Tumor-infiltrating NK cell subsets associated with the magnitude of T cell response in human lung cancer

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1 **Tumor-infiltrating NK cell subsets associated with the magnitude of T cell response in human lung**
2 **cancer**

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25 **ABSTRACT**

26 NK cells are the predominant innate lymphocyte subset that contribute to anti-tumor immunity. The
27 molecular profile of human tumor-infiltrating NK cells, and particularly, their characteristics in relation to CD8⁺
28 cytotoxic T lymphocyte (CTL) density within tumors is poorly understood. Here, we performed single-cell and
29 bulk transcriptomic analysis of NK cells within tumor and adjacent normal lung tissue from patients with
30 treatment-naïve lung cancer. We found divergent NK cell subsets with distinct functional features that were
31 associated with the magnitude of CD8⁺ CTL response in tumors. CD16^{pos} NK cells present in tumors with high
32 density of CD8⁺ CTLs were enriched for transcripts linked to cytotoxicity, pro-inflammatory chemokines and
33 effector function. CD16^{neg} NK cells predominantly infiltrated tumors relative to lung tissue and harbored sub-
34 populations with unique gene expression programs. Notably, tumors with high density of CD8⁺ CTLs were
35 enriched for a CD16^{neg} NK cell subset with features of tissue residency and NK cell memory as well as a
36 CD16^{neg} ILC3-like subset that displayed transcriptional features linked to activation, NFκB signaling, dendritic
37 cell recruitment and lymphoid-tissue inducing properties. These data reveal that a potentially synergistic NK
38 cell innate immune program may co-operate with CD8⁺ CTLs to mediate tumor clearance in tumors with a
39 high tumor-infiltrating lymphocyte (TIL) density.

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52 INTRODUCTION

53 Cancer immunotherapies to potentiate CD8⁺ cytotoxic T lymphocyte (CTL) responses have been
54 integrated as a mainstream treatment modality; however clinical responses occur in only a proportion of
55 patients¹⁻³. Loss of tumor HLA-I expression⁴⁻⁶, lack of neoantigens⁷ and immunoediting-driven emergence of
56 antigen-escape variants⁸ are frequent mechanisms of tumor immune evasion from T cell control. Natural killer
57 (NK) cells, the innate counterpart of CTLs, play a crucial role in this context, owing to their distinct ability to
58 recognize abnormally-expressed stress-induced ligands on cells that have lost MHC class I⁹⁻¹¹. NK cells are
59 cytotoxic innate lymphoid cells (ILCs) that mediate frontline defense against viruses and tumors¹²⁻¹⁴. NK cells
60 express a wide repertoire of germline-encoded activating and inhibitory receptors that generate diversity and
61 maintain tolerance¹⁵. A highly calibrated integration of the signals arising from these receptors enables NK
62 cells to kill target cells expressing low or absent MHC-I while maintaining their tolerance toward self-MHC-
63 expressing cells¹⁶. NK cells possess the intrinsic capacity to kill tumor, infected or stressed cells without prior
64 priming. NK cells also mediate antibody-dependent cellular cytotoxicity (ADCC) following binding of their low-
65 affinity activating receptor FcγRIIIa (CD16) to the Fc portion of immunoglobulin G1 (IgG1)¹⁷. In addition to
66 direct cytotoxicity, NK cells produce pro-inflammatory cytokines and chemokines that not only contribute to
67 NK cell effector function but also promote recruitment and maturation of cross-presenting dendritic cells¹⁸⁻²⁰
68 (DCs) and enhance adaptive immune responses mediated by both CD4⁺ T helper and CD8⁺ T cells²¹⁻²³.

69 NK cell activity has been inversely correlated with cancer incidence, and in several cancers, NK cell
70 infiltration of tumors has been associated with better patient outcomes²⁴⁻²⁹. NK cell anti-tumor responses have
71 been predominantly studied using murine tumor models which have formed the basis for development of NK
72 cell-directed immunotherapies. Multiple NK cell-targeting approaches such as inhibitory receptor blockade,
73 cytokine-driven activation, and adoptive cellular therapies, are being investigated but significant progress in
74 this arena has been hampered by limited understanding of NK cell properties within human tumors³⁰⁻³². In
75 addition to substantial inter-species differences between murine and human NK cells, recent studies have
76 also highlighted the presence of tissue-specific and context-specific differences³³. Transcriptomic studies of
77 NK cells from cancer patients have analyzed cells in peripheral blood or metastatic sites³⁴. However, the
78 precise state of NK cell activation, maturation and function within primary tumors, is poorly understood. NK

cell tumor-specific programs and importantly, how NK cell responses are coordinated with T cell responses, remain unknown. In order to fully characterize the NK cell molecular profile at the tumor site, we have taken an unbiased approach to define the global transcriptional program of purified NK cells from well-characterized cohorts of patients with non-small cell lung cancer (NSCLC).

83

84 RESULTS

85 NK cell activation- and effector function-related genes in tumor-infiltrating NK cells

86 We first generated bulk transcriptomic profiles of tumor-infiltrating NK cells (T-NK) isolated from lung
87 tumor, adjacent non-tumor lung tissue (L-NK) and peripheral blood (P-NK) to discriminate features linked to
88 lung tissue localization from those related to tumor infiltration (**Supplementary Table 1** and **2**). A large
89 number of transcripts were differentially expressed by T-NK when compared to L-NK ($n = 190$) or P-NK ($n =$
90 399), (**Fig. 1a**, **Supplementary Fig. 1a** and **Supplementary Table 3**). T-distributed stochastic neighbor
91 embedding (tSNE) analysis and hierarchical clustering showed that T-NK clustered together, distinct from L-
92 NK and P-NK, which suggested that T-NK was most divergent relative to L-NK or P-NK (**Fig. 1b** and
93 **Supplementary Fig. 1b**).

94 To gain broad insights into the functional relevance of the T-NK transcriptional program, we performed
95 gene pathway analysis (Online Methods) of transcripts differentially expressed in T-NK relative to L-NK or P-
96 NK (**Fig. 1c**, **Supplementary Table 3** and **4**). Interestingly, we observed that the top 5 canonical pathways in
97 T-NK were enriched for overlapping sets of genes encoding products linked to activation such as *CD69*³⁵,
98 *ICOS*³⁶, *CTLA4*³⁷ (which encodes the immunomodulatory receptor CTLA-4 (CD152)), *PDCD1*³⁷ (which
99 encodes for PD-1) and *LAG-3*³⁷. Some of these gene products are also associated with “exhaustion”³⁸⁻⁴⁰,
100 consistent with reports of inhibited lymphocyte function in tumor infiltrating lymphocytes (TILs) consequent to
101 activation. Furthermore, we found enrichment of canonical pathways (Role of NFAT in regulation of the
102 immune response, Granulocyte adhesion and diapedesis) composed of transcripts encoding for cytokines
103 (e.g., *TNF*, *IL32*, *CSF2*, *LTB*)⁴¹, chemokines (e.g., *XCL1*, *XCL2*, *CXCL13*)²⁰ and chemokine receptors (e.g.,
104 *CXCR3*)⁴², with notable functions in dendritic cell (DC) and immune cell recruitment. T-NK cells expressed
105 transcripts from the “NK cell signaling” and “Crosstalk between dendritic cells and NK cells” pathways

