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Tissue-resident memory features are linked to the magnitude of cytotoxic T cell responses in human lung cancer

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Abstract

Therapies that boost the anti-tumor responses of cytotoxic T lymphocytes (CTLs) have shown promise; however, clinical responses to the immunotherapeutic agents currently available vary considerably, and the molecular basis of this is unclear. We performed transcriptomic profiling of tumor-infiltrating CTLs from treatment-naïve patients with lung cancer to define the molecular features associated with the robustness of anti-tumor immune responses. We observed considerable heterogeneity in the expression of molecules associated with activation of the T cell antigen receptor (TCR) and of immunological-checkpoint molecules such as 4-1BB, PD-1 and TIM-3. Tumors with a high density of CTLs showed enrichment for transcripts linked to tissue-resident memory cells (T_{RM} cells), such as *CD103*, and CTLs from CD103^{hi} tumors displayed features of enhanced cytotoxicity. A greater density of T_{RM} cells in tumors was predictive of a better survival outcome in lung cancer, and this effect was independent of that conferred by CTL

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AUTHOR CONTRIBUTIONS

A.-P.G., P.S.F., T.S.-E., P.V. and C.H.O. conceived of the work and designed, performed and analyzed experiments; A.-P.G., D.S.-C. and D.S. performed micro-scaled RNA-Seq experiments and analysis under the supervision of G.S., P.V. and C.H.O.; A.-P.G. performed data analysis and wrote the manuscript under the supervision of P.V. and C.H.O.; J.C., O.W., E.M.G.-M. and T.M. performed the cell isolations and immunohistochemistry data analysis under the supervision of G.J.T. and C.H.O.; and S.J.C., A.A., E.W. and E.V.K. assisted in patient recruitment, obtaining consent and sample collection.

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density. Here we define the ‘molecular fingerprint’ of tumor-infiltrating CTLs and identify potentially new targets for immunotherapy.

Immunotherapy is rapidly gaining its place as a standard treatment for solid tumors^{1,2}, including lung cancer³. Nonetheless, only ~30% of patients benefit from this approach⁴. Much remains to be learned about how immunotherapy works and how to choose the right treatment or combination of treatments for a particular patient. Understanding the mechanisms and molecular basis of effective anti-tumor immune responses will be essential for the development of novel immunotherapeutic agents for those patients who do not respond to the immunotherapies now available.

Immunotherapy is thought to enhance the antitumor responses of cytotoxic T lymphocytes (CTLs); i.e., CD8⁺ T cells that infiltrate into the tumor⁵. Indeed, a high density of tumor-infiltrating lymphocytes (TILs) is predictive of a good prognosis in a wide range of cancers^{6,7}. However, it remains unclear why the degree of infiltration by TILs varies substantially even between people with the same cancer. It is also unknown whether there are merely quantitative differences in TILs or whether qualitative differences also exist in TILs from tumors with a high TIL density that might contribute to the superior outcome seen in patients with such tumors. Understanding of the TIL transcriptome and the molecular basis of TIL heterogeneity could not only lead to novel biomarkers for patient stratification for therapy but also identify novel immunological pathways to be targeted by future immunotherapeutic strategies.

So far, transcriptional studies of CD8⁺ T cells from patients with cancer have analyzed cells in peripheral blood or metastatic sites^{8–11}. The precise state of the activation, differentiation and function of CD8⁺ T cells within primary tumors is poorly understood; however, this must be a key reference point from which to begin elucidating the biology of immunological attack at the time of diagnosis, during tumor progression and after intervention with immunotherapy. To fully characterize the molecular nature of immune responses at the tumor site, we have taken an unbiased approach to define the global transcriptional profile of purified CD8⁺ TILs from well- characterized cohorts of patients with two epithelial cancers: non– small-cell lung cancer (NSCLC), and head-and-neck squamous-cell cancer (HNSCC).

RESULTS

Major transcriptional changes characterize CD8⁺ TILs

To identify the core transcriptional signature of CD8⁺ TILs, we used RNA-based next-generation sequencing (RNA-Seq) to analyze purified populations of CD8⁺ T cells present in tumor samples (CD8⁺ TILs) from patients ($n = 36$) with treatment-naïve early-stage NSCLC (Supplementary Fig. 1a and Supplementary Tables 1 and 2). We also generated matched transcriptional profiles of CD8⁺ T cells isolated from the adjacent non-tumor lung tissue (CD8⁺ N-TILs) to discriminate features linked to lung-tissue residence from those related to tumor infiltration. To assess conservation of the transcriptional program of CD8⁺ TILs in a related solid tumor of epithelial origin, we used a similar data set generated from

patients ($n = 41$) with HNSCC from both human papilloma virus–positive (virus-driven) subtypes and human papilloma virus–negative subtypes.

We identified a large number of transcripts ($n = 1,403$) that were expressed differentially by CD8⁺ TILs relative to their expression by CD8⁺ N-TILs (Fig. 1a and Supplementary Table 3), which suggested major changes in the transcriptional landscape of CD8⁺ TILs in lung tumor tissue. The expression of such lung-cancer ‘CD8⁺ TIL–associated transcripts’ did not differ according to histological subtype (Supplementary Fig. 1b). Principal-component analysis and hierarchical clustering also showed that CD8⁺ TILs from both subtypes of lung cancer mostly clustered together, distinct from the CD8⁺ N-TILs (Fig. 1b and Supplementary Fig. 1c,d). Notably, that set of lung-cancer ‘CD8⁺ TIL–associated transcripts’ was expressed similarly by CD8⁺ TILs in both subtypes of HNSCC (Fig. 1a and Supplementary Fig. 1b), which also clustered together with CD8⁺ TILs from lung cancer (Fig. 1b and Supplementary Fig. 1c,d); this indicated a conserved TIL transcriptome for these two tumor types.

Features associated with inhibited function, anergy and senescence of T cells have been described for TILs^{12–14}. Gene-set–enrichment analysis (GSEA) revealed significantly higher expression of genes encoding molecules linked to the so-called ‘exhaustion stage’, such as *PDCD1* (which encodes the immunological-checkpoint molecule PD-1), *CTLA4* (which encodes the immunomodulatory receptor CTLA-4 (CD152)) and *HAVCR2* (which encodes the cell-surface marker TIM-3) in CD8⁺ TILs than in CD8⁺ N-TILs, while CD8⁺ TILs did not have higher expression of genes encoding molecules associated with T cell anergy and senescence (Fig. 1c,d). Our data set also showed higher expression of T cell–associated genes derived from The Cancer Genome Atlas (TCGA) of lung cancer¹⁵ in CD8⁺ TILs than in CD8⁺ N-TILs (Fig. 1d). Together these findings suggested that our strategy for ‘micro-scaled’ RNA-Seq analysis of freshly purified *ex vivo* CD8⁺ TILs and CD8⁺ N-TILs reliably identified transcripts previously linked to TILs.

