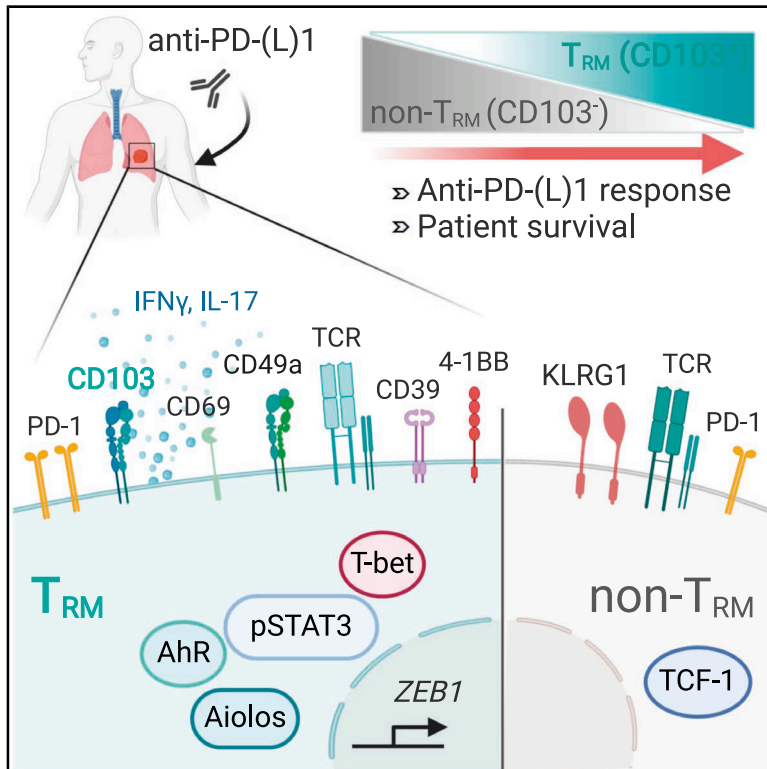


# CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> Cells Accumulate in Tumors of Anti-PD-1-Responder Lung Cancer Patients and Are Tumor-Reactive Lymphocytes Enriched with Tc17

## Graphical Abstract



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## In Brief

Using retrospective cohorts of anti-PD-(L)1-treated NSCLC, Corgnac et al. show that the density of CD103<sup>+</sup>CD8<sup>+</sup> cells in tumors is associated with better progression-free survival. CD103<sup>+</sup>CD8<sup>+</sup> tumor-infiltrating lymphocytes are tumor-specific resident memory T cells (T<sub>RM</sub>) enriched with a subset displaying a unique Tc1/Tc17 differentiation program that differs from CD103<sup>-</sup>CD8<sup>+</sup> T cells (non-T<sub>RM</sub>).

## Highlights

- A high density of CD103<sup>+</sup>CD8<sup>+</sup> cells in tumors correlates with response to anti-PD-(L)1
- The density of CD103<sup>+</sup>CD8<sup>+</sup> cells increases after anti-PD-1 in most responder patients
- CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells are enriched with tumor-specific T cells
- A subset of CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells display a Tc17 differentiation program



## Article

# CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> Cells Accumulate in Tumors of Anti-PD-1-Responder Lung Cancer Patients and Are Tumor-Reactive Lymphocytes Enriched with Tc17

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<https://doi.org/10.1016/j.xcrm.2020.100127>

## SUMMARY

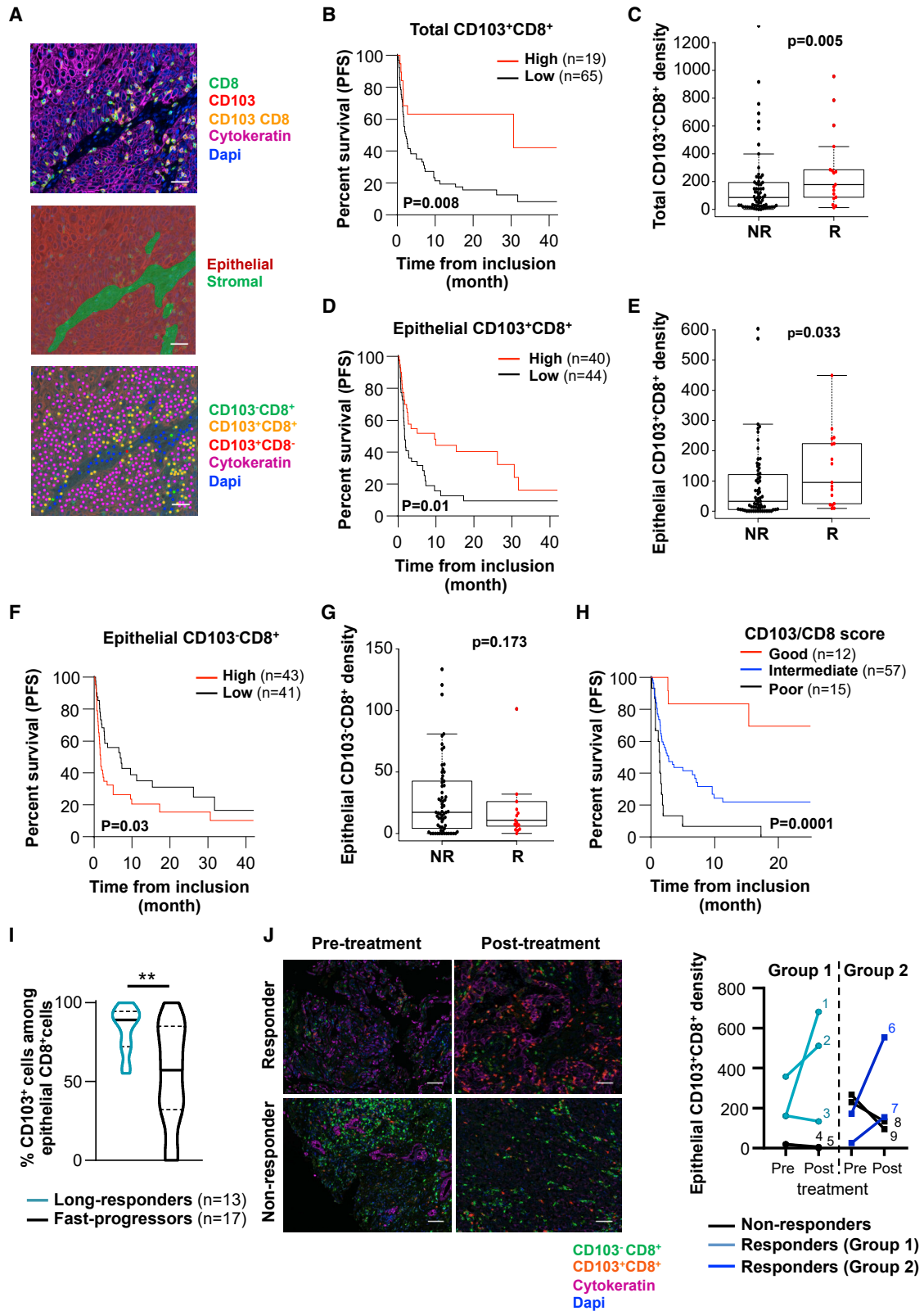
Accumulation of CD103<sup>+</sup>CD8<sup>+</sup> resident memory T (T<sub>RM</sub>) cells in human lung tumors has been associated with a favorable prognosis. However, the contribution of T<sub>RM</sub> to anti-tumor immunity and to the response to immune checkpoint blockade has not been clearly established. Using quantitative multiplex immunofluorescence on cohorts of non-small cell lung cancer patients treated with anti-PD-(L)1, we show that an increased density of CD103<sup>+</sup>CD8<sup>+</sup> lymphocytes in immunotherapy-naïve tumors is associated with greatly improved outcomes. The density of CD103<sup>+</sup>CD8<sup>+</sup> cells increases during immunotherapy in most responder, but not in non-responder, patients. CD103<sup>+</sup>CD8<sup>+</sup> cells co-express CD49a and CD69 and display a molecular profile characterized by the expression of PD-1 and CD39. CD103<sup>+</sup>CD8<sup>+</sup> tumor T<sub>RM</sub>, but not CD103<sup>-</sup>CD8<sup>+</sup> tumor-infiltrating counterparts, express Aiolos, phosphorylated STAT-3, and IL-17; demonstrate enhanced proliferation and cytotoxicity toward autologous cancer cells; and frequently display oligoclonal expansion of TCR-β clonotypes. These results explain why CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> are associated with better outcomes in anti-PD-(L)1-treated patients.

## INTRODUCTION

Effective cancer immunotherapy requires the generation of persistent tumor-reactive cytotoxic T lymphocytes (CTLs) that continuously eliminate primary malignant cells and micro-metastases. Immune checkpoint blockade (ICB), in particular via anti-programmed cell death-1 (PD-1) and anti-PD-L1 neutralizing monoclonal antibodies (mAbs), has led to improved outcomes in non-small cell lung cancer (NSCLC) patients by blocking the interaction of PD-1 inhibitory receptor on tumor-specific T cells with its ligand PD-L1 on target cells, thereby reactivating their anti-tumor effector functions. ICB,

as a single immunotherapy agent, has demonstrated responses in up to 20% of patients with advanced NSCLC who failed platinum-based chemotherapy. In this context, the clinical benefits of anti-PD-(L)1 therapy require that tumors be infiltrated by CD8<sup>+</sup> T lymphocytes expressing high levels of PD-1 and mediating tumor mutation-derived neoantigen-specific responses.<sup>1,2</sup> However, little is known of the nature of these CD8<sup>+</sup> T lymphocytes, whether they belong to the memory pool of T cells, and whether they patrol through blood or are confined to tumor tissues, where they are permanently retained. Recently, a lineage of CD8<sup>+</sup> memory T cells, called resident memory T (T<sub>RM</sub>) cells, was localized in several human





(legend on next page)

solid tumors, including NSCLC, and was associated with a favorable prognosis.<sup>3–8</sup>

T<sub>RM</sub> cells were initially identified in infectious diseases, where they were located at sites of pathogen entry so as to trigger optimized protection against viral re-infections.<sup>9</sup> They are distinct from central memory T (T<sub>CM</sub>) and effector memory T (T<sub>EM</sub>) cells due to their non-recirculating behavior and restricted localization in non-lymphoid peripheral tissues. This memory T cell subset lacks molecules such as S1pr1, Klf2, CCR7, and CD62L, enabling egress from tissue and migration to lymph nodes, and has a unique surface phenotype characterized by the expression of the CD69 activation marker and CD103 ( $\alpha_E$ (CD103)  $\beta_7$ ) and CD49a ( $\alpha_1$ (CD49a)  $\beta_1$ ) integrins, which likely participate in their residency feature by binding to E-cadherin and type IV collagen on epithelial and endothelial cells, respectively.<sup>10,11</sup> T<sub>RM</sub> cells also upregulate lymphocyte function-associated antigen-1 (LFA-1 or  $\alpha_L$ (CD11a)  $\beta_2$ ) integrin, which sustains their residence in liver tissue by binding to intercellular adhesion molecule-1 (ICAM-1) on endothelial cells.<sup>12</sup> Formation and persistence of antiviral T<sub>RM</sub> are regulated by a specific repertoire of transcription factors, including Runx3, Blimp-1, Hobit (homolog of Blimp-1 in T cells), and Notch, and involve a specific set of cytokines, including transforming growth factor  $\beta$  (TGF- $\beta$ ), interleukin-15 (IL-15), type I interferon (IFN), and IL-12.<sup>13–15</sup> T<sub>RM</sub> have been shown to produce pro-inflammatory cytokines such as IFN- $\gamma$  and tumor necrosis factor (TNF) and cytotoxic serine protease granzyme B, which participate in recruiting other immune cells at the sites of infection and mediating cytotoxic function, respectively. With regard to cancer diseases, much less is known about the generation and maintenance of T<sub>RM</sub> at the tumor site and their role in anti-tumor immune responses. Thus far, the phenotypic and functional features of human tumor T<sub>RM</sub> cells and their contribution to T cell immunity to cancer and responses to ICB are poorly understood. In this report, we show that human lung tumor CD8<sup>+</sup> T<sub>RM</sub> cells are a homogeneous CD103<sup>+</sup>CD49<sup>+</sup>CD69<sup>+</sup> population characterized by the expres-

sion of T-bet, phosphorylated (p)STAT-3, and Aiolos transcription factors, and that a subset of them produces IFN- $\gamma$  and IL-17, specific to Tc17 cells. NSCLC CD8<sup>+</sup> T<sub>RM</sub> cells also produce granzyme B, form stable conjugates with autologous tumor cells, and mediate specific cytotoxic activity toward the cognate target. This tumor T<sub>RM</sub> subset, but not its CD103<sup>-</sup>CD8<sup>+</sup> tumor-infiltrating counterpart, proliferate and clonally expand in some tumors, suggesting that it is enriched in tumor-specific CD8<sup>+</sup> T cells. Remarkably, an enhanced CD103<sup>+</sup>CD8<sup>+</sup> subset within the tumor microenvironment (TME) is associated with better progression-free survival (PFS) of anti-PD-(L)1-treated NSCLC patients. This subset is increased upon anti-PD-1 administration in tumors from most responder patients, but not in tumors from non-responder patients, and represents the major CD8<sup>+</sup> tumor-infiltrating lymphocytes (TILs) population in intraepithelial tumor regions. Thus, CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells could be considered potential biomarkers when selecting patients who may benefit from ICB.