106 encoding for NK cell surface receptors (e.g., *RGS1*), NK activating receptor NCR2⁴³ and NK inhibitory
 107 receptors KLRC1, KLRF2, KIR2DL4⁴³. T-NK cells were also enriched for cell cycle-related transcripts such
 108 as *MKI67*, *STMN1*, *BARD1*, *MASTL*, indicating proliferation of T-NK within tumors. Several transcripts
 109 associated with tissue retention of lymphocytes were also increased in T-NK, suggesting that tumors are
 110 enriched for tissue-resident NK (trNK) cells. Examples include, *ITGAE*, which encodes for an integrin molecule
 111 (CD103) that binds the adhesion molecule E-cadherin expressed by epithelial cells in barrier tissues⁴⁴,
 112 *CD69*⁴⁵, *CXCR6*⁴⁶, *ITGA1*⁴⁷(encoding CD49a), *ITGA2* and *ZNF683*, which encodes for the transcription factor
 113 Hobit that imprints tissue-residency program in lymphocytes^{48,49}. Gene set enrichment analysis (GSEA)
 114 revealed that T-NK, relative to L-NK cells, were enriched for tissue-resident memory (T_{RM}) cell signature
 115 genes⁵⁰ and displayed lower expression of genes that are typically downregulated in T_{RM} cells (**Fig. 1d**),
 116 confirming the presence of trNK cells among T-NK cells. Thus, our transcriptional profiling revealed that T-
 117 NK cells display features such as activation, cytokine and chemokine production, proliferation, tumor homing
 118 and tissue residence, suggesting that T-NK are likely to be engaged in anti-tumor immune responses.

119

120 **A tissue-resident population of CD16^{neg} NK cells is present in lung tumors**

121 Circulating NK cells in the blood are known to be predominantly composed of the cytotoxic
 122 CD56^{dim}CD16^{pos} NK cells (~90%) and a smaller proportion of the cytokine-producing CD56^{bright}CD16^{neg} NK
 123 cells (~10%)⁴¹. Similar to blood, the majority of NK cells (up to 80%) in the lung have been shown to be the
 124 CD56^{dim}CD16^{pos} subset that circulates between lung and peripheral blood, whilst a smaller fraction includes
 125 the lung CD16^{neg} NK cells⁵¹. Interestingly, we found that the genes that showed significantly decreased
 126 expression in T-NK relative to L-NK or P-NK included NK marker genes, *FGFBP2* and *FCGR3A* (encodes for
 127 FcγRIIIa (CD16)) and cytotoxicity-related genes such as *PRF1*, *GZMM*, *GZMH*⁵² (**Fig. 1a, Supplementary**
 128 **Table 3**), indicating increased infiltration of CD16^{neg} NK cells in tumor compared to lung tissue. Flow
 129 cytometric analysis of NK cells in peripheral blood (P-NK, *n* = 10), adjacent normal lung (L-NK, *n* = 19), and
 130 lung tumors (T-NK, *n* = 19) confirmed that indeed the proportion of the CD16^{neg} NK subset was increased in
 131 lung tumors relative to adjacent normal lung or peripheral blood (**Fig. 2a**).

Since the lung tumors contained nearly equal proportions of CD16^{neg} and CD16^{pos} NK cells, we asked whether the functional properties of T-NK cells revealed by bulk RNA-sequencing (RNA-seq) analysis were attributes of both or were unique to one population. Therefore, from a second cohort of treatment-naïve NSCLC patients, we performed bulk RNA-seq of CD56⁺ NK cells flow-sorted as CD16^{pos} or CD16^{neg} populations from lung tumors (**Supplementary Table 1 and 2**). We found that the key T-NK features including activation, “exhaustion”, DC recruitment and tissue residence, mapped onto the CD16^{neg} population of T-NK cells (**Fig. 2b, c**). Independent GSEA showed that CD16^{neg} T-NK cells, relative to the CD16^{pos} cells, exhibited high expression of genes typically upregulated among a core T_{RM} cell signature⁵⁰ and low expression of genes that are typically downregulated in T_{RM} cells (**Supplementary Fig. 2**), confirming the presence of tissue-resident NK cells specifically among the CD16^{neg} T-NK cells. Remarkably, although cytotoxicity-related transcripts appeared to be downregulated in total CD56⁺ T-NK cells compared to total CD56⁺ L-NK or P-NK cells (**Fig. 1a**), they were in fact upregulated in CD16^{pos} T-NK relative to CD16^{neg} T-NK cells (**Fig. 2b and Supplementary Table 5**), implying that T-NK with cytotoxic potential were predominantly within the CD16^{pos} T-NK cell population. Taken together, our results indicate that lung tumors are infiltrated with CD16^{pos} T-NK cells with cytotoxic potential. In addition, lung tumors relative to normal lung or blood, are selectively enriched for CD16^{neg} NK cells which display features of tissue-resident NK cells, closely mimicking features of CD8⁺ tissue-resident memory (T_{RM}) cells.

Single cell characterization reveals multiple NK cell subsets

CD8⁺ CTLs play an important role in *de novo* and immunotherapy-induced anti-tumor immune responses. Consequently, patients with high CD8⁺ CTL density in tumors have robust anti-tumor immune responses and better survival outcomes when compared to those with low CD8⁺ CTL density^{53,54}. However, the relative contribution of other immune cell subsets and how they impact CD8⁺ CTLs responses, are not well known. There is greater appreciation of the presence of specialized NK populations that differ not only in NK cell-intrinsic functions but also in their ability and mechanisms for cross-talk with other immune cells. The expression of a wide range of chemotactic, synaptic, effector, and regulatory molecules allows NK cells to interact with various immune cells in the tumor microenvironment and modulate CD8⁺ CTL²¹ function. Recent

studies have also highlighted heterogeneity in human NK cells related to their tissue-specific location and context (homeostatic conditions or pathological states)^{33,55}. Therefore, to determine features of human T-NK cells that are linked to robust anti-tumor immune responses, we compared T-NK cell subsets and their molecular properties at single-cell resolution in tumors with immune infiltration linked to opposing clinical outcomes. We performed single-cell RNA-seq of T-NK and L-NK cells isolated from a cohort of 12 NSCLC patients with either TIL^{high} (high CD8⁺ CTLs density) tumors ($n = 6$) or TIL^{low} (low CD8⁺ CTLs density) tumors ($n = 6$) (**Supplementary Table 1** and **2**). Unbiased single-cell analysis of T-NK and L-NK cell transcriptomes revealed 7 distinct clusters (**Fig. 3a**). Consistent with our bulk RNA-seq data, expression of *FCGR3A* transcripts, encoding for CD16, delineated them into subsets enriched for *CD16* expression (clusters 1-4, referred hereafter as *CD16*^{pos} NK cell clusters) or not (clusters 5-7, *CD16*^{neg} NK cell clusters) (**Fig. 3b**).