Cell-cycle and TCR-activation pathways in CD8⁺ TILs

To gain broad insight into the functional relevance of the CD8⁺ TIL transcriptional program, we performed gene-pathway analysis. Notably, we observed significant enrichment in TILs for transcripts from overlapping sets of genes encoding products involved in cell-cycle control, mitosis, DNA replication and signaling via the pathways of the tumor suppressor p53, the cyclin-dependent kinase ATM and the kinase PLK, relative to the abundance of those transcripts in N-TILs (Fig. 2a–c and Supplementary Table 4); this indicated that the TIL populations (tumors) showed enrichment for proliferating CD8⁺ T cells. Furthermore, we observed enrichment in CD8⁺ TILs, relative to their abundance in N-TILs, for transcripts encoding components of canonical pathways involved in antigen-specific T cell activation, especially the pathway mediated by 4-1BB (encoded by *TNFRSF9*, called ‘4-1BB’ here) and the CD27 co-stimulatory pathway, which are activated following the engagement of TCRs and their co-stimulation by antigen-presenting cells, respectively^{16,17} (Fig. 2a,d). The higher expression of 4-1BB in CD8⁺ TILs than in N-TILs was confirmed at the protein level by flow cytometry (Fig. 2e). Together these data suggested that the engagement TCRs and their co-stimulation, presumably provided by antigen-presenting cells expressing tumor-

associated antigens (TAAs), were probably involved in the antigen-specific activation and proliferation of CD8⁺ TILs, which indicated that the tumor milieu sustained the clonal expansion of presumed TAA-specific CD8⁺ T cells. That suggestion was further supported by analysis of the TCR repertoire, which indicated significantly greater clonal expansion of CD8⁺ TILs than of N-TILs (Fig. 2f and Supplementary Table 5).

Heterogeneity in targets of immunotherapy

The considerable success of immunological-checkpoint blockers, such as agents directed against PD-1 and CTLA-4, in humans and in model organisms^{4,18} suggests that CD8⁺ TILs with features of TCR engagement and strong co-stimulation probably mount robust anti-tumor immune responses. However, the response to such treatment is highly variable and is limited to a minority of patients. We reasoned that the molecular profile of CD8⁺ TILs might explain the inter-person variability in the response to agents directed against PD1 or CTLA-4 and might also reveal alternative immune-system-evasion mechanisms beyond PD-1- and CTLA-4-based pathways. Therefore, we assessed the expression of a spectrum of potential targets of immunotherapy to determine the extent of molecular heterogeneity in CD8⁺ TILs. We observed substantial variability in the expression of transcripts encoding PD-1 and other potential targets of immunotherapy by CD8⁺ TILs from patients with lung cancer (Fig. 3a,b) or HNSCC (Supplementary Fig. 2). We confirmed PD-1 expression at the protein level and showed that the abundance of *PDCD1* transcripts correlated with the average number of PD-1-expressing cells in the tumors (Supplementary Fig. 3a,b). We also found varying combinations of expression of co-inhibitory molecules; for example, CD8⁺ TILs from some patients with lung cancer had upregulation of transcripts encoding four targets of immunotherapy (PD-1, TIM-3, LAG-3 and CTLA-4) relative to the expression of those transcripts by other patients, while some patients showed upregulation of expression of three or two molecules or even a single molecule (Fig. 3a,b). The high molecular resolution and breadth of our data suggested that baseline transcriptional profiling of tumor-infiltrating CD8⁺ T cells might guide the selection of appropriate immunotherapies for each patient and the development of biomarkers that can be used to predict the clinical response to checkpoint blockade with monotherapy or combination therapies.

PDCD1 expression correlates with TIL density

The considerable heterogeneity observed in the abundance of *PDCD1* transcripts led us to investigate factors linked to *PDCD1* expression in CD8⁺ TILs. Despite the perceived role of PD-1 as a negative regulatory immunological checkpoint, it serves as a marker for clonally expanded, antigen-specific T cells capable of lysing autologous tumor cells^{19,20}. Furthermore, we found a strong positive correlation between the expression of *PDCD1* and that of *4-1BB* (Fig. 3c), which is expressed following engagement of the TCR and is thus a marker of antigen-specific T cells^{16,17,21}. The heterogeneity in the expression of these surrogate markers for antigen specificity suggested that not all tumors contained similar numbers of tumor-reactive CD8⁺ TILs. Hence, we sought to determine which factors might influence the enrichment for *PDCD1*- or *4-1BB*-expressing CD8⁺ TILs; i.e., cells we presumed to be TAA specific. We found no correlation between the abundance of *PDCD1* or *4-1BB* transcripts and clinical or pathological characteristics such as patient age, sex, histological subtype, stage of disease, performance status (the ability to perform activities of

daily living without assistance) or smoking status (Supplementary Fig. 3c). However, there was a positive correlation between the abundance of each of those transcripts and the average number of CD8⁺ TILs that infiltrated each tumor sample (Fig. 3c). A similar correlation was also observed between the abundance of each of those transcripts and *CD8A* transcripts (encoding the co-receptor CD8 α) in lung-tumor samples from the TCGA RNA-Seq data set (Fig. 3d).

In addition to their higher expression of *PDCDI* and *4-1BB*, tumors with a high density of TILs ('TIL^{hi}' tumors; tumors were classified as TIL^{hi}, TIL^{int} and TIL^{lo} on the basis of the average number of CD8⁺ T cells that infiltrated the tumors; Supplementary Fig. 4) also had higher expression of transcripts encoding several other targets of immunotherapy, such as TIM-3, LAG-3 or TIGIT, than that of TIL^{lo} tumors (Fig. 3e). Published studies have linked PD-1 and 4-1BB to both exhaustion²² and antigen-specific TCR activation^{19,20}, but the positive correlation of their expression with TIL density indicated that their higher expression probably reflected enrichment for activated TAA-specific CD8⁺ T cells.

TIL^{hi} tumors show enrichment for CD8⁺ T_{RM} cells

Patients with a high density of TILs in tumors have a better survival outcome than that of patients with a low density of TILs⁶. It is not known if beyond the numerical changes in T cells, there are qualitative differences between these groups in tumor-infiltrating CD8⁺ T cells. Defining such features might provide insight into the mechanisms that govern the magnitude and specificity of anti-tumor CD8⁺ T cell responses.

We found 109 transcripts whose expression differed significantly between TIL^{hi} tumors and TIL^{lo} tumors (Fig. 4a and Supplementary Table 6). As expected, transcripts encoding products involved in TCR activation (*4-1BB* and *PDCDI*) were upregulated in TIL^{hi} tumors (Fig. 4a), consistent with enrichment for presumed TAA-specific CD8⁺ T cells, although such specificity would require further experimental confirmation. Several other transcripts encoding products associated with the tissue retention of lymphocytes and tissue-resident memory T cells (T_{RM} cells) were expressed differentially by TIL^{hi} tumors relative to their expression by TIL^{lo} tumors (Supplementary Table 6 and Supplementary Fig. 5a). For example, *ITGAE* encodes the α_E subunit of the integrin $\alpha_E\beta_7$ (CD103), which binds the adhesion molecule E-cadherin expressed by epithelial cells in barrier tissues^{23,24}. TIL^{hi} tumors had higher expression of the gene encoding this marker of T_{RM} cells (CD103) than did TIL^{lo} tumors (Fig. 4a,b), and its expression positively correlated with the average number of CD8⁺ cells within tumors (Fig. 4c). That finding was also confirmed in the TCGA lung-cancer data set (Fig. 4c). We confirmed CD103 expression in CD8⁺ TILs at the protein level by immunohistochemistry and flow cytometry (Fig. 4d,e). Surface molecules linked to T_{RM} cells^{25,26}, such as CD69 and CD49a (ITGA1), were co-expressed with CD103, and surface molecules linked to effector memory cells (KLRG1) and central memory cells (CCR7 and CD62L) had lower expression on CD103⁺CD8⁺ TILs than on CD103⁻CD8⁺ TILs (Fig. 4f and Supplementary Fig. 5b), which suggested that the former population represented T_{RM} cells. We also observed co-expression of PD-1 and 4-1BB in 6% of CD103⁺CD8⁺ TILs and 4% of CD103⁻CD8⁺ TILs, respectively, in a representative patient sample (Fig. 4e).