## RESULTS

### Accumulation of CD103<sup>+</sup>CD8<sup>+</sup> Cells in Tumors Is Associated with Better Outcomes in Anti-PD-(L)1-Treated NSCLC Patients

To determine the contribution of T<sub>RM</sub> cells to the response to ICB, we established a retrospective tumor cohort (discovery cohort) of 111 patients with advanced NSCLC treated with single-agent anti-PD-(L)1 mAbs (Table S1). Tumor sections from formalin-fixed, paraffin-embedded (FFPE) samples were stained simultaneously with anti-CD8, anti-CD103, and anti-cytokeratin mAbs, and the density and distribution of CD103<sup>+</sup>CD8<sup>+</sup> TIL and CD103<sup>-</sup>CD8<sup>+</sup> TIL in epithelial and stromal regions were assessed using InForm software (Figure 1A). Among the 111 analyzed tumor samples, conclusive staining for the quantification of CD103<sup>+</sup>CD8<sup>+</sup> TIL was obtained for 86 patients. Multiplex fluorescent immunohistochemistry (IHC) revealed that the

#### Figure 1. Increased CD103<sup>+</sup>CD8<sup>+</sup> TILs Are Associated with Improved Outcomes of Anti-PD-1-Treated NSCLC

- (A) Fluorescent IHC image of CD8, CD103, cytokeratin, and DAPI staining in lung tumor section (top). Digital markup image shows epithelial and stromal zones of the tumor section defined by cytokeratin staining (center). Digital markup image shows CD103<sup>+</sup>CD8<sup>+</sup> (yellow), CD103<sup>-</sup>CD8<sup>+</sup> (green), CD103<sup>+</sup>CD8<sup>-</sup> (red), and cytokeratin<sup>+</sup> (pink) cells in lung tumors (bottom). Scale bar, 2 cm.
- (B) Kaplan-Meier curve shows iPFS of anti-PD-1-treated patients with tumors harboring high (>252/mm<sup>2</sup>) (n = 19) or low (<252/mm<sup>2</sup>) (n = 65) densities of CD103<sup>+</sup>CD8<sup>+</sup> cells (n = 84).
- (C) Density of total CD103<sup>+</sup>CD8<sup>+</sup> cells in tumors depending on iORRs of non-responders (NR) (n = 65) and responders (R) (n = 17) patients to PD-1 blockade.
- (D) Kaplan-Meier curve shows iPFS of anti-PD-1-treated patients with tumor epithelial regions harboring a high (>48/mm<sup>2</sup>) (n = 40) or low (<48/mm<sup>2</sup>) (n = 44) density of CD103<sup>+</sup>CD8<sup>+</sup> cells (n = 84).
- (E) Density of CD103<sup>+</sup>CD8<sup>+</sup> cells in epithelial tumor regions depending on iORRs of NR (n = 65) and R (n = 17) patients to anti-PD-1.
- (F) Kaplan-Meier curve shows iPFS of anti-PD-1 blockade-treated patients with epithelial regions of tumors harboring a high (>17/mm<sup>2</sup>) (n = 43) or low (<17/mm<sup>2</sup>) (n = 41) density of CD103<sup>-</sup>CD8<sup>+</sup> cells.
- (G) Density of CD103<sup>-</sup>CD8<sup>+</sup> cells in epithelial tumor regions depending on iORRs of NR (n = 65) and R patients (n = 17) to PD-1 blockade.
- (H) Kaplan-Meier curve shows iPFS of anti-PD-1-treated patients with tumors harboring a satisfactory (CD103<sup>+</sup>CD8<sup>high</sup>/CD103<sup>-</sup>CD8<sup>low</sup>, red, n = 12), intermediate (CD103<sup>+</sup>CD8<sup>high</sup> or CD103<sup>-</sup>CD8<sup>low</sup>, blue, n = 57), or poor (CD103<sup>+</sup>CD8<sup>low</sup>/CD103<sup>-</sup>CD8<sup>high</sup>, black, n = 15) CD103/CD8 score.
- (I) Percentages of CD103<sup>+</sup> cells among CD8<sup>+</sup> TILs in epithelial tumor areas of anti-PD-1-treated patients undergoing a long-response (PFS > 6 months and OS > 12 months; n = 13) or a fast-progression (OS < 12 weeks; n = 17). \*\*p < 0.01.
- (J) Fluorescent IHC images show CD103<sup>+</sup>CD8<sup>+</sup> (orange), CD103<sup>-</sup>CD8<sup>+</sup> (green), cytokeratin<sup>+</sup> (pink), and DAPI<sup>+</sup> (blue) cells in tumors from responder and non-responder patients before and after administration of anti-PD-1. Scale bar, 1 cm. Right, density of CD103<sup>+</sup>CD8<sup>+</sup> cells in epithelial tumor regions of tumors before and after the administration of anti-PD-(L)1 in responder (n = 5) or non-responder (n = 4) patients.
- Each symbol represents an individual patient; horizontal lines correspond to mean  $\pm$  standard deviation (SD) (C, E, and G). p value was determined by log-rank test (B, D, F, and H), Chi-square test (C, E, and G) or unpaired t test (I).

See also Figures S1 and S2, and Tables S1–S6.



density of the CD103<sup>+</sup>CD8<sup>+</sup> TIL subset varied between tumors, ranging from 0 to 1,320 cells/mm<sup>2</sup>, with a median of 103 cells/mm<sup>2</sup>. Most CD103<sup>+</sup>CD8<sup>+</sup> TILs were located within the stroma, with a median density of 153 cells/mm<sup>2</sup>. In epithelial tumor regions, there were fewer lymphocytes than in the stroma (median density of 68 cells/mm<sup>2</sup>); however, 76% of these CD8<sup>+</sup> TILs were CD103<sup>+</sup> (median density of 45 cells/mm<sup>2</sup>), whereas only 48% in the stroma (Table S1). Increased intraepithelial lymphocyte infiltration was particularly observed in tumors with a high density of CD103<sup>+</sup>CD8<sup>+</sup> cells in the stromal compartment (Figure S1A).

We then correlated the survival of patients from the date of the first immunotherapy administration with the score of tumor infiltration by CD8<sup>+</sup> TILs. In this cohort, the objective response rate (ORR) was 20% (n = 17/86), corresponding to responders. As expected, patients with strong CD8 cell (CD8<sup>high</sup>) tumor infiltration had increased survival compared to patients with CD8<sup>low</sup> tumors (hazard ratio [HR] = 0.40, 95% confidence interval [CI] 0.20–0.77, p = 0.006), and median immunotherapy progression-free survival (iPFS) of 9.92 months (95% CI 2.73–not reached) (Figure S1B; Table S2). The density of CD8<sup>+</sup> TIL was also higher in responder patients than in non-responders (Figure S1C). Remarkably, when patients were stratified by their tumor CD103<sup>+</sup>CD8<sup>+</sup> cell infiltration, iPFS dramatically increased in the patient population harboring tumors that were strongly infiltrated by CD103<sup>+</sup>CD8<sup>+</sup> cells (CD103<sup>+</sup>CD8<sup>high</sup>), with median iPFS reaching 30 months (95% CI 2.73–not-reached) (Figure 1B and Table S2). The density of total CD103<sup>+</sup>CD8<sup>+</sup> TILs also correlated with the ORR (Figure 1C). In contrast, patients with tumors slightly infiltrated by CD103<sup>+</sup>CD8<sup>+</sup> cells had only a 2.3-month (95% CI 1.68–5.03) median iPFS. Multivariate analyses showed that tumor infiltration by CD103<sup>+</sup>CD8<sup>+</sup> cells was an independent factor in satisfactory prognosis, based on iPFS, with a HR of 0.39 (95% CI 0.18–0.85, p = 0.01) (Table S3). More important, median iPFS in the CD103<sup>+</sup>CD8<sup>high</sup> population was 20 months longer than in the total CD8<sup>high</sup> population (Table S2). As expected, PD-L1 expression by tumor cells correlates with a better PFS (Table S1). These results highlight the strong benefit of an enhanced CD103<sup>+</sup>CD8<sup>+</sup> cell subset in tumors compared to the heterogeneous CD8<sup>+</sup> subset in the response to anti-PD-(L)1.

### Correlation between Intraepithelial CD103<sup>+</sup>CD8<sup>+</sup> TILs, Tumor E-Cadherin, and ICAM-1 Expression and Response to ICB

Next, we examined CD8<sup>+</sup> TILs in epithelial tumor regions where immune cells are in close contact with tumor cells. Patients with high intraepithelial CD103<sup>+</sup>CD8<sup>+</sup> TILs responded more effectively to anti-PD-(L)1 than patients with low intraepithelial CD103<sup>+</sup>CD8<sup>+</sup> infiltration (HR = 0.52, 95% CI 0.31–0.88, p = 0.01) (Figure 1D). The density of intraepithelial CD103<sup>+</sup>CD8<sup>+</sup> TILs also correlated with the ORR (Figure 1E). Similar results were observed when analyzing total CD8<sup>+</sup> cell infiltrate in the epithelial compartment, predominantly composed of CD103<sup>+</sup>CD8<sup>+</sup> TILs (Figures S1D and S1E). Correlation between an enhanced total (Figures S1F and S1G), but to a lesser extent epithelial (Figures S1H and S1I), CD103<sup>+</sup>CD8<sup>+</sup> subset with better PFS of anti-PD-(L)1-treated patients was confirmed in a second cohort (validation cohort) of 41 NSCLC, including 10 (26%) responder patients (Table S4). In contrast, patients with

increased intraepithelial CD103<sup>+</sup>CD8<sup>+</sup> TIL did not respond efficiently to anti-PD-1, with a HR of 1.74 (95% CI 1.05–2.88, p = 0.03), and no correlation was observed between the density of intraepithelial CD103<sup>+</sup>CD8<sup>+</sup> cells and the ORR (Figures 1F and 1G). Since epithelial tumor region infiltration by either high numbers of CD103<sup>+</sup>CD8<sup>+</sup> cells or low numbers of CD103<sup>+</sup>CD8<sup>+</sup> cells was associated with better outcomes, we stratified the cohort into 3 groups based on these 2 parameters. Using a CD103/CD8 score, we considered a group satisfactory if both parameters were present (CD103<sup>+</sup>CD8<sup>high</sup> and CD103<sup>+</sup>CD8<sup>low</sup>), intermediate if only 1 parameter was present (CD103<sup>+</sup>CD8<sup>high</sup> or CD103<sup>+</sup>CD8<sup>low</sup>), and poor if neither was present (CD103<sup>+</sup>CD8<sup>low</sup> and CD103<sup>+</sup>CD8<sup>high</sup>). Interestingly, the group with a satisfactory CD103/CD8 score had a median PFS of 26.2 months (95% CI 15.3–not-reached) versus 2.7 months (95% CI 1.7–7.3) for the group with an intermediate score and 1.38 months for the group with a poor score (95% CI 0.72–1.97, p < 0.0001) (Figure 1H; Table S2). In addition, anti-PD-1-long-responder patients (PFS > 6 months and overall survival [OS] > 12 months) displayed a higher frequency of CD103<sup>+</sup> cells among CD8<sup>+</sup> TILs than fast-progressor patients (defined by an “early death” occurring during the first 12 weeks after the beginning of ICB) (Figure 1I). A higher density of intraepithelial CD103<sup>+</sup>CD8<sup>+</sup> TILs in anti-PD-1-long-responder patients than in fast-progressor patients was also obtained in the validation cohort (Figure S1J). More important, quantitative multiplex fluorescent IHC staining of tumors collected from 9 patients (5 from the validation cohort and 4 additional patients) before (2–25 weeks) and after anti-PD-1 administration showed an increase in the density of epithelial CD103<sup>+</sup>CD8<sup>+</sup> TILs in 4 of 5 responder patients. In contrast, no expansion of this subset was observed in 4 of 4 non-responder patients (Figure 1J; Table S5). An increase in total CD103<sup>+</sup>CD8<sup>+</sup> and total CD8<sup>+</sup> TILs was also observed in responder patients (Table S5). These results highlight the importance of enhanced intratumoral CD103<sup>+</sup>CD8<sup>+</sup> cells in response to anti-PD-(L)1 and support the conclusion of a role of this subset in patient outcomes.

We then assessed the expression of E-cadherin and ICAM-1, the respective ligands of CD103 and LFA-1 integrins, known to play a major role in strengthening the interaction between CTLs and tumor cells following T cell receptor (TCR) recognition of major histocompatibility complex (MHC) class I-peptide complexes.<sup>16,17</sup> The expression of E-cadherin on cancer cells from 105 evaluable tumor sections of the 111 analyzed NSCLCs was scored into 4 groups: scores 0 and 1 were considered low (12%, n = 13/105) and scores 3 and 4 were high (88%, n = 92/105) (Figure S2A; Table S6). Data analyses indicated that E-cadherin expression does not affect PFS, and no effect on the density of intraepithelial CD103<sup>+</sup>CD8<sup>+</sup> TILs was observed (Figures S2B and S2C). Similarly, according to ICAM-1 expression on tumor cells from 101 evaluable tumor sections of the 111 analyzed tumors, the population was classified into low and high groups (Figure S2D), with no impact observed on survival or infiltration by CD103<sup>+</sup>CD8<sup>+</sup> cells in epithelial regions (Figures S2E and S2F). Nevertheless, for tumors combining an epithelial CD103<sup>+</sup>CD8<sup>high</sup> plus ICAM-1<sup>high</sup> profile, median PFS increased up to 15.3 months (95% CI 9.63–not reached) versus a median PFS of 3.8 months (95% CI 2.56–not reached) observed for the

epithelial CD103<sup>+</sup>CD8<sup>+</sup> plus ICAM-1<sup>low</sup> profile (Figure S2G; Table S2). These results support the observation that CD103 promoted CD8 T cell infiltration within epithelial tumor regions, and suggest that these CD103<sup>+</sup>CD8<sup>+</sup> lymphocytes are tumor-reactive T<sub>RM</sub> cells.

### CD8<sup>+</sup> TILs from NSCLC Are Highly Enriched in CD103<sup>+</sup>CD49a<sup>+</sup>CD69<sup>+</sup> T<sub>RM</sub>

Experiments were next performed to delineate the phenotypic and molecular features of CD103<sup>+</sup>CD8<sup>+</sup> NSCLC TILs, and to determine whether they are a homogeneous population or whether heterogeneity exists in the expression of conventional T<sub>RM</sub> surface markers. With this aim, CD8<sup>+</sup> TILs from 39 freshly resected treatment-naïve early-stage NSCLC tumors were isolated and stained for CD3, CD8, CD103, CD49a, and CD69, as well as the effector T cell (T<sub>Eff</sub>) surface marker KLRG1 (Figure S3A). The results indicated that the percentage of CD8<sup>+</sup> T cells displaying a CD103<sup>+</sup>KLRG1<sup>-</sup> phenotype ranged from 18% to 81%, with a mean of 51%, while the percentage of CD8<sup>+</sup> T cells displaying a CD103<sup>-</sup>KLRG1<sup>+</sup>T<sub>Eff</sub> phenotype ranged from 7% to 52%, with a mean percentage of 26% (Figure 2A). The mean percentages of CD103<sup>+</sup>KLRG1<sup>+</sup> and CD103<sup>-</sup>KLRG1<sup>-</sup> T cell subpopulations were only ~10% and 12% of CD8<sup>+</sup> TILs, respectively. Notably, the expression of CD103 inversely correlated with that of KLRG1 on CD8<sup>+</sup> TILs and appeared mutually exclusive (Figure 2B).