TIL^{high} tumors are enriched for *CD16*^{pos} T-NK cells with enhanced cytotoxicity

CD16^{pos} NK cells represent the cytotoxic NK cells as demonstrated by our bulk transcriptomic analysis (**Fig. 2b**), however substantial heterogeneity was observed at the single-cell level (**Fig. 3a**). Among the *CD16*^{pos} NK cell subsets, cluster 1 was mainly composed of L-NK cells (73%), whereas clusters 2, 3, and 4 were enriched for T-NK cells (**Fig. 3b**). Notably, clusters 2 and 3 were enriched for T-NK cells from TIL^{low} tumors whilst cluster 4 was enriched for T-NK cells from TIL^{high} tumors. (**Fig. 3b**). To dissect the molecular properties of *CD16*^{pos} T-NK cells, particularly in relation to TIL density, we performed single-cell differential gene expression analysis of clusters 2,3 (enriched for T-NK from TIL^{low} tumors) versus cluster 4 (enriched for T-NK from TIL^{high} tumors). NK cells in cluster 4 were significantly enriched for the expression of several genes linked to cytotoxicity such as *PRF1*, *GZMA*, *GZMB*, *GZMH*, *GZMM*, *GNLY*, *NKG7*, *FGFBP2* and *CTSW* (**Fig. 3c-e** and **Supplementary Table 6**). Independent GSEA analysis confirmed that cluster 4 was significantly enriched for cytotoxicity signature genes⁵⁶ (**Fig. 3f**). Consistent with this finding, NK cells in cluster 4 also exhibited expression of transcripts encoding transcription factors with critical role in NK cell cytotoxic and effector differentiation such as ID2⁵⁷ and interferon regulatory factor -1 (IRF1) (**Fig. 3c-e** and **Supplementary Table 6**). Particularly, IRF1, an interferon-induced transcription factor, has been shown in murine models to be essential for the induction of NK cell-mediated cytotoxicity, effector functions, and elimination of tumor *in*

186 *vivo*⁵⁸ (**Fig. 3c-e** and **Supplementary Table 6**). Furthermore, cluster 4 NK cells expressed transcripts
 187 encoding NK activating receptor, Killer cell Lectin-like Receptor F1 (KLRF1) which stimulates cytotoxicity,
 188 cytokine release and functions in tumor immunosurveillance^{59,60} (**Fig. 3c, d** and **Supplementary Table 6**). In
 189 addition to effector function, NK cells in cluster 4, relative to clusters 2,3, were enriched for several transcripts
 190 linked to immune cell recruitment such as high mobility group box 1 (HMGB1) and SPON2. NK cell-derived
 191 HMGB1 is pro-inflammatory, recruits activated NK cells to tumor and is known to directly eliminate tumor cells
 192 by inhibiting glycolysis and triggering metabolic cell death^{61,62}. HMGB1 secreted by activated NK cells into the
 193 immunological synapse also induces dendritic cell (DC) maturation and initiation of adaptive immune
 194 responses⁶³. SPON2 serves as an integrin ligand and promotes inflammatory cell recruitment and T cell
 195 priming⁶⁴. Further, NK cells in cluster 4 showed higher levels of transcripts encoding pro-inflammatory
 196 chemokines, CCL3 and CCL4, which promote recruitment of myeloid cells (neutrophils, monocytes,
 197 macrophages) and T cells expressing chemokine receptor (CCR)1, CCR3 and CCR5^{65,66}(**Fig. 3c-e** and
 198 **Supplementary Table 6**). Overall, our single cell analysis demonstrates that TIL^{high} tumors are composed of
 199 functionally specialized *CD16^{pos}* T-NK cells with a distinct gene program that supports cytotoxicity, effector
 200 functions and recruitment of other immune cells within tumors.

201
 202 **Activated ILC3-like cells infiltrate TIL^{high} tumors**

203 The *CD16^{neg}* NK cells clustered into three distinct subsets that were mainly composed of tumor-
 204 infiltrating NK cells (**Fig. 4a, b**). Importantly, TIL^{high} tumors were enriched in NK cells from clusters 5 and 7,
 205 whereas TIL^{low} tumors were predominantly composed of NK cells from cluster 6 (**Fig. 4b**). This distinct pattern
 206 of clustering based on TIL density in tumors suggested that the T-NK molecular profile and CD8⁺ CTL
 207 response was closely inter-linked, reaffirming our observations in *CD16^{pos}* T-NK cells. Pair-wise comparison
 208 of the three *CD16^{neg}* NK cell clusters highlighted several genes with significantly increased expression in each
 209 cluster (**Fig. 4c** and **Supplementary Table 7**). Cells in cluster 5 showed increased expression of several
 210 transcripts linked to innate lymphoid cell (ILC)3 subset such as *IL7R*, *GPR183* and *TCF7*^{67,68}(**Fig. 4c, d**). This
 211 finding is not surprising, as the markers used for the isolation of NK cells (CD56) are also known to be
 212 expressed by innate lymphoid cells (ILCs)⁶⁸. Notably, a recent study employing flow cytometric methods found

that ILC3 cells were increased in tumors and their density was positively associated with that of intra-tumoral tertiary lymphoid structures (TLS) in NSCLC⁶⁹. Consistent with this report, we found that the ILC3-like cells in cluster 5 expressed higher levels of transcripts encoding XCL1 and XCL2²⁰, which are chemokines known to recruit cross-presenting XCR1⁺ DCs that are critical for priming anti-tumor CD8⁺ CTL responses (**Fig. 4c, d**). In addition to their ability to recruit DCs, the ILC3-like cells in cluster 5 were enriched for transcripts linked to TNF α and NF κ B signaling pathway⁷⁰⁻⁷² (*TNFAIP3*, *ZFP36*, *NFKBIA*, *BTG2*, *IER2*, *REL*, *AREG* and *MAFF*) (**Fig. 4c, d**). Notably, activation of NF κ B pathway enables the acquisition and maintenance of effector functions⁷³. Furthermore, ILC3-like cells were also enriched for the expression of genes encoding activation marker CD44, and several immediate early response genes including *NR4A2*, *DUSP2*, *JUND*, which are induced rapidly in response to activation⁷⁴ (**Fig. 4c, d**). Overall, the ILC3-like cells in cluster 5, which were enriched in TIL^{high} tumors, demonstrated evidence of activation, effector function and capacity for DC recruitment.