Another transcript with higher expression in TIL^{hi} tumors than in TIL^{lo} tumors was *CXCR6* (which encodes the chemokine receptor CXCR6) (Fig. 4a,b); not only is CXCR6 expression linked to T_{RM} cells²⁵ but also CXCR6 is important for the localization and function of tissue-residing T cells^{27,28}. *SIPR1* transcripts (encoding the G protein–coupled receptor SIP₁) and *KLF2* transcripts (encoding the transcription factor KLF2), which are known to be downregulated in T_{RM} cells²³, were also diminished in TIL^{hi} tumors relative to their abundance in TIL^{lo} tumors (Fig. 4b). Downregulation of *SIPR1* is necessary for the egress of T cells from the lymph nodes and their subsequent retention in tissues, as T cells with high expression of SIP₁ are retained in the lymph nodes and also easily exit tissues due to the higher concentration of its ligand (S1P) in the lymph nodes and blood. *SIPR1* is a target of KLF2; downregulation of *KLF2* has been shown to result in lower expression of *SIPR1*, and the products of both of these genes together have an important role in the establishment and retention of T_{RM} cells in tissues²⁹. GSEA also revealed that TIL^{hi} tumors exhibited low expression of genes typically downregulated among a core set of T_{RM} cell signature genes, such as *SIPR5*, *STK38* and *FAM65B*^{23,25} (Fig. 4g). Pathway analysis of the genes expressed differentially by TIL^{hi} tumors relative to their expression by TIL^{lo} tumors showed higher expression (by TIL^{hi} tumors than by TIL^{lo} tumors) of genes encoding products involved in the canonical interferon pathway (Supplementary Fig. 5c). Interferon- γ (IFN- γ) was also predicted by Ingenuity pathway analysis to be an upstream regulator of the genes expressed differentially by TIL^{hi} tumors relative to their expression by TIL^{lo} tumors (Fig. 4h). Because IFN- γ produced by T_{RM} cells has been shown to recruit circulating T cells to potentiate robust immune responses in tissues^{30,31}, we inferred that the interferon response signature seen in TIL^{hi} tumors might have been the result of the activation of T_{RM} cells by TAAs (tumor-specific T_{RM} activity). Overall, these results demonstrated that TIL^{hi} tumors showed enrichment for T_{RM} cells.

CD103 density is predictive of survival in lung cancer

We next assessed the transcriptome of CD8⁺ TILs from tumors enriched for T_{RM} cells (CD103^{hi} tumors) for features that would support a robust anti-tumor immune response. Ingenuity pathway analysis of the genes expressed differentially by CD8⁺ TILs from CD103^{hi} tumors relative to their expression by such cells from CD103^{lo} tumors (classified on the basis of the expression of *ITGAE* transcripts (encoding CD103) in CD8⁺ TILs; Supplementary Fig. 6a,b and Supplementary Table 7) indicated cell proliferation and cytotoxicity as the key activated functions (Supplementary Table 8). Consistent with that analysis, the expression of several transcripts encoding products linked to cell cycle and proliferation³² was substantially upregulated in CD8⁺ TILs from CD103^{hi} tumors relative to their expression in such cells from CD103^{lo} tumors (Fig. 5a,b). We confirmed by flow cytometry that CD103⁺CD8⁺ TILs expressed the cell-proliferation marker Ki67 (Fig. 5c). The expression of several transcripts encoding products linked to the cytotoxic function of CD8⁺ T cells (*IFNG*, *GZMA*, *GZMB*, *SEMA7A*, *KLRB1*, *CCL3*, *STAT1*, *RAB27A*, *IL21R* and *FKBP1A*)³² was also significantly upregulated in CD8⁺ TILs from CD103^{hi} tumors relative to their expression in such cells from CD103^{lo} tumors (Fig. 5d,e and Supplementary Table 7). We confirmed at the protein level that CD103⁺CD8⁺ TILs expressed molecules linked to cytotoxicity, such as granzyme B, granzyme A, perforin and CD107a, and produced IFN- γ (Fig. 5f and Supplementary Fig. 7a,b), and demonstrated that

CD103⁺CD8⁺ TILs were the main producers, among CD8⁺ TILs, of both granzyme A and granzyme B (Supplementary Fig. 7c). However, we found no significant difference between CD103⁺CD8⁺ TILs and CD103⁻CD8⁺ TILs from each patient in a comparison of the proportion of cells expressing granzyme A, granzyme B, IFN- γ and CD107a; however, perforin had lower expression in CD103⁺CD8⁺ TILs (Supplementary Fig. 7d). To address the question of whether CD8⁺ TILs from CD103^{hi} tumors had greater effector potential, we compared the mean fluorescence intensity of those molecules and the frequency of cells expressing them in CD103^{hi} tumors relative to that in CD103^{lo} tumors (Fig. 5f and Supplementary Fig. 7e). Notably, we found that CD8⁺ TILs from CD103^{hi} tumors had significantly higher expression of granzyme B than that of CD103^{lo} tumors (Fig. 5f). These results suggested that tumors rich in T_{RM} cells (CD103^{hi} tumors) harbored CD8⁺ T cells that actively proliferated in the tumor milieu and displayed enhanced production of cytotoxic molecules, all hallmarks of robust anti-tumor immunity.

On the basis of the findings reported above, we hypothesized that a high density of CD103⁺ TILs in tumors (tumors enriched for T_{RM} cells) might also confer a survival advantage beyond that previously found to be associated with the density of CD8⁺ TILs^{6,7}. In an independent, large cohort of patients ($n = 689$) with predominantly early-stage lung cancer (83% stage I–IIIA; Supplementary Table 9) monitored from 2007 to 2016, we assessed retrospectively the survival outcome of patients whose tumors were classified on the basis of the density of cells expressing CD8 or CD103 (Supplementary Table 9). A higher density of CD8⁺ TILs was associated with a 28% reduction in mortality ($P = 0.077$ (Cox proportional-hazards model)) and a trend toward improved survival (Fig. 5g). Notably, patients with lung cancer who had CD103^{hi} tumors had significantly lower mortality (34% lower risk of mortality; $P = 0.045$ (Cox proportional-hazards model)) and a better survival outcome than that of patients with CD103^{lo} tumors (Fig. 5h). That finding was also observed in the TCGA data set for lung cancer (Supplementary Fig. 7f). To better understand the dependence on the density of CD103 and CD8 in tumors, we determined the status of the density of cells expressing CD103 (CD103^{hi}, CD103^{int} or CD103^{lo}) in tumors pre-classified on the basis of the density of cells expressing CD8. As expected, the proportion of CD103^{hi} tumors was higher among CD8^{hi} tumors than among CD8^{lo} tumors; however, there was some discordance, as tumors with CD103^{lo} or CD103^{int} status were also observed among CD8^{hi} tumors (Fig. 5i). Notably, even in the subgroup of patients with lung cancer who had a high density of CD8⁺ TILs (CD8^{hi} tumors), patients who had tumors with a higher density of cells expressing CD103 (CD103^{hi} tumors) had significantly lower mortality (60% lower risk of mortality; $P = 0.043$ (Cox proportional-hazards model)) than that of patients with CD103^{lo} tumors and survived significantly longer than did patients with CD103^{lo} tumors (Fig. 5i). Our results suggested that patients with a robust intra-tumoral T_{RM} cell response had better long-term survival outcomes and that this effect was above that conferred by the density of CD8⁺ TILs.

