 |
| IRAK2               | NFE2                 | PI3                  |
| LRP10               | PAD14                | PLAU                 |
| MAFF                | PBX2                 | PPIF                 |
| MAP1LC3B2           | PHOSPHO1             | TGM3                 |
| MCOLN1              | RASGRP4              | TOM1                 |
| MGAM                | REPS2                | UBR5-AS1             |
| MMP25               | SULT1B1              | ZNF267               |
| MSRB1               | TSEN34               |                      |
| NBN                 | XKR8                 |                      |
| NDEL1               |                      |                      |
| NFE2                |                      |                      |
| NOD2                |                      |                      |
| PAD14               |                      |                      |
| PBX2                |                      |                      |
| PHOSPHO1            |                      |                      |
| PI3                 |                      |                      |
| PLAU                |                      |                      |
| PPIF                |                      |                      |
| RASGRP4             |                      |                      |
| REPS2               |                      |                      |
| SULT1B1             |                      |                      |
| TGM3                |                      |                      |
| TOM1                |                      |                      |
| TSEN34              |                      |                      |
| UBR5-AS1            |                      |                      |
| XKR8                |                      |                      |
| ZNF267              |                      |                      |
Figure S7 related to STAR Methods. Flow cytometry gating strategy to define cell populations from NSCLC tumor tissue and normal adjacent tissue.

(A) In initial cleaning steps dead cells, debris and doublets were removed using 7-AAD staining and scatter characteristics. Leukocytes were defined by CD45 staining and sequentially gated into subtypes including neutrophils, monocytes, T cells and B cells. Non-CD45⁺ cells were gated into epithelial cells, endothelial cells and fibroblasts.
### Table S7: related to STAR Methods. Overview of antibody information, multispectral imaging, and immunohistochemistry.

**Antibody cocktails used for flow cytometry:**

| Backbone | Cell definition | Neutrophil Characterization |
|----------|-----------------|----------------------------|
| **Antigen** | **Fluorochrome** | **Antigen** | **Fluorochrome** | **Antigen** | **Fluorochrome** |
| CD56     | BUV395          | CD28       | BUV615P          | CD54        | FITC            |
| CD3      | BUV496          | CD38       | BV421            | CD83        | FITC            |
| CD8      | BUV563          | CD123      | BV650            | CD49b       | PE              |
| CD4      | BUV737          | CD34       | FITC             | CD62L       | PE              |
| CD45     | BUV805          | CD161      | PE               | CD181       | APC             |
| HLA-DR   | BV480           | CD193      | PE-CF594         | CD181       | APC             |
| CD31     | BV605           | TCRgd      | PE-Cy5           |             |                 |
| CD14     | BV711           | CD90       | APC              |             |                 |
| CD15     | BV786           |            |                  |             |                 |
| CD326    | PE-Cy7          |            |                  |             |                 |
| CD19     | APC-R700        |            |                  |             |                 |
| CD16     | APC-eF780       |            |                  |             |                 |

**Antibody-antigen retrieval and opal fluorophore pairing related to multispectral imaging:**

| Antibody | pH (AR) | Opal Pairing | Clone | Provider  | Dilution |
|----------|---------|--------------|-------|-----------|----------|
| CXCR2    | 9       | 570          | EPR22301-103 | Abcam    | 1:500    |
| LOX-1    | 9       | 540          | polyclonal | Sigma-Aldrich | 1:200   |
| CD16     | 9       | 650          | EPR22409-124 | Abcam    | 1:600    |
| CD8      | 9       | 570          | C8'144B   | Dako/Agilent | 1:200    |
| CD3      | 6       | 620          | polyclonal | Dako/Agilent | 1:250    |
| CD68     | 9       | 650          | PG-M1     | Dako/Agilent | 1:200    |
| CD20     | 6       | 540          | L26       | Dako/Agilent | 1:200    |
| DAPI     | 7.4     | 450          | -         | Akoya Biosciences | 1:15  |
| Cytokeratin | 9     | 450          | AE1/AE3   | Dako/Agilent | 1:500    |
| Cytokeratin | 9     | 690          | C-11      | Abcam     | 1:1000   |

**Overview of antibodies used for immunohistochemistry:**

| Antibody | Clone  | Provider  | Dilution  |
|----------|--------|-----------|-----------|
| CD4      | SP35   | Ventana   | pre-diluted |
| CD68     | KP1    | Ventana   | pre-diluted |
| CXCR2    | EPR22301-103 | Abcam   | 1:500      |

### Table S8: related to STAR methods. Quality control thresholds related to datasets integrated into the NSCLC single-cell atlas.

| Dataset              | min counts | max counts | min genes | max genes | max pct_mito |
|----------------------|------------|------------|-----------|-----------|--------------|
| Adams_Kaminski_2020_COPD | 1000       | 35000      | 500       | 10000     | 20           |
| Chen_Zhang_2020:NSCLC | 600        | 30000      | 250       | 10000     | 20           |
| Study                                 | Cell Lines | Sequencing Data |
|--------------------------------------|------------|-----------------|
| Goveia_Carmeliet_2020_NSCLC          | 600        | 30000           |
| Guo_Zhang_2018_NSCLC                 | 20000      | 3000000         |
| Habermann_Kropski_2020_pulmonary-fibrosis | 600      | 30000           |
| Kim_Lee_2020_LUAD                    | 1000       | 35000           |
| He_Fan_2021_LUAD                     | 600        | 30000           |
| Lambrechts_2018_LUAD_6149v1          | 600        | 30000           |
| Lambrechts_2018_LUAD_6149v2          | 600        | 30000           |
| Lambrechts_2018_LUAD_6653            | 1200       | 40000           |
| Laughney_Massague_2020_NSCLC         | 1800       | 40000           |
| Madissoon_Meyer_2020_pulmonary-fibrosis | 600  | 30000           |
| Maier_Merad_2020_NSCLC               | 1000       | 30000           |
| Maynard_Bivona_2020_NSCLC            | 20000      | 20000000        |
| Mayr_Schiller_2020_pulmonary-fibrosis | 600      | 30000           |
| Reyfman_Misharin_2018_pulmonary-fibrosis | 1000  | 30000           |
| Travaglini_Krasnow_2020_Lung_10x     | 1000       | 30000           |
| Travaglini_Krasnow_2020_Lung_SS2     | 20000      | 60000000        |
| UKIM-V                               | 2000       | 100000          |
| Vieira_Teichmann_2019_asthma         | 600        | 30000           |
| Wu_Zhou_2021_NSCLC                   | 600        | 30000           |
| Zilionis_Klein_2019_NSCLC            | 600        | 30000           |
| UKIM-V-2                             | 1000       | 60000           |
| Leader_Merad_2021_10x_3p_v1_sort     | 600        | 30000           |
| Leader_Merad_2021_10x_3p_v2_beads    | 600        | 30000           |
| Leader_Merad_2021_10x_3p_v2_beads_cite | 1000  | 30000           |
| Leader_Merad_2021_10x_3p_v2Digest-deadcell_cite | 1000  | 30000           |
| Leader_Merad_2021_10x_3p_v2_sort     | 600        | 30000           |
| Leader_Merad_2021_10x_3p_v3_beads    | 600        | 30000           |
| Leader_Merad_2021_10x_5p_v1_beads    | 1100       | 30000           |
| Leader_Merad_2021_10x_5p_v1_CD2      | 1100       | 30000           |