We also asked whether the ILC3-like cells in cluster 5 differed qualitatively in TIL^{high} tumors compared to those present within TIL^{low} tumors. Remarkably, differential gene expression analysis showed that the salient features seen in cluster 5 were indeed exhibited by ILC3-like cells from TIL^{high} tumors including expression of transcripts related to NF κ B pathway, activation (*CD44*), immediate early response genes and recruitment of cross-presenting DCs (*XCL2*) (**Fig. 4e** and **Supplementary Table 8**). In addition, we found increased expression of transcripts linked to cytotoxicity (*GZMA*, *GZMK*, *GZMB*, *PRF1*) in ILC3-like cells from TIL^{high} relative to TIL^{low} tumors (**Fig. 4e** and **Supplementary Table 8**). Together, these findings highlight potential effector mechanisms by which ILC3-like cells may promote anti-tumor immune responses within TIL^{high} tumors.

234

235 Tissue-resident NK cells display pro-survival and memory-like properties in TIL^{high} tumors

The composition of cells in the *CD16*^{neg} cluster 7 indicated expansion of this subset in tumor tissue, especially from TIL^{high} tumors (**Fig. 4b**). Transcripts linked to tissue residency⁷⁵ such as *ZNF683*, *ITGB1*, were significantly increased in cluster 7 (**Fig. 5a** and **Supplementary Table 7**). Notably, *ZNF683* encodes for the zinc finger transcription factor Hobit, that has been shown to be essential for the development of tissue-

240 resident NK cells and T cells⁴⁹. GSEA confirmed significant positive enrichment of tissue residency signature
 241 genes⁵⁰ in cluster 7 relative to other CD16^{neg} clusters (clusters 5 & 6) or CD16^{pos} clusters (clusters 1,2,3,4),
 242 which indicated that cluster 7 was mainly composed of tissue-resident NK cells (**Fig. 5b**). We found that NK
 243 cells in cluster 7 were also enriched for transcripts linked to cytotoxicity (*GZMB*, *GZMH*), which is a feature of
 244 tissue-resident lymphocytes (**Fig. 5a**).

245 We next compared the single-cell transcriptomes of tissue-resident NK cells (cluster 7) from TIL^{high}
 246 tumors with those from TIL^{low} tumors to delineate molecular features that are enriched in cells from TIL^{high}
 247 tumors (**Supplementary Table 9**). Tissue-resident lymphocytes orchestrate immune responses by recruiting
 248 circulating immune cells to the tissues⁷⁶. Consistent with this, tissue-resident NK cells from TIL^{high} tumors
 249 showed higher expression of transcripts encoding chemokine CCL3, which plays an important role in the
 250 recruitment of circulating myeloid cells, T cells and NK cells. Similar to our findings in the ILC3-like subset
 251 (cluster 5), we found enrichment of several transcripts linked to activation of NFκB pathway (e.g., *NFKBIA*,
 252 *ZFP36*, *BTG2*, *TNFAIP3*, *PTGER4*) in tissue-resident NK cells from TIL^{high} tumors, suggesting greater
 253 activation of these cells in TIL^{high} tumors. Recent reports have also revealed that NK cell receptors (NKRrs)
 254 might recognize antigen in the context of MHC-I molecules to mediate NK cell activation and enhanced
 255 “memory-like” functional responses⁷⁷. NK cells can also be activated by IL-2^{78,79}, which is known to prime the
 256 cytotoxic function of NK cells toward cancer cells. Furthermore, pre-activation of NK cells with cytokines has
 257 been reported to facilitate formation of ‘memory-like’ NK cells⁸⁰⁻⁸². In support of enhanced IL-2 signaling in
 258 tissue-resident NK cells from TIL^{high} tumors, we found increased expression of several transcripts involved in
 259 IL-2 signaling such as *IL2RB*, *GADD45B*, *ARL4A*, *GPR65*, *CD48* and *KLRC2*⁸³ (encoding for NKG2C) (**Fig.**
 260 **5c**). We also found transcriptional features of increased signalling via the activating NK cell receptor NKG2C
 261 that is mediated by the ITAM protein, DAP12⁸⁴; specifically, several transcripts encoding molecules signalling
 262 downstream from DAP12 such as *FYN*, *LCK*, *PIK3R1*, *RAC2*, were increased in tissue-resident NK cells from
 263 TIL^{high} tumors (**Fig. 5c**). Factors that support the survival of NK cells may also explain why tissue-resident NK
 264 cells are increased in TIL^{high} tumors. Signalling by the pleiotropic cytokine, IL-15, has been shown to increase
 265 the expression of anti-apoptotic protein, myeloid cell leukemia sequence-1 (MCL1), in a STAT5-dependent
 266 manner to promote NK cell survival^{85,86}. Notably, we found that MCL1-encoding transcripts were significantly

increased in tissue-resident NK cells from TIL^{high} tumors compared to TIL^{low} tumors (**Fig. 5c**). Overall, these findings suggest that tissue-resident NK cells from TIL^{high} tumor are more activated, and display pro-survival and enhanced “memory-like” functional properties, which are likely to promote robust anti-tumor immune responses.

We next assessed the molecular properties of the *CD16*^{neg} cluster 6 that was mainly composed of cells from TIL^{low} tumors (**Fig. 4a, b**). NK cells in this cluster, relative to cluster 5, showed increased expression of transcripts related to tissue-residency but not as pronounced as cluster 7 (**Fig. 5a** and **Supplementary Table 7**). GSEA and tissue-residency signature⁵⁰ score confirmed this finding (**Supplementary Fig. 3a, b**), suggesting that cluster 6 was a potential intermediate tissue-resident NK cell subset. Cluster 6 cells showed increased expression of transcripts encoding heat shock proteins such as *HSPA1A*, *HSPA1B*, *HSPA6*, *HSPE1*, *HSPB1*, *HSPD1*, *HSPA8*, suggesting potential stressed cell state (**Supplementary Fig. 3c**). These findings suggest that the subset (cluster 6) of *CD16*^{neg} NK cells enriched in TIL^{low} tumors are likely to be less functional when compared to the other subsets enriched in TIL^{high} tumors.

280

281 DISCUSSION

We have taken an unbiased approach to identify transcriptomic features that are enriched in tumor-infiltrating NK cells and to define NK cell subsets that are linked to robust anti-tumor immune responses and good outcomes. Prior transcriptional studies of tumor-infiltrating NK cells have been restricted to murine tumors⁸⁷ or analysis of NK cells in peripheral blood or metastatic sites in patients³⁴. Therefore, these analyses do not fully evaluate the molecular program of NK cells generated *de novo* at the primary tumor site, which is the focal point for immunotherapies. Furthermore, studies that compare tumor-infiltrating NK cells with their circulating counterparts are most likely to capture features linked to tissue localization rather than those specifically linked to tumor infiltration³⁴. In contrast, our study avoided these confounding factors and surveyed the transcriptomes from purified NK cells derived from tumor tissue (T-NK) and the best relevant control tissue, adjacent uninvolved lung (L-NK), in addition to circulating NK cells in blood (P-NK). Our analyses were centered on treatment-naïve patients where *de novo* anti-tumor responses can be assessed without the caveats introduced by prior treatment.