Molecules linked to tumor immune response

Tumors with CD8^{hi} and CD103^{hi} TIL status had higher expression of transcripts encoding molecules shown to be effective targets of immunotherapy, such as *PDCD1*, *TIM3* and *LAG3*, and CD8^{hi} status and CD103^{hi} status were both independently linked to better anti-

tumor immunity and survival outcome. Therefore, we reasoned that other molecules encoded by the list of genes upregulated in tumors with CD8^{hi} and CD103^{hi} TIL status might also have important functions in modulating the magnitude and specificity of anti-tumor immune responses (Fig. 6a and Supplementary Table 7). An example of this was CD39 (encoded by *ENTPDI*), a cell-surface ectonucleotidase that dephosphorylates ATP to AMP (Fig. 6b,c). We found that the expression of CD39 protein was much higher in CD103⁺CD8⁺ TILs than in CD103⁻CD8⁺ TILs (Fig. 6d). High concentrations of ATP in the tumor microenvironment can have toxic effects on cells via signaling through the purinergic receptor P2RX7 (refs. 33,34). Given that CD8⁺ TILs from CD103^{hi} tumors and those from CD103^{lo} tumors exhibited similar expression of transcripts encoding P2RX7 (Fig. 6c), we speculated that the greater abundance of CD39 probably ‘preferentially’ protected T_{RM} cells (CD103⁺CD8⁺ TILs) from ATP-induced cell death. Notably, however, adenosine produced by CD39 might also suppress the function of natural killer T cells, natural killer cells and probably CD8⁺ T cells^{35,36}. CD38 is another ectonucleotidase and type II trans-membrane glycoprotein with various functions, including regulation of adenosine signaling, adhesion and transduction of activation and proliferation signals^{37,38}. Expression of CD38 protein was also higher in CD103⁺CD8⁺ TILs than in CD103⁻CD8⁺ TILs (Fig. 6d). Given that purinergic receptors can be targeted therapeutically, it might be pertinent to determine how CD39 and CD38 modulate ATP and purinergic signaling pathways to influence the development and function of anti-tumor T_{RM} cells (CD103⁺CD8⁺ TILs).

CD8⁺ TILs from CD103^{hi} tumors had higher expression of several transcripts encoding components of the Notch signaling pathway (*NOTCH*, *RBPI*, *DTX2*, *UBC* and *UBB*), relative to their expression in CD8⁺ TILs from CD103^{lo} tumors (Fig. 6b,c), suggestive of an important role for this pathway in boosting T_{RM} cell responses in lung cancer; this speculation is supported by a report showing that the Notch pathway supports the development of T_{RM} cells in the lungs³⁹. CD8⁺ TILs from CD103^{hi} tumors had higher expression of transcripts encoding two transcription factors (BATF and NAB1) potentially linked to CD4⁺ T cell-mediated help of CD8⁺ T cells, relative to their expression in CD8⁺ TILs from CD103^{lo} tumors (Fig. 6b,c).

Other examples of transcripts upregulated in CD8⁺ TILs from CD103^{hi} tumors included *KIR2DL4*, which encodes the killer-cell immunoglobulin-like receptor KIR2DL4 with activating and inhibitory functions⁴⁰; expression of KIR2DL4 protein was confirmed in CD103⁺CD8⁺ TILs (Fig. 6d). HLA-G, a non-classical major histocompatibility complex class I molecule, has been shown to engage KIR2DL4 and increase the production of cytokines and chemokines by natural killer cells⁴¹. Although the expression of HLA-G is highly restricted, several reports have shown that its expression is increased in tumor tissue, especially in lung cancer⁴², so we speculated that HLA-G expressed in tumors might also convey activation signals via the receptor KIR2DL4 to CTLs. *SIRPG* encodes SIRPG, a member of the immunoglobulin superfamily of signal-regulatory proteins that interact with the ubiquitously expressed signal-regulatory protein CD47 (ref. 43). Notably, SIRPG is the only member of that family that is expressed on T cells, and its interaction with CD47 expressed on antigen-presenting cells has been shown to enhance T cell proliferation and IFN- γ production⁴⁴. Given the higher expression of *SIRPG* transcripts in CD8⁺ TILs from CD103^{hi} tumors than in those from CD103^{lo} tumors (Fig. 6b,c), we speculated that SIRPG

might also serve as an important co-stimulatory molecule and that its function could be exploited to enhance the anti-tumor function of CTLs. Several candidate molecules described here have not been fully assessed for their potential as immunotherapeutic targets in cancer; the importance of these molecules in boosting anti-tumor immune responses should be verified in further functional studies.

DISCUSSION

We have taken an unbiased discovery-based approach to identify transcripts for which CD8⁺ TILs showed enrichment and those that were linked to robust anti-tumor immune responses and good outcomes. Published transcriptional studies of anti-tumor CD8⁺ T cells from patients with cancer have been largely restricted to the analysis of whole tumor tissue or of CD8⁺ T cells from peripheral blood or metastatic sites^{8–11}. Our study surveyed over 100 transcriptomes from purified CD8⁺ TILs and N-TILs derived from tumor tissue and the best control tissue available: the adjacent uninvolved lung. Bioinformatics analysis of those data sets revealed a core CD8⁺ TIL transcriptional profile comprising ~1,400 genes that was shared by various tumor subtypes and was distinct from that of N-TILs. This profile suggested extensive molecular reprogramming within the tumor microenvironment and enrichment for presumably TAA-specific cells that were actively proliferating following TCR engagement and co-stimulation, all hallmarks of effective anti-tumor immunity.

Despite our use of purified CD8⁺ TIL populations for our analyses, we observed substantial heterogeneity among patients in their expression of genes encoding molecules involved in the cell cycle, TCR activation, co-stimulation and inhibition. Such underlying molecular heterogeneity in the anti-tumor CTL response might partly explain the variability in clinical responses to the immunological-checkpoint blockers currently available. We propose that baseline transcriptional profiling of purified tumor-infiltrating CTLs might enable the rational selection of immunotherapies. Our strategy of purifying relevant immune-cell populations from relatively small tumor samples and performing ‘micro-scaled’ RNA-Seq assays to generate high-resolution genome-wide data can be readily applied to any accessible tumor type. This approach can thus be used to develop biomarkers of the response to immunotherapy and to discover novel targets for immunotherapy.