We then further characterized the CD103<sup>+</sup>CD8<sup>+</sup> TIL subset by evaluating the expression of CD69 and CD49a T<sub>RM</sub> markers. The results showed that >90% of CD103<sup>+</sup>CD8<sup>+</sup> T cells expressed CD69 and CD49a (Figures 2C, S3A, and S3B), and that the expression of CD49a correlated with that of CD103 (Figure 2D). In addition, most CD103<sup>+</sup> TILs displayed a CCR7<sup>-</sup>CD45RA<sup>-</sup> profile, which is characteristic of T<sub>EM</sub> cells (Figure S3C). In contrast, only 50% and 35% of KLRG1<sup>+</sup>CD8<sup>+</sup> TILs expressed CD69 and CD49a, respectively (Figure 2C). This T cell subset is more heterogeneous in CD45A expression and is mainly composed of CCR7<sup>-</sup>CD45RA<sup>-</sup> T<sub>EM</sub> and CCR7<sup>-</sup>CD45RA<sup>high</sup> terminally differentiated (EMRA) T cells (Figure S3C). CD103<sup>+</sup>CD49a<sup>+</sup>CD69<sup>+</sup> CD8 T cells, hereafter called T<sub>RM</sub> cells are also present in adjacent healthy lung tissues, but at a lower frequency and lower expression level of CD69 than in autologous tumor tissues (Figures 2E and 2F). In contrast, the KLRG1<sup>+</sup> CD8 T cell subset, hereafter referred to as non-T<sub>RM</sub> cells, is more abundant in healthy lung tissue than in the cognate tumor (Figure 2E). In addition, the number of T<sub>RM</sub> cells per milligram of tissue is much higher in the tumor than in the cognate normal lung, with mean values of ~1,000 T<sub>RM</sub>/mg of tumor versus 100 T<sub>RM</sub>/mg of healthy lung (Figure 2G), with no correlation between the 2 tissue compartments within each patient sample (Table S7). These data support the conclusion that NSCLCs are strongly infiltrated by a homogeneous CD103<sup>+</sup>CD49a<sup>+</sup>CD69<sup>+</sup> CD8 T<sub>RM</sub> subset, which is readily distinguishable from the KLRG1<sup>+</sup> CD8 T<sub>Eff</sub> subset.

### Molecular Features of NSCLC CD8 T<sub>RM</sub> Cells

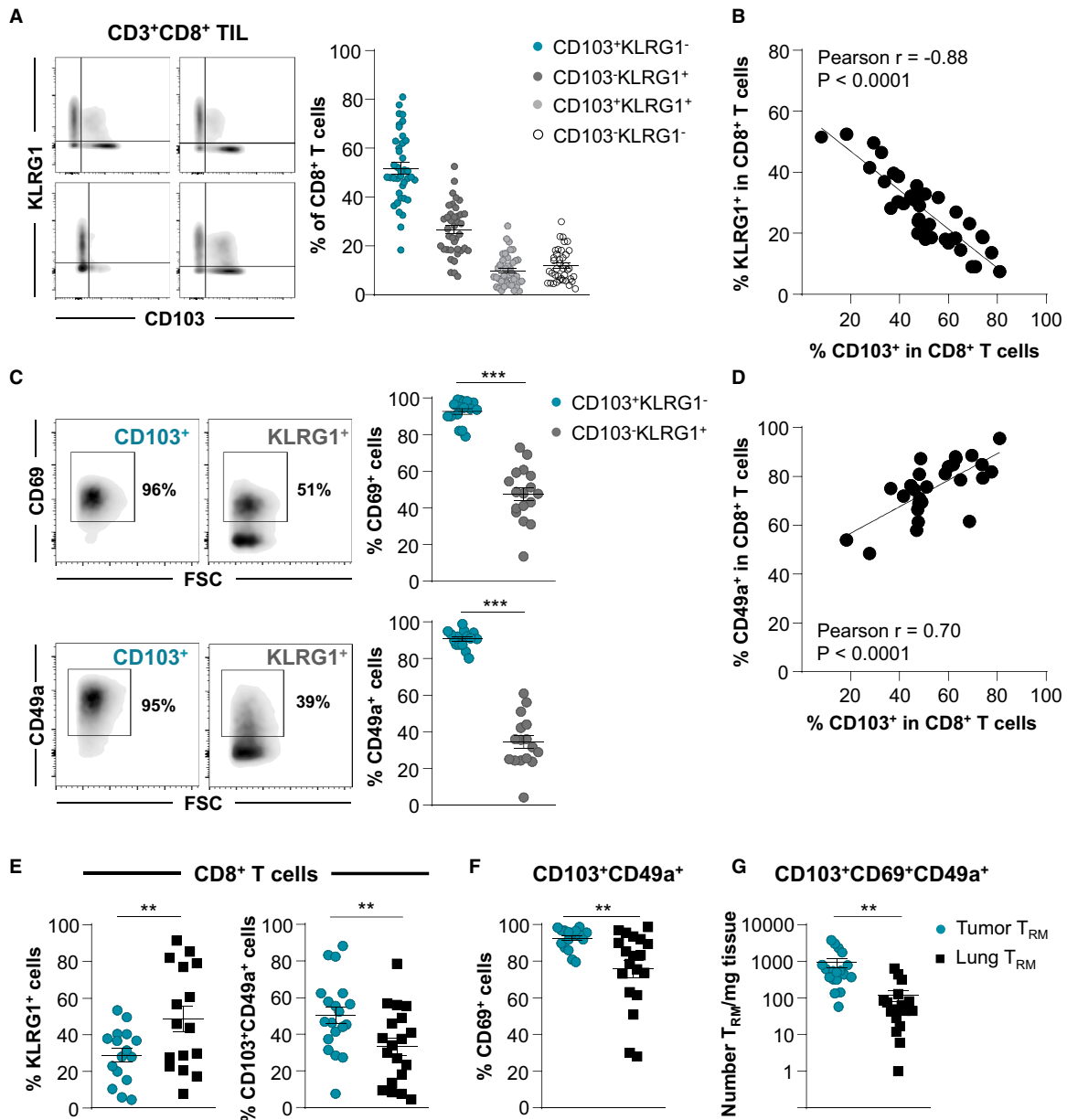
To determine the transcriptional profile of the tumor T<sub>RM</sub> subset, we performed RNA-based next-generation sequencing (RNA-seq) of CD103<sup>+</sup>CD8<sup>+</sup> and matched KLRG1<sup>+</sup>CD8<sup>+</sup> T cells freshly isolated from the TILs of 7 treatment-naïve early-stage NSCLCs.

Gene expression analyses performed with  $p \leq 0.05$  revealed a large number of transcripts ( $n = 2,958$ ) differentially expressed in the 2 T cell subsets, supporting the notion that they correspond to 2 divergent populations (Figure S3D). Among these transcripts, we identified a gene signature that is characteristic of antiviral T<sub>RM</sub> cells and NSCLC T cells,<sup>5,13,18,19</sup> including the upregulation of *CXCR6* and *SIRPG* transcripts (Figure S3E; Table S8). A gene signature with the upregulation of *GPR34*, *ITGAE*, and *CXCR6* genes and the downregulation of *SIPR5* and *SIPR1*, encoding sphingosine phosphate receptors, *KLF2*, encoding Krüppel-like transcription factors that regulate T cell trafficking, and *SELL*, encoding L-selectin CD62L, important in lymphocyte homing to lymphoid organs, was identified in tumor T<sub>RM</sub> cells (Figure S3F; Table S8). The downregulation of S1pr1 in tumor CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells was confirmed by multiparametric flow cytometry, with levels similar to those of healthy lung T<sub>RM</sub> cells (Figure S3G). In addition, gene set enrichment analysis (GSEA) showed that several hallmark gene sets, such as inflammation, cell cycle, TGF- $\beta$  signaling pathways, mammalian target of rapamycin (mTOR), and hypoxia, were enriched in T<sub>RM</sub> cells (Figures S4A and S4B; Table S9).

Among T<sub>RM</sub> signature genes, a panel of genes involved in T cell exhaustion, including *CTLA-4*, *BTLA*, *HAVCR2*, *PDCD1*, *LAG3*, and *TIGIT*, were more strongly expressed in CD103<sup>+</sup> than in KLRG1<sup>+</sup> TIL subsets (Figures 3A and S4C; Table S8). The upregulation of *SPRY1* (Sprouty), *ENTPD1* (CD39), *LAYN* (Layilin), and *TOX* genes was also observed in T<sub>RM</sub> cells (Figure S4C). Flow cytometry analyses confirmed enhanced expression of PD-1 on T<sub>RM</sub> cells from tumors, but not on non-T<sub>RM</sub> cells and T<sub>RM</sub> cells from cognate healthy lung tissue (Figure 3B). Notably, ectonucleotidase CD39 was specifically expressed by T<sub>RM</sub> cells, and its expression was associated with PD-1 (Figures 3C and S4D). Moreover, t-distributed stochastic neighbor embedding (t-SNE) analysis highlighted the strong correlation of expression of CD39 and PD-1 with the T<sub>RM</sub> cluster, whereas the non-T<sub>RM</sub> cluster showed a weak association with these markers (Figure 3D). The expression levels of CD103 on CD8<sup>+</sup> TILs correlated with CD39 and 4-1BB (CD137) levels, and CD103<sup>high</sup> T cells also displayed CD39<sup>high</sup> (Figure 3E) and 4-1BB<sup>high</sup> (Figure 3F) expression profiles characteristic of antigen-experienced T lymphocytes.<sup>20,21</sup> As expected, T<sub>RM</sub> cells from adjacent normal lung expressed only low levels of 4-1BB, excluding the recent engagement of TCR with specific antigen (Figure 3F). These results support the hypothesis that NSCLC CD103<sup>+</sup>CD8<sup>+</sup> TILs were enriched with tumor-reactive T cells harboring all of the features of activated T<sub>RM</sub> cells.

### Lung Tumor T<sub>RM</sub> Cells Express Transcription Factors Involved in Th17 Differentiation

To explore potential pathways involved in T<sub>RM</sub> formation in tumors, we studied genes encoding transcription factors differentially expressed in CD103<sup>+</sup>CD8<sup>+</sup> and KLRG1<sup>+</sup>CD8<sup>+</sup> TILs. RNA-seq analyses indicated that CD103<sup>+</sup>CD8<sup>+</sup> T cells displayed a specific signature characterized by the upregulation of *ZEB1* encoding the zinc-finger E-box binding homeobox-1, *ZNF683* (zinc finger 683) encoding the Blimp1 homolog Hobit, and *PRDM1* (BLIMP1), and the downregulation of *EOMES*, *IKZF2* (*HELIOS*), and *TCF7* genes (Figures 4A, 4B, and S5A; Table S8).



**Figure 2. Expression of CD103, CD49a, and CD69 on CD8 T Cells Infiltrating Treatment-Naive NSCLC and Healthy Lung Tissues**

(A) Freshly resected NSCLC tumors were dissociated and the CD8<sup>+</sup> T cell subset was analyzed for CD103 and KLRG1 expression. Representative dot plots from 4 different TIL samples are included (bi-exponential scale). Right, percentages of CD103<sup>+</sup>KLRG1<sup>-</sup>, CD103<sup>-</sup>KLRG1<sup>+</sup>, CD103<sup>+</sup>KLRG1<sup>+</sup>, and CD103<sup>-</sup>KLRG1<sup>-</sup> TIL subsets among CD3<sup>+</sup>CD8<sup>+</sup> cells (n = 39).

(B) Inverse correlation between percentage of CD103<sup>+</sup> and KLRG1<sup>+</sup> cells among CD8<sup>+</sup> TILs (n = 40). The r value indicates Pearson correlation coefficient.

(C) Expression of CD69 and CD49a on CD103<sup>+</sup> and KLRG1<sup>+</sup> CD8<sup>+</sup> TILs. Representative dot plot from 1 TIL sample is shown. Right, percentages of CD69<sup>+</sup> and CD49a<sup>+</sup> cells among CD103<sup>+</sup> and KLRG1<sup>+</sup> CD8 TILs are presented (n = 17 and n = 16, respectively).

(D) Correlation between percentage of CD103<sup>+</sup> and CD49a<sup>+</sup> cells among CD8<sup>+</sup> TIL (n = 27). The r value indicates Pearson correlation coefficient.

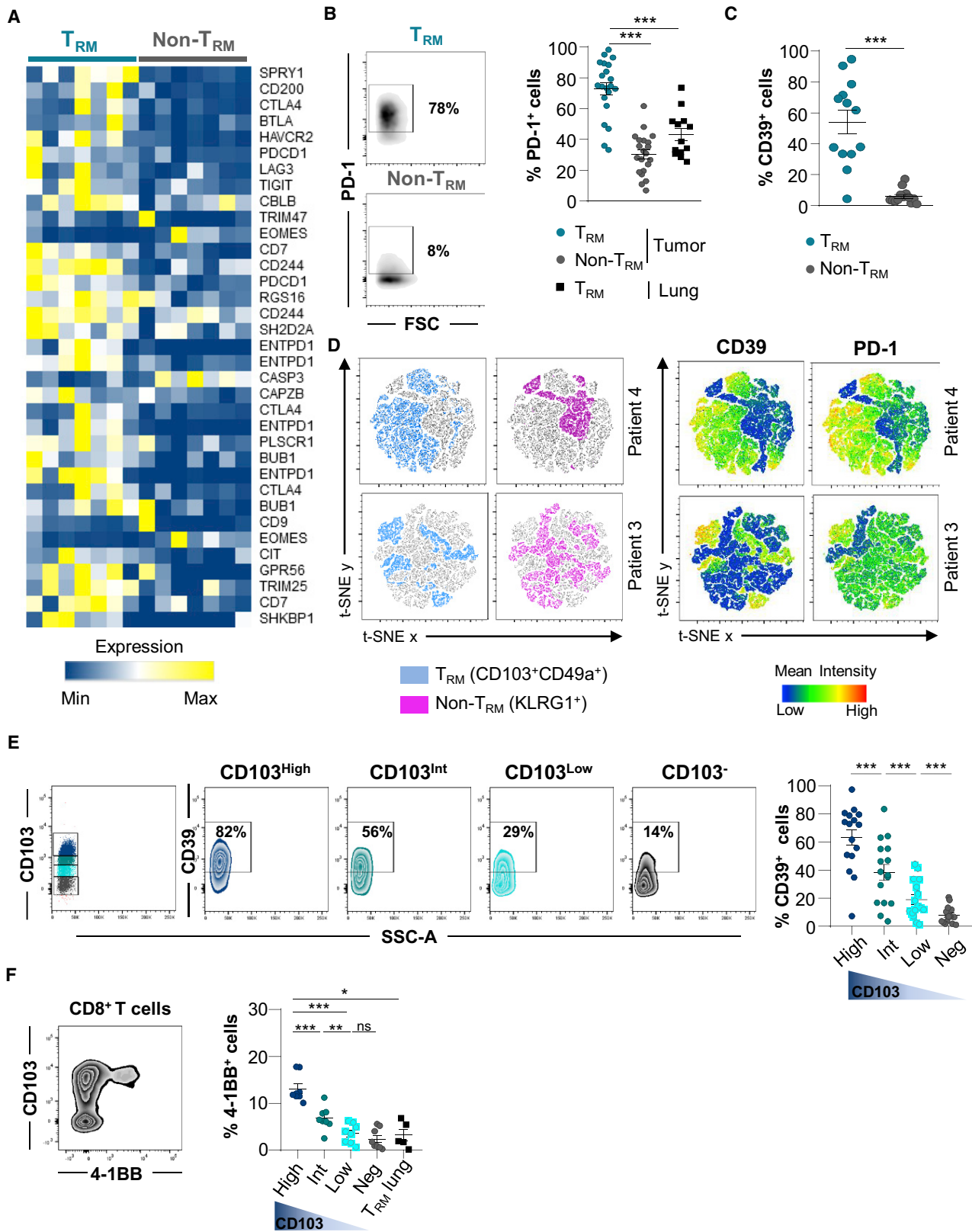
(E) Percentages of KLRG1<sup>+</sup> (n = 16) and CD103<sup>+</sup>CD49<sup>+</sup> cells (n = 19) among CD8<sup>+</sup> T cells infiltrating tumors or autologous healthy lung tissues.