Our studies revealed a core T-NK transcriptional profile, distinct from L-NK and P-NK, which displayed features such as activation, “exhaustion”, cytokine and chemokine production, proliferation, tumor homing and tissue residence, suggesting that T-NK are likely to be engaged in supporting anti-tumor immune responses. Whilst PD-1 expression on CD8⁺ T cells has generally been associated with dysfunctional CTLs termed as “exhausted cells”⁸⁸, recent studies have shown that PD-1-expressing CD8⁺ T_{RM} are in fact, highly functional and clonally expanded⁵⁰. In a similar fashion, it is conceivable that NK cells with an activated and more responsive phenotype may also express PD-1⁸⁹. We demonstrated increased proportions of CD16^{neg} NK cells within tumors, consistent with prior reports⁹⁰. Differential gene expression analysis of CD16^{pos} versus CD16^{neg} T-NK cells uncoupled their distinctive functions; CD16^{pos} T-NK cells were the predominant cytotoxic population while T-NK features including activation, “exhaustion”, DC recruitment and tissue residence, mapped onto the CD16^{neg} T-NK cells.

Given that T-NK cells were not a homogeneous population, we performed transcriptomic profiling of the NK cells at the single-cell level to resolve NK cell heterogeneity. Unbiased clustering of single-cell transcriptomes grouped NK cells into seven distinct clusters, of which four clusters were composed of CD16^{pos} NK cells (clusters 1-4), and three clusters were composed of CD16^{neg} NK cells (clusters 5-7). In addition, the cluster composition of NK cells differed based on the tissue microenvironment (L-NK *versus* T-NK) and TIL density. A unique aspect of our single-cell analyses is the evaluation of NK cell transcriptomes in relation to TIL density, a feature which has been associated with improved survival in multiple cancer types.

Circulating NK cells are known to consist of two main populations, the CD56^{dim}CD16⁺ NK cells which are cytotoxic and the CD56^{bright}CD16^{neg} NK cells that function by secretion of cytokines^{91,92}. Interestingly, among the CD16^{pos} T-NK, we found that NK cells from TIL^{high} tumors specifically displayed enhanced cytotoxicity. In TIL^{high} tumors, NK cell-mediated killing of target cells may enhance the anti-tumor activity of CD8⁺ CTL responses, possibly by promoting antigen cross-presentation. In addition, T-NK cells may be particularly important in targeting tumor cell clones that have lost their MHC class I expression to evade CD8⁺ CTL-mediated killing. Hence, within TIL^{high} tumors, a synergistic immune-promoting program may be operational between tumor-infiltrating NK cells and T cells.

320 The three *CD16^{neg}* NK cell clusters revealed distinct patterns of phenotypic, effector and survival
321 properties, denoting that they are three different subsets. Cells in cluster 5 showed features of ILC3 and,
322 consistent with their reported function in the formation of tertiary lymphoid structures⁶⁷, these ILC3-like cells
323 displayed a chemokine expression profile geared for recruitment of cross-presenting DCs into tumors. ILC3-
324 like cells enriched in cluster 5 showed features of NFκB activation. Recently, in murine tumor-infiltrating NK
325 cells, deletion of Hif1a led to activated NK cells with upregulated NFκB activity and high functional capacity,
326 resulting in tumor reduction⁸⁷. Strikingly, we found that within cluster 5, ILC3-like cells from TIL^{high} tumors
327 relative to TIL^{low} tumors, displayed enhanced NFκB signaling, activation and capacity for DC recruitment.

328 Among the *CD16^{neg}* NK cell clusters, cluster 7 was enriched for tissue-resident NK cells. Whilst most
329 of our knowledge about NK cells is derived from mouse spleen or bone marrow, and human PBMCs
330 representing the circulating NK cells, only recently have studies begun to shed light on the tissue-resident NK
331 (trNK) cells. Using bulk RNA-seq analysis, trNK cells were recently described in the lungs and they shared
332 common core genes when compared to CD8⁺ T_{RM} and trNK cells in other tissues⁵¹. In the present study, we
333 provide the first report of enrichment of trNK cells within tumors. Furthermore, we assessed tr-NK cells in
334 relation to TIL density and demonstrate qualitative differences between TIL^{high} and TIL^{low} tumors. tr-NK cells
335 from TIL^{high} tumors, relative to TIL^{low} tumors, displayed features of NK memory and survival. Recent studies
336 have provided evidence in support of immunological memory in NK cells from both mice and humans, and
337 the unique advantages of NK memory cells in mounting anti-tumor responses⁹³. NK cells stimulated with
338 proinflammatory cytokines (e.g., IL-15, IL-18, type 1 interferons, IFN-γ) have been shown to generate long-
339 lived “memory-like” NK cells that were capable of robust recall responses^{80,81}. CD8⁺ T_{RM} cells, which are
340 abundant in TIL^{high} tumors, are robust producers of IFN-γ and this raises the possibility of whether CD8⁺ T_{RM}
341 may have a role in promoting NK memory in tumors. On the other hand, given that CD8⁺ T_{RM} also require IL-
342 15 for their differentiation⁴⁴, it is conceivable that TIL^{high} tumors harbor the common environmental cues
343 necessary for CD8⁺ T_{RM} and memory NK generation.

344 Our study reveals the transcriptional program of NK cells at the tumor site and underpins, at single-
345 cell resolution, the heterogeneity of NK cells and their differential molecular features associated with the TIL
346 density of the tumors they infiltrate. Future experiments in mouse models are warranted to investigate the *in*

347 *vivo* functional significance of the NK cell subsets, the plasticity among them, and whether and how NK
348 responses are orchestrated in the context of adaptive immunity. Insights into these mechanisms may enable
349 the rational targeting of NK cell pathways as novel immunotherapeutic strategies.

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ONLINE METHODS

Human subjects

Patients with newly diagnosed non-small cell lung cancer (**Supplementary Table 1**) referred to Southampton University Hospitals NHS Foundation Trust and Poole Hospital NHS Foundation Trust (UK) were prospectively recruited following written informed consent. Freshly resected tumor tissue and, where available, matched adjacent non-tumor lung tissue was obtained from patients with lung cancer undergoing surgical resection. The study was approved by the Southampton and South West Hampshire Research Ethics Board (Ref. 14/SC/0186).