Another unique aspect of our study was our evaluation of CD8⁺ TIL transcriptomes relative to TIL density (a feature linked to outcome). This analysis revealed various features linked to robust anti-tumor immune responses, such as TIL density; the most striking of these was tissue residence. CD8⁺ TILs with enrichment for T_{RM} cells (CD103^{hi}) had features of enhanced cytotoxicity and proliferation, which suggested that patients whose tumors had a high density of T_{RM} cell markers, such as CD103, had a more-robust anti-tumor immune response and that this feature in the tumor might independently influence clinical outcome. In a large, independent cohort of patients with lung cancer, we showed that a higher density of cells expressing CD103 was predictive of a better survival outcome. Most notably, we confirmed that this effect was independent of that conferred by the density of CD8⁺ TILs; this finding was biologically relevant and has not been addressed by published studies, to our knowledge^{45–47}. Thus, our study has not only revealed a close link among TIL density, T_{RM} cell features and enhanced survival but has also shed light on the global molecular

features that endow CD8⁺ TILs from T_{RM} cell-rich tumors with robust anti-tumor properties. We speculate that the generation of a robust anti-tumor T_{RM} cell response should be an important goal of vaccination approaches targeting neo antigens or shared tumor antigens.

Since patients with lung cancer who had a high density of CD8⁺ or CD103⁺ TILs had a better survival outcome, our comparison of the transcriptional profiles of CD8⁺ TILs from tumors with either a high density or a low density of cells expressing CD8 or CD103 would probably highlight features linked to the generation of robust anti-tumor immunity. The list of transcripts expressed differentially included those encoding molecules such as PD-1, TIM-3, CTLA-4, LAG-3, CD27, CD8 and OX40, which are effective targets of cancer immunotherapy in humans or in model organisms. Other molecules in that list might also have an important role in modulating the magnitude and specificity of anti-tumor immune response. For example, several promising molecules that we identified, such as CD38, CD39, BATF, NAB1, KIR2DL4, SIPRG and components of Notch signaling, deserve further investigation as immunotherapeutic targets in cancer. BATF has been shown to regulate the metabolism and survival of CD8⁺ T cells and to diminish the inhibited phenotype of CD8⁺ T cells^{48,49}. In a model of infection with lymphocytic choriomeningitis virus, the expression of BATF in CD8⁺ T cells, induced by the cytokine IL-21 derived from CD4⁺ T cells, was shown to be essential for maintaining the effector response of CTLs, and overexpression of BATF restored the effector function of CD8⁺ T cells that had not received help from CD4⁺ T cells⁴⁹. NAB1 is a transcription factor whose mouse homolog (NAB2) is induced in CD8⁺ T cells that have received help from CD4⁺ T cells and is needed to prevent activation-induced cell death of those ‘helped’ CD8⁺ T cells⁵⁰. Thus, we hypothesize that NAB1, which has high sequence homology to NAB2, might also have a similar role in preventing the apoptosis of tumor-infiltrating CTLs and that its increased expression might identify tumors in which CD8⁺ TILs have received help from CD4⁺ T cells. Further functional experiments will be needed to verify the role of these molecules.

Our study has revealed the transcriptional program of CD8⁺ TILs at the tumor site and has identified the inter-patient heterogeneity that presumably underlies the variability in clinical responses to checkpoint blockade. It has provided insight into the molecular mechanisms that govern robust anti-tumor CTL responses and lends support to the proposal that anti-tumor vaccines should be designed to enable the generation of CD8⁺ T_{RM} cells for durable immunity. The ability to perform ‘micro-scaled’ RNA-Seq analysis of purified CD8⁺ TILs from patients’ tumors allowed us to identify gene-expression programs that might inform personalized immunotherapeutic treatment strategies and thereby provide a useful tool for translational application.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

ONLINE METHODS

Patient characteristics and sample processing

The Southampton and South West Hampshire Research Ethics Committee and the Ethics Committee of La Jolla Institute approved the study, and written informed consent was obtained from all subjects. Newly diagnosed, untreated patients with NSCLC and HNSCC (Supplementary Table 1) referred to Southampton University Hospitals NHS Foundation Trust and Poole Hospital NHS Foundation trust, UK, between 2014 and 2016 were prospectively recruited. Freshly resected tumor tissue and matched adjacent non-tumor lung tissue (in the case of patients with NSCLC) was obtained following surgical resection. T cells were isolated from tumor (TILs) or adjacent uninvolved lung (N-TILs) using a combination of mechanical and enzymatic dissociation. In brief, tumor or lung tissue was cut into small fragments and incubated at 37 °C for 15 min in an orbital shaker with 2 ml RPMI-1640 medium (Fisher Scientific) containing 0.15 WU/ml Liberase DL (Roche) and 800 units/ml DNase I (Sigma-Aldrich). Dispersed cells were then passed through a 70-µm filter and centrifuged and were re-suspended in MACS buffer (phosphate-buffered saline containing 2 mM EDTA and 0.5% bovine serum albumin) for sorting or analysis by flow cytometry. For isolating and phenotyping of CD8⁺ T cells from tumor or lung tissue, dispersed cells were first incubated with FcR block (Miltenyi Biotec), then were stained with a mixture of the following fluorescence- conjugated antibodies (each at the concentration recommended by the manufacturer): anti-CD45-FITC (HI30; BioLegend), anti-CD4-PE (RPA-T4; BD Biosciences), anti-CD3-PE-Cy7 (SK7; BioLegend), anti-CD8α-PerCP-Cy5.5 (cSK1; BD Biosciences), anti-HLA-DR-APC (L243; BD Biosciences), anti-CD14-APC-H7 (MφP9; BD Biosciences), anti-CD19-PerCP-Cy5.5 (clone HIB19; BioLegend) and anti-CD20-PerCP-Cy5.5 (clone 2H7; BioLegend). Stained samples were analyzed with a BD FACSaria (BD Biosciences) and FlowJo software (Treestar), and CD8⁺ T cells were sorted into ice-cold TRIzol LS reagent (Ambion)^{51,52}. Phenotypic analysis of CD8⁺ TILs for T_{RM} markers was performed by staining with anti-CD69-BV605 (FN50; BioLegend), anti-CD49a-PE (TS2/7; BioLegend), anti-KLRG1-APC (SA231A2; BioLegend), anti-CD62L-BV510 (DREG-56; BioLegend), anti-CCR7-AF700 (TS2/7; BioLegend) (each at the concentration recommended by the manufacturer). Flow-cytometry analysis of CD8⁺CD103⁺ T cells and intra-cellular assessment of Ki67 were carried out with the following antibodies (each at the concentration recommended by the manufacturer): anti-CD45-FITC (HI30; BioLegend), anti-Ki67-PE (Ki67; BioLegend), anti-CD3-APC-Cy7 (SK7; BioLegend), anti-CD8α-PerCP-Cy5.5 (SK1; BD Biosciences), anti-CD103-APC (Ber-ACT8; BioLegend), anti-PD-1-PE-Cy7 (eBioJ105; eBioscience), anti-4-1BB-Pacific blue (4B4-1; BioLegend). The True-Nuclear Transcription Factor Buffer set (BioLegend) was used for the intracellular staining of Ki67. Flow-cytometry analysis of novel molecules and intracellular assessment of cytotoxic molecules were performed using the following antibodies (each at the concentration recommended by the manufacturer): anti-granzyme A-APC (CB9; BioLegend), anti-granzyme B-PE (REA226; Miltenyi Biotec), anti-Perforin-PE or -BV421 (B-D48; BioLegend), anti-KIR2DL4-PE (mAb33; BD BioLegend), anti-CD38-APC-Cy7 (HB-7; BioLegend), anti-CD39-PE (A1; BioLegend). For cytokine and CD107a assays, CD8⁺ TILs were stimulated *ex vivo* with 20 nM PMA (phorbol 12-myristate 13-acetate) and 1 µM ionomycin for 4 h, and 5 µg/ml brefeldin was added during the final 2 h of

stimulation. Anti-CD107a-PE (H4A3; BioLegend; at the concentration recommended by the manufacturer) was added to the PMA-and-ionomycin stimulation mixture for the final 2 h. Intracellular assessment of interferon- γ was performed using anti-IFNG-BV-421 (4S.B3; BioLegend; at the concentration recommended by the manufacturer) at the end of stimulation. Assays were performed in at least six patients and representative plots are presented. Stained samples were analyzed using a BD FACSCanto II (BD Biosciences). Dead cells were excluded using a LIVE/DEAD Fixable Aqua dead cell stain kit (Life Technologies) or DAPI (4,6-diamidino-2-phenylindole).