(F) Percentages of CD69<sup>+</sup> cells among CD103<sup>+</sup>CD49a<sup>+</sup> CD8 T cells from tumor or healthy lung tissues (n = 19).

(G) Total numbers of CD103<sup>+</sup>CD69<sup>+</sup>CD49a<sup>+</sup> CD8 (T<sub>RM</sub>) cells per milligram of tumor tissues and paired normal lung tissues (n = 18).

Each symbol represents individual TILs or lung sample; horizontal lines correspond to means ± standard errors of the mean (SEM). \*\*p < 0.01, \*\*\*p < 0.001 (paired t test).

See also [Figure S3](#) and [Table S7](#).



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Remarkably, a set of genes encoding transcription factors involved in Th17 differentiation, such as *IKZF3* encoding the Ikaros transcription factor family member Aiolos, and *AHR* encoding an aryl hydrocarbon receptor, were more strongly expressed in  $T_{RM}$  cells than in non- $T_{RM}$  cells (Figure 4B and S5A). A panel of genes associated with the Aiolos pathway, including *SMARCA4*, *UBE2I*, and *GRAP2*, were also upregulated in  $T_{RM}$  cells (Figure S5B).

Flow cytometry analyses showed that tumor  $T_{RM}$  cells expressed lower levels of Hobit protein than non- $T_{RM}$  cells, but levels higher than in healthy lung  $T_{RM}$  cells (Figure 4C). The downregulation of the *TCF7* gene-encoding protein TCF-1 in tumor  $T_{RM}$  as compared to non- $T_{RM}$  cells was confirmed at the protein level (Figure 4D). In contrast, T-bet and Aiolos transcription factors were more strongly expressed in tumor  $T_{RM}$  than in non- $T_{RM}$  cells, at levels similar to or higher than those in healthy lung  $T_{RM}$  cells (Figure 4E). Moreover, AhR protein and phosphorylated-STAT3 (pSTAT3), also known as the master regulators of Th17 differentiation, were more strongly expressed in  $T_{RM}$  than in non- $T_{RM}$  cells, and at levels similar to those of healthy lung  $T_{RM}$  cells (Figures 4F, 4G, and S5C). Notably, tumor  $T_{RM}$  cells displaying high levels of CD103 (CD103<sup>high</sup>) expressed higher levels of pSTAT3 than  $T_{RM}$  cells with intermediate (CD103<sup>int</sup>) and low (CD103<sup>low</sup>) CD103 expression (Figure 4G). In addition, tumor  $T_{RM}$  cells expressed similar levels of pSTAT3 and Aiolos than healthy donor Th17<sup>+</sup> control cells (Figures 4E and 4G). In contrast, AhR was more strongly expressed in Th17<sup>+</sup> controls than in tumor  $T_{RM}$  cells (Figure 4F). The transcription factor ROR $\gamma$ t was equally expressed by  $T_{RM}$  and non- $T_{RM}$  cells, and the chemokine receptor CCR6 was more strongly expressed in  $T_{RM}$  than in non- $T_{RM}$  cells (Figure S5D; Table S8). These results demonstrate that, together with T-bet, human lung tumor CD8<sup>+</sup>  $T_{RM}$  cells express Aiolos, AhR, and pSTAT3, three master regulators of the Th17 pathway, and ultimately suggest that they are enriched with Tc17 lymphocytes.

### Lung Tumor CD8<sup>+</sup> $T_{RM}$ Cells Contain Tc17 and are Tumor-Reactive CTL

To gain further insight into the molecular characteristics of human lung tumor CD8<sup>+</sup>  $T_{RM}$  cells, we studied the expression pro-

files of genes involved in T cell activation. The results showed that  $T_{RM}$  displayed a signature of more activated lymphocytes than non- $T_{RM}$  cells (Figure 5A). Notably, GSEA highlighted the upregulation of several genes belonging to the Th17/Tc17 cell lineage in  $T_{RM}$  cells (Figure 5B; Table S8). Among these genes, *IL17A* and *IL17RA*, encoding IL-17 and the IL-17 receptor (IL-17R), were more strongly expressed in  $T_{RM}$  than in non- $T_{RM}$  cells (Figure 5C; Table S8). The upregulation of *IL17A* transcript in some  $T_{RM}$ , but not in non- $T_{RM}$  cells, was further confirmed by qRT-PCR using paired CD103<sup>+</sup>CD8<sup>+</sup> and KLRG1<sup>+</sup>CD8<sup>+</sup> T cells from 6 additional lung TILs (Figure S5E). Moreover, intracellular flow cytometry analysis of fresh TILs stimulated *ex vivo* with phorbol myristate acetate (PMA) plus ionomycin showed production of IL-17 by  $T_{RM}$  cells and healthy donor Th17<sup>+</sup> control cells, but not by non- $T_{RM}$  cells (Figure 5D). Remarkably, higher percentages of IL-17-producing cells were observed in CD103<sup>high</sup>  $T_{RM}$  cells than in CD103<sup>int</sup> and CD103<sup>low</sup> cells (Figure S5F), and the expression of CD39 and 4-1BB was frequently higher in IL-17<sup>+</sup> than in IL-17<sup>-</sup>  $T_{RM}$  cells (Figure S5G). ELISA also confirmed the restricted secretion of IL-17 in supernatants from  $T_{RM}$ , but not from non- $T_{RM}$  cells (Figure 5E). In contrast, both non- $T_{RM}$  and  $T_{RM}$  cells from tumors were able to produce high levels of IFN- $\gamma$  and TNF- $\alpha$  following *ex vivo* stimulation with PMA plus ionomycin (Figure 5F). Moreover, a subset of  $T_{RM}$  cells from lung tumors produced both IL-17 and IFN- $\gamma$  (Figure 5G). These IL-17-producing CD8<sup>+</sup> T cells were not TCR- $\gamma\delta$  lymphocytes, which often display a CD4<sup>-</sup>CD8<sup>-</sup> profile and represent ~3.6% of CD3<sup>+</sup> TILs, and only 2.2% of them produced IL-17 (Figures S5H and S5I).

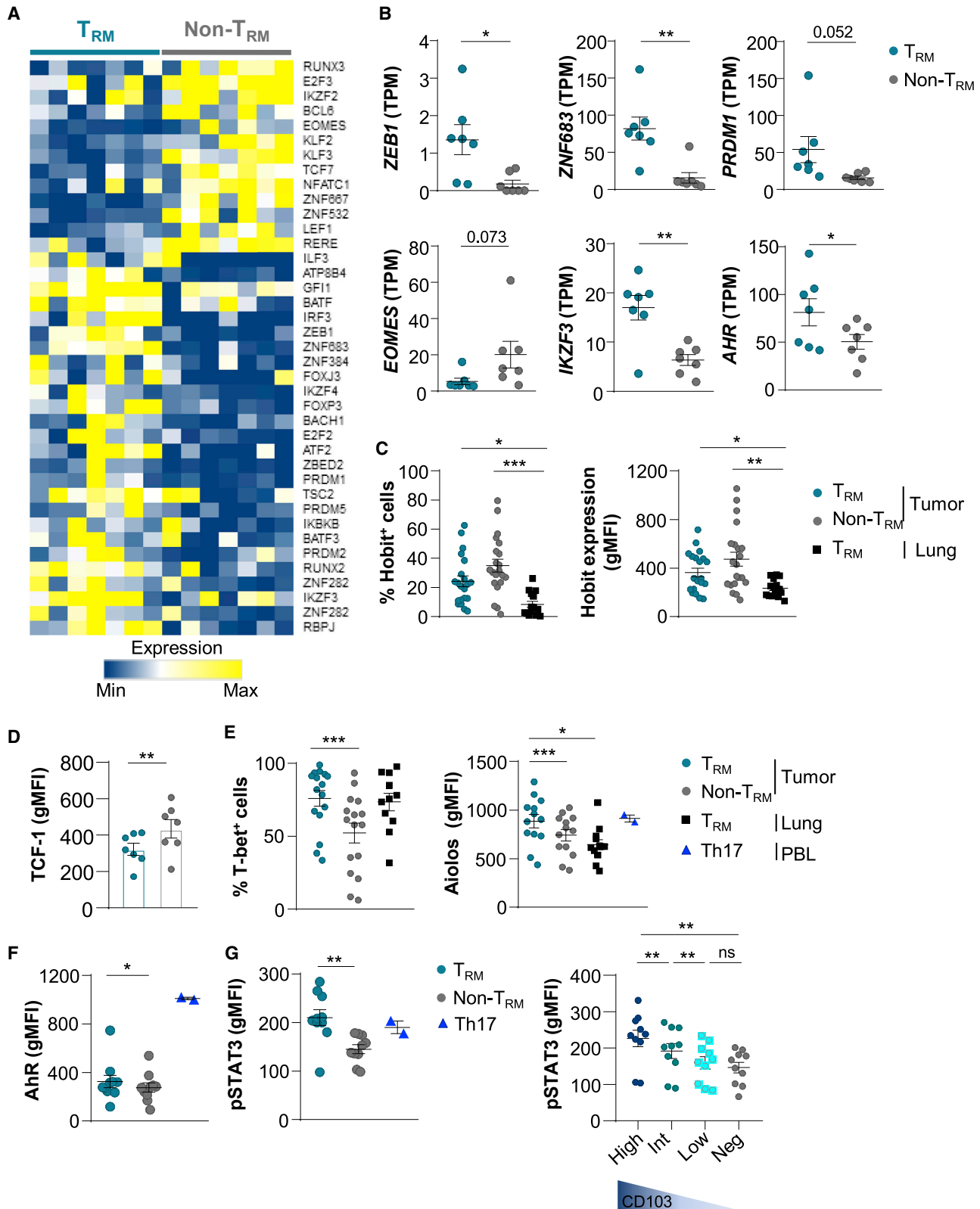
Flow cytometry analyses showed that tumor  $T_{RM}$  cells expressed higher levels of proliferation marker protein Ki67 compared to tumor non- $T_{RM}$  and  $T_{RM}$  cells from adjacent healthy lung (Figure 6A). Intracellular immunofluorescence also revealed that tumor  $T_{RM}$  cells expressed higher levels of granzyme B than non- $T_{RM}$  and healthy lung  $T_{RM}$  cells, supporting the hypothesis that they are tumor-reactive CTLs (Figure 6B). Consistently, further functional studies by confocal microscopy showed that tumor  $T_{RM}$  cells formed more stable conjugates with autologous cancer cells than non- $T_{RM}$  cells, as evaluated by tyrosine phosphorylation (pTyr) at the contact zone between T cells and target cells (Figure 6C). Moreover, tumor  $T_{RM}$  cells

### Figure 3. Expression of T Cell Exhaustion Hallmark in $T_{RM}$ Cells from NSCLC Tumors

- (A) Heatmap of transcripts involved in T cell exhaustion differentially expressed in CD103<sup>+</sup> and KLRG1<sup>+</sup> CD8<sup>+</sup> TILs (n = 7). Different expression patterns correspond to different isoforms of the same gene.
- (B) Expression of PD-1 on CD103<sup>+</sup> and KLRG1<sup>+</sup> CD8<sup>+</sup> TILs. Dot plots of 1 representative patient. Right, percentages of PD-1<sup>+</sup> cells among  $T_{RM}$  and non- $T_{RM}$  (n = 21) and paired  $T_{RM}$  from healthy lung (n = 13).
- (C) Percentages of CD39<sup>+</sup> cells in paired  $T_{RM}$  and non- $T_{RM}$  from NSCLCs (n = 13).
- (D) t-SNE map of CD103<sup>+</sup>CD49a<sup>+</sup> (blue) and KLRG1<sup>+</sup> (pink) cells among CD8<sup>+</sup> TILs. Right, t-SNE analysis of CD39 and PD-1 expression on CD103<sup>+</sup>CD49a<sup>+</sup> ( $T_{RM}$ ) and KLRG1<sup>+</sup> (non- $T_{RM}$ ). The data are from 2 representative TIL samples (patients 3 and 4).
- (E) Dot plots of CD39 expression on CD103<sup>+</sup>CD8<sup>+</sup>  $T_{RM}$ , displaying high (CD103<sup>high</sup>), intermediate (CD103<sup>int</sup>), and low (CD103<sup>low</sup>) CD103 phenotypes, and CD103<sup>-</sup>CD8<sup>+</sup> TIL from 1 representative tumor. Right, percentages of CD39<sup>+</sup> cells among  $T_{RM}$  expressing high, intermediate, and low levels of CD103 and CD103<sup>-</sup>CD8<sup>+</sup> TIL (n = 16).
- (F) Dot plot of 4-1BB expression on CD103<sup>+</sup>CD8<sup>+</sup> TILs from 1 representative tumor. Right, percentages of 4-1BB<sup>+</sup> cells among  $T_{RM}$  displaying high, intermediate, and low CD103 profiles. CD103<sup>-</sup>CD8<sup>+</sup> TIL (n = 7) and CD103<sup>+</sup>CD8<sup>+</sup>  $T_{RM}$  from autologous normal lungs (n = 4) are included. CD103 intensity is shown by a gradient color code.

Symbols represent individual TILs or lung samples; horizontal lines correspond to means  $\pm$  SEMs. \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001 (paired t test or ANOVA with Bonferroni post hoc test); ns, not significant.

See also Figures S3 and S4 and Table S8.



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were able to kill autologous tumor cells much more efficiently than non- $T_{RM}$  cells (Figure 6D). These results further support the conclusion that  $T_{RM}$  cells infiltrating NSCLC tumors are activated tumor-specific cytotoxic effectors.