Flow cytometry

Patient samples were processed as described previously⁹⁴. For sorting of NK cells for bulk transcriptomic analysis, cells were first incubated with FcR block (Miltenyi Biotec), then stained with a mixture of the following fluorescence-conjugated antibodies: anti-CD45 (HI30; BioLegend), anti-CD3 (SK7; BD Biosciences), anti-CD56 (HCD56; BioLegend) and anti-CD16 (3G8; BioLegend) for 30 min at 4°C. Live/dead discrimination was performed by DAPI staining. NK cells were sorted as live, singlet-gated, CD45⁺CD3⁻CD56⁺ cells. For the analysis of CD16^{pos} or CD16^{neg} NK cells, CD16 was additionally used as a marker for sorting. NK cells from tumor or adjacent uninvolved lung or blood of each patient were sorted into ice-cold TRIzol LS reagent (Ambion) using a BD FACS Aria™ (BD Biosciences). For 10x single-cell transcriptomic analysis, cells were incubated with FcR block (Miltenyi Biotec), then stained with a combination of the following fluorescence-conjugated antibodies: anti-CD45 (HI30; BioLegend), anti-CD3 (SK7; BioLegend), anti-CD56 (HCD56; BioLegend), anti-CD16 (3G8; BioLegend), anti-CD8a (SK1; BioLegend), anti-CD19/20 (HIB19/2H7; BioLegend), and anti-CD4 (OKT4; BioLegend) for 30 min at 4°C. Live/dead discrimination was performed using propidium iodide (PI). NK cells from the tumor or adjacent uninvolved lung of each patient were sorted into 50% ice cold PBS, 50% FBS (Sigma) using a BD Aria-III (BD Biosciences). Single-cell RNA-seq of sorted NK cells was performed using the 10x platform.

398 **Histology and immunohistochemistry**

399 Immunohistochemistry (IHC) was performed on FFPE tumor sections against cytokeratin (AE1/AE3;
400 Dako) and CD8a (C8/144B; Dako). TILs were quantified using a Zeiss AxioCam MRc5 microscope (Zeiss,
401 Cambridge, UK) and Zeiss Axiovision software (version 4.8.1.0; Zeiss). An average of 10 high-power (x400)
402 fields across representative areas of each tumor was counted to account for intratumoral heterogeneity; these
403 were averaged to generate an intratumoral TIL score. Tumors with an average CD8 count in the top third or
404 bottom third percentile were classified as TIL^{high} or TIL^{low}, respectively. The lowest CD8 count in the TIL^{high}
405 tumors was at least 2-fold greater than the highest CD8 count in the TIL^{low} tumors.

406
407 **Bulk RNA-sequencing**

408 Bulk RNA-seq of purified cells was performed described previously⁹⁴. Total RNA extraction was
409 performed using a miRNAeasy micro kit (Qiagen, USA). Following quantification, RNA was amplified using
410 the smart-seq2 protocol. DNA was purified using AMPure XP beads (1:1.1 ratio, Beckman Coulter). 1 ng of
411 purified cDNA was used to prepare a standard Nextera XT sequencing library (Nextera XT DNA sample
412 preparation kit and index kit, Illumina). Sequencing was performed using HiSeq2500 (Illumina) to obtain 50-
413 bp single-end reads (**Supplementary Table 2**). Quality control steps were included to determine total RNA
414 quality and quantity, optimal number of PCR pre-amplification cycles, and cDNA fragment size. Samples that
415 failed quality control were eliminated from further downstream analysis.

416
417 **Bulk RNA-sequencing analysis**

418 Bulk RNA-seq data were mapped against the hg19 reference using TopHat (v1.4.1: --library-type fr-
419 unstranded --no-coverage-search) and read counts were calculated using htseq-count -m union -s no -t exon
420 -i gene_id (part of the HTSeq framework, version 0.7.1)). Cutadapt (v1.3) was used to remove adapters. To
421 identify genes differentially expressed between groups, we performed negative binomial tests for paired
422 comparisons by employing the Bioconductor package DESeq2 disabling the default options for independent
423 filtering and Cooks cutoff. We considered genes differentially expressed between any pairwise comparison
424 when the DESeq2 analysis resulted in a Benjamini-Hochberg-adjusted *P* value < 0.05 and log₂ fold change

of 1 (**Supplementary Table 3 and 5**). The Qlucore Omics Explorer 3.2 software package was used for visualization and representation (heat maps) of RNA-seq data. Unsupervised hierarchical clustering of samples was based on the expression of genes (n = 794) with the top 20% variance using DESeq2 (v.1.14.1) method (**Supplementary Fig. 1c**). TPM values of these 794 genes were used to generate hierarchical clustering using Euclidean distance method for distance matrix computation (dist function of R stats) and average linkage method (hclust) of fastcluster (v.1.1.25). The dendrogram was generated using the as.dendrogram function of dendextend (v.1.7.0) R package.

432

433 Ingenuity pathway analysis

434 The biological relevance of differentially expressed genes identified by DESeq2 analysis was further
435 investigated using the Ingenuity Pathways Analysis platform. The enrichment of canonical pathways amongst
436 differentially expressed genes was assessed, with significance determined by right-tailed Fisher's exact test,
437 $P < 0.05$ (**Supplementary Table 4**).

438

439 10x Single-cell RNA-sequencing

440 Samples were processed using 10x v2 chemistry as per manufacturer's recommendations; 11 and 12
441 cycles were used for cDNA amplification and library preparation respectively. Barcoded RNA was collected
442 and processed following manufacturer recommendations, as described previously⁵⁰. Libraries were
443 sequenced on a HiSeq2500 (Illumina) to obtain 100- and 32-bp paired-end reads using the following read
444 length: read 1, 26 cycles; read 2, 98 cycles; and i7 index, 8 cycles (**Supplementary Table 2**).

445

446 Single-cell RNA-sequencing analysis

447 Raw 10x data was processed as previously described⁵⁰, merging multiple sequencing runs using
448 *cellranger count* function in cell ranger, then merging multiple cell types with *cell ranger aggr* (v2.0.2). The
449 merged data was transferred to the R statistical environment for analysis using the package Seurat (v2.1).
450 Only cells expressing more than 200 genes and genes expressed in at least 3 cells were included in the
451 analysis. The data was then log-normalized and scaled per cell and variable genes were detected.

Transcriptomic data from each cell was then further normalized by the number of UMI-detected and mitochondrial genes. A principal component analysis was then run on variable genes, and the first 6 principal components (PCs) were selected for further analyses based on the standard deviation of PCs, as determined by an elbow plot from Seurat. Cells were clustered using the *FindClusters* function from Seurat with default settings, resolution = 0.6. Clusters with less than 50 cells were excluded from analysis.

For differential expression between multiple groups, pairwise differential expression comparisons were performed (**Supplementary Table 7**). A gene was considered differentially expressed (unique to a group), only if the gene was commonly positively enriched in every comparison for a singular group.

The mean CPM and percentage of cells expressing a transcript was calculated with custom R scripts. Further visualizations of exported normalized data were generated using the Seurat package and custom R scripts. Average expression across a cell cluster was calculated using the *AverageExpression* function, and downsampling was achieved using the *SubsetData* function (both in Seurat). Violin plots for differentially expressed genes were created with custom R functions (https://github.com/vijaybioinfo/handy_functions/blob/master/devel/plots.R). Analyses and further data graphics can be found in our GitHub repository (https://github.com/vijaybioinfo/NK_2021).