Histology and immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded] tumor sections with anti-cytokeratin (AE1/AE3; Dako; pre-diluted 'ready to use'), anti-CD8 α (C8/144B; Dako; 1:100), anti-CD103 (EPR4166(2); Abcam; 1:500), anti-PD-1 (NAT105; Abcam; 1:100) and anti-granzyme B (GrB-7; Dako; 1:50). Assays were performed in at least ten patients and representative staining is presented. TILs were quantified using a Zeiss AxioCam MRc5 microscope (Zeiss) and Zeiss Axiovision software (version 4.8.1.0; Zeiss). An average of ten high-powered fields ($\times 400$) across representative areas of each tumor were counted to account for intratumoral heterogeneity; these were averaged to generate an intratumoral TIL score. Tumors with an average quantification of CD8 $^{+}$ cells in the top one-third or bottom one-third percentile were classified as TIL hi or TIL lo , respectively; the lowest quantification of CD8 $^{+}$ cells in the TIL hi tumors was at least twofold greater than the highest quantification of CD8 $^{+}$ cells in the TIL lo tumors (Supplementary Fig. 4). For overall survival analyses (Fig. 5e–g), tumor-tissue microarrays from patients with NSCLC were stained with anti-CD8 α (C8/144B; Dako; 1:100) or anti-CD103 (EPR4166(2); Abcam; 1:500) and were viewed under low-powered magnification (2.5 $\times$ objective) to determine the density of CD8 $^{+}$ or CD103 $^{+}$ cells, as described previously⁵³.

Survival data and analysis

In an independent large cohort of patients with predominantly early-stage NSCLC ($n = 689$; Supplementary Table 9) followed up from January 2007 to June 2016 (minimum follow up, 3.4 years), we retrospectively analyzed survival according to density of CD8 $^{+}$ or CD103 $^{+}$ TILs. The primary endpoint was overall survival, and survival time was measured from the date of diagnosis until the date of death or date last seen alive. Kaplan–Meier plots (with log-rank tests to determine significance of overall survival; P values in Fig. 5g–i) and unadjusted Cox proportional hazards model (to determine relative risk of death) were used to analyze the survival data, as described previously⁵⁴. Patients were excluded from analysis if survival was <30 d, to exclude the possibility of surgery-related mortality. Survival analysis based on the expression of *ITGAE* transcripts (encoding CD103) in tumor samples from patients with lung adenocarcinoma in the TCGA was derived from the OncoLnc tool (<http://www.oncolnc.org>).

RNA sequencing

Total RNA was purified using a miRNAeasy micro kit (Qiagen) and was quantified as described previously⁵⁵ (on average, $\sim 4,230$ CD8 $^{+}$ T cells per sample were processed for

RNA-Seq analysis). Purified total RNA was amplified according to the smart-seq2 protocol⁵⁵. cDNA was purified using AMPure XP beads (1:1.1 ratio, Beckman Coulter). From this step, 1 ng of cDNA was used to prepare a standard Nextera XT sequencing library (Nextera XT DNA sample preparation kit and index kit, Illumina). Samples were sequenced using HiSeq2500 (Illumina) to obtain 50-bp single-end reads. Quality-control steps were included to determine total RNA quality and quantity, optimal number of PCR pre-amplification cycles, and cDNA fragment size⁵⁶. Samples that failed quality control were eliminated from further downstream steps.

RNA-Seq analysis

RNA-Seq data were mapped against the hg19 reference using TopHat⁵⁷ (v1.4.1., –library-type fr-secondstrand-C) and the RefSeq gene annotation downloaded from the University of California Santa Cruz Genome Bioinformatics site. Sequencing read coverage per gene was counted using HTSeq-count (-m union -s yes -t exon -i gene_id, <http://www-huber.embl.de/users/anders/HTSeq/>). To identify genes expressed differentially by various patient groups, we performed negative binomial tests for paired and unpaired comparisons by employing the Bioconductor package DESeq2 disabling the default options for independent filtering and Cooks cutoff⁵⁸. We considered genes to be expressed differentially by any comparison when the DESeq2 analysis resulted in a Benjamini-Hochberg-adjusted *P* value of <0.05. The Qlucore Omics Explorer 3.2 software package was used for visualization and representation (heat maps, principal component analysis) of RNA-Seq data⁵². Unsupervised hierarchical clustering of samples based on the expression of genes (*n* = 1,000) with the highest variance, which accounted for 20% of the total variance, was performed using DESeq package functions and custom scripts on R (Supplementary Fig. 1c). TCR sequences were retrieved from CD8⁺ T cell RNA-Seq data sets, and the frequency of TCRβ chain clonotypes was determined using default parameters of the MiXCR package⁵⁹ (Supplementary Table 5). The CD103 status of TILs was determined on the basis of the transcript abundance of *ITGAE* (*CD103*) in CD8⁺ TILs. Tumors with CD8⁺ TIL expression of *ITGAE* transcripts in the top one-third or bottom one-third percentile were classified as CD103^{hi} or CD103^{lo}, respectively (Supplementary Fig. 6).

Knowledge-based network generation and pathway analysis

The biological relevance of differentially expressed genes identified by DESeq2 analysis was further investigated using the Ingenuity Pathways Analysis platform. The enrichment of canonical pathways (pre-defined, well-described metabolic and signaling pathways curated from literature reviews) among differentially expressed genes was assessed, with significance determined by right-tailed Fisher's exact test (*P* < 0.05). For network analysis, differentially expressed genes were progressively linked together on the basis of a measure of their interconnection, derived from previously characterized functional interactions.

GSEA

The Qlucore Omics Explorer 3.2 software package was used for GSEA. GSEA was used to further assess significant enrichment of specific biological pathways or signatures in one group relative to that in another group. GSEA determines whether an a priori defined 'set' of genes (such as a signature) show significant cumulative changes in gene expression between

phenotypic subgroups⁶⁰. In brief, all genes are ranked on the basis of their differential expression in one group versus their expression in another group. Next, a running enrichment score (RES) is calculated for a given gene set on the basis of how often its members appear at the top or bottom of the ranked differential list. 1,000 random permutations of the phenotypic subgroups are used to establish a null distribution of RES against which a normalized running enrichment score (NES) and false-discovery-rate-corrected *q* values are calculated using Kolmogorov-Smirnov statistic. GSEA was run with a focused group of gene signatures encoding products related to exhaustion²², lung-cancer-associated T cell signature¹⁵, anergy⁶¹, senescence⁶² and tissue residency²⁵. These gene signatures (Figs. 1d and 4g and Supplementary Table 10) were selected to test the null hypothesis that CD8⁺ T cell groups did not show significant enrichment for different CD8⁺ T cell phenotypes.