### Lung Tumor $CD8^+ T_{RM}$ Cells Express a More Clonal TCR Repertoire Than Non- $T_{RM}$ Cells

To assess the clonality of NSCLC  $CD8^+ T_{RM}$  cells, we investigated the TCR- $\beta$  repertoire of 9 freshly sorted  $CD103^+CD8^+$  TILs and autologous  $KLRG1^+CD8^+$  TILs by deep TCR sequencing (TCR-seq). Results indicated that tumor  $T_{RM}$  cells were more oligoclonal than paired non- $T_{RM}$  cells (Figures 7A and S6A). The 10 most frequent (top 10) TCR- $\beta$  clonotypes, defined by hypervariable complementarity-determining region 3 (CDR3) nucleotide sequences, accounted for >60% of the  $T_{RM}$  population and <45% of the non- $T_{RM}$  population (Figure 7B). In contrast, only a marginal difference was observed between TCR- $\beta$  repertoires of  $T_{RM}$  cells from NSCLC tumors and proximal healthy lung (Figure S6B). TCRV- $\beta$  (TCR variable  $\beta$  chain) family distribution within each T cell subset was variable across patients, but it was different between  $T_{RM}$  and non- $T_{RM}$  cells from a given patient (Figure 7C). However,  $T_{RM}$  and non- $T_{RM}$  populations shared some clonotypes, the proportions of which varied from one patient to another and ranged from 4% to 28% (Figures S6C–S6E). Next, we questioned whether the restricted TCR- $\beta$  repertoire observed in  $T_{RM}$  cells correlated with the expression of PD-1, reported to identify the  $CD8^+$  tumor-specific repertoire infiltrating human tumors.<sup>22</sup> The data revealed a correlation between the productive frequency of the top 10 clonotypes and the percentage of  $PD-1^+CD103^+CD8^+$  TILs (Figure 7D), further supporting the conclusion that NSCLC  $CD8^+ T_{RM}$  cells are an oligoclonal subpopulation enriched with activated tumor-specific T cells that underwent antigen-driven expansion at the tumor site. They also explain how this tumor-resident CTL subset can locally participate in anti-tumor  $CD8^+$  T cell immunity and contribute to the response to anti-PD-1 therapy.

## DISCUSSION

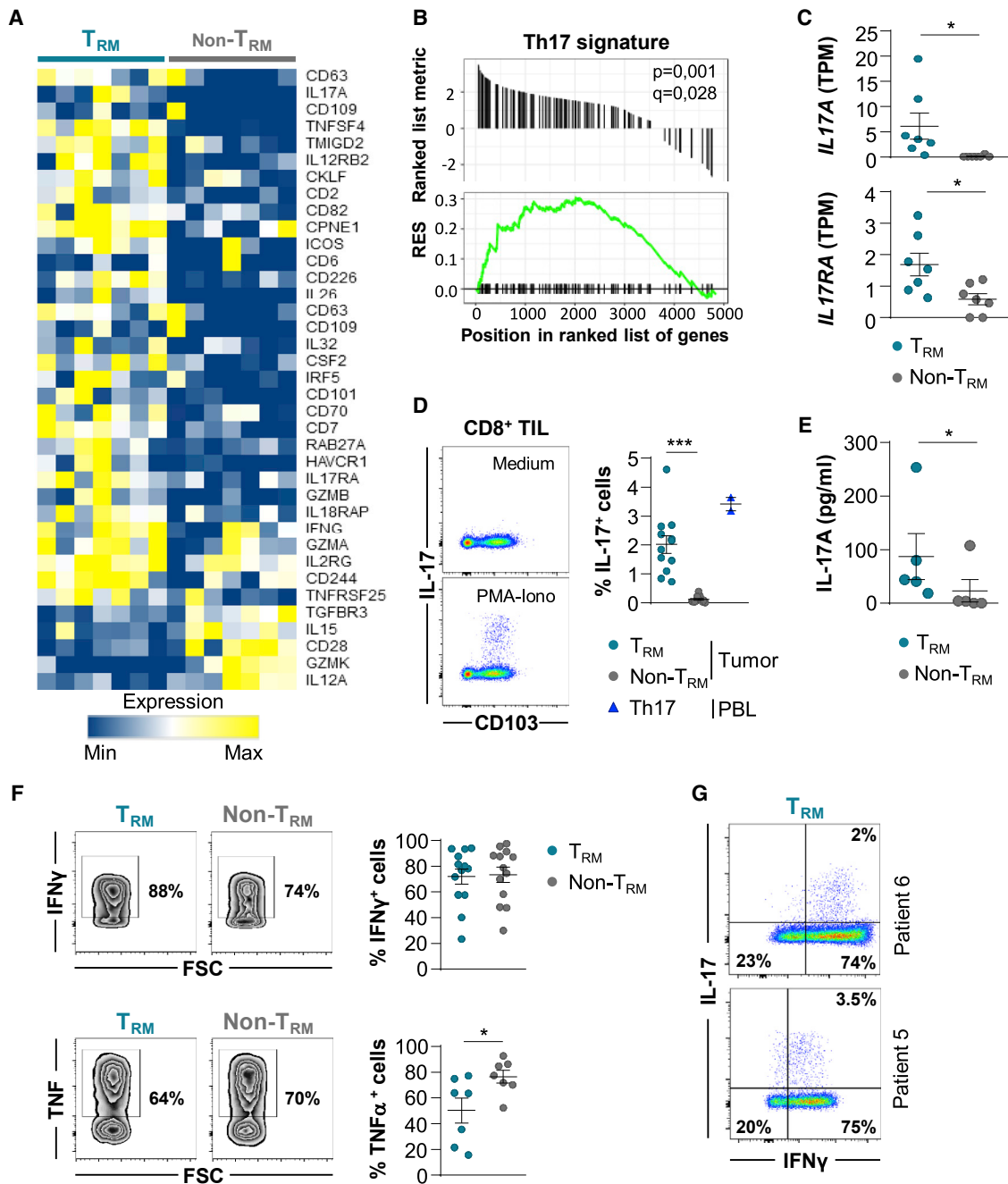
In this report, we show that NSCLC tumors are strongly infiltrated by  $CD8^+ T_{RM}$  cells, most of which express a homogeneous  $CD103^+CD49a^+CD69^+$  phenotype. This  $T_{RM}$  subset was also found in adjacent healthy lung tissues, but at lower densities

than in autologous tumor samples, with no correlation between the frequency of either, suggesting that they are two distinct  $T_{RM}$  subsets. Tumor  $CD103^+CD8^+$  TILs displayed transcriptomic and phenotypic signatures characteristic of  $T_{RM}$  cells, including the downregulation of (1) *Klf2* (regulates genes involved in T cell trafficking such as *SELL*, *CCR7*, and *S1PR1*<sup>23</sup>), (2) *S1pr1* (mediates the egress of T cells from lymphoid organs<sup>24</sup>), and (3) *CD62L* and *CCR7* (key regulators of T cell migration to lymphoid organs) and the upregulation of (1) *CD103* (favors T cell recruitment and retention in epithelial tumor islets through binding to its ligand, epithelial cell marker E-cadherin<sup>25</sup>), (2) *CD49a* (responsible for retaining  $CD8^+$   $T_{RM}$  cells in peripheral tissues via attachment to the extracellular matrix<sup>11</sup>), and (3) *CD69* C-type lectin (modulates the  $T_{RM}$  cell capacity to exit non-lymphoid tissues through interaction and degradation of *S1pr1*<sup>26</sup>). NSCLC  $CD8^+ T_{RM}$  cells also overexpress several T cell inhibitory receptors and exhaustion surface markers, with co-expression of PD-1 and CD39, supporting the conclusion that they are enriched in activated tumor antigen-reactive T cells. In this regard, it has been demonstrated that PD-1 accurately identifies the repertoire of clonally expanded tumor-specific  $CD8^+$  T cells.<sup>22</sup> The expression of CD39, together with *CD103*, has been reported to identify truly tumor-reactive  $CD8^+$  T cells in human solid tumors<sup>20</sup> and to distinguish tumor antigen-specific  $CD8^+$  T cells from viral antigen-specific bystander T cells present in the TME.<sup>27</sup> The *TOX* gene, recently identified in tumor-reactive murine T cells and in human  $PD1^{hi}CD39^{hi}$  TILs,<sup>28</sup> was also upregulated in human NSCLC  $T_{RM}$  cells, further indicating that they are enriched in tumor-specific T cells. NSCLC  $T_{RM}$  cells also frequently expressed 4-1BB (*CD137*) co-stimulatory receptor characteristics of antigen-experienced TILs.<sup>21</sup> These molecular features likely cooperate in retaining  $CD8^+ T_{RM}$  cells in tumor tissues to detect and mount robust local immune responses to transformed cells.

T-bet, together with Eomes T-box transcription factors, were demonstrated to control the development and survival of skin and lung  $CD103^+ T_{RM}$  cells by controlling TGF- $\beta$  and IL-15 expression.<sup>29</sup> However, while extinguishing Eomes is necessary for  $CD103^+CD8^+ T_{RM}$  cell formation, residual T-bet expression is required for IL-15-mediated  $CD103^+CD8^+ T_{RM}$  survival. Consistently, we show that lung tumor  $CD103^+CD8^+ T_{RM}$  cells express T-bet at levels similar to healthy lung  $CD103^+CD8^+ T_{RM}$ , but they only weakly express Eomes. *ZEB1* was strongly

### Figure 4. Transcription Factor Profiles of $T_{RM}$ and non- $T_{RM}$ from NSCLCs

(A) Heatmap of transcripts encoding transcription factors differentially expressed in  $CD103^+$  and  $KLRG1^+ CD8^+$  TIL (n = 7) (p < 0.05).  
 (B) Expression of *ZEB1*, *ZNF683* (HOBIT), *PRDM1* (BLIMP1), *IKZF3* (AILOLOS), *EOMES*, and *AHR* genes in  $T_{RM}$  and non- $T_{RM}$  from NSCLCs (n = 7). Values are transcripts per million (TPM).  
 (C) Percentages of *Hobit*<sup>+</sup> cells among  $T_{RM}$  and non- $T_{RM}$  cells from NSCLCs (n = 21) and among healthy lung  $T_{RM}$  cells (n = 15). Right, expression of *Hobit* (gMFI) in  $T_{RM}$  and non- $T_{RM}$  cells from NSCLCs (n = 21) and in healthy lung  $T_{RM}$  cells (n = 15).  
 (D) Expression of TCF-1 (gMFI) in tumor  $T_{RM}$  and non- $T_{RM}$  cells (n = 7).  
 (E) Percentages of T-bet<sup>+</sup> cells among tumor  $T_{RM}$  and non- $T_{RM}$  cells (n = 21) and among healthy lung  $T_{RM}$  cells (n = 11). Right, expression of *Aiolos* (gMFI) among tumor  $T_{RM}$  and non- $T_{RM}$  and paired healthy lung  $T_{RM}$  (n = 13). Healthy donor Th17 control cells are included (n = 2).  
 (F) Expression of *Ahr* (gMFI) in tumor  $T_{RM}$  and non- $T_{RM}$  (n = 10), and in Th17<sup>+</sup> control cells (n = 2).  
 (G) Expression of pSTAT3 (gMFI) in tumor  $T_{RM}$  and non- $T_{RM}$  cells (n = 10), and in Th17<sup>+</sup> controls (n = 2). Right, expression of pSTAT3 (gMFI) in tumor  $T_{RM}$  cells displaying  $CD103^{high}$  (High),  $CD103^{int}$  (Int), and  $CD103^{low}$  (Low) phenotype, and in  $CD103^+ CD8^+$  TILs (Neg) (n = 10).  
 Each symbol represents an individual donor; horizontal lines are means  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001 (paired t test or ANOVA with Bonferroni post hoc test). gMFI, geometric mean fluorescence intensity; ns: not significant.  
 See also Figure S5 and Tables S8 and S9



**Figure 5. A Subset of Tumor CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> Cells Displays a Tc17-Polarized Pattern**

(A) Heatmap of activation gene signature differentially expressed in paired CD103<sup>+</sup> and KLRG1<sup>+</sup> CD8<sup>+</sup> TIL (n = 7) (p < 0.05).  
 (B) GSEA of gene set from the Th17 signature in the transcriptome of CD103<sup>+</sup>CD8<sup>+</sup> TILs relative to KLRG1<sup>+</sup>CD8<sup>+</sup> TILs (n = 7). Running enrichment score (RES) for the gene set as the analysis “walks down” the ranked list of genes; the position of gene set members (black vertical lines) in the ranked list of genes and the value of ranking metric are shown.  
 (C) Expression of *IL17A* and *IL17RA* transcripts encoding IL-17 and IL-17R, respectively, in tumor T<sub>RM</sub> and non-T<sub>RM</sub> cells (n = 7). TPMs are shown.  
 (D) Intracellular expression of IL-17 in CD8<sup>+</sup> TILs stimulated for 4 h with PMA plus ionomycin. One representative dot plot is shown. Right, percentages of IL-17<sup>+</sup> cells among T<sub>RM</sub> and non-T<sub>RM</sub> cells from tumors (n = 12). Healthy donor Th17<sup>+</sup> control cells are included (n = 2).  
 (E) Production of IL-17 measured by ELISA in supernatant of tumor T<sub>RM</sub> and non-T<sub>RM</sub> cells stimulated overnight with PMA plus ionomycin. The data are concentrations of IL-17 in picograms per milliliter per 100,000 cells (n = 5).  
 (F) Intracellular expression of IFN- $\gamma$  and TNF- $\alpha$  in CD8<sup>+</sup> TILs stimulated with PMA plus ionomycin. The dot plots from 1 representative patient are shown. Right, percentages of IFN- $\gamma$ <sup>+</sup> (n = 13) and TNF- $\alpha$ <sup>+</sup> (n = 7) cells among T<sub>RM</sub> and non-T<sub>RM</sub> cells from tumors.

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expressed in human NSCLC CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells, and this expression is likely induced by TGF- $\beta$  at the tumor site. In this regard, Zeb1 has emerged as a critical transcription factor for memory T cell survival and function.<sup>30</sup> At least a subset of NSCLC CD8<sup>+</sup> T<sub>RM</sub> cells displays a transcription factor profile characteristic of Th17/Tc17 lymphocytes, with expression of Aiolos, pSTAT3, and AhR. The upregulation of Aiolos by STAT3 and AhR was reported to promote Th17 differentiation by silencing IL-2 expression.<sup>31</sup> In contrast, *TCF7/TCF-1* was less abundant in NSCLC T<sub>RM</sub>s than in non-T<sub>RM</sub> cells. The downregulation of *TCF7/TCF-1* in CD103<sup>+</sup>CD8<sup>+</sup> TILs is consistent with previous single-cell analyses of T<sub>RM</sub> cells from breast cancer and NSCLC.<sup>7,8</sup> Exhausted PD1<sup>+</sup>CD8<sup>+</sup> TILs from human melanoma are also *TCF7*<sup>neg</sup> and enriched with CD39- and CD103-expressing cells.<sup>20,32</sup> Notably, the activation of TCR leads to the downregulation of TCF-1 in human CD8<sup>+</sup> T cells,<sup>33</sup> suggesting recent TCR engagement of TCF-1<sup>low</sup> NSCLC T<sub>RM</sub> cells. These TCF-1<sup>low/neg</sup> CD8<sup>+</sup> cells, as opposed to bystander TCF-1<sup>+</sup> TILs, are tumor-reactive CTLs,<sup>8,20,32,34</sup> highlighting their anti-tumor potential and suggesting that TCF-1-expressing (stem-like or memory precursor-like) cells are not the only source of T cell expansion following ICB,<sup>35–40</sup> and that TCF-1<sup>low</sup> (T<sub>RM</sub>) cells are also reactivated to participate in the response to anti-PD-1.