Gene Set Enrichment Analysis (GSEA)

The Qlucore Omics Explorer 3.2 software package was used for GSEA for bulk RNA-seq data as previously described⁹⁴. In brief, all genes are ranked based on their differential expression between two groups. Next, a running enrichment score (RES) is calculated for a given gene set based on how often its members appear at the top or bottom of the ranked differential list. 1000 random permutations of the phenotypic subgroups are used to establish a null distribution of RES against which a normalized running enrichment score (NES) and FDR-corrected q values are calculated using Kolmogorov-Smirnov statistic. For GSEA for single-cell RNA-seq data, the R package fgsea (v1.10.1) was used with the signal-to-noise ratio as a metric calculated using custom R functions (https://raw.githubusercontent.com/vijaybioinfo/handy_functions/master/R/gsea_tests.R). Tailored parameters were minSize = 3 and maxSize = 500. Enrichment plots were created using fgsea's function

479 *plotEnrichment*. The gene signatures used for GSEA are shown in **Supplementary Table 10**.

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481 **Quantification and statistical analysis**

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483 Comparison between two groups was assessed with Mann-Whitney test (**Fig. 2a**) using GraphPad

484 Prism version 9.0.0.

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503 **ACCESSION CODES**

504 Sequencing data is currently being deposited into the Gene Expression Omnibus.

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512

513 **AUTHOR CONTRIBUTIONS**

514 A.A., T.S.E., C.H.O., P.S.F., A.-P.G and P.V. conceived the work, designed and analyzed experiments;
515 A.A. and S.C. assisted in patient recruitment, consenting and sample collection; A.A., O.W. and J.C.
516 performed the cell isolations and sorting under the supervision of C.H.O., P.V. and A.-P.G; A.A. and O.W.
517 performed immunohistochemistry data analysis under the supervision of G.J.T. and C.H.O; A.A., B.P. and
518 C.R.S. performed RNA-seq data analysis under the supervision of A.-P.G and P.V.; P.S.F., P.V. and A.-P.G
519 wrote the manuscript.

520

521 **COMPETING FINANCIAL INTERESTS**

522 The authors declare no competing financial interests.

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Figure Legends

Figure 1. Bulk transcriptional profile of tumor-infiltrating NK cells. (a) RNA-seq analysis showing row-wise z-scores of normalized read counts for each differentially expressed gene (rows) obtained by pairwise comparison of NK cells from blood (P-NK, $n = 4$), lung (L-NK, $n = 9$) or lung tumor (T-NK, $n = 7$) (DESeq2 analysis, Benjamini-Hochberg adjusted $P < 0.05$ and log2 fold change 1) of NSCLC patients; RNA-seq data from each independent sample is shown in columns; right margin, examples of differentially expressed transcripts. (b) t-SNE plot of bulk NK cell transcriptomes (symbol) from blood, lung and lung tumor (key, right) of NSCLC patients; each dot represents data from a patient. (c) Ingenuity Pathway Analysis (IPA) of canonical pathways enriched in T-NK cells, P values calculated by Fisher Exact Test. (d) GSEA plots for T_{RM} gene signature in the transcriptome of T-NK *versus* L-NK cells. The top portion of the plot shows the running enrichment score (RES) for the gene set as the analysis walks down the ranked list of genes and reflects the degree to which the gene set is overrepresented at the top or bottom of the ranked list of genes. The middle portion of the plot shows where the members of the gene set (indicated as blue lines) appear in the ranked list of genes. The bottom portion of the plot shows the value of the ranking metric.

Figure 2. Tissue-resident NK cells are enriched within lung tumors. (a) Representative FACS plots (above) and bar plots (below) show surface expression of CD16 and percentage of CD16^{pos} cells in live, singlet-gated CD45⁺CD3⁻CD56⁺ NK cells obtained from blood ($n = 10$), lung ($n = 19$) or lung tumor ($n = 19$) of NSCLC patients. *** $P = 0.0001$; **** $P < 0.0001$ (Mann-Whitney test); each symbol represents data from a patient; error bars are mean $\pm$ SEM. (b) RNA-seq analysis of CD16^{neg} or CD16^{pos} T-NK cells, showing row-wise z-scores of normalized read counts for genes differentially expressed by T-NK cells as shown in Fig 1a. RNA-seq data from each independent sample is shown in columns. (c) RNA-seq analysis showing expression of transcripts differentially expressed in NK cells from tumor compared pairwise with blood or lung tissue as shown in Fig 1a (right, key); each dot represents data from a patient.

Figure 3. NK cell heterogeneity revealed by single-cell analysis. (a) Seurat clustering of NK single-cell transcriptomes from lung tissue or lung tumor identifying 7 clusters (left); each symbol represents a cell; line

562 delineates clusters based on *FCGR3A* (*CD16*) expression. Violin plots show expression of *FCGR3A*
 563 transcripts in different clusters (right); color indicates percentage of cells expressing the transcript. (b) Pie
 564 charts show cluster-wise composition of L-NK or T-NK cells from TIL^{high} or TIL^{low} tumors (below, key). (c)
 565 Volcano plot shows genes differentially expressed between cluster 4 *versus* clusters 2 and 3 (adjusted P
 566 value < 0.05, log2 fold change 0.25); dot size indicates percentage of cells expressing the transcript; dot color
 567 indicates mean expression. (d) Plot shows mean expression and percentage of cells expressing selected
 568 differentially expressed transcripts in each CD16^{pos} cluster. (e) Violin plots show expression of the indicated
 569 transcripts in NK cells from clusters 2,3 and 4; color indicates percentage of cells expressing indicated
 570 transcript. (f) GSEA plot for cytotoxicity signature genes in cluster 4 compared to clusters 2,3.

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 572
 573 **Figure 4. CD16^{neg} NK cell subsets within tumors.** (a) Seurat clustering of NK single-cell transcriptomes
 574 from lung or lung tumor showing the CD16^{neg} clusters; each symbol represents a cell. (b) Pie chart shows
 575 cluster-wise composition of L-NK or T-NK cells from TIL^{high} or TIL^{low} tumors (right, key). (c) Heatmap shows
 576 expression of the differentially expressed transcripts in pairwise comparison of clusters 5, 6 and 7 (adjusted
 577 P value < 0.05, log2 fold change 0.25); right margin, examples of differentially expressed transcripts. (d) Plot
 578 shows mean expression and percentage of cells expressing selected transcripts that are differentially
 579 expressed in each CD16^{neg} cluster. (e) Violin plots show expression of the indicated transcripts that are
 580 differentially expressed in cluster 5 NK cells from TIL^{high} *versus* TIL^{low} tumors (adjusted P value < 0.05, log2
 581 fold change 0.25); color indicates percentage of cells expressing the indicated transcript.