Statistical analysis

Comparison between two groups was assessed with two-tailed unpaired or paired Student's *t*-test (Figs. 2f and 6d, Supplementary Figs. 5b and 7c,d) or Mann-Whitney test (Fig. 5f and Supplementary Fig. 7e) or Kolmogorov-Smirnov test (Supplementary Fig. 3c) using GraphPad Prism 6. Spearman correlation coefficient (*r* value) was calculated to assess the significance of correlation of the expression of any two transcripts of interest.

Data availability

Sequencing data are deposited into the Gene Expression Omnibus (accession code GSE90730). Source data files for Figures 5 and 6 and Supplementary Figures 5 and 7 are available online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Core transcriptional profile of CD8⁺ TILs. **(a)** RNA-Seq analysis of genes (one per row) expressed differentially by lung CD8⁺ N-TILs (left; $n = 32$ donors) versus NSCLC CD8⁺ TILs (middle and right; $n = 36$ donors) (pairwise comparison; change in expression of 1.5-fold with an adjusted P value of <0.05 (DESeq2 analysis; Benjamini-Hochberg test)), presented as row-wise z -scores of normalized read counts in CD8⁺ TILs from donors with NSCLC adenocarcinoma (red) or squamous carcinoma (pink) or HNSCC negative (light blue) or positive (dark blue) for human papilloma virus (HNSCC TIL; $n = 41$ donors); each column represents an individual sample; right margin, genes encoding exhaustion-associated molecules (vertical lines group genes upregulated (top) or downregulated (bottom) in NSCLC CD8⁺ TILs relative to their expression in lung CD8⁺ N-TILs). **(b)** Principal-component analysis of CD8⁺ T cell core transcriptomes (symbols) in N-TILs and TILs as in **a** (key); numbers along perimeter indicate principal components (PC1–PC3), and numbers in parentheses indicate percent variance for each. HPV, human papilloma virus. **(c)** RNA-Seq analysis of genes encoding exhaustion-associated molecules (as in **a**) in N-TILs and TILs (key in **b**), presented as reads per kilobase per million (RPKM) mapped as University of California Santa Cruz genome browser tracks (top) or as a summary of the results (bottom; log₂ normalized counts). Each symbol (bottom) represents an individual sample; small horizontal lines indicate the mean ($\pm$ s.e.m.). Above plots, position of exons (including untranslated regions) (dark grey) and introns (light grey) in each gene, as well as the chromosome (Chr) on which the gene is present. **(d)** GSEA of various gene sets (above plots) in the transcriptome of CD8⁺ TILs versus that of CD8⁺ N-TILs from donors with NSCLC, presented as the running enrichment score (RES) for the gene set as the analysis 'walks down' the ranked list of genes (reflective of the degree to which the gene set is over-represented at the top or bottom of the ranked list of genes) (top), the position of the gene-

set members (blue vertical lines) in the ranked list of genes (middle), and the value of the ranking metric (bottom). *P* values, Kolmogorov-Smirnov test. Data are from one experiment with $n = 32$ donors (lung N-TILs), $n = 36$ donors (NSCLC TILs) and $n = 41$ donors (HNSCC TILs).

Figure 2.

Pathways for which CD8⁺ TILs show enrichment. (a) Analysis of canonical pathways from the Ingenuity pathway analysis database (horizontal axis; bars in plot) for which CD8⁺ TILs show enrichment, presented as the frequency of differentially expressed genes encoding components of each pathway that are upregulated or downregulated (key) in CD8⁺ TILs relative to their expression in CD8⁺ N-TILs (left vertical axis), and adjusted *P* values (right vertical axis; line; Fisher's exact test); numbers above bars indicate total genes in each pathway. HBCS, hereditary breast cancer signaling; BRCA, tumor suppressor; RA, rheumatoid arthritis; CHK, checkpoint kinase; APRIL, proliferation-inducing ligand; dTMP,

deoxythymidine monophosphate; NF- κ B, transcription factor; iNOS, inducible nitric oxide synthase. **(b)** Overlap of genes encoding components of the cell-cycle and proliferation pathways in CD8⁺ TILs and in CD8⁺ N-TILs: numbers in parentheses indicate total genes in each pathway; numbers along lines indicate total genes shared by the pathways connected by the line. **(c)** RNA-Seq analysis of *PLK1* (encoding the serine-threonine kinase PLK1), *CCNB1* (encoding cyclin B1), *4-1BB*, *CD27* and *JUN* (encoding the transcription factor c-Jun) in lung N-TILs and NSCLC TILs (key in **f**) (presented as in Fig. 1c). **(d)** Ingenuity pathway analysis of genes upregulated in CD8⁺ TILs relative to their expression in N-TILs (yellow), encoding components of the canonical 4-1BB and CD27 signaling pathways (shape indicates function (key)) in lymphocytes. **(e)** Flow-cytometry analysis of the surface expression of 4-1BB and CD8 on live and singlet-gated CD45⁺CD3⁺ T cells obtained from peripheral blood mononuclear cells (PBMC), lung N-TILs and NSCLC TILs (above plots) from the same patient. Numbers in quadrants indicate percent cells in each throughout; red indicates percent cells among TILs throughout. **(f)** Quantification of clonotypes (average values) among CD8⁺ N-TILs and NSCLC CD8⁺ TILs (key) according to their frequency in each donor (horizontal axis), derived from RNA-Seq analysis of genes encoding TCR β -chains. Each symbol (**c,f**) represents an individual sample; small horizontal lines indicate the mean ($\pm$ s.e.m.). * $P < 0.05$ (unpaired Student's two-tailed t -test). Data are from one experiment (**a–d,f**) or are representative of six experiments (**e**).

Figure 3.

Heterogeneity among targets of immunotherapy. **(a)** RNA-Seq analysis (row-wise normalized counts; bottom key) of various transcripts (left margin; one per row) in CD8⁺ TILs from patients with NSCLC (one per column); above, CD8⁺ TIL density (top row; top left key) and tumor stage (bottom row; top right key) for each patient. **(b)** Principal-component analysis of CD8⁺ TILs from patients with NSCLC with TIL^{lo}, TIL^{int} or TIL^{hi} tumors (middle plot), and expression (key) of the transcripts in **a** in CD8⁺ TILs (plots along perimeter). Each symbol represents an individual patient. **(c)** Correlation of the expression of *PDCD1* transcripts and that of *4-1BB* transcripts (log₂ normalized counts) in NSCLC CD8⁺ TILs (left), and correlation of the expression of *PDCD1* transcripts (middle) or *4-1BB* transcripts (right) and the number of tumor-infiltrating CD8⁺ cells (quantified by immunohistochemistry). Each symbol represents an individual patient (colors match those in **b**, middle). **(d)** Correlation of the expression of *PDCD1* transcripts and that of *4-1BB* transcripts (left), and correlation of the expression of *PDCD1* transcripts (middle) or *4-1BB*

transcripts (right) and that of *CD8A* transcripts in the TCGA lung cancer RNA-Seq data set (extreme outliers are not presented here). Each symbol represents an individual patient ($n = 1,013$). (e) RNA-Seq analysis of *PDCD1*, *4-1BB*, *HAVCR2*, *LAG3* and *TIGIT* in N-TILs and TILs from TIL^{hi} or TIL^{lo} tumors (key) (presented as in Fig. 1c). r values (c,d) indicate the Spearman correlation coefficient; P values (c,d), Spearman correlation. Data are from one experiment.