*TCF7* emerges as a central transcription factor in CD8<sup>+</sup> T<sub>RM</sub> differentiation in IL-17-producing lymphocytes, as its downregulation results in enhanced Tc17-cell development.<sup>41</sup> Consistently, our functional experiments showed that a small subset of NSCLC CD8<sup>+</sup> T<sub>RM</sub> cells produces IL-17 after PMA/ionomycin stimulation at frequencies similar to healthy donor Th17/Tc17-polarized T cells. Human CD8<sup>+</sup>CD49a<sup>-</sup> T<sub>RM</sub> cells were also found to produce IL-17 in skin epithelia and psoriasis lesions, promoting local inflammation.<sup>42</sup> Differentiation of a subpopulation of CD8<sup>+</sup> T<sub>RM</sub> cells into the Tc17 lineage is likely associated with the secretion of IL-6 and TGF- $\beta$  by lung tumor cells themselves and the inhibition of IFN $\gamma$ , conditions known to promote the formation of murine CD4<sup>+</sup> Th17 lineage.<sup>43</sup> Murine Tc17/IFN $\gamma$  CD8<sup>+</sup> T cells mediate improved the anti-tumor immunity that is associated with enhanced survival.<sup>44</sup> A small subset of NSCLC CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells also produces IL-17 and IFN- $\gamma$ , displays increased proliferative potential, and mediates cytotoxic function toward autologous cancer cells correlated with the production of granzyme B and the formation of stable conjugates with the cognate target.

The notion that CD103 is a marker of antigen-specific T cells is supported by studies of antiviral and anti-tumor CTL responses, demonstrating that the integrin is induced on CD8<sup>+</sup> T cells specific to influenza virus in the lung,<sup>45</sup> Epstein-Barr virus (EBV) in the tonsil,<sup>46</sup> cancer testis antigen NY-ESO-1 in ovarian cancer,<sup>47</sup> and the  $\alpha$ -actinin 4 (actn-4) neoepitope in NSCLC.<sup>48</sup> The tumor antigen specificity of CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells was further emphasized by TCR-seq analyses revealing

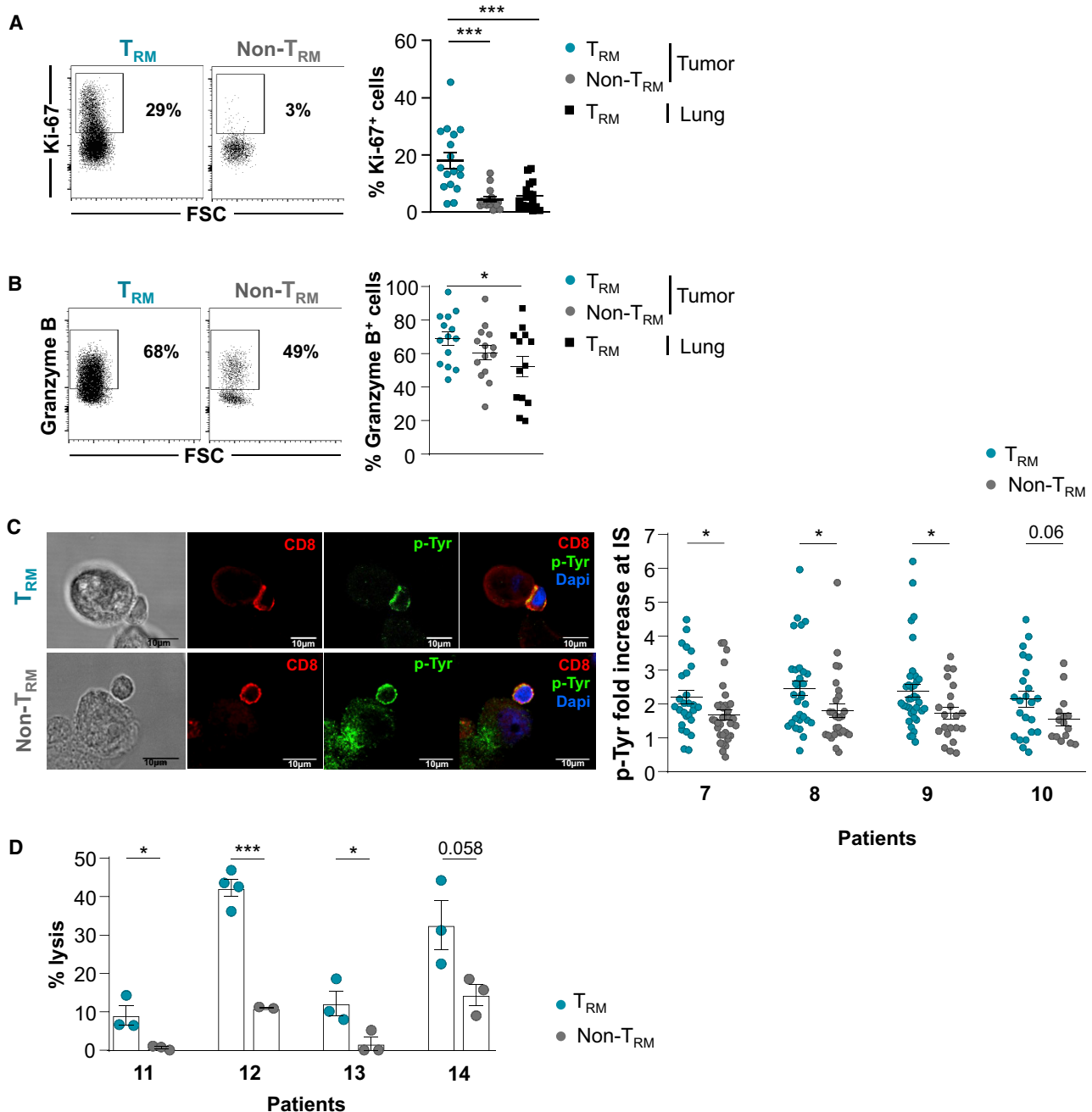
the expansion of particular TCR clonotypes in CD103<sup>+</sup>CD8<sup>+</sup> TILs, but to a lesser extent in KLRG1<sup>+</sup>CD8<sup>+</sup> TILs. TCR clonality in tumor CD8<sup>+</sup> T<sub>RM</sub> cells correlated with high PD-1 expression, which reflects their strong activation state and suggests high TCR avidity for the tumor antigen, resulting in antigen-driving T cell proliferation. Remarkably, CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells were shown to expand during anti-PD-1 immunotherapy of melanoma<sup>49</sup> and lung carcinoma patients.<sup>8</sup> Here, we provide evidence that a high density of CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells in tumors was associated with greatly improved PFS in NSCLC patients treated with anti-PD-(L)1 as a single agent. We also provide evidence that the density of CD103<sup>+</sup>CD8<sup>+</sup> TIL was increased in tumors after anti-PD-1 treatment, suggesting that PD-1 blockade reactivates this tumor-specific T<sub>RM</sub> subset, which proliferates and exerts cytotoxic activity so as to participate in anti-tumor immunity and response to ICB. The reason for intertumor T<sub>RM</sub> density variation is not understood, but it may reflect interpatient variations in the number of antigen-specific T cell precursors, the tumor mutational burden, or the amount of active TGF- $\beta$  required for CD103 expression in TCR-engaged T lymphocytes.<sup>16,48,50</sup> In regard to epithelial tumor regions, enhanced infiltration by CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells did not correlate with E-cadherin expression levels by cancer cells. Consistent results were obtained in ovarian cancer,<sup>3</sup> but not in bladder cancer, where intratumoral infiltration of CD103<sup>+</sup> TIL was associated with the expression of E-cadherin on tumor cells.<sup>51</sup> Although the enhanced expression of ICAM-1 on cancer cells was not associated with the increased density of CD103<sup>+</sup>CD8<sup>+</sup> TIL in epithelial tumor islets, the combination with high T<sub>RM</sub> infiltration improved patient PFS. This may be associated with strong adhesion between tumor cells and specific T<sub>RM</sub> cells provided by the ICAM-1-LFA-1 interaction and required for effective target cell lysis by activated CTLs.<sup>17,52</sup> In contrast, the infiltration of epithelial tumor regions by CD103<sup>-</sup>CD8<sup>+</sup> TIL did not result in the improved survival of anti-PD-1-treated patients. This is surprising, since previous studies reported that CD8<sup>+</sup> T cell infiltration is associated with better clinical outcomes and response to ICB.<sup>1</sup> Although the status of CD8<sup>+</sup> TILs for CD103 expression was not investigated, these T cells likely expressed the integrin, since it is required for their recruitment and functionality within epithelial tumor regions.<sup>53</sup>

Overall, our study demonstrates a major role for CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells in anti-tumor immunity and the response to anti-PD-1 in lung cancer. It also delineates CD103 as a biomarker that is able to accurately identify the repertoire of clonally expanded tumor-reactive CD8<sup>+</sup> T cells. These CD103<sup>+</sup> CD8<sup>+</sup> T cells are activated, proliferate, a subset of which can produce IL-17 and IFN $\gamma$ , and effectively kill autologous malignant cells. Thus, investigating CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells in cancer patients not only contributes to our understanding of the local anti-tumor CTL response but it also provides insight into the role of this T cell population in response to ICB. Improved responses to anti-PD-1 in patients with high

(G) Intracellular co-expression of IL-17 and IFN- $\gamma$  in tumor T<sub>RM</sub> cells stimulated with PMA plus ionomycin. Two representative patients are shown.

Symbols represent individual TILs or lung samples; horizontal lines are means  $\pm$  SEMs (C, D, E, and F). \* $p < 0.05$  and \*\*\* $p < 0.001$  (paired t test or ANOVA with Bonferroni post hoc test).

See also [Figure S5](#) and [Table S8](#).



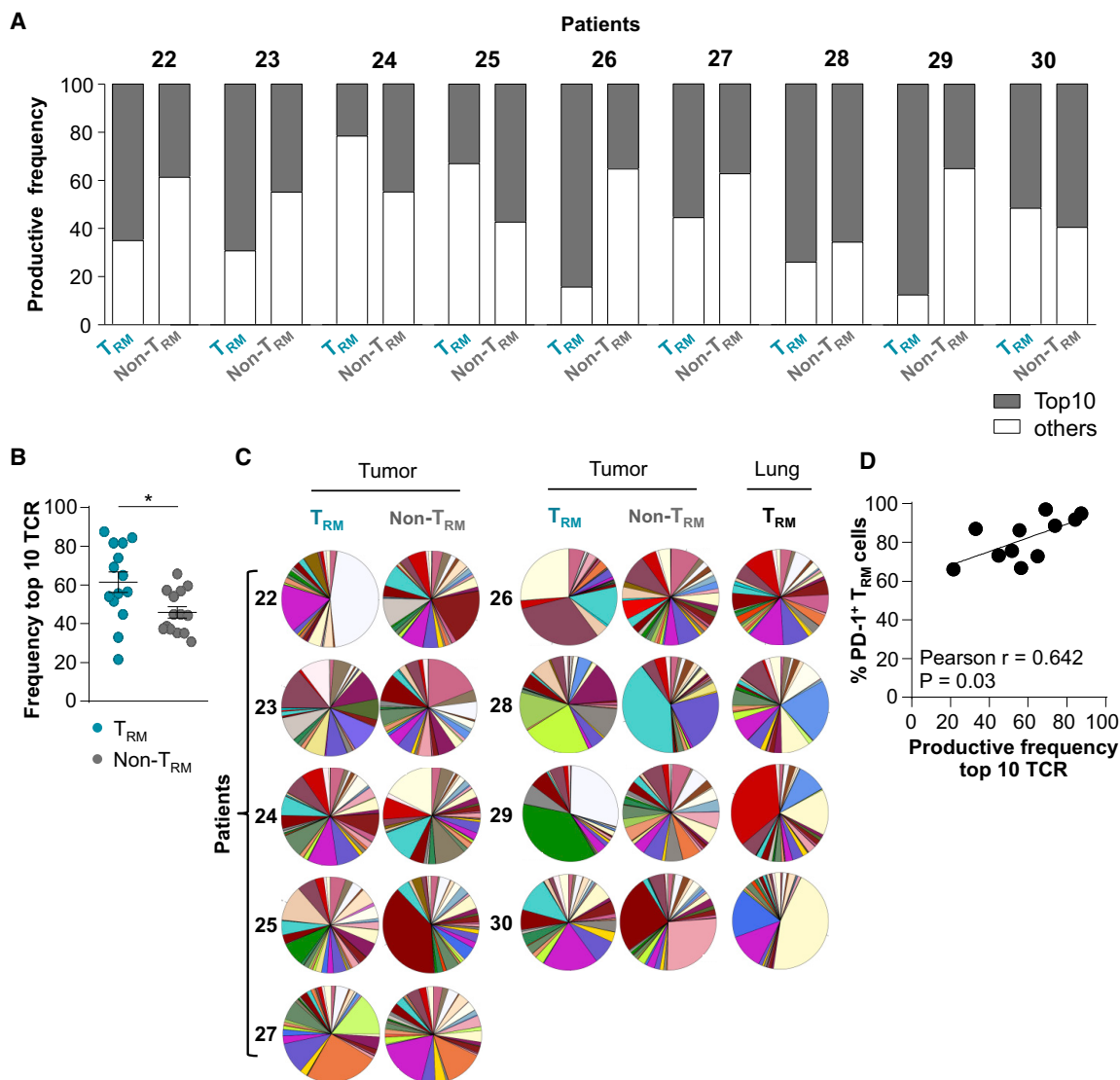
**Figure 6. T<sub>RM</sub> Cells Are Tumor-Specific CD8<sup>+</sup> T Cells**

(A) Intracellular expression of Ki67 in T<sub>RM</sub> and non-T<sub>RM</sub> cells from NSCLCs. The dot plot of 1 representative patient is shown. Right, percentages of Ki67<sup>+</sup> cells in paired T<sub>RM</sub> and non-T<sub>RM</sub> cells from tumors and paired T<sub>RM</sub> cells from healthy lung (n = 18).

(B) Intracellular expression of granzyme B in tumor T<sub>RM</sub> and non-T<sub>RM</sub> cells. The dot plot of 1 representative patient is shown. Right, percentages of granzyme B<sup>+</sup> cells in T<sub>RM</sub> and non-T<sub>RM</sub> cells from tumors and paired T<sub>RM</sub> cells from healthy lung (n = 14).

(C) Conjugates formed between freshly isolated CD8<sup>+</sup> tumor T<sub>RM</sub> or non-T<sub>RM</sub> cells and autologous tumor cells were analyzed by confocal microscopy for p-Tyr (green fluorescence) accumulation in the contact area. T cells were labeled with anti-CD8 mAb (red). Scale bars, 10 μm. Right, quantification of pTyr fluorescence intensity fold increase in CD8 T cells at contact zone with cognate target cells. Four representative patients are included. Each symbol represents an individual synapse from 15 to 37 analyzed conjugates.