582
 583 **Figure 5. Tissue-resident NK cells with distinct properties in TIL^{high} tumors.** (a) Plot shows mean
 584 expression and percentage of cells expressing selected differentially expressed transcripts in each CD16^{neg}
 585 cluster. (b) GSEA plot for T_{RM} signature genes in cluster 7 compared to the other CD16^{neg} clusters 5,6 (left
 586 plot) or compared to CD16^{pos} clusters 1,2,3,4 (right plot). (c) Violin plots show expression of the indicated
 587 transcripts that are differentially expressed in cluster 7 NK cells from TIL^{high} *versus* TIL^{low} tumors (adjusted P
 588 value < 0.05, log2 fold change 0.25); color indicates percentage of cells expressing the indicated transcript.

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Supplementary Figure Legends

Supplementary Figure 1. Unique and shared features of tumor-infiltrating NK cells. (a) Venn diagrams showing overlap of transcripts differentially upregulated (left) or downregulated (right) in pairwise comparison of T-NK, L-NK or P-NK cells. (b) Unsupervised hierarchical clustering of the transcriptomes of NK cells from blood (P-NK, green), lung (L-NK, blue) and lung tumor (T-NK, pink), based on the expression of genes with the top 20% variance ($n = 794$); each line represents an independent sample. Hierarchical clustering was applied based on Euclidean distance (Y-axis) and the average linkage method (hclust).

Supplementary Figure 2. Within lung tumors, tissue-resident NK cells are specifically enriched in the CD16^{neg} NK cell subset. GSEA plots for T_{RM} signature genes in the transcriptome of CD16^{neg} T-NK *versus* CD16^{pos} T-NK cells.

Supplementary Figure 3. Features of tumor-infiltrating CD16^{neg} NK cells in TIL^{low} tumors. (a) GSEA plot for T_{RM} signature genes in cluster 6 compared to the other CD16^{neg} cluster 5 (left plot) or compared to CD16^{pos} clusters 1,2,3,4 (right plot). (b) Violin plot shows enrichment pattern of T_{RM} gene signature for each cluster. Color indicates mean signature scores. (c) Plot shows mean expression and percentage of cells expressing the indicated differentially expressed transcripts in each CD16^{neg} cluster.

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shows the running enrichment score (RES) for the gene set as the analysis walks down the ranked list of genes and reflects the degree to which the gene set is overrepresented at the top or bottom of the ranked list of genes. The middle portion of the plot shows where the members of the gene set (indicated as blue lines) appear in the ranked list of genes. The bottom portion of the plot shows the value of the ranking metric.

Figure 2

Figure 2

Tissue-resident NK cells are enriched within lung tumors. (a) Representative FACS plots (above) and bar plots (below) show surface expression of CD16 and percentage of CD16pos cells in live, singlet-gated CD45+ CD3- CD56+ NK cells obtained from blood (n = 10), lung (n = 19) or lung tumor (n = 19) of NSCLC patients. *** P = 0.0001; **** P < 0.0001 (Mann-Whitney test); each symbol represents data from a patient; error bars are mean ± SEM. (b) RNA-seq analysis of CD16neg or CD16pos T-NK cells, showing row wise z-scores of normalized read counts for genes differentially

expressed by T-NK cells as shown in Fig 1a. RNA-seq data from each independent sample is shown in columns. (c) RNA-seq analysis showing expression of transcripts differentially expressed in NK cells from tumor compared pairwise with blood or lung tissue as shown in Fig 1a (right, key); each dot represents data from a patient.

Figure 3

Figure 3

NK cell heterogeneity revealed by single-cell analysis. (a) Seurat clustering of NK single-cell transcriptomes from lung tissue or lung tumor identifying 7 clusters (left); each symbol represents a cell; line 22 delineates clusters based on FCGR3A (CD16) expression. Violin plots show expression of FCGR3A transcripts in different clusters (right); color indicates percentage of cells expressing the transcript. (b) Pie charts show cluster-wise composition of L-NK or T-NK cells from TILhigh or TILlow tumors (below, key). (c) Volcano plot shows genes differentially expressed between cluster 4 versus clusters 2 and 3 (adjusted P value < 0.05, log2 fold change 0.25); dot size indicates percentage of

cells expressing the transcript; dot color indicates mean expression. (d) Plot shows mean expression and percentage of cells expressing selected differentially expressed transcripts in each CD16pos cluster. (e) Violin plots show expression of the indicated transcripts in NK cells from clusters 2,3 and 4; color indicates percentage of cells expressing indicated transcript. (f) GSEA plot for cytotoxicity signature genes in cluster 4 compared to clusters 2,3.

Figure 4

Figure 4

CD16neg NK cell subsets within tumors. (a) Seurat clustering of NK single-cell transcriptomes from lung or lung tumor showing the CD16neg clusters; each symbol represents a cell. (b) Pie chart shows cluster-wise composition of L-NK or T-NK cells from TILhigh or TILlow tumors (right, key). (c) Heatmap shows expression of the differentially expressed transcripts in pairwise comparison of clusters 5, 6 and 7 (adjusted P value < 0.05, log2 fold change 0.25); right margin, examples of differentially expressed transcripts. (d) Plot shows mean expression and percentage of cells expressing

selected transcripts that are differentially expressed in each CD16neg cluster. (e) Violin plots show expression of the indicated transcripts that are differentially expressed in cluster 5 NK cells from TILhigh versus TILlow tumors (adjusted P value < 0.05, log2 581 fold change 0.25); color indicates percentage of cells expressing the indicated transcript.

Figure 5

Tissue-resident NK cells with distinct properties in TILhigh tumors. (a) Plot shows mean expression and percentage of cells expressing selected differentially expressed transcripts in each CD16neg cluster. (b) GSEA plot for TRM signature genes in cluster 7 compared to the other CD16neg clusters 5,6 (left plot) or compared to CD16pos clusters 1,2,3,4 (right plot). (c) Violin plots show expression of the indicated transcripts that are differentially expressed in cluster 7 NK

cells from TILhigh versus TILlow tumors (adjusted P value < 0.05, log2 fold change 0.25); color indicates percentage of cells expressing the indicated transcript.

Supplementary Files

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- [SupplementaryTable1.ClinicalcharacteristicsofNSCLCpatients.xlsx](#)
- [SupplementaryTable10.GenesignaturesusedforGSEA.xlsx](#)
- [SupplementaryTable2.RNAseqstatistics.xlsx](#)
- [SupplementaryTable3.ListofgenesdifferentiallyexpressedbetweenNKcellsfromtumorlungandblood.xlsx](#)
- [SupplementaryTable4.PathwayanalysisofdifferentiallyexpressedgenesintumorNKcellsfromNSCLC.xlsx](#)
- [SupplementaryTable6.Listofdifferentiallyexpressedgenesincluster4comparedtoclusters2X3.xlsx](#)
- [SupplementaryTable7.ListofgenesdifferentiallyexpressedinCD16clusters567.xlsx](#)
- [SupplementaryTable8.Listofgenesdifferentiallyexpressedincluster5TILhighcomparedtoTILlow.xlsx](#)
- [SupplementaryTable9.Listofgenesdifferentiallyexpressedincluster7TILhighcomparedtoTILlow.xlsx](#)
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