Figure 4.

Tissue-residency features of TIL^{hi} tumors. (a) RNA-Seq analysis of genes (one per row) expressed differentially (P values as in Fig. 1a) by NSCLC CD8⁺ TILs from TIL^{lo} tumors versus those from TIL^{hi} tumors (presented as in Fig. 1a); right margin, genes encoding exhaustion- and T_{RM} cell-associated molecules. (b) RNA-Seq analysis of *ITGAE*, *CXCR6*, *S1PR1*, *KLF2* and *STK38* (presented as in Fig. 1c). Each symbol (bottom) represents an individual sample; small horizontal lines indicate the mean ($\pm$ s.e.m.). (c) Correlation of the expression of *ITGAE* transcripts ($\log_2$ normalized counts) in NSCLC CD8⁺ TILs (key) and

the number of tumor-infiltrating CD8⁺ cells (quantified by immunohistochemistry) (left), and of the expression of *ITGAE* transcripts and that of *CD8A* transcripts in the TCGA lung cancer RNA-Seq data set (right; extreme outliers not presented here) (*r* values and *P* values as in Fig. 3c,d). Each symbol represents an individual patient (*n* = 36 (left) or *n* = 1,013 (right)). (d) Immunohistochemistry microscopy of CD8α, PD-1 and CD103 (above images) in TIL^{lo} and TIL^{hi} NSCLC tumors (left margin). Scale bars, 100 μm. (e) Flow-cytometry analysis of the surface expression of CD8 and CD103 (top), PD-1 and CD103 (middle) and 4-1BB and CD103 (bottom) on live and singlet-gated T cells obtained from peripheral blood mononuclear cells, lung N-TILs and NSCLC TILs (above plots) from the same patient. (f) Flow-cytometry analysis of the expression of CD69 or CD49a versus that of CD103 (top row, left and middle), and of KLRG1, CD62L or CCR7 versus that of CD103 (bottom row) in live and singlet-gated CD45⁺CD3⁺CD8⁺ T cells; top right, overlay of CD103⁺CD8⁺ TILs (red) with CD103⁺CD8⁺ TILs (blue). (g) GSEA of T_{RM} cell signature genes upregulated (top) or downregulated (bottom) in the transcriptome of CD8⁺ TILs from NSCLC TIL^{hi} tumors relative to their expression in other TILs and N-TILs (presented as in Fig. 1d). (h) Ingenuity pathway analysis of transcripts (perimeter) upregulated in NSCLC TIL^{hi} tumors that are regulated by interferon-γ (orange arrows) and encode products with various functions (key); gray arrow indicates an unpredicted effect of IFN-γ. Data are from one experiment (a–c,g,h) or are representative of ten experiments (d) or six experiments (e,f).

Figure 5.

CD103 density predicts survival in lung cancer. **(a)** RNA-Seq analysis of the expression of genes (one per row) encoding products related to cell cycle and proliferation, by NSCLC CD8⁺ TILs from CD103^{lo} or CD103^{hi} tumors (presented as in Fig. 1a). **(b)** RNA-Seq analysis of *DLGAP5*, *CDC20*, *AURKB*, *CCNB2A* and *BIRC5*, all encoding products linked to cell cycle and proliferation (presented as in Fig. 1c). Each symbol (bottom) represents an individual sample; small horizontal lines indicate the mean ($\pm$ s.e.m.). **(c)** Flow-cytometry analysis of the expression of Ki67 and CD103 in live and singlet-gated CD45⁺CD3⁺CD8⁺ T

cells obtained from peripheral blood mononuclear cells, lung N-TILs and NSCLC TILs (above plots) from the same patient. **(d)** Principal-component analysis of CD8⁺ TILs from patients with NSCLC with CD103^{lo}, CD103^{int} or CD103^{hi} tumors (middle plot), and expression (key) of the transcripts encoding cytotoxicity-related products in CD8⁺ TILs (plots along perimeter). Each symbol represents an individual patient. **(e)** Expression of *GZMB*, *GZMA* and *IFNG* transcripts (log₂ normalized counts) in cells as in **b** (key). **(f)** Expression of granzyme B (geometric mean fluorescence intensity (gMFI)) in CD8⁺ TILs from CD103^{lo} tumors ($n = 5$) or CD103^{hi} tumors ($n = 7$) (top left), and flow-cytometry analysis of the expression of granzyme B, granzyme A, perforin, CD107a (LAMP-1) or IFN- γ versus that of CD103 in live and singlet-gated CD45⁺CD3⁺CD8⁺ T cells obtained from NSCLC TILs. $*P = 0.0025$ (Mann-Whitney test). **(g,h)** Survival of patients ($n = 689$) with lung cancer, with a low density (CD8^{lo}) or high density (CD8^{hi}) of CD8⁺ cells (key) in tumors **(g)** or a low density (CD103^{lo}) or high density (CD103^{hi}) of CD103⁺ cells (key) in tumors **(h)**, presented as Kaplan–Meier curves. NS, $P = 0.086$ **(g)**, and $*P = 0.043$ **(h)** (log-rank test). **(i)** Frequency of CD103^{hi}, CD103^{int} or CD103^{lo} tumors (key) among those pre-classified on the basis of CD8 density (horizontal axis) (left), and survival of patients with lung cancer with CD8^{hi} tumors sub-classified according to the density of CD103-expressing cells (key) (right), presented as Kaplan–Meier curves. $*P = 0.036$ (log-rank test). Each symbol **(e,f)** represents an individual sample **(e)** or patient **(f)**; small horizontal lines indicate the mean ($\pm$ s.e.m.). Data are from one experiment **(a,b,d,e,g–i)** or are representative of six experiments **(c)** or twelve experiments **(f)**.

Figure 6.

Molecules newly linked to the tumor immune response. **(a)** RNA-Seq analysis of genes (one per row) expressed differentially by NSCLC CD8⁺ TILs from CD103^{lo} tumors versus those from CD103^{hi} tumors (presented as in Fig. 1a). **(b)** RNA-Seq analysis (z-scores of normalized counts) of various transcripts (horizontal axes) plotted against that of other transcripts (vertical axes) in NSCLC CD8⁺ TILs from CD103^{hi} or CD103^{lo} tumors (key in **c**). **(c)** Expression of the transcripts in **b** (log₂ normalized counts) in N-TILs or in NSCLC CD8⁺ TILs from CD103^{hi} or CD103^{lo} tumors (key). **(d)** Flow-cytometry analysis of the expression of KIR2DL4, CD38 or CD39 versus that of CD103 in live and singlet-gated CD45⁺CD3⁺CD8⁺ T cells obtained from NSCLC TILs (left), and frequency of CD38⁺ cells or CD39⁺ cells among CD8⁺CD103⁻ TILs or CD8⁺CD103⁺ TILs (key). **P* = 0.0006, CD38⁺ cells, or *P* < 0.0001, CD39⁺ cells (paired Student's two-tailed *t*-test). Each symbol (**b–d**) represents an individual patient (**b**) or sample (**c,d**); small horizontal lines (**c**) indicate the mean (± s.e.m.); diagonal lines (**d**) connect data from the same patient (*n* = 11 donors). Data are from one experiment (**a–c**) or are representative of six experiments (**d**, left) or eleven experiments (**d**, right).