(D) Cytotoxic activity of freshly isolated tumor T<sub>RM</sub> and non-T<sub>RM</sub> cells toward autologous cancer cells determined by a conventional 4 h chromium release assay at a 20:1 E:T ratio. The data correspond to 4 independent experiments with 4 different tumor samples. Horizontal lines are means ± SEMs from triplicate. Symbols represent individual TILs or lung samples; horizontal lines are means ± SEMs (A and B). \*p < 0.05 and \*\*\*p < 0.001 (paired t test or ANOVA with Bonferroni post hoc test).



**Figure 7. Tumor CD8<sup>+</sup> T<sub>RM</sub> Cells Display an Oligoclonal TCR Repertoire**

(A) Frequency of productive TCR sequences of the top 10 clonotypes (gray) and all other clonotypes (white) in paired NSCLC T<sub>RM</sub> and non-T<sub>RM</sub> cells from each patient sample (patients 22–30). TCR- $\beta$  CDR3 region in tumor T<sub>RM</sub> and non-T<sub>RM</sub> cells were analyzed by TCR-seq (n = 9).

(B) Frequency of the top 10 TCR sequences in tumor T<sub>RM</sub> and non-T<sub>RM</sub> cells from both TCR-seq and RNA-seq (n = 14).

(C) Pie charts illustrating distribution of TCRV- $\beta$  families in T<sub>RM</sub> and non-T<sub>RM</sub> cells from tumors (n = 9) and autologous T<sub>RM</sub> cells from healthy lungs (n = 4).

(D) Correlation between productive frequency of the top 10 TCR clonotypes and the percentage of PD-1<sup>+</sup> cells in tumor T<sub>RM</sub>s (n = 11).

Horizontal lines correspond to means  $\pm$  SEMs.

\*p < 0.05 paired t test; ns, not significant. The r value indicates the Pearson correlation coefficient.

See also Figure S6.

densities of tumor CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells, even after having undergone resection of their tumor, may be associated with a pool of cancer-specific CD8<sup>+</sup> T cells present in bone marrow, as has been described in infectious diseases.<sup>54</sup> This non-circulating memory CD8<sup>+</sup> T cell subset develops in response to a wide variety of antigens, including tumor antigens, and expands upon antigen rechallenge. Thus, CD103<sup>+</sup>CD8<sup>+</sup> T<sub>RM</sub> cells appear to be crucial components in immune protection against cancer and in the response of NSCLC to ICB, opening up new avenues in cancer immunotherapy.

#### Limitations of Study

The total CD8<sup>high</sup> population in the stroma was as correlative as the CD103<sup>+</sup>CD8<sup>high</sup> population (both with 30.62 months of PFS), most likely due to the frequent expression of CD103 on CD8<sup>+</sup> TILs even in the stroma. The study lacks functional features on IL-17-producing T<sub>RM</sub> cells. Isolation from the same tumor of IL-17<sup>+</sup> and IL-17<sup>-</sup> CD8<sup>+</sup>CD103<sup>+</sup> TILs and sufficient numbers of autologous tumor cells will help to further characterize this small T<sub>RM</sub> cell subset. Single-cell RNA-seq of the tumor T<sub>RM</sub> population may help define the molecular

features of Tc17 cells. TCR- $\gamma\delta$  lymphocytes may contribute to IL-17 production in the TME, even though their total number is very weak.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead Contact
  - Materials Availability
  - Data and Code Availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Patient cohort characteristics and tumor samples
- **METHOD DETAILS**
  - Immunohistochemical staining
  - Tumor cells and tissue-infiltrating lymphocytes isolation
  - RNA and TCR sequencing and analyses
  - Flow cytometry and ELISA
  - Conjugate formation and cytotoxic experiments
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.xcrm.2020.100127>.

### ACKNOWLEDGMENTS

This work was supported by grants from the Institut National du Cancer (INCa; PLBIO016-080 grant no. 10557); the "Association pour la Recherche sur le Cancer (ARC; grant nos. PJA20161204720, SIGN'IT20181007792, and PJA20181208049), Ligue Contre le Cancer (Comité des Yvelines, grant no. 9FI12414QLCZ and Comité du Val de Marne, 2019) and Bristol-Myers Squibb (BMS, France; grant no. CA209-942). S. Corgnac was supported by a grant from the Groupement des Entreprises Françaises dans la Lutte Contre le Cancer (GEFLUC; grant no. 2016-R16180LL) and Cancéropôle Ile de France, and was a recipient of a fellowship from Fondation Recherche Médicale (FRM), Gustave Roussy (SIRIC-SOCRATE), and INCa (PLBIO016-080 grant no. 10557). I.M. was the recipient of a fellowship from the Ligue National Contre le Cancer. M.L. was a recipient of a MENRT fellowship from the French Ministry of Research, a fellowship from the Ligue Contre le Cancer and Gustave Roussy (SIRIC-SOCRATE), and a Taxe d'apprentissage TA-2016 from École des Sciences du Cancer de Gustave Roussy/Université Paris-Sud. We thank Georges Bismuth from Cochin Hospital and Eric Tartour from Hôpital Européen Georges Pompidou for critically reading this manuscript. We also thank Yann Lecluse, Cyril Catelain and Philippe Rameau from the cytometry facility (Plateforme d'Imagerie-Cytométrie), Virginie Marty from the platform of histopathology (Centre de Ressources Biologiques [CRB]), and Yahia Adnani, Aziza Caidi, Guillaume Meurice and Marc Deloger from the platform of bioinformatics (INSERM US23, CNRS UMS3655) of Gustave Roussy for their help with flow cytometry, lung tumor specimens, and RNA-seq analyses and data file deposit, respectively. We are grateful to José-Carlos Benítez-Montanez, Jihène Lahmar-Bach-Hamba, Jordi Remon-Masip, Maud Ngocamus, and Claudio Nicotra from the Department of Cancer Medicine of Gustave Roussy for their help in establishing patient tumor cohorts. We also thank Vassili Soumelis from Institut Curie for help with the RNA extraction protocol and Caroline Comunaux from the CRB of Marie Lannelongue Hospital for help with fresh human NSCLC tumors. The graphical abstract was created with BioRender.

### AUTHOR CONTRIBUTIONS

Conception and design, S. Corgnac and F.M.-C. Development of Methodology, S. Corgnac, I.M., N.D., J.A., and F.M.-C. Data Acquisition (e.g., providing animals, acquiring and managing patients, providing facilities), S. Corgnac, I.M., L.M., E.A., H.H., T.D., M.L., E.V., J.K., L.G., N.S., P.V., V.d.M., O.M., J.-Y.S., C.M., S. Chouaib, D.P., J.A., B.B., and F.M.-C. Data Analysis & Interpretation (e.g., statistical analysis, biostatistics, computational analysis), S. Corgnac, E.A., L.M., J.A., B.B., and F.M.-C. Writing, Reviewing, and/or Revision of Manuscript, S. Corgnac and F.M.-C. Administrative, Technical, and Material Support (e.g., reporting and organizing data, constructing databases), S. Corgnac, I.M., L.M., E.A., P.V., V.d.M., O.M., J.-Y.S., C.M., B.B., and F.M.-C. Study Supervision, F.M.-C.

### DECLARATION OF INTERESTS

B.B.'s sponsored research at Gustave Roussy Cancer Center consists of the following: Abbvie, Amgen, AstraZeneca, BeiGene, Blueprint Medicines, BMS, Boehringer Ingelheim, Celgene, Cristal Therapeutics, Daiichi-Sankyo, Eli Lilly, GSK, Ignyta, IPSEN, Inivata, Janssen, Merck KGaA, MSD, Nektar, Onxeo, OSE Immunotherapeutics, Pfizer, Pharma Mar, Roche-Genentech, Sanofi, Servier, Spectrum Pharmaceuticals, Takeda, Tiziana Pharma, and Tolero Pharmaceuticals.

Received: August 22, 2019

Revised: July 24, 2020

Accepted: September 24, 2020

Published: October 20, 2020

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposit Data		
Raw RNAseq data	This study	EGAS00001004707
Antibodies		
anti-human CD8 Pacific blue (RPA-T8)	BioLegend	Cat# 301023; RRID:AB_493110
anti-human CD103-FITC (Ber-ACT8)	BioLegend	Cat# 350204; RRID:AB_10639865
anti-human KLRG1-PE (13F12F2)	ebioscience	Cat# 12-9488-42; RRID:AB_2572716
anti-human CD3-Alexa700 (UCHT1)	BioLegend	Cat# 300424; RRID:AB_493741
anti-human CD69-APC-Cy7 (FN50)	BioLegend	Cat# 310914; RRID:AB_314849
anti-human granzyme-B-FITC (GB11)	BioLegend	Cat#515403; RRID:AB_2114575
anti-human TNF $\alpha$ -PE/Dazzle594 (Mab11)	BioLegend	Cat# 502945; RRID:AB_2564172
anti-human Aiolos-Alexa488 (16D9C97)	BioLegend	Cat# 371107; RRID:AB_2616975
anti-human V $\alpha$ 7.2-APC-Cy7 (3C10)	BioLegend	Cat# 351713; RRID:AB_2561995
anti-human CD161-Alexa488 (HP-3G10)	BioLegend	Cat# 339923; RRID:AB_2563938
anti-human TCF-1-PE (7F11A10)	BioLegend	Cat#655207; RRID:AB_2728491
anti-human 4-1BB-PE-Dazzle594 (clone 4B4-1)	BioLegend	Cat#309825; RRID:AB_2566259
anti-human TCR $\gamma\delta$ -PerCPeCy5.5 (B1)	BioLegend	Cat# 331223; RRID:AB_2563012
anti-human CD103-BV711 (Ber-ACT8)	BD Biosciences	Cat# 563162; RRID:AB_2738039
anti-human CD49-PerCPeFluor710 (TS2/7)	Thermo Fisher Scientific	Cat# 46-9490-41; RRID:AB_2573890
anti-human S1PR1-eFluor660 (SW4GYPP)	Thermo Fisher Scientific	Cat# 50-3639-41; RRID:AB_2574207
anti-human PD-1-PeCyanine7 (eBioJ105)	Thermo Fisher Scientific	Cat# 25-2799-42; RRID:AB_10853804
anti-human AHR-PerCPeFluor710 (FF3399)	Thermo Fisher Scientific	Cat# 46-9854-41; RRID:AB_2573905
anti-human Ki67-PerCPeFluor710 (20Raj1)	Thermo Fisher Scientific	Cat# 46-5699-41; RRID:AB_10804404
anti-human IFN $\gamma$ -APC (B27)	BD Biosciences	Cat# 554702; RRID:AB_398580
anti-human Hobit-Alexa647 (Sanquin-Hobit/1)	BD Biosciences	Cat# 566250; RRID:AB_2739629
anti-human CD45RA-APC (T6D11)	Miltenyi Biotec	Cat#130-098-187; RRID:AB_2660978
anti-human pSTAT3pS727-APC (REA324)	Miltenyi Biotec	Cat# 130-105-000; RRID:AB_2653576
anti-human CD39-APC (MZ18-23C8)	Miltenyi Biotec	Cat#130-100-459; RRID:AB_2660867
anti-human IL17A-PeVio770	Miltenyi Biotec	Cat#130-100-327; RRID:AB_2659809
anti-human/mouse Tbet-APC (REA102)	Miltenyi Biotec	Cat# 130-119-821; RRID:AB_2784465
mouse anti-human phospho-tyrosine (PY20)	BD Biosciences	Cat# 610000; RRID:AB_397423
rabbit anti-human CD8	Thermo Fisher Scientific	Cat#PA1-37298; RRID:AB_2075396
Goat anti-mouse IgG AlexaFluor-488	Thermo Fisher Scientific	Cat# A-11001; RRID:AB_2534069
Goat anti-rabbit AlexaFluor-647	Thermo Fisher Scientific	Cat# A-21244; RRID:AB_2535812
anti-human Pan-keratin (clones AE1/AE3)	Agilent	Cat# M3515; RRID:AB_2132885
IHC anti-human CD8 (clone SP16)	Spring Bioscience	Cat#M3164; RRID:AB_1660846
IHC anti-human CD103 (clone EPR4166-2)	Abcam	Cat#ab129202; RRID:AB_11142856
anti-human ICAM-1 (clone N1C2)	GeneTex	Cat#GTX100450; RRID:AB_1950536
anti-human E-cadherin (clone EP6)	BioSB	Cat#BSB5466
LEAF Purified anti-human CD3 antibody (OKT3)	BioLegend	Cat#317304; RRID:AB_571925
Purified anti-human CD28 antibody (CD28.2)	BioLegend	Cat#302901; RRID:AB_314303
Biological Samples		
Resected lung tumors and adjacent healthy lung tissue samples	Institut mutualiste Montsouris and the Hôpital Marie-Lannelongue	See <a href="#">Tables S1</a> and <a href="#">S4</a> for details
Human healthy blood	Établissement français du sang, Paris, France	N/A

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	Cat#P1585
ionomycin	Sigma-Aldrich	Cat#10634
recombinant IL-2	Sanofi recherche	Cat#1A1
recombinant human TGF- $\beta$ 1	R&D Systems	Cat#240-B-002
recombinant human IL-6	Miltenyi Biotec	Cat# 130-095-365
BrefeldinA	Thermo Fisher Scientific	Cat#00-4506-51
Chromium-51 Radionuclide	Perkin Elmer	Cat#NEZ030005MC
<b>Critical Commercial Assays</b>		
CD8 Microbeads, human	Miltenyi Biotec	Cat#130-045-201
Tumor cells Isolation Kit, human	Miltenyi Biotec	Cat#130-108-339
Human Tumor dissociation kit	Miltenyi Biotec	Cat#130-095-929
Foxp3/Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	Cat#00-5523-00
Single cell RNA purification kit	Norgen	Cat#51800
SureSelect Automated Strand-Specific RNA Library Preparation Kit	Agilent	Cat#G9691A
QIAamp DNA micro kit	QIAGEN	Cat#56304
Maxima first strand cDNA synthesis kit	Thermo-Fischer Scientific	Cat#K1641
Maxima SYBR Green Master Mix	ThermoFischer Scientific	Cat#4472908
LIVE/DEAD Fixable UV dead cell stain kit	ThermoFischer	Cat#L34962
Human IL-17A ELISA Kit	Biotechne	Cat#DY317-05
Opal 7-color Automation IHC Kit	Akoya/Perkin Elmer	NEL801001KT
<b>Oligonucleotides</b>		
Primer sequences for IKZF3, RORC,AHR, IL17A, TCF7, S1PR1 and 18S	This paper; See <a href="#">Table S10</a>	N/A
<b>Software and Algorithms</b>		
GraphPad Prism software V8	GraphPad Software Inc.	RRID:SCR_002798
FlowJo V10	Tree Star Inc	RRID: SCR_008520
RStudio software	The R Foundation	RRID:SCR_000432
InForm software	Akoya/PerkinElmer	N/A
R (v 3.2.3)	The R Foundation	RRID: SCR_001905
FastQC (v 0.11.3)	Babraham bioinformatics	RRID:SCR_014583
Salmon (v 0.8.2)	<a href="#">55</a>	N/A
sleuth (v 0.28.1)	<a href="#">56</a>	RRID:SCR_002555
clusterProfiler (v 3.8.0)	<a href="#">57</a>	RRID:SCR_016884
MiXCR (v 2.1.10)	<a href="#">58</a>	RRID:SCR_018725
KEGG pathway	<a href="https://www.genome.jp/kegg-bin/show_pathway?hsa04660">https://www.genome.jp/kegg-bin/show_pathway?hsa04660</a>	RRID:SCR_012773
ImmunoSEQ Analyzer Platform Adaptive Biotechnologie	Adaptive Biotechnologie	RRID:SCR_014709

**RESOURCE AVAILABILITY**

**Lead Contact**

Further information and requests for resources and reagents should be directly to and will be fulfilled by the Lead contact, Fathia Mami-Chouaib ([fathia.mami-chouaib@gustaveroussy.fr](mailto:fathia.mami-chouaib@gustaveroussy.fr))

**Materials Availability**

This study did not generate new unique reagents.



### Data and Code Availability

The RNA sequencing dataset comparing the transcriptome of CD8<sup>+</sup>CD103<sup>+</sup> cells with CD8<sup>+</sup>CD103<sup>-</sup> cells, as shown in [Figures 3, 4, 5, S3, and S4](#), Tables generated during this study are available at European Genome-phenome Archive (EGA) under EGAS00001004707 access number study.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Patient cohort characteristics and tumor samples

We conducted a monocentric retrospective study of a discovery cohort of 111 and a validation cohort of 41 advanced NSCLC patients receiving treatment with PD-1/PD-L1 inhibitors in a variety of settings covering routine clinical care, expanded access, compassionate-use programs and clinical trials (nivolumab, pembrolizumab, atezolizumab or durvalumab) at Gustave Roussy between November 2012 and February 2020. Demographic, clinical, pathological and molecular data were also collected ([Tables S1 and S4](#)). Radiological assessments were performed every 8 weeks per RECIST v1.1 and per the investigator's discretion. Fast-progressors were defined as patients who died during the first 12 weeks since the start of immunotherapy. This study was approved by the Institutional Review Board of Gustave Roussy (Commission scientifique des Essais thérapeutiques [CSET]) and informed consent was obtained. Freshly resected lung tumors and adjacent healthy lung tissue samples were obtained from the Institut mutualiste Montsouris and the Hôpital Marie-Lannelongue. For these resected tissues, the sex and the gender of the subjects are not known.

Healthy donor blood samples were collected from the French blood bank (Etablissement Français du Sang (EFS); agreement number N°12/EFS/079). The sex and the gender of the subjects are not known.

## METHOD DETAILS

### Immunohistochemical staining

IHC was performed on archived FFPE tumor tissues using Ventana Benchmark and Discovery automated platforms. Briefly, after deparaffinisation and epitope retrieval in CC1 buffer (pH = 8, 36 min at 95°C), tissue sections were incubated with primary mAb for ICAM-1 (clone N1C2, GeneTex, 1:200 dilution) or E-cadherin (clone EP6, Bio SB, prediluted) for 1 h at room temperature. Amplification and detection steps used the ultraview kit with amplification, and 3,3'-diaminobenzidine was used as a chromogen. ICAM-1 staining was evaluated as H-score (0 to 300) based on the percentage of tumor cells stained at each intensity (scale from 0 to 3). Since the intensity of E-cadherin staining was homogeneous within each individual tumor sample, we scored E-cadherin staining as the prominent intensity for each tumor on a semiquantitative scale from 0 to 3.

Multiplexed fluorescent IHC for CD8 T<sub>RM</sub> was performed by sequential staining of a single tissue section with anti-CD8 (clone SP16, Spring Bioscience, 1:200), anti-CD103 (clone EPR4166-2, Abcam, 1:200) and anti-cytokeratin (clones AE1/AE3, Agilent, 1:100). For each staining, the HRP-conjugated amplification system was associated with a tyramide-coupled fluorophore: Opal 690, Opal 250 and Opal 570, respectively. Multispectral fluorescent images were captured using the Vectra 3 microscope (PerkinElmer) and regions of interest were selected. Image analysis using InForm software (PerkinElmer) included spectral unmixing, tissue segmentation (stromal versus epithelial areas) using a trainable classifier, nuclei detection based on dapi staining and cell segmentation followed by cell phenotyping for identification of cell populations defined by the combination of individual markers. The density (number of cells per square mm) of CD8<sup>+</sup>, CD103<sup>+</sup> CD8<sup>+</sup> and CD103<sup>-</sup>CD8<sup>+</sup> cells was determined for each tumor sample in the total tumor area, as well as in the stromal and intraepithelial compartments, based on tissue segmentation. Results from image analysis were validated for all cases.

### Tumor cells and tissue-infiltrating lymphocytes isolation

Freshly resected lung tumors and adjacent healthy lung tissue samples were immediately cut into small fragments and digested for 40 min at 37°C using the tumor dissociation kit (Miltenyi Biotec). The dissociated samples were smashed on 100 μm cell strainers, washed, and red blood cell lysis was performed. CD8 T lymphocytes were positively selected using CD8 microbeads according to the manufacturer's instructions (Miltenyi Biotec). Recovered cells were either used for phenotypic analyses or further sorted by BD FACSAriaIII or BDFusion cell sorter (BD Biosciences) using anti-CD8-Pacific blue (RPA-T8, Biolegend), anti-CD103-FITC (Ber-ACT8, Biolegend) and anti-KLRG1-PE (clone 13F12F2, ebioscience) mAb. Dead cells were excluded using DAPI. CD103<sup>+</sup>CD8<sup>+</sup> and KLRG1<sup>+</sup>CD8<sup>+</sup> T cell populations were isolated and then either stored at -80°C for further DNA or RNA isolation or cultured for 2-5 days in the presence of low doses of IL-2 (20 U/ml) for functional studies. Tumor cells were recovered from the negative fraction of the CD8 T cell isolation described above, and then purified using a human tumor cell isolation kit (Miltenyi Biotec).

### RNA and TCR sequencing and analyses

Total RNA was extracted from each sorted CD103<sup>+</sup>CD8<sup>+</sup> and KLRG1<sup>+</sup>CD8<sup>+</sup> TIL population pair and autologous healthy lung CD103<sup>+</sup>CD8<sup>+</sup> lymphocytes when available; 150,000 lymphocytes per sample were processed using a single cell RNA purification kit (Norgen) according to the manufacturer's instructions. RNA integrity (RNA Integrity Score ≥ 00007.0) was checked on the Agilent 2100 Bioanalyzer and quantity was determined using Qubit (Invitrogen). The SureSelect Automated Strand-Specific RNA Library

Preparation Kit was used according to the manufacturer's instructions with the Bravo Platform. Briefly, 50 ng of total RNA sample were used for poly-A mRNA selection using oligo(dT) beads and subjected to thermal mRNA fragmentation. The fragmented mRNA samples were subjected to cDNA synthesis and were further converted into double-stranded DNA using the reagents supplied in the kit, and the resulting dsDNA was used for library preparation. The final libraries were bar-coded, purified, pooled in equal concentrations and subjected to paired-end sequencing on a HiSeq-2000 sequencer (Illumina). Fast quality was assessed using FastQC (v 0.11.3) and did not require further trimming or adaptor removal. Counting of reads over the transcriptome was performed over Gencode (v 19, GRCh37.p13) with Salmon<sup>55</sup> (v 0.8.2), using non-oriented library, 100 bootstrap and sequence bias correction. All other parameters were left to default. The pseudo-mapping rates were between 79% and 88% of overall reads. Differential analysis was performed within the R (v 3.2.3) environment with sleuth<sup>56</sup> (v 0.28.1), wasabi (v 0.1) and in-house scripts. PCA highlights inter-individual variations that are far above any variation of interest, which was confirmed by a likelihood-test ratio and taken into account within the Wald test.

Primary functional analyses were performed using Ingenuity Pathway Analysis and GSEA was performed with clusterProfiler<sup>57</sup> (v 3.8.0). RNaseq are available at the European Molecular Biology Laboratory European Bioinformatics Institute database (<https://www.ebi.ac.uk/arrayexpress>). TCR repertoire analysis was performed with a MiXCR package<sup>58</sup> (v 2.1.10). KEGG pathway: [https://www.genome.jp/kegg-bin/show\\_pathway?hsa04660](https://www.genome.jp/kegg-bin/show_pathway?hsa04660).

For TCRseq, total DNA from CD103<sup>+</sup>CD8<sup>+</sup> and KLRG1<sup>+</sup>CD8<sup>+</sup> TIL subset pairs and autologous healthy lung CD103<sup>+</sup>CD8<sup>+</sup> cells when available, sorted from nine NSCLC patient tissues, was purified using a QIAamp DNA micro kit (QIAGEN). DNA was quantified with fluorescence-based measurement Qubit (Life Technologies). TCR $\beta$ -CDR3 sequencing was performed by ImmunoSEQ, Adaptive Biotechnologies (Seattle). Raw data of TCR reads and sequences were uploaded on the ImmunoSEQ Analyzer Platform (Adaptive Biotechnologies).

For quantitative (q) RT-PCR, total RNA was extracted from sorted cell populations using the single cell RNA purification kit (Norgen Biotek). cDNA were synthesized using the Maxima first strand cDNA synthesis kit (Thermo-Fischer Scientific). qRT-PCR was performed on a step-one plus (Applied Biosystems) using Maxima SYBR Green Master Mix (ThermoFischer Scientific). Expression levels of transcripts were normalized to 18S housekeeping gene. PCR primers for human, *TCF7*, *IKZF3*, *AHR*, *RORC*, *IL17* and *18S* genes were designed by Sigma-Aldrich and used according to the manufacturer's recommendations (Table S10).

### Flow cytometry and ELISA

Phenotypic analyses were performed by direct immunofluorescence with a panel of fluorochrome-conjugated antibodies. Anti-CD3-Alexa700 (UCHT1), anti-CD8-PacificBlue (RPA-T8), anti-CD69-APC-Cy7 (FN50), anti-granzyme-B-FITC (GB11), anti-TNF $\alpha$ -PE/Dazzle594 (Mab11), anti-Aiolos-Alexa488 (16D9C97), anti-V $\alpha$ 7.2-APC-Cy7 (3C10), anti-CD161-Alexa488 (HP-3G10), anti-TCF-1-PE (7F11A10), anti-4-1BB-PE-Dazzle594 (clone 4B4-1) and anti-TCR $\gamma\delta$ -PerCPeCy5.5 (B1) were supplied by BioLegend. Anti-CD103-BV711 (Ber-ACT8), anti-IFN $\gamma$ -APC (B27) and anti-Hobit-Alexa647 (Sanquin-Hobit/1) was purchased from BD Biosciences, anti-KLRG1-PE (13F12F2), anti-CD49-PerCPeFluor710 (TS2/7), anti-S1PR1-APC (SW4GYPP), anti-PD-1-PeCy7 (eBioJ105), anti-AHR-PerCPeFluor710 (FF3399), anti-Ki67-PerCPeFluor710 (20Raj1) were supplied by Thermo Fisher Scientific. Anti-CD45RA-APC, anti-pSTAT3pS727-APC, anti-CD39-APC, anti-IL17A-PeVio770 and anti-Tbet-APC (REA102) were purchased from Miltenyi. For intracellular expression of IFN $\gamma$ , TNF $\alpha$  and IL-17A, cells were stimulated for 4 h with PMA (50 ng/ml) plus ionomycin (1  $\mu$ g/ml) in the presence of Brefeldin A (1  $\mu$ g/ml, ebioscience). Cells were fixed, permeabilized (FoxP3 buffers Kit, ebioscience) and then stained with fluorochrome-conjugated mAb. Dead cells were excluded using a LIVE/DEAD Fixable UV dead cell stain kit (Thermo Fisher Scientific). Stained cells were analyzed by flow cytometry using a BD FACS Fortessa flow cytometer (BD Biosciences). Data were processed using FlowJo V10 software (Tree Star Inc.).

For ELISA, sorted CD8<sup>+</sup> T lymphocytes were stimulated overnight with PMA plus ionomycin, and then supernatants were stored until IL-17 dosage (eBiosciences).

Th17-positive control cells were generated from healthy donor's CD4<sup>+</sup> peripheral blood lymphocytes (PBL) stimulated with anti-CD3 and anti-CD28 in the presence of TGF- $\beta$  and IL-6.

### Conjugate formation and cytotoxic experiments

Formation of stable conjugates between T cells and autologous tumor cells was analyzed by confocal microscopy. Effector and target cells were co-cultured for 30 min at 1:1 E:T ratio, and then plated on poly-(L-lysine)-coated coverslips (Sigma-Aldrich, Saint-Louis, MO). Cells were then fixed, permeabilized as described<sup>16</sup> and stained with mouse anti-phospho-tyrosine (PY20, BD Biosciences) and rabbit anti-CD8 (Thermo Fisher Scientific), followed by anti-mouse AlexaFluor-488 and anti-rabbit AlexaFluor-647 (Thermo Fisher Scientific). Coverslips were mounted and analyzed using a fluorescence microscope (Leica, HR Sp8) with x63 lenses, and polarization of phospho-Tyr to the immune synapse between T cells and target cells was calculated. Stable conjugates were defined by polarization of p-Tyr at the contact zone between effector cells and tumor cells. Cytotoxic activity was evaluated using the conventional 4 h <sup>51</sup>Cr-release assay as described.<sup>16</sup>

## QUANTIFICATION AND STATISTICAL ANALYSIS

ORR was defined as complete (CR) plus partial response (PR). OS was calculated from the date of first immunotherapy administration until death due to any cause. PFS was calculated from the date of first immunotherapy until disease progression or death due to any cause. Comparisons between patient characteristics were performed using Chi-square or Fisher's exact test for discrete variables and the unpaired t test, Wilcoxon sign-rank test or analysis of variance for continuous variables. The best cut-point for total CD8<sup>+</sup>, CD103<sup>+</sup> and CD103<sup>-</sup> TIL was assessed using the log-rank maximization method.<sup>59</sup> Survival analyses were performed using the Kaplan-Meier method and the log-rank test. All P values inferior to 0.05 were considered statistically significant.

A Cox proportional hazards regression model was used to evaluate independent prognostic factors for OS and PFS. Variables included in the final multivariate model were selected according to their clinical relevance and statistical significance in univariate analysis (p value cut-off = 0.10). The proportional hazard hypothesis was verified with the Schoenfeld residual method. Predictive factors of disease control were tested with logistic regression in univariate and multivariate analyses. The alpha level was 5%. Statistical analyses were performed with R (free software environment for statistical computing and graphics).

Statistical significance was determined with the paired or unpaired Student t test, or with the one-way analysis of variance (ANOVA) test with Bonferroni correction. Data are presented as mean ± SEM. All statistical details of experiments can be found in Figures and Figure legends; n = number of patient or tumor/lung samples. Statistical analyses were performed with GraphPad Prism software V8 (GraphPad Software Inc., San Diego, CA). \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001 and RStudio software.